UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

FACULTAD DE MEDICINA
DOCTORADO EN CIENCIAS BIOMEDICAS

INSTITUTO NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN *"SALVADOR ZUBIRÁN"*

LA INTERLEUCINA 12 PRODUCIDA EN PLANTAS DE JITOMATE EJERCE UN EFECTO TERAPÉUTICO EN LA TUBERCULOSIS PULMONAR EXPERIMENTAL

T E S I S

QUE PARA OBTENER EL GRADO ACADÉMICO DE:

DOCTORA EN CIENCIAS

P R E S E N T A

M. en C. ANA LUCRECIA ELÍAS LÓPEZ

Facultad de Medicina

DIRECTOR DE TÉSIS:

DR. ROGELIO HERNÁNDEZ PANDO

MÉXICO, D. F. DICIEMBRE DE 2008

SECRETARÍA DE EDUCACIÓN PÚBLICA

INSTITUTO POLITÉCNICO NACIONAL
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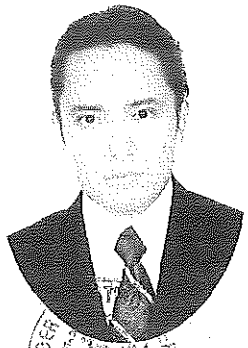
ACTA DE EXAMEN DE GRADO DE MAESTRÍA

En la Ciudad de **México, D.F.,** a las **13:00** horas del día **1** del mes de **julio** del año **2011,** reunidos **en el Aula Magna 4** designada para tal efecto, los C. Profesores de la Sección de Estudios de Posgrado e Investigación.

Dra. Luvia Enid Sánchez Torres, Dra. Sonia Mayra Pérez Tapia, Dra. Iris Citlali Elvira Estrada García, Dr. Rogelio Enrique Hernández Pando, Dra. Dulce Adriana Mata Espinosa y Dra. Jeanet Serafín López.



designados para integrar el Jurado del Examen de Grado de:

MAESTRÍA EN CIENCIAS EN INMUNOLOGÍA

de

Alejandro Francisco Cruz

Con registro **A100169** y considerando que se ha cumplido con los requisitos correspondientes, se procedió a efectuar el examen en los términos que establece el Reglamento de Estudios de Posgrado. Después de concluir la disertación y réplica de rigor, el jurado deliberó, habiéndose obtenido el siguiente resultado:

APROBADO CON MENCION HONORIFICA

Para constancia se levantó la presente acta a las **15:00** horas del día **1** del mes de **julio** del año **2011,** misma que suscriben los sinodales mencionados.

Presidente

Dra. Luvia Enid Sánchez Torres

Secretario

Dra. Sonia Mayra Pérez Tapia

1er. Vocal

Dra. Iris Citlali Elvira Estrada García

2do. Vocal

Dr. Rogelio Enrique Hernández Pando

3er. Vocal

Dra. Dulce Adriana Mata Espinosa

Suplente

Dra. Jeanet Serafín López

Jefe de la Sección

Dr. Manuel Jesús Piñón López

TESIS

Efecto del adenovirus recombinante que codifica al factor estimulante de las colonias de granulocitos y macrófagos en la tuberculosis pulmonar murina

La suscrita directora de **La Escuela Nacional de Ciencias Biológicas** certifica que las firmas que anteceden son auténticas y corresponden a las personas cuyos nombres aparecen en esta acta.

Secretario de Investigación y Posgrado

Dr. Jaime Álvarez Gallegos

M. en C. Rosalía María del Consuelo Torres Bezaury

S. E. P.
INSTITUTO POLITECNICO NACIONAL
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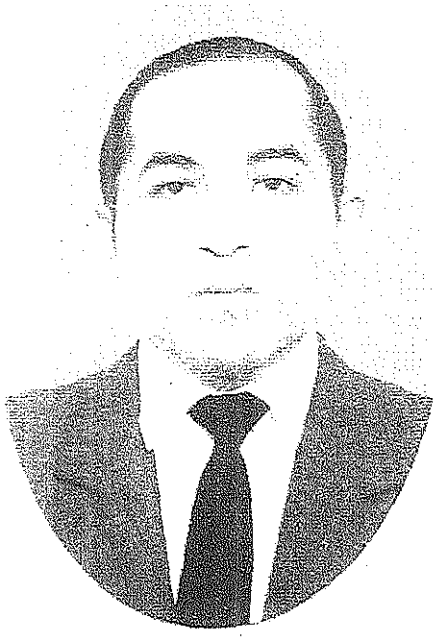
ACTA DE EXAMEN



En Ciudad Universitaria, Distrito Federal, a los 27 días del mes de Enero del 2012, se celebró el examen para obtener el grado de DOCTOR en CIENCIAS, que sustentó CÉSAR ENRIQUE RIVAS SANTIAGO, de nacionalidad MEXICANA, registrado con el número de cuenta 9928007-7, quien cursó los estudios en el periodo comprendido de 2009-II a 2011-II y cumplió los requisitos académicos señalados en el plan de estudio correspondiente, habiendo presentado la tesis: "Péptidos antimicrobianos como inmunoterapia en el tratamiento contra la tuberculosis en modelo animal"

El Comité Académico del Programa designó el jurado formado por los profesores que a continuación se mencionan y que fungieron como:

Presidente: Dra. Yolanda López Vidal

Vocal: Dra. Gloria Ernestina Pinzón

Dr. Manuel Jiménez Estrada

Secretario: Dra. Gloria Soledad Moreno

Al término del examen el jurado resolvió: aprobarlo

Se dio por concluido el acto académico con las firmas de los sinodales que en el intervinieron.

Presidente | Secretario

Vocal

"POR MI RAZA HABLARÁ EL ESPÍRITU"

El suscrito, Coordinador del Programa de Maestría y Doctorado en Ciencias Bioquímicas, certifica que las firmas son auténticas y corresponden al jurado designado.

DR. ROBERTO CORIA ORTEGA

Por la Dirección General de Administración Escolar

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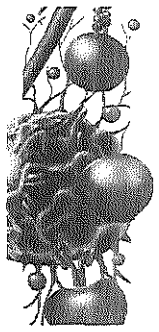

Prophylactic potential of defensins and L-isoleucine in tuberculosis household contacts: an experimental model

Aim: Patients with pulmonary tuberculosis (TB) are the most important source for TB infection, being the risk of infection determined by the source case infectiousness and the contact closeness. Currently, the administration of isoniazid is used to prevent the infection to some extent in household contacts. At experimental level, defensins are efficient molecules for the treatment of TB and other infectious diseases. **Materials & methods:** In this work, we used a model of *Mycobacterium tuberculosis* transmission by long cohabitation of infected and noninfected mice, and treated the latter group with antimicrobial peptides in order to determine the potential capacity of defensins to prevent the infection. **Results:** Our results showed that the intratracheal administration of human neutrophil peptide-1, human β-defensin-2 alone or in combination and the use of L-isoleucine significantly prevents bacterial transmission, diminishing pulmonary lesions and bacterial loads. **Conclusion:** Data suggest the potential use of L-isoleucine as prophylactic for TB household contacts.

Keywords: antimicrobial peptides • defensins • household contact tuberculosis • L-isoleucine • *Mycobacterium tuberculosis* • prophylactic

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Tuberculosis (TB) is one of the most important infectious diseases worldwide; it causes about 1.4 million deaths yearly. Although TB incidence has declined in the past years, multidrug-resistant strains, AIDS and high contagious rates are reversing the trend. To counter-attack this reemerging and expanding disease, it is completely necessary to find new approaches to treat and prevent it [1]. In this context, antimicrobial peptides such as defensins and cathelicidins are a promise for new treatment and prevention. Antimicrobial peptides (AMPs), a diverse group of small molecules, are classified into several categories based on their primary structures and topologies [2–4]. In humans, one important category of AMPs are defensins. This category is characterized by cationic and amphipathic peptides, usually comprising six invariant cysteine residues forming disulfide bridges that assume a conserved structural fold. Based primarily on the spacing between the cysteine residues and the topology of the disulfide bridges, human defensins are organized into three classes, α-, β- and θ-defensins [2]. It has been demonstrated that defensins have antimicrobial effect on *Mycobacterium tuberculosis* (MTB) both *in vitro* [5,6] and *in vivo* [7]. Moreover, our group showed that β-defensins are needed for an efficient bacilli control in pulmonary experimental TB [8], and lung epithelial cells use this antimicrobial peptide to eliminate either intracellular and extracellular mycobacteria [9]. Recently, our group showed that induction of defensins through intratracheal instillation of L-isoleucine in MTB-infected mice reduced drastically pulmonary bacilli loads, either in mice infected with the reference drug sensible strain H37Rv or with a multidrug-resistant strain (MDR) through the induction of defensins [10]. Some other groups have proposed the use of defensins in combination with classic antimycobacterial drugs for treatment, at least *in vitro* have shown remarkable results [11].

Future Medicine part of fsg

Prophylactic potential of defensins and L-isoleucine in tuberculosis household contacts: An experimental model

ARTICLE in IMMUNOTHERAPY · MARCH 2015

Impact Factor: 2.07 · DOI: 10.2217/imt.14.119 · Source: PubMed

TB infected people with acid-fast bacilli in their sputum are the most infectious group in the community; this specific group spreads infection among their household contacts. Household contacts mainly children exposed to adults with smear-positive pulmonary TB have a high risk for infection, and this risk increases with the degree of contact [12,13]. Isolated pulmonary TB cases from household contact infection would be the ideal strategy to interrupt transmission and subsequent TB development; however this is a drastic approach that is not always possible. Although vaccination is the unique known way to prevent TB, it is also accepted that BCG does not prevent efficiently pulmonary TB particularly in adults because its efficacy wanes with time [14]. Administration of isoniazid is another form to prevent infection in household or health workers that have close contact with TB patients, but this approach has the inconvenience of potential hepatotoxicity [15]. Thus, novel strategies are needed in order to prevent household contacts infection. In this study, we used an experimental mouse model of mycobacterial transmission and tested the potential use of β-defensins and L-isoleucine as prophylactic treatment for household contacts infection. The results showed that mice treated with defensins and L-isoleucine had lesser bacilli loads and histopathological damage than nontreated animals.

Materials & methods

Experimental model of progressive pulmonary TB

All animal work was performed in conformity with the institutional Ethics Committee for Experimentation in Animals. Mice were acclimatized for at least 2 weeks before experiments. The laboratory drug-sensitive Mtb strain H37Rv (ATCC number 25618) and MDR strain (clinical isolate, resistant to all first-line antibiotics) were grown in Middlebrook 7H9 broth (Difco Laboratories, MI, USA) supplemented with 0.2% (v/v) glycerol, 10% oleic albumin dextrose catalase enrichment (Difco) and 0.02% (v/v) Tween-80 at 37°C. Mid log-phase cultures were used for all experiments.

Mycobacteria were counted and stored at -80°C until use. Bacterial aliquots were thawed and pulse-sonicated to remove clumps. The experimental model of progressive pulmonary TB has been previously described in detail [16]. Briefly, male BALB/c mice, 6–8 weeks of age, were anaesthetized in a gas chamber using 0.1 ml per mice of sevoflurane and infected through endotracheal instillation with 2.5×10^5 live bacilli. Mice were maintained in the vertical position until spontaneous recovery. Infected mice were maintained in groups of five in cages fitted with micro-isolators.

Household contact mice model

We tried to reproduce the close-contact living conditions between TB patients and their household contacts [17]. Pathogen-free male BALB/c mice were used at 6–8 weeks of age; six noninfected healthy mice were placed in the same microisolator with six mice with 60 days of MTB infection as described above for 30 days, with constant physical contact among them and with bedding, urine and feces. Household contact mice were euthanized by exsanguination at 30 and 60 days after living together with MTB-infected mice. Twelve lungs were prepared for histopathological studies. After eliminating hilar lymph nodes and thymic tissues, 12 lungs were frozen and kept to -70°C for counting CFUs.

Groups & prophylactic treatment

In total there were four different groups of 18 mice, each group was constituted for six MTB-infected mice (60 days postinfection), six healthy mice that co-housed with MTB-infected mice and were treated with defensin or L-isoleucine as prophylactic and six healthy untreated mice (mock-control).

In the first group, six healthy mice were treated with 10 µg/100 µl of recombinant HNP-1 (International peptides, Osaka, Japan), in the second group six healthy mice were treated with 10 µg/100 µl of recombinant HBD-2 (International Peptides), and in the third group six healthy mice were treated with 5 µg/100 µl of recombinant HNP-1 in combination with 5 µg/100 µl of recombinant HBD-2. These groups were treated three-times a week for 4 weeks with their respective peptide.

For the case of L-isoleucine-treated mice group, six mice were treated with 250 µg/100 µl of LPS-free L-isoleucine thrice a week for 1 and 2 months, owing that this concentration was reported by our group previously as a therapeutic dose [10]. Treatments were applied via intratracheal instillation to assure that peptides reach lung, for mock-control mice, isotonic saline solution was instilled.

The treatment was applied 24 h before placing healthy mice with MTB-infected mice. Once mice were living together with MTB-infected mice, treatment was applied three-times a week for 4 and 8 weeks and then animals were euthanized by exsanguination. Twelve lungs from treated mice were prepared for histopathological studies. After eliminating hilar lymph nodes and thymic tissues, ten lungs more were frozen and kept to -70°C for counting colony-forming units (CFUs).

Preparation of lung tissue for histology & automated morphometry

Right or left lung from three different animals per time point and group were perfused intratracheally with

absolute ethyl alcohol (J:T: Baker, Mexico City, Mexico). Lungs were dehydrated and embedded in paraffin (Oxford Labware, MO, USA), sectioned and stained with hematoxylin and eosin. The percentages of the lung surfaces affected by pneumonia were determined using an automated image analyzer, and the inflammatory response in three specific areas of the lung was investigated due their importance in pulmonary TB: peribronchial, perivenular and alveolar-capillary interstitium, measuring with automated morphometry as previously reported [18]. Briefly, at least five airways with a diameter of 150–200 microns and the same number of venules 80–100 microns in diameter were selected per lung and the area in square microns occupied by the inflammatory cells was measured using the equipment of automated morphometry Leica Qwin (Milton Keynes, UK); the area in square microns of ten randomly selected alveolar-interstitial spaces per lung were also measured using the same equipment.

Determination of CFU in infected lungs

Right or left lungs from three mice in each time point in two independent experiments were used. Lungs were homogenized with a polytron (Kinematica, Lucerne, Switzerland) in sterile tubes containing 1 ml PBS, Tween-80 at 0.05%. Five dilutions of each homogenate were spread onto duplicate plates containing Bacto Middlebrook 7H10 agar enriched with oleic acid, albumin, catalase and dextrose-enriched medium (Becton Dickinson, MD, USA). The plates were incubated at 37°C with 5% CO_2. The number of colonies was counted 21 days after plating.

Statistics

Results are expressed as the mean ± SEM and mean ± SD. Data were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons using the software Prism 5.02 (GraphPad, CA, USA). $p \leq 0.05$ was considered statistically significant.

Results

Percentage of pneumonia in different lung areas in treated mice

Areas with pneumonia were characterized by abundant intra-alveolar vacuolated macrophages with some multinucleated giant cells and numerous alveolar and interstitial lymphocytes surrounding perivascular and peribronchial areas. Only control infected animals without treatment showed this abnormality (Figure 1A), while control nontreated animals showed scant inflammation (Figure 1B & C), and treated mice exhibited mild inflammatory infiltrate (Figure 1D–F).

In the peribronchial area, all treatments reduced the inflammation in comparison with control mice ($p < 0.001$), among treatments there were not significant differences. Peribronchial inflammation in control infected mice was abundant (mean–SE, 2282.90–251.87 μm^2), while it was lesser in mice treated with HNP-1 (351.50–39.22 μm^2), HBD-2 (157.76–20.26 μm^2) or in combination (HNP-1/HBD-2 [168.52–32.95 μm^2]; Figure 2A). Perivenular inflammation in control mice (342.54–34.60 μm^2), and in those mice treated with HNP-1 (343.42–49.24 μm^2), HBD-2 (438.13–37.62 μm^2) or defensins combination (HNP-1/HBD-2 [192.50–14.3468 μm^2]) did not show difference (Figure 2B). The treatment increased inflammation in the alveolar-capillary interstitium when compared with control mice ($p < 0.001$). Interstitial inflammation in control mice (180.82–24.20 μm^2) was significantly lower than in mice treated with HNP-1 (310.73–21.94 μm^2), HBD-2 (277.41–54.16 μm^2), or in combination (HNP-1/HBD-2 [300.04–8.19 μm^2]; Figure 2C).

Regarding the use of L-isoleucine as prophylactic, results showed that treatment significatively decreased the inflammatory area either with drug-sensitive strain (Figure 3A) or with MDR strain (Figure 3B) in exposed mice.

Lung live bacilli loads in treated mice

MTB-infected mice had a mean of 2.66 $\log_{10}$ of CFU after 90 days of intratracheal infection. According to the calculated interclass correlation coefficient of the $\log_{10}$-transformed CFU, there was a good concordance of these values among mice defensin or

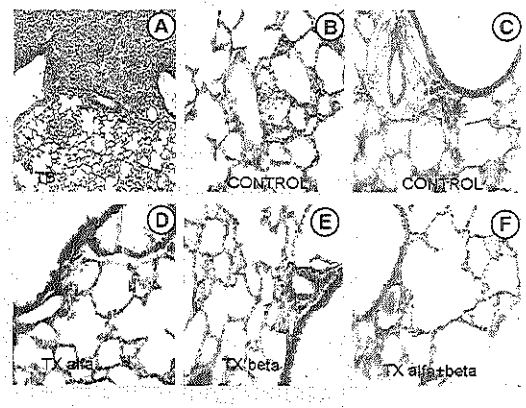

Figure 1. Histopathologic analysis of lungs from recombinant defensin-treated mice. Histology analysis showed characteristic tuberculosis pneumonic areas in infected mice (A), whereas control mock-treated mice show scant infiltration areas in the alveolar capillary interstitium (B), and in the peribronchial and perivascular areas (C). Treated mice showed mild inflammation areas in the alveolar capillary interstitum (D), and in the peribronchial and perivascular areas (E & F).

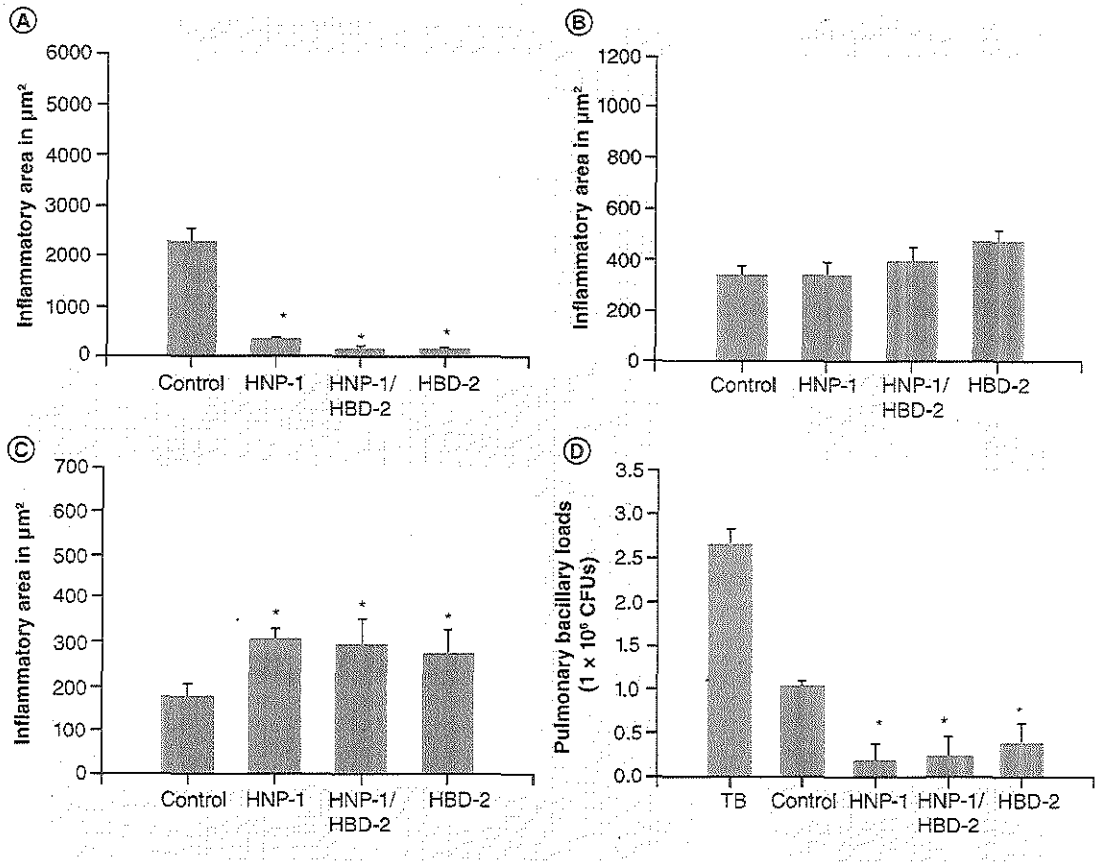

Figure 2. Recombinant defensins decrease inflammation around bronchi and colony forming units. Mice were treated prior to infection with recombinant defensins and after infection three-times a week for a month. Treatment reduced peribronchial inflammation (A), whereas around venules there are not statistical differences when compared with mock-treated mice (B), while a significantly higher inflammation in the alveolar-capillary intertitium was observed in the treated mice (C). A significative reduction in CFU counts was seen in all treated groups when compared with mock-treated mice (control) or infected nontreated mice (D). Data are represented as mean and standard error. n per group = 6.
*$p < 0.05$ when compared with control.
CFU: Colony-forming unit; TB: Tuberculosis.

L-isoleucine-treated in three independent experiments. All defensin-treated mice showed lower CFU counts compared with those of the control group showing a mean of 1.0360 $\log_{10}$ after 30 days living together with MTB-infected mice. Those treated with HNP-1 showed lower CFU (mean, 0.1875 $\log_{10}$) compared with the other groups, followed by the group treated with a combination of HNP-1 and HBD-2 (mean, 0.2375 $\log_{10}$). The group treated with HBD-2 alone, showed higher CFU counts (mean, 0.3800 $\log_{10}$) in comparison with the other treated groups (Figure 2D).

Similar results were shown in the group of prophylactic treatment with L-isoleucine; CFUs from mice infected with drug-sensitive and MDRs strains showed an evident decrease, which was maintained even after 2 months (Figure 3C & D).

Discussion

TB is a very contagious disease; about a third of the world's population is infected with MTB [1,19]. Patients with pulmonary TB are the most important source of infection. The risk of infection is being determined by the infectiousness of the source case and the closeness of contact. Infection occurs by inhalation of droplets which are spread into the air by sputum-positive patients when they cough, sneeze, talk, spit or sing [1]. The closest target for droplets are their household contact, often children [20]. Few strategies are available to avoid infection in TB household contacts; they are limited to the use of BCG vaccine and prophylactic drug administration. Defensins as proposed before [7,11] as therapeutics for lung TB. We sought to determine whether this effect could be achieved in a prophylac-

tic way. We look for HNP-1, since its antimicrobial effects on MTB has been reported both *in vitro* [7] and *in vivo* [11,21,22] and HBD-2 which has been described as an important molecule for pulmonary TB control [8,9,23]. Both defensins were applied intratracheally with a dose 10 µg/mouse each application. This dose has been reported as sufficient to give a therapeutic effect [8,9,23]. One limitation of our study is that antimicrobial peptides or L-isoleucine were administered to the contacts 1 day before the exposition to infected mice, and contacts are likely to be exposed or infected before they are identified for potential treatment. However, it is possible that this kind of treatment could be also efficient in animals already infected after being exposed with tuberculous animals, in a similar way than in mice treated during late progressive disease [24].

Our results show that mice that were living together with MTB-infected mice and were treated with HNP-1 or HBD-2 or HNP-1/HBD-2, showed smaller inflammatory areas around bronchi, while in venules, similar values were observed between treated mice and control group. In contrast, alveolar-capillary interstitium showed higher inflammation when compared with control. This response could be due to higher migration of inflammatory cells via capillaries and venules, considering that antimicrobial peptides are potent chemotactic factors [25]. In general, lungs from mice treated with the different types of defensins showed smaller areas of inflamma-

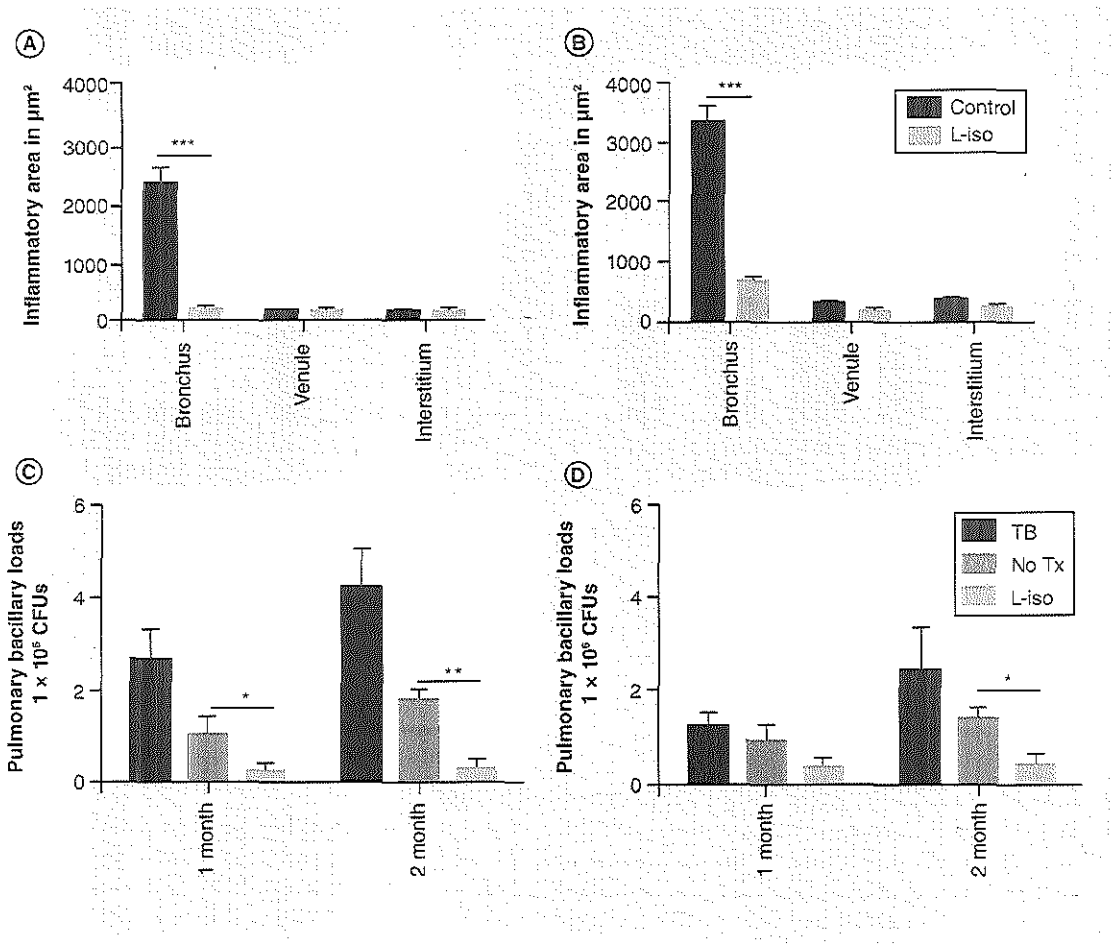

Figure 3. L-Isoleucine reduces inflammatory areas and colony-forming units when used as prophylactic. The prophylactic use of L-isoleucine significatively reduces inflammation in bronchus either in drug-sensitive (A) and multidrug-resistant-infected mice (B). Similar results were observed for CFU counting, treatment with L-isoleucine reduced CFUs after 1 and 2 months of treatment in drug-sensitive strain (C), while contact mice co-housed with multidrug-resistant-infected mice only showed significantly lower bacilli loads after 2 months of treatment (D). Data are represented as mean and standard deviation. n per group = 6.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.
CFU: Colony-forming unit; L-iso: L-isoleucine; TB: Tubeculosis; Tx: Treatment.

tion. For all treatments, CFU counts showed bacterial load reduction in defensin-treated mice compared with control mice. HNP-1 induced the highest lung bacterial load reduction.

It is evident that though the use of recombinant defensins as prophylactic is effective, owing to the high cost of production become into and a nonviable option, mainly in developing countries where TB rates are the highest and income is low. Thus, we decided to use an effective and innocuous defensins inducer such as L-isoleucine. previous studies by our group showed that L-isoleucine induces β-defensins effectively and that this induction promotes MTB elimination in TB mice model [10]. In the present study, we tested L-isoleucine as prophylactic in our transmission model, results showed that the use of this amino acid reduced CFU counts and inflammation in treated mice.

Recently, other antimicrobial peptides such as cathelicidin have shown to have potent effect for MTB killing [26–28]. The cathelicidin effect and the possible synergy with defensins for treatment and prevention of TB need to be elucidated. For further studies, it would be interesting to evaluate installation of L-isoleucine in BCG-vaccinated mice co-treated to prevent MTB.

Conclusion

Altogether our results suggest the potential use of L-isoleucine as prophylactic in household contacts. The efficacy shown in this study and the low cost indicate a suitable approach to avoid pulmonary TB in household contacts, however, further studies are needed to evaluate its use in humans.

Authors' contributions

B Rivas-Santiago performed the experiments, designed the study and wrote the paper. C Rivas-Santiago performed the experiments. E Sada critically reviewed the manuscript and provided financial support. R Hernández-Pando critically reviewed the manuscript, designed the study and provided financial support.

Financial & competing interests disclosure

This work was supported by the Mexican Institute of Social Security (IMSS, FIS//IMSS/PROT/G14/1318), the Mexican National Council of Science and Technology (CONACyT contract 84456) and by the European Community (INCO DC, grant ICA4-CT-2002-10063). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of the manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

- Reporting a household contact experimental model with transmission.
- The use of recombinant antimicrobial peptides decreases colony-forming units from household contact mice but promotes inflammation.
- The use of L-isoleucine as prophylactic in tuberculosis household contact mice reduces colony-forming units and inflammation.
- Prophylactic use of L-isoleucine can be used in reference Mtb strains as with multidrug-resistant Mtb strain.
- The use of L-isoleucine is a plausible unexpensive method to decrease transmission in household contact mice.

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Molecular and Genetic Medicine

Gabriel Del Rio, et al., J Mol Genet Med 2014, 8:3
http://dx.doi.org/10.4172/1747-0862.1000128

Review Article Open Access

Prospective Tuberculosis Treatment: Peptides, Immunity and Autophagy

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Received date: July 29, 2014, **Accepted date:** September 22, 2014, **Published date:** September 30, 2014

Abstract

Tuberculosis (TB) is a world-leading infectious disease caused by *Mycobacterium tuberculosis* (Mtb). The current treatment lasts 6 months and has contributed to the development of multidrug resistant (MDR) strains that nowadays cause almost half a million deaths around the globe. Forty years of research have rendered only 1 new drug to treat the new MDR strains. In the current review we present emerging trends to treat TB particularly focused on natural and synthetic peptides. The ability of some of these peptides to display multifunctional roles in TB treatment, particularly immune system modulation through autophagy and direct antimicrobial activity against Mtb, may present advantages to control the impact of this disease. We review the mechanisms of action relevant in the development of multifunctional peptides that may lead to evaluate new ways to treat TB, a disease that has accompanied human society for centuries

Epidemiology and current treatment of tuberculosis

Tuberculosis (TB) is a chronic infectious disease caused by the bacillus *Mycobacterium tuberculosis*. TB is usually a pulmonary disease but can affect other sites as well (extrapulmonary TB). The disease is spread in the air and in general a relatively small proportion of people infected for the first time with *M. Tuberculosis* will develop active or progressive disease. TB is more common among men than women, and affects mostly adults in the economically productive age groups from developing countries.

TB is a worldwide health problem. Reports by the World Health Organization (WHO) indicate that there were 8.6 million new active cases and 1.3 million deaths during 2012 [1], equivalent to 125 active cases per 100,000 population. Indeed, TB ranks as the second leading cause of death from an infectious agent worldwide, after the human immunodeficiency virus (HIV). Moreover *M. Tuberculosis* is highly infectious: nearly one third of the world's population is latently infected and 10% of this population will develop active disease. An additional significant problem is the association with HIV infection, from 1.3 million deaths caused by TB in 2012, 0.32 million deaths where in HIV positive people [1]. These epidemiological observations hightligths the relevance of the immune system to control TB (see below). Thus, the HIV and TB epidemics create a public health problem of enormous proportions.

TB is an endemic disease in the developing world. The majority of TB active cases worldwide in 2012 were in the South-East Asia (29%), African (27%) and Western Pacific (19%) regions. India and China alone accounted for 26% and 12% of total cases, respectively. A smaller proportion of cases occurred in the Eastern Mediterranean Region (7.7%), the European Region (4.3%) and the Region of the Americas (3%). The TB incidence rate at country level ranges substantially, with around 1000 or more cases per 100 000 people in South Africa and fewer than 10 per 100 000 people in parts of the Americas, several countries in Western Europe, Japan, Australia and New Zealand [1].

Without treatment, TB mortality rates are high. In studies of the natural history of the disease among sputum smear-positive and HIV-negative cases of pulmonary TB, approximately 70% died within 10 years; among culture-positive cases (but smear-negative), 20% died within 10 years. Fortunately there are efficient antibiotics to treat this disease. Treatment for new cases of drug-susceptible TB consists of a 6-month regimen of four first-line drugs: isoniazid, rifampicin, ethambutol and pyrazinamide [2].

The World Health Organization (WHO) declared TB a global public health emergency in 1993. In the mid-1990s, WHO developed the DOTS strategy (direct observed therapy) to improve TB care and control at international level DOTS strategy conforms a short-course of chemotherapy, which comprises an intensive period of two months administering the four primary drugs followed by a period of four months of treatment with isoniazid and rifampicin. Within a decade, almost all countries had adopted this strategy and there was considerable progress; for example, in 2005 the numbers of TB cases reported were over 5 million and treatment success rates reached 85%. However and although TB can be controlled and cured by chemotherapy, treatment usually requires four specific drugs and 6 months of therapy, which produce significant compliance problems. The consequence of this is disease recrudesces and more important the arising of multidrug resistant (MDR) strains (see below).

In the last year MDR strains have increased in frequency afflicting around 450,000 people worldwide and producing 170,000 deaths [1]. In fact, MDR-TB has been identified as a significant problem in every region under the WHO coverage [1]. Treatment of MDR-TB disease is resource intensive and usually requires combination of second line drugs that are more expensive, more toxic, and less effective than drugs used in standard therapy. This problem has accelerated the efforts for new TB drug development and during the last decade has been an intense work in the development and evaluation for regimens to shorten the duration of treatment and reduce the likelihood of the development of resistance [3]. Currently, there are 10 new drugs in

clinical trials and after more than 40 years 1 new anti-TB drug has been approved by the FDA at the end of 2012, this drug is bedaquiline a selective ATP-synthase inhibitor [4].

Regarding protection conferred by vaccination, the Bacille Calmette–Guérin (BCG) vaccine was developed a century ago and is one of the most widely used vaccines globally. Vaccination at birth with BCG is widely applied as part of the Expanded Programme on Immunization of the WHO and billions of people have been vaccinated since 1921 [5]. Except for tuberculous meningitis in children, the capacity of BCG to protect against TB is debated, because randomized clinical trials have provided estimates ranging from 80% to no protection [6]. Several explanations have been suggested for these variations in the protective efficacy of BCG, such as antigenic differences among vaccines, interaction with environmental mycobacteria, nutritional or genetic differences in trial populations and differences in trial methodologies [7–9]. However, there is a lack of compelling evidence in favour of any of these proposed mechanisms. Yet, recent developments in nano-carriers might provide some improvements in the developing of effective vaccination strategies against tuberculosis [10].

Multidrug-Resistant Tuberculosis

The WHO defines MDR to TB caused by strains resistant to at least isoniazid (INH) and rifampicin (RMP), the two most common first line-anti-TB drugs used worldwide.

The mechanisms involved in the development of MDR and extensively drug-resistant (MDR-TB) are complex and determined by the mycobacterium, the host, and iatrogenic factors. Considering that MDR-TB rates are increasing, especially in low-income countries and in high-populated cities, it is important to develop and apply public health programs in TB endemic areas and a comprehensive management structure, including drug management. To increase treatment success of MDR-TB, there are several areas that must be emphasized, for instance, the development of new drugs to reduce the time of treatment and at the same time increase the effectiveness to reduce bacillary loads; furthermore, it is needed the proper follow up of the patients, including monitoring and evaluation [11]. Although in the last decade fluoroquinolones have been used for the treatment of MDR strains, It is likely that *M. Tuberculosis* will develop resistance to this antibiotic, therefore fluoroquinolones and new upcoming drugs must be protected, and their use in the treatment of non-MDR-TB cases must be strongly discouraged and preferably strictly regulated [12,13]. The current rising epidemic of fluoroquinolone-resistant MDR-TB fuelled by careless and indiscriminate use of fluoroquinolone must be used as an experience to both eliminate this practice in current TB treatments and in the controlled administration of novel anti-TB drugs.

In the last few years a new sort of strains has emerged due to the antibiotics evolutive pressure: extensively drug-resistant TB (XDR-TB). XDR-TB strains are defined as any multidrug-resistant strain that is also resistant to any fluoroquinolone and any of the second-line injectable drugs, such as capreomycin, kanamycin, or amikacin. From 2006, when the first report on XDR-TB was published, until the end of 2012, 92 countries had reported the presence of at least one case of XDR-TB. Recently the term totally drug-resistant TB was proposed to define TB cases with a resistance profile beyond XDR-TB, in which the strain would be virtually resistant to all available first- and second-line drugs; however, epidemiological studies are still not abundant on these new class of resitant strains [14]. More recently, it has been shown that resistance to antibiotics may emerge from the natural competition between strains of bacteria that share a niche, such as in the case of *Staphylococcus aureus* [15], highligthing an additional source of pressure for the emergence of natural drug resistance in bacteria.

Alternative treatment for tuberculosis: antimicrobial peptides

Because of the growing and spreading of new MDR-TB strains and its co-evolution with HIV, an urgently need for developing novel compounds and drugs with direct antimicrobial activity and immunomodulatory properties has emerged. Although many proposals have arisen in the last decade, antimicrobial peptides (AMPs) remain to be the best option because of their versatile activity; promoting both direct *M. Tuberculosis* killing through several mechanism and immunomodulation. AMPs are small cationic molecules of a variable length mainly composed by polar-hydrophilic, nonpolar-hydrophobic and positively charged amino acids. This special conformation gives the molecules amphipathic and cationic properties providing them with a partial positive charge; these features are key factors to provide antimicrobial activity [16]. AMPs are broadly distributed in nature. They are an important part of the innate immune response of several living organisms including humans. For instance, it has been observed that either deficiencies or over production of these peptides lead to several infectious and non-infectious diseases which has been reviewed elsewhere [17]. There are more than 40 AMPs in human and two groups (defensin and cathelicidins) are relevant for TB.

The antimicrobial mechanisms of AMPs are conserved among families; when the peptides are at a high concentration, they can insert into the bacterial membrane, causing alterations in the lipid bilayer and making it permeable, hence triggering bacterial death [18,19]. However, this is not the only mechanism of action known for AMPs; it has been shown that members of the buforines and cathelicidins family are able to cross the membrane and, in the cytosol, they can bind to DNA and RNA by electrostatic charges, interfering with vital processes [20]. On the other hand, there are peptides such as mersacidin that inhibits cell wall synthesis by interacting with peptidoglycan precursors [21]. Some other peptides, such as PR 39, HNP 1 and 2, inhibit the synthesis of very important proteins for bacterial viability [18]. Hepcidin, on the other hand, besides damaging the bacterial cell membrane, also decreases the iron levels and down-regulates both protein and mRNA expression of the iron-response element [22] (Figure 1).

It is not clear yet whether AMPs are produced by hosts infected with Mtb in an attempt to eliminate the bacilli during primary infection, but several approaches have been implemented to understand how these innate immunity molecules participate during progressive and latent TB. Although this is not the scope of the present review, it is noteworthy to summarize the studies that catapult the use of AMPs as candidates for TB treatment.

Several antimicrobial peptides from different species have been tested for their activity against *M. Tuberculosis* and so far human neutrophil defensins, synthetic rabbit defensin, and porcine protegrin had the ability to kill *M. Tuberculosis* including clinical isolates [23]. These in vitro observations were consistent with results obtained in animal models of TB: the AMPs tested (HNP-1 and HNP-3) had potent antimicrobial activity against *M. Tuberculosis* in vivo [24,25]. Although these findings encourage translating the use of these

peptides in humans as therapeutics, researchers found two important limitations on these peptides. First, the massive production of the peptides was very costly and second, there is not enough knowledge to determine any secondary effect derived from the physiopathological role of AMPs during TB. Since then, many important studies have emerged answering keystone questions that backup the use of antimicrobial peptides for the treatment of pulmonary TB. For instance, now it is known that defensins are over-produced by lung epithelial cells during *M. Tuberculosis* infection promoting its elimination [26]; this was confirmed through the use of a well-documented TB experimental animal model where susceptible animals that developed TB showed poor expression of defensins whereas resistant mouse strain showed a high and efficient production of defensins [27]. Defensins were the first group described in *M. Tuberculosis* infection and soon after the only cathelicidin in humans, LL-37, was evaluated. This peptide is necessary for *M. Tuberculosis* elimination in infected macrophages [28,29]; importantly, the proper production of LL-37 by macrophages depends on the presence of vitamin D [30]. These observations add to the fact that overexpression of LL-37 eliminates *M. Tuberculosis* during in vitro infection [31]. Now is known that many other molecules besides vitamin D might induce AMPs promoting *M. Tuberculosis* elimination such as L-isoleucine or butyrate and these findings have been reviewed elsewhere [32].

Hence, natural AMPs from humans held the promise to be effective therapeutics to fight TB and other infectious diseases, yet the production of these peptides is cumbersome and expensive. In the last few years several groups worldwide have searched for alternatives to simplify their production; one of these strategies aims to develop synthetic peptides derived from natural AMPs, with the purpose of increasing amphiphacity or by increasing their net positive charge. These modifications have increased the efficiency of these AMPs against bacteria and fungi [33,34]. Recently our group has tested these semisynthetic peptides as promising antimycobacterial compounds in a mouse TB model. Some of these peptides showed good activity to eliminate mycobacteria both in vivo and in vitro [35,36]. Another alternative is the use of antimicrobial peptides produced by bacteria such as the lantibiotics, which are AMPs synthetized by Gram-positive bacteria that are characterized by the presence of post-translationally modified amino acids in their structure, such as lanthionine and methyl lanthionine. The most studied lantibiotic is nisin A. The mechanism of action of this AMP involves the joining of a cell wall precursor to lipid II, allowing pore formation and at the same time inhibiting biosynthesis of the bacterial cell wall. Nisin A and its synthetic derivatives nisin S and nisin T are efficient lantibiotics against *M. Tuberculosis* and non-TB bacteria, and they constitute interesting compounds for clinical studies [37].

AMPs are known mainly because of their antimicrobial activity, however AMPs are not limited to this function. In fact, several authors claim that instead of antimicrobial some of these peptides are immunoregulators, promoting pro-inflamatory and/or anti-inflammatory cytokines, immature dendritic cells maturation through TLR4, chemotaxis [32] and apoptosis (see below). Based on this information some antimicrobial peptides have been modified to increase or decrease immunoregulatory activities, whereas antimicrobial effects remains the same and next we review some recent findings.

Innate Defense Regulator Peptides (IDRs)

IDRs are synthetic immunoregulatory and anti-infective peptides that are based on the sequences of natural human and non-human AMPs [33,34]. These synthetic peptides were designed to selectively modulate the innate immune response to infection, without the potential side effects (mast cell degranulation and enhancement of apoptosis) observed for certain AMPs. In recent studies, it has been demonstrated that the protective activity of IDRs could be solely based on their immunoregulatory properties and that this protection is efficient even in animals infected with MDR strains [35,38]. Besides this immunoregulatory property, the low potential of microbial resistance, lower toxicity and requirement of fewer doses, suggest that IDRs could be used as a treatment and as an adjuvant, as well as for conventional drug-sensitive, but mainly MDR. Several in vitro and in vivo experiments have tested the efficacy of IDRs in experimental TB with pathogenic and MDR strains. In a murine model of progressive pulmonary TB, the intratracheal administration of the IDR peptides E2, E6 (peptides modified from a bovine antimicrobial peptide, bactecin) and CP26 (a synthetic peptide comprising the amphipathic region of cecropin A and the hydrophobic N-terminal of the bee venom peptide melittin), during late disease in mice infected with drug-sensitive *M. Tuberculosis* or MDR strains significantly reduced lung bacillary loads; however, there was no reduction in the inflammatory infiltrate (pneumonia) compared with control non-treated mice [36]. Further experiments showed that the use of others IDRs, such as HH2 or 1018, not only decreased bacillary loads but also pneumonic areas. Conversely, the use of recombinant antimicrobial peptides such as human β-defensin-2 and/or human neutrophil peptide led to an evident reduction in the bacillary loads but a marked pneumonia caused by the non-controlled immune-stimulatory activity of these peptides [35]. Therefore the creation of new synthetic peptides, which modulate specifically immune function, represents a new venue to explore in the treatment of TB.

In this sense, understanding the mechanism to induce regulation of the immune system to fight TB may be relevant to target the action of new compounds. Recent reports have shown that autophagy may be relevant in fighting bacterial infections, particularly TB. The relation between autophagy and immune systems has been reviewed elsewhere [39-41] and in the next section we will review the role of peptidic and non-peptidic compounds in autophagy and their association in TB.

Autophagy: a new mechanism to treat tuberculosis

Autophagy is a highly conserved process occurring inside cells where cytoplasmic constituents including long-lived proteins, protein aggregates, organelles and invading pathogens are sequestered within double-membrane bound compartments that are delivered to the lysosomes for degradation and the products are recycled [42].

Autophagy is important for the innate immunity and pathogen clearance since bacteria and viruses are vulnerable to degradation by this process [43]. Yet, some pathogens have developed strategies to evade autophagy. For instance, Mycobacterium infects permissive macrophages while evading microbicidal ones; this is accomplished by using cell-surface-lipids to hide underlying pathogen-associated molecular patterns and at the same time related phenolic glycolipids induce the recruitment of permissive macrophages [44]. The death is avoided by preventing the normal maturation of the autophagosome into a degradative and microbicidal compartment, and transforming it into a compartment that resembles an early endosome [44,45].

Particularly, *M. Tuberculosis* remains intact in the autophagosome of macrophages by interfering with autophagolysosome biogenesis [46], which involves the inhibition of the fusion between the autophagosome and the lysosome mediated in part by mycobacterial lipids that mimic mammalian phosphatidylinositols and inhibit phosphatidylinositol 3-phosphate (PI3P)-dependent membrane trafficking mechanisms. This blockage can be overcome by the activation of cellular autophagy by different ways, including starvation, drugs, microRNA and peptides [47].

Non-peptidic inducers of autophagy

Starvation

Gutierrez and collaborators demonstrated that stimulation of autophagic pathways by starvation in macrophages causes the maturation of the mycobacterial autophagosomes into autophagolysosomes inducing their acidification, overcoming the trafficking block imposed by *M. Tuberculosis* and culminating in bacterial death [48]. Similar lysosomal mediated killing has also been reported for Streptococcus, Shigella, Legionella, and Salmonella [49-52]; note that in these cases, autophagy may be induced by the bacteria itself.

Vitamin D

It is known that the active form of vitamin D (1, 25-dihydroxyvitamin D3) activates a direct antimicrobial pathway in human macrophages inducing autophagy [53]. This autophagic pathway involves the generation of the peptides cathelicidin and defensin B4, which exert direct antimicrobial activity against *M. Tuberculosis* [29,54,55].

miR-155

The microRNA miR-155 accelerates the autophagic response in macrophages, thus promoting the maturation of mycobacterial phagosomes and reducing the number of intracellular bacteria [56].

Statins

These molecules are cholesterol-lowering drugs but they also can modify immunologic responses. The use of statins in murine TB infection studies showed an increased host protection, with reduced lung burdens and improved histopathologic features. These results have been explained considering that statins might counteract *M. tuberculosis*-induced inhibition of autophagosomal maturation and promote host-induced autophagy, increasing the host protection against TB [57].

ATP

Stimulation of human macrophages with ATP promotes the acidification of Mycobacterium-containing autophagosomes and subsequent killing of *M. tuberculosis*. The acidification of autophagosomes is mediated by ATP stimulation of P2X7, a plasma membrane receptor for extracellular ATP, which is upregulated on mature macrophages [58].

Rapamycin

This drug has been used to induce autophagy and enhance vaccine efficacy against TB in a mouse model. Rapamicyn-induced autophagy enhanced the presentation of the immunodominant mycobacterial antigen Ag85B by macrophages infected with *M. tuberculosis*. Furthermore, rapamycin increased localization of the mycobacteria within autophagosomes and lysosomes [59].

Peptidic inducers of autophagy

Reports of peptides that induce autophagy and some of them with activity against *M. Tuberculosis* are described next.

Apoptosis inhibitor of macrophages (AIM)

AIM is a scavenger protein secreted by tissue macrophages, which enhances macrophage mycobactericidal activity, upregulates the production of reactive oxygen species, increases mRNA levels of the antimicrobial peptides cathelicidin and defensin 4B and acidifies the mycobacterial autophagosomes, leading to bacterial death [60].

DRAM

Damage-Regulated Autophagy Modulator is a lysosomal protein that is induced during DNA damage by p53; in this context, the expression of DRAM leads to macroautophagy and is required for p53-mediated death [61].

FLIP derivated peptides

DED1, an α-helix ten amino-acid (α2) peptide and DED2, an α-helix twelve amino acid (α4) peptide, are two domains of the protein FLIP (FLICE-like inhibitor) capable of binding FLIP itself and Atg3, effectively suppressing Atg3–FLIP interaction without affecting Atg3-LC3 interaction, resulting in robust mammalian cell death with autophagy [62].

Muramyldipeptide

This peptide acts over the nucleotide-binding oligomerization domain–containing-2 (NOD2) protein in dendritic cells inducing autophagy [63].

Tat–beclin 1

Levine and colleagues designed a peptide (Tat-beclin 1) composed with a region from the protein Beclin 1 which is necessary to induce autophagy. To promote cell permeability of this Beclin 1 peptide, it was linked to the HIV-1 Tat protein via a G2 linker (YGRKKRRQRRRGGTNVFNATFEIW). In vitro experiments showed Tat–beclin 1 induced a 10–50-fold reduction titers in HeLa cells infected with the Sindbis virus (SINV), Chikungunya virus (CHIKV), West Nile virus (WNV) and this was not due to the cytotoxicity of the peptide. HIV-1 replication in human monocyte-derived macrophages was also substantially inhibited; increased autophagosome and autolysosome numbers, as well as enhanced protein degradation, were seen in Tat-Beclin 1-treated HeLa cells. Tat-beclin 1 interacts with a previously unknown negative regulator of autophagy, GAPR-1 (also known as GLIPR2). This confirmed that Tat–beclin 1 is an inducer of autophagy. Finally, Tat-beclin 1 showed antibacterial activity in an in vitro model of *Listeria monocytogenes* infection; yet, the reduction of bacteria counts was reported only for a *L. monocytogenes* strain that

lacked the autophagy evasion protein, ActA, thus the real advantage of such peptide in treating bacterial infections remains to be elucidated [64].

Non-Peptidic inducers of autophagy	
Name	Mechanism of action
Starvation	mTOR is inhibited and ATG13 is dephosphorilated during autophagy.
Vitamin D	Activates an antimicrobial pathway (cathelicidin and defensin B4) in human macrophages that induces autophagy.
miR-155	Promotes the maturation of autophagolysosomes.
Statins	Stop the inhibition of autophagosomal maturation and promote host-induced autophagy.
ATP	Stimulates receptor P2X7 and induces the acidification of autophagosomes to kill bacteria.
Rapamycin	Enhances the presentation of the mycobacterial antigen Ag85B by macrophages and induces autophagy.
Peptidic inducers of autophagy	
Name	Mechanism of action
Apoptosis inhibitor of macrophages (AIM)	Upregulates the production of reactive oxygen species, increases mRNA levels of antimicrobial peptides (cathelicidin and defensin 4B) and acidifies the autophagolysosomes, leading to bacterial death.
Damage-Regulated Autophagy Modulator (DRAM)	p53 target; expression of DRAM induces macroautophagy and is required for the cell death induced by p53.
FLIP-derived peptides	Bind FLIP itself and Atg3, suppressing Atg3–FLIP interaction without affecting Atg3–LC3 interaction, resulting in cell death with autophagy.
Muramyldipeptide	Acts over the nucleotide-binding oligomerization domain–containing-2 (NOD2) protein in dendritic cells inducing autophagy.
Tat–beclin 1	Interacts with a previously unknown negative regulator of autophagy, GAPR-1, to induce autophagy.
Indolicidin, SPFK and 27RP	These peptides induce ionic interactions with lipophosphoglycans on the parasite's surface, inducing dissipation of membrane potential and the balance of intracellular pH. Cells treated with these peptides show signs of autophagy.

Table 1: Inducers of autophagy relevant to TB treatment

Indolicidin, SPFK and 27RP

These antimicrobial peptides were tested against *Leishmania donovani*, exhibiting a 50% antileishmanial activity. Their mechanism of action involves ionic interactions with lipophosphoglycans on the parasite's surface, inducing dissipation of membrane potential and the balance of intracellular pH with extracellular environment. By the use of transmission electron microscopy, extensive intracellular damage including cytoplasm vacuolization and degeneration of cellular organization without disruption of the plasma membrane was observed. Indolicidin and the two peptides derived from Seminalplasmin, SPFK and 27RP, induce cell death in *L. donovani* via a non-apoptotic process by activating the pathway(s) of autophagic cell death [65].

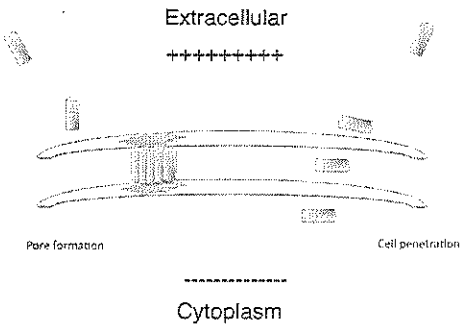

Figure 1: Mechanisms of action on membranes of antibacterial peptides. Biological membranes from both eukaryotes and prokaryotes separate the intracellular from the extracellular spaces: the image represents a biological bilayer membrane by two yellow archs delinated with red lines. A property of these membranes is the presence of an electrochemical potential that it is usually larger for bacterial cells than for eukaryotic cells; the image represents this electrochemical potential by the + and – symbols on both sides of the membrane. Antibacterial peptides are cationic and amphipatic molecules; in the figure antibacterial peptides are represented as cylinders with two colors, red indicates the cationic and blue the hydrophobic charges that in combination with the cationic character renders amphipathicity to these peptides. Antibacterial peptides show some specificity to kill bacterial cells by making pores on their membranes (represented in the image by a blue explosion at the membrane), presumably because these maintain a large chemoelectrical potential at their membrane that is sensed by the cationic peptide. Yet, in membranes with low chemoelectrical potential such as eukaryotic membranes, these peptides may be able to penetrate into them disrupting the membrane. Furthermore, some antibacterial peptides are ligands to the chemokine receptor CCR6 and TLR-4, promoting mobilization and activation of IDC. This multi-functionality may allow antibacterial peptides two kill bacteria and elicit intracellular response on eukaryotic cells, particularly immune system cells.

Mechanisms of action of pro-autophagy and antimicrobial peptides

The use of peptides have some advantages over other molecules decribed above, among others because some peptides besides inducing autophagy also provoke the expression of antibacterial peptides (e.g. AIM), or are antibacterials themselves (e.g. Indolicidin, SPFK and 27RP) which increase their activity in one single molecule. Particularly, small peptides such as indolicidin, SPFK or 27RP are potentially useful therapeutic molecules because they have two activities in a small number of amino acids (13, 12 and 27, resp) overcoming the difficulties of production and displaying polypharmacologic properties.

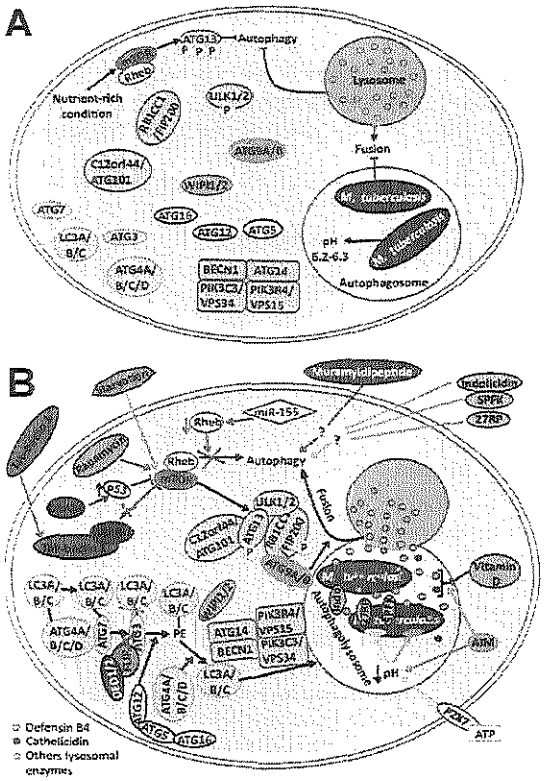

Figure 2: Mechanisms of autophagy inducers. A) In normal cells autophagy is regulated by mTOR. When mTOR is active, phosphorilates and inhibits ATG13 stopping the autophagic process. After having been phagocited by a macrophage, M. Tuberculosis detains the first autophagic stimuli stopping the maturation of the phagosome and preventing its fusion with lysosomes; it also limits the acidification of this vacuole (pH 6.2-6.3), allowing it to reside and multiply inside of the macrophage. B) If a second autophagic stimulus is sensed the autophagic process is restarted and M. Tuberculosis is killed. In the case of starvation or treatment with rapamycin, mTOR is inhibited and ATG13 and ULK1/2 are dephosphorilated starting the autophagic machinery. The microARN: miR-155 diminishes the translation of protein Rheb that together with mTOR inhibit autophagy. Statins induce the incorporation of LC3 proteins to the autophagic membrane improving the fusion with the lysosomes. ATP induces the acidification of phagosomes by the stimulation of the receptor P2X7. Vitamin D and AIM induce the synthesis of the antibacterial peptides: cathelicidin and defensin B4 and AIM acidificates the mycobacterial phagosomes. DRAM induces the expression of p53, which inhibits mTOR. DED1/2 inhibits the protein FLIP that normally prevents the interaction of ATG3 with LC3 for the elongation of the phagosome. Tat-beclin1 inhibits GAPR-1, an activator of mTOR. In the case of muramyldipeptide and the antibacterial peptides indolicidin, SPFK and 27RP it is know that they induce autophagy but the mechanism is unknown.

Beyond natural sources of pro-autophagyc peptides, it is possible to tinker with these to add antibacterial activity to them [66], rendering in this way peptides potentially useful in the treatment of mycobacterial infections. A challenge in these designed peptides is to target specific cells in an organism, avoiding undesired secondary effects. In the case of TB, a possible target would be macrophages. In the case of the peptide Tat-beclin 1 addition of the Tat peptide only improved the penetration of the Beclin peptide into cells in a non-specific way; such design may be targeted by linking the Beclin peptide to a ligand peptide, like Ellerby and collaborators did with their Hunter-Killer peptides [67]. However, it seems that the autophagy-induced by Beclin 1 was not efficient to treat bacterial infections (see above). Thus, it is also important to take into account in these designs the pathway used to induce autophagy (Figure 2).

On the other hand, we have recently pointed out that some cell-penetrating peptides (CPPs) may display direct antimicrobial activity [68] and such peptides may be used to improve the chances of pro-autophagyc peptides such as Tat-Beclin 1, to treat antimicrobial infections. In such case, it is important to consider that some specificty may be lost if the penetrating mechanism is not mediated by receptor-mediated endocytosis or by the emergence of new activities observed when multiple activities are combined into a single peptide [68]. That is, in designing new synthetic peptides useful in the treatment of TB and/or MDR-TB the direct antibacterial mechanism of action of these peptides has to be taken into account as well as the penetrating (Figure 1) and the pro-autophagyc mechanisms (Table 1 and Figure 2). Future research in this direction may provide new tools for the treatment of TB in the developing world.

Acknowledgements

We thank the technical support provided by Dra. Maria Teresa Lara Ortiz. This work was supported in part by Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica Grants IN208014 to G. D. R., Consejo Nacional de Ciencia y Tecnologia (SALUD-2012-01-181008 and 84456) to R. H. P.

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Pulmonary Pharmacology & Therapeutics 32 (2015) 7–14

Contents lists available at ScienceDirect

Pulmonary Pharmacology & Therapeutics

journal homepage: www.elsevier.com/locate/ypupt

Therapeutic efficacy of liposomes containing 4-(5-pentadecyl-1,3,4-oxadiazol-2-yl)pyridine in a murine model of progressive pulmonary tuberculosis

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ARTICLE INFO

Article history:
Received 22 January 2015
Received in revised form
17 March 2015
Accepted 18 March 2015
Available online 2 April 2015

Keywords:
1,3,4-Oxadiazol,4-(5-pentadecyl-1,3,4-oxadiazol-2-yl)pyridine
Lipophilic
Mycobacterium tuberculosis
MDR
Liposomes

ABSTRACT

Background and objectives: Tuberculosis (TB) is one of the deadliest infectious diseases and comprises a global public health concern because co-infection with Human immunodeficiency virus (HIV) and, in particular, the continuous isolation of new Multidrug-resistant strains (MDR), rendering the discovery of novel anti-TB agents a strategic priority. One of the most effective first-line mycobactericidal drugs is Isoniazid (INH). Previously, we reported *in vitro* anti-mycobacterial activity against sensitive and MDR *Mycobacterium tuberculosis* strains of a new oxadiazole obtained from the hybridization of INH and palmitic acid. The present study evaluated the therapeutic potential of liposomes including Phosphatidylcholine (PC) and L-α Phosphatidic acid (PA) or PC and Cholesterol (Chol) containing 4-(5-pentadecyl-1,3,4-oxadiazol-2-yl)pyridine in BALB/c male mice infected by intratracheal (i.t.) route with drug-sensitive or MDR *M. tuberculosis*.

Methods: The lipophilic 4-(5-pentadecyl-1,3,4-oxadiazol-2-yl)pyridine was obtained to mix INH and palmitoyl chloride. The *in vivo* anti-TB effect of this oxadiazole derivative contained in two different liposomes was tested in BALB/c mice infected with a sensitive strain of *M. tuberculosis*, initiating treatment 2 months post-infection, by i.t. route, of 50 μg of oxadiazole derivative for 1 month. In a second stage, mice were infected with an MDR (resistant to first-line drugs) and treated with 150 μg of an oxadiazole derivative carried by PC + Chol liposomes for 2 months. The effect of the oxadiazole derivative *in vivo* was determined by the quantification of lung bacilli loads and histopathology.

Results: In comparison with control animals, drug-sensitive, strain-infected mice treated for 1 month with 50 μg of this oxadiazole derivative contained in the liposomes of PC + Chol showed a significant, 80% decrease of live bacilli in lungs, which correlated with the morphometric observation, and the group of MDR clinical isolate-infected mice treated with 150 μg of the oxadiazole derivative contained in the same type of liposome showed significantly lower lung bacillary loads than control mice, producing 90% of bacilli burden reduction after 2 months of treatment.

Abbreviations: anti-TB, anti-Tuberculosis; CFU, Colony-forming units; Chol, cholesterol; FDA, U.S. Federal Drug Administration; H&E, Hematoxylin and Eosin; HIV, human immunodeficiency virus; INH, isoniazid; MDR, multidrug-resistant; MIC, minimal inhibitory concentration; PA, L-α-Phosphatidic acid; PBS, phosphate-buffered saline solution; PC, L-α-Phosphatidylcholine; Pre-XDR, pre-extensively drug-resistant; SD, standard deviation; TB, tuberculosis; WHO, World Health Organization; XDR, eXtensively drug-resistant.

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http://dx.doi.org/10.1016/j.pupt.2015.03.004

Conclusion: These results confirm and extend the reported highly efficient anti-mycobacterial activity of this lipophilic oxidazole derivative when it is carried by liposomes in mice suffering from late progressive pulmonary TB induced by drug-sensitive, and most prominently by, MDR strains.

1. Introduction

Tuberculosis (TB) is an infectious disease that affects millions of persons each year, ranking in second place after the Human immunodeficiency virus (HIV) infection. Reports by the World Health Organization (WHO) indicate that there were 9.0 million new TB active cases and 1.5 million deaths during 2013 [1]. The epidemiological panorama of TB is aggravated with the emergence of Multidrug-resistant strains (MDR, resistant to at least the first-line drugs Isoniazid (INH) and Rifampin), eXtensively drug-resistant (XDR, resistant to Isoniazid, Rifampin, one fluoroquinolone, and one of three injectable, second-line drugs: Amikacin, Kanamycin, and Capreomycin), and pre-XDR strains (resistant to INH and Rifampin and either a fluoroquinolone or a second-line injectable agent, but not both) [2,3]. In addition, there are several problems with the currently available treatment for TB, such as non-adherence due to its long duration, complexity [4], adverse events [5], and the toxicity profiles of anti-retroviral and anti-TB drugs in patients co-infected with TB and HIV [6]. Thus, there is clearly an urgent need for potential new agents that should reduce the treatment duration, possess an acceptable tolerability profile, and be active against patients with MDR/XDR TB and HIV infection.

In recent years, there has been enhanced activity in the research and development of novel drugs for TB. Several compounds are now under development, while others are being investigated in an effort to discover new molecules for target-based treatment of TB [7–9]. At the time of this writing, there are currently at least 21 drugs at different stages of preclinical or clinical evaluation [10–13]. Moreover, after >40 years without a new anti-TB drug, the mycobacterial selective (ATP)-synthase inhibitor Bedaquiline was approved by the U.S. Federal Drug Administration (FDA) at the end of 2012 [14], and several new targets are being identified and validated for their practical usefulness [15–19].

One of the most effective first-line mycobactericidal drugs is INH (Fig. 1). Several analogues bearing the structure of isonicotinic acid, the central scaffold of INH, have been synthesized and tested as anti-mycobacterials [20]. The conversion has been reported of INH to 1,3,4-oxadiazolone derivatives [21]. The compound 1,3,4-oxadiazole (Fig. 1) is a heterocyclic scaffold containing one oxygen atom and two nitrogen atoms in a five-member ring [22,23]. Compounds containing the 1,3,4-oxadiazole core have a broad pharmacological activity spectrum including anti-diabetic [24], anti-hypertensive [25], anti-inflammatory [26], analgesic [27], anti-convulsant [28], anti-cancer [29], anti-bacterial [30], anti-fungal [31], and anti-viral properties [32]. In addition, 1,3,4-oxadiazole has become an important construction motif for the development of new drugs, such as Raltegravir®, an anti-retroviral, and Zibotenatan®, an anti-cancer drug [22]. In previous studies, we designed diverse 4-(5-substituted-1,3,4-oxadiazol-2-yl)pyridine using INH as central scaffold and several short- and long-chain fatty acids. Diverse compounds were synthesized and tested *in vitro*, determining their anti-mycobacterial activity in several sensitive and MDR *Mycobacterium tuberculosis* strains, as well as their cytotoxicity against the Vero cell line, and primary cultures of human peripheral blood mononuclear cells. Our results showed that the high-lipophilic derivative obtained from the hybridization' of INH and palmitic acid exhibited highest *in vitro* selective antibiotic bioactivity in the low micromolar range [33]. The influence of lipophilic substituents on anti-TB activity coincides with the results reported for different chemical entities, such as 1,5-diphenylpyrrole and 1,4-dihydropyridines derivatives against MDR [34–36], and pentacyclo-undecane-derived cyclic tetra-amines on XDR strains of *M. tuberculosis* [37].

The present study had the aim of evaluating the therapeutic potential of liposomes containing 4-(5-pentadecyl-1,3,4-oxadiazol-2-yl)pyridine (Fig. 1) in a murine model of progressive pulmonary TB produced by drug-sensitive and MDR-TB strains. Due to the highly lipophilic activity of this compound, it exhibited very low solubility and it was necessary to deliver it in liposomes.

2. Materials and methods

2.1. Chemical material

The lipophilic derivative of 1,3,4-oxadiazole was obtained as described previously [33]. Briefly, the mixture of INH (0.0036 mol) and the 1.1 equiv of palmitoyl chloride in 10 mL of dimethylformamide was heated to reflux for 3–4.5 h. Thin layer chromatography was used to monitor the reaction. After cooling, the mixture was neutralized with saturated $NaHCO_3$ solution and the precipitate formed was filtered by suction. The crude product was purified by recrystallization from adequate solvent and the compound 4-(5-pentadecyl-1,3,4-oxadiazol-2-yl)pyridine was identified by spectroscopic (^1H– and ^{13}C Nuclear Magnetic Resonance) and spectrometric data (Mass Spectrometry) [33]. All reagents and laboratory materials were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Liposome preparation

The phospholipids L-α-Phosphatidylcholine (PC) and L-α-Phosphatidic acid (PA), both from egg yolk and Cholesterol (Chol) (all

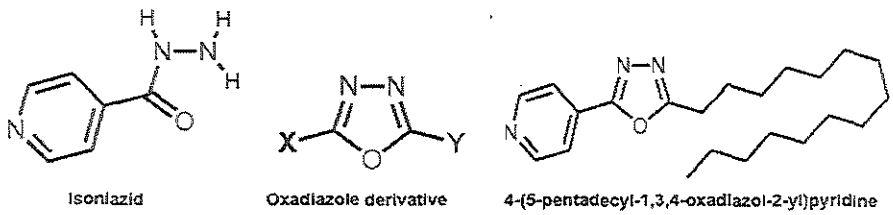

Fig. 1. Chemical structures of Isoniazid (INH), 1,3,4-oxadiazole and 4-(5-pentadecyl-1,3,4-oxadiazol-2-yl)pyridine.

from Sigma Aldrich, St. Louis, MO, USA), were used to prepare two different types of liposomes with the following formulations: PC/PA/4-(5-pentadecyl-1,3,4-oxadiazol-2-yl)pyridine and PC/Chol/4-(5-pentadecyl-1,3,4-oxadiazol-2-yl)pyridine, both at a 0.3:0.7:1 molar ratio. Each molar ratio was the optimal one for the formation of the liposomes. To prepare the liposomes, 1.4 μmol of the phospholipid mixture plus 1.4 μmol of the oxadiazole compound were dissolved in 1 mL diethyl ether; we then added 330 μL of sterile PBS buffer (1 mM NaH_2PO_4/Na_2HPO_4, 1 mM NaCl, pH 7) mixed in a Vortex and sonicated three times (5-s sonication followed by a 30-s resting period), in a Lab Supply G112SPI sonicator (Laboratory Supplies, Hicksville, NY, USA). Then, the diethyl ether was removed under a stream of oxygen-free dry nitrogen at reduced pressure, employing a rotary evaporator at 37 °C [38]. The volume of the liposome suspension was completed to 1 mL with sterile PBS buffer and the liposomes were filtered through MF-Millipore (Millipore, Billerica, MA, USA) membranes with 0.45μm pores to homogenize their size; these liposomes were utilized during 1 week. The phospholipids were at least 97% pure and were negative for lipopolysaccharide contamination, as assessed by the LAL Gel Clot method (Charles River Endosafe, Charleston, SC, USA).

Either the PC/PA or the PC/Chol liposome was centrifuged at 350,000 × *g* for 30 min in the Optima TM MAX/XP Ultracentrifuge (Beckman Coulter, Brea, Cal, USA), and the supernatants were analysed by Mass spectrometry in a Jeol JMS-SX102A spectrometer in order to quantify the amount of the oxadiazole compound outside of the liposomes.

2.3. *Mycobacterium tuberculosis strains*

Evaluation of *in vivo* anti-TB activity was carried out using two strains of *M. tuberculosis*, H37Rv ATTC 27294, susceptible to all five first-line anti-TB drugs (Streptomycin, INH, Rifampin, Ethambutol, and Pyrazinamide), and a clinical isolate that is resistant to all of these drugs, CIBIN 99 (MDR), which was isolated, identified, and characterized in the Mycobacteriology Laboratory of the Centro de Investigación Biomédica del Noreste (CIBIN), Instituto Mexicano del Seguro Social (IMSS) in Monterrey, Nuevo León, Mexico [39].

2.4. *Experimental model of progressive pulmonary tuberculosis in BALB/c mice*

The experimental model of progressive pulmonary TB has been described in detail. Briefly, *M. tuberculosis* strains H37Rv and MDR (CIBIN 99) were grown in 7H9 medium and harvested when they raised the log phase, adjusted to 2.5×10^5 cells in 100 μL of PBS, aliquoted, and maintained at −70 °C until their use. Before use, bacteria were recounted and viability was checked [40]. Pathogen-free male BALB/c mice, 6–8 weeks of age, were anesthetized (Sevoflurane; Abbott Laboratories, IL, USA) and infected by endotracheal (i.t.) cannulation, administering 2.5×10^5 viable bacteria suspended in 100 μL of PBS. Infected mice were maintained in groups of five in cages fitted with microisolators connected to negative pressure. All procedures were performed in a biological security cabinet at a Biosafety level III facility. All the animal work was carried out according to the guidelines and approval of the Ethical Committee for Experimentation in Animals of the National Institute of Medical Sciences and Nutrition (INCMNSZ) in Mexico City, permit number CINVA 224.

2.5. *Oxadiazole administration*

To evaluate the therapeutic effect of 4-(5-pentadecyl-1,3,4-oxadiazol-2-yl)pyridine, two different experiments were performed. In the first, animals surviving 60 days after infection with drug-sensitive strain H37Rv were randomly allocated into two treatment groups: *1)* animals treated every other day with 50 μg of this oxadiazole derivative carried by two different types of liposomes (PC/PA or PC/Chol) suspended in 50 μL of PBS and administered via i.t. route under anesthesia with Sevoflurane, and *2)* infected mice that only received empty liposomes under the same procedure as a control group. Groups of six animals were euthanized on day 30 after treatment.

In the second experiment and considering the results of the first experiment, mice surviving 60 days after infection with the MDR strain were randomly allocated into two experimental groups. The first group was treated with 150 μg of 4-(5-pentadecyl-1,3,4-oxadiazol-2-yl)pyridine carried by liposomes constituted by PC + Chol, administered every other day by i.t. cannula, and the control group, which was solely treated with empty liposomes. Mice were sacrificed by exsanguination under terminal anesthesia after 30 and 60 days of treatment. To determine the effect of treatments, we quantified lung bacillary loads by Colony-forming units (CFU) and the extension of tissue damage by histopathology/automated morphometry as described later.

2.6. *Assessment of Colony-forming units (CFU) in infected lungs and preparation of tissue for histology and morphometry*

Immediately after the animals were euthanized by exsanguination under anesthesia with intraperitoneal (i.p.) Pentobarbital, the lungs were removed; the right lung was immediately frozen by immersion in liquid nitrogen and employed for CFU, while the left lung was perfused with 10% formaldehyde and utilized for histopathology analysis. For CFU determination, frozen lungs were disrupted in a Polytron homogenizer (Kinematica; Lucerne, Switzerland) in sterile 50mL tubes containing 3 mL of isotonic saline solution. Four dilutions of each homogenate were spread onto duplicate plates containing Bacto Middlebrook 7H10 agar (Difco BD, Sparks, MD, USA) enriched with OADC (Difco). The incubation time of the plates was 21 days, and data points are the means of six animals.

For the histological study, after 2 days of fixation, parasaggital sections were taken through the hilum, and these were dehydrated and embedded in paraffin, sectioned at 5 μM, and stained with Hematoxylin and Eosin (H&E). The percentage of lung affected by pneumonia was measured using a Leica Q-win Image Analysis System (Cambridge, UK). Measurements were performed in a blinded manner, and data are expressed as the mean of four animals ± Standard deviation (SD); two independent experiments were performed.

2.7. *Statistical analysis*

One-way Analysis of variance (ANOVA) and Student *t* test were used to compare CFU and morphometry determinations in infected mice treated with the oxadiazole-derivative compound and control animals that received empty liposomes. A difference of $p < 0.05$ was considered significant.

3. Results

3.1. *Effect of oxadiazole derivative in vivo on lung mycobacterial load and histopathological analysis*

Liposomes containing PC/PA or PC/Chol loaded with the oxadiazole derivative at a 0.3:0.7:1 molar ratio was <450 nm wide in size. The drug loading efficiency of either liposome was between 80 and 90%, which is in relation to its high lipophilicity.

In comparison with control mice, animals infected with the drug-sensitive H37Rv strain treated every other day with i.t. doses (50 μg) of the oxidazole derivative as the single drug demonstrated a significant 80% decrease of live bacilli in the lungs after 1 month of treatment, the therapeutic effect similar with either of the two types of liposomes (Fig. 2). The following experiments were carried out using either liposome formulation. The result of bacillary loads correlated well with the morphometric observations, showing a significant decrease of the lung area affected by pneumonia after 30 days of treatment with the oxidazole derivative when compared with those of the control group (Fig. 3).

Due to the emergence of MDR strains and given the good therapeutic effect of our oxidazole derivative in mice infected with the drug-sensitive H37Rv strain, we studied this compound in mice infected with a clinical isolate resistant to all of the first-line anti-TB drugs during late-active disease, tripling the dose because this strain is resistant to INH and this compound is a derivative of this drug. In comparison with control animals, MDR (CIBIN 99) clinical isolate-infected mice treated with 150 μg of this oxidazole derivative showed significantly lower lung bacillary loads than control mice, producing 90% lower bacilli burdens after 2 months of treatment (Fig. 4). These treated animals exhibited lesser but not significant tissue damage than control mice (Fig. 5).

4. Discussion

INH comprises one of the most efficient primary anti-mycobacterial drugs; it acts as a bactericidal agent with an MIC (Minimal inhibitory concentration) of 0.01–0.2 μg/mL for fast replicating mycobacteria [41]. INH is also bacteriostatic to slow-growing or non-dividing mycobacteria; thus, it is also used to treat latent TB. INH is a prodrug that is activated by the mycobacterial enzyme catalase-peroxidase (KatG), which catalyzes the production of the isonicotinic acyl-nicotinamide adenine dinucleotide complex that binds to the enoyl-acyl carrier protein reductase InhA, which block the natural substrate enoyl-AcpM and fatty acid synthase, preventing the synthesis of mycolic acid, an essential component of the mycobacterial cell wall [42].

More than 3000 INH analogues have been synthesized and tested as anti-mycobacterial agents [43]. In this regard, it has been reported that conversion of INH into oxadiazoles produces the corresponding 5-substituted 3H-1,3,4-oxadiazol-2-thione and 3H-1,3,4,-oxadiazol-2-one and their 3-alkyl or aryl derivatives; these compounds possess high activity against *M. tuberculosis* strain H37Rv [44,21]. We synthesized some 4-(5-substituted-1,3,4-oxadiazol-2-yl)pyridine derivatives and tested their activity *in vitro* on two first-line drug-sensitive and on three MDR clinical isolates and the H37Rv strain [33]. Compound 4-(5-pentadecyl-1,3,4-oxadiazol-2-yl)pyridine exhibited similar *in vitro* activity to that of INH against *M. tuberculosis* reference strain H37Rv (0.33 and 0.44 μM, respectively), and more activity against sensitive and MDR clinical isolates, being ten-fold more active than INH [33]. Thus, it appears that this oxadiazole derivative is not a prodrug because INH derivatives cannot be expected to overcome INH-resistance, due to that the molecular action mechanism is identical.

Anti-mycobacterial drugs are partially effective because of the impermeable nature of the *Mycobacterium* cell wall [45]. One important point that could be related with their high efficiency and specificity is that 4-(5-pentadecyl-1,3,4-oxadiazol-2-yl)pyridine is highly lipophilic. This property could facilitate their entrance through the lipid-enriched mycobacterial cell wall but, on the other hand, this lipophilicity was associated with high insolubility, which was a significant inconvenience for its administration by the conventional oral route. In order to overcome this problem, liposomes were used as carriers, employing the aerial route for their administration in tuberculous BALB/c mice infected with a high dose of drug-sensitive H37Rv or MDR strains. Our experimental model of progressive pulmonary TB is highly suitable for exploring the efficiency of novel drugs or immunotherapy, because it is based on the airway route of infection, most common way that disease reaches

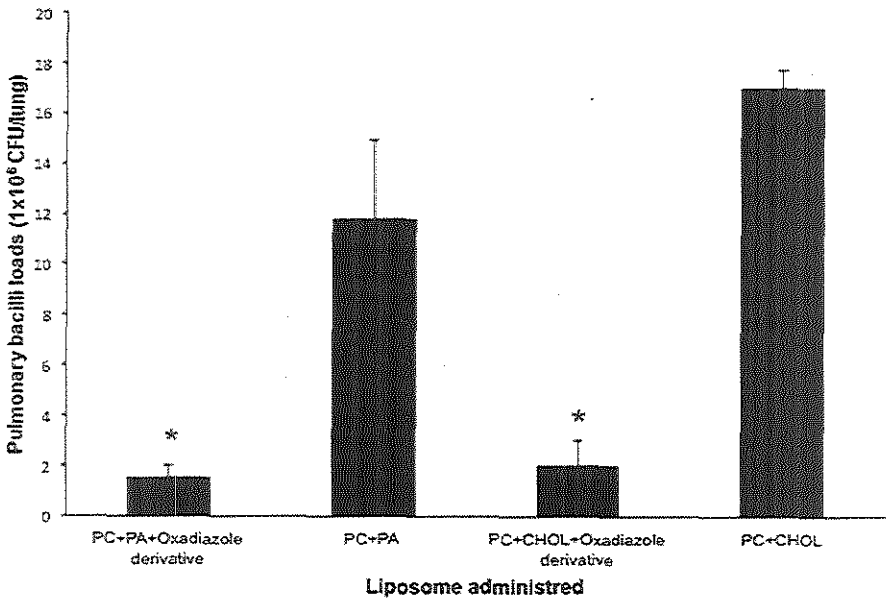

Fig. 2. Effect of 4-[5-pentadecyl-1,3,4-oxadiazol-2-yl]pyridine on the bacterial loads in the lungs from mice infected with the *Mycobacterium tuberculosis* H37Rv strain. This oxadiazole derivative compound was incorporated into liposomes constituted by L-α-Phosphatidylcholine (PC) and L-α-Phosphatidic acid (PA) or liposomes constituted by PC and Cholesterol (Chol), starting the treatment at 60 days after infection and administering, every other day and during 1 month, 50 μg by intratracheal (i.t.) route. There is a decrease of pulmonary bacterial loads when compared with control mice that received empty liposomes. Each bar corresponds to the mean ± Standard deviation (SD) of four mice per group. Asterisks represent statistical significance ($^*P < 0.05$).

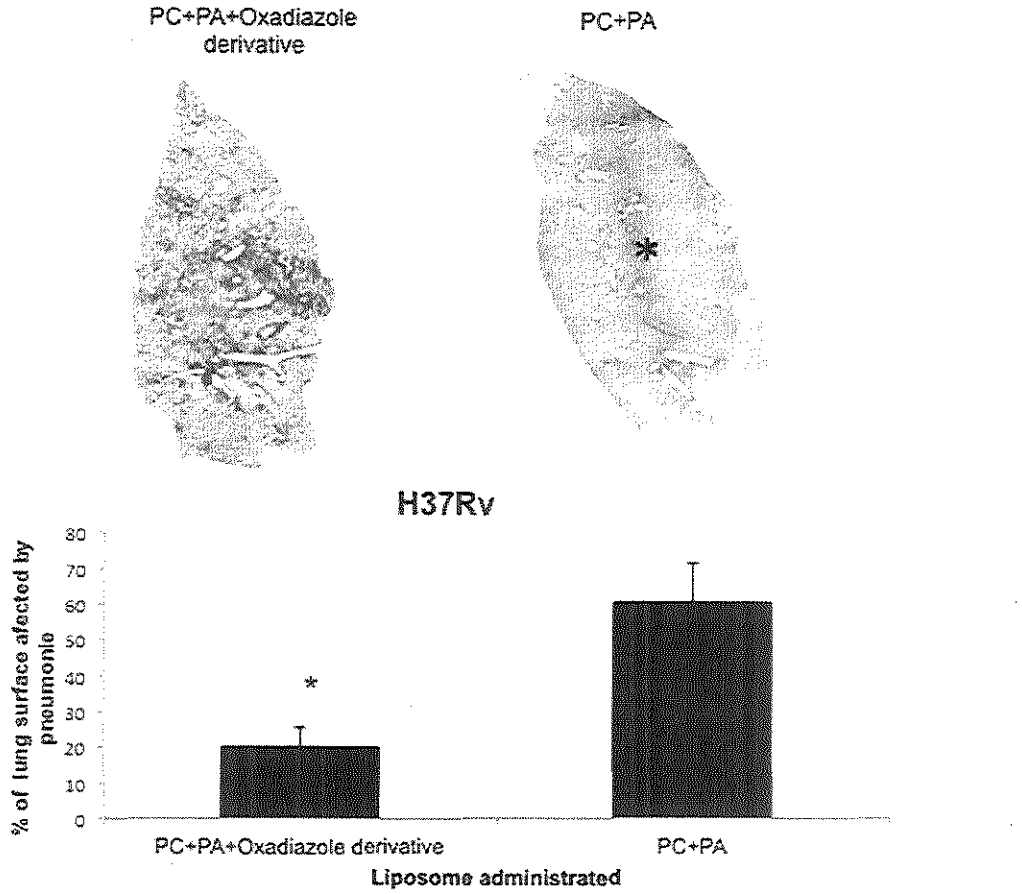

Fig. 3. Representative histology and morphometry of the lungs after 1 month of treatment with 4-[5-pentadecyl-1,3,4-oxadiazol-2-yl]pyridine. BALB/c mice were infected with *Mycobacterium tuberculosis* H37Rv strain by intratracheal (i.t.) route and, after 2 months, the oxadiazole derivative incorporated into liposomes constituted of L-α-Phosphatidylcholine (PC) and Phosphatidic acid (PA) were administered every other day and during 1 month. The upper right panel depicts the lung of control mouse that received empty liposomes (PC + PA) with extensive areas of pneumonia (asterisk), while the upper left panel presents the lung of mouse treated with the oxadiazole compound, exhibiting lesser lung consolidation (Hematoxylin and Eosin [H&E] staining, 25× magnification). Lower panel shows lung area affected by pneumonia determined by automated morphometry, confirming lesser tissue damage in treated animals than in control mice (PC + PA). Each point corresponds to the mean ± Standard deviation (SD) of four mice per group. Asterisks represent statistical significance ($^*P < 0.05$).

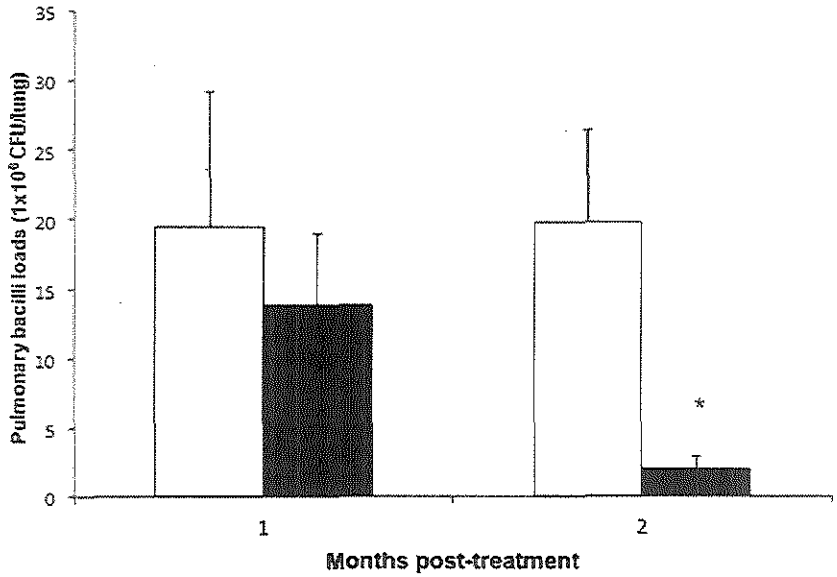

Fig. 4. Effect of 4-[5-pentadecyl-1,3,4-oxadiazol-2-yl]pyridine on bacterial loads in lungs from mice infected with the Multidrug-resistant (MDR) strain. The oxadiazole compound was incorporated into liposomes constituted of L-α-Phosphatidylcholine (PC) and Cholesterol (Chol), starting the treatment at 60 days post-infection and administering 150 μg every other day and during 1 and 2 months by intratracheal (i.t.) route. At both time points, the oxadiazole compound (black bars) decreased pulmonary bacterial loads when compared with control mice that received empty liposomes (white bars), only being significant after 2 months of treatment. Data are presented as the mean ± Standard deviation (SD) of four mice per group. Asterisks represent statistical significance ($^*P < 0.05$).

humans, and the highest rate of bacterial multiplication in the lung correlates with the extent of tissue damage (pneumonia) and death of infected animals [46,47]. We started the treatment 2 months post-infection, because at this time point, infected animals are suffering from ongoing progressive disease with high amounts of live bacilli and lung consolidation. Selection of the appropriate dose was calculated according to the MIC determined *in-vitro* (drug concentration sufficiently efficient to kill 1×10^6 bacilli), adjusting the drug concentration with the estimated number of bacilli in mice lungs after 2 months of infection. Our results showed that in comparison with control animals (treated with empty liposomes), after 1 month of treatment with this oxadiazole derivative, mice infected with H37Rv demonstrated a significant 80% decrease of pulmonary bacilli counts, while animals infected with the MDR strain exhibited, after 2 months of treatment, a significant 90% reduction of bacilli burdens. Thus, 4-(5-pentadecyl-1,3,4-oxadiazol-2-yl)pyridine is highly efficient for treating mice with late, progressive pulmonary TB produced by drug-sensitive or MDR strains. However, due to their insolubility, it was necessary to carry this out by liposomes. These findings also show the high drug-release profile of these liposomes in the cytoplasm of infected cells, an important point, considering that mycobacteria are intracellular facultative organisms. In fact, despite the emergence of new antibiotics, TB treatment remains difficult because the majority of bacilli are localized within phagocytic cells, and the majority of antibiotics, although highly efficient *in vitro*, do not pass through cellular membranes, avoiding the achievement of efficient concentrations within the infected cells [48]. Thus, one important point comprises the design and development of carrier systems for antibiotics that should be efficiently endocytosed by phagocytic cells, and that, once inside the cells, can rapidly eliminate the organisms due to achieving high antibiotic concentration and prolonging its release, permitting the reduction of the number of doses and drug toxicity [49]. In this regard, liposomes are well suited as

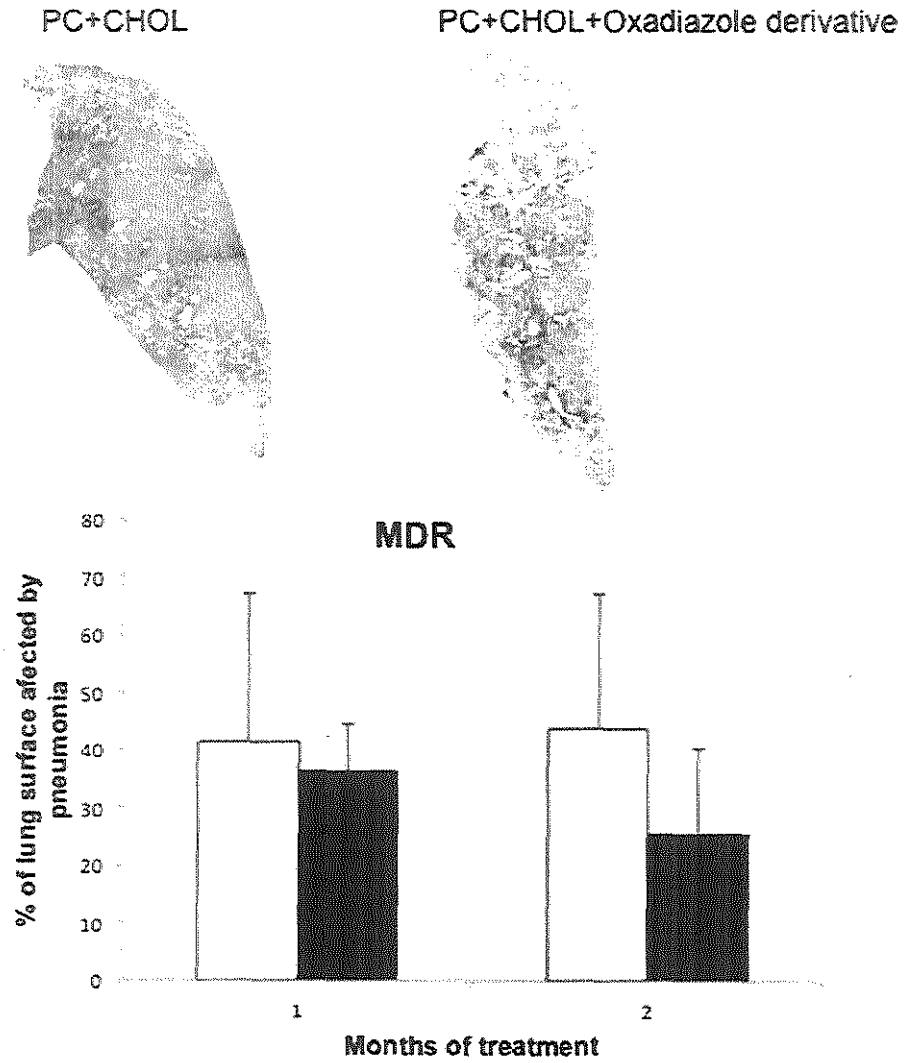

Fig. 5. Representative histology and morphometry of the lungs after 1 and 2 months of treatment with 4-[5-pentadecyl-1,3,4-oxadiazol-2-yl]pyridine in animals infected with the Multidrug-resistant (MDR) strain. BALB/c mice were infected by intratracheal (i.t.) route and, after 2 months, the oxadiazole compound incorporated into liposomes constituted of L-α-Phosphatidylcholine (PC) and Cholesterol (Chol) were administered every other day during 1 and 2 months. Upper left panel shows the lung of control mouse that received, during 2 months, empty liposomes (PC + Chol), exhibiting extensive areas of pneumonia, while the upper right panel depicts the lung of mouse treated during 2 months with the oxadiazole derivative, exhibiting lesser lung consolidation (Hematoxylin & Eosin [H&E] staining, 25× magnification). Lower panel presents the lung area affected by pneumonia determined by automated morphometry, confirming lesser, but not significant, tissue damage in treated animals (black bars) than in control mice (white bars). Each point corresponds to the mean ± Standard deviation (SD) of four mice per group.

vehicles for the delivery of antimicrobial agents because they usually provide sustained drug release effect, minimize the toxicity associated with encapsulated drugs, and increase overall drug efficacy. Moreover, liposomes also protect the drug from premature immunological and enzymatic degradation and could act synergistically with some bactericidal mechanisms of macrophages, such as the production of intracellular reactive oxygen intermediates [49]. Our results clearly illustrate another liposome attribute, which is their use as efficient vehicles of insoluble drugs. Another important point is the drug-administration route, considering that because >80% cases of TB affect the lungs, it is reasonable to propose the aerial drug-administration route, which is very efficient in obtaining high antibiotic concentrations in the lungs and in directly targeting infected alveolar macrophages, maximizing its effect and reducing the number of doses and systemic side effects.

5. Conclusions

Our results confirm and extend the reported highly efficient anti-mycobacterial activity of oxadiazole derivative 4-(5-pentadecyl-1,3,4-oxadiazol-2-yl)pyridine, which showed a significant therapeutic effect when it is carried by liposomes and administered by i.t. route in BALB/c mice suffering from progressive pulmonary TB induced by drug-sensitive or MDR strains.

Conflict of interests

The authors declare that they have no competing interests. All authors read and approved the final paper.

Acknowledgments

This work was supported by Consejo Nacional de Ciencia y Tecnología (CONACyT; contract 84456). GMM-S wishes to thank IMSS for this collaborative work.

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Towards a New Challenge in TB Control: Development of Antibody-Based Protection

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1. Introduction

Throughout history tuberculosis (TB) has been a health problem for humanity. In the beginning of civilization when human population densities were sparse, this disease may have been fairly harmless. However, with the increase in population densities, probably from the 17th to 19th centuries, TB took epidemic proportions [1].

Bacille Calmette Guérin (BCG), the only licensed vaccine against TB, has been shown to be effective in preventing meningeal and miliary TB in children. However, the efficacy of this vaccine in preventing adult pulmonary TB is questionable. Despite widespread vaccination with BCG, nearly 2 million people die each year from TB. Furthermore, the World Health Organization no longer recommends BCG vaccination of children with HIV or HIV-positive mothers due to safety concerns, leaving many infants without any protection against this disease. While drug therapies exist to combat TB infection, the implentation of suitable treatment is often difficult in the countries hardest hit by the disease and a fact complicated by the limited effectiveness of the current therapeutic schemes at treating drug resistant strains of TB [2-4].

Nowadays there is an increasing realization of the need of new animal models to test vaccine efficacy in more realistic scenarios overcoming the limitations of the current models in use. In addition, the elucidation of the significance of humoral defense against intracellular pathogens, in particular against *Mycobacterium tuberculosis*, constitutes an exciting new approach to improve the rational design of new vaccines, therapies and diagnostics.

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2. Reshaping the classical paradigm

In order to develop improved vaccines and new methods for the control of TB, an important element is the discovery of markers to measure the effector mechanisms of the protective immune response against *M. tuberculosis*. For many years Cellular Mediated Immunity (CMI) was attributed as the exclusive defence mechanism against intracellular pathogens. The Th1/Th2 classical paradigm prevailed for a long time and directed the development of vaccines according to this theory [5].

Based on this point of view, only intracellular pathogens could be effectively controlled by granulomatous inflammation induced by a Th1 response whereas a Th2 response induces antibody production that control extracellular pathogens and parasites. However, the question arises of what really constitutes the true demarcation between "extracellular and intracellular"? In the infectious cycle of several intracellular pathogens, they could be found in the extracellular space and *vice versa*. In the specific case of *M. tuberculosis*, it can be localized extracellularly at the beginning of the infection in the upper respiratory tract as well as in advanced stages of the disease after the rupture of granulomatous lesions [6]. In the case of *Erhlichia* spp specific antibodies could mediate protection against [7], possibly by blocking cellular entry or promoting the expression of proinflammatory cytokines [8;9]. It has been demonstrated that this obligate intracellular pathogen has also an extracellular phase that may include replication which could be targeted by specific antibodies [10].

For certain viral pathogens, the induction of Antibody Mediated-Immunity is sufficient to prevent infection, as has been clearly demonstrated by the almost complete eradication of smallpox with the use of vaccines that elicited antibody-mediated immunity [11]. There are several prokaryotic and eukaryotic intracellular pathogens for which antibody have been shown to modify the course of infection by different mechanisms, as reviewed extensively by Casadevall and colleagues [12-14]. Nowadays, it is well established that an efficient combination of both humoral and cellular immune mechanisms could be the best choice to control certain diseases produced by intracellular pathogens [15;16].

In 2005, de Valiere and colleagues reported for the first time that human antimycobacterial antibodies stimulates the Th1 response instead of diminishing it, as was thought previously [17].

3. Protective role of antibodies: Epidemiological evidence

There is accummulated evidence in the last few decades on the influence of antibodies in the development of pulmonary or disseminated TB. Children with low serum IgG against sonicated mycobacterial antigens and LAM, or those who cannot mount antibody responses to these antigens, were predisposed to dissemination of the bacteria [18]. In another report, Kamble and colleagues reported that *M. leprae* reactive salivary IgA antibodies could be quite important in a mucosal protective immunity [19]. In one study carried out on the Mexican Totonaca Indian population, the presence of high antibody titers to Ag85 complex antigens were observed in patients with non-cavitary TB and in patients who were cured with anti-TB chemotherapy. In contrast, patients without such antibodies had a poor outcome of the disease [20].

4. Experimental studies

4.1 Animal models for the evaluation of the role of antibodies in TB infection

One important criterion for the evaluation of the role of specific antibodies in the protection against TB is the use of animal models. Currently, there is no optimal model to reproduce the infection as it occurs in humans [21]. Several animal models have been used to evaluate different aspects. One crucial aspect is the delivery of inoculums, where several routes of inoculation have been employed as intravenous, intraperitoneal, intranasal, aerosol and intratracheal [22]. The geographical location, genetic factors of the host, the presence of environmental mycobacteria and other concomitant infections like helminthiasis, are factors that have to be considered when designing animal experiments [23].

The study of the distribution of monoclonal and polyclonal antibody formulations in different organs and tissues of mice after administration by different routes, including the use of backpack models have been reported [24-26]. Each model has its advantages and drawbacks. For example, the backpack model is very useful for the evaluation of the protective role of IgA, but poses ethical problems in long term experiments due to the increase in tumour size over time produced by the inoculated hybridoma [27].

In prophylactic and therapeutic models, antibody formulations have been administered via the intranasal, intravenous and intraperitoneal routes and combined with cytokines and antibiotics [28,29] before and/or after the infectious challenge.

The administration of *M. tuberculosis* pre-coated with antibodies [30,31] in different models of infection have also contributed to understanding the interactions between host and microbe.

Another approach has been the use of knockout mice models for IgA, polymeric immunoglobulin receptor (pIgR) and B cells, as will be discussed later.

4.2 Experimental studies with antibodies

A great number of studies involving antibodies as inoculum have been conducted as far back as the end of the 19th century. These experiments can be grouped in several categories: serum therapies, mouse polyclonal antibodies, human polyclonal antibodies including commercial human gamma globulins, secretory human IgA (hsIgA) and studies with monoclonal antibodies.

4.2.1 Serum therapies

Serum therapy experiments were conducted from the second half of the 19th century. Immune sera was generated by immunizing animals with different microbial fractions and administered either to animals or humans. The results obtained were either variable, inconclusive or contradictory, due to differences in the methods of serum preparation or its administration, and the lack of appropriate experimental controls [32]. These controversial results led to the perceived minor role of antibodies in the defence against intracellular pathogens.

Why these results were considered "controversial"? Immune serum is a polyclonal preparation that includes antibodies to multiple specificities and isotypes; consequently, polyclonal sera may contain blocking antibodies [33] and antibodies of different functional categories that can affect the outcome of infection. For example, IgG3 murine monoclonal antibodies protects against *Streptococcus pneumoniae* and *M. tuberculosis* but fails to protect against *C. neoformans* [34]. Moreover, results from animal studies are not always reflective of the Ig isotype function in humans. Besides intrinsic factors associated to the antibody structure, other parameters such as the genetic background of the microbe and the immunocompetence of the host could alter the outcome of antibody protection experiments. For some microorganisms (*Legionella pneumophila* and *C. neoformans*), passive antibody therapy efficacy depends on the mouse strain used [35]. In the same way, some microbial strains are more susceptible to the effects of antibodies. The animal model used is another important parameter that varies between different experiments cited in the literature. Timing, the route of infection, the magnitude of the infecting inoculum and the variables to measure efficacy are some of the critical parameters in antibody protection studies [36].

Despite its controversial nature, the results obtained with serum therapy were valuable, demonstrating some beneficial effect of serum on the course of TB in humans, mainly in cases of early or localized TB [37]. Moreover, it was demonstrated that long periods of treatment were necessary to achieve a sustained effect [38].

4.2.2 Polyclonal mouse antibodies

A recent study re-examined the usefulness of immune serum in the context of a therapeutic vaccine against TB [39]. This vaccine, called RUTI, is generated from detoxified *M. tuberculosis* cell fragments that facilitate a balanced T helper response to a wide range of antigens along with intense antibody production. Local accumulation of specific CD8+ T cells and a strong humoral response after immunization are characteristic features of RUTI, features that contribute to its protective properties. In this study, immune serum was generated by immunizing mice with RUTI. Severe Combined Immunodeficiency (SCID) mice were infected with *M. tuberculosis* and treated with chemotherapy for 3-8 weeks. After chemotherapy they were treated for up to 10 weeks with intraperitoneal injections of immune serum. Mice treated with immune serum from RUTI vaccinated animals showed significant decreases in lung CFU as well as reduction in the extent of granulomatous response and abscess formation in comparisson with controls. These results suggest that protective serum antibodies can be elicited by vaccination, and that antibodies may be usefully combined with chemotherapy [29,40].

4.2.3 Human gammaglobulins

4.2.3.1 Human polyclonal antibodies

The first evidence of the stimulatory role upon cellular immunity of specific antibodies in experimental mycobacterial infections was reported by Valiere and colleagues in 2005. In this study, serum samples containing specific antimycobacterial antibodies were obtained from volunteers vaccinated twice with BCG by the intradermal route. Significant titres of IgG antibodies against lipoarabinomannan (LAM) were detected in the volunteers. Moreover, BCG internalization into phagocytic cells was significantly increased in the

presence of BCG induced antibodies as were the inhibitory effects of neutrophils and macrophages on mycobacterial growth. Furthermore, these antibodies induced significant production of IFN-γ by CD4+ and CD8+ T cells [17].

4.2.3.2 IgG formulations

Roy and colleagues demonstrated that the treatment of *M. tuberculosis*-infected mice with a single cycle of human intravenous Ig resulted in substantially reduced bacterial loads in the spleen and lungs when administered either at early or at late stage of infection [41].

The effect of the administration of a commercial preparation of human gammaglobulins in a mouse model of intranasal infection with BCG was evaluated by our group. We demonstrated the passage of specific antibodies to saliva and lung lavage following the intranasal or intraperitoneal administration of human gammaglobulins to mice. This treatment inhibited BCG colonization of the lungs of treated mice. A similar inhibitory effect was observed after infection of mice with gammaglobulin-opsonized BCG [42]. The same formulation was evaluated also in a mouse model of intratracheal infection with *M. tuberculosis*. Animals receiving human gammaglobulins intranasally 2h before intratracheal challenge showed a significant decrease in lung bacilli load compared to non-treated animals. When *M. tuberculosis* was pre-incubated with the gammaglobulin before challenge the same effect was observed. The protective effect of the gammaglobulin formulation was abolished after pre-incubation with *M. tuberculosis* [30]. These results suggest a potential role of specific human antibodies in the defence against mycobacterial infections.

Taken together these studies provide consistent support for the potential use of gammaglobulins and their beneficial immunomodulatory effects in tuberculosis. The results of certain knockout mouse studies and the gammaglobulin experiments indicate that B cells and their products mediate protection against *M. tuberculosis* [43-45]. However, the important question that remains is whether B-cell responses can be augmented to improve immunity against *M. tuberculosis* through immunotherapy or vaccination.

4.2.3.3 Purified human secretory IgA

Human secretory IgA (hsIgA) is the major class of antibody associated with immune protection of the mucosal surfaces [46]. Colostrum volume is above 100 mL in humans during the first three days after delivery [47]. The high percentage of (hsIgA) in human colostrum [48] strongly suggests its important role in passive immune protection against gastrointestinal and respiratory infections [49]. In one study performed by our group, hsIgA from human colostrum was obtained by anion exchange and gel filtration chromatographic methods, using DEAE Sepharose FF and Superose 6 preparative grade, respectively [50]. HsIgA was administered intranasally to BALB/c mice, and the level of this immunoglobulin in several biological fluids was determined by ELISA. The results showed the presence of this antibody in the saliva of animals that received the hsIgA, at all time intervals studied. In tracheobronchial lavage, hsIgA was detected at 2 and 3 hours after inoculation in animals that received the hsIgA [51]. Similar studies were performed by Falero and colleagues with monoclonal antibodies of IgA and IgG class [52]. Following demostration that hsIgA could be detected in several biological secretions after intranasal administration, the protective effect of this formulation against *M. tuberculosis* challenge was evaluated. Mice challenged with *M. tuberculosis* preincubated with hsIgA showed a statistically significant decrease in

the mean number of viable bacteria recovered from the lungs compared to control mice and to the group that received the hsIgA before challenge with *M. tuberculosis*. Moreover, an increased level of iNOS production was also reported (Alvarez et al., mannuscript in preparation). Consistently with this result, a better organization of granulomatous areas with foci of lymphocytes and abundant activated macrophages were observed in the lungs of mice of the group that received *M. tuberculosis* pre-incubated with hsIgA sacrificed at 2 months post-challenge. Untreated animals, however, showed an increased area of bronchiectasis and atelectasis as well as fibrin deposits, accumulation of activated macrophages and lymphocytes. The pneumonic areas were more prominent in the untreated animals than in the groups treated with hsIgA and *M. tuberculosis* pre-incubated with hsIgA (Alvarez et al., mannuscript in preparation)

4.2.4 Studies performed with monoclonal antibodies

Since the first report on the use of the monoclonal antibody Mab 9d8 against *M. tuberculosis*, many similar studies have been reported [53;54]. This IgG3 monoclonal antibody (Mab) generated against arabinomannan (AM) capsular polysaccharide, increased thesurvival of intratracheally infected mice when the *M. tuberculosis* Erdman strain was pre-coated with it. In this study, a longer survival associated with an enhanced granulomatous response in the lungs was found as compared to controls receiving an isotype-specific non-related Mab [31].

Another Mab, SMITB14, directed against the AM portion of LAM prolonged the survival of intravenously infected mice associated with reduced lung CFU and prevention of weight loss. In this study, the authors demonstrated that protection was independent of the antibody Fc portion, because the F(ab')2 fragment also conferred a similar protective effect [55].

In another study, mice receiving the Mab 5c11 (an IgM antibody that recognizes other mycobacterial arabinose-containing carbohydrates in addition to AM) intravenously prior to Mannosylated lipoarabinomannan (ManLAM) administration, showed a significant clearance of ManLAM and redirection of this product to the hepatobiliary system. This study strongly supports an indirect effect of certain antibodies on the course of mycobacterial infection, altering problably the pharmacokinetics of mycobacterial components and contributing to protection against TB [56].

Heparin Binding Hemagglutinin Adhesin (HBHA) is a surface-exposed glycoprotein involved in the mycobacterial binding to epithelial cells and in mycobacterial dissemination [57]. Monoclonal antibodies 3921E4 (IgG2a) and 4057D2 (IgG3) directed against HBHA were used to coat mycobacteria before administration to mice. In this study, spleen CFUs were reduced while lung CFUs did not [58]. These results suggest that binding of these antibodies to HBHA impede mycobacterial dissemination.

The protective efficacy of a monoclonal antibody, TBA61, IgA anti-Acr administered intranasally before and after the intranasal or aerosol challenge with *M. tuberculosis* was demonstrated in a previous work [59]. In another series of experiments carried out by López and colleagues, the protective effect of this Mab administered intratracheally before an intratracheal challenge with virulent mycobacteria was evaluated. At 21 days post-infection, pre-treatment of mice with TBA61 caused a significant decrease in viable bacteria in the lungs compared to control mice or those treated with the Mab against the 38-kDa protein (TBA84). Consistent with the reduction of viable bacteria following treatment with TBA61,

the area of peribronchial inflammation was also statistically smaller in this group compared to the control group [60].

When the lungs of mice were histologically examined, granulomas were better organized in the infected animals that had received TBA61 than in controls or mice treated with TBA84. The reduction of CFU in lungs of the treated group was associated with milder histopathological changes, as indicated by the organization of the granulomas and less pneumonic area. The fact that this Mab promotes granuloma formation in mice infected intratracheally with *M. tuberculosis* strongly suggests the close interaction between antibody-mediated immunity and cell-mediated immunity to induce protection against intracellular pathogens (61). Some of the results obtained in the evaluation of TBA61 monoclonal antibody under different conditions are listed in the Table 1.

MAb, delivery route and inoculation regime	Challenge	Days selected for Organ Harvesting	Parameter measured		References
			CFU reduction	Histopathology	
TBA61 i.n (-3h, +3h, 6h) TBA61 i.n (-3h) TBA61 i.n (+3h) TBA61 i.n (-3h, +3h)	H37Rv i.n, aerosol	9 days	Significant reduction of CFU post-challenge	nd	59
TBA61 i.n + IFN-γ i.n (-3h, -2h, +2h, +7h)	H37Rv i.n, aerosol	9, 21 and 28 days	Significant reduction of CFU post-challenge	Significant reduction of the granulomatous area in the lungs of treated as, compared to untreated mice	28
TBA61 i.t (-3h)	H37Rv i.t	24h, 72h, 21 days	Significant reduction at 21 days post-challenge	Less interstitial and peribronchial inflammation. Well-organized granuloma	60

Table 1. Results from different experimental approaches involving a monoclonal antibody against *M. tuberculosis* 16 kDa protein (TBA61). Note: i.n: intranasal; i.t: intratracheal

The 16 kDa protein (Acr antigen) has been defined as a major membrane protein peripherally associated with the membrane [62] carrying epitopes restricted to tubercle bacilli on the basis of B-cell recognition [63,64]. The Acr antigen is present on the surface of tubercle bacilli and is highly expressed in organisms growing within infected macrophages, allowing it to be potentially targeted by specific antibodies either inside infected cells as well as extracellulary.

A novel immunotherapy, combining treatment with anti-IL-4 antibodies, IgA antibody against 16 kDa protein and IFN-γ, showed the potential for passive immunoprophylaxis against TB. In genetically deficient IL-4-/- BALB/c mice, infection in both lungs and spleen was substantially reduced for up to 8 weeks. Reconstitution of IL-4-/- mice with rIL-4 increased bacterial counts to wild-type levels and making mice refractory to protection by IgA/IFN-γ [65].

More recently, Balu and colleagues reported a novel human IgA1 Mab, constructed using a single-chain variable fragment clone selected from an Ab phage library. The purified Mab monomer revealed high binding affinities for the mycobacterial α-crystallin Ag and for the human FcαRI (CD89) IgA receptor. Intranasal inoculations with the monoclonal antibody and recombinant mouse IFN-γ significantly inhibited pulmonary H37Rv infection in mice transgenic for human CD89 but not in CD89-negative littermate controls, suggesting that binding to CD89 was necessary for the IgA-imparted passive protection. The Mab added to human whole-blood or monocyte cultures inhibited luciferase-tagged H37Rv infection although not for all tested blood donors. Inhibition of the infection by the antibody was synergistic with human rIFN-γ in cultures of purified human monocytes but not in whole-blood cultures. The demonstration of the mandatory role of FcαRI (CD89) for human IgA-mediated protection is important for understanding the mechanisms involved and also for translating this approach towards the development of passive immunotherapy for TB [66].

In all the studies analyzed, it is possible to assert that different mechanisms of action of monoclonal and polyclonal antibodies are involved in the protection against TB. Some of these mechanisms will be discussed later in this chapter.

4.2.5 Studies performed in transgenic mice

Mouse models with deficiency in antibody production can be useful in understanding certain roles of the antibodies in protection against mycobacterial infections. However, knockout mouse studies can lead to premature conclusions regarding the role of a particular component of immunity, if not interpreted carefully. Additionally, experimental conditions can have marked effects on the results.

Rodríguez and colleagues reported that IgA deficient (IgA-/-) mice and wild type non-targeted littermate (IgA+/+) were immunized by intranasal route with the mycobacterium surface antigen PstS-1. These authors showed that IgA-/- mice were more susceptible to BCG infection compared to IgA+/+ mice, as revealed by the higher bacterial loads in the lungs and bronchoalveolar lavage (BAL). More importantly, analysis of the cytokine responses revealed a reduction in the IFN-γ and TNF-α production in the lungs of IgA-/- compared to IgA+/+ mice, suggesting that IgA may play a role in protection against mycobacterial infections in the respiratory tract. Furthermore, these authors demonstrated that immunized pIgR-/- mice were more susceptible to BCG infection than immunized wild-type mice [67].

In the attempt to elucidate whether humoral immunity has a special role in the defence against TB, different experiments with B cell knockout mice were performed by several authors. In 1996, Vordermeier and colleagues developed an infection model of TB in μ chain knockout Ig- mice. Organs from *M. tuberculosis* infected IgG- mice had three to eight fold

elevated counts of viable bacilli compared with those from normal mice. This result suggested that B cells play a role in the containment of murine tuberculous infection [68]. In another study, B cell gene disrupted mice (B cell KO) and controls were infected by aerosol with *M. tuberculosis* to allow the latter group to generate an antibody response in the upper respiratory tract. They were subsequetly given chemotherapy to destroy remaining bacilli and then re-challenged by aerosol exposure. The results of this study, however, revealed no differences in the ability of animals to control this second infection, indicating that, in this low dose pulmonary infection model at least, any local production of antibodies neither impeded nor enhanced the expression of specific acquired resistance [69].

In another series of experiments the role of B cells during early immune responses to infection with a clinical isolate of *M. tuberculosis* (CDC 1551) was evaluated. In this study, despite comparable bacterial loads in the lungs, less severe pulmonary granuloma formation and delayed dissemination of bacteria from lungs to peripheral organs were observed in BKO mice. Additional analysis of lung cell populations revealed greater numbers of lymphocytes, especially CD8+ T cells, macrophages, and neutrophils in wild-type and reconstituted mice than in BKO mice. Thus, less severe lesion formation and delayed dissemination of bacteria found in BKO mice were dependent on B cells, (not antibodies, at least in this study) and were associated with altered cellular infiltrate to the lungs [70].

This latter result differs to the study carried out by Maglione and colleagues in which B cell-/- mice had exacerbated immunopathology corresponding with elevated pulmonary recruitment of neutrophils upon aerosol challenge with *M. tuberculosis* Erdman strain. Infected B cell-/- mice showed increased production of IL-10 in the lungs, whereas IFN-γ, TNF-α, and IL-10R remain unchanged from wild type. B cell-/- mice had enhanced susceptibility to infection when aerogenically challenged with 300 CFU of *M. tuberculosis* corresponding with elevated bacterial burden in the lungs but not in the spleen or liver [43].

Together these studies reveal that B cells may have a greater role in the host defence against *M. tuberculosis* than previously thought.

5. Possible mechanisms of action

Secretions found on mucosal surfaces contain significant levels of Igs, particularly, IgA. This immunoglobin has direct and indirect functional roles to combat infectious agents such as viruses and bacteria that cross the mucosal barrier. Moreover, experimental evidences suggest that the IgA associated with the pIgR may neutralize pathogens and antigens intracellularly during their transport from the basolateral to the apical zone of epithelial cells [71,72]. In addition, as demonstrated previously, IgA may interact with Gal-3 (an intracellular binding β-galactosidase lectin), and interfere with the interaction of mycobacteria with the phagosomal membrane, resulting in the decrease of bacterial survival and replication in the phagosome [73].

As reported by several authors, antibodies may be critical, at least during the extracellular phases of intracellular facultative pathogens. Antibodies may act by interfering with adhesion, neutralizing toxins and activating complement. Moreover, antibodies may be able to penetrate recently infected cells and bind to the internalised pathogen, increasing the antigen processing (74). It is well accepted that antibodies play a crucial role in modulating the immune response

by activating faster secretion of selected cytokines that in turn, contribute to more efficient and rapid Th1 response [74,75], increasing the efficacy of co-stimulatory signals, enhancing Antibody Dependent Cellular Cytotoxicity responses (ADCC) and the homing of immune cells to the lungs after the respiratory infection [13,76-81].

Examples of relevant action mechanisms of antibodies have been discussed by Glatman-Freedman [82].

6. Potential applications

Future applications of antibody formulations for the control of TB may include treatment of patients infected with Multidrug Resistant (MDR) strains, combination with the standard treatment in order to achieve faster therapeutic effects, and administration to recent contacts of TB patients with special attention to risk groups [85].

On the other hand, the induction of specific antibody responses by vaccination in addition to the stimulation of cell mediated immunity could be a novel strategy for the development of new generation prophylactic and therapeutic vaccines against TB.

The prevailing dogma about the uncertain role of antibodies in the protection against TB has somewhat limited the study of B cell immunodominant epitopes which have been mainly related with the development of serodiagnosis assays [86]. Consequently, little information is available on B cell epitopes that could potentially contribute to protection or therapy. With the development of bioinformatics tools for bacterial genome analysis, it has been possible to predict *in silico* microbial regions that trigger immune responses relevant for protection and vaccine development.

Our group is currently developing a candidate experimental vaccine based on proteoliposomes from *M. smegmatis*. In one study, bibliographic search was used to identify highly expressed proteins in active, latent and reactivation phases of TB [87]. The subcellular localization of the selected proteins was defined according to the report on the identification and localization of 1044 *M. tuberculosis* proteins using two-dimensional, capillary high-performance liquid chromatography coupled with mass spectrometry (2DLC/MS) method [88] and using prediction algorithms.

Taking into consideration the cell fractions potentially included in the proteoliposome, from the previously identified proteins, the ones located in the cell membrane and cell wall, as well as those which are secreted and homologous to those of *M. smegmatis* were selected. The regions of the selected proteins containing promiscuous B and T cell epitopes were determined [87]. Thus the *M. smegmatis* proteolipomes were predicted to contain multiple B and T epitopes which are potentially cross reactive with those of *M. tuberculosis*. It is important to note that there could be conformational B epitopes and additional epitopes related with lipids and carbohydrates included in the proteoliposomes that could reinforce the humoral cross reactivity.

Considering the results of the *in silico* analysis, proteoliposomes of *M. smegmatis* were obtained and their immunogenicity was studied in mice [89]. In addition to cellular immune effectors recognizing antigens from *M. tuberculosis*, cross reactive humoral immune responses of several IgG subclasses corresponding with a combined Th1 and Th2 pattern

against antigenic components of *M. tuberculosis* were elicited. These findings were in concordance with the *in silico* predictions [87,89]. It is interesting to note that differences in the pattern of humoral recognition of lipidic components was dependent on the characteristics of the adjuvant used, which could have relevance for the development of vaccines which includes lipidic components [89]. Currently studies are underway to evaluate the protective capacity of *M. smegmatis* proteoliposomes in challenge models with *M. tuberculosis* in mice.

Bioinformatics tools for prediction of T and B epitopes were also employed for the design of multiepitopic constructions, which were used to obtain recombinant BCG strains. Based on this prediction, B cell epitopes from ESAT-6, CFP-10, Ag85B and MTP40 proteins were selected and combined with T cell epitopes of the 85B protein and fused to 8.4 protein [90]. A significant IgG antibody response against specific B cell epitopes of ESAT-6 and CFP-10 was obtained in mice immunized with the recombinant strain. After studying the specific response of spleen cells by lymphoproliferation assay and detection of intracellular cytokines in CD4 + and CD8 + subpopulations, the recognition of T epitopes was also observed. The response showed a Th1 pattern after immunization with this recombinant strain (Mohamud, R, et al. manuscript in preparation). In another series of experiments, recombinant BCG strains expressing several combinations of multiepitopic constructions were used to immunize BALB/c mice subcutaneously and challenged intratracheally with the *M. tuberculosis* H37Rv strain. Recombinant BCG strains expressing T epitopes from 85BAg fused to Mtb8.4 protein and BCG expressing a HSP60 T cell epitope plus different combinations of B cell epitopes from 85BAg, Mce1A, L7/L12, 16 kDa, HBHA, ESAT6, CFP10 and MTP40 and combinations of B cell epitopes alone produced significant reductions in lung CFU compared with BCG (Norazmi MN, manuscript in preparation).

The cumulative works reviewed above related with the use of antibody formulations and vaccines suggest that antibodies if present at the right moment at the site of infection can provide protection against *M. tuberculosis*. This concept opens the way to the development of a new generation of vaccines that elicit specific IgA and/or IgG antibodies able to protect at the port of entry against the infection and directed to bacteria in the infected tissues.

An antibody-based vaccine could be implemented against TB. Such antibodies should recognize the pathogen immediately after its entry into the host, mainly at the mucosal surfaces, where these antibodies must be strategically induced [91]. This vaccine has to induce IgA and IgG antibodies that can inactivate bacterial components essential for the microbial survival in the host, activate complement for direct lysis of the cells, opsonize bacteria to promote their capture by phagocytic cells and induce stimulation of specific cellular immune responses.

Although no serological tests for diagnosis of TB are recommended [92], due to the generation of false results as well as incorrect treatments, for many other pathogens, the availability of serological diagnostic tests has been of great value, in particular in poor countries. In some cases, it constitutes the best protection correlate [93].

In the specific case of TB, several studies of the antibody response have been developed [94]. A number of factors have been described to contribute to the variation of antibody response during the disease. Some of these factors are associated to the pathogen (strain variation,

micro-environment and growth state of bacteria). Not less important are the factors related to the host, mainly the previous exposure to antigen and host genetics [95].

On the other hand, only a small fraction of the genomic regions of *M. tuberculosis* encoding proteins has been explored. Currently, novel immunoassay platforms are being used to dissect the entire proteome of *M. tuberculosis*, including reacting protein microarrays with sera from TB patients and controls [96,97]. These studies could lead to the discovery of new antigens that may constitute a suitable diagnostic marker as well as to the identification of correlates of protection.

The study of the role of specific antibodies in the defense against tuberculosis is opening new possibilities for the future development of new vaccines, diagnostics and therapies against the disease. It is envisaged that new discoveries will arise from the ongoing studies in this area that will expedite the introduction of new strategies in the fight against tuberculosis.

7.Acknowledgements

The authors' work was partly supported by the Ministry of Science, Technology & Innovation, Malaysia [Grant No. 304.PPSK.6150079.N106 & 10-01-05-MEB002] and USM Research University Grant [1001.PPSK.812005] and CONACyT (contract 84456)

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Tuberculosis 95 (2015) 701–706

Contents lists available at ScienceDirect

Tuberculosis

journal homepage: http://intl.elsevierhealth.com/journals/tube

IMMUNOLOGICAL ASPECTS

The implication of pro-inflammatory cytokines in the impaired production of gonadal androgens by patients with pulmonary tuberculosis

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ARTICLE INFO

Article history:
Received 25 March 2015
Received in revised form
12 June 2015
Accepted 21 June 2015

Keywords:
Tuberculosis
Testosterone
Cytokines
Gonadal steroids

SUMMARY

Background: The chronic nature of tuberculosis and the protracted immuno-inflammatory reactions are implied in a series of metabolic and immune-endocrine changes accompanying the disease. We explored components from the hypothalamous-pituitary-gonadal axis and their relationship with cytokines involved in disease immunopathology, in male TB patients.

Methods: Plasma samples from 36 active untreated pulmonary TB male patients were used to determine TNF-α, IFN-γ, TGF-β, IL-6, cortisol, dehydroepiandrosterone, testosterone, progesterone, estradiol, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) by ELISA. Healthy controls corresponded to 21 volunteers without contact with TB patients and similar age (40 ± 16,8 years). Testicular histological samples from necropsies of patients dying from TB were immune-stained for IL-1β, TNF-α, IL-6 and IFN-γ. The TM3 mouse Leydig cell line was incubated with recombinants TNF-α, IFN-γ and TGF-β, supernatants were collected and used to measure testosterone by ELISA.

Results: Patients showed decreased levels of testosterone in presence of high amounts of LH, together with augmented IFN-γ, IL-6 and TGF-β levels. Testicular histological sections showed abundant presence of IL-1β, TNF-α, IL-6 and IFN-γ in interstitial macrophages, Sertoli cells and some spermatogonia. *In vitro* treatment of Leydig cells with these cytokines led to a remarkable reduction of testosterone production.

1. Introduction

One-third of the human population is infected by *M. tuberculosis*, the causative agent of tuberculosis (TB). The development of clinical TB occurs in 5%–10% of them at some point in their lives, for reasons that are not completely understood [1]. As yet, the development of TB seems to depend on a relatively inability of the host to mount an effective response [2]. In individuals wherein the immune response fails to clear the pathogen, a sort of kind of trade-off between the host and microbe takes place, in many cases resulting in a misdirected response which contributes to the development of different levels of tissue damage, as is the case of TB [3,4]. Earlier studies by our group indicate that dysregulated immune responses during human TB translate in an excessive production of pro-inflammatory cytokines which are known to stimulate the endocrine system promoting an unfavorable environment, either for the development of a protective immune response, or the clinical status of patients [5,6]. This bears some relationship with the view that chronic stressful conditions may lead to protracted responses not always beneficial [7], i.e.;

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http://dx.doi.org/10.1016/j.tube.2015.06.002
1472-9792/

endocrine abnormalities involving disturbances of the HP Adrenal (HPA) and Gonadal (HPG) axes, among others.

In this regard, we have shown that patients with newly diagnosed pulmonary TB present augmented systemic concentrations of interferon gamma (IFN-γ), interleukin 10 (IL-10), interleukin 6 (IL-6), and Cortisol, in presence of decreased amounts of adrenal and gonadal androgens [5], as seen in other reports documenting decreased levels of DHEA in serum [8] or urine [9] from TB patients.

As well as modulating each other production, adrenal and sex steroids have important effects on immune cell development and function, mainly because immune cells express diverse hormone receptors [10]. It follows that interactions between the HPG and HPA axes with the immune system are to a great extent involved in the ultimate effects of the anti-infectious response.

In expanding our knowledge into this kind of immune-endocrine communication, the present study was initially addressed to explore the eventual relationship between components mainly from the HPG axis with cytokines involved in the immune and inflammatory response, of male TB patients. One finding to remark was the detection of decreased levels of testosterone in presence of higher amounts of luteinizing hormone (LH), suggesting that testosterone secretion may be modulated by *in situ* influences arisen because of the infectious process, i.e., cytokines. According to this assumption, testicular histological samples from necropsies of patients dying from TB were immune-stained for several pro-inflammatory cytokines which revealed an abundant presence of interleukin 1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), IL-6 and IFN-γ in interstitial macrophages, Sertoli cells and some spermatogonia.

With this evidence in hand, a mouse-derived Leydig cell line was then cultured and exposed to different concentrations of cytokines relevant in TB immunopathology, those were TNF-α, IFN-γ and transforming growth factor beta (TGF-β). It was found that *in vitro* treatment with the three cytokines led to a remarkable reduction of testosterone production.

Taken together, present results point out to a novel and interesting implication of the inflammatory response during tuberculosis in the disturbed production of gonadal steroids.

2. Materials and methods

2.1. Sample population

Thirty six newly diagnosed active pulmonary TB patients were enrolled in this study. All individuals were HIV negative and were untreated at the time of blood collection. All patients were males, and aged 42 (26–55) years (median, 25–75 percentiles), and their sputum was positive for acid-fast bacilli. Disease severity was determined through the X-ray pattern and classified into three categories: mild (a single lobe involved, and without visible cavities, n = 12) moderate (unilateral involvement of 2 or more lobes with cavities, if present, reaching a total diameter no greater than 4 cm; 12 cases) and severe (bilateral disease and multiple cavities, 12 cases). Twenty one volunteers, age-matched [44 (35–55) years] males with no clinical or serological evidence of an associated disease and the antecedent of contact with TB patients (healthy contacts –HCo-) were also included. All individuals gave informed consent for participating in the study and the protocol was approved by the ethical committee at the Medical Sciences School, University of Rosario, Argentina. Exclusion criteria comprised: pathologies affecting the hypothalamus-pituitary-adrenal axis (i.e., tumor, vascular), direct compromise of the adrenal gland, age under 18, or any disorder requiring treatment with corticosteroids, immunosuppressors or immunomodulators.

2.2. Plasma measurements

Blood samples were collected from patients and healthy volunteers at 8:00 am. Plasma was obtained from EDTA-treated blood. Following addition of aprotinin (100 U/ml plasma; Trasylol, Bayer, Germany), samples were preserved at −20 °C. TNF-α, IFN-γ, TGF-β (Pharmingen, Germany), IL-6 (Amersham, UK), cortisol, DHEA, testosterone, progesterone, estradiol, Luteinizing Hormone (LH) and Follicle-Stimulating Hormone (FSH) (DRG Systems, Germany, in all cases) concentrations were determined using commercially available ELISA kits. The detection limits and the Coefficient Variation % (CV) were, respectively: IFN-γ: 4.7 pg/ml; TGF-β: 2 pg/ml, CV: 1; TNF-α: 7.8 pg/ml; IL-6 0.1 pg/ml, cortisol: 2.5 ng/ml, CV: 8.1–5.6; DHEA: 0.1 ng/ml, CV: 3.52–2.64; progesterone 2 ng/ml, CV: 5.4–6.86; testosterone: 0.07 ng/ml, CV: 4.16–3.34; estradiol: 4.6 pg/ml, CV: 6.81–4.13; LH: 0.2 ng/ml (range assay: 0.86–100 mIU/mL), CV: 7.62–4.57; and FSH: 0.4 μg/dl (range assay: 1.27–200 mIU/mL), CV: 7.91–4.18. Results were expressed as the average of two determinations (pg/ml) in an ELISA microplate reader at 450 nm. Cytokines were quantified using reference standard curves generated with human recombinant cytokines. Recent parallel studies by measuring cortisol or DHEAS in plasma (by ELISA with aprotinin) or serum (by electrochemoluminiscence without aprotinin) in the same blood samples, yielded quite similar results (the correlation coefficient was nearly to 1 in both cases).

2.3. Cytokine detection by immunohistochemistry in testicular samples

In order to determine the local cytokine production by immunohistochemistry, paraffin-embedded testicles from three necropsies of patients dying from TB with extensive cavitary bilateral disease and three from non-infective illness as controls (extensive kidney cortical necrosis and two cases of leukemia) were studied. Samples were obtained from files of the Pathology Department at the National Institute of Medical Sciences and Nutrition Salvador Zubirán, México. Tissue samples were obtained during legally authorized autopsies with signed permission by a relative, who agreed to the donation of tissue samples for the present study.

Tissue sections were desparaffinized and maintained in PBS Tween 20, the endogenous peroxidase activity was blocked with peroxidase blocker reagent (BioSB, USA) during 30 min. After blocking with the background sniper (BIOCARE Medical, USA), tissue sections were incubated with primary antibodies overnight at 4 °C at optimal dilutions. The used primary antibodies were to detect: TNF-α (mouse monoclonal antibody; Santa Cruz Biotechnology, USA), IFN-γ (goat polyclonal antibody; Santa Cruz Biotechnology, USA), TGF-β (rabbit polyclonal antibody; Santa Cruz Biotechnology, USA), IL-6 (rat polyclonal IgG; BD, Pharmingen, USA), IL-1β (goat polyclonal antibody; Santa Cruz Biotechnology, USA), and *Mtb* polyclonal antibody against diverse mycobacterial antigens (BIOCARE Medical, USA). Mouse-rabbit immunodetector HRP/DAB (BioSB, USA) detection system and goat on rodent HRP polymer (BIOCARE Medical, USA) were used to develop the reaction. Tissue sections were counterstained with hematoxylin and eosin.

2.4. In vitro production of testosterone by Leydig cells incubated with cytokines

The TM3 cell line, derived from mouse Leydig cells, was purchased from ATCC (ATCC® CRL1714™). Cells were cultured in 1:1 vol of Ham's F12 medium and Dulbecco's modified Eagle's medium, with 2.5 mM L-glutamine, 0.5 mM sodium pyruvate, 1.2 g/L sodium bicarbonate, and 15 mM HEPES (all from Corning Life Technology,

USA), 92.5%; Horse serum, 5%; Fetal bovine serum (both from Thermo Scientific, USA) 2.5%; and 1% of Penicillin-Streptomycin (GYBCO, USA). Cells were maintained at 37 °C in a humidified atmosphere (95%) at 5% CO_2. For assessing testosterone production, TM3 cells were plated in 96-well plates. After 48 h, medium was replaced with medium containing 2.5 UI/ml hCG (Sigma, USA) kindly provided by Dra Lorenza Díaz, Reproductive Biology Department, INCMNSZ, and recombinants TNF-α, IFN-γ and TGF-β kindly provided by Dr F Lopez Casillas form the Cellular Physiology Institute, National University of México; (TNF-α 5 ng/ml, IFN-γ 4000 pM, and TGF-β at 1 ng/ml). Different conditions were settled by triplicate. Leydig cell culture media from each treatment were collected 24 h later and used to assess testosterone by a commercial ELISA kit (DRG systems, USA), according to the manufacturer indications.

2.5. Statistical analysis

Unpaired statistical comparisons were performed by the Mann–Whitney U test or the Kruskall–Wallis followed by post-hoc tests, if applicable. Correlations between hormone and cytokine levels were analyzed by non parametric methods. Statistical significance was inferred for p values < 0.05.

3. Results

Assessment on hormones from the HPG axis showed that TB patients had respectively decreased and increased amounts of testosterone and LH respect to healthy controls (HCo); with a statistically insignificant trend of FSH levels to be a little increased in TB patients (Figure 1, panels a–c). Levels of estradiol and progesterone in TB patients practically overlapped with those seen in HCo, with progesterone showing the lowest amounts in both subject groups (data not shown). In relation to cytokines, there were no gross between-group differences in TNF-α values (data not shown), whereas IFN-γ, IL-6 and TGF-β levels were found augmented in TB, significantly different from HCo (Figure 2, panels a–c). Tested compounds were unrelated to age. Further comparisons within the TB group according to disease severity showed that patients with more progressive forms of TB had a further increase, statistically insignificant, of IFN-γ and IL-6 levels respect cases with mild disease (data not shown).

In line with former findings TB patients continued to showed decreased DHEA levels (3.70 ± 0.57) and augmented cortisol concentrations (186.61 ± 32.48) when compared with values from HCo (DHEA: 6.51 ± 1.56, $p < 0.04$; cortisol: 126.03 ± 10.36, $p < 0.05$).

Pair correlation analysis between hormones and cytokines revealed that levels of TGF-β correlated inversely with DHEA ($r = -0.47$; $p < 0.04$), as did testosterone with either Cortisol ($r = -0.58$; $p < 0.003$) or IFN-γ ($r = -0.32$; $p < 0.035$).

3.1. Histological and immunohistochemistry findings

Tissue sections from all TB autopsy cases showed mild testicular atrophy, manifested by interstitial fibrosis with focal chronic inflammatory infiltrate and detention of spermatogenesis. There was no evidence of local TB infection such as necrosis or granuloma formation. Immunohistochemistry detection of IL-6, IL-1β and TNF-α showed strong positive staining in interstitial macrophages, Sertoli cells and some spermatogonia. Interstitial macrophages showed very strong TNF-α immunostaining, while spermatogonia exhibited intense positivity to IL-6 and Sertoli cells to IL-1β (Figure 3). Some lymphocytes and occasional macrophages from the testicular interstitium showed mild IFN-γ positivity (Figure 3). Occasional macrophages showed immune staining to mycobacterial antigens and non immunoreactivity was seen to TGF-β. Testicle sections from control autopsies showed slight detention of spermatogenesis without infiltration of inflammatory of leukemic cells; all cases were completely negative to all cytokines detection by immunohistochemistry (Figure 3).

3.2. Testosterone production by Leydig cells exposed to different cytokines

In a further experiment, the TM3 cell line, derived from mouse Leydig cells, was cultured in triplicate and exposed to different

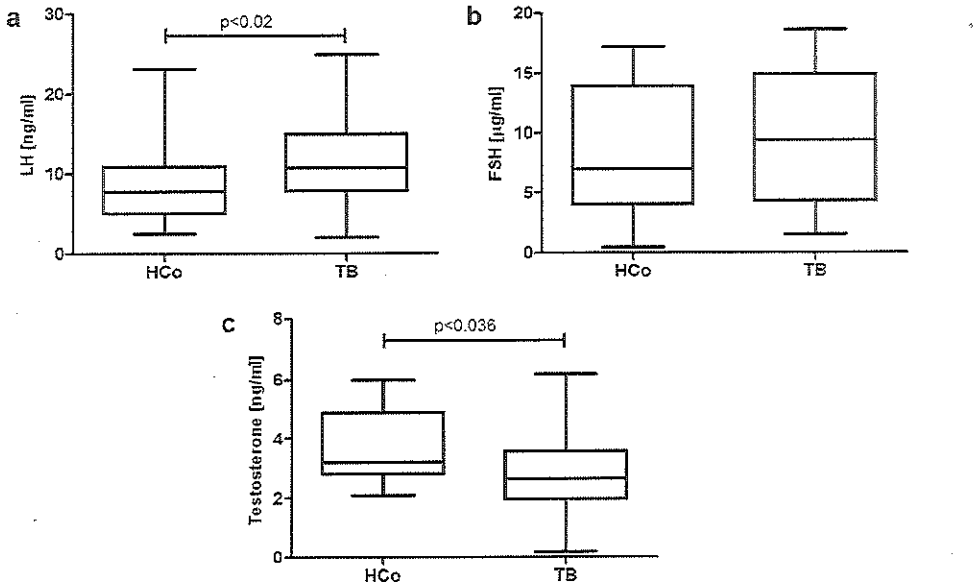

Figure 1. Circulating levels of hormones in patients with active pulmonary TB and healthy controls (HCo). Box plots show 25–75 percentiles of data values in each group with maximum and minimum values. The line represents the median values. Comparisons between groups (TB vs HCo) were performed by non-parametric methods (Mann–Whitney U test).

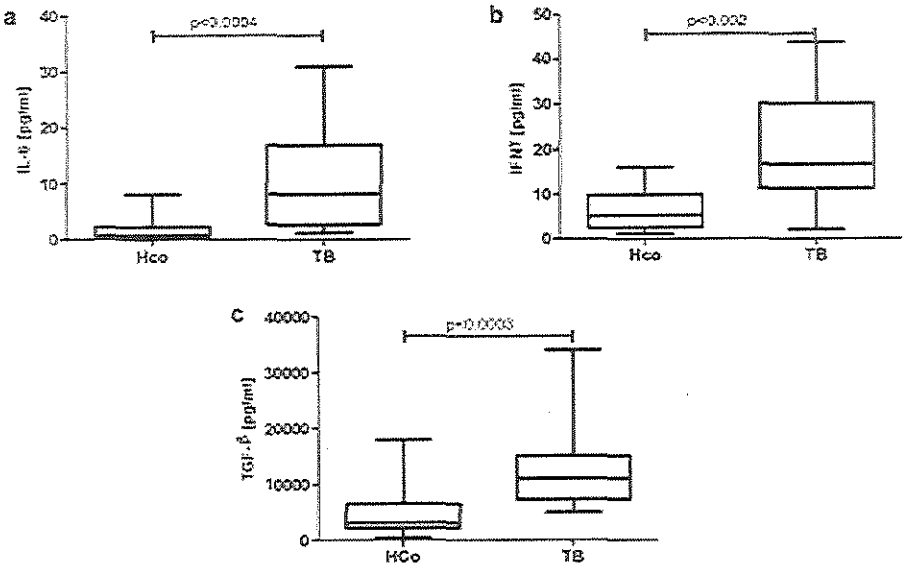

Figure 2. Circulating levels of cytokines in patients with active pulmonary TB and healthy controls. Box plots show 25–75 percentiles of data values in each group with maximum and minimum values. The line represents the median values. Comparisons between groups (TB vs HCo) were performed by non-parametric methods (Mann–Whitney U test).

mouse recombinant cytokines (TNF-α, IFN-γ and TGF-β). Assessment on the levels of testosterone in culture media collected 24 h later revealed that cytokine-treated cultures produced much lesser amounts of testosterone when compared with cultures without cytokines (overall difference, $p < 0.015$, Table 1).

4. Discussion

TB is a disease in which tissue pathology has an immune-mediated component encompassing an excessive and/or protracted cytokine production liable to affect the immune-endocrine communication. In this sense, a variety of factors exogenous to the immune system itself, are likely to play a functional role in regulating the level of immune cell activity by modifying the microenvironment in which the immune cells reside and function. As part of this bi-directional communication, products of the immune response can in turn alter the balance of hormone production and hence affecting different physiological processes.

An interesting finding of present study was the demonstration that active TB patients have decreased levels of testosterone in presence of increased amounts of LH. Production of testosterone by Leydig cells is under the control of LH, which is secreted by the anterior pituitary and reaches the testes via the blood stream. At the gonadal level, LH binds to receptors on the surface of the Leydig cells to stimulate testosterone production by activating an intracellular second messenger system [11]. The present LH/testosterone dissociation implies some degree of testicular resistance to LH and/or suppression of Leydig cells steroidogenesis. Among factors implied in this regard, cytokines were reported to interfere with Leydig cell steroidogenesis and testosterone production [12]. This phenomenon may be linked to infectious stimuli, since LPS-induced inflammation was shown to affect testicular function, including decreased steroidogenesis and impaired spermatogenesis [13,14].

To the best of our knowledge, the present study is the first report documenting the presence of pro-inflammatory cytokines in testes from TB patients. The intimate association between Leydig cells and accessory cells expressing such mediators suggests that they may be functionally linked.

Our findings are in line with experimental studies in testes revealing the presence of pro-inflammatory cytokines like IL-1β and IL-6 from interstitial macrophages [15,16], Leydig cells [17], Sertoli cells [18] and TNF-α from macrophages and spermatocytes [19,20]. Importantly, all these mediators were found to inhibit testosterone production by Leydig cells [21]. At the clinical level, patients with rheumatoid arthritis, a chronic disease exhibiting a protracted inflammatory response are also known for their impairment in gonodal steroid production [22,23]. In the same sense, human volunteers challenged with subcutaneous IL-6 injections (leading to acute elevations in circulating IL-6 levels as seen in severe inflammation), showed decreased testosterone levels without apparent changes in gonadotropin levels [24]. It is worth commenting that pro-inflammatory cytokines were found increased in this series of TB patients, as well as in our former studies in patients with this disease [5,6,25].

Since testes from TB patients contained increased amounts of pro-inflammatory cytokines, an attempt at experimentally reproducing the influence of such situation on testosterone production was carried out. Confirming reports from other laboratories, pro-inflammatory cytokines significantly inhibited testosterone production by Leydig cells (12,21). In analyzing cytokine effects, we also wished to ascertain the effect of TGF-β since this cytokine was also found increased in TB patients [26] being quite relevant in several aspects of TB immunopathogenesis [27]. Our findings revealed that Leydig cells exposed to TGF-β produced lesser amounts of testosterone. Beyond its pro- and anti-inflammatory effects [28], TGF-β was also shown to influence male gonadal function. In the testis, TGF-β regulates the secretor function of Leydig and Sertoli cells, as well as testis development and spermatogenesis [29,30]. TGF-β1 represses testosterone production in Leydig cells through decreasing LH/hCG receptor expression and the expression of steroidogenic genes such as StAR and P450c17 [31]. As well as reducing testosterone production, our former studies also showed that TGF-β was also able to inhibit DHEA by adrenal cells [32], which bears relation with the present demonstration of the inverse association between TGF-β and DHEA. Collectively, it implies a broader range of inhibition of androgen production by this cytokine and the adverse consequences

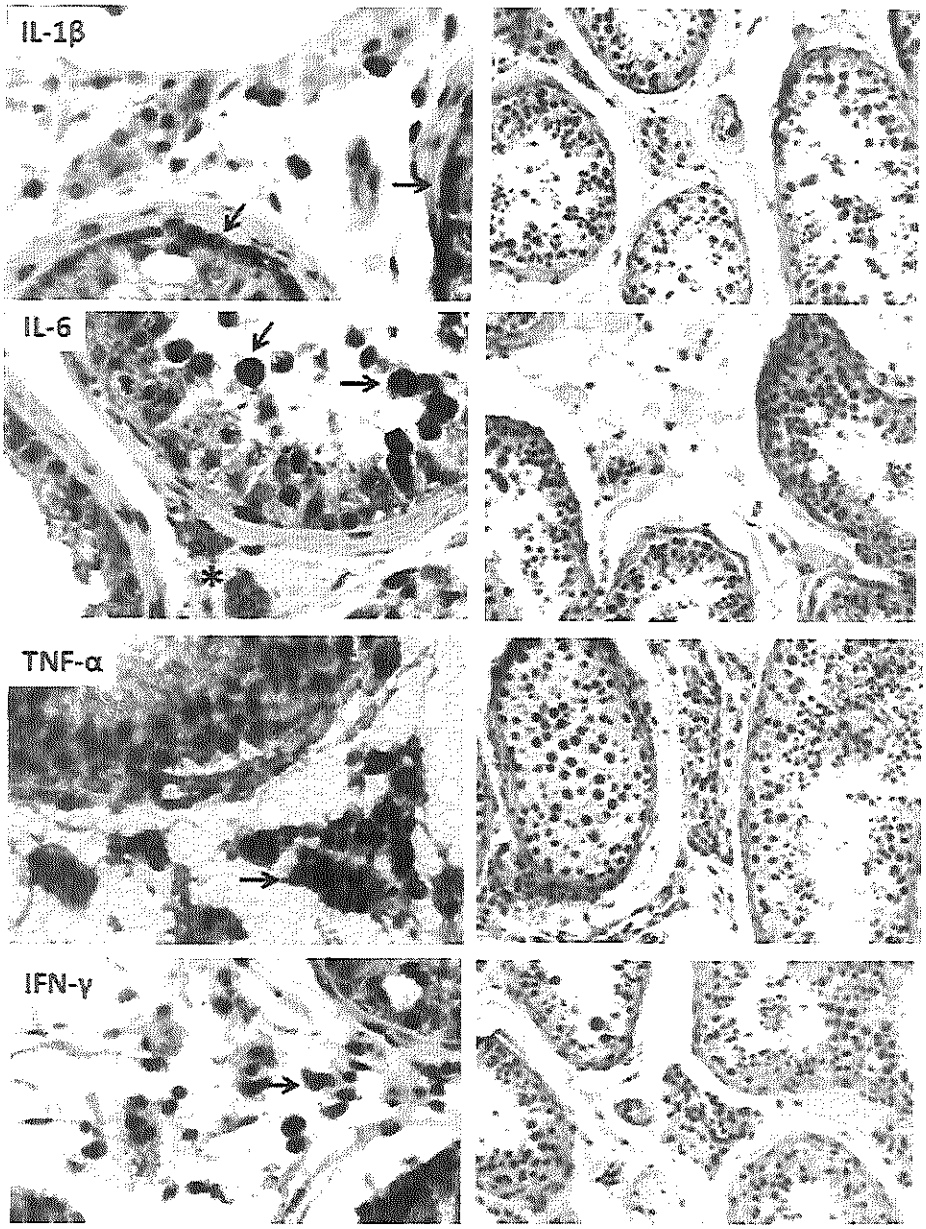

Figure 3. Representative immunohistochemistry for cytokine detection in testicular tissue. Sections from TB autopsy cases (left row) and control cases from subjects dying from causes other than TB (right row), were incubated with specific antibodies to detect the indicated cytokines. Sertoli cells showed strong positivity in their cellular base for IL-1β (arrows), spermatogonia exhibited strong immunostaining to IL-6 (arrows) and mild reactivity in interstitial macrophages (asterisk), while strong TNFα immunostaining was seen in interstitial macrophages (arrow). Occasional macrophages and lymphocytes in the testicular interstitium showed positivity to IFNγ. Control tissues were negative to all tested cytokines (TB tissues magnification 200x, control tissue magnification 100x).

resulting from it; mainly because of the anabolic and anti-inflammatory effects of testosterone [33]. Within this context, diminished amounts of testosterone would not be sufficient to counteract the synthesis of mediators dealing with accompanying inflammatory reaction. Since testosterone and Cortisol display anti-inflammatory effects, the negative correlation between both steroids perhaps may reflect a compensatory interaction between the HPG and HPA axes, to assure some form of a counterbalancing effect for the accompanying inflammation. Also, the inverse correlation between testosterone and IFN-γ levels may be explained in view of the inhibitory effects of the gonadal steroid on Th1 cell differentiation [34]. Another reason for the inhibition of testosterone production may have to do with energy conservation necessary to sustain the immune response. Alternatively, it may represent an adaptive mechanism attempting to avoid the reproduction of the more susceptible people to illness and hence preventing the propagation of this defect.

Whatever the case, it is clear that when the pathogen cannot be contained by defensive mechanisms, as occurs in TB, a systemic response characterized by multiple metabolic and neuroendocrine changes develops. This will affect essential biological functions, like the development of protective responses, control of tissue damage and physiological functions, which in essence are implied in a poorer disease course.

Table 1
Testosterone concentration in culture supernatants from TM3 cells cultured under different conditions.

Treatments	Testosterone levels (ng/ml)[1]
TNF-α	0.119 ± 0.012
TGF-β	0.102 ± 0.042
IFN-γ	0.130 ± 0.026
Control	5.740 ± 0.026*

Recombinant cytokines TNF-α 5 ng/ml, or IFN-γ 4000 pM, or TGF-β 1 ng/ml were added to TM3 cells after 2 hours with hCG to stimulate basal testosterone production. Control cultures were stimulated with hCG. Testosterone concentration in the media in which Leydig cells were cultured was assessed 24 h later in duplicate.

* Significantly different from the remaining groups $p < 0.001$.

[1] Data represent the mean of triplicate determinations ± SEM of a representative experiment from two independent experiments performed under similar conditions.

5. Conclusions

Present results point out to a novel and interesting implication of the inflammatory response during tuberculosis in the disturbed production of gonadal steroids. Patients with severe TB suffer a significant decrease of testosterone production, which is apparently the consequence of testicular production of proinflammatory cytokines produced by diverse cells in absence of local infection. Testosterone is a significant anabolic hormone and its low production could contribute to affect the patient condition worsening the course of the disease.

Acknowledgments

This study was supported by CONACyT (contract: 84456) and Thematic Network N° 253053, CONACyT.

Funding: None declared.

Competing interests: None declared.

Ethical approval: The study protocol was approved by the Ethic Committee of the School of Medical Sciences, National University of Rosario, Argentina.

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Jiménez-Arellanes et al. BMC Complementary and Alternative Medicine 2013, 13:258
http://www.biomedcentral.com/1472-6882/13/258

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RESEARCH ARTICLE Open Access

Ursolic and oleanolic acids as antimicrobial and immunomodulatory compounds for tuberculosis treatment

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Abstract

Background: New alternatives for the treatment of Tuberculosis (TB) are urgently needed and medicinal plants represent a potential option. *Chamaedora tepejilote* and *Lantana hispida* are medicinal plants from Mexico and their hexanic extracts have shown antimycobacterial activity. Bioguided investigation of these extracts showed that the active compounds were ursolic acid (UA) and oleanolic acid (OA).

Methods: The activity of UA and OA against *Mycobacterium tuberculosis* H37Rv, four monoresistant strains, and two drug-resistant clinical isolates were determined by MABA test. The intracellular activity of UA and OA against *M. tuberculosis* H37Rv and a MDR clinical isolate were evaluated in a macrophage cell line. Finally, the antitubercular activity of UA and OA was tested in BALB/c mice infected with *M. tuberculosis* H37Rv or a MDR strain, by determining pulmonary bacilli loads, tissue damage by automated histomorphometry, and expression of IFN-γ, TNF-α, and iNOS by quantitative RT-PCR.

Results: The *in vitro* assay showed that the UA/OA mixture has synergistic activity. The intracellular activity of these compounds against *M. tuberculosis* H37Rv and a MDR clinical isolate in a macrophage cell line showed that both compounds, alone and in combination, were active against intracellular mycobacteria even at low doses. Moreover, when both compounds were used to treat BALB/c mice with TB induced by H37Rv or MDR bacilli, a significant reduction of bacterial loads and pneumonia were observed compared to the control. Interestingly, animals treated with UA and OA showed a higher expression of IFN-γ and TNF-α in their lungs, than control animals.

Conclusion: UA and OA showed antimicrobial activity plus an immune-stimulatory effect that permitted the control of experimental pulmonary TB.

Keywords: Triterpenoids, Antitubercular activity, Antimycobacterial activity, Medicinal plants

Background

At present, Tuberculosis (TB) is the only infectious disease considered by the World Health Organization (WHO) as a health emergency worldwide, because it causes nearly 2 million deaths annually [1]. TB is more frequent in developing countries and its association with human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) renders its control more difficult. In addition, the emergence of multidrug-resistant tuberculosis (MDR-TB, defined as those TB strains simultaneously resistant at least to rifampin and isoniazid) and extensively drug resistant tuberculosis strains (XDR-TB) threaten the success of the directly observed therapy short course (DOTS) and DOTS-Plus treatment programs established by the WHO [2]. Despite all the progress achieved, only one third of patients with TB receive adequate treatment; in the case of MDR, few patients have

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received the DOTS-Plus regimen and only about 70% of MDR-TB cases respond to the current treatment [1,2].

Since the release of rifampicin in 1976, only rifabutin and rifapentin have been approved for TB treatment; unfortunately, these drugs are not yet widely distributed [3]. At present, a number of drugs are under investigation, but only a few compounds are found in preclinical and clinical evaluation (about 10 compounds) [1-4]. Thus, there is an urgent need to discover new antituberculous agents that are effective in the treatment of MDR cases and also novel agents that can shorten the long conventional chemotherapy in drug-sensitive TB. Within this context, not only new synthetic drugs, but also natural products from medicinal plants are potential sources of new anti-mycobacterial products.

Chamaedora tepejilote (*C. tepejilote*) and *Lantana hispida* (*L. hispida*) are widely distributed plants in Mexico known as *"tepejilote, palmita* or *palma camaedor"* and *"cinco negritos* or *verbena"* respectively, both plants have been used in Mexican traditional medicine. Some of their common uses include the treatment of respiratory complaints such as cough, bronchitis, colds and pneumonia [5]. We have previously reported that the hexanic fractions from these plants had *in vitro* antimycobacterial activity and their bioguided fractionation showed that the triterpenic compounds ursolic acid (UA) and oleanolic acid (OA) were the specific agents involved in this activity [6-8]. This effect has been confirmed by other authors [9-11]. These triterpenic acids also have antibacterial [12,13], antiviral [14], antiparasitic [13], antioxidant [15] and antitumoral activities [16], as well as hepatoprotector [17] and gastroprotector [18] effects. Interestingly, UA enhances the production of nitric oxide (NO) and tumor necrosis factor alpha (TNF-α) by activating nuclear factor-kappaB (NF-κB) in mouse macrophages [19,20] and blocking transforming growth factor-beta 1 (TGF-β1) activity [21,22]. The stimulation of NO and TNF-α contributes to their immunoregulatory and antitumoral effects, and could be significant in an immunotherapeutic agent against *M. tuberculosis*. In this study, we report the *in vitro* antimycobacterial activity of UA and OA isolated from the hexanic extract of the aerial parts of *C. tepejilote* and *L. hispida*, against the reference drug-sensitive *M. tuberculosis* strain H37Rv, monoresistant H37Rv strains, several MDR clinical isolates and a group of nontuberculous mycobacteria. The antitubercular activity of both compounds was then confirmed in a well-characterized murine model of progressive pulmonary TB. Our results show therapeutic activity attributable to a combination of bactericidal and immunotherapeutic effects.

Methods

Chemical compounds

Bioguided fractionation of the hexanic extracts from *C. tepejilote* and *L. hispida* aerial parts yielded UA and OA, respectively [6-8]. The plant material was botanically identified by Abigail Aguilar MSc and a voucher of each specimen were deposited at the IMSSM Herbarium with code number 13402 (*L. hispida*) and 140321 (*C. tepejilote*). Both compounds (Figure 1) were structurally characterized by spectroscopic and spectrometric data as compared with those previously reported [23,24].

In vitro antimycobacterial assay

The antimycobacterial activity of the triterpenic acids was evaluated against the *M. tuberculosis* H37Rv (ATCC 27294) reference strain (a pan-sensitive strain) and against four monoresistant strains of *M. tuberculosis* H37Rv [streptomycin-resistant (ATCC 35820), isoniazid-resistant (ATCC 35822), ethambutol-resistant (ATCC 35837) and rifampicin-resistant (ATCC 35838)]. The microorganisms were cultured up to log phase growth at 37°C in Middlebrook 7H12 broth supplemented with 0.2% glycerol and enriched with 10% Oleic acid-albumin, dextrose and catalase (OADC) and further diluted to 1:20. Antimycobacterial activity was determined by using the microplate alamar blue assay (MABA), as previously described [7,8]. In addition, the effect of both terpenoids was also determined against a MDR *M. tuberculosis* strain MTY 147 (resistant to isoniazid, rifampicin, ethambutol, and ethionamide) and against a drug-resistant *M. tuberculosis* strain coded as MMDO that is resistant to isoniazid and ethambutol and five non-tuberculous mycobacteria (*M. avium, M. smegmatis, M. simiae, M. chelonae* and *M. fortuitum*). The compounds were tested at a concentration of 2 mg mL^{-1} in 20% DMSO in Middlebrook 7H9 broth.

In vitro determination of the synergistic antimycobacterial activity of triterpenic acids

The pharmacological synergy of UA and OA was evaluated against *M. tuberculosis* H37Rv by a modification of the MABA assay [25]. Briefly, a stock solution of each compound was prepared in 7H9 broth containing 10% OADC enrichment. A volume of 50 μL of the stock solution of UA (compound A) and 50 μL of OA (compound B) were added simultaneously to the well, having been

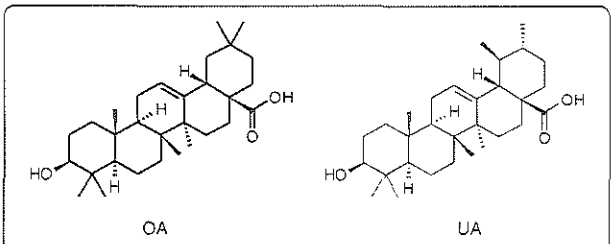

Figure 1 Chemical structure of Ursolic acid (UA) and Olenolic acid (OA).

thoroughly mixed; afterwards, there were added 100 μL of the bacterial suspension adjusted to a McFarland 1 tube and diluted in a ratio of 1:10. Controls for each compound were prepared by adding 50 μL of the corresponding stock solution, 50 μL of the culture medium and 100 μL of the same adjusted bacterial suspension. Control for bacterial growth included 100 μL of 7H9 broth and 100 μL of the bacterial suspension. Plates were incubated for 5 days at 37°C; after this period, 20 μL of alamar blue solution (Trek, USA) and 12 μL of 20% Tween 80 sterile solution were added to the wells, leaving the plates overnight at 37°C. A relative fluorescent unit (RFU) was determined in a fluorometer (Fluoroscank FL, Labsystem). Analysis of pharmacological interactions were carried out by the X/Y quotient analysis, where X represents the RFU value of the drug combination and Y, the lowest RFU value obtained with both pure compounds. Activity was considered synergistic when the X/Y value was <0.5 and additive when X/Y was >0.5 and <1.0. Activity was considered absent when X/Y was 1–2 and antagonistic when X/Y was >2.

Cytotoxicity and intracellular antitubercular activity tested *in vitro*

Cytotoxicity of the triterpenic acids was evaluated by the trypan blue exclusion assay. Briefly, 24 well tissue culture plates were seeded with murine macrophages J774A.1 (ATCC HB-197) in 1 mL of Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) with antibiotics to reach a confluence of at least 80%. Cells were treated with four concentrations of the pure compounds, taking the minimal inhibitory concentration (MIC) of each one as reference. These dilutions were prepared in DMEM with 1% FBS without antibiotics (hereafter denoted working solutions). Before treatment, the wells were washed three times with warm Hank's balanced salt solution (HBSS), and 1 mL of working solution was added to each of the corresponding wells. The percentage of viable cells was determined prior to treatment and after 24, 48, 72, and 96 hrs by adding trypan blue solution to reach a final concentration of 0.2% per well; at least 200 cells per well were counted. Those compound concentrations that after 96 hrs of incubation did not affect cell viability <90% (IC_{90}) were considered non-toxic.

Antimycobacterial intracellular activity was tested in the macrophage cell line J774A.1 infected with *M. tuberculosis* H37Rv and the MDR clinical isolate MTY147, using two non-toxic concentrations: high (12.5 μg mL^{-1} for OA and 6.25 μg mL^{-1} for UA) and low (1.25 and 0.625 μg mL^{-1} for OA and UA, respectively). For this purpose, log phase growth of *M. tuberculosis* H37Rv in Middlebrook 7H9 broth with 10% OADC was washed twice with HBSS and adjusted in DMEM with 1% FBS to reach a bacterial macrophage multiplicity of infection of 10:1. Macrophages were incubated with the bacilli for 2 hrs and non-phagocytosed organisms were removed by three washes with warm HBSS. Then, 1 mL of UA or OA at different concentrations alone or in combination was added to the infected macrophages at 37°C in a 5% CO_2 atmosphere; after 24, 48, 72, and 96 hrs of treatment, the cells from the corresponding wells were lysed with 0.5 mL of 0.25% sodium dodecyl sulfate (SDS) for 3 min and later 0.5 mL of 5% bovine serum albumin (BSA) was added. Control cells contained only the culture medium. Viable bacteria were determined by quantification of colony-forming units (CFU) by plating dilutions of the macrophage lysates on Middlebrook 7H11 agar with 10% BSA.

Experimental model of progressive pulmonary TB in BALB/c mice

The antitubercular activity *in-vivo* of both compounds administered together was determined by using an experimental model of progressive pulmonary TB that was previously described [26]. Briefly, male BALB/c mice at 6–8 weeks of age were used. *M. tuberculosis* H37Rv or MDR clinical isolate (CIBIN/UMF 15:99, strain resistant to rifampicin, ethambutol, streptomycin, pyrazinamide and isoniazid) was cultured in Proskauer and Beck medium as modified by Youmans. After 1 month of culture, the mycobacteria were harvested and adjusted to 2.5×10^5 cells in 100 μL of phosphate buffered saline (PBS), aliquoted and maintained at −70°C until use. Before testing, the bacilli were recounted and the viability was determined.

To induce pulmonary TB, mice were anesthetized with sevofluorane, and 2.5×10^5 viable mycobacteria suspended in 100 μL of PBS were administered intratracheally (i.t.) using a rigid stainless steel cannula and maintained in a vertical position until spontaneous recovery. Infected mice were housed in groups of five in cages fitted with micro-isolators.

Ethics statement

All procedures were performed in a laminar flow cabinet in bio-safety level III facilities. The study with animals was performed according to guidelines of the local Ethical Committee for Experimentation in Animals in Mexico (Ministry of Agriculture, NOM-062-ZOO-1999, Mexico) modified in 2001 and was approved by the Institutional Animal Care and Use Committee, 236. An experimental protocol used in this study was approved by the Comisión Nacional de Investigación Científica (CNIC, IMSS 2006-785-025-028).

Drug administration

Animals surviving 60 days after infection were randomly allocated to the required treatment groups. Thus, treatment began 60 days after infection, and groups of these animals were sacrificed at 1- and 2-month intervals. All data points are the means [± standard deviation (SD)] of

4–6 animals for a representative experiment. The selection of the appropriate dose was calculated according to the MIC determined *in-vitro* (drug concentration efficient to kill 1×10^6 bacilli) by adjusting the drug concentration to the estimated number of bacilli in the lungs of the mice after 2 months of infection; this drug amount was tripled, considering its dilution after absorption and systemic distribution after subcutaneous (s.c.) administration. As shown later in the results section, the pharmacological interaction assay demonstrated synergism of between the two triterpenoids. Thus, 5 mg kg^{-1} of each triterpenic acid was dissolved in ultra-pure olive oil (Sigma) and a total volume of 100 μL was administered s.c.: three parts of UA (75 μg) and one part of OA (25 μg); this mixture was administered 3 times/week for 30 and 60 days. This dose was also used to supplement conventional chemotherapy in order to discover whether it might synergize and shorten the required duration of chemotherapy. Thus, we treated a group of mice with conventional antibiotics (ABS): a combination of 10 μg kg^{-1} rifampicin, 10 μg kg^{-1} isoniazid, and 30 μg kg^{-1} pyrazinamide dissolved in isotonic saline solution (ISS) and intragastrically (i.g.) administered daily; another group was treated with this conventional chemotherapy scheme plus the mixture of both terpenoids. The control group corresponded to infected animals receiving only the vehicle (olive oil) s.c. and ISS by the i.g. route. Groups of six animals were euthanized at 7, 14, 30 and 60 days post-treatment in two independent experiments.

Assessment of colony-forming units (CFU) in infected lungs and preparation of tissue for histology and morphometry

One lung was immediately frozen by immersion in liquid nitrogen and used for colony counting, while the remaining lung was perfused with 10% formaldehyde and used for histopathological analysis. Frozen lungs were disrupted in a Polytron homogenizer (Kinematica, Lucerne, Switzerland) in sterile 50 mL tubes containing 3 mL of isotonic saline solition (ISS). Four dilutions of each homogenate were spread on duplicate plates containing Bacto Middlebrook 7H10 agar (Difco Lab code 0627-17-4) enriched with OADC also from Difco (code 07-22-64-0). Incubation time was 21 days. Four lungs per each group from different animals at each time point in two different experiments were studied.

For the histological study, after 2 days of fixation, parasaggital sections were taken through the hilus, and these were dehydrated and embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin (H&E). The percentage of lung surface affected by pneumonia was measured by using an image analysis system (Q-Win 500 W Leica). Measurements were carried out in blinded fashion and the data are expressed as the mean of four animals' ± SD.

Real time PCR analysis of cytokines in lung homogenates

Total RNA was isolated from cell suspensions using four lungs from the same number of different animals per group after 1 and 2 months of treatment (3 and 4 months of infection). The lung was placed in 2 mL of RPMI medium containing 0.5 mg mL^{-1} collagenase type 2 (Worthington, NJ, USA), and incubated for 1 h at 37°C. It was then passed through a 70 μm cell sieve, crushed with a syringe plunger and rinsed with the medium. The cells were centrifuged, the supernatant was removed, and red cells were eliminated with a lysis buffer. After counting, 350 μL of RLT buffer were added to 5×10^6 cells and RNA was extracted by the RNeasy Plant Mini Kit (Qiagen, Inc., USA) according to the manufacturer's instructions. The quality and quantity of RNA were evaluated through spectrophotometry (260/280) and on agarose gels. Reverse transcription of mRNA was performed using 5 μg RNA, oligo-dt, and the Omniscript kit (Qiagen, Inc). Real-time PCR was performed using the 7500 Real time PCR system (Applied Biosystems, USA) and the QuantiTect SYBR Green Master Mix kit (Qiagen). Standard curves of quantified and diluted PCR product as well as of negative controls were included in each PCR run. Specific primers were designed using the Primer Express (Applied Biosystems, USA) program for the following targets: Glyceraldehyde-3-phosphate dehydrogenase (G3PDH): 5′-cattgtggaaggg ctcatga-3′, 5′-ggaaggccatgccagtgagc-3′; inducible Nitric oxide synthase (iNOS): 5′-agcgaggagcaggtggaag-3′, 5′-catt tcgctgtctccccaa-3′; TNF-α: 5′-tgtggcttcgacctctacctc-3′, 5′-gccgagaaaggctgcttg-3′, and Interferon gamma (IFN-γ): 5′-ggtgacatgaaaatcctgcag-3′, 5′-cctcaaacttggcaatactcatga-3′. The following cycling conditions were employed: an initial denaturation at 95°C for 15 min, followed by 40 cycles at 95°C for 20 sec, at 60°C for 20 sec, and at 72°C for 34 sec. Quantities of the specific mRNA in the sample were measured according to the corresponding gene-specific standard. The mRNA copy number of each cytokine was related to 1 million copies of mRNA encoding the *G3PDH* gene.

Statistics analysis

A one-way analysis of variance (ANOVA) and the Student *t* test were used to compare CFU and morphometry determinations in infected mice treated with terpenoids and in non-treated control animals. A difference of $P < 0.05$ was considered significant.

Results

In vitro determination of antimycobacterial activity and synergism of UA and OA

Table 1 shows the MICs values of UA and OA determined by the MABA assay. When the reference strain H37Rv was used, UA showed a MIC of 25 μg mL^{-1} and OA 50 μg mL^{-1}. Both compounds were also effective

Table 1 *In vitro* antimycobacterial activity of Ursolic acid (UA) and Oleanolic acid (OA) tested by MABA assay

M. tuberculosis strain	MIC (μg mL^{-1})		
(ATCC)	UA	OA	UA/OA
H37Rv (27294)	25	50	12.5
INH-R (35822)	25	25	25
RIF-R (35838)	25	25	25
EMB-R (35837)	25	25	25
STR-R (35820)	12.5	50	25
DR clinical isolates			
MMDO	25	50	ND
MTY147	25	50	ND
Non-tuberculous mycobacterium			
M. chelonae	100	100	100
M. avium	100	100	100
M. smegmatis	>200	100	100
M. fortuitum	100	100	100
M. simiae	>200	100	100

H37Rv: sensitive strain to INH, RIF, EMB, STR, and pyrazinamide; RIF-R: rifampicin-resistant to H37Rv; STR-R: streptomycin-resistant to H37RV; INH-R: isoniazid-resistant to H37Rv and EMB-R: ethambutol-resistant to H37Rv; MMDO: strain resistant to isoniazid and ethambutol; MTY 147: strain resistant to isoniazid, rifampicin, ethambutol and ethionamide. MIC: minimum inhibitory concentration. Data are means of three determinations. UA: ursolic acid; OA: oleanolic acid; UA/OA: mixture of ursolic and oleanolic acids.

against the monoresistant strains (isoniazid-, rifampicin- and ethambutol-resistant) with a MIC of 25 μg mL^{-1}. The streptomycin-resistant *M. tuberculosis* H37Rv strain was more sensitive to UA (MIC = 12.5 μg mL^{-1}) but less sensitive to OA (MIC = 50 μg mL^{-1}). The mixture of both compounds showed a MIC = 12.5 μg mL^{-1} against the H37Rv strain. Terpenoids showed a lesser effect against non-tuberculous mycobacteria, with MICs ranged between 100 to >200 μg mL^{-1}. Interestingly, the combined effect of UA and OA *in vitro* exhibited synergistic activity at a proportion of 0.5 MIC of OA (25 μg mL^{-1}) and 0.5 MIC of UA (12.5 μg mL^{-1}), with an X/Y value of <0.5 (Figure 2).

Cytotoxicity and intracellular activity of UA and OA

Considering the *in vitro* MIC values found for each compound, the intracellular activity of both triterpenoids was evaluated in a macrophage model for both *Mycobacterium* strains (H37Rv and the MDR clinical isolate). The cytotoxicity of these compounds revealed that at concentrations >20 μg mL^{-1}, cell death was above 30% and below 18. Two concentrations below this concentration were used for macrophage treatment: the first was 1/4 of the MIC and second 1/40 of the MIC of each compound (Figure 3). We observed that at a high concentration (1/4 MIC) with both *Mycobacterium* strains there was a statistically significant CFU reduction after

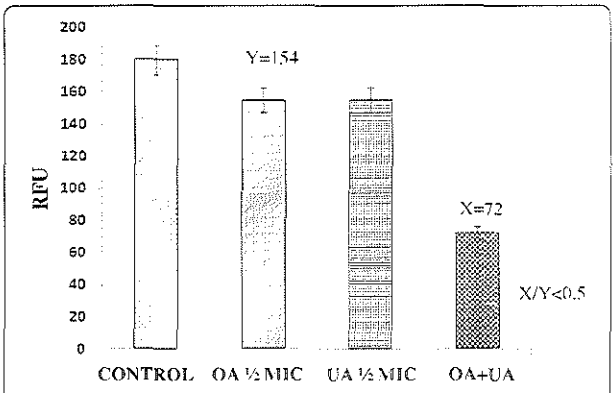

Figure 2 *In vitro* synergistic effect of Ursolic acid (UA) and Oleanolic acid (OA). One half of the *in vitro* MIC of UA and OA alone or in combination were incubated with *M. tuberculosis* H37Rv and RFU were recorded at the end of the incubation period: the X/Y quotient analysis was applied to establish the type of pharmacological interaction between these compounds, where X represents the RFU value obtained with the combination of both compounds, and Y is the RFU value of the compound that presented the lowest value, when alone. Synergy was considered when the X/Y value was <0.5.

UA and OA treatment, but when both compounds were added together greater elimination of bacilli was observed (Figure 3A). Even at a lower concentration (1/40 MIC), there was an efficient antimycobacterial effect of either UA or OA; in the case of the *M. tuberculosis* H37Rv strain, the combined effect of UA and OA at a lower concentrations was still very effective, while for the MDR strain, it was less effective (Figure 3B).

Effects of triterpenic acids *in vivo* on lung bacillary load, histopathology and cytokine gene expression

In comparison with non-treated control mice, animals infected with the drug-sensitive H37Rv strain treated with both OA and UA showed a significant decreased number of live bacilli in the lungs after 1 and 2 months of treatment (Figure 4A). These results in bacillary loads correlated well with the morphometric observations; this showed a significant decrease of the lung area affected by pneumonia in treated animals as compared with those of the non-treated control group (Figure 4B).

Since UA and OA have diverse immunoregulatory activities [19,22], the expression of genes encoding IFN-γ, TNF-α and iNOS was determined by real time PCR. Figure 4C illustrates that animals treated with UA/OA exhibited a higher (but not significantly) expression of both cytokines and a significantly higher expression of iNOS than non-treated control animals.

Animals infected with the drug-sensitive H37Rv strain and treated with both terpenoids in combination with conventional chemotherapy showed pulmonary bacilli burdens and tissue damage similar to that seen in animals treated with chemotherapy only (Figure 5A and 5B). Thus,

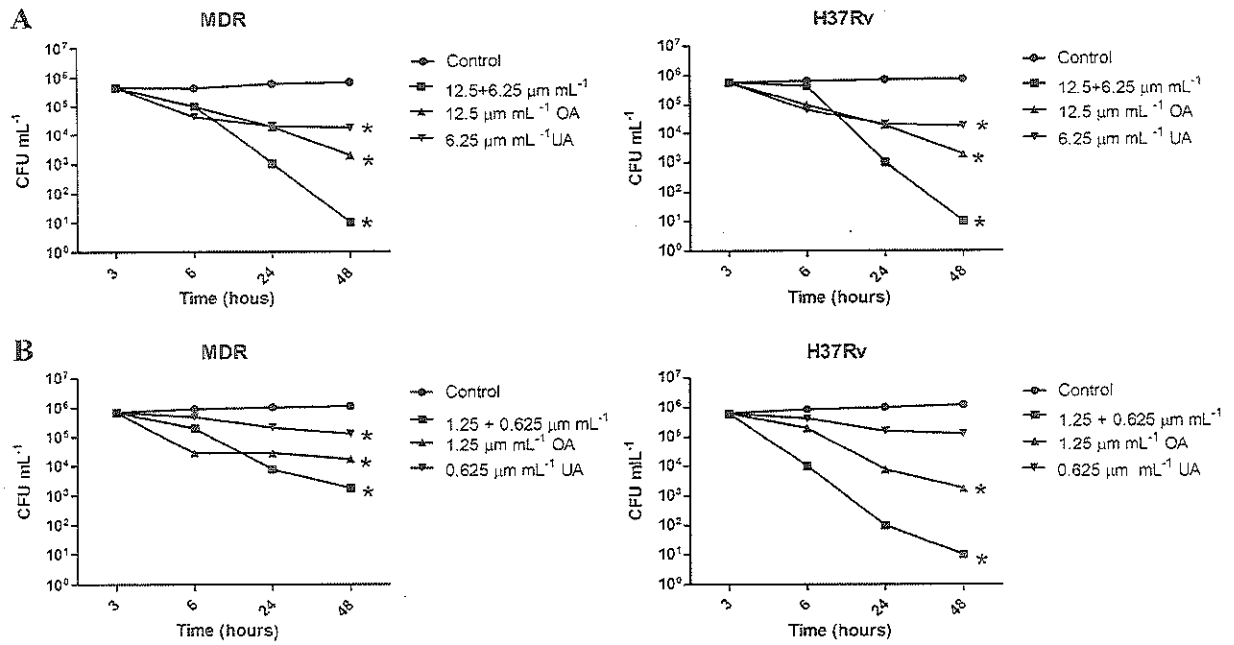

Figure 3 Intracellular activity of Ursolic acid (UA), Oleanolic acid (OA) and their combination against *M. tuberculosis* H37Rv and MDR clinical isolate in the macrophage cell model. Macrophages (cell line J774A.1) were infected (MOI 10:1) with drug-sensitive H37Rv or DR strain and treated with UA, OA, or both together at two concentrations, upper (panel **A**) and lower (panel **B**). After several time points, cells were lysed and intracellular bacillus colony forming units (CFU) were determined. In comparison with non-treated control cells, each terpenoid alone had significant antimycobacterial activity at high or low treatment concentrations, but when OA and UA were incubated together, the antimycobacterial activity was more efficient. *P <0.001.

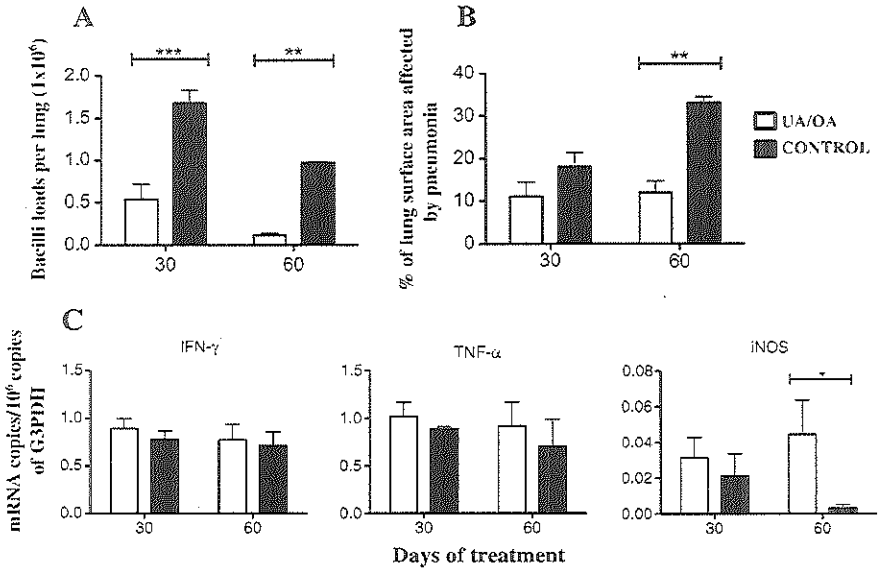

Figure 4 Effect of Ursolic acid (UA)/Oleanolic acid (OA) administration on bacterial loads, pathology and cytokine expression during advanced disease in lungs from mice infected with the *M. tuberculosis* H37Rv strain. (A) UA/OA administration (white bars) starting 60 days after infection decreased pulmonary bacterial loads when compared with those of control mice (black bars). **(B)** UA/OA-treated mice (white bars) at days 30 and 60 post-treatment showed less pneumonic area than the control group. **(C)** UA/OA treatment induced a slightly higher expression of TNF-α and IFN-γ, and a significantly higher expression of inducible iNOS at day 60 when compared with non-treated control mice. ABS: correspond to 10 μg kg^{-1} rifampicin, 10 μg kg^{-1} isoniazid and 30 μg kg^{-1} pyrazinamid. Each point corresponds to the mean ± Standard deviation (SD) of groups of four mice. Asterisks represent the statistical significance between groups (*P <0·05; **P <0·01).

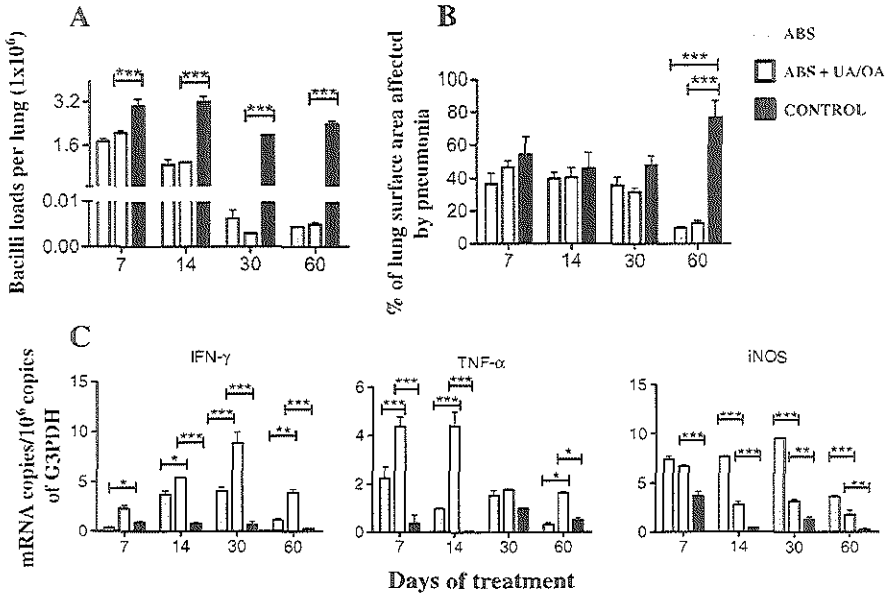

Figure 5 Effect of combined treatment with chemotherapy and Ursolic acid (UA)/Oleanolic acid (OA). **(A)** Shows the number of Colony-forming units (CFU) in lungs of mice infected with *M. tuberculosis* H37Rv sensitive strain. Beginning on day 60 after infection, mice were treated either with conventional chemotherapy alone (gray bars) or with conventional chemotherapy in combination with UA/OA 3 times/week (white bars). Also shown are values for control (untreated) mice (black bars). **(B)** The effect of combined treatment on the percentage of lung surface affected by pneumonia. **(C)** Combined treatment induced significantly higher expressions of TN-α, IFN-γ, and iNOS. Each point corresponds to the mean ± Standard deviation (SD) of four mice groups. Asterisks represent the statistical significance between groups (*P <0·05; **P <0·01).

although there was no apparent synergistic effect, the combined treatment induced a higher expression of IFN-γ, TNF-α, and iNOS than was seen in the group treated only with antibiotics, or in the non-treated control group (Figure 5C).

Due to the emergence of MDR strains and given the improved disease course in UA/OA-treated mice infected with the drug-sensitive H37Rv strain, we decided to study whether this therapy has the ability to produce similar beneficial effects on mice infected with a *M. tuberculosis* clinical isolate resistant to all first-line antibiotics during late active disease. In comparison with control animals, MDR clinical isolate-infected mice treated with UA/OA showed significantly lower lung bacillary loads at 1 month of treatment and reduced, but not significantly, lung bacillary loads at 2 months (Figure 6A). Similarly, improved lung histopathology was observed, with a significant decrease of pneumonia (Figure 6B) at 30 and 60 days of treatment, as compared with the group of non-treated mice (P <0·01). The determination of cytokine gene expression by real-time PCR showed higher IFN-γ expression in the lungs of UA/OA-treated animals (Figure 6C), with statistical significance at 30 days (P <0·05) of treatment. Thus, in addition to modest antimycobacterial activity, both terpenoids also possess immunotherapeutic effects.

Discussion

UA (3β-hydroxy-urs-12-en-28-oic-acid) and its isomer, OA (3β-hydroxy-olea-12-en-28-oic acid) are triterpenoids compounds that are widely distributed in the plant kingdom, in medicinal herbs, and are a common component of the human diet [27]. There are comprehensive reports on their biological activities and beneficial effects in various diseases, including infectious diseases [16,27]. In this regard, there are several reports of their significant antimycobacterial activity when they are primarily purified from diverse plants [9,11,28]. Indeed, the present study comprises part of a research program that involves an ethnopharmacological screening of Mexican medicinal plants in a search for activity against *M. tuberculosis*. Our previous studies showed that UA and OA were in part responsible for the antimycobacterial activity from *L. hispida* (a widely distributed, ornamental Mexican plant used in folk medicine to treat TB) and *C. tepejilote* (a tropical plant from Southern Mexico used to treat respiratory diseases) [6,8]. The results presented here confirm and extend these studies, showing that purified UA and OA have *in vitro* antimycobacterial activity against fully drug-sensitive and monoresistant H37Rv strains, as well as several MDR clinical isolates and to a lesser degree, non-tuberculous mycobacteria. Our results on the *in vitro* activity of UA against *M. tuberculosis* H37Rv were similar

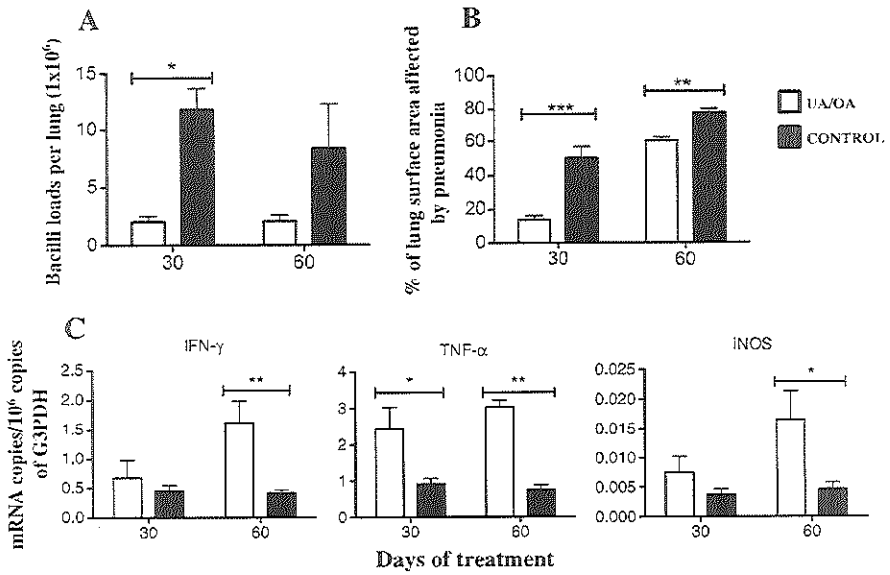

Figure 6 Effect of Ursolic acid (UA)/Oleanolic acid (OA) treatment on lung bacillary loads, pathology and cytokine expression during late disease produced by the multidrug-resistant (MDR) clinical isolate. (A) UA/OA mixture was administered 3 times per week (white bars) for 2 months, starting at day 60 post-infection with the MDR clinical isolate (CIBIN/UMF 15:99), decreased bacterial loads when compared with non-treated control animals (black bars). **(B)** These UA/OA-treated mice displayed a lesser pneumonia area than the control animals. **(C)** Administration of both terpenoids induced higher expression of proinflammatory cytokines and inducible iNOS. Each point corresponds to the mean ± Standard deviation (SD) of groups of four mice. Asterisks represent statistical significance between groups (*P <0·05; **P <0·01).

to those reported previously, with a MIC value of 50 μg mL^{-1} when evaluated by the radiorespirometric Bactec 460, and 31.0 and 41.9 μg mL^{-1} by MABA assay; while MIC values reported for OA were 50 μg mL^{-1} when tested by the radiorespirometry method and 30.0, 28.7, and 25 μg mL^{-1} by MABA [9,29-32]. Both triterpenic acids exhibited less activity against non-tuberculous mycobacteria, with the MIC value of 100 μg mL^{-1}. This is in fact modest antimycobacterial activity. However, one major point of traditional medicine is the use of herb mixtures, which could be more effective than a single product for producing the desired effects [28]. UA and OA are isomers, and our results showed that the combination of both produced *in vitro* intracellular and *in vivo* synergistic effects. Although the molecular mechanism of the antimycobacterial activity has not yet been determined, it has been proposed that UA and OA can produce significant abnormalities in the bacterial cell wall [9,13]. Both terpenoids have efficient antilipidic activity on eukaryotic cells [33], and perhaps this activity can also affect mycobacteria producing damage on the complex cell envelope, which is rich in lipids.

Mycobacterial infections are controlled by the activation of macrophages through type 1 cytokine production by T cells [34-36]. IFN-γ and TNF-α are essential for this process because they promote macrophage activation and iNOS expression. This is clearly observed in our BALB/c mouse model, which is based on infection via the trachea with a high dose of *M. tuberculosis* H37Rv [26,37]. In this model, there is an initial phase of partial resistance dominated by Th1 cytokines plus TNF-α and the expression of iNOS, followed by a late phase of progressive disease after 1 month of infection, characterized by a lower expression of IFN-γ, TNF-α, or iNOS, progressive pneumonia, extensive interstitial fibrosis, high bacillary counts and very high levels of immunosuppressive factors such as TGF-β1 and Prostaglandin E-2 (PGE2) [26,37,38]. This BALB/c tuberculosis model has been used extensively to test different forms of therapy [39-41], thus confirming that it is highly suitable for exploring the efficiency of new natural drugs or immunotherapy based on the airway infection route, which is the most common pathway of infection in humans and the highest rate of bacterial multiplication in the lung correlates with the extent of tissue damage (pneumonia) and death of infected animals [26].

Although contrasting differences in immune responses have been observed that depend on triterpenic concentrations and the biological status of the target cells used in different experimental systems [42], it has been reported that UA and OA stimulate IFN-γ production [43], and also upregulate iNOS and TNF-α expression through NF-kB transactivation in murine resting macrophages [19,20]. More recently, it has been demonstrated

that UA modulates human dendritic cells via activation of IL-12, polarizing the Th-1 response [44]. Tuberculous animals treated with both triterpenic acids showed a higher expression of IFN-γ, TNF-α, and iNOS than non-treated control animals, or even than sick mice successfully treated with conventional chemotherapy, suggesting that UA and OA exert an effect as immunostimulating factors that can restore the protective antimycobacterial cytokine pattern during advanced disease, producing a significant decrease of bacillus loads and tissue damage.

Suppression of T-cell responses to mycobacterial antigens is a consistent feature of TB [45], and *in vitro* and *in vivo* observations indicate that TGF-β participates in these effects [46-50]. It is well established that *M. tuberculosis* and its components are efficient inducers of the TGF-β1 production by macrophages and this cytokine is a significant factor in the suppression of cell-mediated immunity (CMI) and in the induction of fibrosis [49]. Another molecule that is also produced in high amounts during progressive TB and has CMI suppressing activities is PGE-2. In fact, TGF-β and PGE2 share many immunomodulatory functions, such as the inhibition of IFN-γ, interleukin 2 (IL-2) and IL-12 production [50,51] and macrophage deactivation, suppressing TNF-α and iNOS production [52,53]. We have shown, in this experimental model of pulmonary TB, that by blocking TGF-β activity by the administration of its soluble receptor type 3 or betaglycan, while simultaneously suppressing PGE-2 production by the administration of niflumic acid, a specific cyclooxygenase type 2 (COX-2) blocker, we can produce a significant therapeutic benefit associated with restoration of the protective cytokine pattern (41). Interestingly, UA and OA antagonize TGF-β1 activity by blocking the binding of its specific receptor [21,22], which is the same function as the soluble receptor type 3 or betaglycan. Moreover, UA and OA also suppress prostaglandin production by blocking the binding of c-Jun to the response element of the COX-2 promoter, thus preventing the transcription of this enzyme [54], or by irreversible inhibition of secretory phospholipase A2 [55,56]. Thus, the restoration of the protective cytokine pattern observed in animals treated with UA or OA could be attributable to the modulating effect that they have on TGF-β and COX-2 activity. However, there are published reports indicating contrary activities that are receptor- and mouse strain- dependent [57]. Thus, as mentioned previously [58] with respect to the control of the inflammatory response, these triterpenoids can have both positive and negative effects, and further evaluations of their effect on the biological status of target cells or tissues in health and disease are necessary.

It is noteworthy that to date, there are no studies that describe the antituberculous effect of the pure compounds of medicinal plants. Thus, to our knowledge, this study constitutes the first that focuses on evaluating the antituberculous activity *in vivo* of this type of compound. Moreover, we recently published data on the toxicity *in-vivo* of the UA/OA mixture; it is practically innocuous when evaluated by the s.c. route in acute ($LD_{50} > 300$ mg kg^{-1}) and subacute (13 mg kg^{-1} repeated-dose during 28-day) assays in BALB/c mice [59]. Therefore, these compounds can be considered potential candidates to the treatment of TB.

Conclusion

Our results showed that UA and OA obtained from medicinal plants used in Mexican traditional medicine to treat TB have modest antimycobacterial and some immunoregulatory activities that permit the control of pulmonary TB in mice, indicating that research on natural products can produce novel antibiotic and/or immunotherapeutic agents useful for the treatment of this significant infectious disease.

Abbreviations

ABS: Antibiotic; AIDS: Acquired immunodeficiency syndrome; ANOVA: One-way analysis of variance; ATCC: American type culture collection; BSA: Bovine serum albumin; CFU: Colony-forming units; CMI: Cellular mediated immunity; CNIC: Comision nacional de investigación cientifica; COX-2: Cicloxygensase type 2; *C. tepejilote*: *Chamaedora tepejilote*; DMEM: Dulbecco's modified Eagle's medium; DMSO: Dimethyl sulfoxide; DOTS: Directly observed therapy short course; DR: Drug-resistant; EMB: Etambutol; FBS: Fetal bovine serum; HBSS: Hank's balanced salt solutions; H&E: Hematoxylin and eosin; HIV: Human immunodeficiency virus; IFN-γ: Interferon gamma; IL: Interleukin; iNOS: Inducible Nitric oxide synthasa; IMSSM: Instituto Mexicano del Seguro Social Mexico; INH: Isoniazid; ISS: Isotonic saline solition; *i.t.*: *Intratracheally*; *L. hispida*: *Lantana hispida*; MABA: Microplate alamar blue assay; MDR: Multidrug-resistant; MIC: Minimum inhibitory concentration; *M. avium*: *Mycobacterium avium*; *M. chelonae*: *Mycobacterium chelonae*; *M. fortuitum*: *Mycobacterium fortuitum*; *M. smegmatis*: *Mycobacterium smegmatis*; *M. tuberculosis*: *Mycobacterium tuberculosis*; MSc: Master science; NF-kB: Nuclear factor-kappaB; NO: Nitric oxide; OA: Oleanolic acid; OADC: Oleic acid-albumin, dextrose and catalase; PBS: Phosphate buffered saline; PGE2: Prostaglandin E-2; RPMI: Roswell Park Memorial Institute1; RFU: Relative fluorescent unit; RIF: Rifampicin; s.c.: Subcutaneous; SDS: Sodium dodecyl sulfate; SD: Standard deviation; STR: Streptomycin; TB: Tuberculosis; TGF-β1: Transforming growth factor-beta 1, TNF-α: Tumor necrosis factor alpha; UA: Ursolic acid; XDR: Extensively drug-resistant; WHO: World health organization.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AJ-A, RH-P and MMF planned, coordinated the study and wrote manuscript. JC-G, DM-E and BM performed the *in vivo* experiment, analyze and interpretation of data. A.-A, JT and JC-G realized the phytochemical analysis to isolate the ursolic and oleanolic acids from medicinal plants. JL-H, SL-G, MEC-M performed the *in vitro* experiment, analyze and interpretation of data. All authors read and approved the final manuscript.

Acknowledgments

This paper was supported by grant FIS/IMSS/PROT/112 from the Coordinación de Investigación en Salud, IMSS and SEP-CONACyT grants 48339 and 84456 from CONACyT-México (Consejo Nacional de Ciencia y Tecnología-México).

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Received: 21 February 2013 Accepted: 24 September 2013
Published: 7 October 2013

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doi:10.1186/1472-6882-13-258
Cite this article as: Jiménez-Arellanes *et al.*: Ursolic and oleanolic acids as antimicrobial and immunomodulatory compounds for tuberculosis treatment. *BMC Complementary and Alternative Medicine* 2013 **13**:258.

Original Article

Histopathological Study of the Lungs of Mice Receiving Human Secretory IgA and Challenged with *Mycobacterium tuberculosis*

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Submitted: 31 Oct 2013
Accepted: 27 Mar 2014

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Abstract

Background: **Humoral and cellular immune responses are associated with protection against extracellular and intracellular pathogens, respectively. In the present study, we evaluated the effect of receiving human secretory immunoglobulin A (hsIgA) on the histopathology of the lungs of mice challenged with virulent *Mycobacterium tuberculosis*.**

Methods: **The hsIgA was purified from human colostrum and administered to Balb/c mice by the intranasal route prior to infection with *M. tuberculosis* or in a pre-incubated formulation with mycobacteria, with the principal aim to study its effect on qualitative pulmonary histopathology.**

Results: **The intranasal administration of hsIgA and the pre-incubation of mycobacteria with this preparation was associated with the presence of organised granulomas with signs of immune activation and histological features related to efficient disease control. This effect was highly evident during the late stage of infection (60 days), as demonstrated by numerous organised granulomas with numerous activated macrophages in the lungs of treated mice.**

Conclusion: **The administration of hsIgA to mice before intratracheal infection with *M. tuberculosis* or the pre-incubation of the bacteria with the antibody formulation induced the formation of well-organised granulomas and inflammatory lesions in lungs compared with non-treated animals which correlates with the protective effect already demonstrated by these antibody formulations.**

Keywords: colostrums, Mycobacterium tuberculosis, secretory immunoglobulin A

Introduction

It is known that humoral immunity is associated with protection against extracellular pathogens, whereas cellular immune responses are primarily associated with protection against intracellular pathogens (1). Thus, in the case of tuberculosis (TB), in both man and mouse, protective immunity is mediated by activated macrophages and Th1 lymphocytes (2). These cells are the most important constituents of granulomas, which are essential elements in the confinement and control the bacilli growth. These structures are produced by mice and humans with some differences (2). However, it is currently thought that humoral and cell-mediated immune responses collaborate in the protection against intracellular microbial pathogens (1,3–5). Most vaccines that are licensed and used against bacterial infections in humans are aimed at inducing a protective antibody response that allows the elimination of the microorganisms, including intracellular pathogens such as *Salmonella typhi* (6). Antibodies have the ability to control intracellular pathogens. Indeed, the prevalence of antibody susceptibility in the extracellular phase has been documented for certain categories of microbes (7,8). Secretory

antibodies trap exogenous antigens and exclude immune complexes with the assistance of a variety of innate mucosal defence mechanisms (9). SIgA is a highly stable antibody that can preserve its activity for prolonged periods of time even in hostile environments such as the gut lumen (10) and oral cavity (11). In particular, the function of sIgA is most likely enhanced by its high level of cross-reactive activity and its presence in human secretions (12). SIgA is not only present in external secretions but also has antimicrobial properties in epithelial cells during its transport across the epithelium. SIgA is the primary immunoglobulin type found in external secretions at a well-defined quantity, which provides specific immune protection for all mucosal surfaces by blocking the penetration of pathogens into the body (13).

Additional work is needed to understand the molecular mechanisms behind the IgA-mediated inhibition of pulmonary infection caused by *M. tuberculosis*. Reljic et al. proposed that the principal mechanism is the enhancement of the bactericidal functions of IgA on infected macrophages (14,15). IgA may lead to activation-dependent apoptosis causing bacterial death, or the blockage of bacterial interaction with the phagosomal membrane mediated by IgA/Gal-3, which is required for the inhibition of phago-lysosome fusion (15,16). In a previous work, the protective effect of hsIgA administered to mice, including significant decreases in pneumonic areas by morphometric evaluation, was demonstrated (17). To complement these previous results, we present a morphometric evaluation of granuloma areas and perivenular infiltrates as well as a detailed qualitative histopathological study of the effect of hsIgA on tissue damage in the lungs of Balb/c mice challenged with virulent *M. tuberculosis*.

Materials and Methods

HsIgA purification

HsIgA was purified from human colostrum donated by healthy mothers 3 to 5 days after delivery. The purification was performed by anion exchange chromatography and subsequent gel filtration, using DEAE Sepharose Fast Flow and preparative-grade Superose 6 (Pharmacia, Sweden), respectively. The presence of IgA in the chromatographic fractions was identified by dot blot analysis and the purity of hsIgA was verified by 12.5% sodium dodecylsulfate-polyacrylamide gel electrophoresis sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The purity of hsIgA was confirmed by the detection of only IgA heavy and light chains with a migration pattern corresponding to their molecular weights (18). After purification, the reactivity of hsIgA with *M. tuberculosis* antigens was demonstrated (17).

Mycobacterium tuberculosis culture

M. tuberculosis H37Rv (ATCC 27294) was grown to early mid-log phase in Middlebrook 7H9 medium (Difco, Detroit, MI) supplemented with albumin-dextrose-catalase (BBL, Cockeysville, MD) and 0.05% Tween 80 (Sigma Chemical Co., St. Louis, MO). Cultures were incubated at 37 °C with 5% CO_2 and shaken continuously for 28 days. The bacteria were harvested by centrifugation at 5000 × *g* for 15 minutes, resuspended in saline solution, dispensed in aliquots containing 10^6 bacteria/mL, and stored at –70 °C until use.

Inoculation and infection schedule

Three groups (n = 15 in each group) of male Balb/c mice of eight weeks of age were used as follows for inoculation and infection: the non-treated (NT) group, consisting of mice that were intratracheally infected with 2.5 × 10^5 CFU of *M. tuberculosis* in 100 µL of saline solution; the HsIgA-treated group, consisting of mice intranasally inoculated with purified hsIgA (1 mg of hsIgA in 50 µL of saline solution, 25 µL in each nostril) and intratracheally challenged with 2.5 × 10^5 CFU of *M. tuberculosis* 2 h after inoculation with the antibody; and the pre-incubated hsIgA group (Preinc), consisting of mice challenged intratracheally with 2.5 × 10^5 CFU *M. tuberculosis* previously incubated with 1 mg of the purified hsIgA for 4 h at room temperature. Five mice from each group were sacrificed on days 1, 7 and 60 after *M. tuberculosis* challenge and lungs were perfused with 10% formaldehyde dissolved in Phospahate Buffer Saline (PBS) and extracted for histopathological analysis. Infected mice were housed in individual micro-isolator cages in a Biosafety Level 3 (BL3) animal facility. All experimental procedures with animals were performed in a laminar flow cabinet in the BL3 facility, under anaesthesia and according to the guidelines approved by the Animal Ethics Committee of the National Institute of Medical Sciences and Nutrition, Mexico.

Tissue preparation, morphometric evaluation, and lung histopathology

The right lungs of each mouse were fixed with 10% formaldehyde dissolved in PBS solution, dehydrated in alcohol, cleared with xylol, and

embedded in paraffin. Lung tissues were sectioned at 3 μm and stained with hematoxylin and eosin using standard techniques. All lung tissues were mounted in the same orientation and sagittal sections.

For quantitative purpose, three sections with 100 μm of distance separating them were obtained from each mouse. Granulomas were defined as well-delimited nodular structures constituted by lymphocytes and macrophages. Well-formed granulomas were those lesions that included both cellular types, with the latter activated, as defined by the presence of large cells with large and compact cytoplasms and peripheral nuclei with finely dispersed chromatin. In each section, all the granulomas were measured with a determination of their surface area in square microns at 200× magnification using automated morphometry equipment (Leica Microsystems Imaging Solutions LTD, Cambridge, UK). Regarding blood vessels, all venules in transversal sections from 80–100 μm of diameter in each section were considered, with the area occupied by the inflammatory cells around these blood vessels measure in square microns. The data were analysed using one-way ANOVA and a post hoc Tukey multiple comparison procedure. *P* values under 0.05 were considered statistically significant. All data were analysed using GraphPad Prism 4 software.

Results

Histopathological analysis revealed no qualitative differences in the lungs of mice from the different groups at days 1 and 7 after infection. Sixty days after infection, however, there were pneumonic areas constituted by abundant macrophages with cytoplasmic vacuoles (foamy macrophages) and lymphocytes that occupied the alveolar lumen, with some areas of focal necrosis and large perivascular cuffs of lymphocytes in the non-treated group (Figure 1). At this time point, IgA treatment resulted in fewer lung areas affected by pneumonia, and lesions that consisted of predominantly abundant large activated macrophages (Figure 1). More organised granulomas in the Preinc and hsIgA groups were observed, with these lesions constituted by activated macrophages with large and compact cytoplasms and large nuclei with disperse chromatin; these cells were surrounded by numerous lymphocytes (Figure 1). In comparison, the control group showed smaller granulomas constituted predominantly by lymphocytes and activated macrophages. Both groups treated with IgA showed granulomas

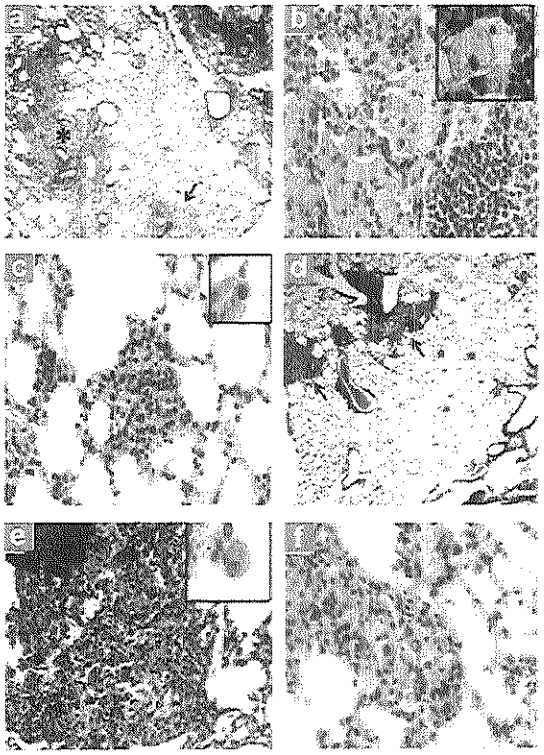

Figure 1: Representative histopathology of the lungs of mice treated with hsIgA in comparison with control non-treated tuberculous mice at day 60 of infection. (a) Low power micrograph shows extensive areas of pneumonia (asterisk) and focal necrosis (arrow) in the lung of a control mouse. (b) A high-power micrograph of these pneumonic areas reveals numerous vacuolated macrophages (inset). (c) A granuloma from the lung of a control mouse comprises small activated macrophages (inset). (d) In contrast, reduced pneumonic areas (arrow) are seen after 60 days of infection in mouse treated with hsIgA. (e) High power of this pneumonic area shows numerous activated macrophages with compact cytoplasms and large nuclei (inset). (f) Granuloma from the lung of mouse treated with hsIgA show numerous activated macrophages and abundant lymphocytes (low power micrograph 25× magnification, high power micrograph 200× magnification, inset 1000× magnification).

with similar cellular constitution and reduced perivascular inflammation from a qualitative point of view, but from the statistical point of view, the morphometry of perivascular inflammation and the total area occupied by granulomas were not significantly different (Figure 2).

Discussion

The qualitative histopathological study showed marked differences in the morphology and cellular composition of pulmonary granulomas between the control non-treated and treated mice with hsIgA. In mice treated with IgA monoclonal antibodies against 16 kDa protein of *M. tuberculosis*, more organised granulomas were reported in the treated group than in the non-treated control group or animals receiving a non related monoclonal antibody (18). Mice challenged with *M. tuberculosis* coated with an IgM monoclonal antibody against mycolyl-arabinogalactan-peptidoglycan complex also showed more organised granulomas than non-treated mice (19). At the same time, bacteria were present only in granulomatous lesions in treated animals compared with controls which showed a random presence of the bacteria in lung tissue (19). In a previous study with hsIgA there was correlation between the decrease bacterial load in lungs and the pneumonic area 60 days after the administration of hsIgA and *M. tuberculosis* pre-incubated with the same antibody formulation compared with non-treated animals (16). These results were confirmed and extended in the present study by the demonstration of different cellular composition in IgA treated animals that showed predominant activated macrophages in the pneumonic areas and granulomas, while in control animals the predominant cells in pneumonia were vacuolated macrophages. Foamy or vacuolated macrophages contain numerous bacilli and they show little immunostaining for the immunoprotective factors tumor necrosis factor alpha (TNF-a) and the induced isoform of nitric oxide synthase (iNOS); but strong immunoreactivity to the immunosuppressive cytokine transforming growth factor beta (TGF) (20,21). Thus, foamy macrophages are related to disease worsening, while activated macrophages show the inverse immunostaining cytokine profile and are associated to efficient bacilli growth control. In comparison with control mice, animals treated with IgA showed also bigger granulomas. In this mice model large granulomas are related to protection (22), and lesser inflammation around airways and blood vessels is also related to efficient bacilli growth control. Thus, IgA treatment well correlated with histological features related with efficient disease control.

It has been suggested that there are

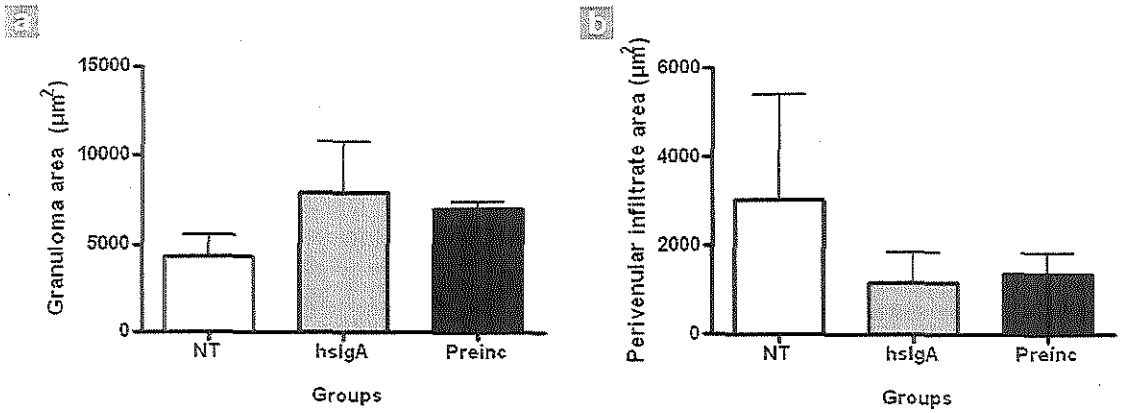

Figure 2: Determination of the granuloma (a) and perivenular inflammation (b) area in the lungs of both non-treated mice and mice treated with hsIgA 1, 7 and 60 days after inoculation with *M. tuberculosis*. NT: non-treated group; hsIgA: mice receiving human secretory IgA intranasally and intratracheally challenged with *M. tuberculosis*; Preinc: mice intratracheally receiving hsIgA preincubated with *M. tuberculosis*. Granulomas and perivenular infiltrate areas were measured, and the data were analysed using one-way ANOVA and a post-hoc Tukey multiple comparison procedure. $P < 0.05$ were considered statistically significant. No significant results between the groups.

essentially two mechanisms by which antibodies mediate protection against *M. tuberculosis* infection: the first is the opsonisation of mycobacteria which improves the processes of phagocytosis and intracellular killing by neutrophils and macrophages, and the second is the activation induced by immunoglobulins in antigen-presenting cells that can enhance the response of specific T cells against mycobacteria (24). Secretory IgA antibodies (sIgA) can operate by other protective mechanisms, such as the inhibition of bacterial or antigen adherence to mucosal surfaces by the properties conferred by the secretory component (25). Other additional mechanisms include the acceleration of immune complex elimination through respiratory ciliary movement and intestinal peristalsis, antibody-dependent cellular cytotoxicity (26), and the stimulation of antigen presenting cells for activation of T cells (27). Indeed, IgA protects the mucosal epithelial barrier through different mechanisms (28). sIgA antibodies interact with antigens at the stromal side of the epithelium, and immune complexes are engulfed and eliminated by phagocytic cells or are incorporated into the vascular system or passed through the epithelium associated with the polymeric immunoglobulin receptor (pIgR) (29,30). This latter process is called sIgA immune exclusion and is able to react to various antigens, including those expressed by bacteria, blocking adherence and microbial penetration of the epithelium and thereby providing an effective means of protection (28). sIgA also prevents mucosal infections by inhibiting the initial pathogen colonisation and eliminating epithelial cells without tissue damage during its transit to the lumen mediated by pIgR (31). In contrast to IgG, IgA is considered an anti-inflammatory element in secretions due to its minimal activating effect on the complement system (31).

The protective effect of IgA in experimental tuberculosis has been previously demonstrated by the intranasal inoculation of monoclonal TBA61 IgA antibodies against the α-crystallin (acr1) antigen of *M. tuberculosis* (32,33). However, it offered only a short duration of protection, which could be prolonged by the administration of IFN-γ three days before infection and further administration of IgA at 2 h before and two and seven days after aerosol infection with *M. tuberculosis* H37Rv (32). Rodriguez et al showed that IgA-deficient mice immunised with the mycobacterium cell surface antigen PstS-1 were more susceptible to intranasal infection with BCG than wildtype non-targeted littermate controls (34). Recently, it was shown that the combined intranasal administration of a novel human IgA monoclonal antibody (2E9IgA1) and recombinant mouse IFN-γ significantly reduced lung infection induced by *M. tuberculosis* H37Rv in CD89 transgenic mice but not in CD89-negative controls, indicating that 2E9IgA1-mediated protection largely depends on its interaction with CD89 (35). Our results on the qualitative evaluation of histopathological lesions after the administration of hsIgA correlate with other reports of the association between granuloma organisation and the presence of activated macrophages with the protective capacity of other antibody formulations (1,15,19).

Conclusion

Human secretory IgA antibodies purified from colostrum can interact with antigens and inhibit the adherence of diverse microrganisms to mucosal surfaces. In comparison with control non-treated mice, the protective role of hsIgA is notable due to the formation and consolidation of well-constituted granulomas with numerous activated macrophages in the lungs of infected mice pre-treated with purified hsIgA or infected with *M. tuberculosis* preincubated with purified hsIgA.

Acknowledgement

None.

Conflict of Interest

None.

Funds

This work was jointly supported by the Ministry of Higher Education, Malaysia LRGS Grant (203.PSK.6722001), Conacyt contract 84456 and the Ministry of Science and Technology, Cuba.

Authors' Contributions

Conception and design and final approval of the article: NA, NMN, MES, RHP, AA
Analysis and interpretation of the data: NA, JFI, MES, RHP, AA
Drafting of the article: NA, JFI, MMH
Critical revision of the article for the important intellectual content: NMN, MES, RHP, AA
Final approval of the article: NA, NMN, MES, RHP, AA

Provision of study materials or patient: DM, JB, RHP
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Virulence and Immune Response Induced by *Mycobacterium avium* Complex Strains in a Model of Progressive Pulmonary Tuberculosis and Subcutaneous Infection in BALB/c Mice

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The genus *Mycobacterium* comprises more than 150 species, including important pathogens for humans which cause major public health problems. The vast majority of efforts to understand the genus have been addressed in studies with *Mycobacterium tuberculosis*. The biological differentiation between *M. tuberculosis* and nontuberculous mycobacteria (NTM) is important because there are distinctions in the sources of infection, treatments, and the course of disease. Likewise, the importance of studying NTM is not only due to its clinical significance but also due to the mechanisms by which some species are pathogenic while others are not. *Mycobacterium avium* complex (MAC) is the most important group of NTM opportunistic pathogens, since it is the second largest medical complex in the genus after the *M. tuberculosis* complex. Here, we evaluated the virulence and immune response of *M. avium* subsp. *avium* and *Mycobacterium colombiense*, using experimental models of progressive pulmonary tuberculosis and subcutaneous infection in BALB/c mice. Mice infected intratracheally with a high dose of MAC strains showed high expression of tumor necrosis factor alpha (TNF-α) and inducible nitric oxide synthase with rapid bacillus elimination and numerous granulomas, but without lung consolidation during late infection in coexistence with high expression of anti-inflammatory cytokines. In contrast, subcutaneous infection showed high production of the proinflammatory cytokines TNF-α and gamma interferon with relatively low production of anti-inflammatory cytokines such as interleukin-10 (IL-10) or IL-4, which efficiently eliminate the bacilli but maintain extensive inflammation and fibrosis. Thus, MAC infection evokes different immune and inflammatory responses depending on the MAC species and affected tissue.

Although the genus *Mycobacterium* was described over a century ago (1, 2), the main research focus has been on *Mycobacterium tuberculosis*, currently considered the most important human bacterial pathogen. Indeed, tuberculosis (TB) is the worldwide leading cause of death produced by bacterial disease and is one of the most important challenges to public health (3–5). However, there are a large number of species known as atypical or nontuberculous mycobacteria (NTM) (6), which include nearly 140 species (1, 2) and although they are not considered a public health problem, their importance is increasing due to their frequent association with immunosuppression, especially in HIV/AIDS patients, which is highly fatal (7–11). Diseases caused by NTM are known collectively as mycobacteriosis, and the symptoms include lung infection, lymphadenitis, soft tissue or skin lesions, and even disseminated disease (12).

The biological differentiation between *M. tuberculosis* and NTM is important because it implies fundamental differences in the source of infection, treatment, and the course of the disease. Likewise, the importance of studying NTM is not only because of its clinical relevance but also because of the involved mechanisms by which some species of the genus are pathogenic while others are not. Although the epidemiology of TB has been extensively studied, the incidence worldwide and the prevalence of mycobacteriosis remains poorly understood, partly due to the fact that NTM diseases are not usually reported to public health centers. Mycobacteriosis estimates are based on occasional laboratory isolates and, in most cases, are suspected of being caused by *M. tuberculosis* (13–15). In addition, because the vast majority of the NTM are naturally resistant to drugs used against *M. tuberculosis* (12), it is not unusual that they are being wrongly identified and reported as multidrug-resistant *M. tuberculosis* strains (16, 17).

Mycobacterium avium complex (MAC) contains clinically important NTM worldwide and is the second largest medical complex in the *Mycobacterium* genus after the *M. tuberculosis* complex. MAC strains are frequently isolated worldwide, and currently *M. tuberculosis* and MAC are the mycobacterial species that require the biggest efforts in care and treatment within the genus (12, 14, 18–20). MAC affects patients with chronic obstructive pulmonary disease, cystic fibrosis, and mainly immunosuppressed individuals with HIV/AIDS (21, 22). MAC is composed of a number of different serovars, strains, subspecies, and morphological forms that differ in virulence (21, 22, 27). MAC is considered the leading cause of highly fatal systemic bacterial infection that affects 40% of

Received 1 February 2013 Returned for modification 18 March 2013
Accepted 29 July 2013
Published ahead of print 19 August 2013
Editor: J. L. Flynn
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doi:10.1128/IAI.00150-13

patients with HIV/AIDS and is the most common NTM pathogen group in the United States (12, 23). In most cases, it is not known which of the MAC species is the pathogen and, unlike the person-person transmission of *M. tuberculosis*, MAC transmission appears to occur from an environmental source (6, 24). *M. avium* is the most widely studied MAC species, is frequently isolated from drinking water, and could be the main source of infection for immunosuppressed individuals (25). Instead, pulmonary disease is the most common manifestation in immunocompetent individuals (26), whereas AIDS patients frequently present with a generalized infection (27).

Mycobacterium colombiense is a MAC species that was isolated from the sputum and blood of HIV/AIDS Colombian patients (28); this strain produced lymphadenopathy in immunocompetent children from France and Spain (29, 30) and was associated with pulmonary infections that complicated cases of cystic fibrosis (31). Therefore, this group of opportunistic pathogens have virulence mechanisms that allow them to adapt, survive, replicate, and produce disease in the host. However, the virulence and immune response evoked *in vivo* by members of these species has not been evaluated. The aim of the present study was to evaluate the virulence and immune response evoked by two MAC species (*M. avium* subsp. *avium* and *M. colombiense*) using experimental models of progressive pulmonary TB and subcutaneous infection in BALB/c mice.

MATERIALS AND METHODS

Selection of study strains. We used two different and well-characterized MAC species from the Spanish Type Culture Collection (CECT); *M. colombiense* CECT 3035 and *M. avium* subsp. *avium* CECT 7407. *M. tuberculosis* H37Rv (American Type Culture Collection [ATCC] 25618) was used as a control for comparison. Bacteria were grown in Middlebrook 7H9 broth (BD Difco) enriched with glycerol and albumin, catalase, and dextrose (Middlebrook ADC; BD Difco), in constant agitation at 37°C and 5% CO_2 during 21 days for *M. tuberculosis* H37Rv and 15 days for MAC. The stock cultures were stored at −70°C in 50% glycerol until use.

Experimental model of progressive pulmonary TB in BALB/c mice. Virulence (as determined by survival, pulmonary histopathology, and bacterial load) and immune response induced by each isolate were evaluated in 8-week-old male BALB/c mice as previously described (32–35). Briefly, bacteria were grown as described above and, as soon as the culture reached the log phase, the bacilli were harvested, and the concentration was adjusted to 2.5×10^5 viable bacilli per 100 μl of phosphate-buffered saline (PBS), as determined by fluorescein diacetate (Sigma-Aldrich) incorporation. Progressive pulmonary TB induction was performed as follows: mice were anesthetized with sevoflurane vapors and inoculated intratracheally using a sterile cannula (Thomas Scientific, catalog no. 1121A12, straight, 22G×1"), with 2.5×10^5 bacilli in 100 μl of PBS. Injected mice were kept in a vertical position until the effect of anesthesia passed. Two independent experiments were performed; in each experiment three groups of 50 mice were infected with either MAC species or H37Rv, and 10 mice more from each group were left undisturbed to record survival from day 1 up to day 120 after infection. Six animals from each group were sacrificed by exsanguination at 1, 3, 7, 14, 21, 28, 60, and 120 days after infection. One lung lobe, right or left, was perfused with ethanol and prepared for histopathological studies. The other lobe and other samples (whole blood, spleen, and mediastinal lymph nodes) were snap-frozen in liquid nitrogen and stored at −70°C for microbiological and immunological analyses. Infected mice were kept in cages fitted with microisolators connected to negative pressure. All procedures were performed in a class III cabinet in a biosafety level III facility according to the guidelines and approval by the Animal Experimentation Ethics Committee of the National Institute of Medical Sciences and Nutrition of Mexico.

Experimental model of subcutaneous infection in BALB/c mice. The experimental model was set up in 6- to 8-week-old male BALB/c mice. Bacteria were grown as described above and, as soon as the culture reached log phase, the bacilli were harvested, and the concentration was adjusted to 2.5×10^5 viable bacilli per 100 μl of PBS, as determined by diacetate of fluorescein incorporation (Sigma-Aldrich). As reported previously (36), groups of 50 animals were inoculated subcutaneously with 2.5×10^5 bacilli suspended in 40 μl of PBS for each individual strain utilizing a sterile syringe and needle in each footpad, and 10 mice from each group were left undisturbed to record survival from day 1 up to day 120 after infection. Three animals from each group were sacrificed by exsanguination at 3, 14, 21, 28, 60, and 120 days after infection. One footpad, right or left, was prepared for histopathological studies, and the other footpad was snap-frozen in liquid nitrogen and stored at −70°C for microbiological and immunological analysis. Infected mice were kept in cages fitted with microisolators connected to negative pressure.

Tissue preparation for histology and automated morphometry. One lung lobe from each mouse was fixed by intratracheal perfusion with 10% formaldehyde dissolved in PBS for 24 h and then sectioned through the hilus and embedded in paraffin. Sections, 5 μm thick, were stained with hematoxylin and eosin for the histological and morphometric analysis using an automated image analyzer (Carl Zeiss, Ltd., Herts, United Kingdom) as previously described (33).

Regarding the subcutaneous tissue analysis, one footpad from each of the three mice per time point was obtained, immediately fixed by immersion in 10% formaldehyde dissolved in PBS during 24 h, and then sectioned longitudinally and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin and analyzed.

Determination of CFU in infected tissues. Right or left lung lobes, whole blood, mediastinal lymph nodes, and spleens from three mice at each time point and in two independent experiments were used for CFU counting. The tissues were homogenized with a Polytron (PT 3100-Laboratoryhomogenizer; Kinematica Dispersing and Mixing Technology) in sterile 50-ml tubes containing 1 ml of PBS–1× Tween 80 (0.05%). Three dilutions of each homogenate were spread onto duplicate plates containing Middlebrook 7H10 agar (BD Difco) enriched with glycerol, albumin, oleic acid, dextrose, and catalase (Middlebrook OADC; BD Difco). Colonies were counted twice under a stereoscopic microscope (STEMI 2000 MICR-PA 085; Carl Zeiss) after 21 days of incubation for *M. tuberculosis* H37Rv and 15 days for MAC (33).

Three footpads, right or left, collected from infected and control mice at each time point were used for colony counting. Tissues were ground with a mortar and then homogenized with a Polytron; dilutions of each homogenate were spread, incubated, and counted as described above.

Kinetics of cytokines gene expression determined by real-time PCR in tissue homogenates. Right or left lung lobes and footpads from three different mice per group were used to isolate total RNA using an RNeasy minikit (Qiagen Sample & Assay Technologies) according to the manufacturer's recommendations. Total RNA quality and quantity were evaluated through spectrophotometry (using a 260/280 absorbance ratio) and on agarose gels. Reverse transcription was performed using Omniscript reverse transcriptase (Qiagen) from 100 ng of total RNA, 150 μg of Oligo(dT) 15 primer (Promega Corp.)/ml, 10 U of RNase inhibitor (Invitrogen/Life Technologies), 1× reverse transcriptase buffer, 0.5 mM concentrations of each deoxynucleoside triphosphate, and 4 U of Omniscript reverse transcriptase. Real-time PCR was performed using the 7500 real-time PCR system (Applied Biosystems) and a QuantiTect SYBR green PCR kit (Qiagen). Standard curves of quantified and diluted PCR product, as well as negative controls, were included in each PCR run. Specific primers were used for the following targets: GAPDH (glyceraldehyde-3-phosphate dehydrogenase), 5′-CATTGTGGAAGGGTCCATGA-3′ and 5′-GGAAGGCCATGCCAGTGAGC-3′; tumor necrosis factor alpha (TNF-α), 5′-TGTGGCTTCGACCTCTACCTC-3′ and 5′-GCCGAGAAA GGCTGCTTG-3′; gamma interferon (IFN-γ), 5′-CCTCAAACTTGGCA ATACTCAT-3′ and 5′-GGTGACATGAAAATCCTGCAG-3′; inducible

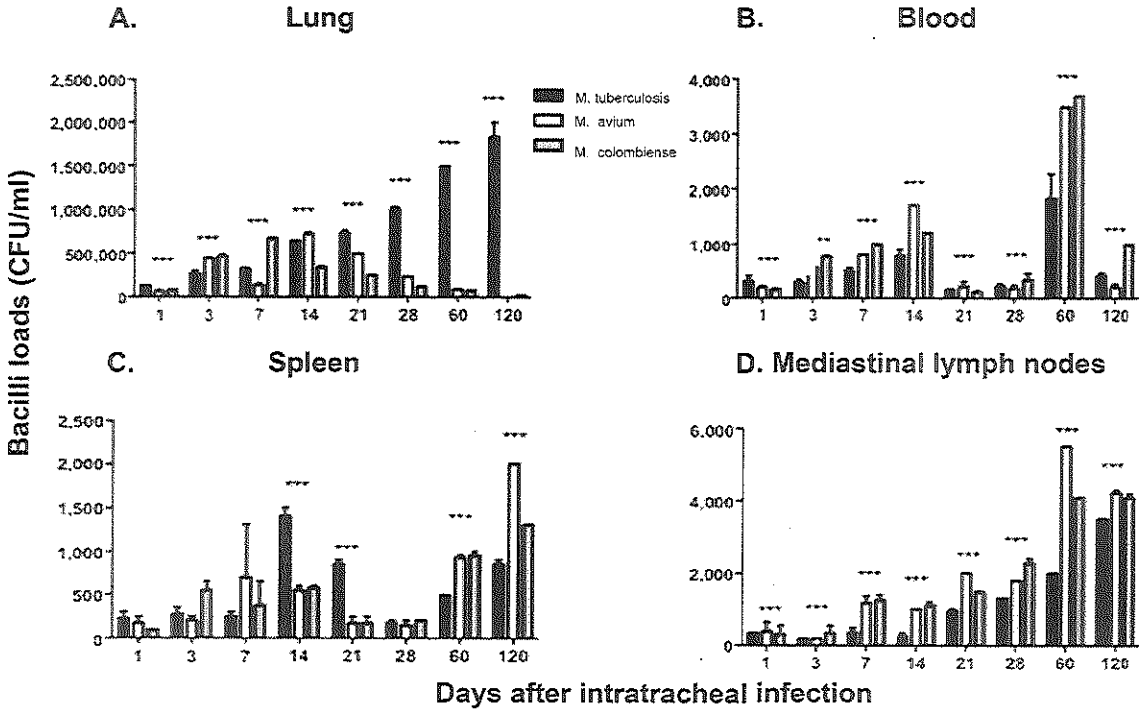

FIG 1 Lung bacillus loads in BALB/c mice infected by intratracheal injection. Mice were infected with *M. colombiense* (grey), *M. avium* (white), or *M. tuberculosis* strain H37Rv (black) and euthanized at different time points after infection, and the indicated organs were used to determine the number of CFU. Asterisks represent statistical significance ($P < 0.005$) comparing MAC strains with *M. tuberculosis* H37Rv.

nitric oxide synthase (iNOS), 5′-CATTTCGCTGTCTCCCCAA-3′ and 5′-AGCGAGGAGCAGGTGGAAG-3′; interleukin-4 (IL-4), 5′-CGTCCT CACAGCAACGGAGA-3′ and 5′-GCAGCTTATCGATGAATCCAGG-3′; transforming growth factor (TGF), 5′-AGGGCTACCATGCCAACTT CT-3′ and 5′-CCGGGTTGTGTTGGTTGTACA-3′; and IL-10, 5′-AAAG GCACTGCACGACATAGC-3′ and 5′-TGCGGAGAACGTGGAAAAA C-3′. The cycling conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles at 95 for 20 s, 58 to 60°C for 20 s, and 72°C for 35 s. Quantities of the specific mRNA in the sample were measured according to the corresponding gene-specific standard. The mRNA copy number of each cytokine was related to one million copies of mRNA encoding the GAPDH gene (37). H37Rv was used as a control for all experiments in the BALB/c model.

Cytokine production determined by immunohistochemistry and digital automated morphometry. The same paraffin-embedded material prepared for histopathological studies was used to determine the local cytokine production by immunohistochemistry. Lung and footpad sections from mice infected with either MAC or *M. tuberculosis* H37Rv strain, obtained at early (day 21) and late (day 120) infection, were deparaffinized and maintained in 1× HCN buffer (HEPES, NaCl, and $CaCl_2$). Sections were washed with 1× HCN plus 0.05% Tween 20, and the endogenous peroxidase activity was blocked with 6% H_2O_2 dissolved in 1× PBS plus 0.1% sodium azide, followed by incubation for 1 h. After blocking with normal swine serum, tissue sections were incubated with primary antibodies overnight at 4°C at optimal dilutions, which had been determined previously. We used primary antibodies against TNF-α (rabbit polyclonal IgG clone H-156; Santa Cruz Biotechnology), IFN-γ (goat polyclonal IgG clone D-17; Santa Cruz Biotechnology), IL-4 (goat polyclonal IgG; Santa Cruz Biotechnology), and IL-10 (goat polyclonal IgG clone M-18; Santa Cruz Biotechnology). Secondary biotinylated antibodies (biotin–anti-rabbit IgG or biotin–anti-goat IgG) were used to detect the binding of the primary antibodies. Finally, horseradish peroxidase-conjugated avidin and 3,3-diaminobenzidine–hydrogen peroxide were used to develop the reaction. Tissue sections were counterstained with hematoxylin.

For digital automated morphometry, slides were scanned for each strain and tissue using an Aperio ScanScope (Aperio Technologies, Vista, CA). After saving each digital image, all of the lung area or the footpad inflammatory infiltrate were selected for analysis. Aperio ImageScope software (Aperio) was used with the application of the Aperio Pixel Count v9 algorithm, which is based on the spectral differentiation between brown (positive) and blue (counter) staining and provide a number of 1+, 2+, and 3+ intensity positive pixels, a mean of intensity was determined from each slide that corresponded to the estimated concentration of each selected cytokine.

Statistical analysis. Two-way analysis of variance was used to determine the statistical significance of CFU and cytokines. A P value of <0.05 was considered significant.

RESULTS

Survival, histopathology, and bacillary loads in mice infected by the intratracheal route. In order to characterize the *M. avium* and *M. colombiense* virulence in the model of pulmonary TB, groups of BALB/c mice were infected intratracheally with 2.5×10^5 bacilli of either MAC species or *M. tuberculosis* H37Rv strain as a comparative control. All of the animals infected with *M. avium* or *M. colombiense* survived after 4 months of infection. In contrast, mice inoculated with *M. tuberculosis* started to die at 8 weeks postinfection, and 50% survived after 120 days of infection (data not shown). These survival rates associated well with the CFU quantifications in lung homogenates where, by the third week postinfection, a lower bacterial load was found in mice infected with *M. colombiense* even though similar numbers of CFU had been detected in the three groups during the first week of infection (Fig. 1A). In comparison to animals infected with *M. tuberculosis*, at days 21, 28, and 60 postinfection, significantly lower bacterial loads were found in the lungs of mice infected with *M. colombiense*

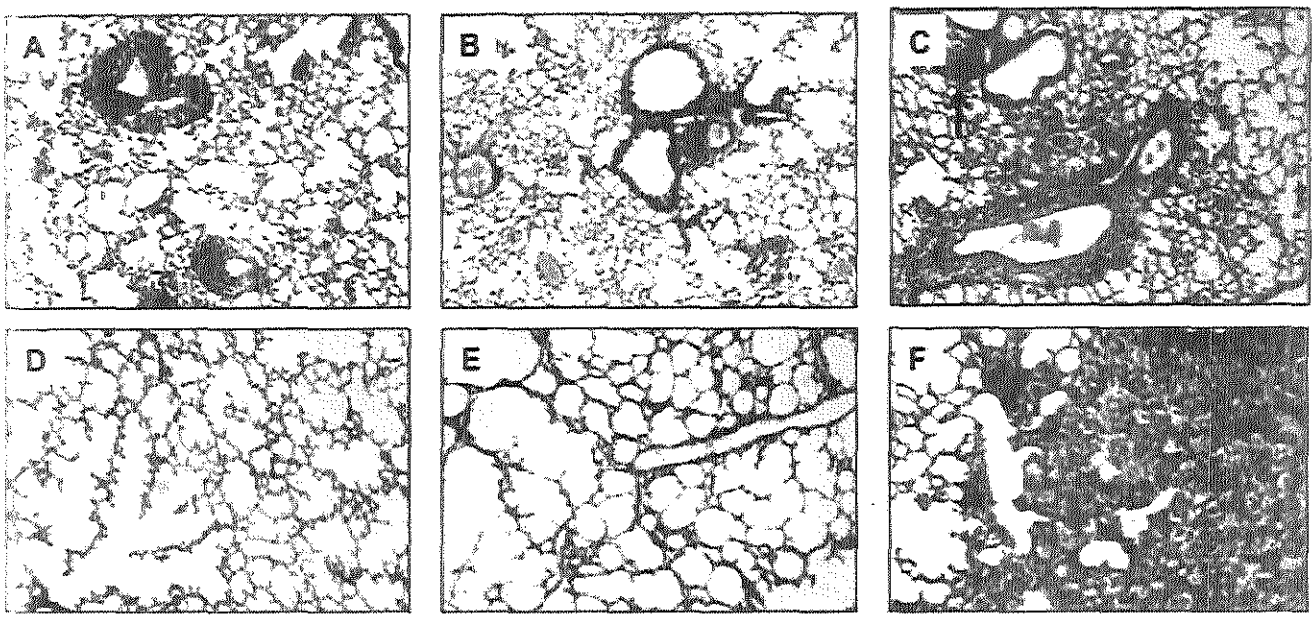

FIG 2 Representative lung histopathology in mice infected by the intratracheal route with MAC strains and *M. tuberculosis*. (A and B) *M. colombiense* (A) and *M. avium* (B) induced extensive inflammatory infiltrate around blood vessels and bronchioles after 21 days of infection. (C) *M. tuberculosis* infection induced perivascular inflammation with well-formed granulomas (arrow). (D) At day 120 after infection, there is almost normal histological appearance in the lungs of a mouse infected with *M. colombiense*. (E) Slight inflammation around small blood vessels was observed after 120 days of infection with *M. avium*. (F) Extensive pneumonia was evidenced after 4 months of infection with *M. tuberculosis* H37Rv. Hematoxylin and eosin staining; all micrographs, ×200 magnification.

or *M. avium*, being fewer in the former, while at day 120 almost undetectable live bacilli were observed in animals infected with either MAC species. In contrast, a progressive increase of bacillus loads in the lung was seen in mice infected with *M. tuberculosis*, raising its peak at day 120 postinfection (Fig. 1A). In blood, after 3 days of infection, mice infected with either MAC species showed a progressive increase of the bacillus load for 2 weeks, followed by a transient decrease by days 21 and 28 postinfection and a new increase at day 60 postinfection. At almost all of these time points, the bacillus loads in blood from mice infected with *M. avium* or *M. colombiense* were significantly higher than in mice infected with *M. tuberculosis* (Fig. 1B). A similar trend was observed in spleens and mediastinal lymph nodes (Fig. 1C and D, respectively), where, after the first week during late infection at days 60 and 120, higher bacillus loads were determined in animals infected with MAC strains than with *M. tuberculosis*.

The histopathological analysis of the lungs showed after 2 and 3 weeks of infection with MAC, extensive inflammatory infiltrate constituted by lymphocytes and macrophages located in the alveolar-capillary interstitium, as well as around venules and bronchioles (Fig. 2A and B). At this point during the infection, larger granulomas (12,703 ± 1,000 μm^2) were formed than those produced by *M. tuberculosis* (4,025 ± 670 μm^2); 2 weeks later, granulomas induced by MAC (9,215 ± 435 μm^2) were similar in size to those in the lungs of *M. tuberculosis*-infected mice (9,963 ± 650 μm^2). During late infection, granulomas progressively declined in number and size, being very few in number and small at day 120 postinfection with either MAC strain (2,844 ± 320 μm^2). It is important emphasize that there was little pneumonia in MAC-infected mice (<5%) and that the inflammatory response decreased substantially after 1 month of infection, with only occasional cuffs of lymphocytes around blood vessels and mild hyperplasia of the lymphoid tissue associated with bronchial epithelium observed at day 120 postinfection (Fig. 2D). As shown in Fig. 2D and E, at day 120 there was almost normal histological appearance in the lung of mouse infected with *M. colombiense* and *M. avium*. In contrast, *M. tuberculosis*-infected mice showed progressive pneumonia after 28 days postinfection, reaching its peak at day 120 when 80 ± 10% of the lung surface was affected (Fig. 2F).

Cytokine gene expression and production in the lungs of infected mice. TNF-α gene expression in mice infected with *M. tuberculosis* was rapid and higher during early infection and was followed by a progressive decrease during late disease (Fig. 3A). In contrast, whereas infection with *M. colombiense* induced the highest expression of TNF-α during early infection at days 3 and 7, and this peak was followed by a progressive decrease, in mice infected with *M. avium* TNF-α showed progressive expression peaking at day 120 (Fig. 3A). Similar kinetics were observed in iNOS gene expression (Fig. 3B). The kinetics of IFN-γ gene expression were similar among the three groups, with higher expression during early infection peaking at day 14, followed by progressive decrease, being higher in mice infected with *M. colombiense* strain (Fig. 3C). Regarding the expression of anti-inflammatory cytokines, mice infected with *M. colombiense* showed the highest TGF-β expression from days 3 to 21, whereas animals infected with *M. avium* exhibited the highest TGF-β expression at days 28 and 60, and both MAC species induced higher TGF-β expression than did *M. tuberculosis* (Fig. 3D). *M. tuberculosis* infection induced progressive IL-4 expression, whereas *M. colombiense* induced lower and stable IL-4 expression, and animals infected with *M. avium* exhibited higher IL-4 expression during early infection (Fig. 3E). During the first week of infection, *M. colombiense* induced high expression of IL-10, whereas *M. avium* and *M. tuberculosis* induced

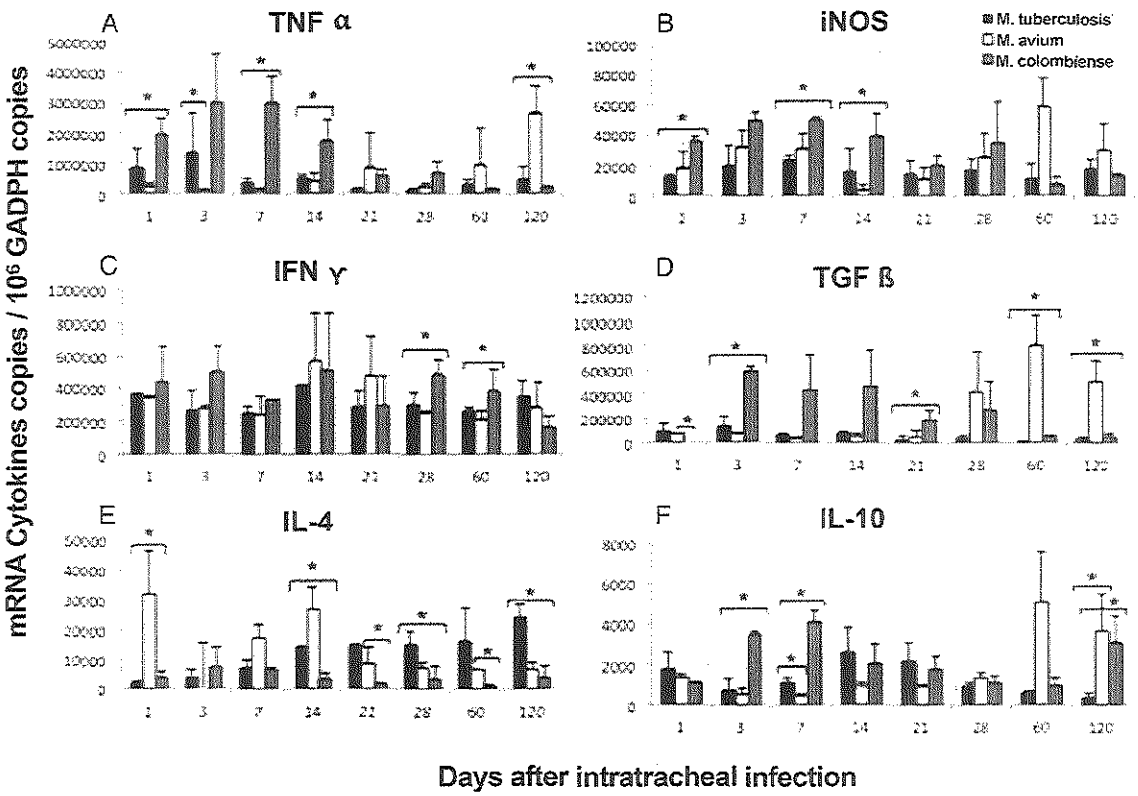

FIG 3 Kinetics of cytokines and iNOS gene expression in mice infected by the intratracheal route. BALB/c mice were infected with *M. colombiense* (gre... *M. avium* (white), or *M. tuberculosis* strain H37Rv (black). Groups of three animals were sacrificed at different time points; gene expression of iNOS and of t... indicated cytokines in the lungs was determined by real-time PCR. The results are expressed as means and the standard deviations from three mouse group... Asterisks represent statistical significance ($P < 0.05$) of MAC strains compared to *M. tuberculosis* H37Rv.

similar mild expression during late infection at day 120, and both MAC species induced higher expression of IL-10 (Fig. 3F).

Considering that the cytokine gene expression determined by real-time reverse transcription-PCR cannot inform the cellular source and since total tissue homogenates used to isolate RNA may not reflect protein levels, we performed immunohistochemistry and digital quantitative image analysis of lung sections comparing early infection, specifically examining when protective immunity against *M. tuberculosis* is maximal in this murine model (day 21) and for late disease (day 60) (37). Activated macrophages were the principal source of TNF-α, being 2-fold higher after 21 days of infection in animals infected with *M. tuberculosis* than with either MAC strain, whereas similar low TNF-α production was seen at day 120 postinfection (Fig. 4). At day 21 postinfection, a trend similar to that seen with TNF-α was observed for IFN-γ production, with lymphocytes being the most common immunostained cells, while at day 60 of infection mice infected with *M. tuberculosis* showed lower IFN-γ production than at day 21, but it was still significantly higher than in mice infected with either MAC strain. Regarding anti-inflammatory cytokines, low production of both IL-10 and IL-4 was determined at day 21 of infection with either MAC strain or *M. tuberculosis*, while at day 60 the lungs of mice infected with *M. tuberculosis* showed 2-fold more IL-10 and IL-4 production than in mice infected with MAC, with lymphocytes being the predominant immunostained cells located in the pneumonic areas in *M. tuberculosis* infection and in perivascular inflammation in MAC-infected animals (Fig. 4).

Survival, histopathology, and bacillary loads in mice infected subcutaneously. Although it is not common, *M. avium* may penetrate the subcutaneous tissue following traumatic inoculation through the skin (37, 38). To characterize the *M. avium* and *M. colombiense* virulence in subcutaneous tissue infection, groups of BALB/c mice (60 per group) were infected in both footpads with either MAC strains or *M. tuberculosis* H37Rv as comparative control. All of the animals survived after 4 months of infection (data not shown).

Both NTM strains showed progressive increases in the bacillus burden, reaching a peak at day 28 that was higher than in the *M. avium* infection; this peak was followed by a pronounced decrease until day 120, when the lowest level was detected (Fig. 5A). In contrast, animals infected with *M. tuberculosis* showed bacillus burdens characterized by a progressive decrease throughout the time points measured, and these burdens were consistently lower than in mice infected with either MAC strain (Fig. 5A). In the regional lymph nodes (inguinal) at any time point measured—except at day 28 postinfection—the bacillus loads were higher in animals infected with MAC than in mice infected with *M. tuberculosis*. Late during infection, at days 60 and 120, almost 5-fold more CFU were counted in MAC-infected mice than in *M. tuberculosis*-infected mice, the counts being highest in mice infected with *M. avium* (Fig. 5B).

Extensive and progressive chronic inflammatory infiltrate was seen in the footpad subcutaneous tissue of mice infected with MAC strains at day 60 (data not shown) and 120 when some

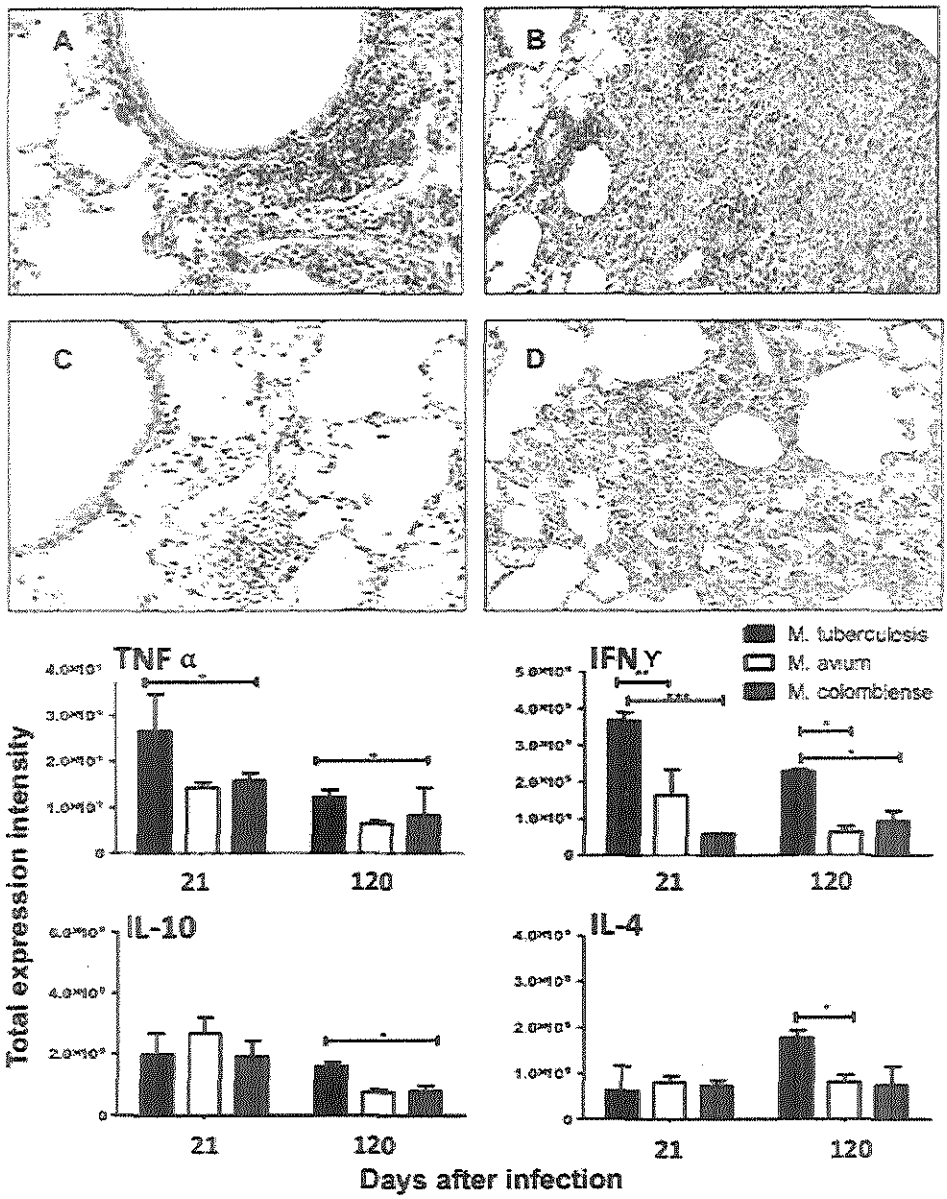

FIG 4 Representative micrographs of cytokine detection (top) and automated morphometry analysis (bottom) from the lungs of infected mice. (A) Numerous IFN-γ immunostained lymphocytes around the bronchial wall and blood vessels after 120 days of infection with *M. colombiense*. (B) Pneumonic areas after 120 days of infection with *M. tuberculosis* show abundant IFN-γ immunostained cells. (C) Numerous IL-4 immunoreactive lymphocytes are located in the inflammatory infiltrate around the bronchial wall and blood vessels after 120 days of infection with *M. colombiense*. (D) Numerous IL-4 immunostained lymphocytes in the pneumonic areas after 120 days of infection with *M. tuberculosis* strain H37Rv. The bottom panels show quantification of the indicated cytokines by the determination of staining intensity using digital automated morphometry. Results are expressed as means and the standard deviations from three mice per group at days 21 and 120 of infection. Asterisks represent statistical significance ($P < 0.05$) comparing MAC strains with *M. tuberculosis* H37Rv.

animals showed focal areas of necrosis and fibrosis, with numerous lymphocytes infiltrating not only the connective tissue but also the muscle and adipose tissues (Fig. 5C and D). In contrast, since the third day of infection, *M. tuberculosis* induced mild inflammation that consisted of lymphocytes and macrophages spread in the connective tissue, with occasional granulomas seen after 28 days (data not shown). However, the infection did not spread beyond the limb with any of the mycobacterial strains studied. As shown in Fig. 5E, at day 120 of *M. tuberculosis* infection, scarce chronic inflammatory infiltrate in the connective tissue and around the blood vessels was observed.

Cytokine gene expression and production in the subcutaneous tissues of infected mice. The expression of the proinflammatory cytokines TNF-α and IFN-γ in the subcutaneous tissues of mice infected with *M. colombiense* was rapid, stable, and highest throughout the course of infection; iNOS exhibited similar kinetics but with fewer transcripts. Subcutaneous infection with *M. avium* induced low and stable expression of TNF-α, iNOS, and

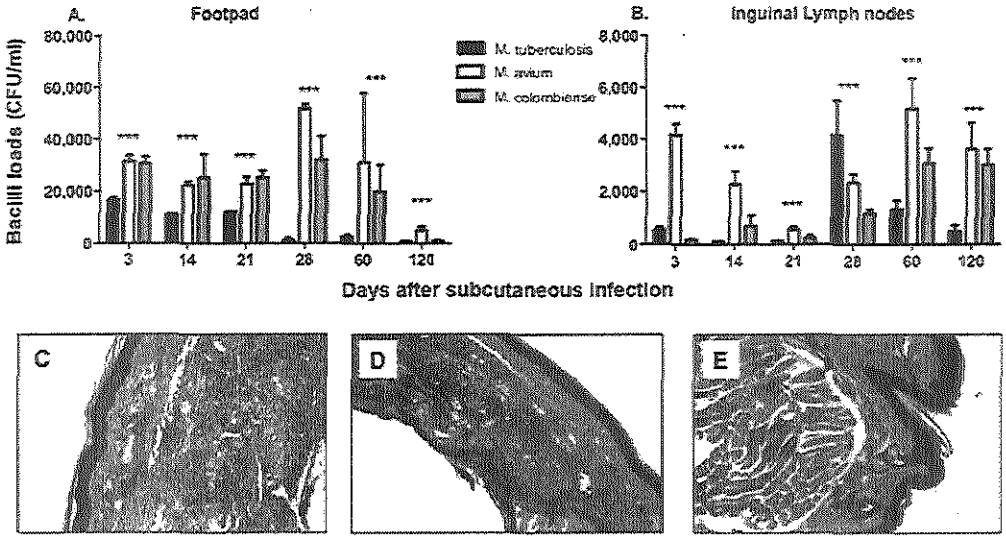

FIG 5 Bacillus loads and representative histopathology in BALB/c mice infected in the footpad subcutaneous tissue. Mice were infected with *M. colombiense* (grey), *M. avium* (white), or *M. tuberculosis* H37Rv (black) and euthanized at different time points after infection, and the footpads (A) and inguinal lymph nodes (B) were used to determine the numbers of CFU. Asterisks represent statistical significance ($P < 0.005$) comparing MAC strains with *M. tuberculosis*. Subcutaneous tissues of a mouse after 120 days of infection with *M. colombiense* (C) and *M. avium* (D) show the extensive chronic inflammatory infiltrate. (E) Mild inflammation is shown in a mouse 120 days after subcutaneous infection with *M. tuberculosis* H37Rv (hematoxylin and eosin staining; magnification, ×20)

IFN-γ during early infection, similarly to *M. colombiense* during late infection at days 60 and 120 (Fig. 6A to C). Animals subcutaneously infected with *M. tuberculosis* exhibited the lowest levels of transcripts encoding TNF-α and iNOS proinflammatory factors (Fig. 6A and B). Although the levels of transcripts for IFN-γ in the footpads of mice infected with the three strains were rather similar throughout the first month, late in the infection (day 120) the levels were higher in footpads infected with *M. tuberculosis* than in those infected with MAC (Fig. 6C). With regard to the levels of transcripts encoding anti-inflammatory cytokines, during the first month of infection there was similar low expression in animals infected with either strain. At days 60 and 120, there was higher expression, with the levels being similar in the case of TGF-β, and animals infected with *M. avium* showed the highest IL-10 expression but relatively low numbers of transcripts and similar higher levels for IL-4 in MAC infections (Fig. 6).

Quantitative immunohistochemistry analysis showed at day 21 postinfection a similar high expression of TNF-α production in mice infected with *M. tuberculosis* or either MAC strain, with macrophages being the most commonly immunostained cells, while at day 60 of infection animals infected with *M. tuberculosis* showed significantly lower TNF-α production than did MAC-infected mice, the highest levels being detected in *M. avium*-infected footpads (Fig. 7). Animals infected with *M. avium* showed the highest production of IFN-γ at days 21 and 60 of infection, while *M. colombiense* induced similarly high production: 2-fold higher than *M. tuberculosis* at day 60 of infection (Fig. 7). Regarding anti-inflammatory cytokines, at day 21, higher production of IL-10 was determined in the footpads of mice infected with *M. tuberculosis* or *M. avium* than in the footpads of mice infected with *M. colombiense*, while at day 120 postinfection, similar low production levels were observed in the footpads of MAC- or *M. tuberculosis*-infected animals. A comparable trend was observed for IL-4 production (Fig. 7).

DISCUSSION

The *Mycobacterium avium* complex (MAC) consists of nine recognized species and a variety of strains that may be members of undescribed taxa (12, 28, 38–41). MAC can induce four distinct clinical syndromes: pulmonary disease, lymphadenitis, disseminated disease, and skin disease (12). Human infection by MAC is believed to be initiated by the respiratory airways or the intestinal tract. We used intratracheal infection with a high bacillus dose in BALB/c mice in order to reproduce one of the most common infection routes in humans and compared two MAC species and *M. tuberculosis* H37Rv, which has been extensively studied in this mouse model (32, 33, 35, 37). When BALB/c mice are infected with *M. tuberculosis* H37Rv, a T helper cell type 1 response is developed that peaks at 2 to 3 weeks, temporarily controlling bacterial growth (33). After this control, bacterial proliferation recommences, accompanied by increasing anti-inflammatory cytokine expression, such as TGF-β, IL-10, and IL-4, and decreasing IFN-γ, TNF-α, and iNOS expression, along with extensive tissue damage and death of the animals (33, 37). Both MAC species induced different disease evolution with total mice survival and scarce pneumonia (<5%) without necrosis or fibrosis. However, both MAC strains induced moderate inflammatory infiltrate around middle-size blood vessels and airways during the first month of infection, as well as granulomas. During the late stage of the infection, the inflammation was almost totally cleared. In addition, mild perivascular inflammatory cuffs and hyperplasia of the lymphoid tissue associated with bronchial mucosa with some intra-alveolar activated macrophages were seen. MAC-infected mice showed an increase in CFU during the first 2 weeks of infection, followed by a progressive decline that was almost undetectable after 4 months of infection. Although the bacillus load kinetics were similar, mice infected with *M. avium* had higher bacterial burdens than animals infected with *M. colombiense*, indicating a

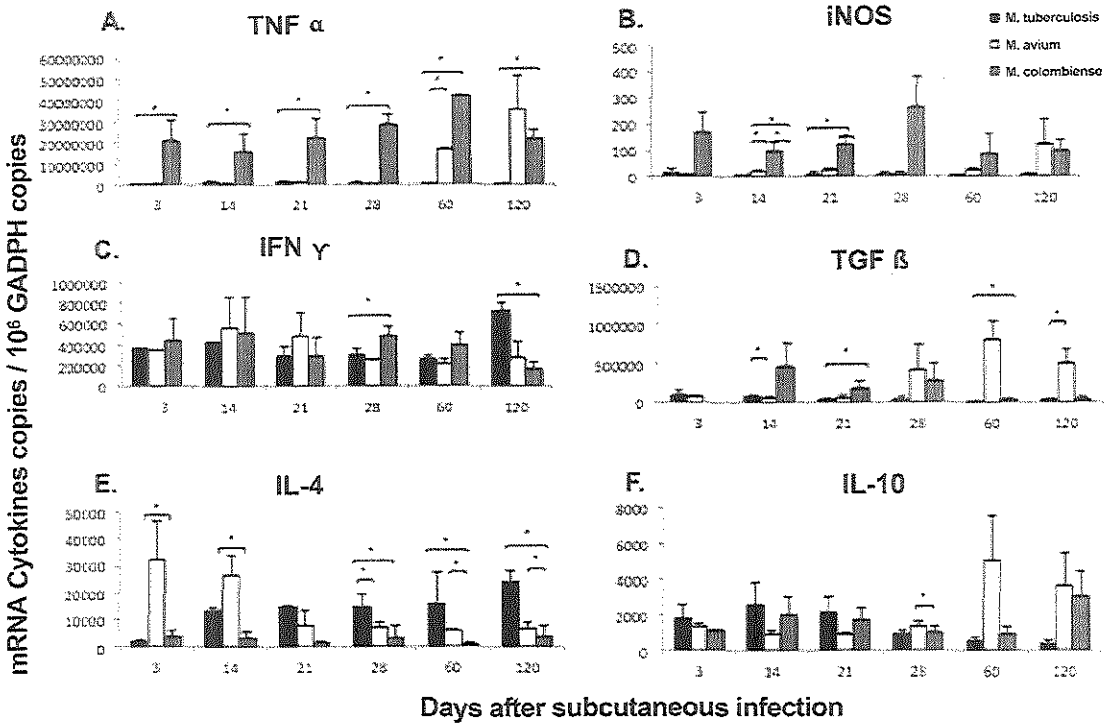

FIG 6 Kinetics of cytokines and iNOS gene expression in mice infected in the footpad subcutaneous tissue. BALB/c mice were infected with *M. colombiense* (grey), *M. avium* (white), or *M. tuberculosis* H37Rv (black). Groups of three animals were sacrificed at different time points; the gene expressions of iNOS and of the indicated cytokines were determined by real-time PCR. The results are expressed as means and the standard deviations from three mice per group. Asterisks represent statistical significance ($P < 0.05$) comparing MAC strains with *M. tuberculosis*.

higher virulence of the former, but both MAC strains were clearly attenuated compared to *M. tuberculosis* H37Rv. It is interesting that in spite of its attenuation, higher bacillus loads were observed in the blood of mice infected with either MAC species than in *M. tuberculosis*-infected animals. During the late stage of the infection with MAC, there were also higher bacillus loads in the spleen and mediastinal lymph nodes. It appears that MAC strains disseminate more efficiently than *M. tuberculosis* H37Rv and, perhaps due to this ability, systemic MAC infections are common in immunodeficient patients.

The cytokine expression profiles in infected lungs were quite different. Intratracheal infection with *M. colombiense* induced rapid and very high expression of TNF-α during the first and second weeks of infection, followed by a progressive decrease. This TNF-α expression was highest at days 3 and 7 postinfection and exhibited a trend similar to that of iNOS expression. In contrast, *M. avium* induced low and stable TNF-α expression during the first month of infection, followed by high expression during late infection, peaking at day 120. TNF-α production in *M. avium* determined by immunohistochemistry, however, was similar to *M. colombiense*. The iNOS gene expression kinetics were similar to those of TNF-α, which coincided with very low bacillus burdens during late infection. Thus, MAC infection was efficiently controlled, with complete animal survival and minimal tissue damage. TNF-α is one of the most important cytokines produced by macrophages after *M. avium* recognition and ingestion (42), and the *M. avium* morphotype and virulence often determine the amount of the cytokine secreted (42, 43). Although some studies show that nitric oxide is relatively ineffective in *M. avium* removal, since most strains are not susceptible to its toxic effects (44), our results showed that iNOS gene expression kinetics are similar to those of TNF-α, suggesting that nitric oxide may participate in the control of bacillus growth.

Adaptive immunity to *M. avium* is centered on $CD4^+$ T cells and IFN-γ production. Depletion studies have shown that $CD4^+$ but not $CD8^+$ T lymphocytes are required for adaptive immunity against *M. avium* (45). Antibody blocking of IFN-γ exacerbates *M. avium* infection (45), and mice deficient in the expression of this cytokine are more susceptible to the infection (46, 47). Our results showed that the lungs of animals infected with *M. avium* displayed IFN-γ expression kinetics and protein production similar to that of mice infected with *M. tuberculosis*, exhibiting higher expression during early infection, followed by a progressive decrease. *M. colombiense* induced slightly higher IFN-γ production in the earlier stage of the infection rather than later, suggesting that this strain is more efficient at maintaining Th1 responses than is *M. avium*, an observation that could indicate a higher immunogenicity and lower virulence for *M. colombiense* than for *M. avium*. What was not expected was high IFN expression through the course of the infection in BALB/c mice infected with *M. tuberculosis*. The reason for this finding is unknown. An uninfected control group was not included in the present study that would have allowed us to evaluate this result.

The type 2 response mediated by IL-4, IL-13, IL-10, and TGF-β does not seem to have a significant role in determining susceptibility to infection (48). *M. avium* infection in C57BL mice does

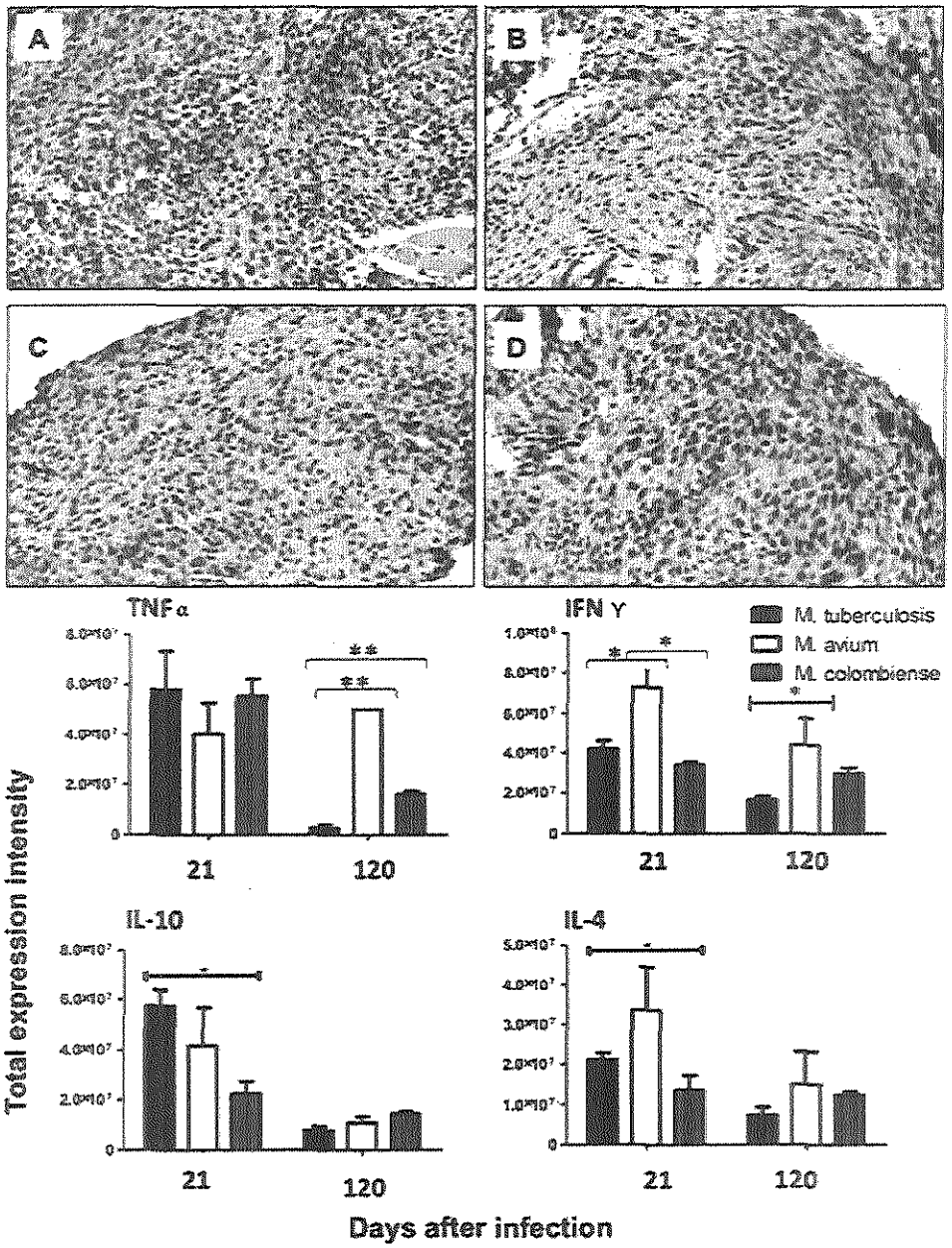

FIG 7 Representative micrographs of cytokine detection (top) and automated morphometry analysis (bottom) from the footpads of infected mice. (A) Numerous TNF-α immunostained cells in the subcutaneous inflammatory infiltrate induced after 120 days of infection with *M. avium*. (B) In contrast, at the same time point, the subcutaneous inflammation induced by *M. tuberculosis* shows fewer inflammatory cells and fewer TNF-α immunostained cells. (C) Occasional IL-10 immunoreactive lymphocytes are located in the subcutaneous inflammatory infiltrate after 120 days of infection with *M. avium*. (D) Scat IL-10 immunostained lymphocytes in the subcutaneous inflammatory infiltrate after 120 days of infection with *M. tuberculosis* strain H37Rv. The bottom panels show quantification of the indicated cytokines by the determination of staining intensity using digital automated morphometry. Results are expressed as mea and the standard deviations from three mice per group at days 21 and 120 of infection. Asterisks represent statistical significance ($P < 0.05$) comparing MA strains with *M. tuberculosis*.

not induce detectable IL-4 responses, and antibody or genetic depletion of IL-10 have little or no impact on the course of *M. avium* infection (49). Although it was reported that disease was more severe in transgenic mice that overproduce human IL-10 (50), our results suggest that these cytokines should participate in clearing inflammation during the late stage of the infection when MAC bacillus loads are very low. In consequence, this high expression will not have a detrimental effect on bacillus growth control due to their antagonistic effect on Th1 cells. This coincides with previous observations in BALB/c mice that did not develop granuloma ne crosis after intravenous infection with virulent *M. avium* ATC 25291, and this mouse phenotype was reverted after the genetic disruption of the IL-10 gene (51). In addition, in response to the stress caused by the immune response and hypoxia, the major

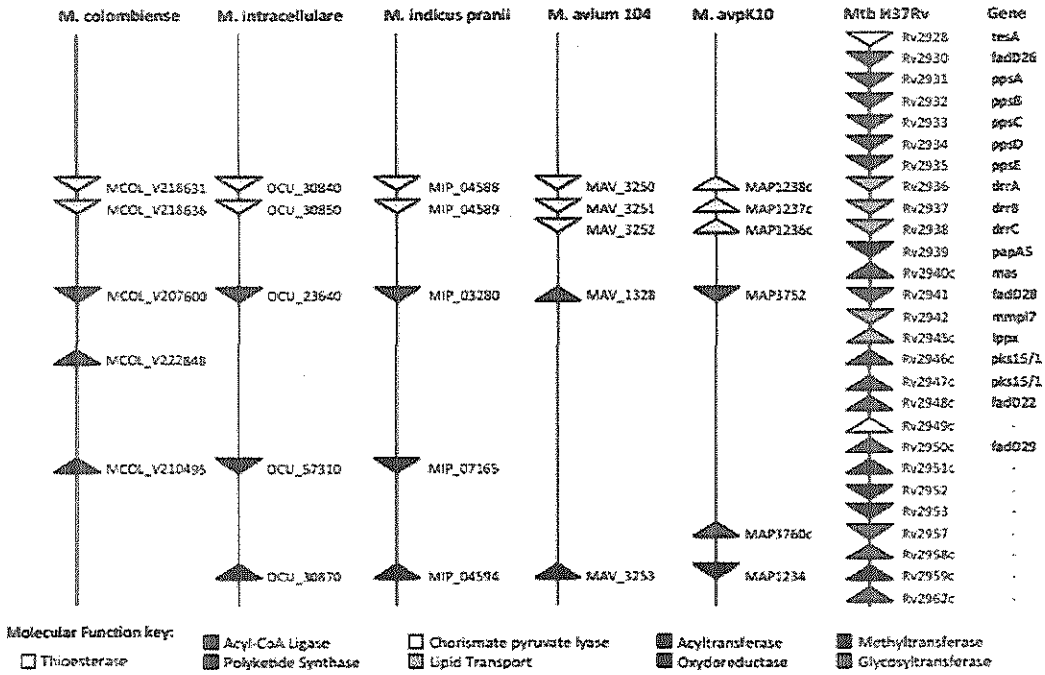

FIG 8 Comparative genomics of the PDIM/PGL/p-HBAD locus between MAC species and *M. tuberculosis* H37Rv. Many genomes of the MAC members (i.e., *M. colombiense* CECT 3035, *M. intracellulare* ATCC 13950, *M. indicus pranii* MTCC 9506, *M. avium* 104, and *M. avium* subsp. *paratuberculosis* k-10) lack several important genes necessary for the biosynthesis of these lipid molecules.

of mycobacteria are thought to enter a dormant state in human TB; however, it is not known whether NTM, as MAC members, can develop this state during pulmonary infection. Detection of culturable *M. tuberculosis* from latently infected individuals is extremely difficult. Our results in this model of pulmonary infection in BALB/c mice suggest that the MAC species used here (*M. avium* and *M. colombiense*) developed a viable but not culturable state (52) with mild levels of pro- and anti-inflammatory cytokines (TNF-α versus IL-10) characterized by minimal pulmonary inflammation and very low bacillus burdens. Additional experiments are needed to investigate this hypothesis.

MAC can also enter the host by the intradermal route through cuts and skin punctures (53, 54). Experimental work showed that intradermal bacillus administration resulted in infection of cervical and/or axillary lymph nodes in both BALB/c and nude mice, suggesting that skin lesions may also be responsible for the cervical lymphadenitis seen in humans (53). Our results confirmed and extended these findings by the demonstration that subcutaneous infection in the footpads of BALB/c mice induced local bacillus growth. This growth was efficiently controlled after 4 months of infection and was able to disseminate to local lymph nodes (inguinal) and shown to be higher in mice infected with *M. avium*. One significant difference with pulmonary infection despite low bacillus loads was the extensive and constant inflammation with fibrosis produced by MAC infection in the subcutaneous tissue. This tissue response corresponds with the high and stable expression and production of TNF-α and IFN-γ during the later stage of infection with MAC along with the production of relatively low anti-inflammatory cytokines. Although MAC induced a high expression of TNF-α and IFN-γ during late subcutaneous infection, iNOS expression was slightly higher. Nitric oxide is an inhibitory factor in the production of fibrosis, as shown in iNOS-deficient mice infected with mycobacteria that developed more fibrosis (55). Thus, the fibrosis observed in the subcutaneous tissue during the late stage of the MAC infection in BALB/c mice could be influenced by the low expression of iNOS.

Few mycobacterial species, most of which are pathogenic for humans, produce a unique array of complex cell wall-associated lipids, such as phthiocerol dimycocerosates (PDIMs) and phenolglycolipids (PGLs), two groups of molecules shown to be important virulence factors (56, 57). Experimental studies demonstrated that *M. tuberculosis* strains deficient in the production or surface localization of PDIMs are markedly attenuated for growth in the lungs of intravenously or intranasally infected mice (58–60), and both PDIMs and PGLs are required for full virulence of *M. marinum* in the zebrafish model (61). Members of *M. tuberculosis* complex also produce *p*-hydroxybenzoic acid derivatives (p-HBADs), which are precursors of PGL biosynthesis (62). Mutants of *M. tuberculosis* that lack production of some or all forms of p-HBADs were shown to induce histological differences in lung tissue of infected BALB/c mice with extensive and diffuse inflammation (63). These *M. tuberculosis* mutants also induce an increased secretion of the proinflammatory cytokines TNF-α, IL-6, and IL-12 compared to the *M. tuberculosis* strain Mt103 (63). These studies could indicate that while PGL and PDIM are associated with virulence, p-HBAD is associated with tissue damage. The biosynthesis of PDIM/PGL/p-HBAD is a very complex pathway that involves more than 15 enzymatic steps and more than 27 genes, most clustered on a 70-kb region of the chromosome (56, 64), and MAC members lack several important genes necessary for the biosynthesis of these lipid molecules (65–67; see also the Virulence Factors of Pathogenic Bacteria database [http://www.mgc.ac.cn/cgi-bin/VFs/compvfs.cgi?Genus=Mycobacterium]) (Fig. 8). We hypothesized

that MAC members and *M. tuberculosis* have different pathogenicities and trigger different immune responses and inflammation as a result of differences in their cell envelopes. Thus, in the murine model of infection with MAC strains, the low virulence could be associated with the lack of production of PDIMs and/or PGLs, and the high chronic inflammation could be associated with the lack of production of p-HBADs.

In conclusion, MAC strains differ in their level of virulence and type of immune response. *M. colombiense* and *M. avium* demonstrate low virulence in BALB/c mice infected by the intratracheal route. Infection in subcutaneous tissue by either MAC strain was also efficiently controlled, but they each induced high expression of proinflammatory cytokines during the later stage of the infection and relatively low production of anti-inflammatory cytokines, producing extensive and constant inflammation. In addition to the mycobacterial antigenic constitution and the genetic background of mice, the site of infection is important in the type of evoked immune response.

ACKNOWLEDGMENTS

This study was supported in part by the Intramural Research Program of the National Institutes of Health, National Library of Medicine, National Center for Biotechnology Information, and CONACyT (grant 84456). M.G.-P. was supported by the National University of Colombia (Convocatoria Apoyo de la DIB a Tesis de Investigacion en Posgrados–Quinto Corte; Proyecto 15129).

We thank Jian Yang (Institute of Pathogen Biology, CAMS&PUMC) for the analysis of PDIM/PGL locus in *Mycobacterium* genomes.

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Pathogens and Disease ISSN 2049-632X

RESEARCH ARTICLE

The protective effect of immunoglobulin in murine tuberculosis is dependent on IgG glycosylation

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This work compares conventional fully glycosylated and deglycosylated IVIg determining their mycobacterial antigen recognition by two dimensional Western-blotting, specific mycobacterial antigen recognition by ELISA, kinetics of distribution after intraperitoneal administration, and protective efficiency by evaluating pulmonary bacilli loads and tissue damage after i.p. administration during early infection in a model of progressive pulmonary tuberculosis. The results add to the growing body of information that antibodies have a protective effect at least in animal models of tuberculosis.

Keywords
tuberculosis; antibodies; glycosylation; experimental models; IVIG: Immunotherapy.

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Received 25 April 2013; revised 29 June 2013; accepted 1 July 2013. Final version published online 19 August 2013.

doi:10.1111/2049-632X.12069

Editor: Patrick Brennan

Abstract

Antibodies have demonstrated having a protective effect in animal models of tuberculosis (TB). These experiments have considered the specificity of antigen recognition and the different isotypes and subclasses as significant contributors of this effect. However, the carbohydrate chain heterogeneity on the Fc region of IgG (Fc-IgG) can play an important role in modulating the immune response. Patients with TB usually have high titers of specific IgG; however, the carbohydrate associated with Fc-IgG usually lacks galactose. To assess the effect of this abnormal IgG in murine pulmonary TB, we evaluated the specificity of recognition to *Mycobacterium tuberculosis* antigens *in vitro* and protective effects *in vivo* comparing human intravenous immunoglobulin (IVIg) and IVIg treated with an endoglycosidase to remove the glycan residues (EndoS-treated IVIg). Our results showed similar antigen recognition. The study of distribution and kinetics of IVIg in serum and bronchial lavage after intraperitoneal (i.p.) administration in mice showed that IVIg circulates for 21 days. Finally, the protective effect of intact and EndoS-treated IVIg administered by i.p was studied in a murine model of progressive TB. IVIg treatment caused reduction in pulmonary bacilli loads, larger granulomas, and less pneumonia, while animals treated with EndoS-treated IVIg were not protected compared with control animals. Thus, IVIg has a protective activity in experimental pulmonary TB, and this effect requires intact Fc oligosaccharides.

Introduction

During the early part of the last century, some preclinical and clinical experiments were performed to find successful forms of serum therapy against TB. This type of research was abandoned because of its inconsistent results, as well as the discovery of Bacillus Calmette–Guérin (BCG) to prevent TB and efficient chemotherapy (Glatman-Freedman, 2006). Nonetheless, during the last two decades, the use of diverse forms of serum therapy to treat other medical problems has been increasing with good results, such as the replacement therapy for primary humoral immunodeficiency or as anti-inflammatory therapy in a variety of autoimmune diseases (Abdou *et al.*, 2009; Magy, 2012).

Currently, TB still represents a significant world health public problem with 1.2 million deaths and 9 million cases of active disease each year, and the number of patients with multidrug resistant disease is increasing (World Health

Organization, 2012). This has motivated studies about TB pathogenesis and testing different approaches to control this infectious disease. Considering that TB is caused by a facultative intracellular pathogen, much of the research has focused on cell-mediated immunity; however, the study of the contribution of humoral immunity in its pathogenesis and therapeutic benefit has resurged during the last decade (Glatman-Freedman, 2010). In this regard, monoclonal antibodies against mycobacterial polysaccharides such as arabinomannan or lipoarabinomannan (LAM), as well as against proteins such as the 16 kDa, have demonstrated some protective activity (Teitelbaum *et al.*, 1998; Hamasur *et al.*, 2004; Williams *et al.*, 2004; López *et al.*, 2009). Besides their specificity of antigen recognition, different isotypes and subclasses of antibodies were evaluated to determine the mechanism of their beneficial activity (Glatman-Freedman, 2010). Polyclonal antibodies such as intravenous human immunoglobulins (IVIg) have also been tested in mouse BCG models (Olivares *et al.*, 2006). IVIg preparations are pooled from many subjects, some of whom will have been latently infected with mycobacteria, or exposed to environmental mycobacteria or BCG-vaccinated. Thus, the IVIg protective effect is probably due to the recognition of diverse mycobacterial antigens by many polyclonal specific antibodies (Olivares *et al.*, 2006), although there might also be pharmacological effects of different patterns of glycosylation, as outlined below.

Human immunoglobulin G (IgG) isotype antibodies normally have a complex oligosaccharide (glycan) chain covalently joined to an asparagine (Asn) residue in the second constant domain of the heavy chain. This N-linked glycan has a core region with two GlcNAc and three mannose (Man) residues branching into a bi-antennary structure (Fig. 1a). Many different carbohydrate residues can be associated with this core determining glycosylation heterogeneity (Stadlmann *et al.*, 2010). These glycoforms are involved in the interaction between the IgG-Fc and its receptor (FcR), and although their physical contact with the receptor is minimal, the carbohydrate moieties have a significant influence on this interaction affecting the response of significant IgG functions, such as cytotoxicity mediated by complement (CDC) or antibody-dependent cellular cytotoxicity (ADCC) (Nimmerjahn & Ravetch, 2005, 2008). Another recently described activity of the IgG-Fc oligosaccharide chain is the interaction with the DC-SIGN receptor (CD 209) (Anthony & Ravetch, 2010). DC-SIGN is a transmembrane C-type lectin receptor whose carboxyl-terminal domain binds mannose residues from glycoconjugates with high affinity. DC-SIGN has significant immunoregulatory functions in the inflammatory response, such as migration and adhesion of dendritic cells (DC), activation of T cells, and participation in the immune escape of pathogens and tumor cells (Zhou *et al.*, 2006). IVIg formulations have high amounts of IgG with sialic acid (Sia) in its Fc oligosaccharide chain, which are recognized by macrophages from the marginal zone of the spleen through the DC-SIGN receptor. This stimulates the release of anti-inflammatory factors by macrophages, which reach the inflamed tissues causing increased expression of the inhibitory type of FcγR on macrophages provoking attenuation of the inflammation and significant protective effect in autoimmune diseases (Anthony *et al.*, 2011).

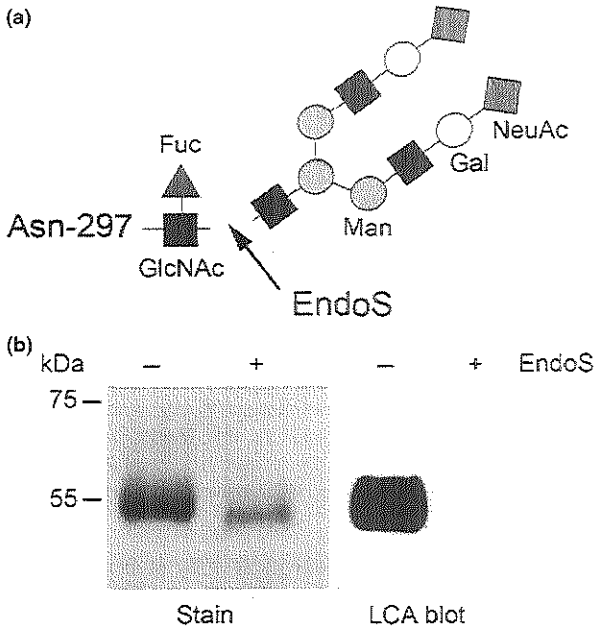

Fig. 1 EndoS-mediated hydrolysis of the IgG-associated glycan moiety. Representation of the fully substituted Asn-297 attached sugar moiety of IgG. As indicated EndoS cleaves after the first GlcNAc, resulting in the generation of a minimal sugar moiety containing only one GlcNAc with or without a branching fucose residue; adapted from reference 20 (a). Analysis of IVIG incubated with (–) or without ( ) EndoS and separated by 10% SDS-PAGE. The proteins were detected by gel staining (Stain), or by blotting onto a PVDF membrane probed with LCA (b).

Patients with active TB usually have high titers of IgG lacking the terminal galactose (Gal) and Sia (Parekh *et al.*, 1989; McCulloch *et al.*, 1995). In contrast to fully sialylated IgG, agalactosyl IgG has pro-inflammatory activity (Rademacher *et al.*, 1994) that could contribute to immunopathology in advanced TB, and high doses of IVIg could replace this abnormal IgG providing a protective effect (Anthony *et al.*, 2011). The aim of this work was to compare conventional fully glycosylated and deglycosylated IVIg determining their mycobacterial antigens recognition by two-dimensional Western blotting, specific mycobacterial antigen recognition by enzyme-linked immunosorbent assay (ELISA), kinetics of distribution after intraperitoneal administration, and protective efficiency by evaluating pulmonary bacilli loads and tissue damage after i.p. administration during early infection in a murine model of progressive pulmonary TB.

Materials and methods

EndoS hydrolysis of IVIg

Human IVIg (Octagam, 50 mg mL^{-1}; Octapharma, Stockholm Sweden) was hydrolyzed with recombinant EndoS

fused to GST (GST-EndoS) prepared as previously described (Collin & Olsén, 2001a). Five micrograms of recombinant GST-EndoS in phosphate-buffered saline (PBS) was added per mg of IVIg followed by incubation for 16 h at 37 °C. GST-EndoS was completely removed by three serial passages over fresh Glutathione–Sepharose 4B columns with a 1000-fold overcapacity of GST binding (GE Healthcare, Uppsala, Sweden). SDS-PAGE and *Lens culinaris* agglutinin (LCA) lectin blotting were used to assess the purity and efficacy of EndoS cleavage. Briefly, 2 μg of EndoS-treated and untreated IgG was separated on 10% SDS-PAGE followed by staining with PageBlue protein stain (Thermo Scientific, Rockford, IL), or blotted to PVDF using TransBlot Turbo transfer packs and apparatus (Bio-Rad, Hercules, CA). Membranes were blocked with 10 mM HEPES (pH 7.5) with 0.15 M NaCl, 0.01 mM $MnCl_2$, 0.1 mM $CaCl_2$, and 0.1% Tween 20 (HBST) and incubated with 1 μg mL^{-1} of biotinylated LCA lectin (Vector Laboratories, Burlingame, CA). After washing in HBST, membranes were incubated with 200 ng mL^{-1} of peroxidase-labeled streptavidin (Vector Laboratories). After washing, membranes were developed using SuperSignal West Pico chemiluminescent substrate (Thermo Scientific Pierce) and developed using a ChemiDocXRS imaging system (Bio-Rad).

Pattern of mycobacterial antigens recognized by intact IVIg and EndoS-treated IVIg

The IVIg preparation Octagam® (Octapharma) was used as intact formulation (intact IVIg) and as modified formulation through the IgG-specific endoglycosidase EndoS (EndoS-treated IVIg). Two-dimensional immunoelectrophoresis was performed to determine the specificity of these preparations against proteins from *Mycobacterium tuberculosis*. Briefly, total proteins from a filtrate of *M. tuberculosis* H37Rv culture at 6 weeks of growth were separated by two-dimensional electrophoresis and then transferred to Immobilon-P PVDF membrane (Millipore, Bedford, MA) as previously described (Xolalpa *et al.*, 2007). Then, membranes were incubated with intact IVIg or EndoS-treated IVIg diluted 1 : 800 for 1 h at 25 °C. Bound IgG was detected with anti-human IgG labeled with horseradish peroxidase (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). The color was developed with diaminobenzidine as substrate.

The recognition of specific recombinant *M. tuberculosis* antigens by intact IVIg and the EndoS-treated IVIg was determined by IgG ELISA. Briefly, MaxiSorp microtiter plates (Nunc, Roskilde, Denmark) were coated with 5 μg mL^{-1} of recombinant immunodominant proteins from *M. tuberculosis:* Hsp 79, Esat-6, 85 B, P 38 kDa, P 27 kDa, PGRS 33, Apa, PGRS 1, P 16 kDa, hsp 65, HBHA, and P 19 kDa. After washing and blocking, dilutions 1 : 800 from intact IVIg and the EndoS-treated IVIg samples were incubated. Bound antibodies were detected using anti-human IgG labeled with horseradish peroxidase (Sigma-Aldrich Chemie GmbH). The reaction was developed with a solution of o-phenylenediamine as substrate and the absorbance measured at 450 nm in an ELISA reader (Modulus™ II, Microplate Multimode Reader; Turner BioSystems). Results are expressed as units of optical density (OD). As a negative control, we used an IVIg sample absorbed with *M. tuberculosis*, as previously described (Olivares *et al.*, 2009). As a positive control, we used a pool of samples from PPD-positive patients who had high titers of each individual recombinant antigen. Cutoff values were means of negative control OD ± 2 SD.

Pharmacokinetics of IVIg in serum and bronchial lavage from BALB/c mice after intraperitoneal administration

IVIg (1 mL) was administered by the i.p. route to noninfected male BALB/c mice. Serial samples of serum and bronchial lavage were obtained from four different animals at 1, 2, 3, 6, 10, 24, 48, 72 h, and 21 days after i.p. administration, and human antibodies against mycobacterial antigens from culture filtrate were determined by IgG ELISA. Briefly, MaxiSorp microtiter plates (Nunc) were coated with proteins from the filtrate of *M. tuberculosis* H37Rv culture at 6 weeks (0.32 μg mL^{-1}), and after washing and blocking, double serial dilutions from serum and bronchial lavage samples were incubated. Bound antibodies were detected using anti-human IgG labeled with horseradish peroxidase (Sigma-Aldrich Chemie GmbH). The reaction was developed with a solution of o-phenylenediamine as substrate and the absorbance measured at 450 nm in an ELISA reader (Modulus™ II, Microplate Multimode Reader; Turner BioSystems). Results are expressed as the titer, calculated as the inverse of the last positive dilution. For a negative control, we used samples from mice not subjected to IVIg administration. Cutoff values were means of negative control OD ± 2 SD.

Protection mediated by IVIg and EndoS-treated IVIg in experimental pulmonary tuberculosis

Mycobacterium tuberculosis H37Rv was grown in 7H9 Middlebrook medium supplemented with 0.05% Tween 80% and 10% Middlebrook OADC enrichment (Difco Laboratories, Sparks) at 37 °C for 14 days, aliquoted, and stored at −80 °C. Aliquots were thawed and diluted in PBS prior to use. Male BALB/c mice aged 8 weeks were anesthetized with sevoflurane vapor (Abbott Laboratories, Mexico City, Mexico) in a sealed acrylic cage, immobilized, and infected with *M. tuberculosis* (H37Rv) by the intratracheal route, using a rigid stainless steel cannula (Thomas Scientific, Swedesboro, NJ) connected to an insulin syringe. Each animal received 250 000 live bacteria suspended in 100 μL of PBS.

Groups of twelve mice were treated with intact IVIg or EndoS-treated IVIg by i.p. route at days 3 and 5 after infection (final total dose of 2 g kg^{-1} body weight). Controls received saline solution (SS) by i.p. route. Groups of six mice were euthanized by exsanguination at days 14 and 28 postinfection. One lung was removed and snap-frozen in liquid nitrogen for the determination of bacillary loads by colony-forming units (CFU). The other lung was perfused with 10% formaldehyde via the trachea and removed for

histological analysis. All the animal work was done according to the guidelines and approval of the Ethical Committee for Experimentation in Animals of the National Institute of Medical Sciences and Nutrition in Mexico, code 268. Two independent experiments were performed.

For pulmonary bacilli load determinations, frozen lungs were homogenized using a polytron (Kinematica, Luzern, Switzerland). The suspensions were then diluted in 1 mL of PBS (pH 7.2) with 0.05% Tween 80. Three consecutive logarithmic dilutions were made with this homogenate; 10 μL of each dilution was plated by duplicate on Bacto Middlebrook 7H10 agar (Difco, Detroit, MI) enriched with oleic acid, albumin, dextrose, and catalase. Plates were then incubated at 37 °C in 5% of CO_2 for 21 days to quantify the CFUs, which are expressed as millions of bacteria per lung.

For histological analysis, lungs were perfused via the trachea with 10% formaldehyde dissolved in PBS. Parasagittal sections were dehydrated and embedded in paraffin, sectioned, and stained with hematoxylin–eosin and Ziehl–Neelsen. The granuloma area and percentage of lung surface affected by pneumonia were determined using an automated image analyzer (Q Win Leica; Milton Keynes, Cambridge, United Kingdom).

Statistical analysis

The results are expressed as mean ± SE, and statistical analysis was performed with one-way or two-way ANOVA as appropriate, followed by Bonferroni's multiple comparison test if the ANOVA was significant.

Results

EndoS hydrolysis of IVIg

The endoglycosidase EndoS secreted from *Streptococcus pyogenes* hydrolyzes the glycan on native human IgG, leaving an N-acetylglucosamine with a core fucose if originally present (Fig. 1a) (Collin & Olsén, 2001b). To prepare for analysis of the glycan dependence of IVIg effects on tuberculosis, IVIg was hydrolyzed with recombinant EndoS fused to GST. Subsequently, the enzyme was completely removed from the IVIg by serial affinity removal using the GST tag, generating EndoS-treated IVIg. Efficient hydrolysis of the IgG glycans in the IVIg preparation was revealed in SDS-PAGE with a typical approximately 3-kDa size shift and by loss of reactivity with LCA in lectin blotting (Fig. 1b).

Antigen recognition by intact and EndoS-treated IVIg

To exclude altered antigen recognition in EndoS-treated IVIg compared with original IVIg, two sets of experiments were performed. First, reactivity toward total proteins from a filtrate of *M. tuberculosis* H37Rv was tested using two-dimensional (2-D) immunoelectrophoresis. This 2D analysis demonstrated that both intact IVIg and EndoS-treated IVIg recognized a similar pattern of mycobacterial antigens (Fig. 2).

Secondly, reactivity of IVIg and EndoS-treated IVIg against a set of specific immunodominant *M. tuberculosis* antigens was tested by ELISA. This revealed that intact IVIg and EndoS-treated IVIg had similar titers against the mycobacterial antigens Hsp 79, Esat-6, 85 B, P 38 kDa, P 27 kDa, PGRS 33, Apa, PGRS 1, P 16 kDa, hsp 65, HBHA, and P 19 kDa (Fig. 3). Taken together, these two experiments clearly indicate that EndoS hydrolysis does not alter the Fab-mediated recognition of mycobacterial antigens.

Kinetics of IVIg distribution in sera and bronchial lavage after intraperitoneal administration

In order for IVIg to have an effect in the animals, antibodies have to reach the relevant compartments following injection via the i.p. route in mice. Therefore, IgG concentrations were followed in noninfected BALB/c from 1 h to 21 days after IVIg injection using an IgG ELISA. This revealed that IVIg rapidly arrives to the airways and maintains constant concentrations from 1 to 10 h, and then, after 1 day, it almost disappears in the lungs. In contrast, IVIg concentrations in serum were much higher, peaking at 6 h and progressively declining, but even after 21 days, a relatively high concentration was detected (Fig. 4). Thus, there is a high and constant serum concentration of antibodies that specifically recognize mycobacterial antigens from the IVIg preparation after i.p administration.

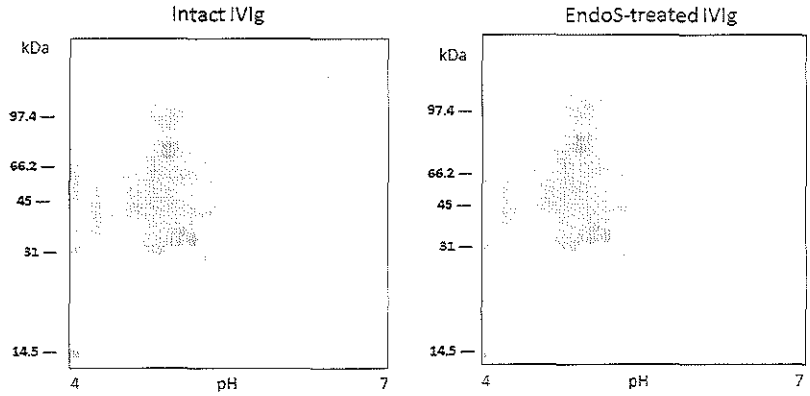

Fig. 2 Conventional two-dimensional immunoelectrophoresis shows equal recognition against proteins from *Mycobacterium tuberculosis* culture filtrate by the intact IVIg and EndoS-treated IVIg.

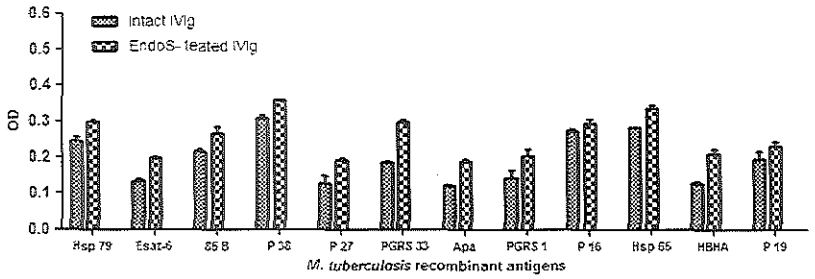

Fig. 3 Intact IVIg and EndoS- treated IVIg recognition against *Mycobacterium tuberculosis* recombinant antigens. OD values showed are lecture minus the cut off for each recombinant specific *M. tuberculosis* antigens.

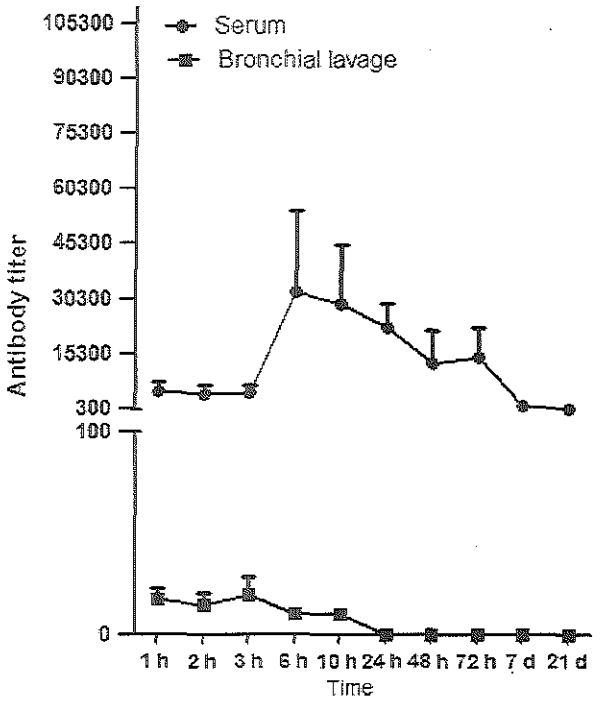

Fig. 4 Kinetics of IVIg distribution in sera and bronchial lavage after intraperitoneal administration. Each point represents the mean value of the titers.

Effect of intact and EndoS-treated IVIg administered during the early phase of experimental pulmonary tuberculosis

To investigate the effect of intact IVIg and EndoS-treated IVIg during tuberculosis, a high-dose mouse infection model was used. BALB/c mice were infected by the intratracheal route with a high dose of *M. tuberculosis* strain H37Rv and treated with intact or EndoS-treated IVIg administered in two doses by i.p route after 3 and 5 days of infection. IVIg produced a significant reduction in pulmonary bacilli loads at days 14 and 28 postinfection when compared with control nontreated animals. In contrast, infected mice treated with EndoS-IVIg using the same dose and route showed similar pulmonary bacilli burdens as control nontreated mice (Fig. 5a).

The morphometry and histopathology analysis showed bigger granulomas at day 14 of infection in mice treated with intact IVIg than in animals treated with deglycosylated IVIg or control nontreated mice, this difference did not reach statistical significance (Fig. 5b), while the percentage of lung area affected by pneumonia was significantly lower in mice treated with intact IVIg than in the other groups (Fig. 5f). Acid-fast staining showed similar bacilli distribution, at day 14 bacilli were found in the cytoplasm of macrophages located in granulomas, while at day 28 mycobacteria were seen in macrophages from granulomas and pneumonia patches, being apparently lesser in infected mice treated with IVIg (data not shown).

Discussion

The role of antibodies in the pathogenesis and control of TB has been controversial for a long time (Glatman-Freedman, 2010). From the pathogenic perspective, it is clear that not only the bacilli burden but also the inflammatory response is significant participants. This latter factor is crucial because excess of inflammation causes structural and functional pulmonary damage (Surkova & Dius'mikeeva, 2003). Theoretically, antibodies could participate in both events, decreasing bacilli loads by direct recognition of bacterial antigens by the Fab region through diverse mechanisms: and by regulating cell-mediated immunity and inflammation by the Fc region through their direct interaction with specific receptors. The Fc glycosylation is a significant modifier of these mechanisms. Indeed, through post-translational glycosylation processes, terminal sugar residues are added to antibodies that have an important effect on their biological function. For example, an increase in GlcNAc residues produces lectin complement activation due to an increased affinity of the IgG for the ficolins in serum (Malhotra *et al.*, 1995) and reduces CDC by decreasing the affinity of the IgG to C1q (Hodoniczky *et al.*, 2005). An increase in Sia residues decreases the ADCC through a negative effect on the binding of IgG to FcRγRIIa (Scallon *et al.*, 2007). On the other hand, an increase in Man residues produces a rise in ADCC by increasing the affinity of the IgG to FcRγRIIa and decreases CDC by lowering the affinity of the IgG to C1q (Zhou *et al.*, 2008).

Previous studies conducted by Roy and colleagues using BALB/c mice infected with 2.5×10^5 bacteria through the intravenous route showed protective effect of IVIg administered during early and late disease (Roy *et al.*, 2005). Using the same mouse strain but infected intratracheal route, which is less artificial, our results confirm the protected effect of IVIg during early infection and extend the informa-

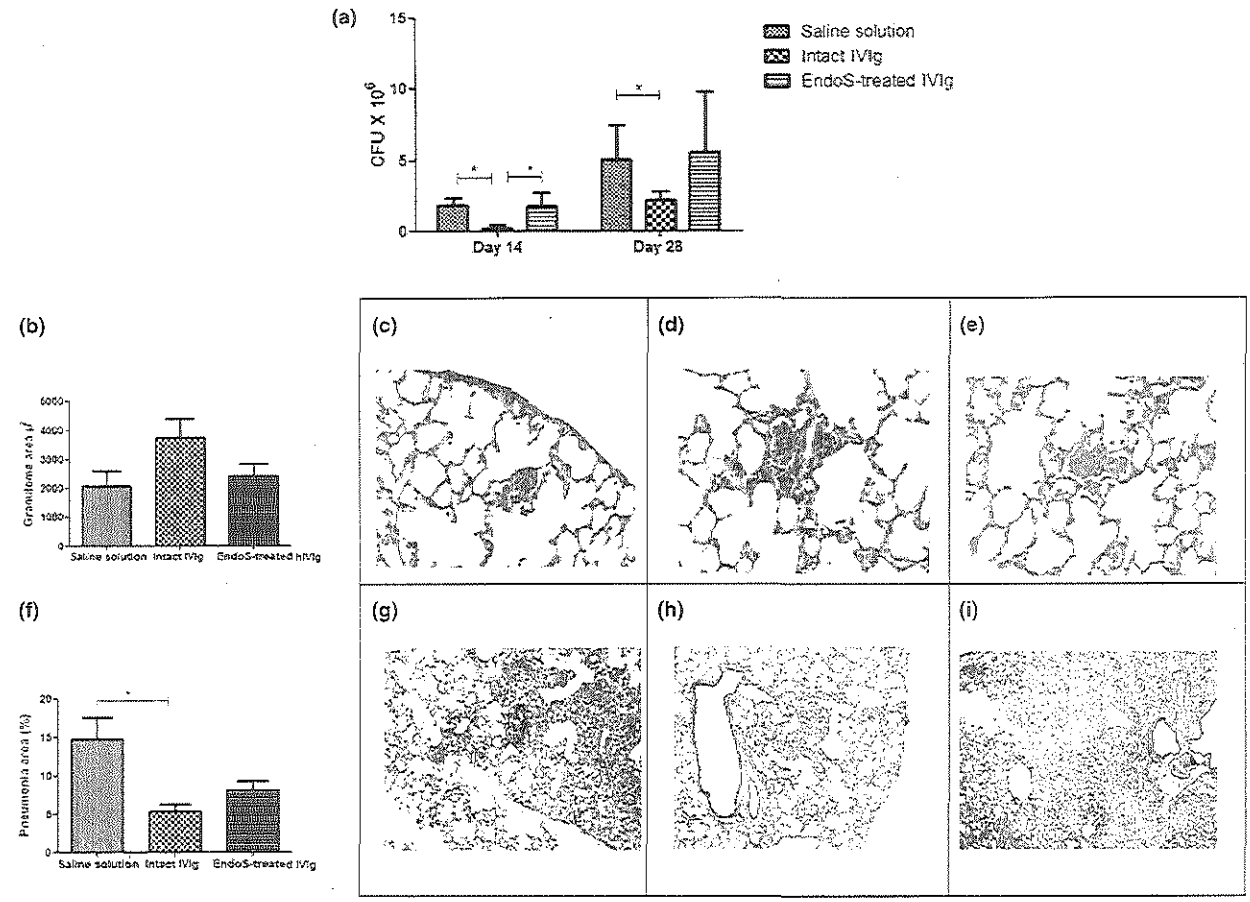

Fig. 5 Effect of the treatment with intact IVIg, EndoS-treated IVIg and saline solution on lung CFU count in mice at 14 and 28 days after infection with *Mycobacterium tuberculosis* H37Rv ($P < 0.05$) (a). Morphometric and histopathology analysis. Granuloma area at 14 days post-infection (b), representative granulomas in lungs of mice treated with saline solution (c), intact IVIg (d) and EndoS-treated IVIg (e). Percentage of lung area affected by pneumonia at 28 days post-infection (f), representative pneumonic lung area of mice treated with saline solution (g), intact IVIg (h) and EndoS-treated IVIg (i).

tion by the demonstration that the oligosaccharide chain associated with the Fc of IgG is essential for this beneficial effect. According to information from the Global Octapharma Web site, the IVIg preparation Octagam® was obtained from 45 plasma donation centers in U.S.A. and 9 centers in Germany. We considered that the donor population, especially from U.S.A., would include immigrants or American tourists previously exposed to mycobacterial antigens. This assumption was originally based on the similarity of mycobacterial antigens recognition determined by ELISA of Octagam®(Octapharma) and Intacglobin® (Blood Products Center, Havana, Cuba) (data not showed). Intacglobín® is obtained from a donor Cuban population of persons highly exposed to mycobacterial antigens from environmental mycobacteria, latent infection, or vaccinated at birth with BCG (Olivares *et al.*, 2006). Furthermore, we observed that both intact IVIg and the EndoS-treated IVIg recognized a similar wide pattern of mycobacterial antigens by immunoelectrophoresis, particularly over the 31-kDa region that matches with the 85-KDa complex previously reported by proteomic analysis (Xolalpa *et al.*, 2007) (Fig. 2). These results were confirmed by ELISA using purified *M. tuberculosis*-specific antigens (Fig. 3); however, *M. tuberculosis*, environmental mycobacteria, and *M. bovis* share many surface antigens, so we cannot exclude the possibility of cross-reaction after IVIg administration to mice. Nonetheless, the presence of antibodies that bind to *M. tuberculosis* is very important to obtain a protective effect in animal models. This statement is supported by a previous study that found a protective effect after giving IVIg intranasally 2 h before intratracheal infection; this effect was abrogated when IVIg was previously absorbed with *M. tuberculosis*, eliminating specific antibodies from the preparation (Olivares *et al.*, 2009). Thus, it is considered that the protective effect of IVIg is mediated by the binding to *M. tuberculosis* via their antigen-binding sites and subsequent IgG-Fc interaction with FcRs and complement (Olivares *et al.*, 2009; Glatman-Freedman, 2010). An additional mechanism of Ab-mediated protection was reported for intracellular pathogens that normally evade lysosomal fusion, such as

M. bovis BCG. It was shown that Fc receptor engagement by antibodies, which can be temporally and spatially separated from BCG, renders the host cell nonpermissive for bacterial replication and targets the pathogens to lysosomes. This process is strictly dependent on kinases involved in FcR signaling, but not on host cell protein synthesis or protease activation (Joller *et al.*, 2010). A direct lytic effect of antibodies against the bacilli is also possible, as has been recently demonstrated with the intracellular fungus *Cryptococcus neoformans*, which after incubation with specific antibodies showed up-regulation or down-regulation of many different genes that affect the synthesis of significant metabolism proteins (McClelland *et al.*, 2010; Achkar & Casadevall, 2013).

The distribution and kinetics of IVIG in serum and bronchial lavage showed high Abs concentrations in the site of infection for long periods of time, which is relevant in order to implement an efficient form of serum therapy against TB in future controlled clinical trials. After the glycan hydrolysis of IVIg using EndoS, no changes were reported to its chemical properties such as solubility, stability, or formation of immune aggregates. In fact, EndoS has been administered intravenously with the purpose of hydrolyzing the glycans on serum IgG in autoimmune animal models, producing antirheumatic effect attributed to an IgG with intact chemical properties (Allhorn & Collin, 2009). Thus, it is possible that EndoS-treated IVIg has kinetics and distribution comparable to intact IVIg.

Our results suggest a cell-mediated immunoregulatory protective effect by IVIg, as showed by lung histopathology that was different in treated mice, and the trend toward an increase in granuloma area on day 14 in the intact IVIg-treated group is considered beneficial in this murine model as an indicator of increase immune protective response (Hernández-Pando *et al.*, 1996). Interestingly, mice treated with intact IVIg resulted in a significant reduction in the percentage of pneumonia, which is the histological parameter most closely related to disease progression and mortality (Hernández-Pando *et al.*, 1996). It is possible that this result points to the immunoregulatory mechanism described by Ravetch and colleagues, thought to involve an anti-inflammatory effect secondary to an interaction between the fully sialylated Fc oligosaccharides and the receptor SIGN-R1 in mice or DC-SIGN in humans (Anthony *et al.*, 2008). This immunoregulatory mechanism could be supported because in high dose, this formulation provides enough IgG rich in sialic acid to obtain the observed anti-inflammatory effect, while in normal serum, the amount of IgG with high sialic acid content is only about 3–10% (Käsermann *et al.*, 2012); however, this requires further investigation.

Interpretation of results using human IgG in a mouse model should be made cautiously, because the interaction between human IgG and murine Fc receptors most likely differs from interactions with human FcRs (Shashidharamurthy *et al.*, 2010). However, the results from the present study indicate that removing the Fc glycan from human IgG reduces its effector functions in the mouse. It should be mentioned that EndoS has been used with success in other cross-species models, for instance where EndoS has been shown to abolish pathogenicity of rabbit anti-mouse platelet IgG in a mouse model of immune thrombocytopenic purpura (Collin *et al.*, 2008; Andersen *et al.*, 2012).

In conclusion, we have observed a protective effect of high-dose IVIg after intratracheal infection of BALB/c mice with *M. tuberculosis*, and this effect was not observed using EndoS-treated IVIg, clearly showing that the glycosylation state of the IVIg is crucial for antituberculosis activity.

Acknowledgements

Ms Ulla Johannesson is acknowledged for excellent technical support. M.C. is supported by grants from the Swedish Research Council (2010-57X-20240), the Foundations of Wiberg, Kock, Bergvall, Österlund, Hedberg, Hedlund, Groschinsky, Söderberg, the Swedish Society for Medicine, the Royal Physiografic Society, King Gustaf V's Memorial Fund, the Medical Faculty at Lund University, and Hansa Medical AB. R.H.P. is supported by the Mexican Council of Science and Technology (CONACyT, contract 84456). N.O. is on a PhD program in Biomedical Sciences from National Autonomous University of Mexico, funded by the same Mexican Council. The funders had no role in preparation of the manuscript or decision to publish. Hansa Medical AB has filed patent applications for using EndoS as a treatment for antibody-mediated diseases. M.C. is listed as one of the inventors on these applications and is a Scientific Consultant for Hansa Medical AB.

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Archives of Medical Research 44 (2013) 99–104

Archives of Medical Research

ORIGINAL ARTICLE

Antitubercular Activity and the Subacute Toxicity of (−)-Licarin A in BALB/c Mice: A Neolignan Isolated from *Aristolochia taliscana*

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Received for publication August 11, 2012; accepted December 11, 2012 (ARCMED-D-12-00408).

Background and Aims. Tuberculosis remains a worldwide health problem and requires long-term treatment with several antibiotics; therefore, compliance problems and the emergence of multidrug resistance (MDR) are involved. (−)-Licarin A (LA) was isolated from diverse plants such as *Aristolochia taliscana* and possesses antimycobacterial, anti-inflammatory, trypanocidal, and neuroprotective activities. The aim of the study was to determine the antitubercular and subacute toxicity of LA isolated from *A. taliscana* in BALB/c mice.

Methods. The antitubercular activity of LA was tested in a TB murine model inducing disease with *M. tuberculosis* H37Rv or MDR. Mice were treated with LA (5 mg/kg) for 30 and 60 days; post/treatment, lung bacilli loads and pneumonia percentage were determined. The subacute toxicity of LA (21 days) was evaluated in healthy mice. After treatment, biochemical and hematological parameters were determined and main organs were analyzed histologically.

Results. In animals infected with drug-sensitive or MDR strains, LA produced a significant decrease of pulmonary bacillary burdens at day 30 of treatment, and a significant pneumonia reduction at days 30 and 60 of treatment. Regarding subacute toxicity, LA administration during 21 days showed no abnormalities in main-organ macro- and micro-architecture. Biochemical and hematological parameters analyzed showed no statistical differences between control and treated groups.

Conclusions. (−)-Licarin A reduces pneumonia of mice infected with both mycobacterium strains. Also, subacute toxicity of LA exhibits no major signs of damage. Biochemical and hematological parameters and histological analyses indicate that LA caused no significant changes at the doses assayed. © 2013 IMSS. Published by Elsevier Inc.

Key Words: Tuberculosis, (−)-Licarin A, Subacute toxicity, MDR, *Mycobacterium tuberculosis*.

Introduction

Tuberculosis (TB) is currently the only infectious disease considered by the World Health Organization (WHO) as a worldwide emergency, mainly in developing countries, with an estimated nine million new cases each year and two million deaths annually. However, the TB/human immunodeficiency virus (HIV) association renders control of the disease difficult (1,2). Despite the progress attained,

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0188-4409/$ - see front matter.
http://dx.doi.org/10.1016/j.arcmed.2012.12.006

only one third of patients with TB receive adequate treatment, and in multidrug resistance (MDR) cases a scarce number of these patients have access to Directly Observed Treatment, Short-course (DOTS)-Plus (with drug susceptibility testing). Four drugs comprise the current treatment for newly diagnosed patients with TB and for sensitive cases. Treatment duration is 6–9 months, which enhances patient noncompliance and contributes to the incidence of MDR strains. In addition, MDR and extensively drug-resistant (XDR) cases do not respond to the standard treatment regimen established with first- and second-line drugs, making this treatment longer, more difficult, and more expensive. In consequence, compliance with the DOTS and DOTS-Plus programs established by the WHO is poor (3), and the need for searching novel drugs is urgent.

Since the release of rifampicin in 1976, only rifabutin and rifapentine have been approved for TB treatment; unfortunately these drugs have not yet achieved extended distribution for clinical application. At present, numerous drugs (>8,000) are under investigation, but some compounds (ca. 10) are in preclinical and clinical phases, most notably TMC207, PA824, SQ109, OPC 67683, PNU 100480, AZD 5847, and others (4).

The increasing prevalence of TB and the emergence of cases of patients with MDR-TB and XDR-TB underscore the importance of having either new and efficient antitubercular agents that shorten treatment or agents with a longer half-life to avoid resistance (5). Within this context, medicinal plants comprise a potential resource for obtaining new antitubercular molecules that are structurally different from the current therapeutic drugs. Furthermore, WHO suggests developing novel drugs with the following characteristics: low toxicity, selective effect on *M. tuberculosis*, minimum secondary effects, and novel or different mechanisms of action. Recent reports in the literature have described the possible use of some immunomodulatory compounds in TB treatment (6,7).

(−)-Licarin A (LA) is a neolignan that has been isolated from numerous species such as *A. taliscana*. Diverse pharmacological activities, for instance, the antiinflammatory effect of this compound, have been addressed by several authors (8–13). Recently, our research group reported its moderate antimycobacterial *in vitro* activity against *M. tuberculosis* H37Rv (minimum inhibitory concentration [MIC] = 25 μg/mL). Furthermore, this compound showed significant activity against certain MDR *M. tuberculosis* clinical isolates (MIC <6.25 μg/mL) and against a group of nontuberculous mycobacteria. Additionally, the median lethal dose (LD_{50}) value >1,706 mg/kg determined in BALB/c mice was reported; these data suggest low toxicity of the compound (12).

Regardless of pharmacological information of LA and its therapeutic potential *in vitro*, to our knowledge a subacute toxicological and *in vivo* antitubercular studies have not yet been provided. In this study, we report the therapeutic effect of LA in a well-characterized model of progressive pulmonary TB in BALB/c mice infected with drug-sensitive *M. tuberculosis* reference strain H37Rv and MDR *M. tuberculosis* clinical isolate after 30 and 60 days of treatment, determining pulmonary bacilli burdens and tissue damage. The effect of LA on the subacute toxicity caused by 21-days s.c. administration in healthy BALB/c mice was also studied.

Materials and Methods

Isolation of (−)-Licarin A

This compound was isolated from the hexanic extract of *A. taliscana* roots following the procedure previously described by León-Díaz et al. (12). Chemical identification was performed by comparison of spectroscopic and spectrometric data with those described in the literature (12,13).

Experimental Model of Pulmonary TB

All *in vivo* experimental studies were performed according to the local Ethics Committee for Experimentation in Animals in Mexico (NOM-062-ZOO-1999) guidelines (14).

The therapeutic activity of LA was determined using the previously described experimental model of pulmonary TB (15). Briefly, groups of six male BALB/c mice 6–8 weeks of age were used. To induce pulmonary TB, mice were anesthetized with 56 mg/kg of i.v. pentothal; the trachea was exposed via a small midline incision and 2.5×10^5 viable mycobacteria H37Rv or MDR strains, suspended in 100 μl of PBS were injected. The incision was then sutured and the mice were maintained in a vertical position until spontaneous recovery. All procedures were performed in a laminar flow cabinet with bio-safety level III facilities. The infected mice were housed in cages fitted with micro-isolators.

Drug Administration

Animals surviving 60 days after infection were randomly allocated to the required treatment groups. LA (5 mg/kg) was dissolved in ultrapure olive oil (Sigma) and was s.c. administered daily for 30 and 60 days. The animals of each group (controls and treated) were sacrificed at 90 and 120 days after infection, which corresponds to 30 and 60 days of treatment, respectively. Two independent experiments were performed. Selection of the appropriate dose (5 mg/kg) was calculated according to the MIC determined *in vitro* (drug concentration efficient to kill 1×10^6 bacilli) by adjusting the drug concentration to the estimated number of bacilli in lungs of mice after 2 months of infection; this drug amount was triplicated considering its dilution after absorption and systemic distribution after s.c. administration.

Quantification of Colony-forming Units (CFU) in Infected Lungs

Three animals per group were sacrificed by exsanguination in two independent experiments, and the right or the left lung was removed, deposited in sterile 2.5 mL cryotubes containing 1 mL of PBS and homogenized in a Polytron (Kinematica, Lucerne, Switzerland). Four dilutions of each homogenate were spread onto duplicate Petri plates containing Bacto Middlebrook 7H10 agar enriched with OADC (both from Difco Laboratories, Franklin Lakes, NJ). The plates were incubated for 21 days at 37°C in a 5% CO_2 atmosphere for assessment of CFU. CFU for each point are reported as the mean ± standard deviation (SD) of measurements from three mice.

Histopathology and Morphometry of the Infected Lung

The right or the left lung was intratracheally perfused with 100% ethanol and immersed for 24 h in the same fixative. Parasagittal sections (4 μm) were taken through the hilus, dehydrated, embedded in paraffin, and stained with hematoxylin/eosin (H&E). The percentage of lung affected by pneumonia was measured by automated morphometry (Zidas image-analysis system, Zeiss, Jena Germany). Measurements were taken blind and data are reported as the mean ± SD of measurements from three mice.

The same procedures were performed for MDR *M. tuberculosis* (CIBIN/UMF 15:99)-infected animals; this clinical isolate is resistant to rifampicin, ethambutol, streptomycin, pyrazinamide, and isoniazid, all first-line antituberculous drugs (16).

Subacute Toxicity

BALB/c mice (weighing 20 ± 2.0 g) were randomly assigned to three groups of eight animals each: 1) negative control; 2) vehicle control (ultrapure olive oil SIGMA), and 3) mice treated with 5 mg/kg of LA. The test dose of the compound is five times higher than the MIC determined *in vitro* assay (12). Animals were maintained under standard environmental conditions at 12 h light/dark photoperiods and were allowed free access to food and water. LA solubilized in ultrapure olive oil (Sigma) was administered s.c. for 21 days. Animals were weighed weekly and daily observed for physiological and behavioral changes. At the end of the experimentation period, the surviving mice were weighed, anesthetized with 5 mg/kg xylazine (Procin) and 50 mg/kg ketamine hydrochloride (Inoketam), and sacrificed by exsanguination. Blood samples were obtained for hematological and clinical chemical analyses and complete autopsy was performed, the main organs (liver, lung, kidney, brain, heart, spleen, and skeletal muscle) were weighed and stored in 10% aqueous formaldehyde for histological examination.

Heparinized blood samples were taken for determining complete blood count (CBC), red blood cell count (RBC), hemoglobin concentration (Hb), hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), platelets (Pt), white blood cell count (WBC), percentage of segmented leukocytes (Seg), and lymphocytes (Lymp). Serum was carefully collected for blood chemistry and enzyme analyses of the following: glucose (Glu); uric acid (UA); creatinine (Creat); total cholesterol (TCHOL); triglycerides (TRI); high-density lipoproteins (HDL); aspartate aminotransferase (AST); alanine aminotranferase (ALT); alkaline phosphatase (ALP), and gamma-glutamyl transferase (γ-GT). Analyses were performed utilizing automated equipment (Coulter T890 and Selectra II, respectively). Results were expressed as mean ± SD. Statistical significance between controls and experimental groups was assessed by one-way analysis of variance (ANOVA) and the post-hoc least-significant difference (LSD) test; p values <0.05 were considered significant. Parasagittal section of each organ was dehydrated and embedded in Paraplast (Kendall, Tyco Healthcare group, Oxford, UK), histological sections (3 μm) were stained with H&E.

Results

Figure 1 presents the chemical structure of (−)-Licarin A, and Figure 2 presents the results obtained of the LA treatment on BALB/c mice infected with drug-susceptible *M. tuberculosis* H37Rv. At the first month of treatment, the control group showed pulmonary bacilli loads significantly higher ($7_{,} \times 10^6 \pm 1.32$ CFU/lung) than the group treated with LA ($2.3 \times 10^6 \pm 1.75$ CFU/lung). After 2 months of treatment, there were higher, but not significantly, bacilli loads in the control group ($7.3 \times 10^6 \pm 1.53$ CFU/lung) than in the LA treated group ($4.9 \times 10^6 \pm 1.32$ CFU/lung) (Figure 2A).

At 1 month of treatment, the percentage of pneumonia-affected pulmonary area in the LA-treated group was significantly lower (3.19 ± 1.69%) than the control group (46% ± 15.73). At the second month of treatment (120 days post-infection), this difference was maintained being double in the control mice (56% ± 6.48) than in the LA-treated animals (22.8% ± 8.36) (Figure 2B).

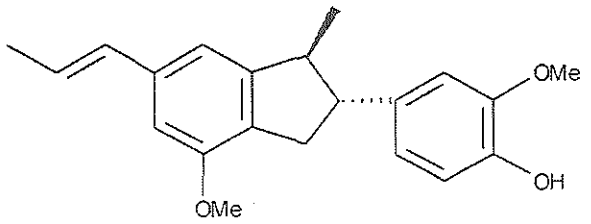

Figure 1. Chemical structure of (−)-Licarin A.

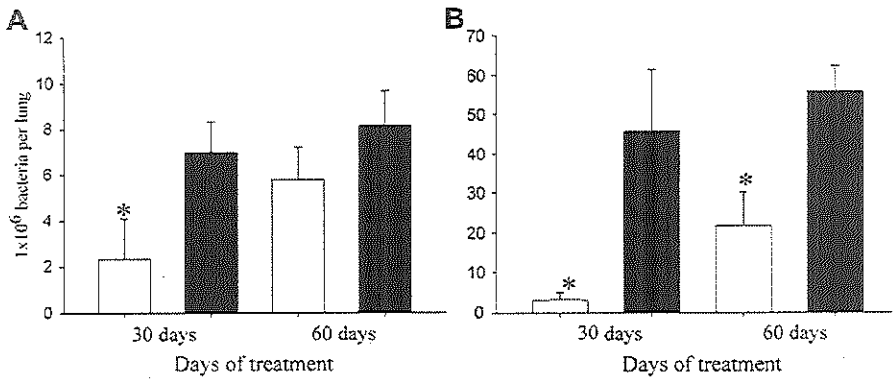

Figure 2. Antitubercular effect of (−)-Licarin A on BALB/c-infected mice with *M. tuberculosis* H37Rv. White bars correspond to the group treated with LA and black bars correspond to the control mice group. (A) Results of the bacterial burden in the infected lungs. (B) Comparison of the percentage of pneumonia area in the mice-infected lungs. Data are expressed as mean ± SD, three mice per time point; asterisks represent statistical significance ($p < 0.05$).

Regarding BALB/C mice infected with MDR *M. tuberculosis* (strain CIBIN/UMF 15:99), mice treated with LA for 1 month showed significantly lower bacilli loads ($0.28 \times 10^6 \pm 0.06$ CFU/lung) than control mice ($1.5 \times 10^6 \pm 0.08$ CFU/lung). During the second month of treatment, the antitubercular effect of LA was reduced and was similar to the control group (Figure 3A). In contrast, the percentage of pneumonia in animals treated with LA was significantly lower than in control animals after 1 month (22.28 vs. 43.56%). The difference was higher after 2 months of treatment (29.15 vs. 71.97%) (Figure 3B).

Healthy BALB/c mice treated with 5 mg/kg LA s.c. for 21 days did not show behavioral changes or clinical signs or deaths. A normal increase in body weight was registered in both the treated and control groups. Relative organ weights were also determined in all of these groups and no statistical differences were found (data not shown). Data from hematological and biochemical analyses are presented in Tables 1 and 2, respectively. Statistically analyzed data did not show differences between the control and treated groups. Autopsy study did not reveal gross lesions and no histological abnormalities were observed in main organs such as liver, kidney, spleen, lung, brain, heart, and muscle.

Discussion

As far as we know, this is the first report on a natural compound, (−)-Licarin A, displaying antitubercular and anti-inflammatory properties in a murine TB model. Our results show a significant reduction of the bacterial burden in the lungs of mice infected with *M. tuberculosis* H37Rv and MDR *M. tuberculosis* only during the first month of the LA treatment. In addition, the percentage of pneumonia was significantly reduced in animals treated with the natural compound; this effect was more significant in H37Rv-infected mice.

Despite the fact that TB is a worldwide problem and the task undertaken by several research groups has been the search

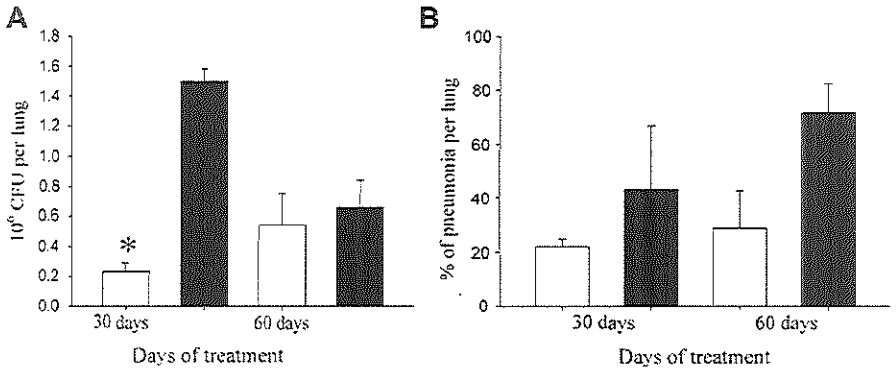

Figure 3. Antitubercular effect of (−)-Licarin A on BALB/c infected mice with *M. tuberculosis* MDR (CIBIN/UMF 15:99 resistant to rifampicin, ethambutol, streptomycin, pyrazinamide and isoniazid). White bars correspond to the group treated with LA and black bars correspond to the control mice group. (A) Results of the bacterial burden in the infected lungs. (B) Comparison of the percentage of pneumonia area in the mice-infected lungs. Data are expressed as mean ± SD, three mice per time point; asterisks represent statistical significance ($p < 0.05$).

Table 1. Hematological parameters of healthy BALB/c mice treated s.c. with 5 mg/kg (−)-Licarin A

Parameters	Control	Vehicle	(−)-Licarin A
RBC × 10^6/μL	10.22 ± 0.29	10.27 ± 0.39	10.23 ± 0.49
Hb (g/dL)	16.58 ± 0.58	17.36 ± 0.44	16.91 ± 0.90
Ht (%)	45.01 ± 1.57	45.36 ± 1.92	45.06 ± 2.33
MCV (fL)	44.00 ± 0.52	44.16 ± 0.53	44.03 ± 0.51
MCHC (g/dL)	36.85 ± 0.38	38.26 ± 0.70	37.55 ± 0.58
WBC × 10^{-3}/μL	7.06 ± 2.57	9.42 ± 1.84	6.96 ± 2.05
Seg (%)	17.83 ± 3.60	15.00 ± 2.55	13.80 ± 2.05
Lymp (%)	82.16 ± 3.60	86.50 ± 4.32	87.66 ± 6.59
Pt 10^5/μL	4.10 ± 0.41	4.49 ± 0.37	4.26 ± 0.30

RBC, red blood count; Hb, hemoglobin; Ht, hematocrit; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; WBC, white blood count; Seg, segmented leukocytes; Lymph, lymphocytes; Pt, platelets.

Values are expressed as mean ± standard deviation (SD) (n = 6). (−)-Licarin A (5 mg/kg/21 days) solubilized in olive oil (vehicle) was administrated s.c. to BALB/c mice.

for antimycobacterial compounds, only 10 of the >8,000 active molecules found are currently being evaluated in clinical trials (4,5). Novel, potential antimycobacterial molecules isolated from medicinal plants have been reported in the literature. Some examples are *E*-phytol, 12-dimethylmulticauline, multihorminone, 12-demethylmultiortho-quinone, 12-methyl-5-dehydrohorminone, 12-methyl-5-dehydroacetylhorminone, and others; these compounds possess *in vitro* MIC values of <2 μg/mL (12,17). However, the antitubercular activity of these compounds has not been corroborated *in vivo*. The murine TB model has been widely used to explore the effects of novel synthetic drugs (6,18,19); on the other hand, LA inhibits *in vitro* the growth of *M. tuberculosis* H37Rv, several MDR *M. tuberculosis* clinical isolates, and non-tuberculous mycobacterium strains with MIC values of <50 μg/mL (12).

Table 2. Biochemical parameters of healthy BALB/c mice treated s.c. with 5 mg/kg (−)-Licarin A

Parameter	Control	Vehicle	(−)-Licarin A
GLU (mg/dL)	209.16 ± 25.40	207.50 ± 32.67	229.50 ± 36.64
TCHOL (mg/dL)	33.50 ± 2.88	33.16 ± 1.72	31.66 ± 1.63
TRI (mg/dL)	2.43 ± 0.23	2.91 ± 0.81	2.28 ± 0.44
HDL (mg/dL)	14.85 ± 0.52	15.06 ± 0.59	14.15 ± 1.51
UA (mg/dL)	3.78 ± 1.71	2.56 ± 1.30	3.43 ± 1.47
Creat (mg/dL)	0.45 ± 0.05	0.42 ± 0.04	0.41 ± 0.02
ALP (IU/L)	299.66 ± 25.18	340.83 ± 30.13	321.66 ± 34.42
AST (IU/L)	110.25 ± 13.72	127.50 ± 11.93	193.00 ± 30.91
ALT (IU/L)	35.83 ± 4.99	37.16 ± 2.99	34.33 ± 2.58
γ-GT (IU/L)	4.61 ± 0.94	4.36 ± 0.68	4.8 ± 0.54

Glu, glucose; TCHOL, total cholesterol; TRI, triglycerides; HDL, high-density lipoproteins; UA, uric acid; Creat, creatinine; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; γ-GT, gamma-glutamyl transferase.

Values are expressed as mean ± standard deviation (SD) (n = 6). (−)-Licarin A (5 mg/kg/21days) solubilized in olive oil (vehicle) was administrated s.c. to BALBc mice.

In addition to the direct effect that (−)-Licarin A has on mycobacteria, the reduction in bacilli burdens and pneumonia produced by the LA treatment could be also ascribed to the anti-inflammatory effect exerted by this compound through inhibition of cyclooxygenase-2 (COX-2) activation (20,21) and the production of prostaglandin E_2 (PGE_2) (10). In this experimental model of TB, there is a high production of PGE_2 during late infection, which induces a significant decrease in protective immunity against *M. tuberculosis* by deactivating macrophages and T lymphocytes suppressing the production of significant factors such as IFN, TNF and iNOS (22). Although the reduction of bacterial burdens detected the second month during treatment with LA was not high, we observed in this group of mice a significant reduction of the percentage of pneumonia; thus, progression of tissue damage was decreased and this effect was likely a major factor that enhanced mice survival (15). In the search for new drugs, the anti-inflammatory properties of microbicidal molecules are important to consider as they prevent tissue damage progression or can provide disease contention (23). LA is an interesting natural product with a dual activity as antibiotic and immunoregulator.

Previous *in vitro* assays demonstrated (−)-Licarin A activity against mono- and MDR *M. tuberculosis* (12); thus, evaluation of the activity in the *in vivo* model is an important and necessary step contributing to the search for new antitubercular agents, specially considering the toxicity of the current drugs administered in pan-sensitive, MDR and XDR cases. Current TB treatment involves first- and second-line drugs that cause several adverse effects; for example, kanamycin and amikacin cause nephrotoxicity, fluoroquinolones cause neurotoxicity and induce drug-resistance, and some first-line drugs such as rifampicin, pyrazinamide, and ethambutol are hepatotoxic (4,24,25). LA toxicity studies showed normal histological architecture of the liver and kidney, and functional biochemical tests were also normal. Thus, LA produced no toxic effects in our experimental model.

Low toxicity together with the discrete bacteriostatic activity and significant anti-inflammatory effect, as well as the previously anti-mycobacterial effect described *in vitro* (12), makes LA a prototype for the development of new antitubercular agents. This work contributes to the knowledge of the toxic and therapeutic potential of a compound isolated from a Mexican medicinal plant. Ongoing evaluation is conducted to determine the antitubercular effect of LA in combination with antibiotics in the same model *in vivo* in order to evaluate its ability to shorten conventional chemotherapy.

Acknowledgments

This work was supported by grant FIS/IMSS/PROT/112 from the Coordinación de Investigación en Salud. IMSS and grant SEP-CONACyT 48339 and 84456 from CONACyT-México (Consejo

Nacional de Ciencia y Tecnología-México). RL-D is grateful to CONACyT-México for the scholarship (No. 172436) and to the National Autonomous University of Mexico (UNAM) Biological Sciences Postgraduate Program.

Conflict of Interest

The authors declare no conflict of interest.

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Vaccine 31 (2013) 676–684

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Prime-boost BCG vaccination with DNA vaccines based in β-defensin-2 and mycobacterial antigens ESAT6 or Ag85B improve protection in a tuberculosis experimental model

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ARTICLE INFO

Article history:
Received 17 August 2012
Received in revised form 2 November 2012
Accepted 13 November 2012
Available online 26 November 2012

Keywords:
Tuberculosis
DNA vaccine
BCG
Defensins
Ag85B
ESAT6

ABSTRACT

The World Health Organization (WHO) has estimated that there are about 8 million new cases annually of active Tuberculosis (TB). Despite its irregular effectiveness (0–89%), the Bacillus Calmette-Guérin) BCG is the only vaccine available worldwide for prevention of TB; thus, the design is important of novel and more efficient vaccination strategies. Considering that β-defensin-2 is an antimicrobial peptide that induces dendritic cell maturation through the TLR-4 receptor and that both ESAT-6 and Ag85B are immunodominant mycobacterial antigens and efficient activators of the protective immune response, we constructed two DNA vaccines by the fusion of the gene encoding β-defensin-2 and antigens ESAT6 (pDE) and 85B (pDA). After confirming efficient local antigen expression that induced high and stable Interferon gamma (IFN-γ) production in intramuscular (i.m.) vaccinated Balb/c mice, groups of mice were vaccinated with DNA vaccines in a prime-boost regimen with BCG and with BCG alone, and 2 months later were challenged with the mild virulence reference strain H37Rv and the highly virulent clinical isolate LAM 5186. The level of protection was evaluated by survival, lung bacilli burdens, and extension of tissue damage (pneumonia). Vaccination with both DNA vaccines showed similar protection to that of BCG. After the challenge with the highly virulent *Mycobacterium tuberculosis* strain, animals that were prime-boosted with BCG and then boosted with both DNA vaccines showed significant higher survival and less tissue damage than mice vaccinated only with BCG. These results suggest that improvement of BCG vaccination, such as the prime-boost DNA vaccine, represents a more efficient vaccination scheme against TB.

1. Introduction

Tuberculosis (TB) is an infection that produces 8.8 million new cases of active disease worldwide, 1.4 million deaths annually [1], and is considered a global emergency due to the increased appearance of new highly virulent [2] and drug-resistant strains [3,4]. Therefore, it is urgent to create new vaccines and/or vaccination schemes that can generate an efficient prophylactic effect or that can boost protective immunity in Bacillus Calmette-Guérin (BCG)-vaccinated individuals.

Mycobacterium bovis BCG, a live attenuated mycobacterial strain, is the sole vaccine available against TB to date. It has been used for nearly 100 years and its protection is extremely variable, from 0–89% [5–7]. Variability in BCG efficacy is associated with multiple factors [8–10]. Different approaches have been proposed to generate new and more effective vaccines, such as *Mycobacterium tuberculosis* (Mtb) mutants [11,12], recombinant BCG, which expresses highly immunogenic antigens [13,14], subunit vaccines based on the majority of immunogenic Mtb antigens [15,16], and DNA vaccines [17,18]. However, limited success has been achieved in this matter.

Recently, we have been working on the role that some antimicrobial peptides play in the innate immune response and activation

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http://dx.doi.org/10.1016/j.vaccine.2012.11.042

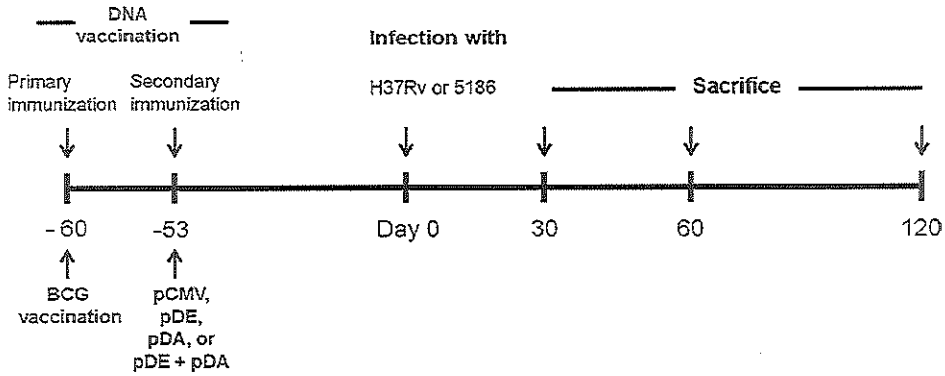

Fig. 1. Timeline of the animal vaccination, infection and sacrifice.
Groups of at least 6 animals were immunized by administration of 50 μg of DNA vaccine in thigh muscles. Electroporation was applied at the vaccination site. Bacillus Calmette-Guérin (BCG) vaccination was performed with 8×10^3 viable bacilli at day 60 prior to infection as shown in the time-line graph. Sixty days after immunization with DNA vaccine, vehicle, BCG, or a combination of any of the latter, the mice were infected with either of the two Mtb strains: H37Rv, and LAM 5186. Infected mice were sacrificed at different time points after Mtb infection, depending on the virulence of each strain. This strategy was used for all of the experiments. At least three independent experiments were conducted.

of the immune responses acquired in experimental TB. This is the specific case of β-defensin-2, an antimicrobial peptide that induces dendritic cell maturation in a Toll-like (TLR) receptor 4-dependent manner [19–21]. Interestingly, genetic construct coding sequences of β-defensin-2 generate a polarized and antigen-specific Th1 immune response [22–24]. This is important because multiple reports indicate that a Th1/CD8+ cytotoxic cellular immune response is essential for Mtb growth control [25]. Several Mtb antigens induce a strong Th1 response, such as the Early Secretory Antigenic Target-6 kDa (ESAT-6) protein, which is a potent immunogen encoded by the RD1 gene complex of Mtb [26] that is absent in BCG [27]. Similarly, Ag85B is a protein related with the mycolyl transferase secreted by Mtb and is a highly immunogenic antigen that induces a cytotoxic immune response [28–31]. Thus, in this work we designed DNA vaccines based on β-defensin-2 fused with ESAT6 or Ag85B, which actually induce a polarized Th1 immune response, and their efficiency was evaluated in a murine model of pulmonary TB challenged with Mtb strains, which possess diverse virulence levels.

2. Materials and methods

2.1. Gene cloning, fusion, and plasmid constructions

DNA constructs were made by amplification and cloning of the gene-of-interest; the gene for mature murine β-defensin-2 (mBD2) was cloned from mouse skin treated with LPS (10 ng/ml) by RT-PCR from total RNA utilizing specific primers: β-defensin-2 (*Defb2*) *F-5′-ACCATGGAACTTGACCACTGCCACACC-3′,R-5′-TGAATTCAAGATCTTTCATGTACTTG CAACAGGGGTTGTT, ESAT6 (esxA) F-5′-TATCTCGAGACCACC-3′, R-5′-CACCACCATCACCATCACT-AAGGATCCCGG GTAA-3′, Ag85B (fbpB) F-5′-ATGGATCCTATGTCG-ACCACATGACAGACGT GAGCCGAAAGATT-3′, R-5′-ATCCCGGGAAGGGT-CCTTAGTGATGGTGATG GTGGTGGCCGGCGCCTAACGAACTCTGCA-3′,* and *GAPDH F-5′-CTGGTGCTGAGTATGTCGTG-3′ R-5′-CAGTCTTCTG-AGTGGCAGTG-3′*. Amplification of the *esxA* and *fbpB* genes was performed from genomic DNA of Mtb H37Rv strain, isolated as reported elsewhere [32]; these sequences encode for ESAT6 or Ag85B antigens, respectively. The DNA constructs are based on a pCMV vector; the specific characteristics of the construct were reported previously by our group [23,33]. The following four constructs were generated: pCMV-*mDF2B-esxA* (pDE); pCMV-*mDF2B-fbpB* (pDA); pCMV-*esxA* (pE); pCMV-*fbpB* (pA), and the empty pCMV vector that was used as control.

The constructs were analyzed by PCR, enzyme digestion, and sequencing in order to confirm insertion and open reading frame (ORF). XL10 Gold bacteria (Invitrogen, Carlsbad, CA, USA) were transformed with each construct and grown in LB broth base medium (Invitrogen). Plasmid purification was performed with the Endofree Plasmid Maxi kit as referred by the supplier (QIAGEN, Hilden, Germany). The plasmids were eluted in sterile pyrogen-free phosphate buffer (SIGMA, Steinheim, Germany).

2.2. Vaccination

All animal studies were approved by the Institutional Ethics Committee in accordance with the guidelines of the Mexican National Regulations on Animal Care and Experimentation NOM 062-ZOO-1999. BALB/c mice aged between 6 and 8 weeks of age were anesthetized with sevoflurane (Abbott, Quebec, Canada). Then, the respective plasmid was administered intramuscularly (i.m.) in the right thigh, using 100 μl of sterile PBS as vehicle. In order to increase the efficiency of the DNA vaccination, an electric shock was applied at the injection site with the CYTOPULSE pulseAgile® Model PA-3000 electroporator system, according to the manufacturer's suggestions. The amount of DNA vaccine to be employed was determined with a dose-response curve. Best expression profile was observed at 50 μg for DNA vaccine alone and for co-administration, this was 25 μg DNA of each vaccine. A second dose of the DNA vaccine was applied 8 days after the first vaccination as a boost. In the case of BCG vaccination, 8×10^3 viable bacterial cells BCG substrain Phipps were injected subcutaneously (s.c.) in the base of the tail. This BCG substrain was the most protective of 10 strains tested in the BALB/c mouse model of progressive pulmonary tuberculosis [34]. The immunization schedule is depicted in detail in Fig. 1.

2.3. RT-PCR for mRNA expression assessment of DNA constructs

One, 3, 8, and 14 days post-vaccination, the animals were euthanized and thigh muscles were immediately excised; one fragment was fixed by immersion in 10% formaldehyde dissolved in PBS for immunohistochemistry, and the remaining tissue fragment was preserved in TRIzol (Invitrogen) for gene analysis. For nucleic acid purification, tissue was homogenated in Ultra-Turrax® T-10 apparatus (IKA, Wilmington, NC, USA). The RNAeasy mini kit with DNAase (QIAGEN, Düsseldorf, Germany) was employed for RNA isolation according to the manufacturer's instructions. One hundred

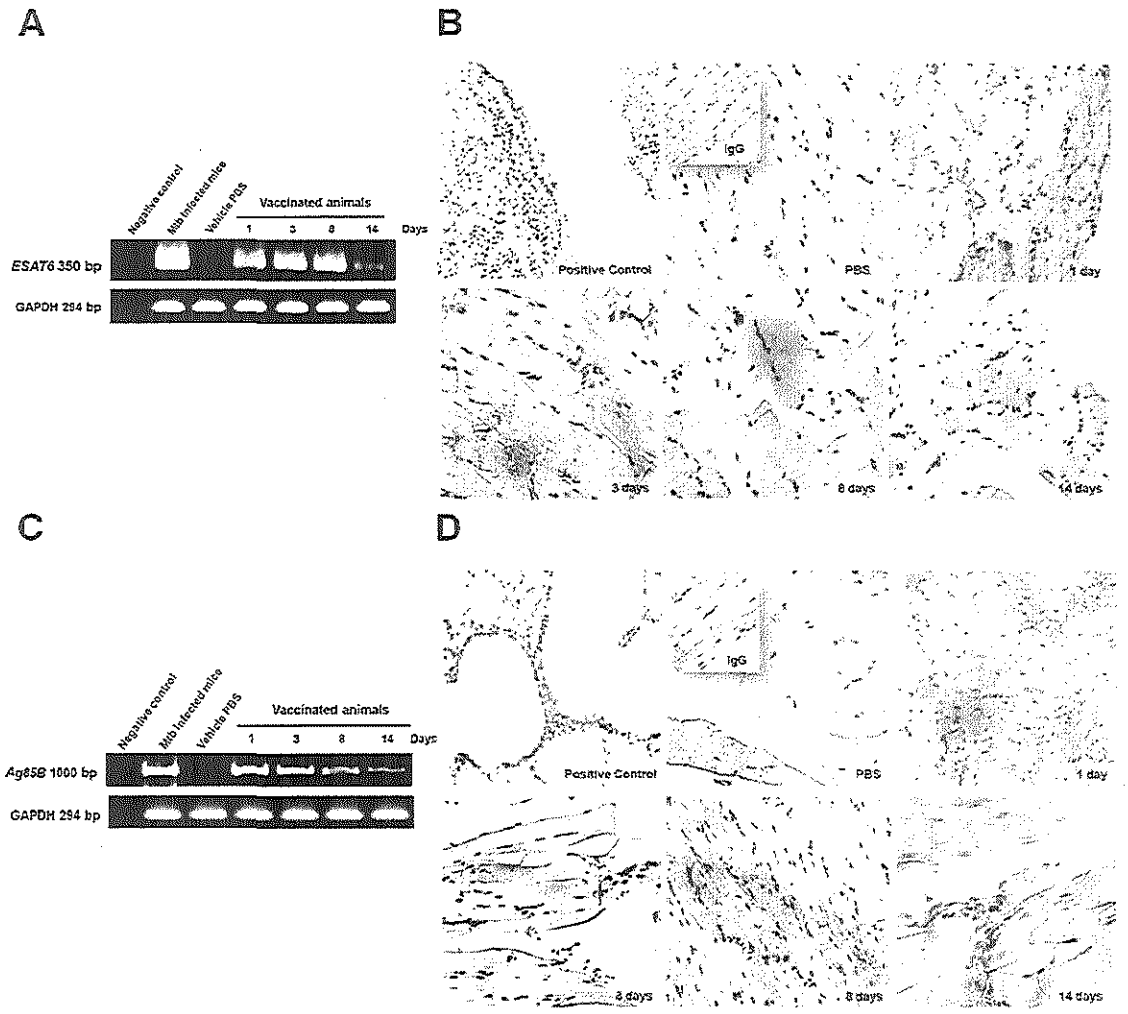

Fig. 2. Kinetics of ESAT6 and Ag85B mRNA and protein expression in the muscle of immunized mice.
Two groups of six BALB/c mice per group were immunized with either pDE or pDA and sacrificed at 1, 3, 8, and 14 days. (A) RT-PCR for ESAT6 mRNA expression from thigh muscle samples extracted from vaccinated mice. GAPDH mRNA expression by RT-PCR was used for determination of basal expression; (B) Micrographs showing antigenic protein expression and immunohistochemistry for ESAT6 at 20× magnification. Positive control was from mice infected with H37Rv strain at a 10× magnification. Similarly, panels depict protein expression at 1, 3, 8, and 14 days after vaccination; (C) RT-PCR for expression of Ag85B mRNA extracted from the muscle of immunized mice. GAPDH mRNA expression by RT-PCR was utilized for determination of basal expression; (D) Ag85B antigenic protein expression analyzed by immunohistochemistry at 20× magnification. Positive control from mice infected with H37Rv at 10× magnification. Protein expression at 1, 3, 8, and 14 days after vaccination is illustrated. Representative results from three independent experiments are shown.

nanograms of purified RNA was used for cDNA synthesis utilizing the Omniscript cDNA synthesis kit (QIAGEN) and submitted to PCR.

2.4. Immunohistochemistry

For immunohistochemistry, 5-μm-thick sections were deparaffinized and the endogenous peroxidase quenched with 0.03% H_2O_2. Then, the sections were blocked with PBS supplemented with 2% human pool serum. Muscle sections were incubated with rabbit polyclonal anti ESAT6 and rabbit polyclonal anti Ag85B (Abcam, Cambridge, U.K.) and subsequently incubated with a biotin-labeled anti rabbit IgG antibody. Bound antibodies were detected with avidin–biotin peroxidase (Biocare Medical, Concord, CA, USA) and counterstained with hematoxylin.

2.5. Immunogenicity of DNA vaccines

Groups of six BALB/c mice were vaccinated with either pDE, pDA, pE, pA, pCMV, or PBS and sacrificed at 14, 21, and 40 days post-vaccination. Cell suspensions from inguinal lymph nodes, spleen, and lungs were cultured and stimulated with mycobacterial Culture filtrate antigens (CFA), rESAT-6, or rAg85B, as previously reported [35]. Cultures for cytokine production (1×10^5 cells in 100 μl of culture medium) were performed in flat-bottom, 96-well plates with 5 μg CFA, rESAT6, or rAg85B. After 72 h, the supernatants were collected and utilized for Interferon gamma (IFN-γ) quantification by means of a commercial Enzyme-linked immunosorbent assay (ELISA) test kit (Pharmingen, San Diego, CA, USA).

2.6. Experimental model of progressive pulmonary TB

The experimental model of progressive pulmonary TB has been previously described in detail [14]. Groups of vaccinated mice were challenged 60 days after the first immunization with *M. tuberculosis* strains H37Rv or with the Latin-American Mediterranean (LAM) clinical isolate (5186 strain), which is highly virulent in this mouse model [2,36]. Each animal was anesthetized with

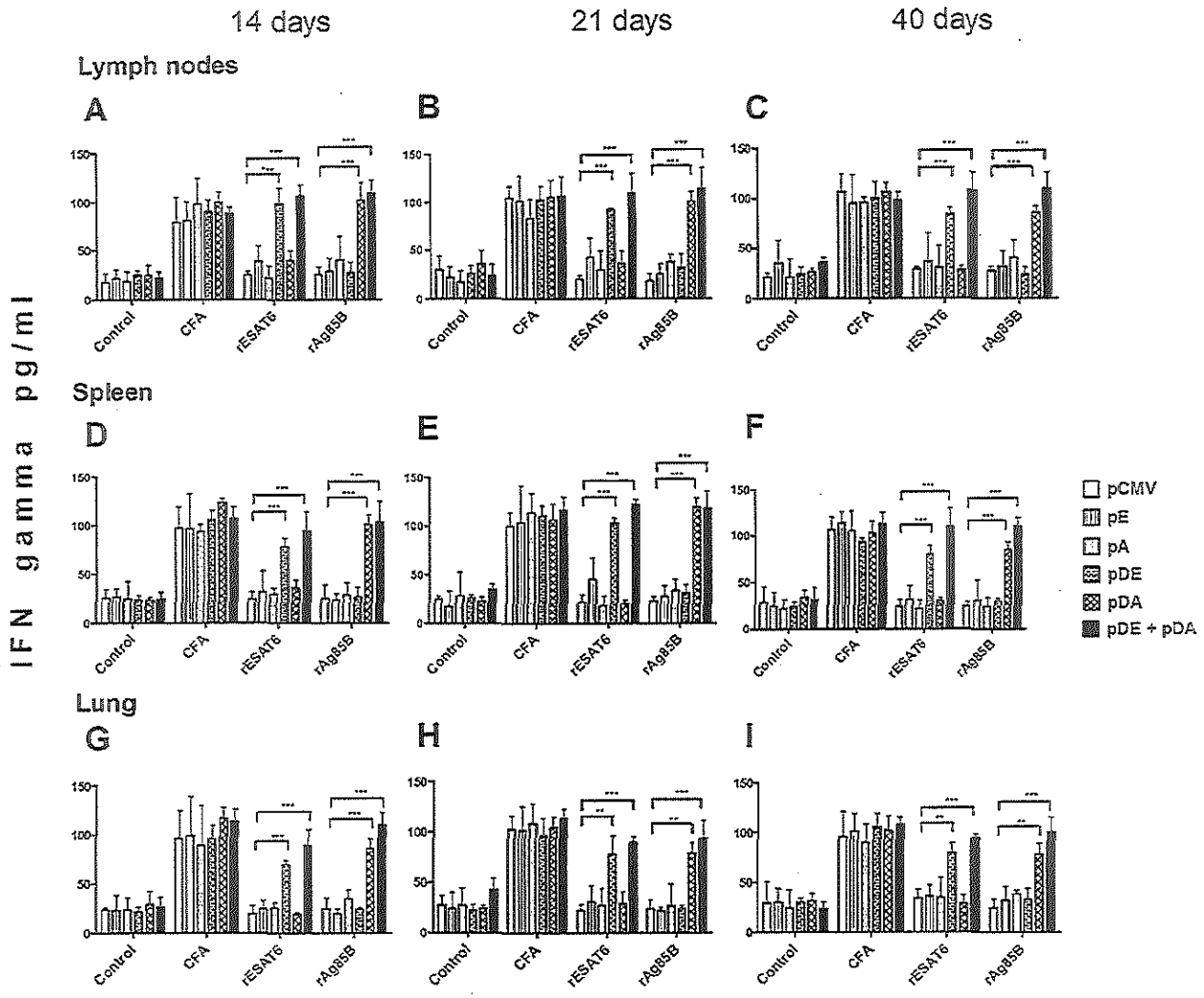

Fig. 3. Interferon gamma (IFN-γ) quantification in culture supernatants of mononuclear cells from immunized mice stimulated with specific mycobacterial antigens. Mice were immunized with pE, pA, pDE, pDA, or pCMV. Mononuclear cells extracted from immunized mice were cultured in 96-well plates and exposed to antigens such as culture filtrate antigen (CFA), rESAT6, or rAg85B proteins for 72 h. The cell supernatants were employed to determine IFN-γ production by ELISA. Cells were isolated from the inguinal lymph node at 14, 21, and 40 days post-vaccination (A, B, and C, respectively); spleen cells were isolated at days 14, 21, and 40 days post-vaccination (D, E, and F, respectively). The same procedure was performed for cells isolated from the lungs of vaccinated mice (G, H, and I). Each group of animals consisted of 6 mice in two independent experiments. Two-way ANOVA and Bonferroni's post-test was performed in order to identify differences among groups; p values of <0.05 are considered statistically significant.

sevoflurane and was intratracheally (i.t.) instillated with 2.5×10^5 viable bacterial cells suspended in 100 μl of sterile, pyrogen-free PBS. To determine vaccination effectiveness, groups of six mice in two independent experiments were euthanized after 30, 60, or 120 days of challenge; their lungs were employed to determine bacilli burdens by colony forming unit (CFU) quantification and histology/morphometry, thus determining the percentage of lung surface affected by pneumonia. Infected mice were sacrificed at different time points after Mtb infection, depending on the virulence of each strain. Another group of 10 mice was left untouched and mortality was recorded to construct survival curves.

2.7. Determination of colony-forming units and histopathological analysis of infected lungs

The right lungs of six mice at each time point in at least two independent experiments were utilized for CFU quantifications. Briefly, the lungs were homogenized with a polytron (Kinematica, Lucerne, Switzerland) in sterile 50-ml tubes containing 3 ml of isotonic saline. Four dilutions of each homogenate were spread onto duplicates containing Bacto Middlebrook 7H10 agar plates enriched with OADC. The plates were incubated at 37 °C and the CFU were counted at day 21.

For histopathological analysis, lungs were perfused i.t. with absolute ethanol, immersed for 24 h, and embedded in paraffin. Five-μm-thick sections taken through the hilus were stained with H&E. In these slides, the area (μm^2) occupied by the inflammatory infiltrate was determined using an image analyzer (Axiovert 200 M with Axiovision ver.4.3; Carl Zeiss, Jena, Germany).

2.8. Statistical analysis

Data normality was assessed through the Kolmogorov-Smirnov test. Normal distribution data were analyzed with one-way Analysis of variance (ANOVA) and Bonferroni's post-test. For CFU nonparametric data, a Kruskal–Wallis multiple comparisons test was employed with Dunn's post-test. Immunogenicity assays based in gamma interferon production data were analyzed with

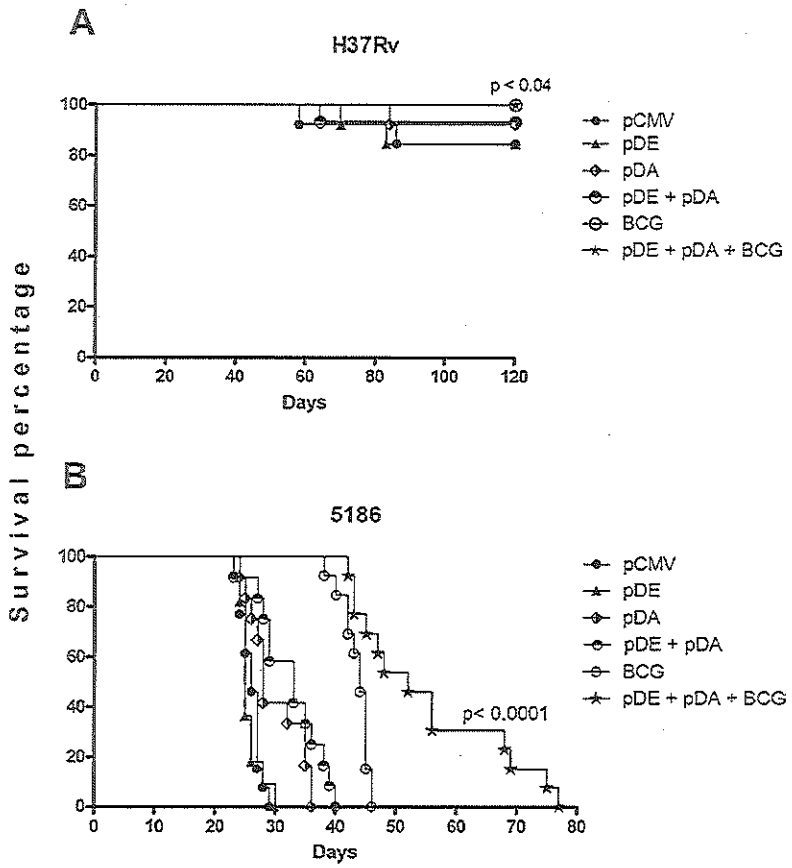

Fig. 4. Survival rate of vaccinated animals challenged with different Mtb strains.
Several groups of animals were vaccinated with DNA constructs, controls, and BCG. The immunized animals were challenged with the H37Rv strain (A), and the 5186 strain (B). Death of animals was recorded daily. Twenty animals were included in each group in two independent experiments. Kaplan–Meier survival curves were performed, in addition to statistical analysis using the log-rank test; a p value of <0.05 was considered significant.

two-way ANOVA using Bonferroni's post-test. Statistical analysis of Kaplan–Meier survival curves was performed using Log-rank test. P values of ≤ 0.05 were considered significant.

3. Results

3.1. DNA vaccine constructs are expressed and translated in the muscle cells of mice

In order to determine whether muscle cells were efficiently transfected by DNA vaccine electroporation, the expression *in situ* of mRNA and antigen protein were evaluated. The kinetics for ESAT6 or Ag85B mRNA are depicted in Fig. 2A and C, respectively. The expression of both constructs is stronger at day 1, exhibiting a progressive decrease until day 14, when lowest expression was observed. Similar protein expression kinetics determined by immunohistochemistry is illustrated in Fig. 2B and D; both proteins were specifically detected in the cytoplasm of striated muscle cells located at the vaccination site.

3.2. pDA and pDE dna vaccines induce a specific Th1 immune response against mycobacterial antigens

Subsequently, we tested the immunogenicity of vaccine constructs. Mice were DNA-immunized with pE, pA, pDE, pDA, and pDE + pDA constructs by assessing IFN-γ production from T-cells 14, 21, and 40 days after immunization, measuring cytokine levels in the supernatants of cell suspensions from inguinal lymph nodes, spleen, and lung after stimulation with rESAT6, rAg85B, or CFA. IFN-γ production from inguinal lymph node cells from animals vaccinated with either or both pDE and pDA DNA vaccines showed that there is a specific response from these cultured cells to these mycobacterial antigens. Significant differences are demonstrated in animals vaccinated with pCMV, pE, and pA compared with those vaccinated with pDE and pDA ($p < 0.0001$), confirming the efficient role of mBD2 as an adjuvant inducing a stronger immune response. Similar levels of IFN-γ were detected in the different organs along the experiment, indicating that there is a sustained effect on the production and elicitation of this cytokine (Fig. 3A–C).

3.3. Protective effect of DNA vaccines

After confirming the immunogenicity efficiency of our DNA vaccines, pE and pA vaccines were not employed for further experiments because they had non-significant differences with the control (pCMV). Animals were vaccinated and challenged (Fig. 1) with strains of different virulence and genotype levels. Survival, lung bacilli burdens, and extensions of tissue damage (pneumonia) were analyzed at each sacrifice point.

Fig. 4 shows the survival curves: control animals that received only the empty vector and that were challenged with the H37Rv reference strain began to die after 2 months and 85% survived to the end of the experiment. Animals vaccinated with pDE exhibited similar responses to pCMV, while mice vaccinated with pDA or both pDE + pDA exhibited better survival (90%) than those of the control group. Mice vaccinated with BCG and boosted with both DNA

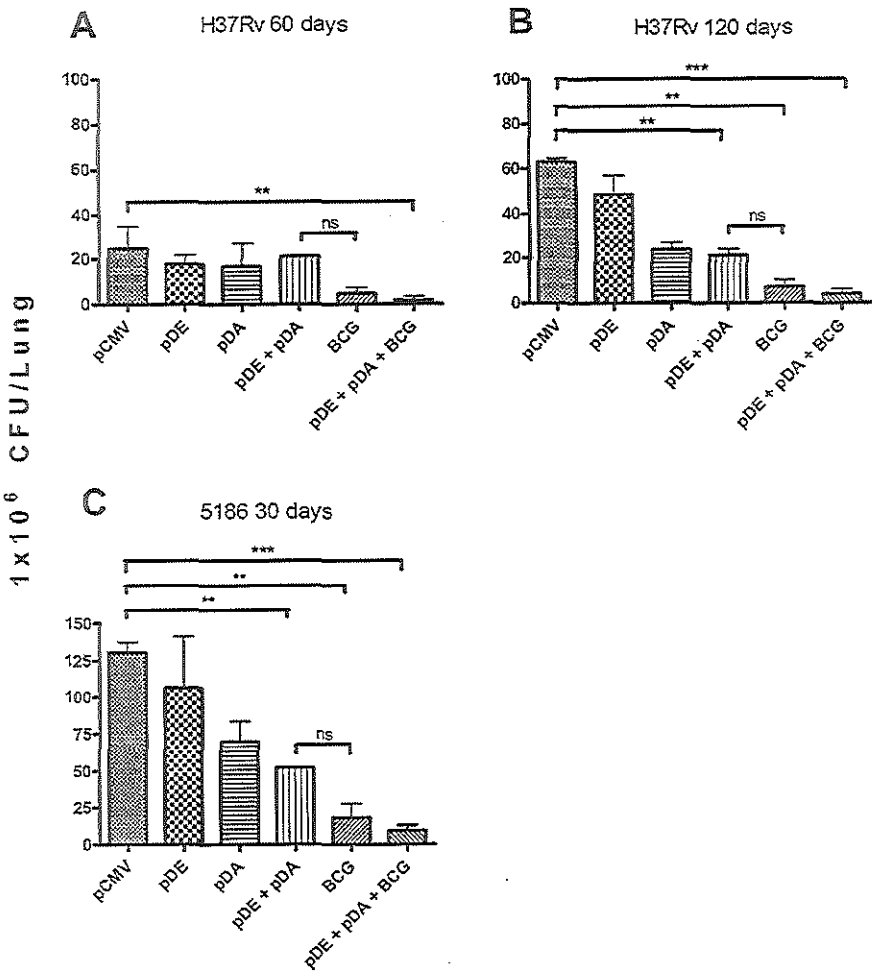

Fig. 5. Determination of Colony-forming units (CFU) in mice immunized and challenged with two different *Mycobacterium tuberculosis* strains.
Groups of mice were immunized with DNA vaccines, BCG, and controls according to the schematic description in Fig. 1. Then, animals were challenged with several Mtb strains and CFU determination was performed in the lungs of infected animals at different time points. (A) Animals were challenged with the H37Rv strain and sacrificed at days 60 and (B) 120 post-infection; (C) Mice were immunized and later challenged with the 5186 strain and sacrificed at 30 days post-infection. Groups of six mice per group were sacrificed at the specified times, and the experiments were repeated three times independently. One-way ANOVA with Bonferroni's multiple comparison test was performed to establish statistical significance. *P* values of <0.05 were considered statistically significant.

vaccines showed 100% survival after 4 months of challenge and there was a statistical difference ($p < 0.04$) when these were compared with pCMV-vaccinated mice (Fig. 4A). Similar results were observed when mice were challenged with the highly virulent LAM 5186 strain; pCMV-vaccinated mice died after 30 days of i.t. infection, and similar survival trends were observed in mice vaccinated with pDE. The animals vaccinated with pDA, BCG, or both pDE + pDA died at 40 ± 5 days post-challenge. In contrast, significant survival was observed in animals vaccinated first with BCG and boosted with pDA and pDE, which demonstrated two-fold survival (80 days; $p < 0.0001$) compared with BCG alone.

Survival curves were consistent with lung bacilli burdens, because mice immunized with any vaccine type and challenged with strain H37Rv showed significantly lower bacilli loads than the control group at both time points, with the lowest in the prime-boosted group (Fig. 5A and B).

Similar results were observed in vaccinated animals challenged with the highly virulent LAM 5186 strain. The group of animals vaccinated with pDA + pDE showed higher, but non-significant, lung bacilli loads than the BCG-vaccinated group, while this latter group showed higher, but non-significant, bacilli burdens than the group previously vaccinated with BCG and boosted with both DNA vaccines (Fig. 5C).

All groups showed a considerable reduction in pneumonic area when compared with control mice, this more evident at day 120. Similar protection was observed between the groups vaccinated with both DNA vaccines and the BCG-vaccinated group, while this latter group showed a significant, five-fold higher pneumonic area than the group previously vaccinated with BCG and boosted with both DNA vaccines (Fig. 6A and B). Comparable results were observed in vaccinated mice challenged with the highly virulent strain (Fig. 6C). Thus, vaccination with both DNA vaccines and prime-boosted with BCG confers greater significant tissue damage protection than the BCG vaccination alone.

4. Discussion

In this work, a new vaccination strategy was used to induce a greater protective response against Mtb based on the use of a DNA vaccine conformed of mBD-2 and the immunodominant mycobacterial antigens ESAT-6 and Ag85B. Our immunogenicity results based on the release of IFN-γ from antigen-specific T-cells derived from inguinal lymph nodes, spleen, and lung confirm this property by demonstrating that in the immediate vicinity of the vaccination site (inguinal lymph node), in the systemic milieu (spleen),

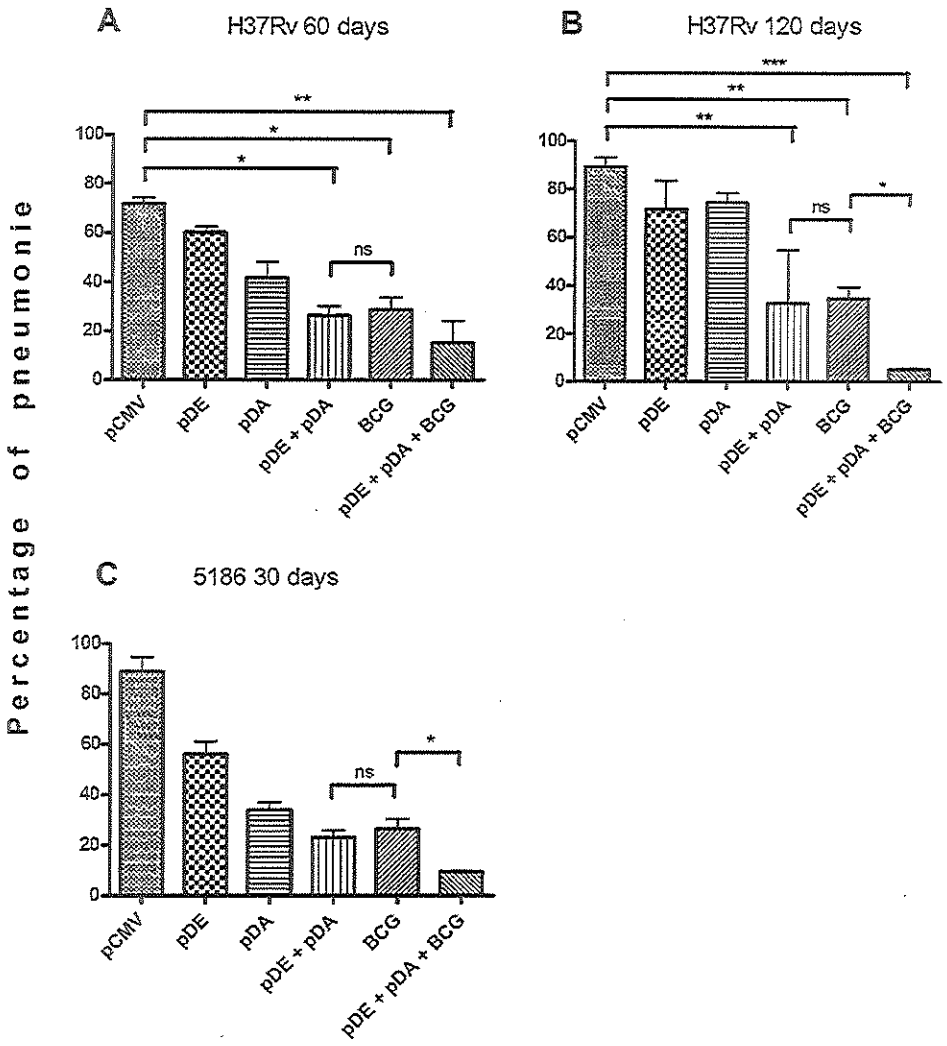

Fig. 6. Pneumonic area in mice immunized and challenged with Mtb strains.
Mice were immunized with pDE, pDA, or pCMV DNA vaccines, employing the vehicle as control. Sixty days after vaccination, the animals were infected with either the H37Rv or the 5186 strain. Animals were then sacrificed and the left lungs were ethanol-fixated and paraffin- embedded. After Hematoxylin–eosin (H&E) staining, the pneumonic area was determined in imaging analysis software. (A) Shows pneumonic area analysis of mice challenged with the H37Rv strain and sacrificed at days 60 and (B) 120 post-infection; (C) After immunization, mice were challenged with the 5186 strain and sacrificed at 30 days post-infection. Each group consisted of six mice, and three experiments were conducted independently. One-way ANOVA with Bonferroni's post-test was performed for assessment of differences. *P* values of $p < 0.05$ were considered statistically significant.

and in the lung, both of the pDE or pDA constructs induced a better immune response compared with constructs coding for Ag85B (pA) and ESAT6 (pE) antigens alone, indicating that β-defensin-2 increased the specific immune response when employed as an adjuvant in the DNA construct, similarly to data previously reported for other antigen targets, such as the HIV gp120 protein [22]. This effect is probably due to the more efficient maturation and activation of immature dendritic cells (iDC) mediated by mBD-2 in a TLR4-dependent manner [20]. These results are also consistent with published reports in cancer models, in which DNA vaccines expressing β-defensin-2 increased the immune response specifically against strong or poorly immunogenic neoplastic antigens in different cancer types [23,24,37].

It has been demonstrated in experimental models that BCG protection depends on the virulence of the infecting organism [38,39]. In agreement with this, both pneumonia and bacilli burdens determined from pDE- or pDA-vaccinated mice showed differences depending on the Mtb strain utilized for the challenge. These differences could probably also be due to differences in the expression of ESAT6 or Ag85B, considering that strains with higher expression of either ESAT6 or Ag85B antigens would be better recognized by animals vaccinated with pDE and pDA. However, more studies are needed for clarify concerning this issue.

When pDA and pDE were co-administrated, they showed a similar protective effect in animals compared with BCG-vaccinated mice, particularly when the animals were infected with the highly virulent 5186 strain. This is probably due to higher expression of ESAT-6 and Ag85B by this strain and perhaps by the role of both antigens in mycobacterial virulence [40,41]. Moreover, several reports indicate that one participating factor in the variability of the BCG efficacy is probably associated with the low expression and absence of Ag85B and ESAT6, respectively [9,42].

Because the majority of the human population in the developing world is already BCG-vaccinated, an attractive strategy would be to boost this existing immune response. Moreover, children in endemic countries where BCG vaccination is a generalized practice are highly sensitized due to the combination of BCG vaccination, environmental mycobacteria, and latent TB infection. In these

settings, BCG revaccination did not increase protection and is generally not recommended [43]. This is in agreement with animal studies showing that revaccination with BCG lowers effectiveness [44] or can produce by Interleukin (IL)-17-mediated necrosis [45]. Thus, BCG, despite its variable effectiveness, can be employed as a priming agent for a booster vaccination scheme [46]. Therefore, we performed the co-administration of BCG plus pDE + pDA in order to complement the antigenic repertoire required to generate a greater immune response. Considering the genetic diversity among the different BCG sub-strains that have showed diverse levels of protection, choice of the BCG strain utilized for vaccination is a very important issue; we used substrain Phipps because it was the most efficient in conferring protection among 10 different BCG strains in our murine model [34]. Interestingly, mice vaccinated with BCG Phipps boosted with both DNA vaccines and challenged with the highly virulent 5186 strain showed significant higher protection than mice with a single vaccination of BCG, suggesting an improvement of the antigenic repertoire by the mBD2 Th-1 polarizing immune response to ESAT6, Ag85B, plus the BCG antigens.

5. Conclusions

Our data suggest that use of DNA vaccines containing coding sequences for β-defensin-2 induces a Th1 adaptive response against highly immunogenic antigens from Mtb, and when these DNA vaccines were used for booster immunization after BCG vaccination, a significant improvement of protection against Mtb strains was produced. This new strategy could greatly improve BCG vaccination efficacy against highly transmittable and virulent strains, such as the LAM 5186 strain, suggesting that improvement of BCG vaccination combined with DNA vaccines in a prime-boost scheme is a good choice for the rational design of a more efficient vaccine against TB.

Conflicts of interest

The authors declare no conflict of interest

Acknowledgments

This work was supported by Consejo Nacional de Ciencia y Tecnologia-México (CONACyT-México grant CB-2007-01-82424 to BR-S and 84456 to RH-P.

The authors acknowledge Dr. Felipe Villagrana-Félix for his help in animal anesthesia. ARC-V acknowledges CONACyT-México for scholarship number 203661/CVU 208395.

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International Journal of Antimicrobial Agents 41 (2013) 143–148

Contents lists available at SciVerse ScienceDirect

International Journal of Antimicrobial Agents

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Activity of LL-37, CRAMP and antimicrobial peptide-derived compounds E2, E6 and CP26 against *Mycobacterium tuberculosis*

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ARTICLE INFO

Article history:
Received 25 June 2012
Received in revised form
13 September 2012
Accepted 26 September 2012

Keywords:
Tuberculosis
Treatment
Antimicrobial peptides

ABSTRACT

Tuberculosis (TB) is a major worldwide health problem in part due to the lack of development of new treatments and the emergence of new strains such as multidrug-resistant (MDR) and extensively drug-resistant strains that are threatening and impairing the control of this disease. In this study, the efficacy of natural and synthetic cationic antimicrobial (host defence) peptides that have been shown often to possess broad-spectrum antimicrobial activity was tested. The natural antimicrobial peptides human LL-37 and mouse CRAMP as well as synthetic peptides E2, E6 and CP26 were tested for their activity against *Mycobacterium tuberculosis* both in in vitro and in vivo models. The peptides had moderate antimicrobial activities, with minimum inhibitory concentrations ranging from 2 μg/mL to 10 μg/mL. In a virulent model of *M. tuberculosis* lung infection, intratracheal therapeutic application of these peptides three times a week at doses of ca. 1 mg/kg led to significant 3–10-fold reductions in lung bacilli after 28–30 days of treatment. The treatments worked both against the drug-sensitive H37Rv strain and a MDR strain. These results indicate that antimicrobial peptides might constitute a novel therapy against TB.

1. Introduction

Tuberculosis (TB) [1], caused by the bacterium *Mycobacterium tuberculosis*, remains one of the leading causes of disease and mortality due to an infectious agent. According to recent data from the World Health Organization (WHO), in 2010 there were 8.8 million active TB cases worldwide and nearly 1.5 million deaths. It has been estimated that one-third of the human population carries *M. tuberculosis* and 10% of these people will develop active disease at some time in their lives, creating an enormous reservoir [2].

Treatment of pulmonary TB has become increasingly challenging due in part to the required long duration of therapy and the advent of multiple drug resistance. One of the most important factors is the emergence of multidrug-resistant (MDR) bacilli that has been associated with inadequate use of antibiotics and poor adherence to recommended treatment regimens [3]. In recent years, new strains have emerged, termed extensively drug-resistant (XDR), that are also resistant to second-line antibiotics such as fluoroquinolones and either kanamycin, amikacin or capreomycin. These strains lead to poor treatment outcomes and a considerably increased rate of mortality [4]. Recent reports suggest the possible existence of cases of completely resistant TB in the Middle East, raising concerns regarding how to treat these TB cases effectively [5].

In the past 40 years, no broadly successful new TB drug has been developed. Therefore, there is a strong drive to develop new treatments for TB and/or to improve those currently in use. Important advances have been made and there are several clinical trials underway that have utilised fluoroquinolones in place of ethambutol, leading to preliminary indications of a significant reduction in the duration of therapy and encouraging the possibility of an improvement in patient survival [6].

Antimicrobial peptides (AMPs) are gene-encoded, amphipathic, cationic peptides that are produced by several species of mammals, birds, reptiles and amphibians. These peptides can inhibit microbial growth through a variety of often complex mechanisms, including membrane interactions that lead to permeabilisation of cells, inhibition of cell wall synthesis, and entry into cells leading to inhibition

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http://dx.doi.org/10.1016/j.ijantimicag.2012.09.015

of macromolecular synthesis [7–9]. In addition, these peptides, also termed host defence peptides, can profoundly and favourably modulate innate immunity, upregulating protective immunity such as increasing the production of chemokines to recruit immune cells whilst dampening potentially harmful inflammation [9,10]. The major groups of AMPs in humans are the defensins and a single cathelicidin, LL-37. It has been reported that alterations in the production of these molecules increase susceptibility to infectious diseases, including TB [11]. Conversely, upregulation of cathelicidin LL-37 through use of vitamin D supplementation has been considered to be a potential strategy to improve TB infection outcomes, although current data do not necessarily favour this possibility [12].

Previous studies by our group have reported that during *M. tuberculosis* infection of lung epithelial cells, there was a high production of β-defensins-3 and -4, and both were associated with mycobacteria in the lung, suggesting their possible participation in clearance of *M. tuberculosis* [13,14]. Subsequently, it was reported that in murine TB models, BALB/c mice produced low quantities of murine β-defensins-3 and -4 during late progressive TB, and when both defensins were overproduced by intratracheal administration of isoleucine (a defensin inducer) these animals efficiently controlled infection both by drug-sensitive and drug-resistant bacilli [14,15]. In addition, it has been shown that the interaction of a 19-kDa lipopeptide of *M. tuberculosis* with Toll-like receptor-2 on the macrophage surface upregulated the expression of vitamin D receptor leading to the induction of cathelicidin LL-37, promoting the killing of intracellular *M. tuberculosis* [16,17].

Recently, methodologies have been developed to enable the enhanced design of AMPs (e.g. [18]). Rational substitution studies led to an enhanced 26-amino-acid β-helical peptide CP26 derived from a hybrid peptide comprising the amphipathic α-helical N-terminal region of cecropin A and the hydrophobic N-terminal α-helix of the bee venom peptide melittin [17]. Peptide array methods and substitution studies, starting from the smallest known broad-spectrum natural AMP bactenecin (also known as bovine dodecapeptide), led to peptides E2 (also known as Bac8c), an 8-amino-acid peptide, as well as E6 (also called Sub3), a 12-amino-acid peptide, both of which demonstrated enhanced activity against a range of pathogenic Gram-positive and Gram-negative bacteria and the yeast *Candida albicans* [18,19].

In this study, the antimicrobial activity of five natural and synthetic peptides against *M. tuberculosis* was evaluated in an in vitro setting.

2. Materials and methods

2.1. Peptide synthesis and design

Peptides were synthesised by the Peptide Synthesis Facility, Biomedical Research Centre at the University of British Columbia (Vancouver, Canada) using tertiary butyloxycarbonyl (tBOC) solid-phase synthesis. Peptides were purified by high-performance liquid chromatography to >95% purity and were confirmed by mass spectrometry.

The following peptides were utilised: mouse CRAMP (GLLRKGGEKIGEKLKKIGQKIKNFFQKLVPQPEQ) [20]; human LL-37 [20,21] (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES); E2 (also termed Bac8c; RIWVIWRR-NH_2) [18,19]; E6 (also termed Sub3; RRWRIVVIRVRR-NH_2) [18,19]; and CP26 (KWKSFIKKLTSAAKKVVTTAKPLISS) [22]. Briefly, these peptides were selected as either natural peptides with moderate antimicrobial activity (LL-37 and CRAMP) or as broad-spectrum synthetic peptides with moderate to good antimicrobial activity (E2, E6 and CP26).

2.2. Mycobacterium tuberculosis strain growth

The drug-sensitive *M. tuberculosis* strain H37Rv (ATCC) and a MDR strain (clinical isolate, resistant to first-line antibiotics) were grown in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI) supplemented with 0.2% glycerol, 10% oleic acid–albumin–dextrose–catalase (OADC enrichment media; Becton Dickinson, Franklin Lakes, NJ) and 0.02% Tween 80 at 37 °C. Mid-log phase cultures were used for all experiments.

2.3. Microdilution colorimetric reduction assay

Susceptibility testing utilising resazurin (Trek Diagnostic, Westlake, OH) as an indicator of residual bacterial viability was performed in Costar® 96-well flat-bottom plates (Corning Inc., Corning, NY) as described previously [23]. Briefly, all test wells contained 100 μL of OADC-supplemented Middlebrook 7H9 growth medium. Then, 100 μL of the diluted peptide at the highest concentration starting at 12.8 μg/mL was added to one well. The contents of the wells were mixed thoroughly and 100 μL was transferred into the next well; the process was then repeated, thus creating serial two-fold dilutions. In addition to the tested peptides, rifampicin (8.0 μg/mL) was used as a positive control, and medium without any compound was used as a negative control in each plate. Peptides were tested in the concentration range 0.4–12.8 μg/mL.

Plates were incubated at 37 °C for 5 days. On Day 5, 20 μL of 0.01% resazurin solution and 12 μL of sterile 10% Tween 80 solution were added to several control wells containing *M. tuberculosis* but no antibacterial agent and plates were incubated again for 24 h under the same conditions. If the *M. tuberculosis* viability controls tested positive for resazurin reduction, resazurin was added to all wells. The minimum inhibitory concentration (MIC) was defined as the lowest peptide concentration that prevented the reduction of resazurin and therefore a colour change from blue to pink. Previous studies by our group suggest that some AMPs may induce dormancy or a bacteriostatic state in *M. tuberculosis* [24]. To examine this, 10 μL from the lowest concentration that did not reduce resazurin was serially diluted and seeded onto 7H10 agar plates supplemented with Middlebrook OADC enrichment media and incubated for ≥21 days at 37 °C to observe whether *M. tuberculosis* re-growth occurred.

2.4. Experimental model of progressive pulmonary tuberculosis in BALB/c mice

The experimental model of progressive pulmonary TB has been described in detail elsewhere [25]. Briefly, male BALB/c mice aged 6–8 weeks were anaesthetised in a gas chamber using 0.1 mL per mouse of sevoflurane, and each mouse was infected by endotracheal instillation with 2.5×10^5 live bacilli. Mice were maintained in the vertical position until they underwent spontaneous recovery. Infected mice were maintained in groups of five in cages fitted with micro-isolators. Animal work was performed in accordance with Mexican national regulations on Animal Care and Experimentation (NOM 062-ZOO-1999).

2.5. Treatment of infected mice with peptides

After 60 days of infection, animals were arbitrarily allocated into four groups. Peptide treatment started 60 days after infection, when advanced progressive disease was well established. In the first experiments conducted to determine the in vitro MIC of each peptide, it was determined that doses at or near to 3.2 μg/mL for all peptides were able to kill *M. tuberculosis*. Thus, a dose of 32 μg in 100 μL of saline solution (ca. 1 mg/kg) was used for the therapeutic experiments. Three independent experiments were performed. All

groups of animals received the corresponding dose three times a week for up to 4 weeks by intratracheal instillation, since preliminary studies indicated no efficacy via the intraperitoneal delivery route. Six animals in each group were sacrificed at 7, 14 and 28 days after starting treatment. The efficiency of each peptide treatment was determined by quantifying the lung bacillary loads by assessing CFUs and the extent of tissue damage by histopathology.

2.6. Determination of CFUs in infected lungs

Lungs were homogenised with a Polytron® homogeniser (Kinematica, Lucerne, Switzerland) in sterile tubes containing 1 mL of 0.05% Tween 80 in phosphate-buffered saline (PBS). Five dilutions of each homogenate were spread onto duplicate plates containing Bacto Middlebrook 7H10 agar (Difco Laboratories) enriched with OADC-enriched medium (Becton Dickinson, Sparks, MD). The number of colonies was counted after 21 days of incubation.

2.7. Preparation of lung tissue for histology

The lungs from each of three different animals per time point and group were perfused intratracheally with ethyl alcohol (J.T. Baker, Mexico City, Mexico). Lungs were then dehydrated and embedded in paraffin (Oxford Labware, St Louis, MO), sectioned and stained with haematoxylin and eosin. The percentage of the lung surface affected by pneumonia was determined using an automated image analyser (Axiovert M200; Carl Zeiss, Oberkochen, Germany).

2.8. Ultrastructural analysis of treated Mycobacterium tuberculosis

Determination of the ultrastructural damage to *M. tuberculosis* caused by treatment with the different AMPs was evaluated using transmission electron microscopy. Briefly, bacilli were cultured in Middlebrook 7H9 broth (Difco Laboratories) supplemented with Middlebrook OADC enrichment media (BBL; BD, Franklin Lakes, NJ) until logarithmic phase was achieved. Viable bacilli (1×10^7) were placed in the wells of 96-well plates and were exposed to the corresponding AMP for 18 h at the MICs determined previously using the resazurin assay. Subsequently, fixation was performed with 4% paraformaldehyde in PBS and the fixed bacilli suspension was treated with 0.05 mM NH_4Cl in PBS to block free aldehyde groups. The bacterial suspension was then centrifuged to form a pellet that was later dehydrated with graded ethyl alcohol solutions and embedded in LR White hydrosoluble resin (London Resin Company, London, UK). Thin sections of 70–90 nm width were placed on nickel grids and were contrasted with uranium salts (Electron Microscopy Sciences, Fort Washington, PA) and were examined with a Zeiss M-10 electron microscope (Carl Zeiss).

2.9. Statistical analysis

Data were analysed by parametric two-way analysis of variance (ANOVA) with Tukey's post-test or a non-parametric Kruskal–Wallis multiple comparisons test with Dunn's post-test. GraphPad 5.02 software (GraphPad Inc., La Jolla, CA) was used to perform the analysis. For all analyses, a P-value of <0.05 was considered statistically significant.

3. Results

3.1. Antimicrobial activity of CRAMP, LL-37, E2, E6 and CP26 in vitro

For pre-clinical testing of antimycobacterial drugs, the most versatile and efficient technique utilises resazurin for determining residual *M. tuberculosis* viability [23].

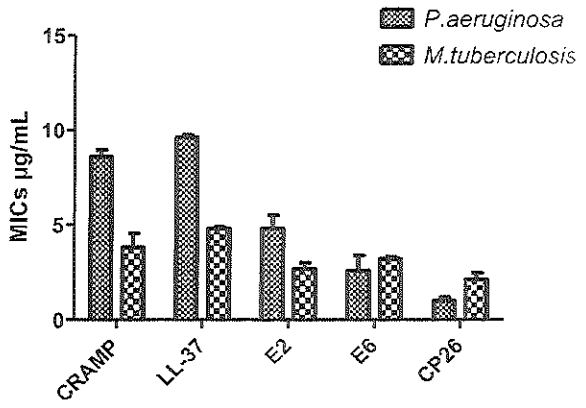

Fig. 1. The effect of antimicrobial peptides (AMPs) human LL-37, mouse CRAMP, E2, E6 and CP26 on the growth of *Mycobacterium tuberculosis*. *Mycobacterium tuberculosis* strain H37Rv was incubated with increasing concentrations of the indicated AMP to determine the minimum inhibitory concentration (MIC). Data are expressed as the mean ± standard deviation of three independent experiments, each performed in duplicate.

This assay was performed here to evaluate the capacity of selected AMPs to inhibit the growth of *M. tuberculosis* strains. Fig. 1 shows that all peptides had strong antimicrobial activity against *M. tuberculosis*, with CP26 being the most efficient (MIC = 2.1 ± 0.33 µg/mL), followed by E2 and E6 (MICs = 2.6 ± 0.34 µg/mL and 3.2 ± 0.10 µg/mL, respectively). Interestingly, these three optimised synthetic peptides all showed higher activity than the natural human (LL-37) and mice (CRAMP) cathelicidins, with 1.5–2-fold lower MICs. The fast-growing bacteria *Pseudomonas aeruginosa* was included as a control under the same conditions, showing similar results as those obtained for *M. tuberculosis*.

3.2. Ultrastructural changes in Mycobacterium tuberculosis in response to antimicrobial peptides

To examine the cytotoxic effect of these AMPs against *M. tuberculosis*, the ultrastructure of bacilli after treatment with these peptides was examined. In previous studies, it was demonstrated that antimicrobial cationic peptides bind to negatively charged molecules of the membrane and cell wall components such as lipoarabinomannan in *M. tuberculosis* and lead to membrane disruption [26]. To investigate the effects of semisynthetic peptides E2, E6, LL-37, CRAMP and CP26 on *M. tuberculosis*, bacilli incubated with these peptides were studied using electron microscopy.

Control untreated bacilli showed a well-defined, homogeneous and slightly electron-lucent cell wall, whilst the cytoplasm was generally electron dense with some medium-sized vacuoles (Fig. 2). Incubation with peptide E2 produced substantial abnormalities in the cell wall, including thinning, budding and thickening of the wall as well as condensation of the cytoplasm producing an electron-lucent area under the wall that was more prominent at one pole (Fig. 2). Incubation with peptide CP26 led to an almost complete disappearance of the cell wall, with only a thin superficial rim of electron-dense material evident around the bacteria, whilst the cytoplasm exhibited large vacuoles or was condensed leading to a shrinking of the whole bacillus (Fig. 2). Incubation with peptide E6 also induced significant abnormalities in the cell wall, including extreme thinning of the wall which alternated with thickened areas and regions of vesicular budding that could be visualised as an irregular surface coexisting with extreme cytoplasmic condensation leading to a large

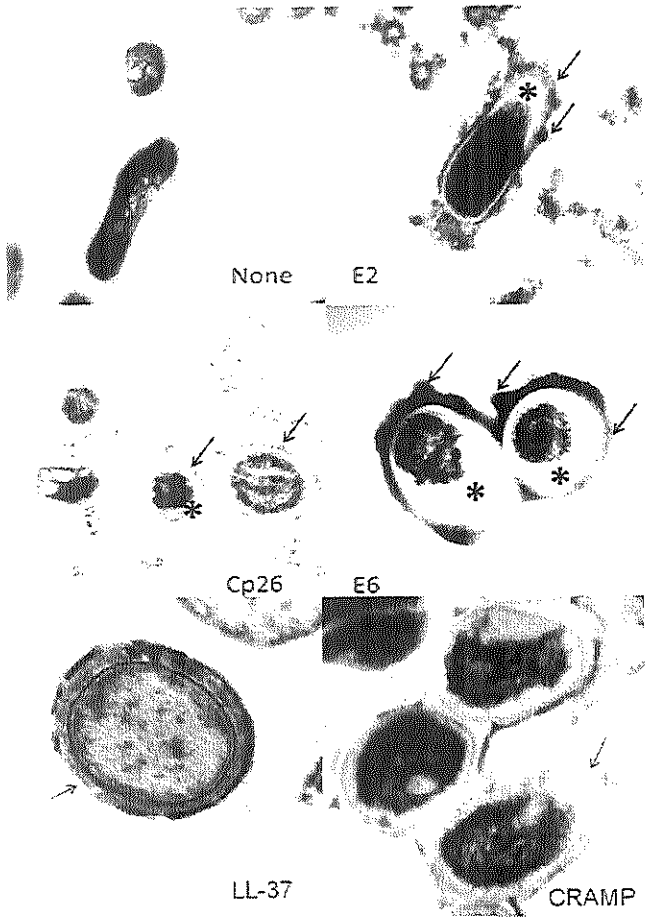

Fig. 2. Representative micrographs of conventional electron microscopy and immunoelectron microscopy of *Mycobacterium tuberculosis* strain H37Rv treated with antimicrobial peptides. In contrast to the untreated control bacteria, bacilli incubated with a minimum inhibitory concentration of peptides E2, CP26, E6, LL-37 or CRAMP showed cell wall thinning, budding or disruption (arrows), and cytoplasmic condensation producing a broad electron-lucent halo between the wall and the condensed cytoplasm (*).

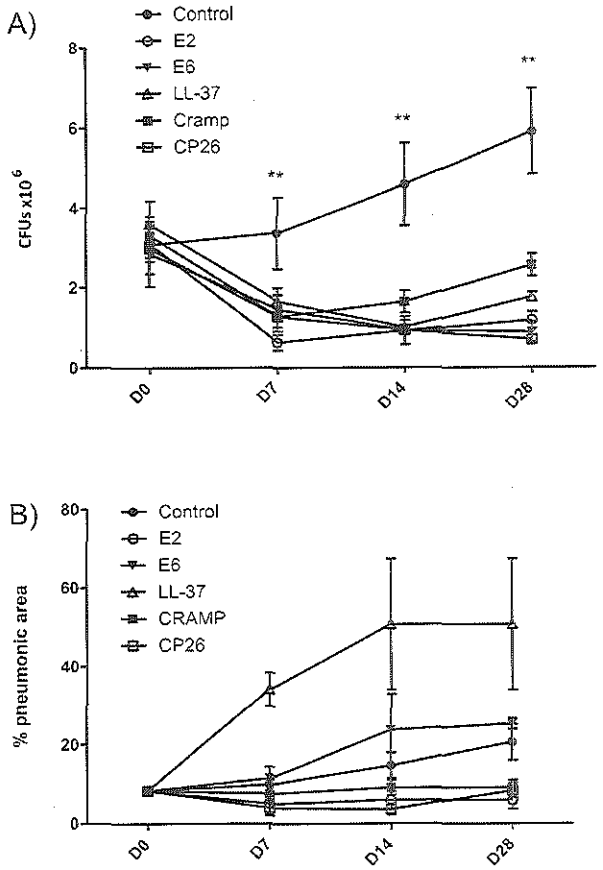

Fig. 3. Effect of antimicrobial peptide (AMP) treatment on (A) pulmonary bacilli burden and (B) tissue damage (pneumonia) during advanced experimental tuberculosis. (A) Mice were infected with the drug-sensitive H37Rv *Mycobacterium tuberculosis* strain and after 60 days were treated three times per week with 32 μg of the indicated AMP in 100 μL of saline solution. All AMPs decreased the lung bacillary loads in comparison with non-treated mice. (B) Percentage of lung surface affected by pneumonia determined by automated morphometry. Results are expressed as the mean ± standard deviation. $P < 0.05$ was considered statistically significant. Asterisks show the kinetic points where there was statistical difference in CFUs between the control group and the rest of the groups. None of the AMPs induced a statistically significant decrease or increase in the pneumonic area compared with the control group.

electron-lucent space between the cell wall and the cytoplasm (Fig. 2). E6 induced the most striking subcellular abnormalities, however the general theme of cell wall destruction/modification and cytoplasmic condensation was evident for all peptides, including LL-37 and CRAMP, which induced a homogeneous increase of the electron-lucent cell wall surrounded by a thin electron-dense rim (Fig. 2).

Overall, these observations indicated that the cell wall and membrane are important targets of these peptides, whilst the observation of a condensed cytoplasm is consistent with osmotic activity and perhaps also DNA binding. Overall, these observations mirrored those for other peptides in Gram-positive bacteria [22].

3.3. *Effect of intratracheal administration of LL-37, CRAMP, E2, E6 and CP26 during late progressive tuberculosis produced by the drug-sensitive strain H37Rv*

The mean of the highest MIC for all peptides against *M. tuberculosis* in vitro was 3.2 μg/mL, thus we decided to use a dose of 32 μg in 100 μL of saline solution (ca. 1 mg/kg), which was administered intratracheally three times a week. Treatment was started 60 days post infection, when advanced active TB was well established. In comparison with control mice in which bacilli numbers increased progressively over the 28 days with a net doubling over this time, animals treated with each of the tested peptides showed a significant reduction in bacilli loads during the entire treatment (Fig. 3A). For the mouse and human cathelicidin peptides CRAMP and LL-37, there was a similar initial decrease in bacterial load after 7 days but the bacteria appeared to grow thereafter, albeit at a slower rate than the untreated control, consistent with the weaker antimicrobial activity of these peptides. For the three synthetic peptides, the bacilli did not re-grow, and for E6 and CP26 there was an apparently increasing therapeutic effect between 7 days and 28 days.

Consistent with these findings, after 4 weeks of treatment with E2, CRAMP or CP26, histological examination revealed that the lung area affected by pneumonia tended to be smaller than in control mice but not significantly. In contrast, mice treated with LL-37 showed a modest but non-significant increase in lung area affected by pneumonia, whilst those treated with E6 showed a slight or no increase in the pneumonic area (Fig. 3B).

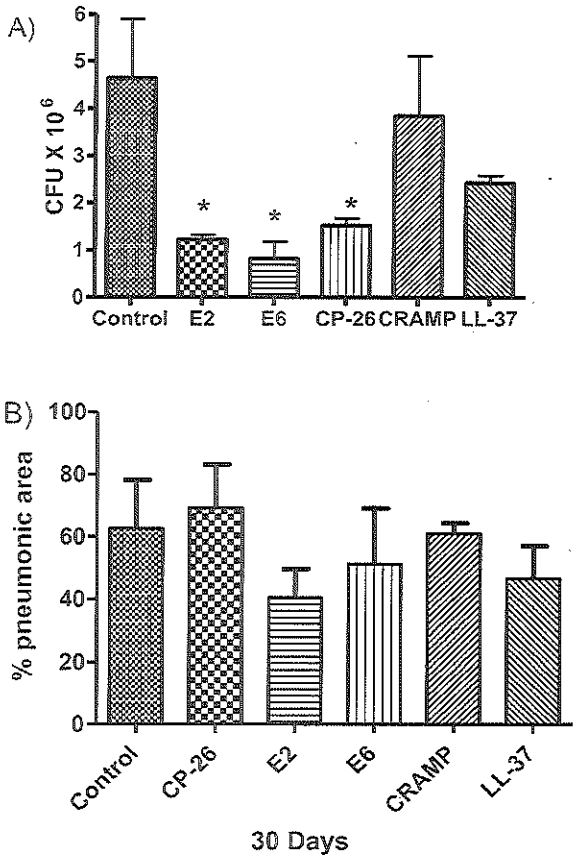

Fig. 4. Effect of antimicrobial peptides (AMPs) in the treatment of mice infected with a multidrug-resistant (MDR) strain of *Mycobacterium tuberculosis*. (A) Animals were infected with MDR strain and after 60 days were treated three times per week with 32 μg of the indicated AMP in 100 μL of saline solution. In comparison with the non-treated control animals, all of the semisynthetic peptides induced a significant decrease in the lung bacillary loads, whilst cathelicidins both from human and mouse induced a non-significant reduction of bacilli burdens. (B) In contrast, similar lung consolidation determined by automated morphometry was seen between treated and control groups. Results are expressed as the mean ± standard deviation. $^{*}P < 0.05$ was considered statistically significant.

3.4. Effect of intratracheal administration of LL-37, CRAMP, E2, E6 and CP26 during late progressive tuberculosis produced by a multidrug-resistant strain

Owing to the emergence of MDR strains worldwide as well as the results observed in mice treated with the different peptides during infection with the drug-sensitive H37Rv strain, we studied whether this therapy had the ability to induce similar beneficial effects during late active disease in mice infected with a clinical isolate resistant to all first-line antibiotics. In comparison with control animals, MDR-infected mice treated with all three synthetic peptides demonstrated a significant 2.5–4.5-fold reduction in CFU counts (Fig. 4A). In contrast, whilst there was a trend towards activity for the natural peptides LL-37 and CRAMP, this was not significant. Although treatment with the different peptides led to a slight decrease in pneumonic area, these differences were again not statistically significant when compared with mice that received only saline solution (Fig. 4B).

4. Discussion

In the past decade, an increasing number of publications have suggested AMPs as molecules with great potential for the treatment of TB [11,27–29]. The present study demonstrates that several different AMPs showed a notable antimicrobial effect against the drug-sensitive *M. tuberculosis* strain H37Rv, in some cases even more than observed for a *P. aeruginosa* clinical isolate. Since previous studies by our group showed that β-defensin-2 and -3 might be involved in the maintenance of latency using a murine model [24], we wanted to assess whether treatment with these peptides would led to a decrease in metabolic activity in *M. tuberculosis*, making the mycobacterium unable to reduce resazurin. However, bacteria could not be re-grown from the wells containing the lowest inhibitory concentrations in the MIC assays, indicating that these peptides were in fact bactericidal rather than merely inducing a reduction of the metabolic activity in *M. tuberculosis*.

AMPs have complex multimodal mechanisms of action that have been proposed to involve several targets, including cell membrane-associated and intracellular targets [8]. These mechanisms have in common the initial interaction of positively charged peptides with the negatively charged cytoplasmic membrane and the insertion of peptides in the membrane owing to their amphipathic nature, leading to either membrane perturbation or translocation to cytoplasmic targets. The electron microscopy study, which showed disruption, thinning and budding of the bacterial cell wall after incubation of bacilli with the different peptides, suggests that their interaction with the membrane and/or cell wall might be an important mechanism to produce bacterial damage. This damage could in turn relate to triggering of autolytic mechanisms or interference with cell wall biosynthesis, both of which have been reported to be mechanisms by which peptides can act [8,9]. In addition, abnormalities such as bacterial cytoplasmic shrinkage are consistent with the peptide being taken up by cells [8]. Overall, we can conclude that peptides utilise complex mechanisms to produce *M. tuberculosis* damage, as observed for several peptides in other bacteria [22]. These peptides did not cause lysis of red blood cells at very high concentrations [18,19,22].

Treatment with the different peptides was initiated after 8 weeks of infection, when active disease was occurring, mimicking a common clinical situation in developing countries. Intratracheal instillation of the different peptides led to decreased lung bacillary loads. The activity of the peptides did not seem to be strongly related to their origin in that human LL-37, mouse CRAMP, bovine-derived E2 and E6 and insect-derived CP26 all had rather similar initial activities, with the natural peptides allowing slight re-growth of the bacillus. This indicates that neither the specific sequence not the origin of the peptides determined their activity, but rather their antimicrobial properties. Although none of the results were statistically significant, for three peptides (mouse CRAMP, E2 and CP26) slightly decreased pneumonia was observed, whilst LL-37 led to a modest but insignificant increase in pneumonia and E6 showed a slight or no effect. The modest suppression of pneumonia by mouse CRAMP, E2 and CP26 cannot be just due to the antimicrobial activity, which was similar for both the natural peptides and all three synthetic peptides. Indeed, it might suggest that there is another property of peptides that contributes to the suppression of pneumonia, such as an immunomodulatory, anti-inflammatory activity [9,10] that might differ among the peptides.

Cationic peptides like these have a variety of relevant properties, including suppression of inflammation, enhancement of cellular recruitment and a wound healing function. Thus, whilst the peptides were selected for their antimicrobial activities, other properties may assist in the control of *M. tuberculosis* infection. Intriguingly, the enhanced AMPs demonstrated apparently superior activities to the natural peptides, whilst LL-37 showed an increased (but not significantly) pneumonic area, perhaps due to the fact that it tends to be more cytotoxic. To evaluate the potential role of immune modulation, we are currently investigating peptides with enhanced immunomodulatory activities [30] to see

whether these are more successful at reducing the pneumonic area.

With regard to the MDR strain, the current results showed that intratracheal administration of peptides E2, E6 and CP26 in mice infected with this strain during the advanced phase of infection could significantly reduce lung bacillary loads. However, the reduction of pneumonia did not demonstrate significant differences when compared with control mice. Thus, similar to the H37Rv-infected mice, these results indicate that these peptides have an effective antimicrobial effect against MDR infection without affecting pneumonia.

In conclusion, these results show that repeated intrapulmonary administration of AMPs permits an efficient method of suppressing the growth of bacilli when they are administered during the late progressive disease induced by drug-sensitive or drug-resistant virulent mycobacteria. Although this treatment was not completely curative, these results suggest that, in conjunction with other more conventional treatments, inhalation therapy with AMPs would be a feasible treatment option in developing countries where there is an urgent need for new treatment options.

Funding: REWH acknowledges funding from the Grand Challenges in Global Health Research programme through the Foundation of the National Institutes of Health and the Canadian Institutes for Health Research, and holds a Canada Research Chair in antimicrobials and genomics. BR-S acknowledges the Mexican Social Security Institute grant project FIS/IMSS/PROT/G10/832. RH-P acknowledges CONACyT (contract: 84456).

Competing interests: None declared.

Ethical approval: Animal work was performed in accordance with Mexican national regulations on Animal Care and Experimentation (NOM 062-ZOO-1999).

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Clinical & Experimental Immunology
The Journal of Translational Immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/cei.12015

Immunotherapeutic effects of recombinant adenovirus encoding granulocyte–macrophage colony-stimulating factor in experimental pulmonary tuberculosis

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Accepted for publication 16 October 2012
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Summary

BALB/c mice with pulmonary tuberculosis (TB) develop a T helper cell type 1 that temporarily controls bacterial growth. Bacterial proliferation increases, accompanied by decreasing expression of interferon (IFN)-γ, tumour necrosis factor (TNF)-α and inducible nitric oxide synthase (iNOS). Activation of dendritic cells (DCs) is delayed. Intratracheal administration of only one dose of recombinant adenoviruses encoding granulocyte–macrophage colony-stimulating factor (AdGM-CSF) 1 day before *Mycobacterium tuberculosis* (Mtb) infection produced a significant decrease of pulmonary bacterial loads, higher activated DCs and increased expression of TNF-α, IFN-γ and iNOS. When AdGM-CSF was given in female mice B6D2F1 (C57BL/6J X DBA/2J) infected with a low Mtb dose to induce chronic infection similar to latent infection and corticosterone was used to induce reactivation, a very low bacilli burden in lungs was detected, and the same effect was observed in healthy mice co-housed with mice infected with mild and highly virulent bacteria in a model of transmissibility. Thus, GM-CSF is a significant cytokine in the immune protection against Mtb and gene therapy with AdGM-CSF increased protective immunity when administered in a single dose 1 day before Mtb infection in a model of progressive disease, and when used to prevent reactivation of latent infection or transmission.

Keywords: gene immunotherapy, latent tuberculosis, lung immunology, transmissibility

Introduction

With more than 1·7 million deaths annually in the world, tuberculosis (TB) is the leading cause of death by a single infectious agent in the history of humanity, and one of the most important causes of mortality in adults infected with human immunodeficiency virus (HIV) [1]. Although efficient chemotherapy is available, TB treatment is long-term and based on several antibiotics, which results in poor compliance, recidivism, toxicity and emergence of multi-drug-resistant (MDR) strains. *Mycobacterium tuberculosis* (Mtb) can produce progressive disease or latent infection [2]. Indeed, in highly endemic areas infection occurs first in childhood, and in most cases is controlled. Only 10% of these primary infections lead to progressive disease [2,3]. However, some bacilli remain in tissues in a non-replicating dormant or slowly replicating stage for the rest of the life of the individual. This latent TB (LTB) is clinically asymptomatic, and in countries with low or moderate endemicity most active TB cases arise as a result of reactivation of latent bacilli [2,3]. It is estimated that one-third of the world's population carries latent Mtb, and millions of TB reactivation cases are predicted in the coming years [4].

Patients with pulmonary TB are the most important source for Mtb transmission; the risk of infection is determined by the source case infectiousness and the contact closeness. Household contacts, mainly children exposed to adults with TB, have a high risk of infection, and this risk increases with the degree of contact [5,6]. Avoiding house contact infection would be the most appropriate strategy to interrupt transmission and subsequent TB development. Another alternative is preventive chemoprophylaxis based on isoniazid (INH), which is prolonged (6–12 months) [7,8] with low completion rates, reinfection risk [9] and selection of MDR strains [10]. Thus, it is important to develop new and more efficient therapeutic strategies to

treat active TB with lower toxicity and simpler administration, as well as to develop new therapies to prevent LTB reactivation and protect healthy close contacts against Mtb transmission.

Granulocyte–macrophage colony-stimulating factor (GM-CSF) is a pleiotropic cytokine able to induce effects on survival, proliferation and differentiation priming of myeloid and non-myeloid precursor cells [11]. Furthermore, GM-CSF exerts a specific physiological role regulating the surfactant replacement priming for the efficient function of alveolar macrophages (AMs) [11]. GM-CSF regulates the inflammatory response in pulmonary infections by activation of the Jak kinase–signal transducer and activator of transcription (STAT) factor pathway, inducing the expression of interferon-regulating factor 5 (IRF5). High expression of IRF5 results in classical macrophage activation (M1) or inflammatory macrophage induction [12], and absolute or relative GM-CSF deficiencies produce severe AM dysfunction with a phenotype of pulmonary alveolar proteinosis and abnormalities in host defence [13]. Exogenous GM-CSF administration through aerosol induces an increased number of AMs [14]. Therefore, GM-CSF has a specific role in lung physiology and would mediate the control of intracellular infections such as TB, as it is able to induce the production of interleukin (IL)-12, tumour necrosis factor (TNF)-α and interferon (IFN)-γ [11,12]. Furthermore, *in-vivo* pulmonary over-expression of GM-CSF by local administration of recombinant adenoviruses encoding this cytokine (AdGM-CSF) induces early differentiation and activation of dendritic cells (DCs) with potent immunostimulatory function [15,16]. This is important, considering that in experimental murine pulmonary TB there is a delay in DC recruitment and maturation in both lungs and mediastinal lymph nodes, which apparently contributes to early Mtb immune evasion [17,18]. Thus, GM-CSF should be a significant participant in the immune response against Mtb and could be a target for immunotherapy. The aim of the present study was to determine in a murine model of progressive pulmonary TB the effect of the intratracheal (i.t.) administration of AdGM-CSF 1 day before infection, as well as its effect in preventing reactivation in a mouse model of LTB and avoiding infection of healthy mice co-housed with tuberculous animals in a transmissibility model.

Materials and methods

Kinetics of GM-CSF gene expression and cytokine location during progressive pulmonary TB in BALB/c mice

We used the murine model of i.t. infection described previously [19,20]. Briefly, virulent Mtb strain H37Rv was cultured in Proskauer and Beck medium. After 1 month of culture, mycobacteria were harvested and adjusted to $2{\cdot}5 \times 10^5$ cells in 100 μl of phosphate-buffered saline (PBS), aliquoted and maintained at −70°C until used. Pathogen-free male BALB/c mice, 6–8 weeks old, were anaesthetized (sevoflurane; Abbott Laboratories, Abbott Park, IL, USA) and 100 μl of isotonic sterile endotoxin-free saline solution with $2{\cdot}5 \times 10^5$ viable bacilli were inoculated i.t. using a stainless steel cannula. Animals were then maintained in cages fitted with micro-isolators in a P-3 biosecurity level facility. Following infection, mice were killed by exsanguination under anaesthesia at days 1, 3, 7, 14, 21, 28, 60 and 120 post-infection; lungs were collected immediately to perform quantitative reverse transcription–polymerase chain reaction (RT–PCR) and immunohistochemistry (IHC).

mRNA extraction and reverse transcription (RT)

Five lungs per group per day of killing were placed into 2 ml of RPMI-1640 medium (Invitrogen Life Technologies, Carlsbad, CA, USA) containing 0·5 mg/ml of collagenase type 2 (Worthington, Lakewood, NJ, USA) and incubated for 1 h at 37°C; the lung was then macerated and passed through a sterile 70-μm cell sieve (BD Biosciences, Bedford, MA, USA). The cell suspension was centrifuged at 250 *g* for 1 min at 4°C and washed with RPMI-1640 medium. The supernatant was removed and red cells were lysed with 1 ml of lysis buffer [0·34 M ammonium chloride, 0·12 mM ethylenediamine tetraacetic acid (EDTA) and 1 mM potassium carbonate], and finally the cells were washed and centrifuged under the same conditions. Five × 10^6 cells were counted, and 350 μl of buffer RLT was added (Qiagen, Hilden Germany) with β-mercaptoethanol. RNA was isolated using the RNeasy minikit (Qiagen, Hilden, Germany), passing the sample through a column, centrifuged at 10 000 *g* for 1 min at 4°C. The RNA bound to the column was washed with 700 μl of two other buffers of the mini RNeasy kit (Qiagen, Hilden, Germany) and finally eluted with 50 μl of RNase-free water. The RNA was treated with one unit of DNase (Invitrogen Life Technologies) per microgram of RNA. The quality and quantity of RNA were also evaluated by spectrophotometry (260 nm and $A_{260/280}$ ratio, NanoDrop-1000; Thermo Fisher Scientific, Waltham, MA, USA) and agarose gels. The cDNA was synthesized using Omniscript RT kit (Qiagen, Hilden, Germany), oligo dT (Promega Corporation, Madison, WI, USA) and 100 ng of RNA. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was confirmed by conventional PCR and the cDNAs were amplified with *Taq* DNA polymerase Hot Start (Qiagen, Germantown, MD, USA).

Real-time PCR

Real-time PCR was developed using the computer real-time PCR system 7500 (Applied Biosystems, Bedford, MA, USA).

We used 100 ng of cDNA, 12·5 μl of the mix Quantitect SYBR Green PCR (Qiagen, USA): QuantiTect SYBR Green PCR buffer containing Tris-Cl, KCl, $(NH_4)_2SO_4$, 5 mM $MgCl_2$, pH 8·7, the mixture of dNTPs (dATP, dCTP, dGTP, dTTP/dUTP), SYBR green I and ROX, plus primer sense and primer anti-sense (50 pmol of each). The formation of a single PCR product and the expected amplicon size were confirmed previously by electrophoresis of the conventional PCR product. The standard curves of PCR products, quantified and diluted, and negative controls were included in each real-time PCR run. The specific primers were designed using Primer Express software (Applied Biosystems) for the following targets: GAPDH: 5′-GGCGCTCACCAAAACAT CA-3′, 5′-CCGGAATGCCATTCCTGTTA-3′ [232 base pairs (bp) expected amplicon size]; inducible NO synthase (iNOS): 5′-AGCGAGGAGCAGGTGGAAG-3′, 5′-CATTTC GCTGTCTCCCCAA-3′ (206 bp expected amplicon size); TNF-α: 5′-TGTGGCTTCGACCTCTACCTC-3′, 5′-GCCG AGAAAGGCTGCTTG-3′ (205 bp expected amplicon size); IFN-γ: 5′-GGTGACATGAAAATCCTGCAG-3′, 5′-CCTCA AACTTGGCAATACTCATGA-3′ (180 bp expected amplicon size); GM-CSF: 5′-GCCATCAAAGAAGCCCTGAA-3′, 5′-GCGGGTCTGCACACATGTTA-3′ (114 bp expected amplicon size); and IL-12: 5′-GGATGGAAGAGTCC CCCAAA-3′, 5′-GCTCTGCGGGCATTTAACAT-3′ (125 bp expected amplicon size).

Conditions used were: initial denaturation at 95°C for 15 min, followed by 40 cycles at 95°C for 20 s, 60°C or 58°C for 20 s and 72°C for 34 s. The number of copies of each cytokine mRNA were related to a million copies of GAPDH mRNA. Data were reported as mean ± standard deviation (s.d.) of five different mice for each of two independent experiments.

IHC for F4/80 and GM-CSF

The same paraffin-embedded tissues were used for IHC; 5-μm sections were obtained on slides loaded with poly L-lysine (Biocare Medical, Lake Concord, CA, USA). For dewaxing, the slides were placed at 60–70°C for 20 min, then incubated for 5 min into xylene. The slides were changed five times into the medium in the following sequence: (i) xylene-alcohol (1:1), (ii) absolute alcohol, (iii) alcohol 96% and (iv) distilled H_2O. Once hydrated, endogenous peroxidase was blocked with methanol–10% H_2O_2. The washings were performed with HEPES-buffered saline (HBS)-Tween 20 (10 mM HEPES, 150 mM NaCl, 2 mM $CaCl_2$, 0·05% Tween 20). The areas of tissue were delineated and then blocked with 100 μl of HBS with 2% background sniper (Biocare Medical) and incubated for 30 min in a humid chamber. The slides were then incubated with monoclonal antibody anti-F4/80 conjugated with biotin (eBioscience, San Diego, CA, USA) for 12 h at room temperature. Subsequently, slides were washed and 100 μl of antibody–horseradish peroxidase (AB/HRP) complex (Vectastain ABC System, Burlingame, CA, USA) was added and incubated for 30 min to be revealed with 100 μl of diaminobenzidine/H_2O_2 (0·004 g diaminobenzidine + 10 ml HBS + 4 μl H_2O_2). Slides were washed and contrasted with haematoxylin. Staining for GM-CSF took a primary antibody monoclonal anti-GM-CSF (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and incubated subsequently with biotin-conjugated secondary antibody (Invitrogen) for 1 h, washed, and the processing was performed based on the same procedure as above. To obtain the percentage of F4/80-positive AMs or GM-CSF-positive cells, an image analyser was used (Q Win Leica, Milton Keynes, UK). We analysed 50 different random fields with an increase of ×100 in interstitial, pneumonic, peribronchial and perivascular areas as well as all the granulomas found per slide. Measurements were taken by investigators who were blinded to the treatment groups, and the data are reported as the mean values ± s.d. from five different mice at each time-point in each of two different experiments.

Determination of the most effective dose of AdGM-CSF to induce GM-CSF transcription and cytokine production in healthy BALB/c mice

The construction, expression, biological effect and titration of AdGM-CSF and its vector control Addl70-3 have been reported previously [15]. Groups of five healthy male 8-week-old BALB/c mice were anaesthetized with sevoflurane vapour (Abbott Laboratories, Mexico City, Mexico) in a sealed acrylic cage. Anaesthetized mouse was fixed on cardboard and recombinant adenoviruses were administered through a stainless steel cannula (Thomas Scientific, Swedesboro, NJ, USA) connected to an insulin syringe. The cannula was introduced first into the mouth and then directed into the trachea, where the selected dose of recombinant adenoviruses suspended in 100 μl of isotonic sterile endotoxin-free saline solution was injected. Three different doses were tested: 1×10^7, 5×10^7 and 1×10^8 plaque-forming units (pfu). Groups of five mice per each dose were euthanized by exsanguination under terminal anaesthesia after 1 and 7 days post-administration; one lung lobe, right or left, was collected immediately and frozen in liquid nitrogen for total RNA isolation and determination of GM-CSF gene expression by quantitative RT–PCR (qRT-PCR), as described above, while the other lung was perfused with absolute ethanol for fixation and embedded in paraffin for histological evaluation and GM-CSF detection by IHC.

Studies on the effect of AdGM-CSF administration in the experimental model of progressive pulmonary TB

We used the previously described model of progressive TB to study the effect of IT AdGM-CSF [19,20]. Male BALB/c 6–8-week-old mice were treated with 1×10^8 pfu of AdGM-CSF or Addl70-3 i.t., as described above, then 1 day later

animals were anaesthetized with sevoflurane, and 100 µl of isotonic sterile endotoxin-free saline solution with $2{\cdot}5 \times 10^5$ viable bacilli was inoculated i.t. Following infection, mice were killed by exsanguination under anaesthesia at days 1, 3, 7, 14, 21, 28, 60 and 120 post-infection to obtain the lungs and perform bacteriological, histomorphometric and molecular biology studies. Five mice per group were euthanized at every time-point selected for the various analyses.

For quantification of bacilli loads by colony-forming units (CFUs), lungs were collected at the selected times, as mentioned, and homogenized using a polytron (Kinematica, Lucerne, Switzerland) homogenizer. The suspensions were then diluted with 0·05% Tween-80 to a final 1-ml volume. Three consecutive logarithmic dilutions were made from this homogenate. Ten µl of each dilution were plated in duplicate on BactoMiddlebrook 7H10 agar (Difco, Detroit, MI, USA) enriched with oleic acid, albumin, dextrose and catalase. Plates were then incubated at 37°C and 5% CO_2 for 21 days to quantify the CFUs.

For histological study, the right or left lungs from five different mice per group were perfused i.t. with absolute ethanol. Parasaggital sections were dehydrated and embedded in paraffin (Oxford Labware, St Louis, MO, USA), sectioned and stained with haematoxylin and eosin (H&E). The granuloma area (measured in square microns) and percentage of lung surface affected by pneumonia were determined using an automated image analyser (Q Win Leica), as described previously [20]. Measurements were performed blind, and the data are reported as the mean values ± s.d. from five different mice at each time-point in each of two different experiments. For IHC detection of GM-CSF, the procedure was as described above.

For quantification of activated DCs by cytofluorometry, five lungs per group per day of killing were placed into 2 ml of RPMI-1640 medium (Invitrogen Life Technologies) containing 0·5 mg/ml of collagenase type 2 (Worthington) and incubated for 1 h at 37°C; the lung was then macerated and passed through a 70-µm sterile strainer (BD Biosciences). The cell suspension was centrifuged at 250 *g* for 1 min at 4°C and washed with RPMI-1640 medium. The supernatant was removed and red cells were lysed with 1 ml of lysis buffer (0·34 M ammonium chloride, 0·12 mM EDTA and 1 mM potassium carbonate). Cells were washed and centrifuged under the same conditions; 1×10^6 viable cells were counted (by trypan blue exclusion) and stained for activated DCs determination [major histocompatibility complex class II (MHC II)$^+$CD11c$^+$CD86$^-$]. The antibodies used were: fluorescein isothiocyanate-labelled anti-I-A/I-E (MHC II-FITC; BD Pharmingen), allophycocyanin-labelled anti-CD11c (CD11c-APC; BD Pharmingen), phycoerythrin-labelled anti-CD86 (CD86-PE; BD Pharmingen). In each cell suspension for each day of sacrifice, staining controls were included to check specificity. Individual stains were made with each of the antibodies used as positive controls and their respective isotype controls. Finally, we included negative controls (unstained) and a dual control mark (MHC II$^+$CD11c$^+$). The double-positive control was the negative parameter for determining the zone of histogram considered positive for CD86; 1×10^5 events were acquired for each sample using fluorescence activated cell sorter (FACS-)Calibur cytofluorometer and CellQuest software (BD Biosciences). The data collected were analysed using FlowJo software version 6·1. Data were reported as mean ± s.d. of five different mice for each of two independent experiments.

For cytokines and iNOS gene expression determined by qRT–PCR, five lungs, right or left, from two different experiments were removed and used for isolating RNA from the different groups, following the protocol as published previously [21].

Experimental model of chronic infection similar to LTB

The murine model of LTB has been described previously [22]. Two groups of five 8-week-old female mice B6D2F1 (C57BL/6J X DBA/2J) were infected i.t. with 4000 viable bacteria H37Rv strain suspended in 100 µl of isotonic sterile endotoxin-free saline solution. After 7 months of infection, groups of five mice were treated with AdGM-CSF (1×10^8 pfu) or Addl70-3 (1×10^8 pfu) administered by i.t. instillation as described above. One month later, corticosterone was then administered in drinking water (3 mg/l) for 1 month. Mice were killed under terminal anaesthesia and the lungs were obtained and used to determine bacilli burdens and pneumonia by CFU quantification and automated histomorphometry, as described above.

Mtb transmissibility experimental model in BALB/c mice

The transmissibility experimental model has been described previously [23]. Five BALB/c mice infected ($2{\cdot}5 \times 10^5$ bacilli) with Mtb H37Rv or highly virulent Beijing strain 9001000 were co-housed in the same micro-isolator from the first day of infection with five healthy non-infected BALB/c mice (contacts), which received AdGM-CSF (1×10^8 pfu IT) by the i.t. route. Control groups received Addl70-3 (1×10^8 pfu IT) or INH by intragastric cannulation (0·2 mg/day). Delayed-type hypersensitivity (DTH) to mycobacterial antigens was performed in the footpads after 2 months of co-housing, following the reported method [20]. Animals were killed after 2 months of co-housing and their lungs were collected and homogenized for CFU determination. The suspensions were diluted with 0·05% Tween-80 to a final volume of 1 ml. One part of the lung suspension was diluted into three parts of PBS and 100 µl of each dilution were plated in triplicate on BactoMiddlebrook 7H10 agar enriched with oleic acid, albumin, dextrose and

catalase. Plates were then incubated at 37°C and 5% of CO_2 for 21–45 days to quantify the CFU.

Statistical analysis

Results are expressed as mean ± s.d. Student's two-tailed *t*-test was used for comparing experimental groups, with a $P < 0.05$ value considered significant.

Ethical approval

Animal studies were approved by the Institutional Ethics Committee of the National Institute of Medical Sciences and Nutrition 'Salvador Zubirán' in accordance to the guidelines of the Mexican national regulations on Animal Care and Experimentation NOM 062-ZOO-1999.

Results

Kinetics of endogenous GM-CSF gene expression during progressive pulmonary TB

When BALB/c mice are infected by the i.t. route with a high dose of the reference Mtb strain H37Rv, an early phase of temporal bacilli growth control is produced and dominated by high expression of TNF-α and IFN-γ with granuloma formation. After 3 weeks of infection, a progressive disease phase develops, characterized by high pulmonary bacilli burdens, tissue damage (progressive pneumonia), lower production of TNF-α and IFN-γ with high expression of T helper type 2 (Th2) cytokines, such as IL-4 and IL-13 [19,20]. In order to investigate the potential role of GM-CSF in this model, gene expression kinetics was determined by qRT–PCR. After Mtb infection a low but progressive increase of GM-CSF gene expression was seen peaking at day 7, followed by a progressive decrease to day 120, when the lowest level was detected (Fig. 1a). IHC and automated morphometry showed that during the course of infection the principal GM-CSF cellular source was the bronchial and bronchiolar epithelium; from days 1 to 14 after infection 10–20% of airway epithelial cells showed strong GM-CSF immunostaining, rising to maximal percentage at day 21 (90%) but with lower staining per cell, followed by progressive decrease until day 120, when 30% of these cells were immunostained (Fig. 1b). Macrophages also showed GM-CSF immunostaining in the alveolar–capillary interstitium at day 14 and during late infection at days 60 and 120, when 10–14% showed positive staining. Granulomas at days 60 and 120 also showed immunostained macrophages (Fig. 1b–d).

Determination of the most suitable AdGM-CSF dose to induce transgenic GM-CSF expression in the lungs of healthy mice

Three different doses of AdGM-CSF administered by the i.t. route in healthy BALB/c mice were used to define the most suitable dose to induce GM-CSF gene expression. GM-CSF expression was dose-dependent, with the 1×10^8 pfu dose inducing the highest transgene expression (Fig. 2a). The control group received the empty adenovirus vector (Addl70-3). The cellular source of GM-CSF was determined by IHC; at day 1 post-treatment it was detectable and the bronchiolar epithelium showed strong immunostaining. No detectable basal expression was seen in the alveolar epithelium or AMs. Addl70-3 did not induce GM-CSF production detectable by IHC in healthy mice (Fig. 2b,c).

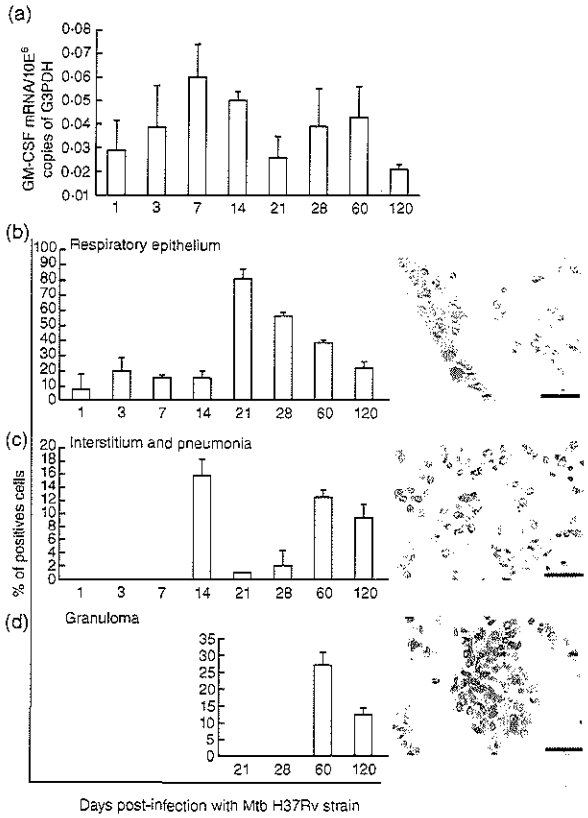

Fig. 1. Kinetics of granulocyte–macrophage colony-stimulating factor (GM-CSF) during progressive pulmonary tuberculosis (TB). (a) Five lungs from the same number of *Mycobacterium tuberculosis* (Mtb)-infected mice at each indicated time-point were used to isolate total RNA and determine the gene expression of GM-CSF by quantitative reverse transcription–polymerase chain reaction (RT–PCR). (b–d) GM-CSF protein expression was detected by immunohistochemistry and the percentage of positive cells was determined in the indicated lung compartments by automated morphometry; representative histological figures of each compartment are to the right of the morphometry graphs (scale bar represents 20 μm). All values are means ± standard deviation (s.d.) from two independent experiments with five mice per group (408 × 552 mm; 150 × 150 DPI).

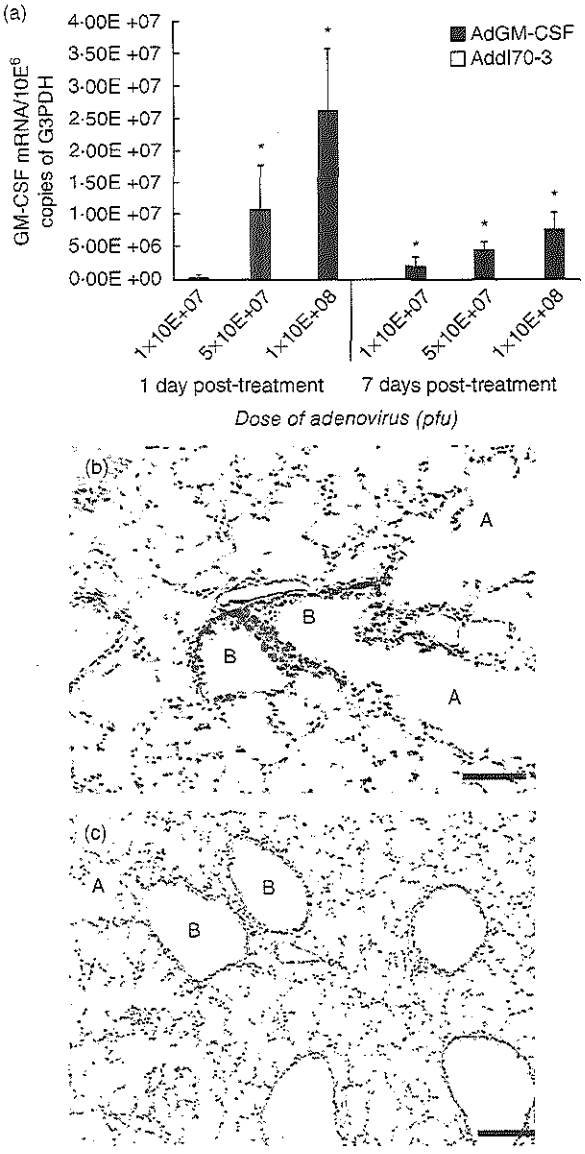

Fig. 2. Granulocyte–macrophage colony-stimulating factor (GM-CSF) expression after intratracheal (i.t.) administration of different doses of adenoviruses encoding GM-CSF (AdGM-CSF). (a) Groups of healthy mice were treated with the indicated dose of AdGM-CSF (black bars) or the control adenoviruses Addl70-3 (white bars, undetectable levels), and euthanized after 1 and 7 days; the lungs were used to determine the expression of GM-CSF reverse transcription–polymerase chain reaction (RT–PCR), values are means ± standard deviation (s.d.) from two independent experiments with five mice per group; $^{*}P < 0{\cdot}05$. (b) GM-CSF protein expression was detected by immunohistochemistry; the mouse lung after 1 day of 1×10^{7} plaque-forming units (pfu) i.t. administration shows strong immunostaining in the airways epithelium in bronchioles (B) and negative immunostaining in alveolar walls (A). (c) In contrast, the mouse lung treated with the same dose of control adenovirus Addl70-3 does not show immunostaining (scale bar represents 60 μm) (207 × 386 mm; 300 × 300 DPI).

Effect of transgenic GM-CSF expression by AdGM-CSF delivered 1 day before infection in the murine model of progressive pulmonary TB

Considering the low percentage of GM-CSF immunostained cells during early infection, two independent experiments were performed to assess the effect of i.t. administration of AdGM-CSF or vector control Addl70-3 1 day before i.t. infection with Mtb H37Rv. The AdGM-CSF administration effect was determined by changes in lung bacterial loads, the histomorphometry of tissue damage (percentage of pneumonic area) and number and size of granulomas. A single dose of AdGM-CSF (1×10^{8} pfu) 1 day before infection induced a significant decrease of bacterial loads from day 14 post-infection until the end of the experiment (day 120) (Fig. 3a). With regard to the histological changes, animals that received AdGM-CSF exhibited a significantly lower pneumonic area and more and bigger granulomas than mice treated with Addl70-3 (Fig. 3b–h).

In this model of murine pulmonary TB there is a delay in DC activation [17]. Considering that GM-CSF is a significant factor in promoting DC activation, this effect could be a mechanism which explains the observed protective effect of AdGM-CSF. To this end, flow cytometry was used to quantify activated DCs (MHC II^{+}CD11c^{+}CD86^{+}) in lung cell suspensions from each selected day after infection. Mice treated with AdGM-CSF showed a significantly higher number of lung-activated DCs on day 7 post-infection compared with the group treated with Addl70-3, in which there was an increase of these cells until day 21 post-infection (Fig. 4a). Both groups showed a progressive decrease of activated DC during late disease.

GM-CSF induces the local differentiation and activation of AMs [14], and this could be another participating mechanism in the observed efficient protective activity of AdGM-CSF. High expression of F4/80 has been associated with the presence of inflammatory macrophages, overexpression of MHC II, CD80, CD11b and increased production of NO and IL-12 [24,25]. Similarly, F4/80 has been related to the early formation of granulomas induced by bacillus Calmette–Guérin (BCG) [26]. Thus, we used the F4/80 marker to monitor macrophages determined by IHC in different histological compartments (perivascular, peribronchial and alveolar–capillary interstitium areas) and lesions (pneumonia and granulomas). In comparison with the control group, mice treated with AdGM-CSF showed significant earlier and higher recruitment of activated AM F4/80^{+} in perivascular, peribronchial and interstitial areas at day 1. This significantly higher recruitment of AM F4/80^{+} remained in perivascular and peribronchial areas at day 3 (Fig. 4b). At day 14 post-infection, significantly higher AM F4/80^{+} was detected only in the interstitial area in the group treated with AdGM-CSF compared with the control group. At day 28, in the peribronchial and interstitial areas a significantly higher number of F4/80-positive cells was seen in

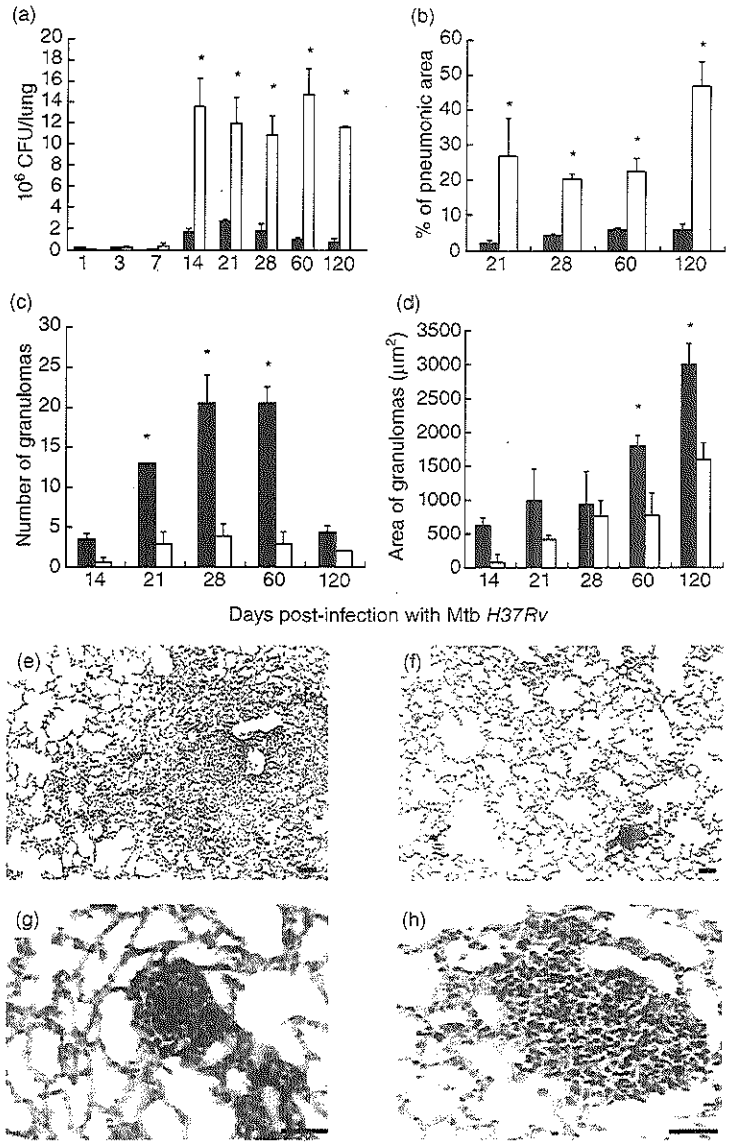

Fig. 3. Effect of a single administration of adenoviruses encoding granulocyte–macrophage colony-stimulating factor (AdGM-CSF) 1 day before infection in the murine model of progressive pulmonary tuberculosis (TB). (a) Groups of mice were treated with AdGM-CSF (black bars) or control virus Addl70-3 (white bars) 1 day before intratracheal (i.t.) infection with a high dose of *Mycobacterium tuberculosis* (Mtb) H37Rv; five mice were killed at each day indicated and the lungs were used to determine bacterial loads by colony-forming units (CFU). A significant CFU decrease was produced by AdGM-CSF administration. (b) The morphometry study showed a significantly lower percentage of lung surface affected by pneumonia in animals treated with AdGM-CSF. (c) The number of granulomas at days 21, 28 and 60 was significantly higher in the AdGM-CSF-treated group, as well as the granuloma size at days 60 and 120 (d). Representative histopathology of the lung from treated mice after 120 days of infection with Mtb, (e) the mouse lung treated with Addl70-3 shows extensive areas of pneumonia. (f) In contrast, mouse treated with AdGM-CSF shows scarce inflammation. (g) Small granulomas are seen in mice treated with Addl70-3. (h) In comparison, bigger granulomas are formed in mouse treated with AdGM-CSF; scale bars represent 60 μm (e) and (f), and 20 μm (g) and (h). All values of bacilli loads and histomorphometry are means ± standard deviation (s.d.) of two independent experiments with five mice per group; $^*P < 0{\cdot}05$ (466 × 771 mm; 150 × 150 DPI).

the AdGM-CSF treated group. At late infection, day 60, in the peribronchial and pneumonic areas the number of F4/80-positive AMs was significantly higher in the AdGM-CSF-treated group (Fig. 4b,c), while in granulomas this group showed a significantly earlier and higher recruitment of AMs at days 21 and 28 (Fig. 4b,d).

In human and murine TB, protective immunity is mediated by Th1 responses (IFN-γ, IL-12) and activated macrophages which produce TNF-α and NO by activity of the enzyme-iNOS [27]. GM-CSF promotes the production of Th1 cytokines [11,12]. Thus, higher induction of the Th1 response is another mechanism which could be involved in the AdGM-CSF protective activity. Murine lungs from each treated group were homogenized for total RNA extraction and subsequent retrotranscription and real-time PCR (qRT–PCR) to quantify the transcription of GM-CSF, IFN-γ, IL-12, TNF-α and iNOS. In comparison with the control group, mice treated with AdGM-CSF showed higher transcription of GM-CSF, which began at the same time as IL-12 expression (Fig. 5a). Similarly, an earlier and significantly higher transcription of IFN-γ, TNF-α and iNOS was seen in the AdGM-CSF-treated group, showing a peak at days 21 and 90 post-infection (Fig. 5a). The cellular production of GM-CSF protein was evaluated by IHC. In the group treated with AdGM-CSF, the bronchial and bronchiolar epithelium showed strong GM-CSF immunostaining at various examined time-points, while by comparison with the control group the immunostaining was much weaker at these time-points (Fig. 5b). Animals treated with AdGM-CSF showed GM-CSF immunostaining at days 1 and 3

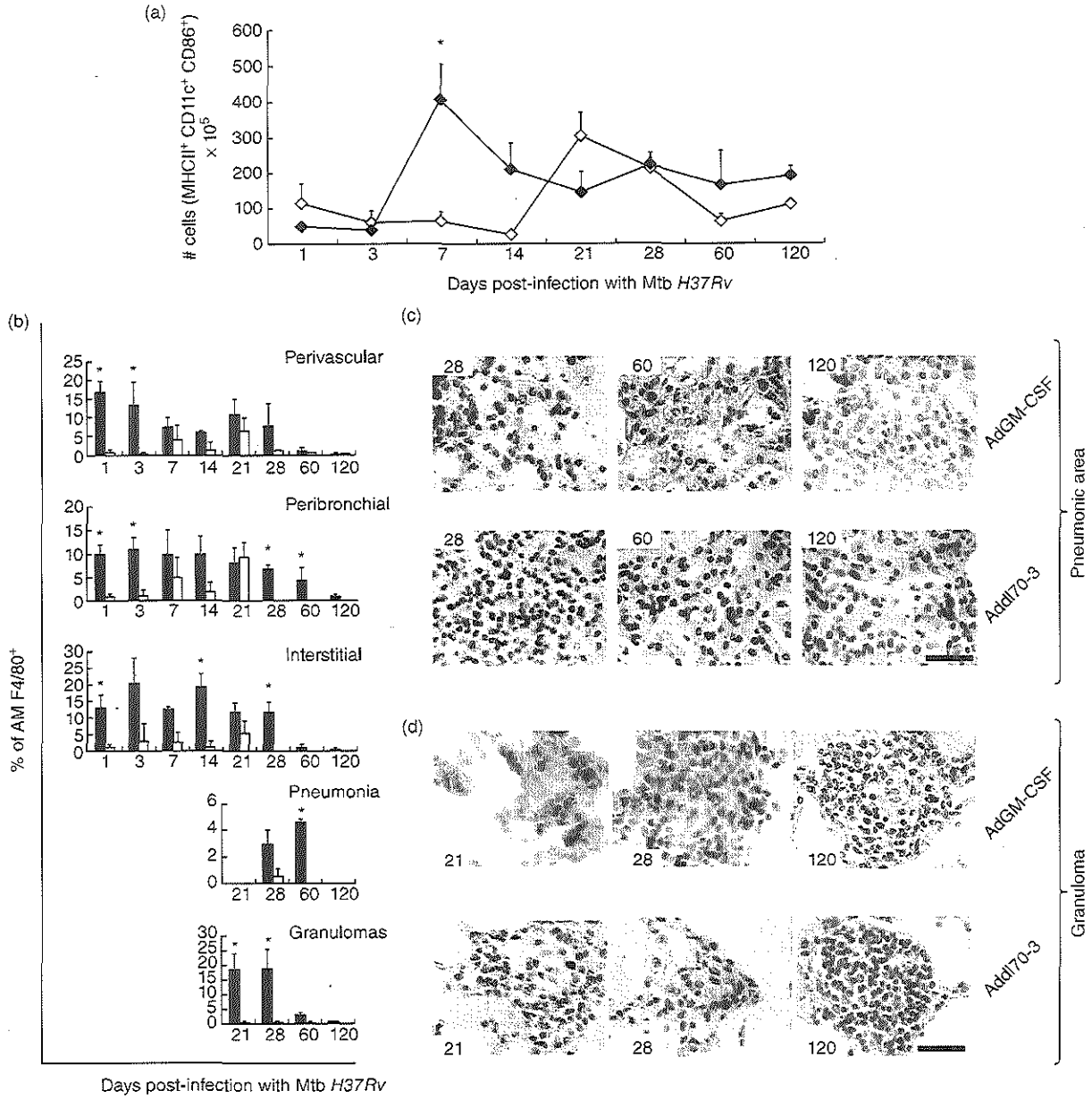

Fig. 4. Effect of a single administration of adenoviruses encoding granulocyte–macrophage colony-stimulating factor (AdGM-CSF) on the production of activated dendritic cells and macrophages in the murine model of progressive pulmonary tuberculosis (TB). (a) Groups of mice were treated with AdGM-CSF (black symbols) or Addl70-3 (white symbols) 1 day before infection with *Mycobacterium tuberculosis* (Mtb) H37Rv; lungs were used to obtain cell suspensions and the number of activated dendritic cells was determined by flow cytometry using antibodies against MHC II-fluorescein isothiocyanate (FITC), CD11c-allophycocyanin (APC) and CD86-phycoerythrin (PE). For each lung sample 1×10^5 events were acquired and the population of cells positive for FITC (FL1) and APC (FL4) (i.e. MHC II$^+$ CD11c$^+$ cells) were selected for obtaining the histogram for the number of positive events for PE (FL2) (i.e. MHC II$^+$ CD11c$^+$ CD86$^+$ cells). The treatment with AdGM-CSF induced more rapid and higher numbers of activated dendritic cells. (b) Lung sections from treated animals with adenoviruses were used to detect the marker of activated macrophages F4/80 by immunohistochemistry, and the percentage of immunostained cells were determined in the indicated lung compartments and lesions. Results are expressed as means ± standard deviation (s.d.) from five mice per time-point and per group in two independent experiments. Representative immunohistochemistry detection of F4/80$^+$ cells in the lungs of animals treated with AdGM-CSF or Addl70-3 at the indicated time-point (left corner) in pneumonic areas (c) or granulomas (d). There are more F4/80$^+$ cells in animals treated with AdGM-CSF; scale bar represents 20 μm (518 × 553 mm; 200 × 200 DPI).

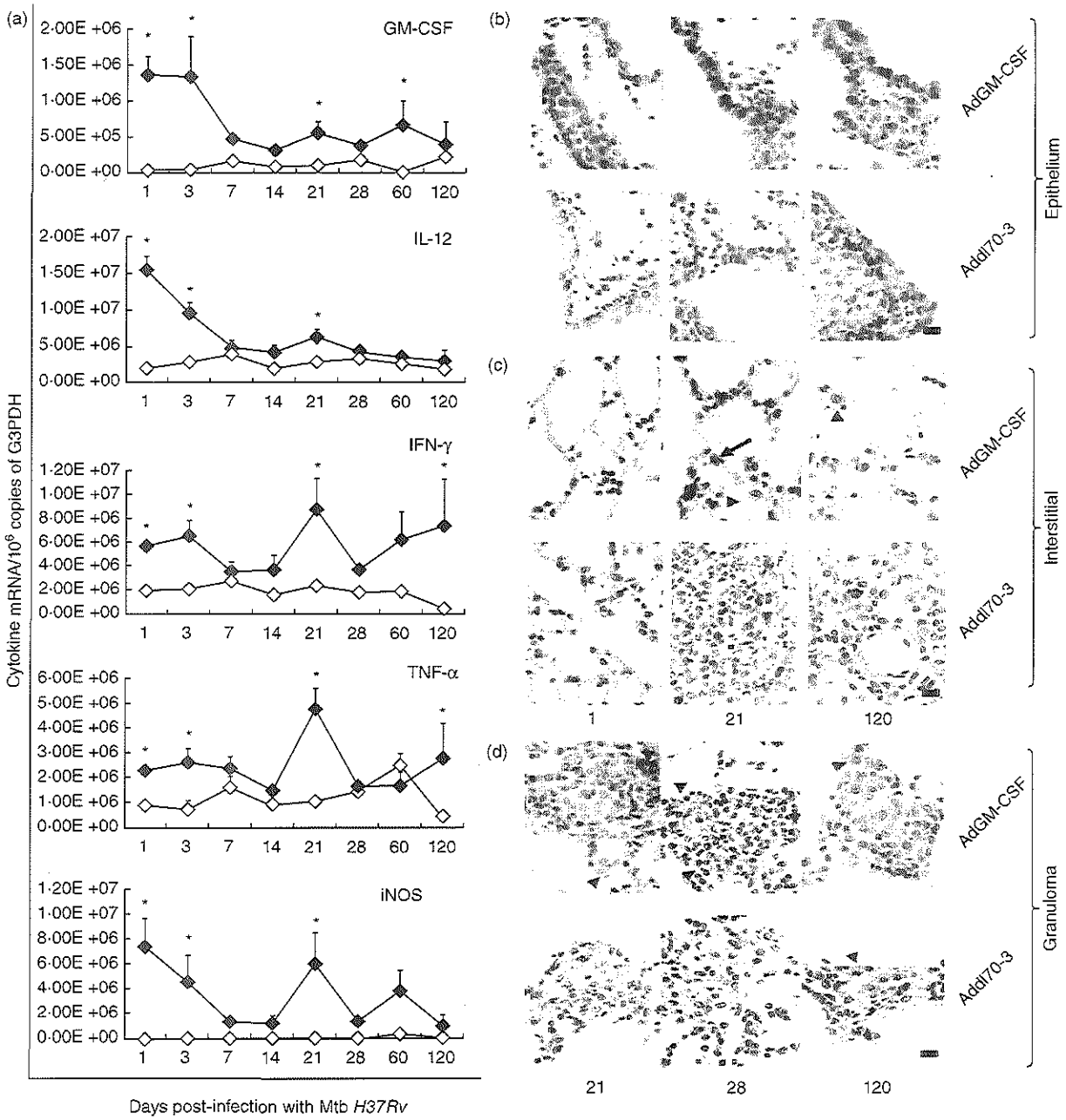

Fig. 5. Effect of a single administration of adenoviruses encoding granulocyte–macrophage colony-stimulating factor (AdGM-CSF) on the cytokines and inducible nitric oxide synthase (iNOS) gene transcription in the murine model of progressive pulmonary tuberculosis (TB). (a) Groups of mice infected by the intratracheal (i.t.) route with a high dose of *Mycobacterium tuberculosis* (Mtb) to induce progressive TB were treated 1 day before with AdGM-CSF (black symbols) or Add170-3 (white symbols), and their lungs were used to isolate total RNA and quantify the expression of the indicated cytokines by real-time reverse transcription–polymerase chain reaction (RT–PCR). Animals treated with AdGM-CSF showed a higher expression of cytokines and iNOS. Results are expressed as means ± standard deviation (s.d.) of two independent experiments with five mice per group; $^{*}P < 0{\cdot}05$. Representative immunohistochemistry of GM-CSF cellular detection in treated animals with AdGM-CSF or Add170-3 at different time-points in bronchial epithelium (b), alveolar–capillary interstitium (c) and granulomas (d); more immunostained cells are observed in animals treated with AdGM-CSF, the strongest being labelled the airways epithelium; scale bar represents 20 μm (439 × 475 mm; 200 × 200 DPI).

post-infection in pneumocytes type II, while AMs were negative at the same time-points and showed the strongest positivity later, suggesting that these cells were the most important GM-CSF cellular source. In contrast, the lungs from the control group showed GM-CSF positivity until 14 day post-infection in type II pneumocytes and AMs (Fig. 5c). In granulomas, mice treated with AdGM-CSF showed immunostained macrophages with heterogeneous distribution, predominantly in the granuloma periphery, until day 28 post-infection. A similar distribution was seen in the control group until day 60 post-infection (Fig. 5d).

Effect of transgenic GM-CSF expression on reactivation of chronic LTB infection

When female B6D2F1 mice are infected by the i.t. route with a low dose of Mtb strain H37Rv, a chronic form of TB, similar to latent infection, is produced [22]. It is characterized by a low and stable lung bacillary load (fewer than 500 CFU) without weight loss, tissue damage, spontaneous reactivation or death. If a 3-mg/l concentration of corticosterone is administered by drinking water during late infection (7 months), supraphysiological plasma levels are reached, and the disease reactivation is then manifested by an increment of bacilli growth and progressive pneumonia. This chronic/latent infection model was used to determine if transgenic expression of GM-CSF by AdGM-CSF delivered at 7 months post-infection could prevent reactivation. One month after AdGM-CSF delivery, reactivation was induced with cortisone administered for 1 month in drinking water. Pulmonary Mtb burdens and histopathology were evaluated at 9 months post-infection. Mice treated with AdGM-CSF showed significantly lesser bacterial loads and exhibited lower pneumonic areas than mice that received the control vector Addl70-3 (Fig. 6a,b).

Effect of transgenic GM-CSF expression on the transmission of Mtb from infected to healthy mice

Our transmissibility model is based on long co-housing between infected and healthy mice [23]. This model was used to determine whether or not AdGM-CSF treatment was able to prevent infection of healthy mice co-housed with animals infected with moderate (H37Rv) or highly virulent (Beijing 900–1000) strains. The negative control group was represented by mice treated with Addl70-3, while the positive control animals received antibiotic INH treatment. Cultures of lung homogenates from healthy mice (contacts) treated with AdGM-CSF or INH did not show Mtb growth after 2 months of co-housing with infected mice with H37Rv (Fig. 6c) or Beijing (Fig. 6d) strains. In contrast, contact mice treated with Addl70-3 showed bacillary loads after 2 months of co-housing, being highest in the group that co-housed with mice infected with the highly virulent strain Beijing 900–1000. As an independent verification of the status of Mtb transmission, cutaneous DTH response to mycobacterial antigens was also performed in healthy contact mice after 2 months of co-housing with infected mice. Co-housed contact mice with H37Rv-infected animals treated with INH or AdGM-CSF showed a significantly lower DTH response than contact mice treated with Addl70-3 (Fig. 6e). Co-housed contact mice with animals infected with the highly virulent Beijing strain and treated with AdGM-CSF showed significantly less DTH than contact mice treated with INH or Addl70-3, and no difference was found between these control groups (Fig. 6f).

Discussion

GM-CSF was first identified in mouse lung following lipopolysaccharide injection by its ability to stimulate proliferation of bone marrow cells and generate colonies of granulocytes and macrophages [28]. Interestingly, mice with homozygous deletion of the GM-CSF gene develop normally without significant alteration of haematopoiesis, but they develop lung abnormalities such as extensive accumulation of pulmonary surfactant phospholipids and increased susceptibility to opportunistic bacterial and fungal infections [13]. Thus, GM-CSF has significant physiological and immunological regulatory activity in the lung, such as increasing myeloperoxidase activity in neutrophils [29], stimulating differentiation and activation of macrophages and Toll-like receptor 4 (TLR-4) expression and T cell activation [30]. Indeed, most of the GM-CSF-mediated effects on T cells are believed to be exerted indirectly through antigen-presenting cells (APCs). Regarding its participation in mycobacterial infection, it was reported that mice lacking GM-CSF died rapidly, showing severe necrosis when exposed to an aerosol-delivered Mtb infection because of their inability to mount a Th1 response [31]. Our results confirm and extend these observations, by showing progressive GM-CSF expression during early infection peaking at day 7 and maintaining high levels until day 28, which coincide with the transitory bacilli growth control in this model mediated by a predominant Th1 response and proinflammatory cytokine expression [19,20].

GM-CSF can be produced by a wide variety of cells, including macrophages, fibroblasts, endothelial cells, T cells, mesothelial and epithelial cells, among others [32]. In these cells, bacterial antigens and inflammatory cytokines such as IL-1, IL-6 and TNF-α are potent inducers [33,34]. Thus, these factors should contribute to the observed higher expression of this growth factor, considering that all these cytokines are produced preferentially during the first month after infection in this model [20]. During late stages of the disease there was a progressive decrease of GM-CSF expression, showing its lowest level at day 120 post-infection. The expression of GM-CSF can be inhibited by IL-10 and IL-4 [35] or glucocorticoids [36]. These anti-inflammatory

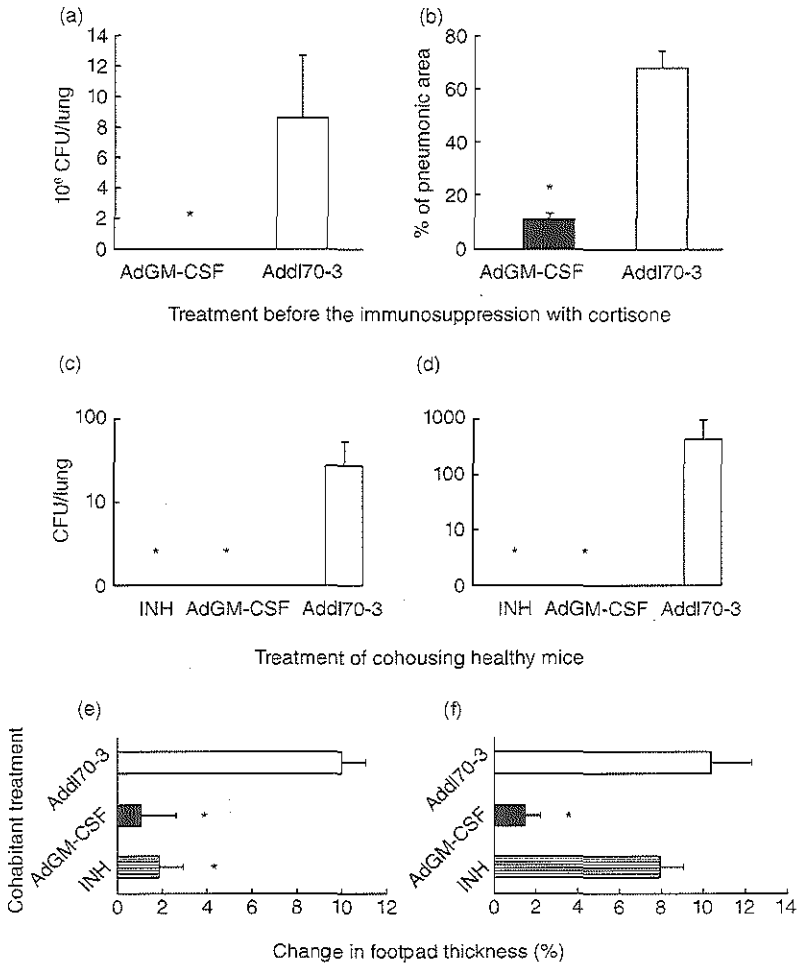

Fig. 6. Adenovirus encoding granulocyte–macrophage colony-stimulating factor (AdGM-CSF) treatment prevents reactivation of chronic tuberculosis (TB) similar to latent infection following immunosuppression and prevention of transmission in co-housed healthy mice. (a) Mice were infected with low-dose *Mycobacterium tuberculosis* (Mtb) H37Rv to induce chronic infection similar to latent infection, and after 7 months animals were treated with AdGM-CSF (black bars) or Addl70-3 (white bars); after 1 month mice were treated with cortisone for 1 month to induce disease reactivation. Animals treated with AdGM-CSF showed lower pulmonary bacilli loads and (b) lesser tissue damage (pneumonia) than mice treated with Addl70-3. (c) With regard to evaluation of the effect of AdGM-CSF treatment in the prevention of transmission, groups of mice were infected with mild virulent Mtb strain H37Rv or (d) with the highly virulent Beijing strain 9001000 and co-housed with healthy mice treated with isoniazid (INH; hatched bar), AdGM-CSF (black bar) or Addl70-3(white bar) for 2 months; the lungs were used for bacilli burden determination by quantification of colony-forming units (CFUs). In comparison with animals treated with Addl70-3, the lungs of animals treated with INH or AdGM-CSF do not show bacilli growth. (e) Significantly lower delayed-type hypersensitivity (DTH) was measured in animals treated with INH or AdGM-CSF than mice treated with Addl70-3 that were co-housed with mice infected with Mtb strain H37Rv. (f) In contrast, there was no DTH difference in animals treated with INH or Addl70-3 when co-housed with mice infected with highly virulent Beijing strain, while animals treated with AdGM-CSF showed significantly lower DTH. All values are means ± standard deviation (s.d.) from two independent experiments with five mice per group;* $P < 0.05$ (561 × 632 mm; 200 × 200 DPI).

factors might be related to GM-CSF decrease during advanced disease, when there is a significant decline in immune protection in this model co-existing with extensive tissue damage and high bacilli burdens [19,20].

The significant participation of GM-CSF in Mtb infection was supported by our current results obtained after the administration of only one dose of recombinant adenoviruses expressing this cytokine, 1 day before infection with Mtb. This pretreatment resulted in efficient GM-CSF transgenic expression in the airways epithelium that induced fourfold more activated DCs and significantly higher expression of the immune protective factors TNF-α, IL-12, IFN-γ and iNOS, which led to more and bigger granulomas, more AMs F4/80$^+$, lesser pneumonia and a prolonged decrease in bacilli loads. Thus, although GM-CSF is produced preferentially in our model during early infection it

appears not to be enough, as is indicated by the late emergence of activated DCs, and overproduction by AdGM-CSF administration boosts the Th1 cells and DC activation, producing a significant improvement in the immune protective response.

Cytokines involved in the activation of Th1 lymphocytes can be used clinically as a form of immunotherapy to increase anti-mycobacterial activity through activation of DCs, AMs and T lymphocytes. In this regard, the use of recombinant cytokines such as IFN-γ [37] or aerosolized IFN-α [38], in conjunction with antibiotics, produced clinical improvement in patients with TB. Using the same murine TB model, it was showed that gene therapy based on adenoviruses encoding IFN-γ controlled disease progression successfully in mice infected with drug-sensible and drug-resistant strains [21]. Furthermore, vaccines based on recombinant viral vectors encoding mycobacterial antigens [39] or vaccines based on recombinant BCG [40,41] have shown effectiveness in experimental models of TB, and have also demonstrated that the respiratory mucosal route of administration is the best way to induce an efficient protective immune response against respiratory infections. This is greatly augmented when GM-CSF is used as adjuvant [42], reaching improved protection against disseminated infection associated with expansion and activation of APCs [40,41]. Moreover, it has been shown recently that recombinant BCG expressing GM-CSF is highly efficient in preventing TB in a mouse model [41], inducing a similar cytokine response that we observed when AdGM-CSF was administered 1 day before infection. In fact, administration of GM-CSF, or inducing its expression, has been useful in the treatment against other pulmonary infections such as aspergillosis [43], *Chlamydia trachomatis* [44] and pneumococcal pneumonia [45]. Moreover, the delivery of recombinant human GM-CSF by inhalation was shown to be beneficial in the treatment of alveolar proteinosis and it was well tolerated in healthy subjects [46]. However, there are no available data about their prophylactic use to augment anti-micobacterial host defence for mainly immunodeficient patients. In line with this, one disadvantage is that recombinant cytokines are extremely expensive and have a short *in-vivo* half-life. Thus, as shown in our current study, the use of only one dose of adenoviruses encoding GM-CSF could have a potential benefit during early Mtb infection.

Both adenoviral vectors AdGM-CSF and Addl70-3 have been well characterized; they are expressed temporarily in the lung (for 12–14 days) [47] and essentially infect the respiratory epithelium resulting in detectable GM-CSF in the lung until the third week post-administration, without evidence of serum increments, when administered in healthy mice. This transient expression is important, considering that transgenic mice that over-express GM-CSF showed macrophage accumulation, blindness and severe damage to various tissues [48]; these mice also exhibited a significant increment of many cytokines and inflammatory mediators, and failed to focus T cells and macrophages into sites of Mtb infection [31], suggesting that high and permanent expression of GM-CSF leads to defects in cytokine and chemokine regulation. Therefore, excess of GM-CSF does not induce an overly Th1 response, and very fine control of this cytokine is needed to fight infections. With regard to Addl70-3, they did not induce detectable levels of GM-CSF and did not cause significant viral-mediated inflammation [47].

Our experimental results suggest that the immunostimulatory effect of GM-CSF promotes not only protection against primary TB, but also prevention of LTB reactivation. Compared with mice treated with Addl70-3, a single dose of AdGM-CSF was able to prevent reactivation after immunosuppression induced by the administration of corticosterone in mice, with chronic infection similar to latent infection. These preclinical results offer an alternative for future immunotherapeutic trials in high-risk individuals preventing reactivation of LTB, such as patients with rheumatological diseases treated with anti-TNF-α therapy [49], or HIV/AIDS patients who can develop TB after primary infection or after LTB reactivation. However, this therapeutic approach in HIV infection should be taken with some caution, considering that some studies have shown an increase of viral replication in HIV-infected patients due to higher immune cell activation induced by GM-CSF [50], while others report beneficial therapeutic effects induced by this cytokine [51].

Another clinical scenario for the potential application of gene therapy codifying cytokines such as GM-CSF is to prevent infection in the healthy contacts of TB patients. The World Health Organization (WHO) recommends the screening of household contacts from a TB source case to identify infected individuals, principally children under 6 years of age, in order to supply prompt treatment to ill individuals and provide preventive chemotherapy to subjects who do not develop the disease [1]. The only current alternative to BCG vaccination for preventing TB is chemoprophylaxis, particularly with INH administered for at least 6 months. However, a systematic review of TB prophylaxis in HIV-infected adults showed that INH reduced the incidence of TB in those subjects with positive DTH, but was ineffective in those with negative DTH, suggesting that chemoprophylaxis does not prevent primary infection [7]. Our current results in the BALB/c mouse model of transmissibility confirm this suggestion, particularly in animals co-housed with mice infected with the highly virulent Beijing strain, as contact mice that were treated with INH chemoprophylaxis did not show any statistically significant difference in DTH compared with the Addl70-3 control group, confirming that INH did not prevent primary infection, although it inhibited the growth and viability of Mtb efficiently. In contrast, administration of AdGM-CSF was more efficient in preventing transmission, as suggested by the lower DTH without detectable CFU exhibited by treated

contact mice. These results are in agreement with clinical trials which have shown that administration of INH as chemoprophylaxis in children living in a TB-endemic zone did not reduce the risk of infection, illness or death due to TB [52]. However, INH has shown effectiveness in preventing active TB in individuals who have had contact with TB patients [53]. Considering the increased prevalence of MDR and XDR strains, our results, using the murine transmissibility model, suggest that gene-based immunotherapeutics using adenoviruses encoding GM-CSF could be useful to prevent infection in close contacts of TB patients. In addition, chemoprophylaxis with INH takes 6–9 months and is potentially hepatotoxic; substitution by only one administration of AdGM-CSF without toxic effects could have significant implications in the control of the transmissibility of this disease.

In conclusion, GM-CSF is a significant cytokine in the immune protection against Mtb. Gene therapy based on adenoviruses encoding GM-CSF increased protective immunity when administered in a single dose 1 day before Mtb infection in BALB/c mice, after recombinant adenoviruses infected the airways epithelium and macrophages increased production of GM-CSF, which induced rapid and efficient activation of DCs that enhanced the production of IFN-γ, TNF-α and iNOS, permitting the efficient control of bacilli growth. The same treatment was effective in preventing LTB reactivation and transmission. Whether administration of AdGM-CSF during late progressive disease could also be beneficial in drug-susceptible and drug-resistant disease is currently under investigation in our laboratory.

Acknowledgements

We thank I. Estrada-García, Y. Serafín-López, L. Sánchez-Torres, M. Perez-Tapia, J. Luna and L. Flores-Romo for valuable suggestions and help. A.F.C. was responsible for design, performance and data interpretation of the study, and wrote the primary writing of the manuscript. D.A.M.E. performed and analysed experiments. Z.X. kindly provided and characterized AdGM-CSF and Add170-3. R.H.P conceived, designed and provided funding of the study and reviewed the final manuscript. The authors thank Cristina Parada for her technical assistance. The study was supported by Consejo Nacional de Ciencia y Tecnología (CONACyT; contract 84456). A.F.C. is a recipient of post-graduate fellowship by CONACyT.

Disclosure

The authors have no conflicting financial interests.

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Immunobiology xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Immunobiology

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Mycobacterium tuberculosis manipulates pulmonary APCs subverting early immune responses

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ARTICLE INFO

Article history:
Received 6 April 2011
Accepted 20 May 2012

Keywords:
Alveolar macrophages
Dendritic cells
In vivo
Tuberculosis

ABSTRACT

Alveolar macrophages (AM) and dendritic cells (DCs) are the main antigen presenting cells (APCs) in the respiratory tract. Whereas macrophages have been extensively studied in tuberculosis, *in situ* interactions of DC with *Mycobacterium tuberculosis* (Mtb) are poorly explored. We aimed to characterize lung APCs during pulmonary tuberculosis in Balb/C mice infected with *Mtb* H37Rv.

Mtb-infection via the airways induced a delayed and continuous accumulation of DCs and AM in the lungs. While lung DCs increased after day 3 post-infection, macrophages increased after 2–3 weeks. Although both populations accumulated in lungs during the infection, DCs decreased in the late stages. Infection induced differential expression of co-stimulatory molecules in these lung APCs, decreasing to basal levels in both APCs in the late stages. A remarkable segregation was found regarding bacillary burden. Many macrophages contained numerous bacilli, but DC contained scarce mycobacteria or none. Mtb-infection also induced delayed accumulation of DC in draining lymph nodes. This delayed recruitment was not associated with a lack of IL-12p40, which was detected from day 3 post-infection. Although AM and lung DCs behave differently during pulmonary tuberculosis, Mtb apparently manipulates both lung APCs subverting early protective responses resulting in disease progression.

Introduction

DCs, the most potent antigen presenting cells, are the only APCs able to stimulate naïve T lymphocytes and are involved in both tolerance and immunity (Steinman and Cohn 1973; Steinman and Banchereau 2007). However, their interactions with microbial pathogens of human relevance are poorly understood, especially *in vivo*. *Mycobacterium tuberculosis*, one of the most fearsome infectious agents worldwide, is able to survive and grow inside non-activated macrophages causing pulmonary tuberculosis (Leemans et al. 2005, 2001). DCs are present throughout the respiratory tract and play important roles in the regulation of local immune responses to inhaled antigens (Hammad and Lambrecht 2008), but their potential role and interactions with *Mycobacterium* bacilli still are not well elucidated. AM and DCs are the main APC of the respiratory tract, but it has been observed that AM express limited APC activity, and in some circumstances they can suppress T cell activation (Lambrecht 2006; Bilyk and Holt 1993; Holt et al. 1988). *In vitro* studies have shown that both DCs and macrophages can internalize *Mycobacteria*, but bacilli grow more easily in macrophages than in DCs (Bodnar et al. 2001; Tailleux et al. 2003). Furthermore, these APCs responded with a differential cytokine production since DCs produced mainly IL-12, while macrophages produced IL-10 (Bodnar et al. 2001; Mohagheghpour et al. 2000; Giacomini et al. 2001). *Mycobacterium bovis* has often been used *in vivo* for systemic infection to study the functions of DCs (Jiao et al. 2001; Lagranderie et al. 2003). However, to date there is scarce research in animal models using virulent Mtb infection via the respiratory tract to evaluate the potential role of pulmonary DCs during tuberculosis. We therefore aimed to characterize the behavior *in situ* of lung DCs compared to AM during the course of experimental pulmonary tuberculosis.

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http://dx.doi.org/10.1016/j.imbio.2012.05.022

Materials and methods

Experimental model of pulmonary tuberculosis

The experimental model of pulmonary tuberculosis has been previously described in detail (Hernandez-Pando et al. 1995, 1996). Briefly, male Balb/C mice 6- to 8-week old were intratracheally injected with 2.5×10^5 viable *M. tuberculosis* H37Rv. Control mice were inoculated only with sterile saline. Experiments were performed in P3 biosecurity facilities in accordance with the institutional guidelines for animal care and experimentation. Three separate experiments were performed, and at least 3 mice were sacrificed at each selected time point. In some experiments mice were intratracheally inoculated with heat-inactivated Mtb H37RV or with soluble mycobacterial antigens (1 tuberculin unit of PPD).

Cell suspensions of lungs and draining lymph nodes

Upon sacrificing the animals, the respiratory tract was surgically exposed and mediastinal lymph nodes (MLN) were carefully dissected and collected for digestion in RPMI 1640 with 5% of fetal calf serum (Invitrogen, USA), 1 mg/ml collagenase type 2 (Worthington Biochemical Corporation, USA), and 2 U/ml of DNase I (Invitrogen, USA) for 30 min at 37 C. To remove AM from lungs, bronchoalveolar lavage (BAL) was performed with 5×1 ml of PBS. Then, lungs were perfused via the right ventricular cavity of the heart with 10 ml of sterile PBS to remove peripheral blood cells from lung vasculature. Lungs were carefully removed and cut in small pieces which were digested for 1 h at 37 °C as described above. Tissues were homogenized and cell suspensions were passed through cell strainers. Single cell suspensions were subjected to red blood cell lysis and viability was determined by trypan blue exclusion.

Flow cytometry analysis

Cell suspensions from lungs, MLN and BAL were analyzed by flow cytometry. Antibodies used were: anti-CD11c-APC (Armenian hamster IgG, clone HL3, BD Biosciences), anti-I-A/I-E FITC (Rat IgG_{2a}, clone 2G9, BD Biosciences), anti-CD40-biotinylated (Rat IgG_{2a}, clone 3/23, BD Biosciences), anti-CD80-PE (Hamster IgG, clone 16-10AI, BD Biosciences), anti-CD86-PE (Rat IgG_{2a}, clone GL1 BD Biosciences) and anti-DEC205 (Rat IgG_{2a}, clone NLDC145, kindly donated by Ralph Steinman, NY, USA). Cells were incubated with antibodies for 30 min at 4 °C in the dark, and then washed three times. In the case of biotinylated antibodies, incubation with streptavidin-PE or Cy-Chrome followed for 15 min at 4 °C. Finally, cells were washed and fixed with 1% paraformaldehyde and analyzed in a BD FACScalibur. In addition of removal of BAL cells, FL-3 channel was always used to exclude auto-fluorescent cells in lung parenchyma cell suspensions.

Immunohistochemistry

Lungs were perfused and fixed with absolute ethanol and embedded in paraffin. Lung sections of 5 μm thickness were mounted on slides and deparaffinized. Antigen retrieval was achieved with citrate buffer (Dako, USA) in a pressure cooker for 8 min. Endogenous peroxidase was blocked with 6% of H_2O_2 in PBS for 15 min. Lung sections were incubated with the primary antibody (rat anti-human IL-12 p40, Santa Cruz Biotechnology, USA) overnight at room temperature. A secondary biotinylated antibody was used to detect the primary bound antibody, followed by HRP-conjugated streptavidin. Enzyme-linked antibody was revealed with 3,3′-diaminobenzidine/H_2O_2 for 10–15 min at room temperature. As a final point, tissue sections were stained with Ziehl–Neelsen and counterstained with hematoxylin. Frozen sections of MLN and lung tissue were used to evaluate $DEC205^+$ and $CD11c^+$ cells. Positive cells were counted in at least 10 fields, and the percentages of positive cells were calculated relative to the total number of cells per field.

Immunolabeling of cells in cytospin preparations

Lung cell suspensions from control and Mtb-infected mice were subjected to metrizamide gradients to enrich lung APC. Enriched cells were fixed with paraformaldehyde (to inactivate the bacilli), and then spun onto slides. Cytospin preparations were used for immunolabeling of MHC-CII molecules (I-A/I-E). Ziehl–Neelsen staining was subsequently performed to reveal the bacilli.

Statistical analysis

Differences between mean values were analyzed for statistical significance with GraphPad Prism Software (version 4.0), using unpaired *t* tests. *P* values less than 0.01 were considered statistically significant ($^*P < 0.01$; $^{**}P < 0.001$; $^{***}P < 0.0001$).

Results

Alveolar macrophages in tuberculosis

AM expressed CD11c and were highly auto-fluorescent (Fig. 1A). During the first weeks of pulmonary tuberculosis, there was a slight decrease in the absolute number of AM, but they increased after 21 days, reaching their maximum in the late stage (Fig. 1B). In animals inoculated with either dead Mtb H37Rv or PPD, there were no overt changes in AM, except for a decrease seen only at day 1 with dead Mtb and PPD (Fig. 1B and C). The proportion of AM decreased during the course of pulmonary tuberculosis (Fig. 1C), suggesting that other populations might be recruited to the alveolar spaces during infection.

In Mtb-infected mice the activation state of AM was modified progressively (Fig. 2). CD40 expression increased late around day 28 while CD80 increased from day 1, reaching its maximum at day 3, and was maintained for at least 3 weeks. CD86 was poorly expressed in AM from control mice and started to be up-regulated in infected mice at day 3. Significant expression was observed after 1 month of infection. In the late phase of pulmonary tuberculosis the expression of these three markers decreased. CD40 fell to near basal level, while CD80 was even lower than the baseline.

Lung DCs during pulmonary tuberculosis

Characterization of lung DCs was first performed in healthy non-infected mice. Single cell suspensions of lung tissue were labeled for MHC-CII and CD11c. There were two populations regarding MHC-CII expression, $CD11c^+$/MCH-$CII^{-/low}$ and $CD11c^+$/MHC-CII^{high}, region R2 and R3, respectively (Fig. 3A). Trying to get a better discrimination between these two subsets, mice were treated intraperitoneally with FLT3-L, a factor known to selectively increase the number of DCs. After 9 days of treatment, $CD11c^+$/MHC-CII^{high} cells increased in lung tissue and MLN, around sevenfold compared with control mice (Fig. 3B). In contrast, the lung $CD11c^+$/MHC-$CII^{-/low}$ subset did not change significantly under this treatment. Furthermore, when AM were excluded either by using FL-3 channel for auto-fluorescent cells or by removing BAL cells, only region R2 was affected (Fig. 3A). Altogether the data indicate that R2 corresponds to lung monocyte/macrophages (and perhaps some very immature DCs), whereas R3 most likely corresponds to lung DCs. We thus used these settings and this approach for the subsequent analyses of lung DC populations during the infection.

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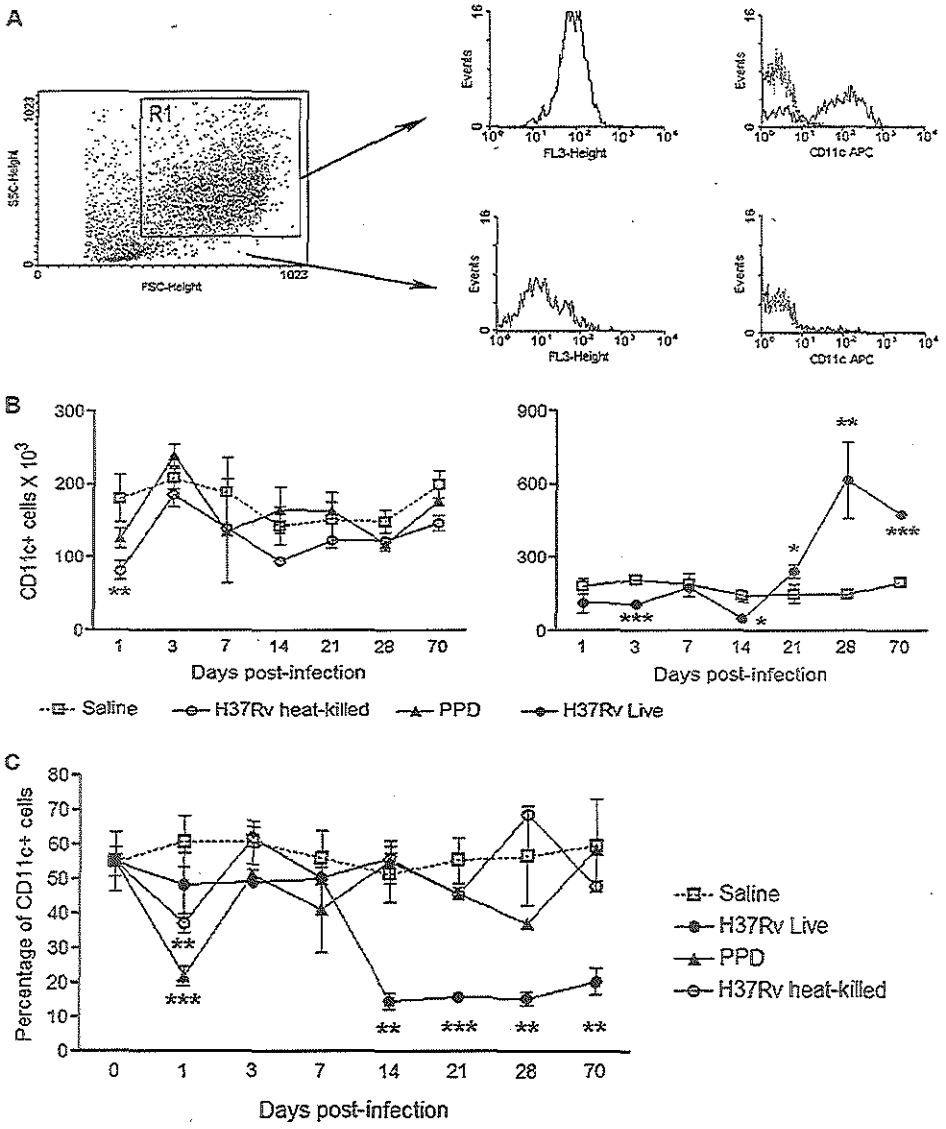

Fig. 1. AM accumulated in infected lungs. Alveolar cells obtained by bronchoalveolar lavage (BAL) were analyzed by flow cytometry. (A) Unlabeled cells were gated in the region of macrophages, based on their size and complexity (R1), showing auto-fluorescence in the FL-3 channel compared with the rest of the cells. Since auto-fluorescence was not detected in the FL-4 channel, this was then used to assess the expression of CD11c in these cells (right histograms). (B and C) $CD11c^+$/Auto-flourescent AM were measured at different time points in mice inoculated intra-tracheally with different stimuli: saline solution, live Mtb H37Rv; dead Mtb H37Rv or soluble mycobacterial antigens (PPD). Absolute numbers and proportions of AM are represented in (B and C), respectively. Absolute numbers were calculated by multiplying the percentage of AM obtained through flow cytometry by the total number of live cells in the BAL.

During pulmonary tuberculosis the number and proportion of lung DCs changed dramatically increasing from day 3 post-infection, doubling their basal number by day 7, reaching the highest level at day 21. Interestingly, in the late infection DCs decreased notably (Fig. 3C and D). We compared the numbers of lung DC from mice inoculated either with intact live Mtb H37Rv, dead Mtb or PPD. Upon inoculating dead Mtb the number of DCs decreased slightly at day 1, returned to basal levels at day 3, while with PPD there was a significant increase between days 1 and 3, and then cells returned later to normal levels. Thus, compared to the other conditions, only live Mtb was able to induce a delayed accumulation of DCs in the lungs until day 28.

Increased expression of CD40, CD80 and CD86 was observed in lung DCs during pulmonary tuberculosis. CD40 started to increase as early as day 3 post-infection and was maintained throughout the infection (Fig. 4). CD80 and CD86 increased later by day 14, but after 2 months of infection they were down-regulated.

Mtb infection up-regulates DEC-205 expression in lung APCs

The macrophage mannose receptor (MMR) is apparently involved in interactions with virulent strains of Mtb (Schlesinger 1993). We wanted to know whether expression of DEC205, a C-type lectin evolutionarily related to MMR, changes during pulmonary tuberculosis. We thus analyzed the expression of DEC205 in lung sections from non-infected and Mtb-infected mice. In normal lung tissue $DEC205^+$ cells were observed in the alveolar spaces and in the interstitial alveolar walls (Fig. 5A). $DEC205^+$ cells clearly increased during Mtb infection. In fact, $DEC205^+$ cells were present within granulomas in tuberculous lesions (Fig. 5B), and also in the pneumonic areas (Fig. 5C). A remarkable $60.9 \pm 10.9\%$ of $DEC205^+$ cells

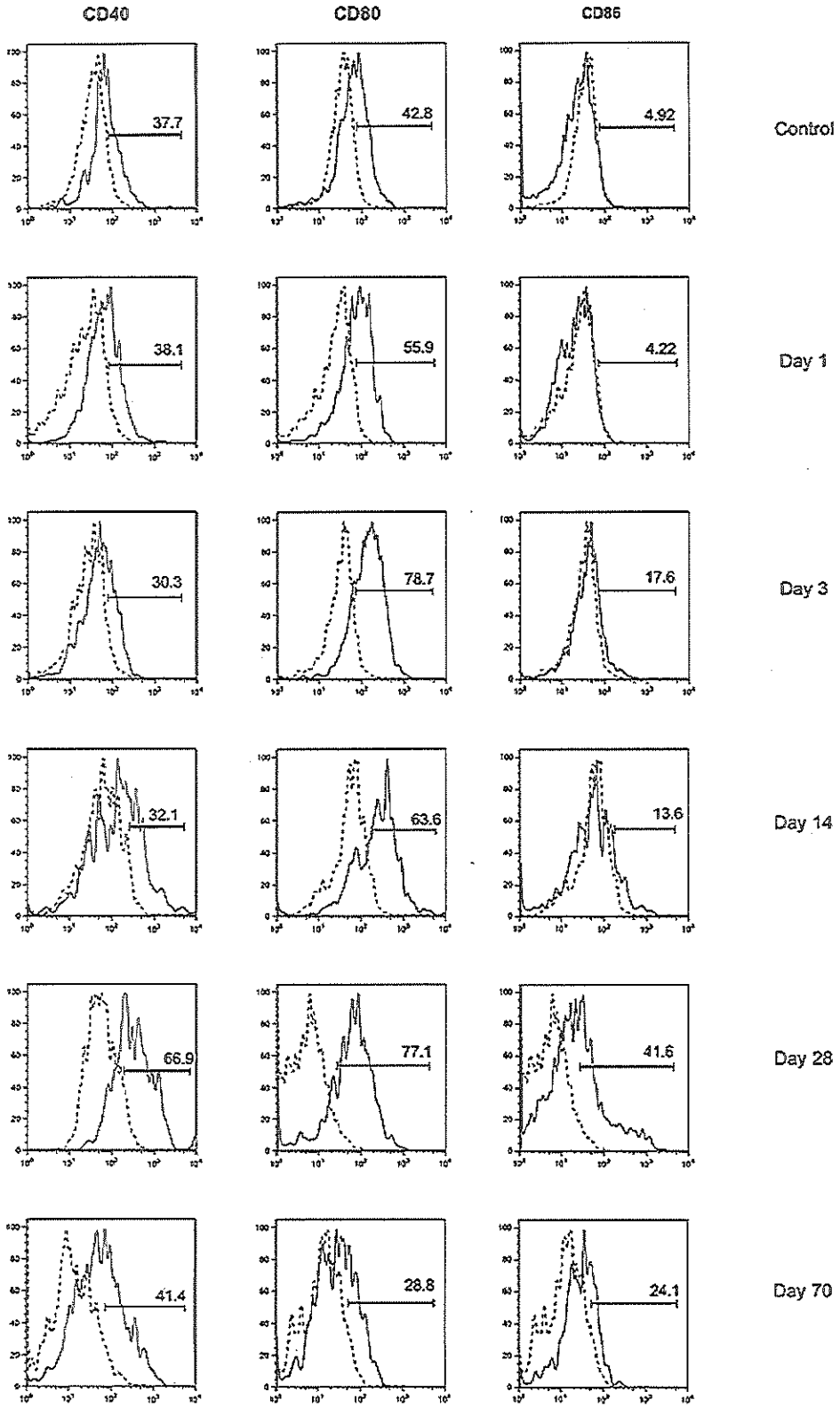

Fig. 2. Activation state of AM during pulmonary tuberculosis. BAL cells were stained for CD11c and for the activation markers CD40, CD80 and CD86. Activation of AM was monitored at the indicated time points during the infection. Mice inoculated with saline were used as a control group. Thin dashed lines depict unlabeled cells, while thick lines indicate the positive cells for each marker.

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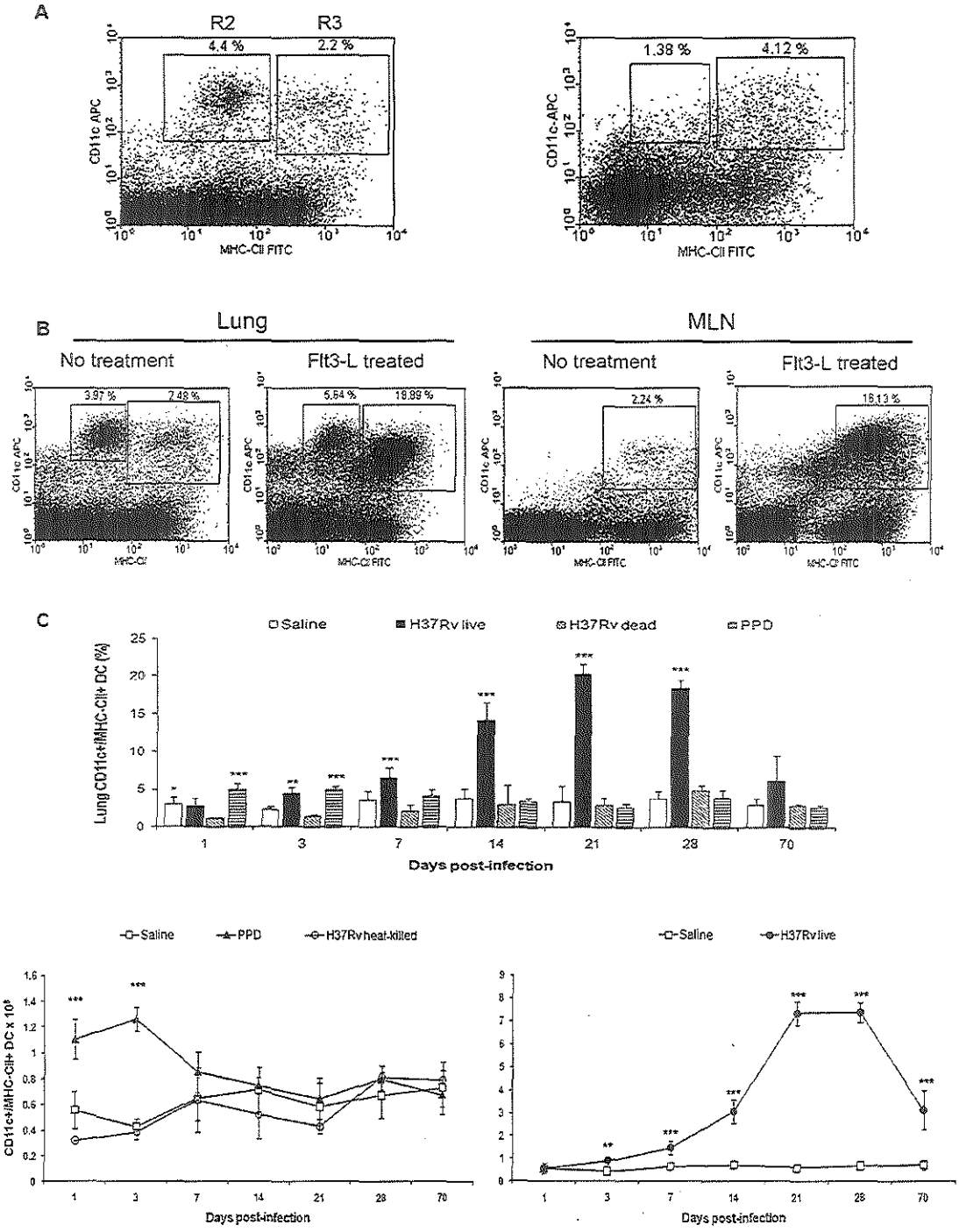

Fig. 3. Influx of lung dendritic cells during Mtb infection. (A) Lung cell suspensions of healthy non-infected mice were analyzed by flow cytometry using antibodies to CD11c and MHC-CII. $CD11c^{+}/MHC\text{-}CII^{-/low}$ cells (R1) were practically absent in lung cell suspensions when BAL and auto-fluorescent cells were excluded (right dot-plot). (B Mice injected 9 days IP with 10 μg of Flt3L showed a significant augmentation in the number of DCs in the MLN. In the lung of Flt3-L treated mice $CD11^{+}/MCH\text{-}CII^{high}$ cells (R2) increased considerably. Proportions (C) and absolute numbers (D) of lung $CD11c^{+}/MHC\text{-}CII^{high}$ cells (lung DCs) were monitored for 70 days in mice inoculated intra-tracheally with saline solution, live Mtb H37Rv, dead Mtb H37Rv or PPD.

was positive for the bacilli by Ziehl–Neelsen staining and contained 4.4 ± 2.6 bacilli per cell. Expression of DEC205 on lung DCs was analyzed by flow cytometry. They expressed DEC205 throughout the infection (Fig. 5E). However expression decreased in advanced infection in accordance with the lower number of these cells in the late phase (Fig. 3). These data suggest that $DEC205^{+}$ cells observed in pneumonic areas in the chronic stage might correspond mainly to lung macrophages.

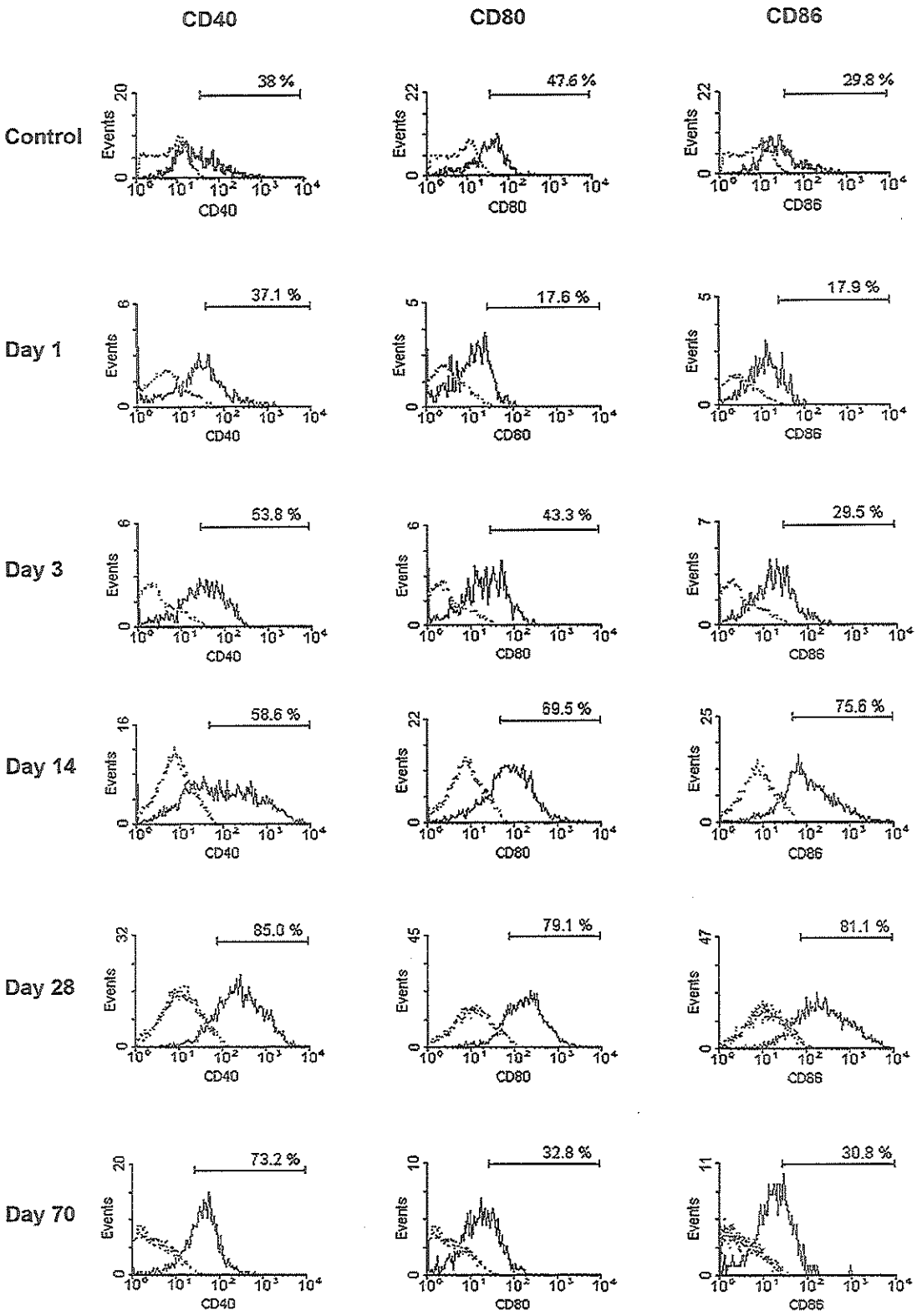

Fig. 4. Late activation of lung DCs during pulmonary tuberculosis. In lung cell suspensions CD40, CD80 and CD86 molecules were measured on $CD11c^{+}$/MHC-CII^{high} dendritic cells during pulmonary tuberculosis by flow cytometry. Thin dashed lines depict control isotype antibodies.

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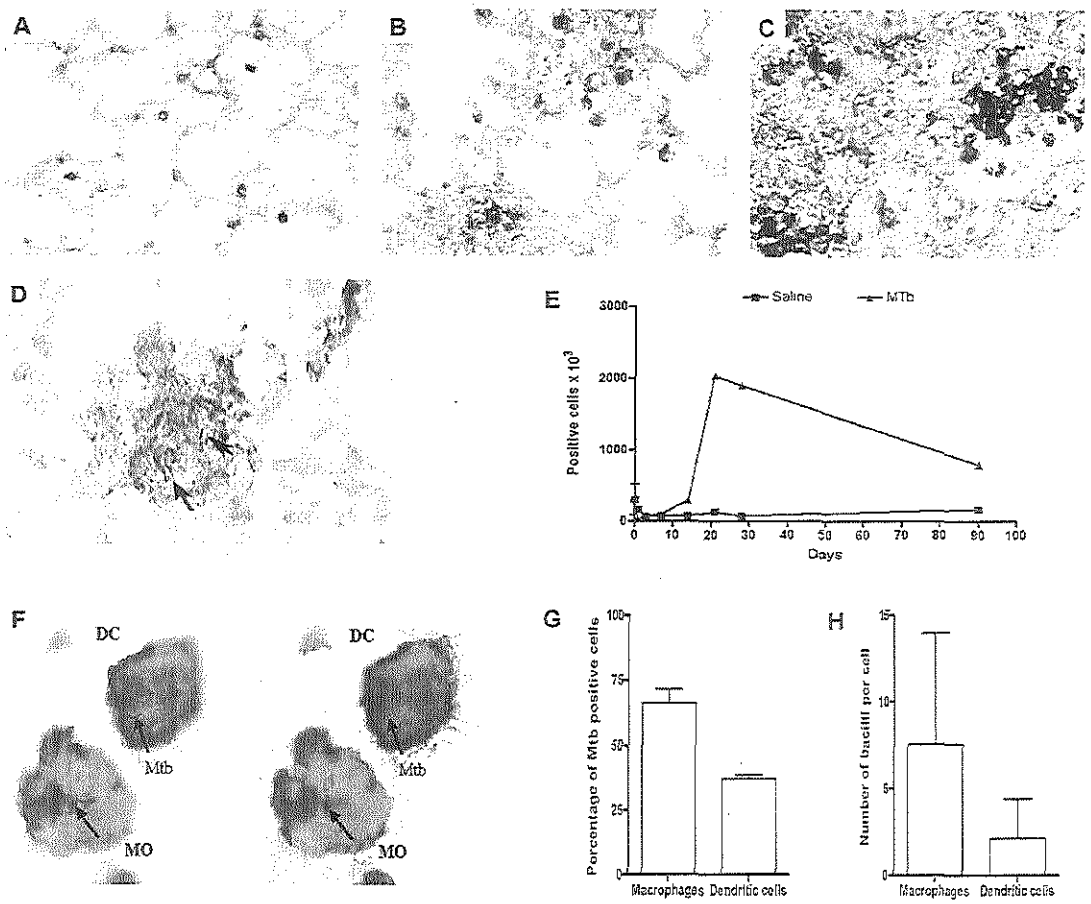

Fig. 5. *In vivo* interaction of lung APCs containing Mtb. Immunolabeled lung sections from healthy (A), and Mtb-infected mice at different times post-infection (B and C) were used to evaluate DEC205 expression at days 0, 21, and 60, respectively. DEC205⁺ cells were found to bear intracellular bacilli (D); (A–C) 200× and (D) 1000×. (E) Expression of DEC205 on lung CD11c⁺ DCs was analyzed by flow cytometry in cell suspensions, excluding BAL and auto-fluorescent cells. (F–H) Lung APCs were enriched by metrizamide gradients from lung cell suspensions of Mtb-infected mice (day 28 post-infection). Cells were cytospun onto slides and stained for MHC-CII and Ziehl–Neelsen. (F) The image on the right illustrates the intracellular bacilli while the one on the left illustrates the morphology of the same cells. (G) Shows the percentages of Mtb⁺ cells and (H) the number of bacilli per cell.

Lung DCs and AM exhibit a differential interaction with Mtb in vivo

Previously, we reported that CD14⁺ cells in the lungs of Mtb-infected mice had the higher burden on intracellular bacilli (Pedroza-Gonzalez et al. 2004). To extend this analysis, the interaction of Mtb bacilli with lung APCs *in vivo* was evaluated by immunolabeling MHC-CII molecules and Ziehl–Neelsen staining using cytospin preparations of enriched lung APCs. Lung DCs and macrophages were distinguished both by the morphology and by the relative expression of MHC-CII (Fig. 5F). Intracellular bacilli were quantified at day 28, when they are more abundantly found in lung tissue in this experimental model (Lopez et al. 2003). Over 70% of macrophages were positive for the bacilli. They contained an average of 8 bacilli per cell, with a broad range from 1 to 30 (Fig. 5G–H). Interestingly, the cells with the highest number of intracellular bacilli showed numerous cytoplasmic vacuoles corresponding to the so-called foamy macrophages. In contrast, only 37% of lung DCs were Ziehl–Neelsen positive, with an average of 2 bacilli per cell, and a much narrower range, from 1 to 7.

Delayed arrive of DCs to MLN was observed during MTb infection

We quantified the DCs in the MLN which drain the respiratory tract, and found a delayed accumulation of DCs in mice infected with live Mtb in comparison with mice inoculated with PPD (Fig. 6A). PPD induced an increase of DCs in MLN at early time points post-inoculation (from days 1 to 7), while in Mtb-infected mice DCs numbers started to have a very slight increase between days 3 and 7, and the maximum number was observed at day 21. At this time point the presence of CD11c⁺ cells bearing Mtb bacilli was observed in MLN (Supplementary Fig. 1). Similarly to the lung, the number of DCs in MLN decreased nearly to basal levels in the advanced stage of the disease (day 70).

IL-12p40 is actively produced by infected lung APCs

IL-12 has been shown to be critical in tuberculosis (Nolt and Flynn 2004; Flynn et al. 1995; Cooper et al. 1997), and IL-12p40 seems to be involved in the migration of lung DCs to MLN, required for the activation of naïve T cells (Khader et al. 2006; Wolf et al. 2008). For these reasons we were interested in determining the *in situ* production of IL-12p40 during the course of pulmonary tuberculosis. IL-12p40 was detected from day 3 post-infection and increased throughout the infection (Fig. 6B). Immunohistochemistry analysis of lung sections from control and Mtb-infected mice showed that IL-12p40 was clearly detected only in Mtb-infected mice (Supplementary Fig. 2A and B). IL-12p40⁺ cells were localized in a rather concentric way inside the tuberculous lesions, and in the first days of infection they were localized mainly in the

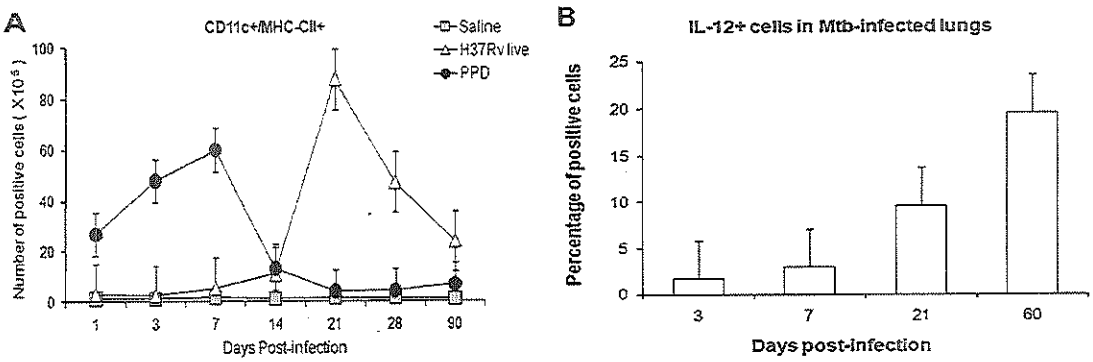

Fig. 6. Delayed accumulation of DCs in MLN and production of IL-12 during pulmonary tuberculosis. (A) CD11c$^+$/MHC-CII$^+$ DCs were analyzed by flow cytometry in MLN cell suspensions from mice intratracheally infected with live Mtb H37Rv, PPD or saline solution. (B) IL-12p40 production was assessed by immunohistochemistry in lung sections from saline inoculated and Mtb-infected mice at different times post-infection. The percentage of IL-12p40$^+$ cells in Mtb-infected lung tissue at different times post-infection was quantified by counting at least five different fields for each slide.

alveolar spaces (Supplementary Fig. 2C and D). To determine whether Mtb bacilli were associated with the production of IL-12p40 we stained IL-12p40 together with Ziehl–Neelsen and found that the majority of the cells expressing IL-12p40 were also positive for Mtb bacilli (Supplementary Fig. 2C).

Discussion

When BALB/c mice are infected intratracheally with a high dose of virulent Mtb H37Rv, an initial phase is dominated by high production of Th1 cytokines that, together with high levels of tumor necrosis factor (TNF) and inducible nitric oxide synthase (iNOS), temporally control the infection (Hernandez-Pando et al. 1997, 2001). One month after infection the expression of Th-1 cytokines, TNF and iNOS start to decline. Gradually, pneumonic areas prevail over granulomas. Pneumonia in co-existence with a high burden of bacteria causes death. In the present study we extended the immunological characterization of this model by studying the kinetics and *in situ* localization of macrophages and dendritic cells in the lung and MLN. Our results showed that AM were rapidly infected by Mtb and their number increased dramatically in the advanced phase, when the bacillary burden is high. AM up-regulated the expression of co-stimulatory molecules during the first month of infection, while in the late stage, when they were heavily infected, a down-regulation of these molecules was observed. Interestingly, many macrophages collected during late infection showed numerous cytoplasmic vacuoles (foamy macrophages), which are predominant in the pneumonic areas (Pedroza-Gonzalez et al. 2004; Hernandez-Pando et al. 1997). Besides their high content of cytoplasmic bacilli, these foamy macrophages show little immunostaining for TNF or iNOS, but strong transforming growth factor beta (TGFβ) immunoreactivity (Hernandez-Pando et al. 2001). Thus, the low expression of CD40 and CD86 molecules by AM during late infection, plus their ability to produce immunosuppressive cytokines could potentially hamper an effective protective response. They might induce local anergy to bacillary antigens by presenting these antigens without an adequate co-stimulatory function (Gimmi et al. 1993).

In pulmonary Mtb-infection we found a delayed accumulation of lung DCs in comparison with mice inoculated with PPD. Early DCs recruitment has been observed in other infections through the airways (Lagranderie et al. 2003; Jakubzick et al. 2006; McGill et al. 2008; Muralimohan et al. 2008) and also with BCG infection (Lagranderie et al. 2003; Reljic et al. 2005). This delayed DCs accumulation in the lungs could be an Mtb evasion/pathogenic mechanism to avoid the early generation of protective responses, by delaying the early expansion of truly effector T cells. In this context, it has been shown that Mtb-infected DCs had low capacity to present mycobacterial antigens and stimulate Mtb-specific CD4$^+$ T cells *in vivo* (Wolf et al. 2008; Balboa et al. 2010). Furthermore, Mtb-infection induced the production of several cytokines like IL-10 which can decrease DC trafficking to draining lymph nodes (Demangel et al. 2005). We have found a similar delay in the recruitment of DCs into MLN during pulmonary infection with virulent Mtb. Delayed recruitment of DCs was not associated with a miss-production of IL-12p40, which has been reported to be involved with the migration of lung DCs to the MLN (Khader et al. 2006; Robinson et al. 2008). Thus, perhaps the accumulation of DCs in the infected lungs could account at least partially for the delayed arrival of these cells in the MLN. In accordance with our results there are reports showing that the onset of immune response in tuberculosis is delayed and depends of the arrival of the antigen to the draining lymph nodes (Wolf et al. 2008; Garcia-Romo et al. 2004; Humphreys et al. 2006; Bhatt et al. 2004). Furthermore, the delay of Mtb-specific T cell response led to impaired control of bacillary replication (Wolf et al. 2007; Tian et al. 2005).

In the advanced phase of Mtb-infection the number of lung DCs decreased importantly and their activation status was down-regulated. Several cytokines produced during Mtb-infection can modulate the differentiation of DCs. These cytokines can induce the preferential development of macrophages instead of DCs (Balboa et al. 2010; Fortsch et al. 2000; Mariotti et al. 2002, 2004; Hanekom et al. 2003), favoring Mtb survival and dissemination. Thus it is possible that this might be happening in the lung tissue infected by Mtb. Of note, we observed an important decrease in lung DCs, but not of AM in the late stage of Mtb-infection. Furthermore, lung DCs from Mtb-infected mice contained intracellular Mtb bacilli, but in much smaller numbers than AM. Thus, we hypothesize that one of the mechanisms that Mtb might use to progress is by inducing the differentiation of macrophages in preference to DCs in lung tissue during active infection, promoting an uncontrolled bacillary growth in non-activated macrophages which, eventually results in progressive tissue damage, respiratory insufficiency and death.

AM and lung DCs behave differently during pulmonary tuberculosis. This seems in accordance with the long held assumption that AM are focused in controlling the bacterial growth, while DCs initiate and regulate the response against the infection by transporting and presenting mycobacterial antigens to lymphocytes in the MLN. These crucial functions can be subjected to alterations/manipulations by virulent Mtb to induce disease progression.

Acknowledgments

We thank Victor Rosales-Garcia, Juana Calderon-Amador and Luis Donis-Maturano for technical assistance. We are grateful to

Please cite this article in press as: Garcia-Romo, G.S., et al., *Mycobacterium tuberculosis* manipulates pulmonary APCs subverting early immune responses. Immunobiology (2012), http://dx.doi.org/10.1016/j.imbio.2012.05.022

Dr. Graham A.W. Rook for reviewing and correcting the manuscript. This work was supported by the National Council for Science and Technology, CONACyT (Grant 104667 to Flores-Romo L and Grant 84456 to R. Hernandez-Pando).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.imbio.2012.05.022.

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Journal of Infectious Diseases Advance Access published August 21, 2012

MAJOR ARTICLE

Extrapulmonary Locations of *Mycobacterium tuberculosis* DNA During Latent Infection

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Background. One-third of the world's population has latent infection with *Mycobacterium tuberculosis*, and 10%–15% of cases of reactivation occur at extrapulmonary sites without active pulmonary tuberculosis.

Methods. To establish the frequency and location of mycobacterial DNA, organ specimens from 49 individuals who died from causes other than tuberculosis were studied by means of polymerase chain reaction (PCR), PCR plus DNA hybridization, in situ PCR, real-time PCR, and spoligotyping.

Results. Lung specimens from most subjects (36) were positive for *M. tuberculosis*, as were specimens from the spleen (from 35 subjects), kidney (from 34), and liver (from 33). By in situ PCR, mycobacterial DNA was found in endothelium, pneumocytes, and macrophages from the lung and in Bowman's parietal cells and convoluted proximal tubules from the kidney. In spleen, macrophages and sinusoidal endothelial cells were positive, whereas in liver, Kupffer cells and sinusoidal endothelium were commonly positive. Spoligotyping of 54 pulmonary and extrapulmonary positive tissues from 30 subjects showed 43 different genotypes, including 36 orphan types. To confirm the viability of mycobacteria, 10 positive tissue samples were selected for isolation of mycobacterial RNA. All samples showed 16S ribosomal RNA expression, while 8 and 4 samples showed expression of the latent infection genes encoding isocitrate lyase and α-crystallin, respectively.

Conclusions. *M. tuberculosis* persists in several sites and cell types that might constitute reservoirs that can reactivate infection, producing extrapulmonary tuberculosis without lung involvement.

Mycobacterium tuberculosis (Mtb) can produce progressive disease or latent infection [1]. In areas of high endemicity, infection first occurs in childhood and in most cases is controlled. Only 10% of these primary infections lead to progressive disease [1, 2]. However, some bacilli remain in tissues in a nonreplicating dormant or slowly replicating stage for the rest of the individual's life. This latent form of tuberculosis (TB) is clinically asymptomatic. In countries with low or moderate endemicity, most active TB cases arise as a result of reactivation of latent bacilli [1, 2]. It is estimated that one-third of the world's population carries latent Mtb, and millions of cases of reactivated TB are predicted in the coming years [3, 4].

One important point is the location of the bacilli during latent infection [1]. It has been assumed that latent bacilli are located in old fibrotic pulmonary granulomas, where the low nutrient supply and hypoxic microenvironment induce the low-level metabolism characteristic of latent bacilli [1]. However, we have detected mycobacterial DNA by conventional and in situ polymerase chain reaction (PCR) analysis in histologically normal lung tissue specimens from humans and mice during latent infection [5, 6]. This suggests that latent mycobacteria can reside not only in macrophages from old granulomas, but also in nonprofessional phagocytic cells from superficially normal tissue. However, recent results have shown mycobacterial DNA in apparently histologically normal adipose tissue from nontuberculous persons living in countries where TB is endemic (eg, Mexico) or nonendemic

Received 10 August 2011; accepted 18 January 2012.

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The Journal of Infectious Diseases

DOI: 10.1093/infdis/jis381

(eg, France), as well as in adipose tissue from mice with latent infection [7]. This is important because approximately 15% of cases of reactivation occur at extrapulmonary sites (ie, liver, spine, kidney, spleen, and brain) without active pulmonary TB [8, 9]. These findings suggest extrapulmonary reactivation rather than reactivation from pulmonary sites and subsequent migration of bacilli to other sites. The aims of this study were to determine whether Mtb DNA and RNA are present in extrapulmonary tissue specimens (from the liver, kidney, and spleen) from individuals who had no histopathologic findings specific for TB and died from causes other than TB and, if Mtb DNA and RNA are present, to identify their cellular locations. We confirmed our findings for humans in a murine model of chronic infection that is similar to latent infection.

METHODS

Human Samples

Forty-nine subjects who died from causes other than TB were included. The necropsy and tissue samples were collected at the Department of Pathology of the General Hospital of Mexico. Sex, age, and causes of death are presented in Table 1. Subjects with human immunodeficiency virus infection or immunosuppressive treatment were excluded. Tissue samples were obtained during legally authorized autopsies with signed permission by a relative, who agreed to the donation of additional samples for the present study. The Ethics Committee of the General Hospital of México approved the study (code DI/11/310/03/083).

Between 4 and 6 hours after death, tissue samples were obtained from the lung, liver, spleen, and kidney. Samples were taken from random sites of all organs except lungs, in which samples were obtained from the base and apex. Samples were taken using sterile technique (ie, new scalpel blades, sterile clothes, and a flame) and divided into 2 fragments. One fragment was frozen in liquid nitrogen for DNA and RNA extraction and culture of Mtb in Middlebrook 7H9 broth (Difco Becton Dickinson, Sparks, MD), while the other fragment was fixed in 10% formaldehyde for histological analysis, acid-fast staining, and mycobacterial DNA detection by in situ PCR.

Detection of Mycobacterial DNA by Conventional and In Situ PCR

A fragment of each frozen sample was homogenized in a mini-bead beater (Biospec Products, Bartlesville, OK). DNA was extracted by the phenol-chloroform-isoamyl alcohol method [10]. Strict procedures and controls were followed to avoid cross-contamination between samples during DNA extraction. To detect mycobacterial DNA, conventional PCR for the specific insertion sequence *IS6110* was carried out as previously described [11]. Considering that >90% of newborn infants in Mexico receive BCG vaccine, it was important to confirm that mycobacterial nucleic acids in positive samples were not derived from *Mycobacterium bovis*. We used conventional PCR to amplify the *oxyR* gene, highly specific for *M. bovis*, using primers JB21 (5′-TCGTCCGCTGATGCAAGTGC-3′) and JB22 (5′-CGTCCGCTGACCTCAAGAAG-3′) [12]. We used DNA from Mtb H37Rv and *M. bovis* BCG Danish 1331 as positive controls and DNA from *Homo sapiens* and *Escherichia coli* as negative controls. In negative samples, PCRs were carried out twice to confirm the result.

To increase the sensitivity of *IS6110* detection, DNA hybridization was applied to all negative or weakly positive samples. In brief, Hybond N+ membrane was sensitized with denatured *IS6110*–PCR product from each necropsy, incubated for 2 hours with blocking reagent (Roche Applied Science, Indianapolis, IN), and incubated overnight with the *IS6110*-Biot probe. The membrane was washed with 2 × SSPE/0.5% sodium dodecyl sulfate and incubated with streptavidin-HRPO (Invitrogen, Camarillo, CA). Probe detection was performed with Immobilon Western Chemiluminescent HRP substrate (Millipore, Billerica, MA). The *IS6110*-Biot probe was made using the INS-1 primer 5′-CCTGCGAGCGTAGGCG TCGG-3′ labeled with biotin at the 5′ end, using as controls the DNA from *H. sapiens*, *E. coli*, and *Haemophilus influenzae*.

For in situ PCR detection of mycobacterial DNA, 5-μm sections from each paraffin block were examined, following the protocol previously described [5–7]. Lung sections from 1 TB case were used as positive control, and lung sections from a noninfected mouse were used as a negative control.

Spoligotyping

To determine whether the detected mycobacterial DNA was related to a particular genotype, as well as whether >1 genotype was in each necropsy specimen, spoligotyping was carried out for every positive sample, according to the manufacturer's recommendations (Ocimum Biosolution, Hyderabad, India). In all experiments, DNA from Mtb H37Rv and *M. bovis* BCG was used as a positive control. The reaction mixture was prepared with AmpliTaq Gold fast PCR Master Mix, (Applied Biosystems, Carlsbad, CA), using DRa 5′-GGTTTTGGGTCTGAC GAC-3′ biotinylated at the 5′ end and DRb 5′-CCGAGAGGGG ACGGAAAC-3′ primers and 1 μg of total DNA as a template. Spoligotypes in octal code were compared with the SPOLDB4.0 database of the Pasteur Institute of Guadeloupe (available at: http://www.pasteur-guadeloupe.fr/tb/bd_myco.html).

Quantification by Real-Time PCR of Mycobacterial Gene Expression

To define bacterial viability in tissue samples in which we detected mycobacterial DNA, we used a previously described technique with some modification to determine mycobacterial gene expression by real-time PCR after isolation of putative mycobacterial RNA [13, 14]. Ten samples from different

Table 1. Detection of Mycobacterial DNA in Pulmonary and Extrapulmonary Tissues by Conventional Polymerase Chain Reaction (PCR) Plus DNA Hybridization

Subject	Sex	Age, Years	Cause of Death	PCR Hybridization Finding			
				Lung	Spleen	Kidney	Liver
1	M	61	Emphysema	+	+	−	−
2	F	62	Systemic arterial hypertension	+	−	−	+
3	M	18	Kidney failure	+	+	−	+
4	F	52	Sepsis	+	−	+	+
5	F	27	Systemic erythematous lupus	+	+	+	+
6	M	73	Kidney failure	−	+	+	+
7	F	57	Systemic arterial hypertension	−	−	+	+
8	M	55	Lymphoblastic acute leukemia	−	+	+	−
9	M	40	Diabetes	+	+	+	+
10	F	24	Chronic kidney failure	+	+	+	+
11	F	84	Systemic arterial hypertension	+	+	+	+
12	M	22	Myeloblastic acute leukemia	+	+	+	+
13	M	93	Systemic arterial hypertension	+	+	+	+
14	M	33	Fusiform cell sarcoma	+	+	+	+
15	M	61	Acute hemorrhagic pancreatitis	−	+	+	−
16	M	60	Multiple myeloma	+	−	+	+
17	F	28	Ovarian cancer	−	−	−	+
18	F	76	Cancer	+	+	+	+
19	F	16	Sepsis	+	+	+	+
20	M	35	Cardiac malformation	+	+	+	−
21	F	20	Systemic arterial hypertension	+	+	−	+
22	F	58	Systemic arterial hypertension	−	−	−	+
23	M	42	Chronic kidney failure	+	−	−	−
24	F	70	Hypovolemic shock	+	+	+	−
25	F	45	Lung cancer	+	+	+	+
26	F	69	Malignant glioma	+	+	+	+
27	F	18	Sepsis	+	+	+	−
28	M	35	Pneumonia	+	+	+	−
29	M	64	Myeloblastic acute leukemia	+	+	−	+
30	M	42	Kidney cancer	+	+	+	+
31	F	57	Emphysema	+	−	+	+
32	M	89	Hypovolemic shock	+	+	+	−
33	M	29	Brain hemorrhage	+	+	+	+
34	F	29	Diabetes mellitus	+	+	+	+
35	M	80	Brain hemorrhage	+	+	+	+
36	F	29	Breast cancer	+	+	+	−
37	F	83	Chronic kidney failure	+	+	+	+
38	F	22	Brain hemorrhage	+	+	+	+
39	M	36	Breast cancer	+	+	+	+
40	F	70	Heart failure	−	+	+	+
41	F	50	Myocardial infarction	+	+	−	+
42	M	76	Chronic kidney failure	+	+	+	+
43	F	59	Sepsis	+	+	+	+
44	F	14	Brain hemorrhage	−	−	−	−
45	F	40	Systemic arterial hypertension	−	−	−	−
46	F	84	Emphysema	−	−	−	−
47	M	52	Pneumonia	−	−	−	−
48	M	45	Disseminated intravascular coagulation	−	−	−	−
49	M	37	Pneumonia	−	−	−	−

Abbreviations: −, negative; +, positive.

subjects that showed the strongest PCR positivity for *IS6110* were selected. The tissue was homogenized using a Multi-Gen 7 mm generator and centrifuged; the supernatant contained eukaryotic RNA, while the pelleted material contained the bacilli. To isolate the bacterial RNA, the pellets suspended in TRIzol (Gibco BRL, Camarillo, CA) were disrupted using a Mini-Bead Beater-8 (Biospec Products, Bartlesville, OK) and zirconia and silica beads. After vortexing and centrifugation, the supernatant contained Mtb RNA. Bacterial RNA was isolated using 4 cycles of purification with an RNeasy column (Qiagen, Valencia, CA), followed by DNase incubation. We used mycobacterial 16S ribosomal RNA (rRNA) for reference constitutive gene expression and as a viability marker because this gene is expressed in all growth conditions [13]. Primers for 16S rRNA, isocitrate lyase (*icl*), and α-crystallin (*acr*) mycobacterial genes were designed with Primer Express software, version 2.0 (Applied Biosystems, Carlsbad, CA). The nucleotide sequences of the forward and reverse primers were as follows: for 16S rRNA, 5′-TCCCGGGCCTTGTACACA-3′ (forward) and 5′-CCACTGGCTTCGGGTGTAA-3′ (reverse); for *icl*, 5′-ACACCTACCCCGACCAGAG-3′ (forward) and 5′-TG CAGCTCGTAGACGTTGAG-3′ (reverse); and for *acr*, 5′-CGAGAAGGACGTCGACATTA-3′ (forward) and 5′-CC TTGTCGTAGGTGGCCTTA-3′ (reverse). The quality and quantity of RNA were evaluated by spectrophotometry and electrophoresis. Complementary DNA (cDNA) synthesis was performed using 5 μg of total RNA, 2 μM of random primers (Promega, Madison, WI), 10 U/μL of ribonuclease inhibitor

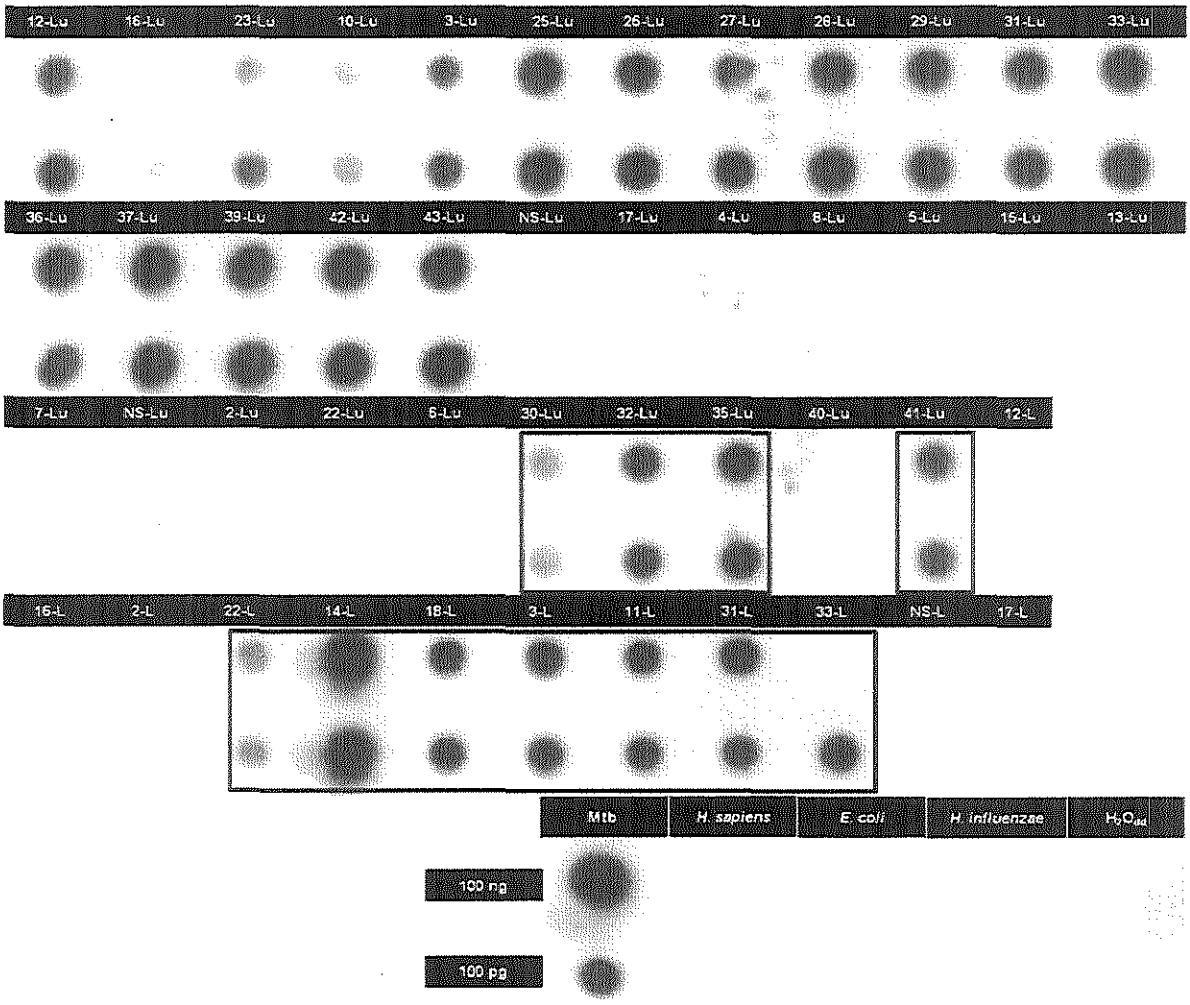

Figure 1. Detection of mycobacterial DNA in lung and liver samples by conventional polymerase chain reaction (PCR) plus DNA hybridization. To increase the sensitivity of *IS6110* detection, DNA hybridization was applied to all negative (*box*) or weakly positive samples by conventional and in situ PCR. Hybridization was performed in duplicate, using the PCR product; the number corresponds to the patient referred in Table 1. *Bottom,* A total of 100 ng and 100 pg of *Mycobacterium tuberculosis* (Mtb) DNA (positive control) and 100 ng of negative controls DNA (from *Homo sapiens* [*H. sapiens*], *Escherichia coli* [*E. coli*], and *Haemophilus influenzae* [*H. influenzae*] and water). Abbreviations: L, liver sample; Lu, lung sample.

(Invitrogen, Camarillo, CA), 1 μL of buffer RT, 0.5 mM of dNTP, and 4 units of Omniscript reverse transcriptase (Qiagen, Valencia, CA). A preliminary conventional PCR using 16S rRNA primers was carried out with an aliquot of cDNA. Real-time quantitative PCR was performed with the QuantiTect SYBR Green PCR Master Mix (Qiagen, Valencia, CA). To obtain a standard curve, 4 different PCRs were performed in parallel, using as a template 10-fold dilutions of known amounts of the Mtb H37Rv 16S rRNA gene (10^8–10^2 copies) together with the experimental sample. Reactions were performed in a 7500 Prism spectrofluorometric thermal cycler (Applied Biosystems, Carlsbad, CA). The reaction mixtures consisted of 0.5 μM of target and control primers, 12.5 μL of Master Mix, and 1 μg of cDNA. Cycling conditions were as follows: initial denaturation for 15 minutes at 95°C, followed by 40 cycles at 95°C for 20 seconds, at 59°C for 20 seconds, and at 72°C for 34 seconds. An independent real-time PCR assay was carried out in triplicate for each tissue sample in 2 separate experiments. The messenger RNA copy number of mycobacterial genes was related to 1 million copies of RNA encoding the 16S rRNA gene. PCR fragments amplified from *acr* and 16S rRNA genes from Mtb were identified by means of a genetic analyzer, ABI PRISM 310 PE (Applied Biosystems, Carlsbad, CA). RNA from spleen tissue of infected mice was used as a positive control, and RNA from lung tissue from noninfected mice and lung tissue obtained during necropsy of newborn mice were used as negative controls.

Murine Model of Latent Infection

To evaluate the presence of extrapulmonary Mtb in apparently histologically normal tissues, we used a murine model of chronic infection that is similar to latent infection [6]. Hybrid F1 C57BL/DBA mice were infected intratracheally with 1×10 live Mtb H37Rv. Groups of 5 mice were sacrificed at days 30, 90, 150, and 210 after infection. The kidney, spleen, and liver were collected, and fragments were prepared as described above

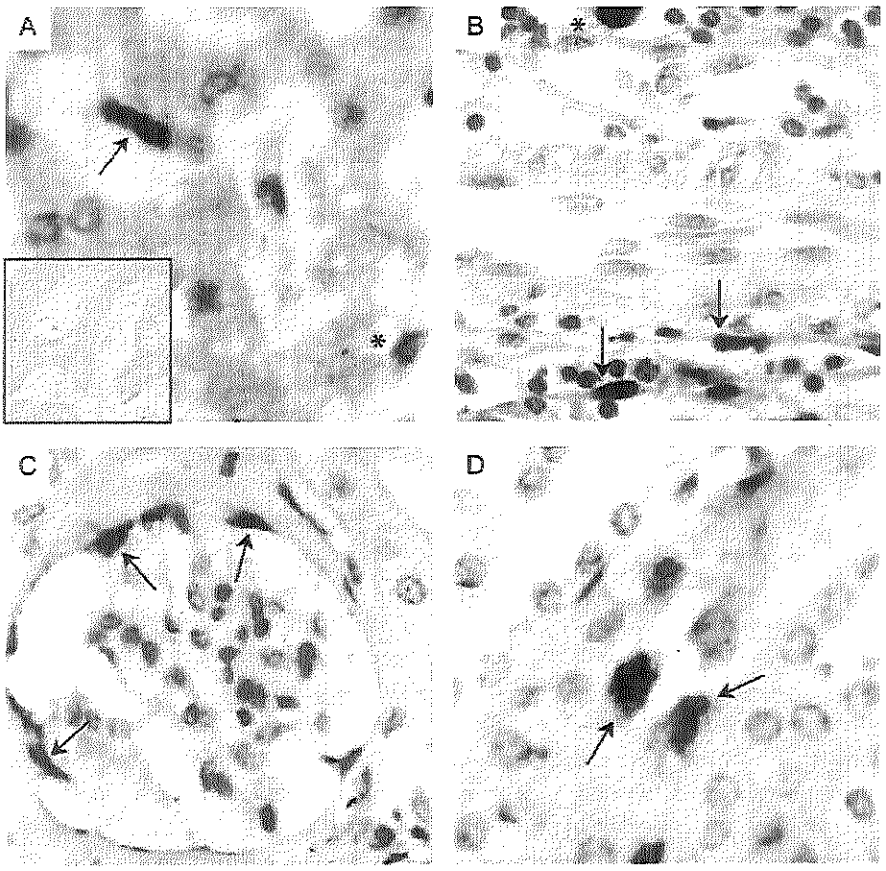

Figure 2. Representative micrographs of the localization of mycobacterial DNA detected by in situ polymerase chain reaction. *A*, Kupffer cell from hepatic sinusoids shows strong positivity (*arrow*), as well as occasional hepatocytes (*asterisk*). *Inset*, liver section from normal noninfected mouse as a negative control. *B*, In spleen, endothelial cells from sinusoids (*arrows*) and red pulp macrophages (*asterisk*) are positive. *C*, In the kidney, strong positivity is seen in the parietal cells from the glomerular capsule and in epithelial cells from the proximal convoluted tubules (*arrows*; *D*). For all micrographs, original magnification ×400.

for histological analysis and *IS6110* detection. Serial sections were stained with Ziehl-Neelsen. Another tissue fragment was used for quantification of colony-forming units (CFUs) [6].

RESULTS

Mycobacterial DNA in Pulmonary and Extrapulmonary Tissues

A total of 43 necropsy specimens (from 19 of 23 males and 24 of 26 females) were positive for mycobacterial DNA (Table 1). We considered necropsy specimens as positive when *IS6110* was detected in at least 1 tissue by conventional PCR, PCR plus DNA hybridization, or in situ PCR. As expected, the lung was the organ that most commonly yielded specimens positive for *IS6110* (in 36 cases), but spleen specimens (from 35), kidney specimens (from 34), and liver specimens (from 33) were also positive (Table 1). In 35 subjects (70%), we found mycobacterial DNA in lung and extrapulmonary tissues, but in 7 (14%), detection was exclusively in extrapulmonary tissues. When the result was difficult to interpret, we performed PCR plus DNA hybridization (Figure 1). Thus, 34 of 141 samples (3 lung specimens, 13 liver specimens, 16 spleen specimens, and 2 kidney specimens) that were negative by conventional PCR yielded positive results after hybridization. No samples were positive for *M. bovis*.

By use of in situ PCR, we detected mycobacterial DNA in endothelial cells, type II pneumocytes, and alveolar macrophages (data not shown), which corroborates previous findings for lung tissue [5]. In kidney, positivity was localized in the parietal cells of Bowman's capsule and, on occasion, in epithelial cells from convoluted proximal tubules (Figure 2). In spleen, *IS6110* positivity was found in red pulp macrophages and sinusoidal endothelial cells, while in liver, positivity was located in Kupffer cells and sinusoidal endothelium. On occasion, hepatocytes and portal biliary duct epithelial cells were also positive (Figure 2). None of these positive tissues showed any apparent histological abnormality, such as granulomas, inflammatory infiltrates, or fibrosis.

Spoligotyping

In 54 positive tissue specimens from pulmonary and extrapulmonary sites of 30 subjects, we found 42 different genotypes, but only 8 genotypes had a match in the SPOLDB4.0 database. The most frequently detected genotype (in 7 samples from 6 subjects) corresponded to shared international type (SIT) 53. This profile has been reported 2380 times in the SITVIT database (54 of which were reported from Mexico). Other profiles found were SIT 523 (in 3 samples), SIT 291 (in 2), and SITs 245, 521, 1166, 1196, and 1690 (in 1 each). With the exception of SIT 53, none of the profiles found was previously reported in Mexico. We found 34 orphan types from different organs. They had spoligotyping codes, but the codes did not have matches in the database (Table 2). In 15 of 30 subjects, we found 1 genotype; 9 of 30 subjects had 2 genotypes, and 6 of 30 carried ≥3 genotypes.

Detection of Mycobacterial Gene Transcription by Real-Time PCR

To confirm the viability of mycobacteria, we selected 10 tissue samples (4 from lungs, 4 from kidneys, and 2 from spleens) with the highest positivity to *IS6110* detection by PCR. Spleen taken from chronically infected mice was the positive control. We selected the mycobacterial 16S rRNA as a viability marker, and for latency we selected α-crystallin (encoded by *acr*), since this protein is expressed during stressful conditions [14], and isocitrate lyase (encoded by *icl*), because Mtb uses this enzyme in the metabolism of fatty acids during chronic infection [13].

In the 10 selected samples, we detected 16S rRNA (Figure 3*A*), while *icl* was detected in 8 samples (4 from kidneys, 3 from lungs, and 1 from spleen) at levels varying from 1×10^1 to 1×10^6 copies per 10^6 copies of 16S RNA (Figure 3). In 4 samples that were positive for *icl*, we also detected *acr* (in 2 kidney specimens, 1 lung specimen, and 1 spleen specimen). The expression of *acr* was higher in all cases (range, 1×10^7–1×10^9 copies).

Murine Model

We used a mouse model that mimics latency, with a low, stable lung bacillary load without weight loss, spontaneous reactivation, or death [6]. Low and stable numbers of CFUs were detected only in liver and spleen (Figure 4). After histological analysis, acid-fast staining showed bacilli in tissue sections from liver and spleen, specifically in macrophages, hepatocytes, and endothelial cells from apparently normal tissue (Figure 4). In situ PCR showed positivity in the same cells as in the human tissues, but the labeling was stronger, and the cellular distribution was much wider (Figure 4).

DISCUSSION

Previous work has demonstrated the presence of mycobacterial DNA in macroscopically and histologically normal lung and adipose tissue from people who died from causes other than TB, suggesting latent infection [5, 7]. Here, we confirm and extend these observations by demonstrating the presence of mycobacterial DNA in pulmonary and multiple extrapulmonary tissues from people who died from causes other than TB and had no history of this disease. This agrees with many reports in which Mtb DNA was detected in extrapulmonary samples from patients in a wide range of geographical locations who were putatively without illness [15–19].

We found a higher percentage of pulmonary and extrapulmonary specimens positive for mycobacterial DNA than in our previous study [5]. This difference can be explained by the use of DNA hybridization in addition to PCR, which greatly increased sensitivity. We detected mycobacterial DNA in both

Table 2. Spoligotyping Results

Specimen Source	Sex	Age, Years	Organ	Octal	Spoligotype	SIT
M. tuberculosis H37Rv	...	...	...	777777477760771		451
M. bovis from BCG vaccine	...	...	...	676773777777600		482
Subject 2	F	62	L	777777677760771		291
Subject 3[a]	M	18	Lu	777777777760671		245
			S	777777777760771		53
			L	777777777760611		521
Subject 5	F	27	S	777346377560771		...
Subject 6	M	73	L	777777777761771		1196
Subject 7	F	57	K	777777777760771		53
Subject 9	M	40	Lu	777777777421771		...
Subject 10[b]	F	24	S	757377477760771		...
			K	777777777777771		523
Subject 11[b]	F	84	S	777777777760771		53
			K	777777777760771		53
			L	777377777760771		1166
Subject 12[b]	M	22	Lu	777001314521770		...
			L	337046377760771		...
Subject 13[b]	M	93	S	776363767760671		...
			L	777777777760771		53
Subject 14	M	33	S	777777377160771		...
Subject 16	M	60	Lu	777777776420730		...
Subject 18[b]	F	76	S	777777777100771		...
			K	777777677760771		291
Subject 19[a]	F	16	Lu	777777777760771		53
			K	777777776561771		...
			L	777365574760771		...
Subject 21	F	20	S	777377376700771		...
Subject 22	F	58	Lu	777777777760771		53
Subject 28	M	35	K	774357777777761		...
Subject 29	M	64	S	777377377765661		...

Table 2 continued.

Specimen Source	Sex	Age, Years	Organ	Octal	Spoligotype	SIT
Subject 30	M	42	S	777277777777620		...
Subject 31[a]	F	57	S	777777577774771		...
			K	777357477760771		...
			L	417243077760771		...
Subject 32[a]	M	89	Lu	777357476766771		...
			K	777777677761771		...
			L	777357777777771		...
Subject 33[a]	M	29	Lu	777357476760771		...
			S	777777777762771		1690
			K	737377477760771		...
Subject 34[b]	F	29	S	777357477760771		...
			L	777777476773771		...
Subject 35[b]	M	80	S	777377477760771		...
			L	777377677770771		...
Subject 36[a]	F	29	Lu	777357676761771		...
			S	777357477760771		...
			K	777757477760771		...
			L	777777777777771		523
Subject 37	F	83	S	777377677763771		...
Subject 38[b]	F	22	Lu	777355476360671		...
			L	777377477723771		...
Subject 39	M	36	S	773257676763771		...
Subject 40	F	70	L	777777777777771		523
Subject 43[b]	F	59	Lu	757057777777631		...
			L	777357777777671		

Data are for 54 tissue specimens from the lung (Lu), liver (L) kidney (K) and spleen (S) that positive for IS6110 by polymerase chain reaction. Forty-two different genotypes were detected, of which 34 were orphan types whose codes did not have a match in the SPOLDB4.0 database. No sample from subjects had the pattern of *Mycobacterium tuberculosis* H37Rv or *Mycobacterium bovis* BCG.

Abbreviation: SIT, shared international type.

[a] Had ≥3 genotypes.

[b] Had 2 genotypes.

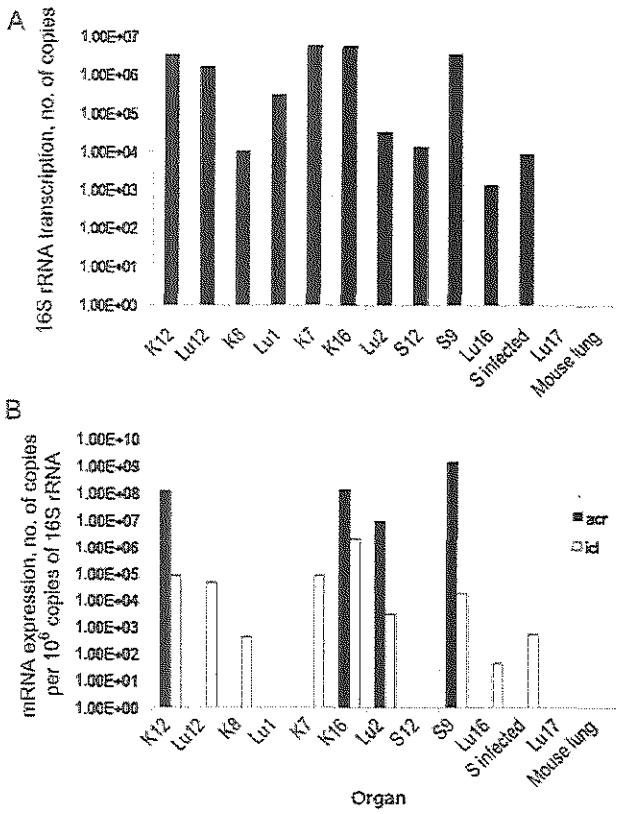

Figure 3. Mycobacterial gene transcription determined by quantitative real-time polymerase chain reaction (PCR). Ten tissue samples were selected from lungs (L), kidneys (K), and spleens (S) (for each, the number corresponds to the patient referred in Table 1) according their high positivity to IS6110, as determined by conventional PCR. *A*, Transcription of 16S ribosomal RNA (rRNA) as a viability marker. *B*, Expression of 2 related latency genes, *acr* (which encodes alpha-crystallin) and *icl* (which encodes isocitrate lyase). The messenger RNA (mRNA) copy numbers for *acr* and *icl* are given relative to 1 million copies of 16S RNA.

pulmonary and extrapulmonary sites in 84% of the studied cases and exclusively in extrapulmonary sites in 14%. The organ that was most commonly positive was the lung (72%), but the frequency of positive findings was similarly high for the spleen (70%), the kidney (68%), and the liver (66%). All of these organs have high blood throughput, which facilitates infection. Similarly the in situ PCR study showed mycobacterial DNA preferentially in Kupffer cells and red pulp macrophages. Thus, Mtb may be phagocytosed during hematogenous dissemination by cells from the mononuclear phagocytic system, as well as by nonprofessional phagocytic cells, such as endothelial cells, which also frequently showed *IS6110* positivity. Endothelial cells are easily infected by Mtb in vitro, and recent microarray studies showed an extensive shutdown of diverse bacterial genes related to metabolic pathways, which suggests bacillary dormancy, or at least greatly reduced activity [20].

Kidney samples commonly showed mycobacterial DNA. In situ PCR revealed positivity in epithelial cells, particularly Bowman's parietal cells. Renal glomeruli filter a high volume of blood, and the parietal Bowman's epithelium is exposed to the glomerular filtrate. This suggests that, despite their size, mycobacteria can pass through capillary walls and infect epithelial cells from glomeruli and proximal tubules. At present we have no insights into how this might occur or whether they are transported within cells. Interestingly, there were no histological abnormalities in any of these extrapulmonary tissues, as previously reported for the lung [5]. Since all these positive cells are heavily exposed to circulating blood, hypoxia might not be a significant factor in the induction of bacterial dormancy. Nitric oxide could be important in these cellular settings. Nitric oxide is produced by endothelial cells and macrophages, and treatment with nitric oxide–blocker drugs in animal models provokes mycobacterial reactivation [21].

Because extrapulmonary TB occurs commonly in lymph nodes, genitourinary tract, meninges, and pericardium, it will be important to extend these studies to material from these sites. We do not know whether reactivation in these other sites is local or due to spread from sites such as those we have studied.

The use of PCR-based genotyping methods such as spoligotyping to differentiate Mtb strains has revealed multiple strains within single patients in several geographical areas [22–24]. These studies have been performed in patients with active infection. Our spoligotyping study showed 42 different genotypes from 30 individuals, including 34 orphan strains. This variability suggests chronic sporadic infection. Interestingly, 50% of the studied individuals showed only 1 genotype while the other 50% showed 2 or 3 genotypes. Thus, mixed infection is a common condition in latency, and latent infection with 1 strain does not elicit protection from infection with another strain. Moreover, our genotyping studies showed that all the positive cases corresponded to Mtb infection. No samples were positive for *M. bovis*, which is important considering that BCG vaccination is a prevalent in Mexico.

Detection of mycobacterial DNA does not mean that the organisms are alive. Several studies have focused on understanding the quorum-sensing signals used by Mtb for resuscitation from the nonculturable state. During active growth, Mtb organisms secrete resuscitation-promoting factor (Rpf), which is required for growth of in vitro–induced vegetative cells and for resuscitation of dormant cells [25, 26]. Latent Mtb has extremely low metabolic activity and needs Rpf and special requirements to promote resuscitation. However, no strains could be recovered from autopsy specimens cultured with Rpf according the conditions described by Mukamolova et al (data not shown). Therefore, we used a recently reported technique for isolating mycobacterial RNA from infected tissues [26–28]. Ten samples were studied, and all showed expression of the constitutive mycobacterial gene encoding

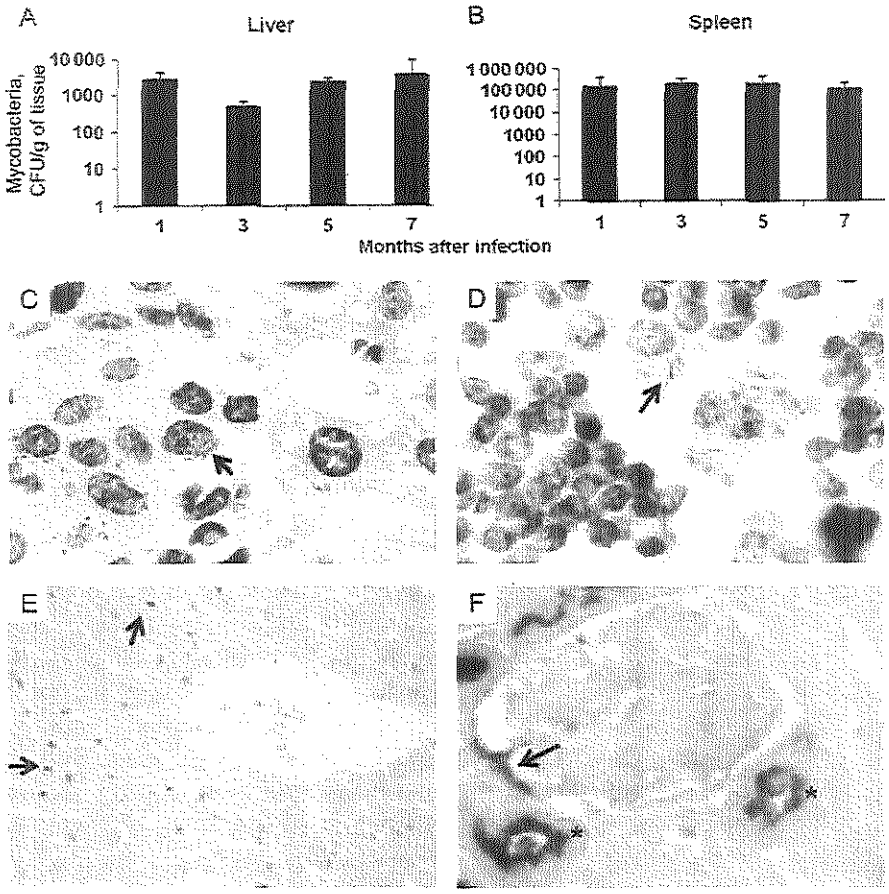

Figure 4. Determination of extrapulmonary burdens of bacilli, and representative micrographs of acid-fast staining and *IS-6110* mycobacterial transposon detection by in situ polymerase chain reaction in organs from mice infected with low doses of *Mycobacterium tuberculosis* to induce chronic infection similar to latent infection. Kinetics of bacilli burdens in tissue from liver (*A*) and spleen (*B*). Bars represent means and SDs of 5 mice per time point. CFU, colony-forming units. *C*, After 5 months of infection, liver sections shows acid-fast bacilli in the cytoplasm of a hepatocyte (*arrow*; original magnification, ×40 000). *D*, At the same time point, there are Ziehl-Neelsen stain–positive bacilli in splenic red pulp macrophages (*arrow*; original magnification, ×40 000). *E*, After 5 months of infection, low-power micrograph of a liver specimen shows numerous Kupffer cells and endothelial cells positive for the mycobacterial IS-6110 transposon (*arrows*). Note that there is no inflammation or other evident histological abnormality (original magnification, ×1000). *F*, Strong IS-6110 positivity in parietal Bowman cells (*arrow*) and proximal tubular epithelium (*asterisks*) in the kidney from mouse after 5 months of infection (original magnification, ×40 000).

ribosomal 16S, suggesting that organisms were viable in a nonculturable state [26, 29]. We extended this study by determining the expression of factors associated with dormancy, such as *acr* and *icl* [30, 31]. acr is a prominent stationary phase-induced protein produced by mycobacteria in humans during infection [1, 32]. It is upregulated in vitro after exposure of Mtb to hypoxia or nitric oxide precursors and during treatment [14, 33]. We detected *acr* expression in 4 samples, but the high copy numbers suggest that bacilli were stressed. Biochemical studies indicate that, in chronically infected lung tissue, fatty acids are a major carbon source for Mtb [34]. In Mtb strain H37Rv (but not in nonvirulent strains), *icl* activity has been reported to increase in proportion to the age of the culture. Fatty acids are available within the macrophage phagosome, and Mtb might use these as a carbon source [31]. Thus, *icl* promotes persistence by enhancing bacterial survival within inflammatory macrophages [35]. We detected some expression of *icl* in 8 of 10 tissue samples.

By use of a murine model of chronic infection similar to latent infection, we confirmed the data found in the human necropsy specimens. In contrast with latent infection in humans, in the murine model the bacilli were not in a truly dormant state because they grew in culture. However, hematogenous dissemination of bacilli and infection of nonprofessional phagocytic cells in superficially normal tissue was seen. Acid fast bacilli were detected in splenic macrophages and in hepatocytes, and the cellular location of mycobacterial DNA in murine tissues was the same as in human tissue.

Table 3. Guidance for Laboratory Management and Quality Control to Prevent Contamination of Specimens

Process	Guidance
Sample collection	Aseptic surgical instruments (new scalpel blades, sterile clothes) and aseptic surgical techniques were used to avoid microbiological contamination of samples and cross-contamination between samples.
Standardized phenol/chloroform DNA extraction [5]	DNA from each sample was extracted on different days, using new sterile material and molecular biology grade chemicals. To avoid cross-contamination, samples were manipulated under biosafety level II cabinets until the alcohol precipitation step, which was performed in a laboratory where mycobacterial DNA had never been isolated.
	DNA from tissues, bacteria, and the cell line used as negative controls was extracted under the same conditions.
IS*6110* amplification [6]	DNA from *M. tuberculosis* complex was detected by a highly specific PCR method based on direct amplification of insertion sequence IS*6110* (1 to >25 copies in genome).
	IS*6110*-PCR is a highly specific method for detection of *M. tuberculosis*, with the ability to detect 1 fg of H37Rv DNA (corresponding to 1 genome).
	The sensitivity of the method has been reported to be 100% for smear-positive clinical samples and at least 59% for smear-negative and extrapulmonary clinical samples. All of our negative controls (human DNA and DNA from other nonrelated organisms) were negative by *IS6110* PCR.
	M. bovis infection was discounted by PCR amplification of the *oxyR* gene, which is specific for *M. bovis* and attenuated BCG vaccine substrains [12].
IS*6110*-Hybridization	Sequence-specific hybridization of IS*6110* with a PCR-generated single-strand probe increases the threshold of detection and might reduce false-negative results (ie, weakly positive and negative samples) obtained by IS*6110* PCR.
	High-stringency conditions decrease the chance of nonspecific binding of the probe to human DNA; to bacterial DNA, such as that from *E. coli* and *H. influenzae*; or to the oligonucleotides used for IS*6110* PCR.
In situ IS*6110*-PCR	This has a lower sensitivity than conventional *IS6110* PCR, but the intracellular localization of the DNA signal as small dots in professional and nonprofessional phagocytes in positive samples with correct positive and negative controls directly disproves cross-contamination, which would yield a random distribution of the DNA signals.
Spoligotyping	This is a genotyping method used for simultaneous detection and typing of *M. tuberculosis* complex bacteria. The obtained SITs did not correspond to spoligotype patterns of the commonly used laboratory reference strain, *M. tuberculosis* H37Rv, or the common vaccine strain, *M. bovis* BCG. Human DNA did not show any band.

Abbreviations: E. coli, Escherichia coli; H. influenzae; Haemophilus influenzae; M. bovis, Mycobacterium bovis; M. tuberculosis, Mycobacterium tuberculosis; PCR, polymerase chain reaction; SIT, shared international type.

A major concern in this study is the possibility of contamination, especially as the conditions in the autopsy room were not ideal. We conducted tissue sample collection and the associated techniques with strict procedures to avoid contamination (Table 3). We emphasize in particular the following 2 facts. First, contamination from other activities in the laboratory would yield spoligotypes characteristic of BCG and of H37Rv, but these were not found. Second, in situ PCR showed DNA in professional and nonprofessional phagocytes, whereas contamination would give a random distribution. These and other points are highlighted in Table 3. Thus, we believe that the results are reliable.

In conclusion, we have confirmed the often forgotten classical studies by Opie and Aronson, who demonstrated in guinea pigs in 1927 that latent Mtb survives in histologically normal tissue, not in old granulomas [36]. Moreover, we have shown that the bacteria persist in extrapulmonary tissues almost as frequently as in lung and that they are located within cells with limited antigen-presentation abilities, such as epithelial cells. This may be of great advantage for the tubercle bacillus and can explain the lack of local histological reaction. The frequent presence of bacteria in endothelial cells in various organs suggests that hematogenous dissemination of free Mtb occurs during the infection process.

Another important finding is that there can be latent infection with >1 strain of Mtb in the same individual. Therefore latent infection with one strain does not elicit protection from infection with another strain. Moreover the bacterial strains in different individuals were of different genotypes, indicating that the ability to disseminate and establish latent infection in multiple organs is a fundamental strategy used by Mtb for survival and not an unusual property of a particular strain. Clearly, there is more to Mtb infection than phagocytosis by macrophages, survival in these cells, and granuloma formation.

Note

Financial support. This work was supported by the European Community (ICA4.CT-2002-10063), by the European Community's Sixth Framework Programme (contracts LSHP-CT-2006-037566 and LSHP-CT-2006-036871), by CONACyT Mexico (contract 84456), and by ICyTDF (contract PIFUTP08-101).

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Clinical & Experimental Immunology
The Journal of Translational Immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/j.1365-2249.2010.04313.x

Induction of β-defensins by L-isoleucine as novel immunotherapy in experimental murine tuberculosis

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Summary

Tuberculosis is a worldwide health problem, and multidrug-resistant (MDR) and extensively multidrug-resistant (XMDR) strains are rapidly emerging and threatening the control of this disease. These problems motivate the search for new treatment strategies. One potential strategy is immunotherapy using cationic anti-microbial peptides. The capacity of L-isoleucine to induce beta-defensin expression and its potential therapeutic efficiency were studied in a mouse model of progressive pulmonary tuberculosis. BALB/c mice were infected with *Mycobacterium tuberculosis* strain H37Rv or with a MDR clinical isolate by the intratracheal route. After 60 days of infection, when disease was in its progressive phase, mice were treated with 250 μg of intratracheal L-isoleucine every 48 h. Bacillary loads were determined by colony-forming units, protein and cytokine gene expression were determined by immunohistochemistry and reverse transcription–quantitative polymerase chain reaction (RT–qPCR), respectively, and tissue damage was quantified by automated morphometry. Administration of L-isoleucine induced a significant increase of beta-defensins 3 and 4 which was associated with decreased bacillary loads and tissue damage. This was seen in animals infected with the antibiotic-sensitive strain H37Rv and with the MDR clinical isolate. Thus, induction of beta-defensins might be a potential therapy that can aid in the control of this significant infectious disease.

Keywords: anti-microbial peptides, defensins, L-isoleucine, therapy, tuberculosis

Introduction

Tuberculosis (TB) is a worldwide health problem. Reports by the World Health Organization indicate that there are 8 million new cases and 1·6 million deaths yearly due to this disease [1,2]. Moreover, *Mycobacterium tuberculosis* (Mtb) is highly infectious. It has been reported that nearly one-third of the world's population is latently infected, but only about 10% of these infected individuals will develop active disease [2].

Although TB can be controlled and cured by chemotherapy, treatment requires at least four specific drugs and 6 months of therapy, which produce problems in compliance. The consequence of this is disease relapse, and more importantly the development of multidrug-resistant (MDR) and extensively multidrug-resistant (XMDR) strains. In the last few years these strains have increased in frequency and now afflict approximately 2 million people worldwide [3]. These problems have motivated the search for new treatment strategies. One such strategy is immunotherapy, which requires a better understanding of the immune response against Mtb. Innate immunity has been recognized as a significant participant in the control of mycobacterial growth [4]. In this regard, it is considered that lung epithelial cells and macrophages are the first cells that encounter Mtb during primary infection [4–6]. Interestingly, not only macrophages but also bronchial cells can participate in the elimination of bacilli because epithelial cells can produce molecules of innate immunity such as β-defensins and cathelicidins, which are small cationic anti-microbial peptides [4,7–12]. Defensins contribute directly to defence against pathogens by killing microbes and chemoattracting and activating inflammatory cells in the infection site [8,9,13–15]. Defensins are divided into three subfamilies: α, β and θ, that differ in the position of their disulphide bridges. There are four different human β-defensins (HBD

1–4) that are expressed largely in epithelial cells from different organs, and except for HBD-1, which is expressed constitutively, all β-defensins are induced [16–18]. The expression and up-regulation of these anti-microbial peptides can be induced by pathogen-associated molecular patterns such as lipopolysaccharides (LPS) or by some proinflammatory cytokines [tumour necrosis factor (TNF)-α, interleukin (IL)-1β, interferon (IFN)-γ] [19,20]. Interestingly, recent reports demonstrated that amino acids such as L-isoleucine or proteins such as albumin can induce the expression of these peptides [18,21], and a small amount of defensins can control infections efficiently in experimental animal models [22].

In a murine model of progressive pulmonary tuberculosis, we recently showed rapid and stable expression of murine beta defensins (mBD) 3 and 4 by the bronchial epithelium during the early phase of infection, when control of bacterial growth is efficient. Then, during the late progressive phase of the disease when uncontrolled bacillary proliferation occurs, a pronounced decrease of both mBD was detected. These observations provided circumstantial evidence that mBD-3 and mBD-4 provide significant control of bacterial growth during the early phase of experimental tuberculosis [12]. To confirm this, and to determine if mBD could be a novel form of immunotherapy, we sought to determine whether it is possible to reinduce expression of these anti-microbial peptides with L-isoleucine during the late progressive phase of experimental tuberculosis and, if so, whether induction of mBD leads to control of bacterial growth.

Materials and methods

Induction of β-defensins in type II pneumocytes by L-isoleucine *in vitro*

To determine if L-isoleucine is able to induce β-defensin production in lung epithelial cells *in vitro*, human type II alveolar pneumocytes [A549; American Type Culture Collection (ATCC) reference number CCL185] were first grown in 75 cm^2 culture flasks (Costar, Ontario, Canada) with antibiotic-free RPMI-1640 medium (GIBCO BRL, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS) (HyClone Laboratories, Logan, UT, USA) at 37°C with 5% CO_2. Then A549 cells were seeded into 24-well plates at a concentration of 10^5 cells per ml of culture medium with 1% of FCS and after 24 h they were stimulated with different concentrations of L-isoleucine (3, 7, 12, 25, 50 and 100 μg/ml) in the presence of 5% CO_2 at 37°C. After 1, 6, 12 and 18 h of incubation, A549 cells were collected and lysed in 350 μl RLT buffer (Qiagen, Valencia, CA, USA) for each 10^5 cells and kept at −70°C until use. Published results have shown that very high concentrations of the enantiomer D-isoleucine are necessary to induce β-defensin production [21]. Thus, we used selected concentrations of D-isoleucine as negative control (50 μg/ml per well).

Human defensin-2 (HBD-2) gene expression was determined by real-time polymerase chain reaction (PCR) following the method described previously [4], and protein production by immunohistochemistry.

For immunohistochemistry, A549 cells were grown to confluence (95%) on four-well chamber slides (Costar) with F-10 medium. Subsequent to L-isoleucine stimuli, as reported above, cells were fixed with formaldehyde 10% for 2 h and stored at 4°C in phosphate-buffered saline solution (PBS). Slides were blocked with 5% goat serum for 20 min, and incubated subsequently with 1 : 5000 dilution of HBD-2 antibody (Peptide International, Osaka, Japan) in 5% goat serum at 4°C for 18 h. Slides were then developed with biotinylated goat anti-rabbit immunoglobulin (Ig)G using Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). Then, slides were counterstained with haematoxylin and visualized with a light microscopy axiovert 200 m (Carl Zeiss, Gena, Germany).

Induction of β-defensins in murine lung by L-isoleucine

To test whether L-isoleucine induced β-defensin production *in vivo*, we dissolved it in physiological saline solution obtaining different concentrations from 25 μg/100 μl to 1 mg/100 μl. These preparations were then administered to male BALB/c mice by the intratracheal route. After 12, 18, 24 and 48 h animals were euthanized and their lungs removed immediately for analysis of defensin production. As control, we used the vehicle (saline solution) and selected concentrations of D-isoleucine (250 μg/100 μl). Due to the high number of samples, conventional reverse transcription (RT)–PCR was used to determine defensin expression. After the mice were euthanized, lungs were removed, hilar lymph nodes and thymus were eliminated and the tissue was frozen immediately by immersion in liquid nitrogen. Three lungs, right or left, from different mice were used to isolate mRNA from each group at each time-point, and the cDNA from the three mice was analysed separately. mRNA was isolated by use of Trizol (GIBCO BRL); cDNA was synthesized by use of Maloney murine leukemia virus reverse transcriptase (GIBCO BRL) and priming with oligo dT. The PCR products were electrophoresed on 6% polyacrylamide gels, and molecular-weight standards with known DNA mass concentrations (low DNA mass ladder; GIBCO BRL) were run. The PCR products were then analysed by use of an image-analysis densitometer linked to a computer program (ID image-analysis software; Kodak Digital Science, Sn Leandro, CA, USA). To determine, in nanograms, the quantity of PCR product the computer program compared the optical densities from the experimental samples with the molecular-marker bands, the DNA content of which was provided by the manufacturer. To correct for errors in the quantity of starting material, the densitometer reading of the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) PCR

product was used. Production of mBD was confirmed by immunohistochemistry following the procedure described below.

Experimental model of progressive pulmonary TB in BALB/c mice

The experimental model of progressive pulmonary TB has been described in detail elsewhere [12,23,24]. Briefly, the laboratory drug-sensitive Mtb strain H37Rv (ATCC no. 25618) and MDR strain (clinical isolate, resistant to all first-line antibiotics) were grown in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI, USA) supplemented with 0·2% (v/v) glycerol, 10% oleic albumin dextrose catalase (OADC) enrichment (Difco) and 0·02% (v/v) Tween-80 at 37°C. Mid log-phase cultures were used for all experiments. Mycobacteria were counted and stored at –80°C until use. Bacterial aliquots were thawed and pulse-sonicated to remove clumps [25].

Male BALB/c mice, 6–8 weeks of age, were anaesthetized in a gas chamber using 0·1 ml per mice of sevofluorane and infected through endotracheal instillation with $2{\cdot}5 \times 10^5$ live bacilli. Mice were maintained in the vertical position until spontaneous recovery. Infected mice were maintained in groups of five in cages fitted with micro-isolators. Animal work was performed in accordance with the national regulations on Animal Care and Experimentation (NOM 062-ZOO-1999).

Treatment of the infected mice with L-isoleucine

After 60 days of infection, survivor animals were allocated arbitrarily into four groups of 20 animals each. L-isoleucine treatment was started 60 days after infection, when advanced progressive disease is well established. The experiments conducted to determine *in vivo* the efficiency of mBD production by L-isolucine showed that 250 μg/100 μl was the most efficient concentration to induce mBD in non-infected mice. Thus, we used this dose for the therapeutic experiments, performing two separate experiments.

Six animals in each group were euthanized at 15, 30 and 60 days after starting treatment. The first group infected with the drug-sensitive H37Rv strain received L-isoleucine (250 μg/100 μl), dissolved in saline solution, every 48 h by intratracheal instillation. A second group (controls) was infected with H37Rv strain and received only the vehicle (saline solution) by the same route and timing. The third group was infected with the MDR strain, and received L-isoleucine by the same route and schedule. The fourth group was infected with the MDR strain but served as a control, receiving only the vehicle. The efficiency of the L-isoleucine treatment was determined by quantifying the lung bacillary loads by counting colony-forming units (CFU), extent of tissue damage by histopathology and automated morphometry and lung cytokine gene expression by real-time RT–PCR.

Determination of CFU in infected lungs

Right or left lungs from three mice in each time-point in two different experiments were used. Lungs were homogenized with a polytron (Kinematica, Lucerne, Switzerland) in sterile tubes containing 1 ml PBS, Tween-80 at 0·05%. Five dilutions of each homogenate were spread onto duplicate plates containing Bacto Middlebrook 7H10 agar (Difco) enriched with oleic acid, albumin, catalase and dextrose-enriched medium (Becton Dickinson, Sparks, MD, USA). The plates were incubated at 37°C with 5% CO_2. The number of colonies was counted 21 days after plating.

Preparation of lung tissue for histology/morphometry and mBD detection by immunohistochemistry

Right or left lungs from three different animals per time-point and group were perfused intratracheally with ethyl alcohol (J:T: Baker, Mexico City, Mèxico). Lungs were dehydrated and embedded in paraffin (Oxford Labware, St Louis, MO, USA), sectioned and stained with haematoxylin and eosin. The percentages of the lung surfaces affected by pneumonia were determined using an automated image analyser (Q Win Leica, Milton Keynes, Cambridge, UK).

For immunohistochemical detection of mBD-3, 5-μm-thick sections were mounted on silane-coated slides, deparaffinized, the endogenous peroxidase quenched with 0·03% H_2O_2 in absolute methanol and blocked with 2% human serum dissolved in PBS. Lung sections were incubated for 18 h with goat anti-mouse mBD-3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing, sections were incubated for 2 h with a donkey anti-goat IgG biotin-labelled antibody. Bound antibodies were detected with avidin–biotin peroxidase (Biocare Medical, Concord, CA, USA) and counterstained with haematoxylin.

Expression of anti-microbial peptides and cytokines determined by real-time RT–PCR

Three lungs, right or left, from two different experiments were used for isolating RNA and synthesis of cDNA as described previously [12]. Real-time PCR was performed using the 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) and Quantitect SYBR Green Mastermix kit (Qiagen, Hilden, Germany). Standard curves of quantified and diluted PCR product, as well as negative controls, were included in each PCR run. The primers used for the real-time PCR analysis were: mBD-3: 5′- ATC CAT TAC CTT CTG TTT GCA TTT C-3′ and 5′- TGT AGG TGG AGA CAG CAG C-3′; mBD-4: 5′-CAC ATT TCT CCT GGT GCT GCT-3′ and 5′-TGA TAA TTT GGG TAA AGG CTG CA-3′; IFN-γ: 5′-GGTGACATGAAAATCCTGCAG-3′ and 5′-CC TCAAACTTGGCAATACTCATGA-3′; TNF-α: 5′-TGTGG CTTCGACCTCTACCTC-3′, 5′-GCCGAGAAAGGCTGCT

TG-3′; and glyceraldehyde-3-phosphate dehydrogenase (G3PDH): 5′-GGC GCT CAC CAA AAC ATC A-3′ and 5′-CCG GAA TGC CAT TCC TGT TA-3′. The specificity of each product was confirmed by PCR in agarose gels. The cell cycling conditions used were initial denaturation at 95°C for 15 min, followed by 40 cycles each at 95°C for 20 s, 60°C or 58°C, respectively, for 20 s, and 72°C for 34 s. Quantities of the specific mRNA in the sample were measured in accordance with the corresponding gene specific standard. The mRNA copy number of each cytokine was related to 1 million copies of mRNA encoding the G3PDH gene.

Statistical analysis

Data from the qPCR assays as well as CFUs and histopathology were analysed as follows. Kolmogorov–Smirnov normality tests were performed for each data set to choose the appropriate group comparison test. For each treatment the dependent variable was the expression, CFUs or variable of interest compared among different time-periods, treatment or not with L-isoleucine and the interaction of both in an interaction model. Results that were significant overall with a regular two-way analysis of variance (ANOVA) (not repeated-measures) were submitted to pairwise comparisons by Bonferroni's post-test. Two-sided P-values of $< 0{\cdot}05$ were considered statistically significant. Statistical analyses were performed using the GraphPad Prism version 5·02 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Induction of β-defensin by L-isoleucine in the lung epithelial cell line A549 *in vitro*

It has been demonstrated that L-isoleucine induces the production of β-defensins efficiently in bovine kidney epithelial cells *in vitro*, while its enantiomer D-isoleucine required much higher concentrations to achieve this [21]. In order to evaluate if there is a similar effect on human pulmonary epithelial cells, we determined the expression of HBD-2 (of which the homologue in mouse is mBD-3) by real-time RT–PCR using the lung epithelial cell line A549. Our results showed that the expression of this anti-microbial peptide increased with the concentration and time of exposure to L-isoleucine. The highest gene expression was seen after exposure to 25 μg/ml of L-isoleucine for 18 h (Fig. 1a). Immunohistochemistry confirmed the high production of this anti-bacterial peptide induced by L-isoleucine at this time-point (Fig. 1b). In order to confirm the specificity of mBD production by L-isoleucine, A549 cells were incubated during 48 h with 50 μg/ml of D-isoleucine. Figure 1c shows that in these conditions D-isoleucine did not induce the expression of HBD-2.

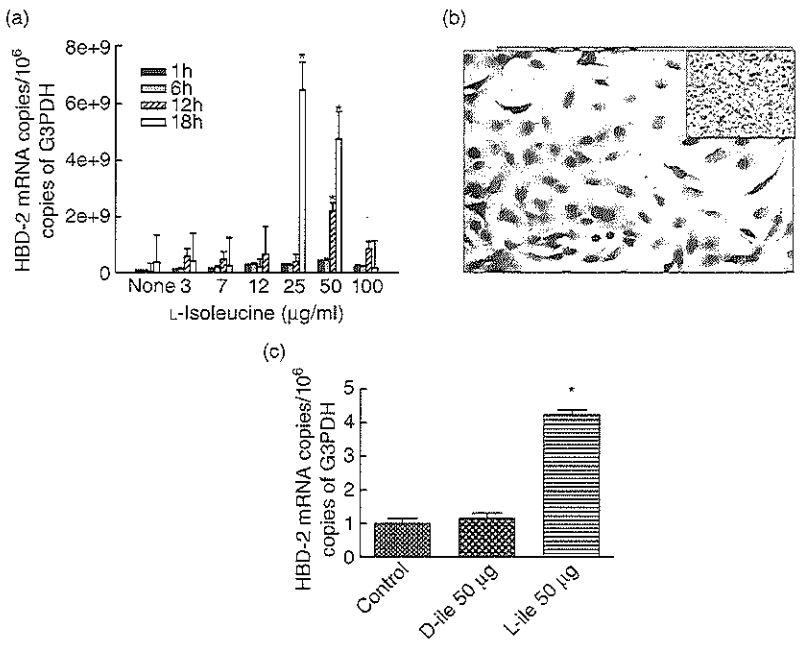

Fig. 1. Kinetics of human β-defensin 2 gene expression in the lung epithelial cell line A549 after stimulation with different concentrations of L-isoleucine. (a) A549 cells were stimulated with L-isoleucine using the indicated concentrations. At the indicated time-points the cells were collected, the RNA was isolated and the number of mRNA copies of β-defensin was quantified by real-time reverse transcription–polymerase chain reaction (RT–PCR). All values are mean ± standard deviation of five different experiments. Asterisks represent statistical significance when compared with the control non-stimulated cells. (b) Immunocytochemistry confirmed protein production in the condition in which the cells showed the highest β-defensin expression (25 μg/ml for 18 h), while non-stimulated cells do not show immunostaining (inset). (c) In comparison with L-isoleucine, A549 cells stimulated with 50 μg of D-isoleucine during 48 h did not induce human β-defensin 2 gene expression. Asterisks represent statistical significance when compared with the control non-stimulated cells.

Induction *in vivo* of β-defensins by L-isoleucine in the lung of non-infected BALB/c mice

After the confirming *in vitro* that L-isoleucine induced β-defensins efficiently in pulmonary epithelial cells, we tested if the intratracheal instillation of this amino acid had a similar effect in the lung of non-infected mice. We tested multiple concentrations of L-isoleucine at several time-points. Both mBD-3 and mBD-4 were induced efficiently using this treatment (Fig. 2). The highest expression of mBD3 was seen after 12 h of stimulation with 250 μg/100 μl of L-isoleucine, while for mBD4 the peak was at 48 h with the same concentration. In contrast with L-isoleucine, either saline solution or 250 μg/100 μl of D-isoleucine instilled in control animals induced significantly lower expression of both mBD (Fig. 2).

Effect of intratracheal L-isoleucine administration during late progressive tuberculosis produced by the drug-sensitive strain H37Rv

Because 250 μg/100 μl of L-isoleucine administered by intratracheal instillation induced β-defensin production efficiently during 24 to 48 h, this concentration was administered intratracheally every 48 h for 2 months. This treatment was started after 60 days post-infection, when advanced active TB was well established and production of β-defensins decreased. In comparison with control mice, animals treated with L-isoleucine showed significantly higher expression of β-defensins 3 and 4 and lower lung bacillary loads during the whole treatment (Fig. 3). Consistent with these findings, after 4 weeks of treatment, histological examination revealed that the lung areas affected by pneumonia were smaller than in control mice, and the bronchial epithelium in the lungs of treated mice showed strong mBD-3 immunostaining (Figs 3 and 5).

Considering that β-defensins are chemotactic and can activate T helper type 1 (Th1) cells and macrophages [26,27], we determined the expression of IFN-γ and TNF-α in these animals. In comparison with control mice, there was a significant increment of IFN-γ and TNF-α expression in the L-isoleucine-treated group (Fig. 3).

Effect of intratracheal L-isoleucine administration during late progressive tuberculosis produced by multidrug-resistant strain

Due to the emergence of MDR strains, and given the improved course in L-isoleucine-treated mice infected with the drug-sensitive H37Rv strain, we decided to study

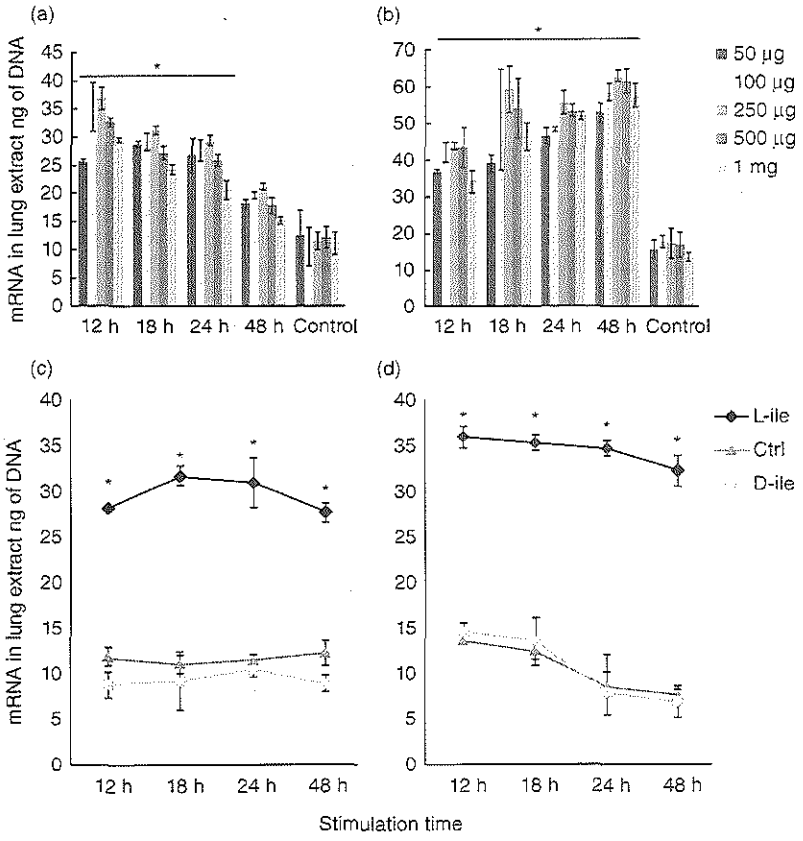

Fig. 2. Effect on the β-defensin gene expression in non-infected mice after the administration of L-isoleucine. Groups of BALB/c mice received the indicated amount of L-isoleucine by the intratracheal route, and were euthanized at different time-points and their lungs were used to isolate total mRNA to determine the expression of murine β-defensin 3 (a) and 4 (b). In comparison with L-isoleucine, administration of 250 μg of D-isoleucine or the vehicle saline solution (control group) did not induce gene expression of β-defensin 3 (c) or β-defensin 4 (d). All values are mean ± standard deviation of five mice from three independent experiments. Asterisks represent statistical significance when compared with the control group that received only the vehicle ($P < 0.005$).

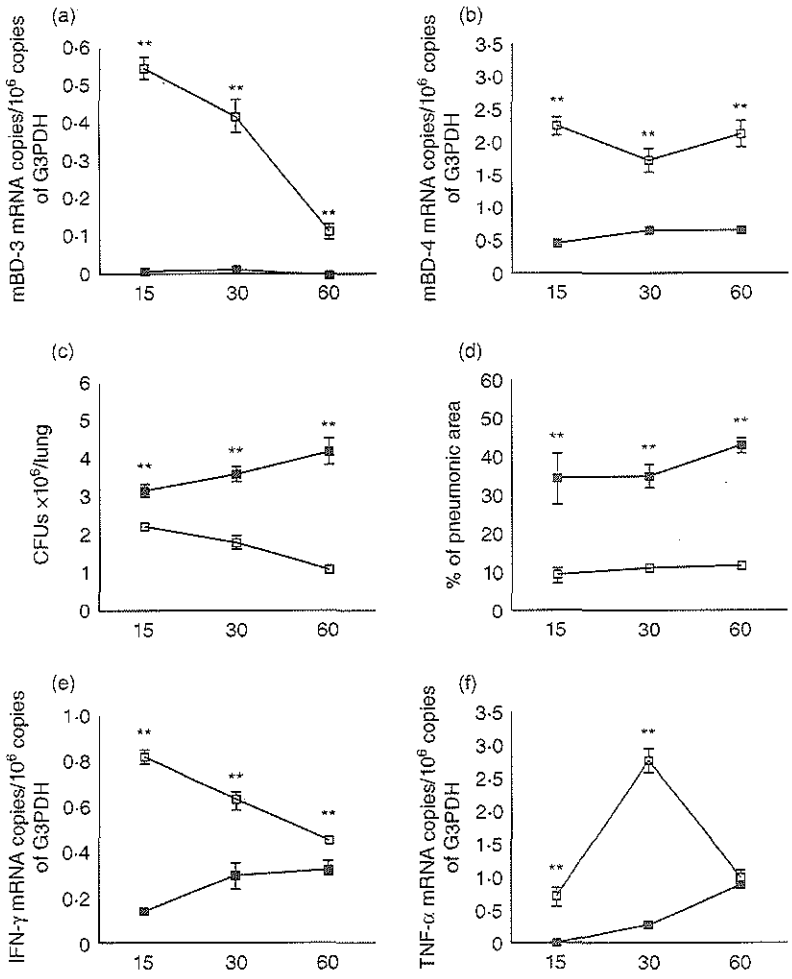

Fig. 3. Effect of L-isoleucine administration during advanced disease in the lungs of mice infected with drug-sensitive H37Rv strain. (a) L-isoleucine administration (white symbols) starting 60 days after infection increased mBD3 and mBD4 (b) gene expression when compared with control mice (black symbols). (c) L-isoleucine treatment also decreased pulmonary bacterial loads and (d) the area of pneumonia. (e) In comparison with the control group, L-isoleucine treatment increased the expression of interferon-γ and tumour necrosis factor-α (f). Each point corresponds to the mean and standard deviation of five mice group in one representative experiment. Asterisks represent statistical significance when compared to the control non-treated group (*$P < 0.05$; **$P < 0.01$).

whether this therapy has the ability to produce similar beneficial effects on mice infected with a clinical isolate resistant to all first-line antibiotics during late active disease. In comparison with control animals, MDR-infected mice treated with L-isoleucine showed a significant increase of mBD-3 and 4 and lower lung bacillary loads ($P < 0.01$), compatible with the participation of β-defensins in the control of bacillary growth and supporting the beneficial effect of this therapy (Fig. 4). Similarly, improved lung histopathology was seen, with a significant decrease of pneumonia (Figs 4 and 5) at 60 days of treatment ($P < 0.01$). Determination of cytokine gene expression by real-time PCR showed higher IFN-γ and TNF-α expression in the lungs of L-isoleucine-treated animals (Fig. 4).

Discussion

Defensins are anti-microbial peptides that are considered as a prototype family of mediators of innate immunity. They have efficient anti-microbial activity on a broad spectrum of organisms, including Mtb [26,27]. Besides direct bactericidal activity, anti-microbial peptides also have immunoregulatory functions, such as chemotaxis [28], immature dendritic cell activation [29] and activation of other immune cells [30,31]. Mtb induces production of HBD-2 in lung epithelial cells and it seems that these peptides could contribute to bacteria killing [6]. In murine models, expression of mBD-3 and mBD-4 correlates with control of mycobacterial growth in progressive pulmonary tuberculosis [12] and latent infection [10]. Our results confirm and extend these observations by demonstrating that stimulating β-defensin production during late active disease significantly increases control of bacillary growth.

Both mBD-3 and mBD-4 are inducible mainly through pathogen-associated molecular patterns (PAMPs) such as lipoarabinomannan [4] or lipopolysaccharide (LPS) [32] and proinflamatory cytokines [33,34]. However, none of these molecules can be used as therapeutic inducers of defensins because they can produce collateral toxic effects. Conversely, administration of recombinant defensins is not practical due to its high cost and the short half-life of the peptides. Thus, the use of defensin inducers is an attractive

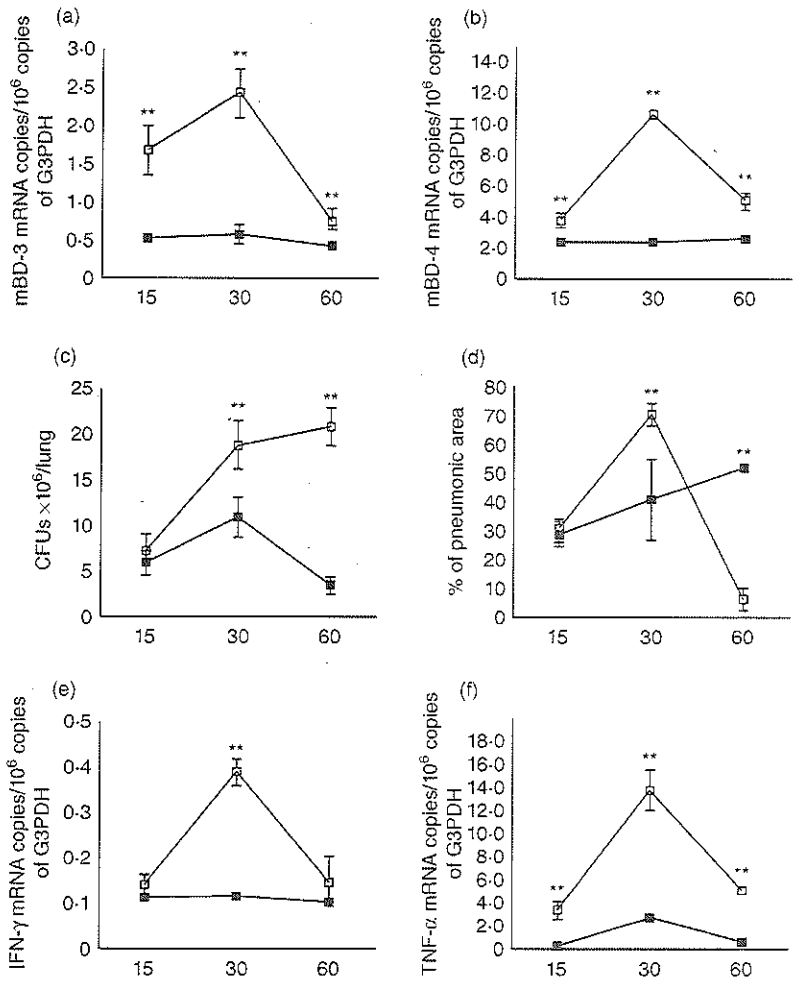

Fig. 4. Effect of L-isoleucine administration during advanced disease in the lungs of mice infected with a multidrug-resistant strain. (a) L-isoleucine administration (white symbols) starting 60 days after infection increased mBD3 and (b) mBD4 gene expression when compared with control mice (black symbols). (c) L-isoleucine treatment also decreased pulmonary bacilli burdens, and after 60 days of treatment the lung surface affected by pneumonia (d) when compared with the control group. L-isoleucine treatment increased the expression of interferon-γ (e) and tumour necrosis factor-α (f). Each point corresponds to the mean and standard deviation of five mice group in one representative experiment. Asterisks represent statistical significance when compared to the control non-treated group ($^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$).

and low-cost immunotherapeutical alternative. Interestingly, Fehlbaum and coworkers showed that the essential amino acid L-isoleucine induces β-defensin production efficiently in Madin–Darby bovine kidney epithelial cells through a chiral receptor or enzyme and nuclear factor (NF)-κB/rel species activation, after reaction with a recognition site in an isoleucine-inducible defensin promoter [21]. Here we demonstrate that lung epithelial cells (type II pneumocytes) stimulated with L-isoleucine also produce high amounts of β-defensin. Furthermore, L-isoleucine caused no cytotoxicity even at concentrations as high as 1 mg/ml (data not shown). Similar results were obtained *in vivo* after intratracheal instillation of this essential amino acid in non-infected mice. In contrast, in the tested concentration of 50 μg/ml or 250 μg/100 μl, D-isoleucine did not induce β-defensin production, respectively, in *in-vitro* and *in-vivo* experiments, supporting the specificity of L-isoleucine. Thus, L-isoleucine can be considered as a novel immunostimulant with very limited toxic activity and low cost, which are important attributes considering that TB is a devastating disease in poor countries.

When BALB/c mice are infected by the intratracheal route with a high dose of the drug-sensitive H37Rv strain, there is an early high production of β-defensins and Th1 cytokines which, together with high levels of TNF-α, temporarily controls the infection. After 4 weeks of infection, there is a decrease in the levels of β-defensins, IFN-γ and TNF-α. Gradually, pneumonic areas prevail over granulomas. Extensive pneumonia plus a high burden of bacteria cause death [24]. We started the treatment with L-isoleucine after 8 weeks of infection, when active disease was in course and the expression of β-defensins was very low. This treatment efficiently restored high expression of mBD-3 and -4, and there was a simultaneous decrease in lung bacillary loads and tissue damage. This is compatible with the view that the re-establishment of β-defensin production permitted significant control of bacillary growth. This could be attributable to its direct anti-microbial activity [35], or to blockage of bacterial DNA replication [36], or to its well-known property of immune cell activation [36–38], including production of Th1 cytokines [29], which we confirmed by the high IFN-γ expression observed in L-isoleucine-treated animals.

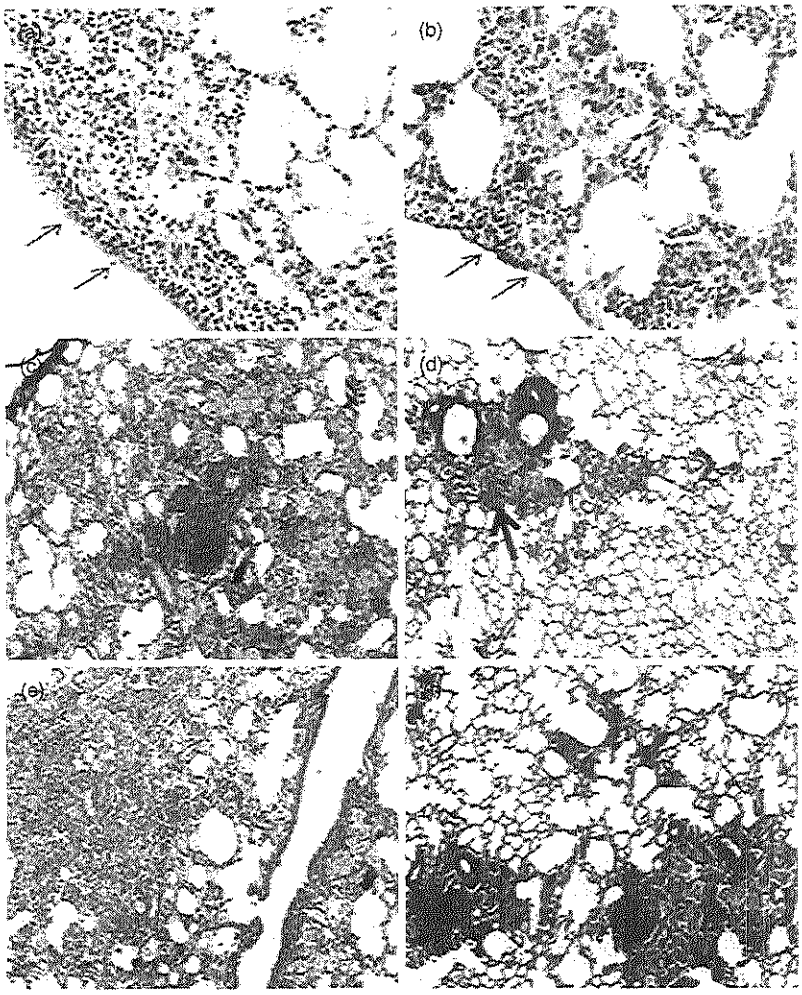

Fig. 5. Representative lung histopathology and immunohistochemistry after 2 months of treatment with L-isoleucine and in a control non-treated mouse. (a) Very low expression of mBD3 in the bronchial epithelium (arrows) in the lung of control mouse after 4 months of infection with H37Rv strain. (b) In contrast, there is strong mBD3 immunostaining in the bronchial epithelium (arrows) and in some macrophages in the lung of the mouse infected with strain H37Rv and treated with L-isoleucine. (c) The control animal shows extensive pneumonia after 4 months of infection with drug-sensitive strain H37Rv. (d) In comparison, the L-isoleucine treated mouse shows less lung surface area affected by pneumonia (arrow). (e) Representative micrograph showing extensive pneumonia after 4 months of infection with the multidrug-resistant clinical isolate, while the L-isoleucine-treated mouse shows less lung consolidation (f).

Moreover, alveolar macrophages can take external defensins and use them as anti-microbial effectors to eliminate intracellular mycobacteria [37,38]. Thus, several β-defensin-dependent mechanisms could explain the observed control of murine tuberculosis produced by treatment with L-isoleucine. However, at this stage we cannot assess formally the possible roles of other mediators or biological effects that might be caused by L-isoleucine.

Another important problem in the control of TB is the emergence of MDR strains. Approximately 400 000 new cases of MDR Mtb emerge worldwide each year, and this form of TB has been identified as a significant problem in every region under World Health Organization surveillance. Treatment of MDR strains is resource-intensive and usually requires a combination of second-line drugs that are more expensive, more toxic and less effective than drugs used in standard therapy. Our results showed that intratracheal administration of L-isoleucine in mice infected with MDR bacilli during the advanced phase of infection reduced significantly lung bacillary loads and tissue damage. Similarly to H37Rv strain-infected mice, 1 month after treatment the expression of IFN-γ and TNF-α was higher than in control mice. Thus, L-isoleucine administration was also able to stimulate the production of protective cytokines during MDR progressive disease, reducing disease severity as occurred with the drug-sensitive H37Rv strain infection.

In conclusion, our results show that repeated intrapulmonary administration of L-isoleucine induced β-defensin production *in vivo*, and that this correlated with improved protective immunity and higher resistance to mycobacterial infection when administered during late progressive disease induced by drug-sensitive or drug-resistant virulent mycobacteria. Although this treatment was not completely curative, these results suggest that continuous administration of L-isoleucine by the respiratory route is a potential therapy that might aid the control of this significant infectious disease. Moreover, there are efficient devices for deep administration of aerosols to human lungs that might reach the infected areas more reliably than the simple intratracheal injection used here.

Acknowledgements

C.R.-S. was supported by the National University of Mexico (UNAM) and a scholarship of the National Council for Science and Technology (CONACyT) Mexico. This study was supported by CONACyT (contract: 84456).

Disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in, or financial conflict with, the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Journal of Infectious Diseases Advance Access published August 21, 2012

MAJOR ARTICLE

Extrapulmonary Locations of *Mycobacterium tuberculosis* DNA During Latent Infection

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Background. One-third of the world's population has latent infection with *Mycobacterium tuberculosis*, and 10%–15% of cases of reactivation occur at extrapulmonary sites without active pulmonary tuberculosis.

Methods. To establish the frequency and location of mycobacterial DNA, organ specimens from 49 individuals who died from causes other than tuberculosis were studied by means of polymerase chain reaction (PCR), PCR plus DNA hybridization, in situ PCR, real-time PCR, and spoligotyping.

Results. Lung specimens from most subjects (36) were positive for *M. tuberculosis*, as were specimens from the spleen (from 35 subjects), kidney (from 34), and liver (from 33). By in situ PCR, mycobacterial DNA was found in endothelium, pneumocytes, and macrophages from the lung and in Bowman's parietal cells and convoluted proximal tubules from the kidney. In spleen, macrophages and sinusoidal endothelial cells were positive, whereas in liver, Kupffer cells and sinusoidal endothelium were commonly positive. Spoligotyping of 54 pulmonary and extrapulmonary positive tissues from 30 subjects showed 43 different genotypes, including 36 orphan types. To confirm the viability of mycobacteria, 10 positive tissue samples were selected for isolation of mycobacterial RNA. All samples showed 16S ribosomal RNA expression, while 8 and 4 samples showed expression of the latent infection genes encoding isocitrate lyase and α-crystallin, respectively.

Conclusions. *M. tuberculosis* persists in several sites and cell types that might constitute reservoirs that can reactivate infection, producing extrapulmonary tuberculosis without lung involvement.

Mycobacterium tuberculosis (Mtb) can produce progressive disease or latent infection [1]. In areas of high endemicity, infection first occurs in childhood and in most cases is controlled. Only 10% of these primary infections lead to progressive disease [1, 2]. However, some bacilli remain in tissues in a nonreplicating dormant or slowly replicating stage for the rest of the individual's life. This latent form of tuberculosis (TB) is clinically asymptomatic. In countries with low or moderate endemicity, most active TB cases arise as a result of reactivation of latent bacilli [1, 2]. It is estimated that one-third of the world's population carries latent Mtb, and millions of cases of reactivated TB are predicted in the coming years [3, 4].

One important point is the location of the bacilli during latent infection [1]. It has been assumed that latent bacilli are located in old fibrotic pulmonary granulomas, where the low nutrient supply and hypoxic microenvironment induce the low-level metabolism characteristic of latent bacilli [1]. However, we have detected mycobacterial DNA by conventional and in situ polymerase chain reaction (PCR) analysis in histologically normal lung tissue specimens from humans and mice during latent infection [5, 6]. This suggests that latent mycobacteria can reside not only in macrophages from old granulomas, but also in nonprofessional phagocytic cells from superficially normal tissue. However, recent results have shown mycobacterial DNA in apparently histologically normal adipose tissue from nontuberculous persons living in countries where TB is endemic (eg, Mexico) or nonendemic

Received 10 August 2011; accepted 18 January 2012.

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The Journal of Infectious Diseases

DOI: 10.1093/infdis/jis381

(eg, France), as well as in adipose tissue from mice with latent infection [7]. This is important because approximately 15% of cases of reactivation occur at extrapulmonary sites (ie, liver, spine, kidney, spleen, and brain) without active pulmonary TB [8, 9]. These findings suggest extrapulmonary reactivation rather than reactivation from pulmonary sites and subsequent migration of bacilli to other sites. The aims of this study were to determine whether Mtb DNA and RNA are present in extrapulmonary tissue specimens (from the liver, kidney, and spleen) from individuals who had no histopathologic findings specific for TB and died from causes other than TB and, if Mtb DNA and RNA are present, to identify their cellular locations. We confirmed our findings for humans in a murine model of chronic infection that is similar to latent infection.

METHODS

Human Samples

Forty-nine subjects who died from causes other than TB were included. The necropsy and tissue samples were collected at the Department of Pathology of the General Hospital of Mexico. Sex, age, and causes of death are presented in Table 1. Subjects with human immunodeficiency virus infection or immunosuppressive treatment were excluded. Tissue samples were obtained during legally authorized autopsies with signed permission by a relative, who agreed to the donation of additional samples for the present study. The Ethics Committee of the General Hospital of México approved the study (code DI/11/310/03/083).

Between 4 and 6 hours after death, tissue samples were obtained from the lung, liver, spleen, and kidney. Samples were taken from random sites of all organs except lungs, in which samples were obtained from the base and apex. Samples were taken using sterile technique (ie, new scalpel blades, sterile clothes, and a flame) and divided into 2 fragments. One fragment was frozen in liquid nitrogen for DNA and RNA extraction and culture of Mtb in Middlebrook 7H9 broth (Difco Becton Dickinson, Sparks, MD), while the other fragment was fixed in 10% formaldehyde for histological analysis, acid-fast staining, and mycobacterial DNA detection by in situ PCR.

Detection of Mycobacterial DNA by Conventional and In Situ PCR

A fragment of each frozen sample was homogenized in a mini-bead beater (Biospec Products, Bartlesville, OK). DNA was extracted by the phenol-chloroform-isoamyl alcohol method [10]. Strict procedures and controls were followed to avoid cross-contamination between samples during DNA extraction. To detect mycobacterial DNA, conventional PCR for the specific insertion sequence *IS6110* was carried out as previously described [11]. Considering that >90% of newborn infants in Mexico receive BCG vaccine, it was important to confirm that mycobacterial nucleic acids in positive samples were not derived from *Mycobacterium bovis*. We used conventional PCR to amplify the *oxyR* gene, highly specific for *M. bovis*, using primers JB21 (5′-TCGTCCGCTGATGCAAGTGC-3′) and JB22 (5′-CGTCCGCTGACCTCAAGAAG-3′) [12]. We used DNA from Mtb H37Rv and *M. bovis* BCG Danish 1331 as positive controls and DNA from *Homo sapiens* and *Escherichia coli* as negative controls. In negative samples, PCRs were carried out twice to confirm the result.

To increase the sensitivity of *IS6110* detection, DNA hybridization was applied to all negative or weakly positive samples. In brief, Hybond N+ membrane was sensitized with denatured *IS6110*–PCR product from each necropsy, incubated for 2 hours with blocking reagent (Roche Applied Science, Indianapolis, IN), and incubated overnight with the *IS6110*-Biot probe. The membrane was washed with 2 × SSPE/0.5% sodium dodecyl sulfate and incubated with streptavidin-HRPO (Invitrogen, Camarillo, CA). Probe detection was performed with Immobilon Western Chemiluminescent HRP substrate (Millipore, Billerica, MA). The *IS6110*-Biot probe was made using the INS-1 primer 5′-CCTGCGAGCGTAGGCG TCGG-3′ labeled with biotin at the 5′ end, using as controls the DNA from *H. sapiens*, *E. coli*, and *Haemophilus influenzae*.

For in situ PCR detection of mycobacterial DNA, 5-μm sections from each paraffin block were examined, following the protocol previously described [5–7]. Lung sections from 1 TB case were used as positive control, and lung sections from a noninfected mouse were used as a negative control.

Spoligotyping

To determine whether the detected mycobacterial DNA was related to a particular genotype, as well as whether >1 genotype was in each necropsy specimen, spoligotyping was carried out for every positive sample, according to the manufacturer's recommendations (Ocimum Biosolution, Hyderabad, India). In all experiments, DNA from Mtb H37Rv and *M. bovis* BCG was used as a positive control. The reaction mixture was prepared with AmpliTaq Gold fast PCR Master Mix, (Applied Biosystems, Carlsbad, CA), using DRa 5′-GGTTTTGGGTCTGAC GAC-3′ biotinylated at the 5′ end and DRb 5′-CCGAGAGGGG ACGGAAAC-3′ primers and 1 μg of total DNA as a template. Spoligotypes in octal code were compared with the SPOLDB4.0 database of the Pasteur Institute of Guadeloupe (available at: http://www.pasteur-guadeloupe.fr/tb/bd_myco.html).

Quantification by Real-Time PCR of Mycobacterial Gene Expression

To define bacterial viability in tissue samples in which we detected mycobacterial DNA, we used a previously described technique with some modification to determine mycobacterial gene expression by real-time PCR after isolation of putative mycobacterial RNA [13, 14]. Ten samples from different

Table 1. Detection of Mycobacterial DNA in Pulmonary and Extrapulmonary Tissues by Conventional Polymerase Chain Reaction (PCR) Plus DNA Hybridization

Subject	Sex	Age, Years	Cause of Death	PCR Hybridization Finding			
				Lung	Spleen	Kidney	Liver
1	M	61	Emphysema	+	+	−	−
2	F	62	Systemic arterial hypertension	+	−	−	+
3	M	18	Kidney failure	+	+	−	+
4	F	52	Sepsis	+	−	+	+
5	F	27	Systemic erythematous lupus	+	+	+	+
6	M	73	Kidney failure	−	+	+	+
7	F	57	Systemic arterial hypertension	−	−	+	+
8	M	55	Lymphoblastic acute leukemia	−	+	+	−
9	M	40	Diabetes	+	+	+	+
10	F	24	Chronic kidney failure	+	+	+	+
11	F	84	Systemic arterial hypertension	+	+	+	+
12	M	22	Myeloblastic acute leukemia	+	+	+	+
13	M	93	Systemic arterial hypertension	+	+	+	+
14	M	33	Fusiform cell sarcoma	+	+	+	+
15	M	61	Acute hemorrhagic pancreatitis	−	+	+	−
16	M	60	Multiple myeloma	+	−	+	+
17	F	28	Ovarian cancer	−	−	−	+
18	F	76	Cancer	+	+	+	+
19	F	16	Sepsis	+	+	+	+
20	M	35	Cardiac malformation	+	+	+	−
21	F	20	Systemic arterial hypertension	+	+	−	+
22	F	58	Systemic arterial hypertension	−	−	−	+
23	M	42	Chronic kidney failure	+	−	−	−
24	F	70	Hypovolemic shock	+	+	+	−
25	F	45	Lung cancer	+	+	+	+
26	F	69	Malignant glioma	+	+	+	+
27	F	18	Sepsis	+	+	+	−
28	M	35	Pneumonia	+	+	+	−
29	M	64	Myeloblastic acute leukemia	+	+	−	+
30	M	42	Kidney cancer	+	+	+	+
31	F	57	Emphysema	+	−	+	+
32	M	89	Hypovolemic shock	+	+	+	−
33	M	29	Brain hemorrhage	+	+	+	+
34	F	29	Diabetes mellitus	+	+	+	+
35	M	80	Brain hemorrhage	+	+	+	+
36	F	29	Breast cancer	+	+	+	−
37	F	83	Chronic kidney failure	+	+	+	+
38	F	22	Brain hemorrhage	+	+	+	+
39	M	36	Breast cancer	+	+	+	+
40	F	70	Heart failure	−	+	+	+
41	F	50	Myocardial infarction	+	+	−	+
42	M	76	Chronic kidney failure	+	+	+	+
43	F	59	Sepsis	+	+	+	+
44	F	14	Brain hemorrhage	−	−	−	−
45	F	40	Systemic arterial hypertension	−	−	−	−
46	F	84	Emphysema	−	−	−	−
47	M	52	Pneumonia	−	−	−	−
48	M	45	Disseminated intravascular coagulation	−	−	−	−
49	M	37	Pneumonia	−	−	−	−

Abbreviations: −, negative; +, positive.

subjects that showed the strongest PCR positivity for *IS6110* were selected. The tissue was homogenized using a Multi-Gen mm generator and centrifuged; the supernatant contained eukaryotic RNA, while the pelleted material contained the bacilli. To isolate the bacterial RNA, the pellets suspended in TRIzol (Gibco BRL, Camarillo, CA) were disrupted using a Mini-Bead Beater-8 (Biospec Products, Bartlesville, OK) and zirconia and silica beads. After vortexing and centrifugation, the supernatant contained Mtb RNA. Bacterial RNA was isolated using 4 cycles of purification with an RNeasy column (Qiagen, Valencia, CA), followed by DNase incubation. We used mycobacterial 16S ribosomal RNA (rRNA) for reference constitutive gene expression and as a viability marker because this gene is expressed in all growth conditions [13]. Primers for 16S rRNA, isocitrate lyase (*icl*), and α-crystallin (*acr*) mycobacterial genes were designed with Primer Express software, version 2.0 (Applied Biosystems, Carlsbad, CA). The nucleotide sequences of the forward and reverse primers were as follows: for 16S rRNA, 5′-TCCCGGGCCTTGTACACA-3′ (forward) and 5′-CCACTGGCTTCGGGTGTAA-3′ (reverse); for *icl*, 5′-ACACCTACCCCGACCAGAG-3′ (forward) and 5′-TG CAGCTCGTAGACGTTGAG-3′ (reverse); and for *acr*, 5′-CGAGAAGGACGTCGACATTA-3′ (forward) and 5′-CC TTGTCGTAGGTGGCCTTA-3′ (reverse). The quality and quantity of RNA were evaluated by spectrophotometry and electrophoresis. Complementary DNA (cDNA) synthesis was performed using 5 μg of total RNA, 2 μM of random primers (Promega, Madison, WI), 10 U/μL of ribonuclease inhibitor

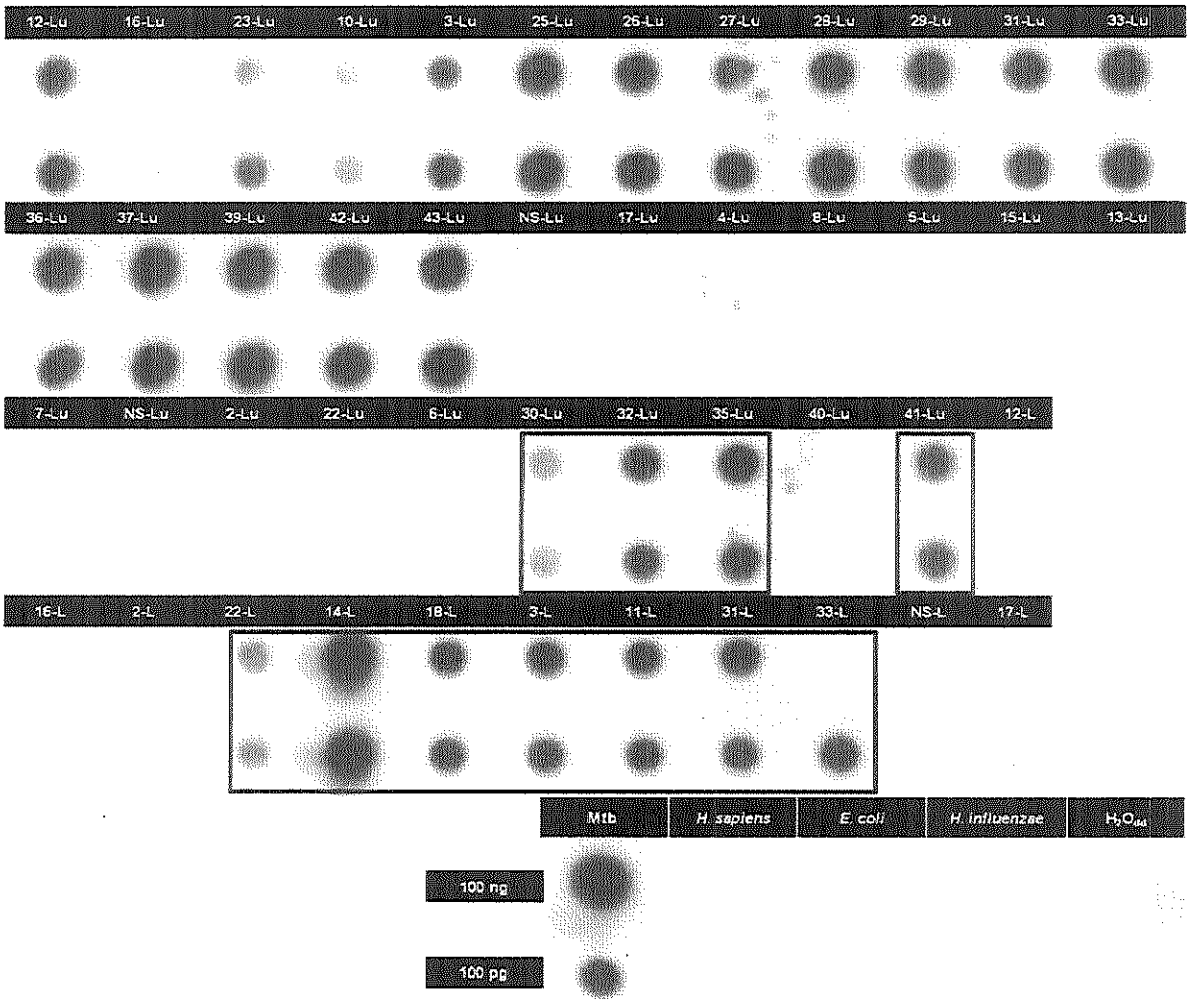


Figure 1. Detection of mycobacterial DNA in lung and liver samples by conventional polymerase chain reaction (PCR) plus DNA hybridization. To increase the sensitivity of *IS6110* detection, DNA hybridization was applied to all negative (*box*) or weakly positive samples by conventional and in situ PCR. Hybridization was performed in duplicate, using the PCR product; the number corresponds to the patient referred in Table 1. *Bottom*, A total of 100 ng and 100 pg of *Mycobacterium tuberculosis* (Mtb) DNA (positive control) and 100 ng of negative controls DNA (from *Homo sapiens* [*H. sapiens*], *Escherichia coli* [*E. coli*], and *Haemophilus influenzae* [*H. influenzae*] and water). Abbreviations: L, liver sample; Lu, lung sample.

(Invitrogen, Camarillo, CA), 1 μL of buffer RT, 0.5 mM of dNTP, and 4 units of Omniscript reverse transcriptase (Qiagen, Valencia, CA). A preliminary conventional PCR using 16S rRNA primers was carried out with an aliquot of cDNA. Real-time quantitative PCR was performed with the QuantiTect SYBR Green PCR Master Mix (Qiagen, Valencia, CA). To obtain a standard curve, 4 different PCRs were performed in parallel, using as a template 10-fold dilutions of known amounts of the Mtb H37Rv 16S rRNA gene (10^8–10^2 copies) together with the experimental sample. Reactions were performed in a 7500 Prism spectrofluorometric thermal cycler (Applied Biosystems, Carlsbad, CA). The reaction mixtures consisted of 0.5 μM of target and control primers, 12.5 μL of Master Mix, and 1 μg of cDNA. Cycling conditions were as follows: initial denaturation for 15 minutes at 95°C, followed by 40 cycles at 95°C for 20 seconds, at 59°C for 20 seconds, and at 72°C for 34 seconds. An independent real-time PCR assay was carried out in triplicate for each tissue sample in 2 separate experiments. The messenger RNA copy number of mycobacterial genes was related to 1 million copies of RNA encoding the 16S rRNA gene. PCR fragments amplified from *acr* and 16S rRNA genes from Mtb were identified by means of a genetic analyzer, ABI PRISM 310 PE (Applied Biosystems, Carlsbad, CA). RNA from spleen tissue of infected mice was used as a positive control, and RNA from lung tissue from noninfected mice and lung tissue obtained during necropsy of newborn mice were used as negative controls.

Murine Model of Latent Infection

To evaluate the presence of extrapulmonary Mtb in apparently histologically normal tissues, we used a murine model of chronic infection that is similar to latent infection [6]. Hybrid F1 C57BL/DBA mice were infected intratracheally with 1×10 live Mtb H37Rv. Groups of 5 mice were sacrificed at days 30, 90, 150, and 210 after infection. The kidney, spleen, and liver were collected, and fragments were prepared as described above

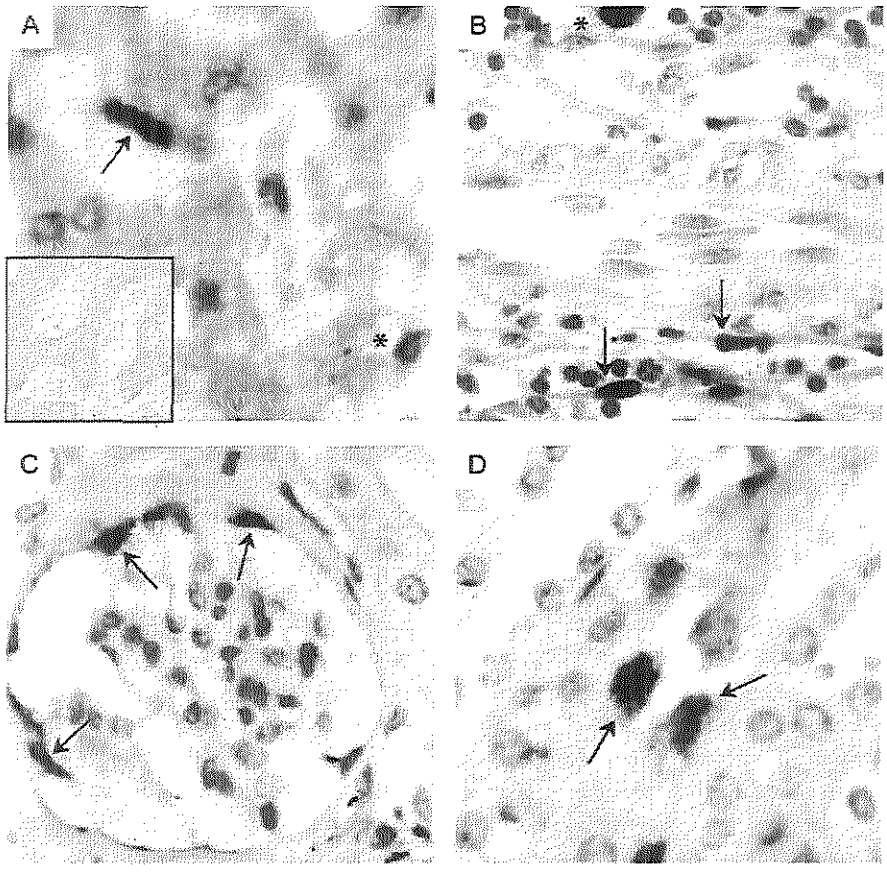


Figure 2. Representative micrographs of the localization of mycobacterial DNA detected by in situ polymerase chain reaction. *A*, Kupffer cell from hepatic sinusoids shows strong positivity (*arrow*), as well as occasional hepatocytes (*asterisk*). *Inset*, liver section from normal noninfected mouse as a negative control. *B*, In spleen, endothelial cells from sinusoids (*arrows*) and red pulp macrophages (*asterisk*) are positive. *C*, In the kidney, strong positivity is seen in the parietal cells from the glomerular capsule and in epithelial cells from the proximal convoluted tubules (*arrows*; *D*). For all micrographs, original magnification ×400.

for histological analysis and *IS6110* detection. Serial sections were stained with Ziehl-Neelsen. Another tissue fragment was used for quantification of colony-forming units (CFUs) [6].

RESULTS

Mycobacterial DNA in Pulmonary and Extrapulmonary Tissues

A total of 43 necropsy specimens (from 19 of 23 males and 24 of 26 females) were positive for mycobacterial DNA (Table 1). We considered necropsy specimens as positive when *IS6110* was detected in at least 1 tissue by conventional PCR, PCR plus DNA hybridization, or in situ PCR. As expected, the lung was the organ that most commonly yielded specimens positive for *IS6110* (in 36 cases), but spleen specimens (from 35), kidney specimens (from 34), and liver specimens (from 33) were also positive (Table 1). In 35 subjects (70%), we found mycobacterial DNA in lung and extrapulmonary tissues, but in 7 (14%), detection was exclusively in extrapulmonary tissues. When the result was difficult to interpret, we performed PCR plus DNA hybridization (Figure 1). Thus, 34 of 141 samples (3 lung specimens, 13 liver specimens, 16 spleen specimens, and 2 kidney specimens) that were negative by conventional PCR yielded positive results after hybridization. No samples were positive for *M. bovis*.

By use of in situ PCR, we detected mycobacterial DNA in endothelial cells, type II pneumocytes, and alveolar macrophages (data not shown), which corroborates previous findings for lung tissue [5]. In kidney, positivity was localized in the parietal cells of Bowman's capsule and, on occasion, in epithelial cells from convoluted proximal tubules (Figure 2). In spleen, *IS6110* positivity was found in red pulp macrophages and sinusoidal endothelial cells, while in liver, positivity was located in Kupffer cells and sinusoidal endothelium. On occasion, hepatocytes and portal biliary duct epithelial cells were also positive (Figure 2). None of these positive tissues showed any apparent histological abnormality, such as granulomas, inflammatory infiltrates, or fibrosis.

Spoligotyping

In 54 positive tissue specimens from pulmonary and extrapulmonary sites of 30 subjects, we found 42 different genotypes, but only 8 genotypes had a match in the SPOLDB4.0 database. The most frequently detected genotype (in 7 samples from 6 subjects) corresponded to shared international type (SIT) 53. This profile has been reported 2380 times in the SITVIT database (54 of which were reported from Mexico). Other profiles found were SIT 523 (in 3 samples), SIT 291 (in 2), and SITs 245, 521, 1166, 1196, and 1690 (in 1 each). With the exception of SIT 53, none of the profiles found was previously reported in Mexico. We found 34 orphan types from different organs. They had spoligotyping codes, but the codes did not have matches in the database (Table 2). In 15 of 30 subjects, we found 1 genotype; 9 of 30 subjects had 2 genotypes, and 6 of 30 carried ≥3 genotypes.

Detection of Mycobacterial Gene Transcription by Real-Time PCR

To confirm the viability of mycobacteria, we selected 10 tissue samples (4 from lungs, 4 from kidneys, and 2 from spleens) with the highest positivity to *IS6110* detection by PCR. Spleen taken from chronically infected mice was the positive control. We selected the mycobacterial 16S rRNA as a viability marker, and for latency we selected α-crystallin (encoded by *acr*), since this protein is expressed during stressful conditions [14], and isocitrate lyase (encoded by *icl*), because Mtb uses this enzyme in the metabolism of fatty acids during chronic infection [13].

In the 10 selected samples, we detected 16S rRNA (Figure 3*A*), while *icl* was detected in 8 samples (4 from kidneys, 3 from lungs, and 1 from spleen) at levels varying from 1×10^1 to 1×10^6 copies per 10^6 copies of 16S RNA (Figure 3). In 4 samples that were positive for *icl*, we also detected *acr* (in 2 kidney specimens, 1 lung specimen, and 1 spleen specimen). The expression of *acr* was higher in all cases (range, 1×10^7–1×10^9 copies).

Murine Model

We used a mouse model that mimics latency, with a low, stable lung bacillary load without weight loss, spontaneous reactivation, or death [6]. Low and stable numbers of CFUs were detected only in liver and spleen (Figure 4). After histological analysis, acid-fast staining showed bacilli in tissue sections from liver and spleen, specifically in macrophages, hepatocytes, and endothelial cells from apparently normal tissue (Figure 4). In situ PCR showed positivity in the same cells as in the human tissues, but the labeling was stronger, and the cellular distribution was much wider (Figure 4).

DISCUSSION

Previous work has demonstrated the presence of mycobacterial DNA in macroscopically and histologically normal lung and adipose tissue from people who died from causes other than TB, suggesting latent infection [5, 7]. Here, we confirm and extend these observations by demonstrating the presence of mycobacterial DNA in pulmonary and multiple extrapulmonary tissues from people who died from causes other than TB and had no history of this disease. This agrees with many reports in which Mtb DNA was detected in extrapulmonary samples from patients in a wide range of geographical locations who were putatively without illness [15–19].

We found a higher percentage of pulmonary and extrapulmonary specimens positive for mycobacterial DNA than in our previous study [5]. This difference can be explained by the use of DNA hybridization in addition to PCR, which greatly increased sensitivity. We detected mycobacterial DNA in both

Table 2. Spoligotyping Results

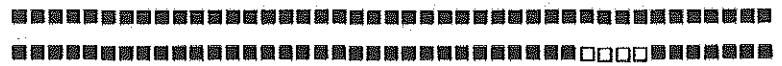
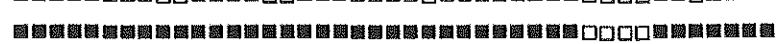
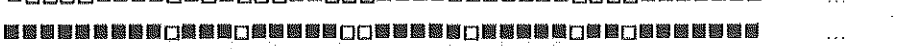
Specimen Source	Sex	Age, Years	Organ	Octal	Spoligotype	SIT
M. tuberculosis H37Rv	...	...	...	777777477760771		451
M. bovis from BCG vaccine	...	...	...	676773777777600		482
Subject 2	F	62	L	777777677760771		291
Subject 3[a]	M	18	Lu	777777777760671		245
			S	777777777760771		53
			L	777777777760611		521
Subject 5	F	27	S	777346377560771		...
Subject 6	M	73	L	777777777761771		1196
Subject 7	F	57	K	777777777760771		53
Subject 9	M	40	Lu	777777777421771		...
Subject 10[b]	F	24	S	757377477760771		...
			K	777777777777771		523
Subject 11[b]	F	84	S	777777777760771		53
			K	777777777760771		53
			L	777377777760771		1166
Subject 12[b]	M	22	Lu	777001314521770		...
			L	337046377760771		...
Subject 13[b]	M	93	S	776363767760671		...
			L	777777777760771		53
Subject 14	M	33	S	777777377160771		...
Subject 16	M	60	Lu	777777776420730		...
Subject 18[b]	F	76	S	777777777100771		...
			K	777777677760771		291
Subject 19[a]	F	16	Lu	777777777760771		53
			K	777777776561771		...
			L	777365574760771		...
Subject 21	F	20	S	777377376700771		...
Subject 22	F	58	Lu	777777777760771		53
Subject 28	M	35	K	774357777777761		...
Subject 29	M	64	S	777377377765661		...

Table 2 continued.

Specimen Source	Sex	Age, Years	Organ	Octal	Spoligotype	SIT
Subject 30	M	42	S	777277777777620		...
Subject 31[a]	F	57	S	777777577774771		...
			K	777357477760771		...
			L	417243077760771		...
Subject 32[a]	M	89	Lu	777357476766771		...
			K	777777677761771		...
			L	777357777777771		...
Subject 33[a]	M	29	Lu	777357476760771		...
			S	777777777762771		1690
			K	737377477760771		...
Subject 34[b]	F	29	S	777357477760771		...
			L	777777476773771		...
Subject 35[b]	M	80	S	777377477760771		...
			L	777377677770771		...
Subject 36[a]	F	29	Lu	777357676761771		...
			S	777357477760771		...
			K	777757477760771		...
			L	777777777777771		523
Subject 37	F	83	S	777377677763771		...
Subject 38[b]	F	22	Lu	777355476360671		...
			L	777377477723771		...
Subject 39	M	36	S	773257676763771		...
Subject 40	F	70	L	777777777777771		523
Subject 43[b]	F	59	Lu	757057777777631		...
			L	777357777777671		

Data are for 54 tissue specimens from the lung (Lu), liver (L) kidney (K) and spleen (S) that positive for IS6110 by polymerase chain reaction. Forty-two different genotypes were detected, of which 34 were orphan types whose codes did not have a match in the SPOLDB4.0 database. No sample from subjects had the pattern of *Mycobacterium tuberculosis* H37Rv or *Mycobacterium bovis* BCG.

Abbreviation: SIT, shared international type.

[a] Had ≥3 genotypes.

[b] Had 2 genotypes.

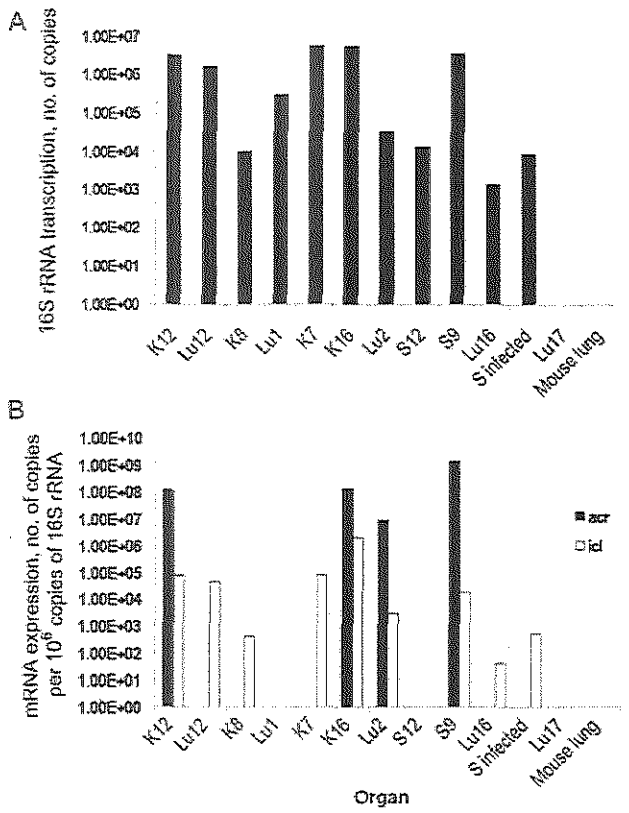


Figure 3. Mycobacterial gene transcription determined by quantitative real-time polymerase chain reaction (PCR). Ten tissue samples were selected from lungs (L), kidneys (K), and spleens (S) (for each, the number corresponds to the patient referred in Table 1) according their high positivity to IS6110, as determined by conventional PCR. *A*, Transcription of 16S ribosomal RNA (rRNA) as a viability marker. *B*, Expression of 2 related latency genes, *acr* (which encodes alpha-crystallin) and *icl* (which encodes isocitrate lyase). The messenger RNA (mRNA) copy numbers for *acr* and *icl* are given relative to 1 million copies of 16S RNA.

pulmonary and extrapulmonary sites in 84% of the studied cases and exclusively in extrapulmonary sites in 14%. The organ that was most commonly positive was the lung (72%), but the frequency of positive findings was similarly high for the spleen (70%), the kidney (68%), and the liver (66%). All of these organs have high blood throughput, which facilitates infection. Similarly the in situ PCR study showed mycobacterial DNA preferentially in Kupffer cells and red pulp macrophages. Thus, Mtb may be phagocytosed during hematogenous dissemination by cells from the mononuclear phagocytic system, as well as by nonprofessional phagocytic cells, such as endothelial cells, which also frequently showed *IS6110* positivity. Endothelial cells are easily infected by Mtb in vitro, and recent microarray studies showed an extensive shutdown of diverse bacterial genes related to metabolic pathways, which suggests bacillary dormancy, or at least greatly reduced activity [20].

Kidney samples commonly showed mycobacterial DNA. In situ PCR revealed positivity in epithelial cells, particularly Bowman's parietal cells. Renal glomeruli filter a high volume of blood, and the parietal Bowman's epithelium is exposed to the glomerular filtrate. This suggests that, despite their size, mycobacteria can pass through capillary walls and infect epithelial cells from glomeruli and proximal tubules. At present we have no insights into how this might occur or whether they are transported within cells. Interestingly, there were no histological abnormalities in any of these extrapulmonary tissues, as previously reported for the lung [5]. Since all these positive cells are heavily exposed to circulating blood, hypoxia might not be a significant factor in the induction of bacterial dormancy. Nitric oxide could be important in these cellular settings. Nitric oxide is produced by endothelial cells and macrophages, and treatment with nitric oxide–blocker drugs in animal models provokes mycobacterial reactivation [21].

Because extrapulmonary TB occurs commonly in lymph nodes, genitourinary tract, meninges, and pericardium, it will be important to extend these studies to material from these sites. We do not know whether reactivation in these other sites is local or due to spread from sites such as those we have studied.

The use of PCR-based genotyping methods such as spoligotyping to differentiate Mtb strains has revealed multiple strains within single patients in several geographical areas [22–24]. These studies have been performed in patients with active infection. Our spoligotyping study showed 42 different genotypes from 30 individuals, including 34 orphan strains. This variability suggests chronic sporadic infection. Interestingly, 50% of the studied individuals showed only 1 genotype, while the other 50% showed 2 or 3 genotypes. Thus, mixed infection is a common condition in latency, and latent infection with 1 strain does not elicit protection from infection with another strain. Moreover, our genotyping studies showed that all the positive cases corresponded to Mtb infection. No samples were positive for *M. bovis*, which is important considering that BCG vaccination is a prevalent in Mexico.

Detection of mycobacterial DNA does not mean that the organisms are alive. Several studies have focused on understanding the quorum-sensing signals used by Mtb for resuscitation from the nonculturable state. During active growth, Mtb organisms secrete resuscitation-promoting factor (Rpf), which is required for growth of in vitro–induced vegetative cells and for resuscitation of dormant cells [25, 26]. Latent Mtb has extremely low metabolic activity and needs Rpf and special requirements to promote resuscitation. However, no strains could be recovered from autopsy specimens cultured with Rpf according the conditions described by Mukamolova et al (data not shown). Therefore, we used a recently reported technique for isolating mycobacterial RNA from infected tissues [26–28]. Ten samples were studied, and all showed expression of the constitutive mycobacterial gene encoding

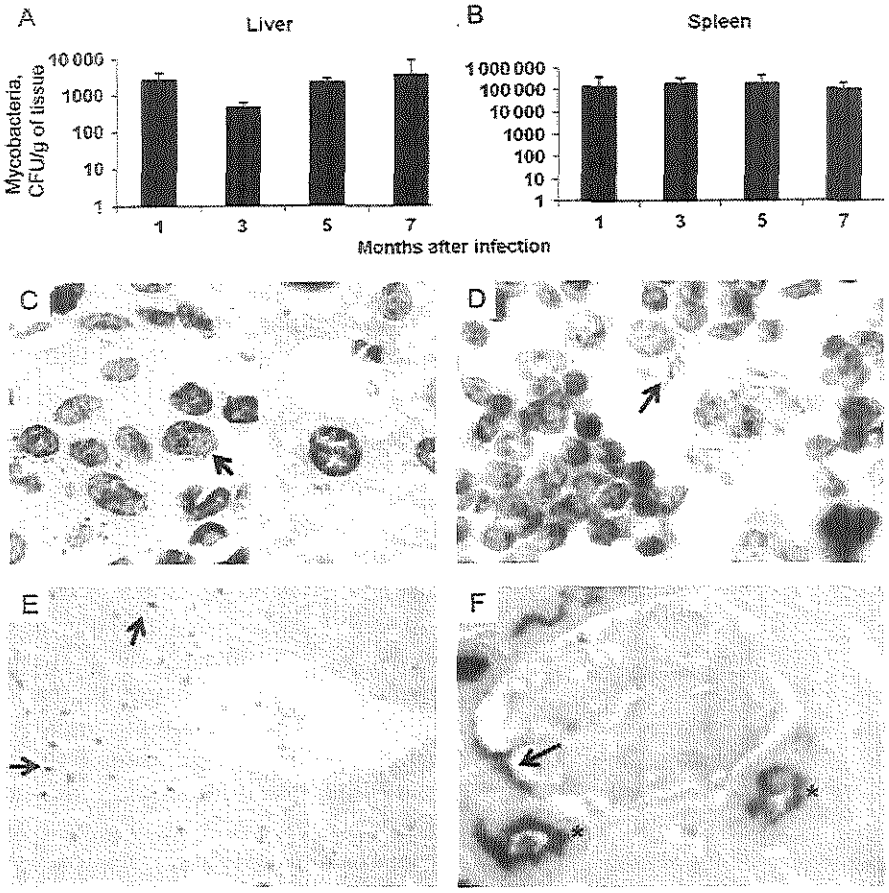


Figure 4. Determination of extrapulmonary burdens of bacilli, and representative micrographs of acid-fast staining and *IS-6110* mycobacterial transposon detection by in situ polymerase chain reaction in organs from mice infected with low doses of *Mycobacterium tuberculosis* to induce chronic infection similar to latent infection. Kinetics of bacilli burdens in tissue from liver (*A*) and spleen (*B*). Bars represent means and SDs of 5 mice per time point. CFU, colony-forming units. *C*, After 5 months of infection, liver sections shows acid-fast bacilli in the cytoplasm of a hepatocyte (*arrow*; original magnification, ×40 000). *D*, At the same time point, there are Ziehl-Neelsen stain–positive bacilli in splenic red pulp macrophages (*arrow*; original magnification, ×40 000). *E*, After 5 months of infection, low-power micrograph of a liver specimen shows numerous Kupffer cells and endothelial cells positive for the mycobacterial IS-6110 transposon (*arrows*). Note that there is no inflammation or other evident histological abnormality (original magnification, ×1000). *F*, Strong IS-6110 positivity in parietal Bowman cells (*arrow*) and proximal tubular epithelium (*asterisks*) in the kidney from mouse after 5 months of infection (original magnification, ×40 000).

ribosomal 16S, suggesting that organisms were viable in a nonculturable state [26, 29]. We extended this study by determining the expression of factors associated with dormancy, such as *acr* and *icl* [30, 31]. acr is a prominent stationary phase-induced protein produced by mycobacteria in humans during infection [1, 32]. It is upregulated in vitro after exposure of Mtb to hypoxia or nitric oxide precursors and during treatment [14, 33]. We detected *acr* expression in 4 samples, but the high copy numbers suggest that bacilli were stressed. Biochemical studies indicate that, in chronically infected lung tissue, fatty acids are a major carbon source for Mtb [34]. In Mtb strain H37Rv (but not in nonvirulent strains), *icl* activity has been reported to increase in proportion to the age of the culture. Fatty acids are available within the macrophage phagosome, and Mtb might use these as a carbon source [31]. Thus, *icl* promotes persistence by enhancing bacterial survival within inflammatory macrophages [35]. We detected some expression of *icl* in 8 of 10 tissue samples.

By use of a murine model of chronic infection similar to latent infection, we confirmed the data found in the human necropsy specimens. In contrast with latent infection in humans, in the murine model the bacilli were not in a truly dormant state because they grew in culture. However, hematogenous dissemination of bacilli and infection of nonprofessional phagocytic cells in superficially normal tissue was seen. Acid fast bacilli were detected in splenic macrophages and in hepatocytes, and the cellular location of mycobacterial DNA in murine tissues was the same as in human tissue.

Table 3. Guidance for Laboratory Management and Quality Control to Prevent Contamination of Specimens

Process	Guidance
Sample collection	Aseptic surgical instruments (new scalpel blades, sterile clothes) and aseptic surgical techniques were used to avoid microbiological contamination of samples and cross-contamination between samples.
Standardized phenol/chloroform DNA extraction [5]	DNA from each sample was extracted on different days, using new sterile material and molecular biology grade chemicals. To avoid cross-contamination, samples were manipulated under biosafety level II cabinets until the alcohol precipitation step, which was performed in a laboratory where mycobacterial DNA had never been isolated.
	DNA from tissues, bacteria, and the cell line used as negative controls was extracted under the same conditions.
IS*6110* amplification [6]	DNA from *M. tuberculosis* complex was detected by a highly specific PCR method based on direct amplification of insertion sequence IS*6110* (1 to >25 copies in genome).
	IS*6110*-PCR is a highly specific method for detection of *M. tuberculosis*, with the ability to detect 1 fg of H37Rv DNA (corresponding to 1 genome).
	The sensitivity of the method has been reported to be 100% for smear-positive clinical samples and at least 59% for smear-negative and extrapulmonary clinical samples. All of our negative controls (human DNA and DNA from other nonrelated organisms) were negative by *IS6110* PCR.
	M. bovis infection was discounted by PCR amplification of the *oxyR* gene, which is specific for *M. bovis* and attenuated BCG vaccine substrains [12].
IS*6110*-Hybridization	Sequence-specific hybridization of IS*6110* with a PCR-generated single-strand probe increases the threshold of detection and might reduce false-negative results (ie, weakly positive and negative samples) obtained by IS*6110* PCR.
	High-stringency conditions decrease the chance of nonspecific binding of the probe to human DNA; to bacterial DNA, such as that from *E. coli* and *H. influenzae*; or to the oligonucleotides used for IS*6110* PCR.
In situ IS*6110*-PCR	This has a lower sensitivity than conventional *IS6110* PCR, but the intracellular localization of the DNA signal as small dots in professional and nonprofessional phagocytes in positive samples with correct positive and negative controls directly disproves cross-contamination, which would yield a random distribution of the DNA signals.
Spoligotyping	This is a genotyping method used for simultaneous detection and typing of *M. tuberculosis* complex bacteria. The obtained SITs did not correspond to spoligotype patterns of the commonly used laboratory reference strain, *M. tuberculosis* H37Rv, or the common vaccine strain, *M. bovis* BCG. Human DNA did not show any band.

Abbreviations: E. coli, Escherichia coli; H. influenzae; Haemophilus influenzae; M. bovis, Mycobacterium bovis; M. tuberculosis, Mycobacterium tuberculosis; PCR, polymerase chain reaction; SIT, shared international type.

A major concern in this study is the possibility of contamination, especially as the conditions in the autopsy room were not ideal. We conducted tissue sample collection and the associated techniques with strict procedures to avoid contamination (Table 3). We emphasize in particular the following 2 facts. First, contamination from other activities in the laboratory would yield spoligotypes characteristic of BCG and of H37Rv, but these were not found. Second, in situ PCR showed DNA in professional and nonprofessional phagocytes, whereas contamination would give a random distribution. These and other points are highlighted in Table 3. Thus, we believe that the results are reliable.

In conclusion, we have confirmed the often forgotten classical studies by Opie and Aronson, who demonstrated in guinea pigs in 1927 that latent Mtb survives in histologically normal tissue, not in old granulomas [36]. Moreover, we have shown that the bacteria persist in extrapulmonary tissues almost as frequently as in lung and that they are located within cells with limited antigen-presentation abilities, such as epithelial cells. This may be of great advantage for the tubercle bacillus and can explain the lack of local histological reaction. The frequent presence of bacteria in endothelial cells in various organs suggests that hematogenous dissemination of free Mtb occurs during the infection process.

Another important finding is that there can be latent infection with >1 strain of Mtb in the same individual. Therefore latent infection with one strain does not elicit protection from infection with another strain. Moreover the bacterial strains in different individuals were of different genotypes, indicating that the ability to disseminate and establish latent infection in multiple organs is a fundamental strategy used by Mtb for survival and not an unusual property of a particular strain. Clearly, there is more to Mtb infection than phagocytosis by macrophages, survival in these cells, and granuloma formation.

Note

Financial support. This work was supported by the European Community (ICA4.CT-2002-10063), by the European Community's Sixth Framework Programme (contracts LSHP-CT-2006-037566 and LSHP-CT-2006-036871), by CONACyT Mexico (contract 84456), and by ICyTDF (contract PIFUTP08-101).

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Reg. CONACYT 84456

Reporte final del proyecto: Diseño y prueba de nuevos regímenes inmunoterapéuticos para tratar a la tuberculosis

Introducción

El objetivo del presente proyecto fue diseñar y probar nuevos regímenes inmunoterapéuticos que tuvieran un efecto significativo en el tratamiento de *M. tuberculosis* multidrogoresistente (MDR) y en acortar la antibioticoterapia convencional para un mejor control de la tuberculosis drogo-sensible; bajo la hipótesis de que la estimulación de la respuesta Th-1 y de la activación macrofágica, así como la inhibición de la respuesta Th-2 y de otros factores inmunosupresores tendría un efecto terapéutico significativo en la tuberculosis producida por bacterias drogo sensibles y MDR.

De acuerdo al proyecto original se propusieron los siguientes regímenes que fueron probadas en un modelo experimental de tuberculosis pulmonar progresiva en ratones BALB/c

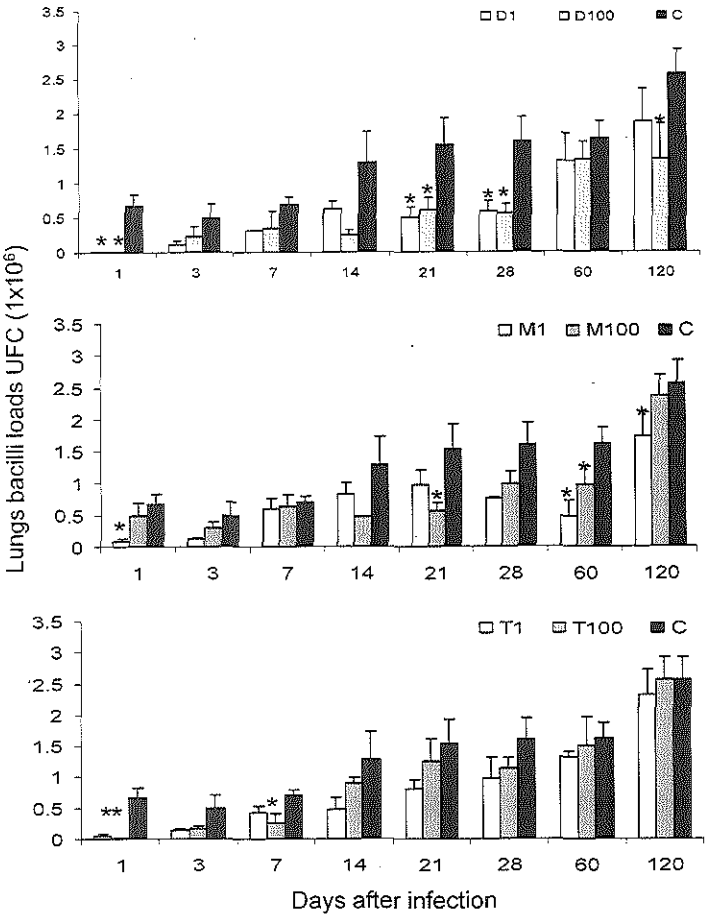
1.- Administración de micobacterias saprófitas y actinomicetos.

Se propuso usar a una micobacteria saprofita muy inmunogenica (*M. vaccae*) que induce una intensa estimulación de linfocitos Th-1 y células T citotoxicas y también activa células T reguladoras que inhiben respuesta Th2. Cuando se administró *M. vaccae* por vía oral con sonda nasogástrica un día antes o 30 días después de la infección, se produjo una disminución significativa de la carga bacilar y de la expresión de IL-4 en asociación con mayor expresión de TNF, IFN e iNOS, demostrando así que esta forma de inmunoterapia es altamente eficiente para controlar la tuberculosis pulmonar experimental. Los resultados fueron publicados en: Hernández-Pando R, Aguilar D, Orozco H, Cortez Y, Brunet LR, Rook GA. Orally administered Mycobacterium vaccae modulates expression of immunoregulatory molecules in BALB/c mice with pulmonary tuberculosis. Clin Vaccine Immunol 2008 Nov; 15(11):1730-6.

La otra proposición fue utilizar Actinomicetos que son organismos similares a *M. tuberculosis* como un posible tratamiento. Considerando que estos organismos tienen contacto muy temprano en la vida de los seres humanos sobre todo en regiones tropicales, se administraron varias preparaciones desde el destete y en dos ocasiones con una semana de intervalo a ratones BALB/c por vía gástrica usando dos dosis 10,000 y 100,000 bacterias y después de dos meses se infectaron con una dosis alta de *M. tuberculosis* cepa H37Rv de acuerdo al modelo experimental de tuberculosis progresiva.

Grupos de 6 ratones se sacrificaron a diferentes tiempos y en ellos se determino la cantidad de bacterias vivas por la cuantificación de unidades formadoras de colonia UFC. Dos preparaciones que corresponden al género Rodococus BE-D101 (representada como D in la grafica y la BE-M102 representada como M) produjeron una disminución significativa en la carga bacilar. Actualmente se está concluyendo la expresión de citocinas por RT-PCR en

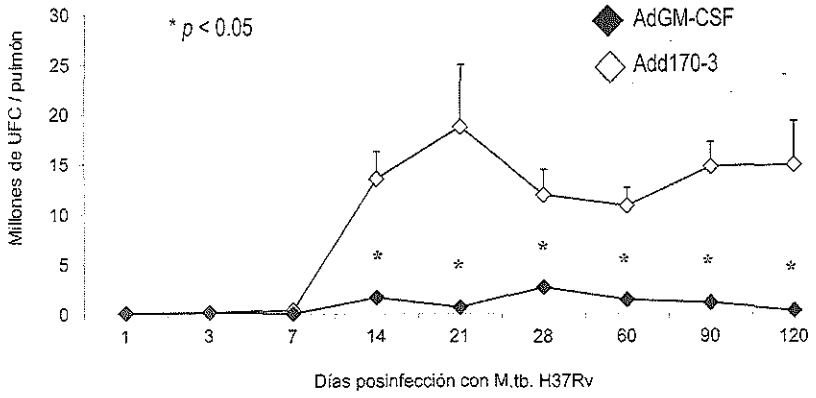
tiempo real para concluir y escribir el trabajo el cual se realizo en colaboración con el Prod John Stanford de la Universidad de Londres.



En otra parte de este trabajo se compararon los proteomas de *Streptomyces coelicolor* con *M. tuberculosis* y *M. bovis* por sistemas computacionales y se encontró una alta homología a nivel genómico y de proteínas de superficie. Posteriormente se administró *S coelicolor* por vía intraperitoneal y se determino su distribución. No se encontró que estos organismos se diseminaran incluso probando otras vías de inoculación, pero si se produjo una importante cantidad de anticuerpos que cruzaron con antígenos micobacterianos y cuando a estos animales se les administro *M. bovis*, controlaron la infección significativamente mejor que los animales control no sensibilizados en Streptomices. Estos resultados indican que Streptomyces puede ser usado para proteger en contra de la infección con micobacterias y puede incluso ser un vector vacunal, pues es fácilmente manipulado genéticamente y por ende se pueden sobrexpresar antígenos micobacterianos. Estos resultados se publicaron en: Arzuaga NO, Vila Granda A, Gómez JC, San Miguel ME, Bourzac JF, Hernández YL, Elías López AL, Pólux CR, Mesa LG, Hernández-Pando R, Domínguez AA. The use of Streptomyces for immunization against mycobacterial infection. Human Vaccine. 2011 Sep;7(9):934-40.

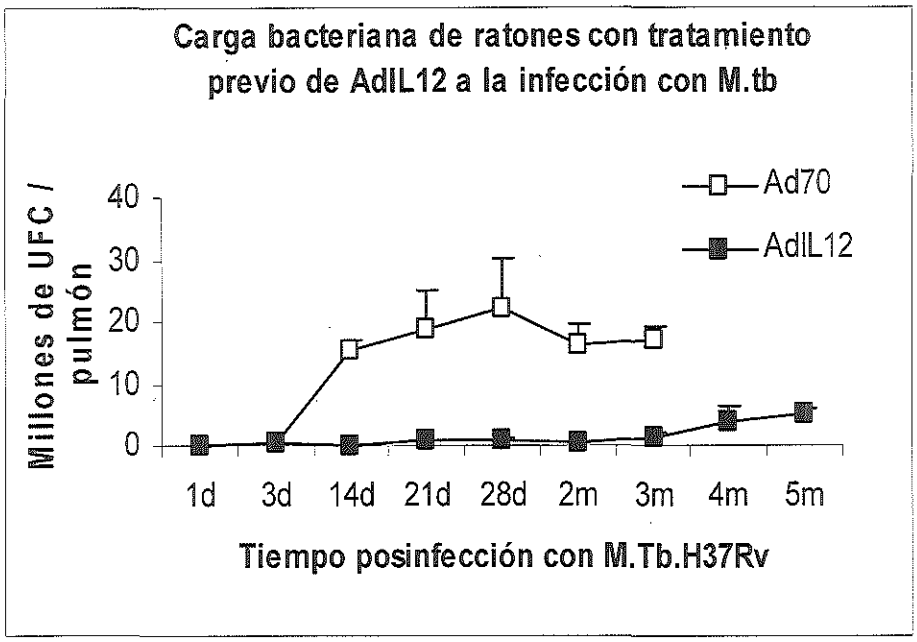
2.- Activación de células dendríticas

En nuestro modelo experimental de tuberculosis progresiva, hemos demostrado que una característica importante desde el punto de vista inmunopatogénico es el retraso en la activaciòn de las células dendríticas, tanto en el pulmón como en los ganglios linfáticos mediastinales. Estudios de la cinética de las células dendríticas durante el curso de la enfermedad utilizando lavados bronquiales y citometría de flujo, mostraron que estas células aparecen una semana después de la infección, alcanzan su máximo número en el día 21 y después declinan paulatinamente durante la fase progresiva de la enfermedad. Esto contrasta con la instilación vía intratraqueal de péptidos como los contenidos en el derivado proteico purificado (PPD) de cultivo de *M. tuberculosis*, el cual induce en horas la aparición de un gran número de células dendríticas activadas. Por lo tanto, el retraso en la activación de células dendríticas es un mecanismo de evasión inmunológica de *M. tuberculosis* y la posibilidad de activar a estas células de manera más temprana y eficiente es una estrategia potencialmente útil de inmunoterapia. Para lograr esto usamos citocinas altamente eficientes en la activación de células dendríticas, como el factor estimulante de colonias granulocítico y macrofágica (SCF-GM por sus iniciales en inglés). Nuestros resultados mostraron que la administración de adenovirus recombinantes que expresan esta citocina por vía intratraqueal en una sola dosis de $1x10^8$ un día antes de la infección con *M. tuberculosis* genero un significativa reducción en la carga bacilar, con menos daño neumónico y una alta activación temprana de células dendríticas y macrófagos activados así como un incremento significativo en la expresión de las citocinas protectoras IFN y TNF. Cuando se administraron 3 dosis en la etapa avanzada de la enfermedad (2 meses postinfección) también se obtuvo un notable efecto inmunoterapéuticos. Más aun, la administración de los adenovirus en nuestro modelo de infección latente previno la reactivación inducida por corticosterona y también evito el contagio de animales sanos que convivieron con enfermos en un modelo de transmisibilidad. Estos resultados se están preparando para ser enviados a publicación y este trabajo gano el segundo lugar del premio Glaxo del área de investigación básica en 2011. El MC Alejandro Francisco Cruz obtuvo con este trabajo el grado de Maestría con mención honorifica en Inmunología por el IPN, actualmente está cursando su Doctorado con el mismo tema sobre terapia génica en tuberculosis experimental.



Efecto de AdGM-CSF (adenovirus recombinante que expresa GM-CSF) y Addl70-3 (virus control) en la carga bacteriana pulmonar. Una sola administración IT de AdGM-CSF, évitó el crecimiento micobacteriano a partir del día 14 postinfección con H37Rv, hasta el día 120.

La interleucina 12 tiene también un efecto eficiente en la activación de las células dendríticas, se realizaron experimentos similares a los anteriormente descritos con adenovirus recombinante que expresa IL-12. La administración un día antes y en una sola dosis también produjo activación más temprana de células dendríticas que permitió un mejor control de la infección.



Cuando los adenovirus se administraron a los dos meses después de la infección, la respuesta terapéutica no fue adecuada pues indujo disminución temporal de la carga bacilar, actualmente se está probando la administración de varias dosis del adenovirus para tratar de obtener efecto terapéutico durante la enfermedad tardía. Pero otra posibilidad interesante fue administrar la IL-12 en vegetales transgénicos, con la colaboración del Dr. Gómez Lim del CINVESTAV sede Irapuato primero se administró extractos de jitomate recombinante que expresaban IL-12 por vía intratraqueal para evaluar su efecto in-vivo, los resultados mostraron que la citocina mantenía su actividad al ser producida en jitomate y administrarla en ratones BALB/c por vía intratraqueal, estos resultados se publicaron en: Sánchez Hernandez C, Aguilar León D, Hernandez Pando R, Gómez Lim M, Gómez García B, Gutiérrez Ortega A. In vivo activity of plant-based interleukin-12 in the lung of Balb/c mouse. BMC Research Notes 2010, May 27;3: 151. El jitomate transgénico que expresa IL-12 fue administrado por vía intragástrica diario a ratones BALB/c infectados con M tuberculosis drogosensible y MDR tanto en la fase temprana (desde el primer dia de infección) y durante la fase progresiva de la enfermedad (60 días), los resultados mostraron una actividad terapéutica significativa en todos los casos, estos resultados fueron publicados en: Elías López A; Marquina Castillo B; Gutiérrez-Ortega A; Aguilar D; Gómez Lim M; Hernandez Pando R. Transgenic tomato expressing interleukin-12 has therapeutic effect in a murine model of progressive pulmonary tuberculosis. Clinical and Experimental Immunology. 2008 Oct;154(1):123-33.
Con este trabajo la M en C Ana Elías obtuvo el grado de Doctor en Investigación Biomédica Básica en el programa del mismo nombre de la UNAM. Este trabajo obtuvo el premio Roberto Kreshmer otorgado por la Academia Nacional de Medicina al mejor trabajo publicado en Inmunología en 2010.

3.- **Promoción de apoptosis macrofágica.**

La infección de macrófagos por *M. tuberculosis* induce apoptosis y esta produce no solo la muerte del macrófago, sino también de la bacteria. En nuestro modelo experimental existe una gran cantidad de macrófagos activados apoptosicos después del primer y tercer día de la infección, mientras que durante la fase progresiva de la enfermedad los macrófagos vacuolados expresan mucho la molécula anti-apoptósica Bcl-2, lo cual los hace muy resistentes a morir por apoptosis, permitiendo así a la bacteria tener un hábitat celular de larga vida, que además es una fuente muy importante de moléculas inmunosupresoras. Por lo tanto, probamos el uso de moléculas o fármacos que promuevan la apoptosis macrofágica y así eliminen el nicho de la bacteria y la fuente de moléculas inmunosupresoras.

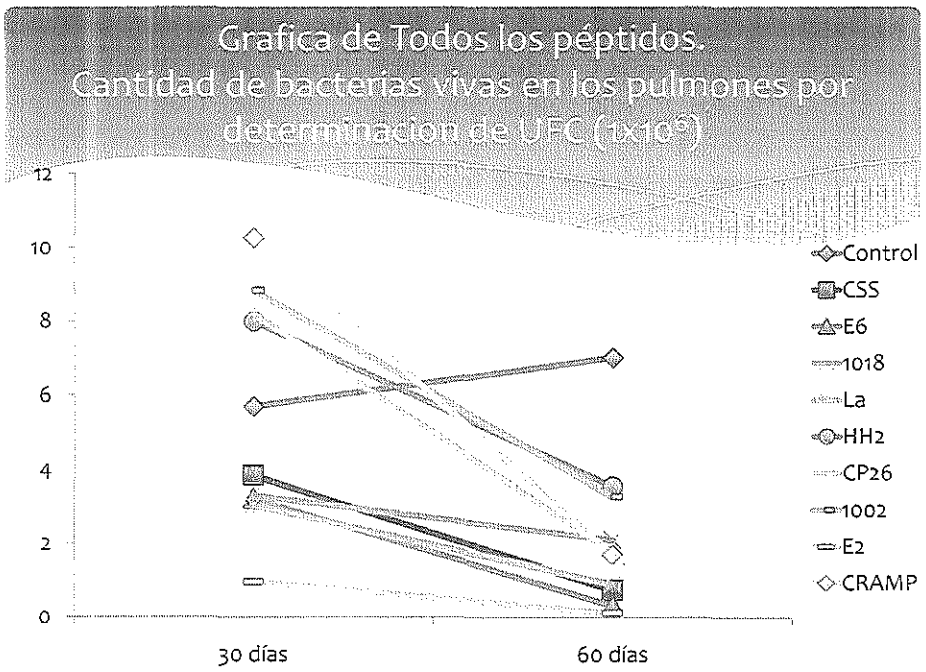
El abundante infiltrado inflamatorio intralveolar consecuencia del crecimiento de la bacteria es un aspecto distintivo de nuestro modelo experimental, lo cual produce insuficiencia respiratoria e hipoxia. En nuestro modelo la neumonía induce la expresión del factor relacionado a hipoxia (HIF), específicamente en los macrófagos vacuolados. El factor HIF entre otras funciones, induce la expresión de Bcl-2 previniendo la apoptosis de células inflamatorias. El 2-metoxiestradiol es un eficiente y selectivo inactivador de HIF y su administración intratraqueal durante la fase progresiva en nuestro modelo, disminuyó significativamente la carga bacilar, en coexistencia con abundantes macrófagos apoptósicos en las áreas neumónicas. Este trabajo está en su última etapa de revisión del manuscrito para ser enviado a publicación y obtuvo el premio CANIFARMA de investigación básica en 2010.

4.- **Péptidos antimicrobianos como agentes terapéuticos en la tuberculosis.**

En nuestros modelos experimentales de tuberculosis progresiva y latente hemos observado que el epitelio bronquial produce gran cantidad de defensinas beta 3 y 4, ambos péptidos se producen durante la fase temprana de nuestro modelo de tuberculosis progresiva y después durante la enfermedad progresiva disminuyen significativamente, lo cual sugiere que este tipo de péptidos antimicrobianos participan en el control del crecimiento bacilar. Las defensinas beta recombinantes están disponibles comercialmente pero son muy costosas por lo que usamos inductores de la expresión de defensinas beta como el aminoácido isoleucina. Utilizando una línea celular de neumocitos tipo II la cual es susceptible a la infección por M. tuberculosis y en consecuencia produce una gran cantidad de defensinas beta se confirmó que isoleucina es capaz de inducir la producción de defensinas beta 3 y 4 y su administración intratraqueal en ratones Balb/c no infectados confirmo que este aminoácido es también capaz de inducir la producción de defensinas in-vivo. La administración cada 72 hr de isoleucina intratraquealmente en ratones Balb/c con tuberculosis avanzada (dos meses post-infección) indujo una alta producción de defensinas lo cual permitió un control eficiente de la enfermedad constatado por una menor cantidad de UFC y neumonía en ratones infectados con bacterias drogosensibles o MDR, estos resultados se publicaron en: Rivas-Santiago C, Rivas-Santiago B, Aguilar León D, Castañeda-Delgado J, Hernández Pando R. Induction of β-Defensins by L-isoleucine as novel immunotherapy in experimental murine tuberculosis. Clinical and Experimental Immunology 2011, 164: 80-89. El QFB Cesar Santiago obtendrá con este trabajo próximamente el grado de Doctor en Ciencias Bioquímicas por la UNAM y ha sido contratado por la Universidad de Nueva Jersey USA para realizar un postdoctoral.
Otro péptido antimicrobiano de importancia en tuberculosis es la cathelicidina, la cual se estudió su cinética de expresión y fuente celular en nuestros modelos experimentales, de forma similar a las defensinas, la cathelicidina también se produce en mayor cantidad durante la fase temprana de la infección y durante la enfermedad progresiva sus niveles disminuyen bastante, estos resultados fueron publicados en: Castañeda-Delgado J, Hernández-Pando R, Serrano C, Aguilar-León D, León-Contreras J, Rivas Santiago C, Méndez R, González- Curiel I, Enciso-Moreno A, Rivas-

Santiago B. Kinetics and cellular sources of cathelicidin during the course of experimental latent tuberculous infection and progressive pulmonary tuberculosis. Clin Exp Immunol 2010, 161: 542–550.

Actualmente en colaboración con los Dres. Bruno Santiago del IMSS y B Hancock de la Universidad British Columbia en Canadá se están estudiando varios péptidos como la cathalicidina de ratón (CRAMP) y los péptidos sintéticos derivados de esta y defensinas: E6, E2, 1018, 10002, HH2, todos ellos mostraron se eficientes en un primer experimento en los que se administraron intratraquealmente a ratones BALB/c con dos meses de infección por la cepa drogosensible H37Rv. También se están estudiando bajo el mismo esquema experimental péptidos antimicrobianos obtenidos de arácnidos (péptidos SSC y La) en colaboración con el Dr. Corzo del Instituto de Biotecnología de la UNAM y el Dr. G Sandoval de la Compañía Silanes, los cuales también mostraron ser muy eficientes para disminuir la carga bacteriana pulmonar y actualmente se están realizando experimentos con ratones infectados con la cepa MDR. Esta es en realidad toda una línea de investigación que surgió de este proyecto, pues además de los péptidos mencionados existen algunos más obtenidos de animales marinos específicamente de caracoles en colaboración con los Dres. Alexei Licea del CICESE en Ensenada BC y G Sandoval de Silanes. También estamos empezando a estudiar péptidos antimicrobianos obtenidos de la saliva de perros y hienas en colaboración con el Dr. T Melgarejo de la Universidad Estatal de Kansas USA.



5.- Uso de anticuerpos como agentes inmunoterapéuticos en tuberculosis

Siempre se ha considerado que la inmunidad celular es la única que confiere protección en las infecciones por organismos intracelulares facultativos como M. tuberculosis. Sin embargo, estudios en modelos experimentales de tuberculosis incluido el nuestro han mostrado que la administración de anticuerpos es una eficiente forma de inmunoterapia. En un primer estudio se incubaron bacterias con anticuerpos policlonales humanos y así se administraron intratraquealmente a ratones BALB/c, después de 21 y 60 días postinfección se sacrificaron y se determino la cantidad de bacterias vivas, en el día 21 se observó una disminución significativa pero al dia 60 no existió diferencia, parece entonces que los anticuerpos durante la fase temprana de la infección pueden tener efecto terapéutico por actividad directa en contra de la bacteria o por mediar actividad

opsonica estos resultados fueron publicados en: Olivares N, Puig A, Aguilar D, Moya A, Cádiz A, Otero O, Izquierdo L, Falero G, Solis RL, Orozco H, Sarmiento ME, Norazmi MN, Hernández-Pando R, Acosta A. Prophylactic effect of administration of human gamma globulins in a mouse model of tuberculosis. Tuberculosis (Edinb). 2009 May;89(3):218-20.

Para estudiar el efecto directo de los anticuerpos en contra de los antígenos de la bacteria, se seleccione un antígeno de secreción que se expresa en situaciones de stress bacteriano, este es la proteína alfa cristalina y en contra de esta se produjeron anticuerpos monoclonales de clase IgA para la cual no hay receptores de su porción FC, al administrarlos a ratones infectados se mostro un efecto eficiente en la eliminación de la bacteria, estos resultados se publicaron en: López Y, Yero D, Falero-Diaz G, Olivares N, Sarmiento ME, Sifontes S, Solis RL, Barrios JA, Aguilar D, Hernández-Pando R, Acosta A. Induction of a protective response with an IgA monoclonal antibody against Mycobacterium tuberculosis 16kDa protein in a model of progressive pulmonary infection. Int J Med Microbiol. 2009 Aug;299(6):447-52

La actividad terapéutica puede depender de la porción FAB que reconoce epitopes de la bacteria o como recientemente empieza a evidenciarse, de los residuos de carbohidratos que existen en el dominio constante 2 del FC y que son reconocidos por receptores en células dendríticas (DC sign) o por receptores FC y de complemento en macrófagos y linfocitos. En pacientes con TB avanzada se ha descrito que dichas cadenas de azucares carecen de galactosa lo cual les confiere actividades proinflamatorias. Recientemente concluimos experimentos en los cuales a ratones infectados con M. tuberculosis se les administro uno y tres días después de la infección anticuerpos policlonales usados para terapia en humanos y a otro grupo se les administro anticuerpos del mismo tipo pero a los que se les eliminaron los azucares de los por tratamiento enzimático. Los animales tratados con los anticuerpos completos mostraron una disminución significativa de la carga bacilar 14 días después de la infección, mientras que los ratones tratados con los anticuerpos sin azucares mostraron una disminución minima de la carga bacilar. El manuscrito con estos resultados ha sido enviado recientemente a publicación a la revista PLos One, el título es: Olivares N, Marquina B, Mata Espinoza D, Hernandez Pando R, Collin M, Rook G. The effects of high dose human intravenous immunoglobulin in a model of pulmonary tuberculosis in BALB/c mice is highly dependent on IgG glycosylation

Artículos publicados

1.- Elias Lopez A; Marquina Castillo B; Gutierrez-Ortega A; Aguilar D; Gómez Lim M; Hernandez Pando R. Transgenic tomato expressing interleukin-12 has therapeutic effect in a murine model of progressive pulmonary tuberculosis. Clinical and Experimental Immunology. 2008 Oct;154(1):123-33.

2.- Hernández-Pando R, Aguilar D, Orozco H, Cortez Y, Brunet LR, Rook GA. Orally administered Mycobacterium vaccae modulates expression of immunoregulatory molecules in BALB/c mice with pulmonary tuberculosis. Clin Vaccine Immunol 2008 Nov;15(11):1730-6.

3.- López Y, Yero D, Falero-Diaz G, Olivares N, Sarmiento ME, Sifontes S, Solis RL, Barrios JA, Aguilar D, Hernández-Pando R, Acosta A. Induction of a protective response with an IgA monoclonal antibody against Mycobacterium tuberculosis 16kDa protein in a model of progressive pulmonary infection. Int J Med Microbiol. 2009 Aug;299(6):447-52

4.- Olivares N, Puig A, Aguilar D, Moya A, Cádiz A, Otero O, Izquierdo L, Falero G, Solis RL, Orozco H, Sarmiento ME, Norazmi MN, Hernández-Pando R, Acosta A. Prophylactic effect of administration of human gamma globulins in a mouse model of tuberculosis. Tuberculosis (Edinb). 2009 May;89(3):218-20

5.- Sanchez Hernandez C, Aguilar Leon D, Hernandez Pando R, Gomez Lim M, Gomez Garcia B, Gutierrez Ortega A. In vivo activity of plant-based interleukin-12 in the lung of Balb/c mouse. BMC Research Notes 2010, May 27;3:151.

6.- Castañeda-Delgado J, Hernández-Pando R, Serrano C, Aguilar-León D, León-Contreras J, Rivas Santiago C, Méndez R, González- Curiel I, Enciso-Moreno A, Rivas-Santiago B. Kinetics and cellular sources of cathelicidin during the course of experimental latent tuberculous infection and progressive pulmonary tuberculosis. Clin Exp Immunol 2010, 161: 542–550.

7.- Rivas-Santiago C, Rivas-Santiago B, Aguilar León D, Castañeda-Delgado J, Hernández Pando R.

Induction of β-Defensins by L-isoleucine as novel immunotherapy in experimental murine tuberculosis. Clinical and Experimental Immunology 2011, 164: 80-89.
8.- Arzuaga NO, Vila Granda A, Gómez JC, San Miguel ME, Bourzac JF, Hernández YL, Elías López AL, Pólux CR, Mesa LG, Hernández-Pando R, Domínguez AA. The use of Streptomyces for immunization against mycobacterial infection. Human Vaccine. 2011 Sep;7(9):934-40.

Capítulo de libro:
Título: Towards a New Challenge in TB Control: Development of Antibody-Based Protection
Autores: Armando Acosta, Yamile Lopez, Norazmi Mohd Nor, Rogelio Hernadez Pando, Nadine Alvarez, Maria Elena Sarmiento. En prensa para el libro: Tuberculosis, Editor: P Cardona

Presentaciones en Congresos:

1. Rogelio Hernández Pando. Experimental immunotherapy in tuberculosis. World TB Day Conference, Tuberculosis: Still a Global emergency. New drugs, New vaccine, New tools for TB diagnosis. Institute of Tropical Medicine Antwerp, Belgium. 24th March 2009.
2. Rogelio Hernández Pando. The role of chronic hipoxia in the himmunopathogenesis of experimental tuberculosis. IV Simposio fronteras del Conocimiento en Tuberculosis y otras Micobacteriosis Dr. Joseph Colston. V Reunión de la Sociedad Latinoamericana de Tuberculosis y otras Micobacteriosis, del 22 al 25 de agosto del 2010. En Zacatecas Zac. Mex.
3. Rivas-Santiago C.E., Rivas Santiago B., Aguilar D., Hernández R. B-Defensins as potentialimmunotherapy in thetreatment of tuberculosis in a murine model. Aplicaciones Médicas de la Biotecnología. Habana 2-5 de noviembre del 2009.
4. Hernandez Pando. Inmunoterapia en tuberculosis experimental. Congreso Franco-Argentino de Inmunologia, Nov 1-3, Buenos Aires Argentina 2010
5. Hernandez Pando R. Peptidos antimicrobianos en tuberculosis. Primera Reunion de la Sociedad Latinoamericana de Inmunologia de Mucosas. Buenos Aires, Argentina Nov 2, 2010
6. Rogelio Hernández Pando. Inmunoterapia experimental en tuberculosis. 6° Simposio Internacional de Inmunoterapia en Cáncer y Enfermedades Infecciosas realizado por el Cuerpo Académico Inmunología de la Facultad de Ciencias Biológicas, Universidad Autonoma de Nuevo Leon, 16 -18 de noviembre del 2011.

Formación de recursos humanos

1.- Titulo de tesis: Efecto inmunoterapéutico de la administración de jitomate transgénico que expresa interleucina 12 en la tuberculosis pulmonar experimental.

Tesista: **Ana Elías.** Doctorado en Investigación Biomédica Básica. Instituto de Investigaciones Biomédicas UNAM.

Fecha de obtención Del grado: Febrero 2009

2.- título de tesis: Efecto del adenovirus recombinante que codifica al factor estimulante de las colonias de granulocitos y macrófagos en la tuberculosis pulmonar murina.

Tesista: **Alejandro Francisco Cruz**

Maestría en Ciencias con Especialidad en Inmunología. Departamento de Inmunología, Escuela Nacional de Ciencias Biológicas. Instituto Politécnico Nacional

Fecha de obtención de grado: Junio 2011

3.- Titulo de tesis: Inducción de Péptidos antimicrobianos como inmunoterapia en el tratamiento contra la tuberculosis en modelo animal

Tesista: **Cesar Rivas Santiago**

Doctorado en Ciencias Bioquímicas, Facultad de Química, Universidad Nacional Autónoma de México.

Fecha de examen: Febrero 2012

Indique si se dio cumplimiento a los objetivos, metas y/o productos comprometidos (Fundamente/Justifique

Se considera que se cumplieron los objetivos y metas en un 90%, pues solo quedo por concluir la caracterización de 11 péptidos obtenidos del Factor de Transferencia en su mecanismo inmunológico, aspecto que está actualmente en marcha, se graduaron los estudiantes de posgrado y se generaron el número de publicaciones propuestas, pero aún quedan por enviar cuando menos otras 3 este año y ya se tiene una estudiante de doctorado más que esta en su 2ª semestre. Además se amplió significativamente el horizonte de algunos de las estrategias inmunoterapéuticas propuestas, en especial lo que corresponde a péptidos antimicrobianos y anticuerpos, también se tiene otro adenovirus recombinante que expresa osteonectina y el receptor tipo 3 de TGF para continuar con los experimentos de terapia génica para lo cual tenemos un estudiante de doctorado más.

Con base en los productos generados, señale los alcances en: a) Generación del conocimiento, b) Formación de recursos humanos especializados y c) Creación y/o fortalecimiento de grupos de investigación

En cuanto a generación de conocimientos, por primera vez se demuestra que experimentalmente la terapia génica basada en adenovirus recombinantes que codifican para citocinas protectoras administradas en una sola dosis es una forma eficiente para controlar la tuberculosis drogosensible y resistente. También se demostró por primera vez que la alimentación con vegetales transgénicos (jitomate) que expresa citocina protectora (IL-12) es una novedosa modalidad terapéutica para tratar la tuberculosis. También por primera vez se demostró que la administración aérea de inductores de péptidos antimicrobianos del tipo de las beta-defensinas con isoleucina es eficiente para controlar el crecimiento de bacterias drogosensibles y resistentes. Por primera vez se demuestra que los anticuerpos tienen la capacidad de suprimir el crecimiento bacteriano y esto depende en gran parte de residuos de azucares que tienen en el dominio Fc, los cuales son ligandos para receptores tipo DCsign, Fc y del complemento. Los experimentos que generaron este conocimiento fueron realizados por 4 estudiantes de postgrado, 3 se recibieron)1 maestría y 2 doctorado) y una estudiante de doctorado continua trabajando, uno de los graduados de Doctorado ha sido aceptado por la U de Nueva Jersey USA para realizar un postdoctorado y otra es posible que se contrate por la U de Carolina USA, esperamos que a su regreso se incorporen y fortalezcan grupos de investigación, como el grupo del Dr. Bruno Rivas que fue contratado por el IMSS y actualmente tiene como línea de trabajo defensinas para tratamiento y vacunas, el Dr. Rivas se graduó poco después de que empezó el presente proyecto y se ha establecido una colaboración solida con mi grupo en esta importante línea de investigación.

En términos de impacto, destaque las principales contribuciones de su investigación

La principal contribución del presente proyecto fue la demostración a nivel experimental que la activación de respuestas protectoras de la inmunidad innata (péptidos antimicrobianos) o adquirida (activación Th-1, inhibición Th-2) a través de diversos procedimientos como terapia génica con adenovirus, vegetales transgénicos, anticuerpos monoclonales o policlonales, inductores de péptidos antimicrobianos etc, son eficientes para controlar la tuberculosis producida por bacterias drogosensibles y resistentes y algunos de ellos son inclusos capaces de acortar el tiempo de antibioticoterapia

Cuáles argumentos plantearía como sustantivos para integrar su investigación dentro de los CASOS DE ÉXITO

Considero el presente proyecto como un caso de éxito porque más del 90% de los objetivos y metas propuestas fueron alcanzadas, generando publicaciones en revistas internacionales (9) y recursos humanos altamente especializados (2 doctores, un maestro), y actualmente hay otros dos estudiantes de doctorado de recién ingreso que continúan trabajando en este proyecto, además se pudo establecer una red extensa de colaboración con colegas a nivel nacional como con el Dr. A Licea del CISESE Ensenada BC, G Corzo (IBT UNAM), F López Casillas (IFC UNAM), I y E Estrada (Esc Ciencias Biol IPN), y con empresa nacional G Sandoval Silanes, además de grupos en el extranjero: Dr Hancock y Xing de Canada, T Melgarejo U Estatal de Kansas USA, G Rook de Inglaterra, M Collin de Suecia.

INSTITUTO NACIONAL DE
CIENCIAS MÉDICAS
Y NUTRICIÓN
SALVADOR ZUBIRÁN

México D. F. a 11 de febrero de 2016.

Dra. Norma Bobadilla Sandoval
Coordinadora de la CINVA
Presente

Estimada Dra. Bobadilla:

Por este conducto me permito solicitar el cierre del protocolo: "DISEÑO Y PRUEBA DE NUEVOS REGIMENES INMUNOTERAPEUTICOS PARA TRATAR LA TUBERCULOSIS", con No. de Registro Clave: PAT-043-09-12-1, CINVA **268** debido a que el protocolo ha concluido.

Sin otro particular por el momento, quedo de usted.

Atentamente,

Dr. Rogelio Hernández Pando
Investigador en Ciencias Médicas F
Departamento de Patología
Sección de Patología Experimental

INSTITUTO NACIONAL DE CIENCIAS MEDICAS Y NUTRICION "SALVADOR ZUBIRAN"
DEPARTAMENTO DE NEFROLOGÍA Y METABOLISMO MINERAL

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C.c.p: **Dr. Gerardo Gamba Ayala** – Director de Investigación
Dra. Ma. Elena Flores Carrasco – Encargada del Depto. de Invest. Exp. Y Bioterio

INSTITUTO NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN SALVADOR ZUBIRÁN

México, D.F. a 10 de Febrero de 2016

Dr. Rogelio Hernández Pando
Depto. Patología Experimental
Presente

INS. NACIONAL DE CIENCIAS MEDICAS Y NUTRICIÓN "SALVADOR ZUBIRÁN"
10 FEB 2016
DIRECCIÓN DE INVESTIGACIÓN

Estimado Dr. Hernández:

Por este conducto me permito solicitar el cierre del Protocolo: "**DISEÑO DE UNA PRUEBA DE NUEVOS REGIMENES INMUNOTERAPEÙTICOS PARA TRATAR A LA TUBERCULOSIS.**", con registro **CINVA 268.**, debido a que el periodo de realización y la prórroga correspondiente autorizado por la CINVA ha concluido. Favor de llenar el formato de cierre del protocolo que se anexa a la presente. De no recibir el formato de su parte en el plazo de 30 días, el protocolo se dará por cerrado.

Sin otro particular por el momento, quedo de usted.

INSTITUTO NACIONAL DE CIENCIAS MEDICAS Y NUTRICIÓN
10 FEB 2016
INVESTIGACIÓN EXPERIMENTAL

Atentamente,

Dra. Norma A. Bobadilla Sandoval
Coordinadora de la Comisión de Investigación en Animales

c.c.p. Dr. Gerardo Gamba Ayala, Director de Investigación
MVZ Mariela Contreras Escamilla, Jefa del DIEB

NAB/nom

Avenida Vasco de Quiroga No. 15
Colonia Belisario Domínguez Sección XVI
Delegación Tlalpan
Código Postal 14080
México, Distrito Federal
Tel. (52)54870900
www.incmnsz.mx

INSTITUTO NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN SALVADOR ZUBIRÁN

"2014, Año de Octavio Paz"

Acuse
16/DIC/14

México, D. F., a 10 de Diciembre del 2014.

Recibí 16/12/14

DR. ROGELIO HERNÁNDEZ PANDO
Depto. de Patología Experimental
P r e s e n t e .

REF.: CINVA 268, Clave: PAT-268-

Estimado Dr. Hernández Pando:

Habiendo analizado detalladamente el Protocolo de Investigación Experimental titulado:

"DISEÑO DE UNA PRUEBA DE NUEVOS REGIMENES INMUNOTERAPEÙTICOS PARA TRATAR A LA TUBERCULOSIS."

Este comité ha dictaminado **aprobar** la prórroga solicitada por un año a partir del 1º. de Enero del 2015 condicionado a la congruencia de los nuevos planteamientos propuestos con el protocolo original.

Sin más por el momento quedo de usted.

Atentamente,

Dra. Norma A. Bobadilla Sandoval
Coordinadora de la Comisión de Investigación en Animales

INS. NACIONAL DE CIENCIAS MEDICAS Y NUTRICIÓN "SALVADOR ZUBIRÁN"
15 DIC 2014
DIRECCIÓN DE INVESTIGACIÓN

c.c.p. Dr. Gerardo Gamba, Director de Investigación
Dra. Ma. Elena Flores, Encargada del Bioterio

Vasco de Quiroga No. 15
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INSTITUTO NACIONAL DE
CIENCIAS MÉDICAS
Y NUTRICIÓN
SALVADOR ZUBIRÁN

México D. F., a 24 de noviembre del 2014. .

Dra. Norma A. Bobadilla Sandoval
Coordinadora de la CINVA
Presente

En relación a su oficio con fecha 28 de octubre del año en curso, en el que nos solicita le notifiquemos si el proyecto titulado: DISEÑO Y PRUEBA DE NUEVOS REGIMENES INMUNOTERAPEUTICOS PARA TRATAR LA TUBERCULOSIS, con registro **CINVA 268**, requiere de una prórroga; le solicito de la manera más atenta sea concedida por 1 año a partir del 1 de enero de 2015, dicha solicitud obedece a nuestra necesidad de continua con nuestra investigación debido a que los resultados arrojados por este protocolo son publicables y de un alto interés para la comunidad científica, los que nos ha llevado a volverlo una línea de investigación.

Las líneas que están directamente relacionadas con este protocolo son varias, ejemplificadas en la siguiente tabla:

Tratamientos	Cinética de sacrificios	Reto con bacterias	Análisis	No. de animales	Repetición
Péptidos antimicrobianos de arácnidos	1,3,7,14,21,28, 60 Y 120	H37Rv,5186,583 y MDR	Carga Bacilar, PCR en tiempo real, Histología, ELISA y Citometría	200	200
Tratamiento con fármacos convencionales	1,3,7,14,21,28, 60 Y 120	H37Rv,5186,583 y MDR	Carga Bacilar, PCR en tiempo real, Histología, ELISA y Citometría	200	200
Factor de transferencia	1,3,7,14,21,28, 60 Y 120	H37Rv,5186,583 y MDR	Carga Bacilar, PCR en tiempo real, Histología, ELISA y Citometría	200	200
Nanoparticulas asociadas a almidón	1,3,7,14,21,28, 60 Y 120	H37Rv,5186,583 y MDR	Carga Bacilar, PCR en tiempo real, Histología, ELISA y Citometría	200	200
Péptidos aislados de toxinas de caracol	1,3,7,14,21,28, 60 Y 120	H37Rv,5186,583 y MDR	Carga Bacilar, PCR en tiempo real, Histología, ELISA y Citometría	200	200

Solicitamos que se nos otorgue un 10% más a la cantidad solicitada para cubrir pérdidas durante el proceso de infección.

Sin más por el momento me despido con cordiales saludos.

Atentamente,

Dr. Rogelio Hernández Pando
Investigador en Ciencia Médicas F
Departamento de Patología
Sección de Patología Experimental

Recibí 3/12/14

Vasco de Quiroga No. 15
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México, D. F. 14000
Tel.: (52) 54870900
www.incmnsz.mx

C.c.p: **Dr. Gerardo Gamba Ayala** – Director de Investigación
Dra. Ma. Elena Flores Carrasco – Encargada del Depto. de Invest. Exp. Y Bioterio

INSTITUTO NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN SALVADOR ZUBIRÁN

"2014, Año de Octavio Paz"

México, D.F. a 28 de Octubre del 2014

INSTITUTO NACIONAL DE CIENCIAS MEDICAS Y NUTRICION "SALVADOR ZUBIRAN" RECIBIDO DEPARTAMENTO DE ANATOMIA PATOLOGICA

Dr. Rogelio Hernández Pando
Depto. de Patología Experimental
Presente

Estimado Dr. Hernández:

Por este conducto le informo que su proyecto: **"DISEÑO DE UNA PRUEBA DE NUEVOS REGIMENES INMUNOTERAPEÙTICOS PARA TRATAR A LA TUBERCULOSIS"**, con registro **CINVA 268** finaliza en el mes de diciembre del año en curso. Por lo que le solicito de la manera más atenta me haga saber si el proyecto requerirá una prórroga. En caso afirmativo, favor de enviar a la **CINVA** el periodo de extensión que solicita y de requerir un mayor número de animales especificar y justificar como se utilizarán y los procedimientos experimentales que se llevarán a cabo con los mismos. En caso de no requerir una prórroga favor de llenar el formato de cierre del protocolo que se anexa a la presente.

Sin otro particular por el momento, quedo de usted.

Atentamente,

Dra. Norma A. Bobadilla Sandoval
Coordinadora de la CINVA

INS. NACIONAL DE CIENCIAS MEDICAS Y NUTRICIÓN "SALVADOR ZUBIRÁN" 31 OCT 2014 DIRECCIÓN DE INVESTIGACIÓN

c.c.p. Dr. Gerardo Gamba Ayala, Director de Investigación.
c.c.p. Dra. María Elena Flores Carrasco, Encargada del Departamento de Investigación Experimental y Bioterio.

INSTITUTO NACIONAL DE CIENCIAS MEDICAS Y NUTRICIÓN SALVADOR ZUBIRÁN INCMNSZ M.E. Flores DEPARTAMENTO DE INVESTIGACIÓN EXPERIMENTAL Y BIOTERIO

Vasco de Quiroga No. 15
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11 dic/2014 ✓

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INSTITUTO NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN
SALVADOR ZUBIRÁN

Abril 02, 2013

Dr. Rogelio Hernández Pando
Investigador en Ciencias Médica "F"
Departamento de Patología
Sección de Patología Experimental
P r e s e n t e.

Por este medio me permito informar a usted que se aprueba su solicitud de ampliación de fecha de término a diciembre de 2014 del proyecto "Diseño y prueba de nuevos regímenes inmunoterapéuticos para tratar a la tuberculosis", con registro CINVA 268. Así mismo, le solicito enviar a esta Jefatura, información a cerca de la cantidad de animales de laboratorio que requerirá para el desarrollo del proyecto.

A t e n t a m e n t e

Dr. Rafael Hernández González
Coordinador de la Comisión de Investigación en Animales
Jefe del Departamento de Investigación Experimental y Bioterio

ccp. Dr. Rubén Lisker Y. Director de Investigación.
MVZ. Griselda Salmerón Estrada. Secretaria CINVA
MVZ. M.en C. Ma. de la Luz Streber Jiménez. CINVA
Dr. Gonzalo M. Torres Villalobos. CINVA
Dra. Nimbe Torres y Torres. CINVA
Dr. Emiliano Tesoro Cruz. CINVA

Recibí
Roxana Gutiérrez Vidal
03/03/13

Investigación
Tradición Servicio
Asistencia Docencia

• Vasco de Quiroga 15,
• Delegación Tlalpan
• C. P. 14000 México, D. F.
• Tel. 54-87-09-00

20007700

"DIC/2014 P"

INSTITUTO NACIONAL DE
CIENCIAS MÉDICAS
Y NUTRICIÓN
SALVADOR ZUBIRÁN

"2013, Año de la Lealtad Institucional y Centenario del Ejército Mexicano"

México D. F., a 25 de marzo de 2013.

Dr. Rafael Hernández González
Jefe del Departamento de Investigación
Experimental y Bioterio
Presente

Por medio de este conducto solicito a usted de la manera más atente, una prórroga del proyecto titulado: "Diseño y prueba de nuevos regímenes inmunoterapéuticos para tratar a la tuberculosis", con número de CINVA 268, del cual soy el investigador responsable, para con concluir el 31 de diciembre del año 2014, dicha solicitud obedece a nuestra necesidad de continuar con nuestra investigación debido a que el proyecto antes mencionado ha arrojado resultados publicables y de un alto interés para la comunidad científica, situación que nos alienta a solicitarle dicha prorroga.

Sin más por el momento me despido enviándole cordiales saludos.

Atentamente,

Dr. Rogelio Hernández Pando
Investigador en Ciencias Médicas "F"
Departamento de Patología
Sección de Patología Experimental

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INSTITUTO NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN SALVADOR ZUBIRÁN

23 de junio de 2009

Dr. Rogelio Hernández Pando
Departamento de Patología

Estimado Dr. Hernández Pando:

En referencia con el proyecto de investigación titulado: "**Diseño y Prueba de Nuevos Regimenes Inmunoterapeúticos para Tratar a la Tuberculosis**"

Registro CINVA: **268**

El Comité de Investigación en Animales ha revisado su respuesta a las observaciones emitidas por el Comité y decidió **APROBARLO.**

Recibí
Forma Única

Atentamente

MVZ. M.Sc. Rafael Hernández González
Coordinador del Comité de Investigación en Animales

INSTITUTO NACIONAL DE CIENCIAS MEDICAS Y NUTRICION INNSZ "SALVADOR ZUBIRAN"
30 JUN 2009
DIRECCIÓN DE INVESTIGACIÓN

Friéndira Angeles
29/06/09.

cop. Dr. Rubén Lisker Y Director de Investigación
MVZ., M.en C. Octavio Villanueva Sánchez. Secretario CINVA
Dr. Patricio Santillán Doherty. Comité de Investigación en Animales.
Dr. Gerardo Gamba Ayala. Comite de Investigación en Animales
MVZ . M. en C. Ma. de la Luz Streber J Comite de Investigación en Animales
MVZ. Griselda Salmeron Estrada Comité de Investigación en Animales.
Dra. Nimbe Torres y Torres Comite de Investigación en Animales.

Investigación

Tradición Servicio

Asistencia Docencia

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- Delegación Tlalpan
- C. P. 14000 México, D. F.
- Tel. 54-87-09-00

20007700

"OIC/2014-4"

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INSTITUTO NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN
SALVADOR ZUBIRÁN

22/Junio

Dr Rafael Hernández González
Coordinador del CINVA.

Estimado Dr Hernández

Por medio de la presente me permito contestar a las observaciones realizadas a nuestro proyecto "Diseño y prueba de nuevos regimenes inmunoterapeuticos para tratar a la tuberculosis", con registro CINVA 268.

1.- El procedimiento analgésico para los animales en el caso de la anestesia para la infeccion intratraqueal es exponer al raton a vapores de sevofluorano, empapando una gasa con un ml de este agente y exponiendo al animal por un periodo de aproximadamente un minuto en una caja de acrilico, durante este tiempo el animal alcanza un estado de relajación aceptable para ingresar la cánula a la boca y después a la traquea, sin producirle ningún tipo de sufrimiento. Para el sacrificio, se administra pentotal sódico por via intraperitoneal (56 mg/kg), después de aproximadamente un minuto se obtiene anestesia total y se secciona la arteria axilar para producir eutanasia por exsanguinación.

2.- La disminución del peso en los animales control no tratados al final del experimento es de aproximadamente 5-10%. En los animales tratados, en la mayoria de los protocolos expuestos en el presente proyecto y de acuerdo a resultados de experimentos preliminares, muestran ganancia y no perdida de peso.

3.- De acuerdo con el número de animales por microaislador.

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INSTITUTO NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN
SALVADOR ZUBIRÁN

4.- El procedimiento de bioseguridad para el personal participante consiste en el uso de uniforme quirurgico, cubrebocas de alta seguridad y guantes quirúrgicos. La infección y sacrifico de los animales de experimentación se realiza en campana de bioseguridad 3, todos los desechos son almacenados en las bolsas correspondientes y destinados a incineración. Los animales son albergados en el sistema de bioseguridad consistente en microaisladores conectados a presión negativa, con circulación y filtración constante de aire, que garantiza la ausencia total de salida de bacterias desde el interior de los microaisladores hacia el cuarto de trabajo. Todo el personal que participa en este proyecto ha sido entrenado en estas medidas y tiene mas de 10 años de experiencia, siguiendo las indicaciones implementadas por el CINVA desde hace 15 años lo cual ha permitido la ausencia total de accidentes en el personal desde que se empezaron a realizar este tipo de experimentos.

En espera de que estas respuestas hayan aclarado los comentarios del CINVA, me permito aprovechar la ocasión para enviarle afectuosos saludos.

Atentamente

Dr. Rogelio Hernández Pando
Investigador en Ciencias Médicas F.
Sección de Patología Experimental

• Vasco de Quiroga 15,
• Delegación Tlalpan
• C. P. 14000 México, D. F.
• Tel. 54-87-09-00

11 OIC/2014-IV

INNSZ

INSTITUTO NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN
SALVADOR ZUBIRÁN

México D.F., a 18 de junio de 2007

Dr. Rogelio Hernández Pando
Departamento De Patología experimental

En relación con la revisión de protocolo remitido por usted para su revisón cor el
Comité de Investigación en Animales
Registro CINVA 268
"Diseño y prueba de nuevos regimenes inmunoterapeúticos para tratar a la tuberculosis"

El Comité requiere d ela siguiente información para emitir su ditamen.

Observaciones:

1. Describir el procedimiento analgésico para los animales.
2. Es necesario establecer la variación en porcentaje que existirá entre peso inicial de los animales y peso final al término del experimento. Con base en la NOM-062.
3. No se debe exceder la capacidad máxima de los microaisladores que manejan la cual es de 5 animales por caja.
4. Especificar las normas de bioseguridad que se seguirán para el personal.

Sin otro particular

Atentamente

INSTITUTO NACIONAL DE CIENCIAS MEDICAS Y NUTRICION "SALVADOR ZUBIRAN"
RECIBIDO 19 JUN 2007
DEPARTAMENTO DE ANATOMIA PATOLOGICA

M.V.Z., M.Sc. Rafael Hernández González
Coordinador del CINVA

• Vasco de Quiroga 15,
• Delegación Tlalpan
• C. P. 14000 México, D. F.
• Tel. 54-87-09-00

"1 DIC/2014 Y"

FECHA DE CLASIFICACIÓN: 28-05-2009
ÁREA: Invest. Exp. y Bioterio
CONFIDENCIAL: ☐
RESERVADA: ☒ TOTAL ☐ PARCIAL ☐
PERÍODO DE RESERVA: 5 Años
PARTES O SECCIONES: completo
FUNDAMENTO LEGAL. Art. 13, 14 y 18 de la L.F.T.A.I.P.G.
FIRMA DEL TITULAR DEL ÁREA:

INSTITUTO NACIONAL DE CIENCIAS MEDICAS Y NUTRICIÓN
"SALVADOR ZUBIRAN"
Dirección de Investigación
Comité de Investigación en Animales (CINVA)

INCMNSZ

SOLICITUD DE EVALUACION DE PROYECTOS

No invada las zonas sombreadas

No. CINVA: 268

CLAVE: [ ] [ ] [ ] [ ] [ ]

Fecha de Recepción: [ ] Fecha de revisión: [ ]

TITULO DEL PROYECTO: DISEÑO Y PRUEBA DE NUEVOS REGIMENES INMUNOTERAPEUTICOS PARA TRATAR A LA TUBERCULOSIS

INVESTIGADOR PRINCIPAL (IP): DR. ROGELIO HERNÁNDEZ PANDO
DEPARTAMENTO DE ADSCRIPCION: PATOLOGÍA EXT. 2194
TELEFONOS PARA EMERGANCIAS: 58480481
CORREO ELECTRÓNICO: [redacted] OBSERVACIONES:

Articulo 113 Fracción I de LFTAIP eleminado correo electronico por tratrarse de un dato personal

Personal que trabajará directamente con los animales (investigadores asociados, alumnos, tesistas, etc).

Nombre	Puesto en el INCMNSZ	Ext.	Tel. en caso de emergencia
1. Dra. Diana E. Aguilar León	Invest. en Ciencias Medicas D	2194	56587556
2. Dr. Héctor Orozco Estévez	Invest. en Ciencias Medicas C	2194	55737454
3. Sr. Leonardo Ortiz Romero	Técnico	2194	
4.			

Los animales utilizados en el proyecto de investigación serán adquiridos, mantenidos, manejados y utilizados de acuerdo al reglamento, a los manuales y guías de procedimientos del Departamento de Investigación Experimental y Bioterio, mismos que contienen la Norma Oficial Mexicana NOM-062-ZOO-1999, Especificaciones técnicas para la producción, cuidados y uso de los animales de laboratorio. Publicada por la SAGARPA en el Diario Oficial el miércoles 22 de agosto del 2001, y los lineamientos nacionales e internacionales para el buen uso de los animales de experimentación.

Sólo el suscrito, los investigadores y personal registrado y autorizado por el CINVA trabajarán con los animales, siendo el investigador principal el responsable de que cada uno de ellos cuente con los conocimientos, habilidades y experiencia en el manejo de los animales para realizar los maniobras experimentales descritas en el proyecto.

Los animales serán sometidos exclusivamente a los procedimientos especificados en este protocolo, siendo indispensable solicitar por escrito al CINVA cualquier modificación, incluyendo las fechas de inicio y terminación del estudio.

Nombre y Firma del Investigador Principal

El presente formulario presente facilitar la evaluación de su proyecto e identificar las necesidades de animales de laboratorio, equipo y manejo para su proyecto de investigación. Para el llenado de los cuadros consulte la información que describe cada sección de los mismos:

Fecha de inicio del estudio:	01-06-2009	Fecha de terminación del estudio	31-05-2012

Indique con el inciso correspondiente las características de los animales, condiciones de alojamiento y maniobras experimentales que requiere el proyecto:

1. Especie	2. Raza cepa	3. Condición Microbiol.	4. No. H	Total M	5. Distribución	6. Alojamiento	7. Densidad	8. Nivel Biosegur.	9. Nivel de afeccion	10. Destino	11. Eutanasia
RATON	BALB/C	SPF		3000	500 AL MES	D	5	III	C	D	

1. Nombre genérico o especie: Escriba el nombre común o científico de los animales que empleará en su estudio.

2. Raza, cepa, o tipo genético: escriba la nomenclatura que mejor describe las características genéricas del animal que necesita (ej: ratón, BALB/c, C57BL&, Cd1, un/un, Rata: wistar, Fischer 344, sprague-dawley, NIH, Conejo. Nueva Zelanda albino, Hámster: dorado)

3. Tipo o condición microbiológico: A) Convencional: animal con flora microbiológica desconocida, sin signos aparentes de enfermedad, B) SPF: (specific pathogen free) libre de patógenos específicos (indicar el tipo de patógenos indeseable ej: virus, bacterias, hongos, parásitos), C) Otro: especifique.

4. Número total: indique el número de animales que utilizará en el estudios, H: hembras, M: machos, incluyendo grupos piloto. En caso de utilizar animales de un solo sexo favor de invalidar la columna correspondiente.

5. Distribución: Indique la cantidad y la frecuencia en que requiere se le entreguen los animales ej: todos en una entrega, 10 cada semana, al mes, bimestre, trimestre, semestre, etc.

6. Alojamiento: Indique con la letra el tipo que corresponda:
A) Caja de policarbonato de piso sólido
B) Jaula con piso de malla o rejilla
C) Jaula metabólica
D) Microaislador
E) Caja de policarbonato de piso sólido con filtro
F) Perrera
G) Corral
H) Corraleta metabólica
I) Pecera

7. Densidad poblacional: Indique como alojará a los animales ej: un animal por caja, parejas, 3, 5, etc. Consultar la Norma Oficial Mexicana NOM-062-ZOO-1999, Especificaciones técnicas para la producción, cuidado y uso de los animales de laboratorio. Publicada por la SAGARPA en el Diario Oficial el miércoles 22 de agosto del 2001.

8. Nivel de bioseguridad: deberá indicar el nivel de riesgo biológico que existe para el personal que maneja a los animales o sus desechos, tanto para ivestigadores, alumnos y técnicos de bioterio.
Nivel I) Trabajo con agentes químicos, físicos o biológicos que no producen enfermedad y no son un riesgo para la salud de personas sanas y el medio ambiente.
Nivel II) Trabajo con agentes químicos, físicos o biológicos que tienen un peligro potencial bajo o moderado para la salud del personal y el medio ambiente)Ej: Salmonelosis, Toxoplasmosis, Hepatitis B).
Nivel III) trabajo con agentes químicos, físicos o biológicos que tienen un peligro potencial alto para la salud humana y animal o pueden producir la muerte, cuando se inhalan (Ej: Tuberculosis, Coxiella burnetti)
Nivel IV) Trabajo con agentes químicos, físicos o biológicos exóticos transmisibles por aerosoles y mortales para seres humanos y animales (Ej: virus Ébola, virus hanta).

9. Nivel de afectación de los animales: Indique el nivel de invasividad y el grado de dolor que sentirá el animal durante los procedimientos experimentales o manipulación:
Categoría A) Experimentos con invertebrados, huevos, protozoarios, organismos unicelulares. Uso de metazoarios, cultivo de tejidos u órganos obtenidos después de la muerte del animal.
Categoría B) Experimentos que causen molestias o estrés minimo (inyección no dolorosa, restricción de movimiento, marcado o aretado de orejas).
Categoría C) Experimentos que causan estrés menos o dolor de corta duración, realizados con analgesia o anestesia (colocación de cánulas, biopsia, cirugía menor)
Categoría D) Experimentos que causan estrés o dolor de moderado a severo contratado con anestesía (procedimientos quirúrgicos mayores)

10. Destino final: indicar el destino final de los animales al término de los experimentos:
A) Vivo sin cirugía
B) Vivo post-cirugía
C) Cirugía terminal (no despierta de la anestesia)
D) Eutanasia

11. Eutanasia: Indique el método empleado para dar muerte al animal. Consultar la Norma Oficial Mexicana NOM-062-ZOO-1999. especificaciones técnicas para la producción, cuidado y uso de los animales de laboratorio. Publicada por la SAGARPA en el Diario Oficial el miércoles 22 de agosto del 2001, capítulo 9, eutanasia.

En el siguiente cuadro marque con la clave que se idica el nivel de habilidad y experiencia de usted y su personal para realizar las maniobras experimentales mencionadas en la columna de la izquierda.

Clave:

A) Entrenado, hábil y con experiencia
B) Será entrenado y supervisado por el I.P.
C) Requiere instrucción, entrenamiento y supervisión por el personal del D.I.E.B.

En caso de que las maniobras experimentales sean realizadas por el personal del bioterio se marcará con una X el espacio correspondiente en la columna D.I.E.B.

Investigador ó personal que trabajará directamente con los animales (investigadores asociados, alumnos, tesistas), etc

MANIOBRA	I. P.	1	2	3	4	D.I.E.B.
Inmovilización	A			A		
Anestesia	A	A	A			
Medicación Enteral	A	A	A			
Medicación Parental	A	A	A			
Toma de sangre						
Otras muestras*	A	A	A			
Cirugía	A	A	A			
Eutanasia	A	A	A			
Otras ***						

*Especificar de qué, cantidad y frecuencia de muestreo:

*** Especificar:

Dr. Rogelio Hernández Pando
Nombre y firma del Investigador Princilal

RHG/mlsj, agosto del 2001

0:(2441)

INSTITUTO NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN
SALVADOR ZUBIRAN

INCMNSZ

Dirección de Investigación

FORMA ÚNICA PARA REGISTRO DE PROYECTOS

No invada las zonas sombreadas

CLAVE: PAT-043-09-12-1

FECHA DE RECEPCIÓN : _____

TÍTULO: DISEÑO Y PRUEBA DE NUEVOS REGIMENES INMUNOTERAPEUTICOS PARA TRATAR A LA TUBERCULOSIS

INVESTIGADOR RESPONSABLE: DR. ROGELIO HERNANDEZ PANDO

DEPARTAMENTO O SERVICIO: DEPARTAMENTO DE PATOLOGIA

TIPO DE INVESTIGACIÓN:

	1. Investigación Clínica	(incluye seres humanos o sus productos biológicos)
X	2. Investigación Experimental	(incluye animales de investigación o sus productos biológicos)
	3. Investigación Documental	(revisión de expedientes, revisión bibliográfica, informe de casos, etc.)
	4. Desarrollo Tecnológico	(instrumental, equipo, metodos diagnósticos, drogas nuevas, etc)
	5. Investigación Epidemiológica	(estudios en poblaciones, en comunidad o en hospital)
	6. Otros	(organización de eventos, asistencia a reuniones, donativos, etc)

PATROCINADORES:

	Cantidad:	
CONACyT	$3,300,000.00	$3,300,000.00
		TOTAL
		Fondo de Apoyo

PERÍODO DE UTILIZACIÓN DE LOS RECURSOS: de mes: 06 año: 2009 a mes: 05 año: 2012

FORMA EN LA QUE SE RECIBIRÁN LOS FONDOS:

Primer año:	$2,600.000.00			
Segundo año:	$300.000.00			
Tercer año:	$300.000.00			
Cuarto año:				
Quinto año:				
	primer trimestre	segundo trimestre	tercer trimestre	cuarto trimestre

COSTOS TOTALES DE LA INVESTIGACIÓN (ver instrucciones al reverso)	
1. Personal: (sueldos y sobresueldos al personal)	
2. Equipos: (de laboratorio, cómputo, transporte, etc.)	$2,600.000.00
3. Materiales: (reactivos, consumibles, desechables, etc.)	$700.000.00
4. Animales: (adquisición, cuidado, procedimientos, etc.)	
5. Estudios: (de laboratorio, gabinete, especiales, etc.)	
6. Viáticos: (reuniones científicas y trabajo de campo)	
7. Publicaciones: (costos directos de publicación, sobretiros)	
8. Suscripción: (libros, revistas, software, periodicc, etc.)	
9. Varios: (teléfono, fax, fotocopias, mensajeria, etc.)	
10. Fondo de Apoyo: (15% de la cantidad total del proyecto)	
TOTAL:	$3,300,000.00

INSTITUCIONES PARTICIPANTES

INSTITUTO NACIONAL DE CIENCIAS MEDICAS Y NUTRICION

INSTITUTO NACIONAL DE PSIQUIATRIA

UNIVERSIDAD DE SALAMANCA, ESPAÑA

FIRMAS

Investigador Responsable

Jefe del Departamento

Comité de Investigación en Humanos

Comité de Investigación en Animales

Director de Investigación

Director General

22/07/09

23 junio de 2009

Fecha de Resolución

RESUMEN

ANEXO

1. **TÍTULO DEL PROYECTO: Diseño y prueba de nuevos regimenes inmunoterapeuticos para tratar a la tuberculosis**

2. INVESTIGADORES

2a. IDENTIFICACIÓN DE INVESTIGADORES

Nombre, firma y adscripción de cada uno de los investigadores participantes. El investigador principal deberá ser un profesional adscrito a la Institución(médico de base o investigador y no un alumno de curso, residente, pasante, interno,etc).

INVESTIGADOR	POSICIÓN INSTITUCIONAL	POSICIÓN EN EL PROYECTO	EXTENSIÓN	CORREO-E
Rogelio Hdez	Inv CM-F	Invest principa	2194	rhpando@quetz
Diana Aguilar	Inv CM-D	Invest Asocia	2194	[redacted]
Hector Orozc	Inv CM-D	Invest Asocia	2194	[redacted]
Leonardo Orti	Tecnico	Tec bioterio	2194	

Articulo 113 Fracción I de LFTAIP eleminado correo electronico por tratrarse de un dato personal

2b. PERTINENCIA DEL GRUPO DE INVESTIGADORES CON RESPECTO DEL PROYECTO

Brevemente describa las calificaciones del grupo investigador con respecto del proceso de investigación científica en general y con respecto del proyecto presentado (v.gr.:grado académico, experiencia laboral, miembro del sistema de investigadores de los INS, del SNI, etc).

El Dr Hernández Pando es patologo y doctor en inmunologia, durante 20 años ha realizado investigación en inmunopatologia experimental de la tuberculosis, es investigador F y SNI-3. La Dra Aguilar Leon es QFB y doctora en biología experimental, experta en el manejo de animales de experimentación y diversas tecnicas inmunologicas y moleculares, es investigadora D y SNI-1. El Dr Hector Orozco es médico, experto en manejo de animales de experimentación y diversas tecnicas morfologicas, es investigador C. El Sr L Ortiz es el unico tecnico de biotero en el pais con experiencia en el cuidado de animales de experimentación infectados con germenes patogenos. Los Dres Aguilar y Orozco realizaran el trabajo experimental supervisando a los estudiantes asociados al proyecto

3. INSTITUCIONES PARTICIPANTES

Nombre y dirección de la o las instituciones participantes. Para estudios multicéntricos añadir los datos del centro

Instituto Nacional de Ciencias Medicas y Nutrición. Departamento de Patología, Seccion de Patología Experimental. Direccion: Vasco de Quiroga 15, Tlalpan. Telefono 54870900 ext2194. Investigador responsable y coordinador del proyecto: Dr Rogelio Hernandez Pando

Instituto Nacional de Psiquiatria. Periferico, Investigador responsable Dr Benito Antón

Universidad de Salamanca, Facultad de Farmacia Departamento de Quimica Farmaceutica. Direccion: Campus Miguel de Unamuno, 37007 Salamanca España.

PATROCINIO

4b. ORGANISMOS PATROCINADORES

Nombre, dirección y teléfono de la o las organizaciones, instituciones o laboratorios que aportarán recursos

Consejo Nacional de Ciencia y Tecnología. Av Insurgentes Sur 1582. Col Credito Constructor. Benito Juarez 03940, Mexico DF

MARCO TEÓRICO

Explicar detalladamente los fundamentos disponibles a la fecha en los que se basa el estudio que se propone(sentido biológico, datos de experimentos en animales o en humanos):

a) Antecedentes:

b) Definición del problema:

c) Justificación:

La tuberculosis es una importante enfermedad infecto-contagiosa que afecta principalmente a los pulmones y produce profundas alteraciones en el sistema inmunològico. El agente causal *Mycobacterium tuberculosis* es un microorganismo patógeno intracelular facultativo que puede producir tanto enfermedad progresiva como infección latente asintomática. Generalmente la primoinfección tuberculosa se lleva a cabo en los pulmones durante la niñez y en la mayoría de los casos es controlada por el sistema inmunológico, solo el 10 % de estos casos sufrirán la enfermedad progresiva En esta primoinfección tuberculosa, aun en aquellos casos que son controlados por el sistema inmunológico, no todas las bacterias son eliminadas, algunos bacilos permanecen en los tejidos en un estado quiescente con poca o nula actividad reproductiva por el resto de la vida del individuo infectado, lo cual se denomina infección latente. Actualmente se calcula que anualmente esta enfermedad produce 2 millones de defunciones a nivel mundial, con 8 millones de nuevos casos y un tercio de la humanidad tiene infecciòn latente, lo cual la convierte en la enfermedad infecto-contagiosa más relevante a nivel mundial. Un aspecto de gran importancia es que a pesar de que actualmente es curable, se necesita de una terapia combinada de 4 antibióticos por 6 a 9 meses, lo que redunda en una alta tasa de abandono del tratamiento, lo que promueve recaidas y el surgimiento de cepas resistentes a múltiples antibióticos (MDR, XDR), lo cual complica aun mas el tratamiento al incrementar su costo y generar mas efectos nocivos para el paciente, mientras que para las cepas XDR no existe actualmente forma de curarlas, por esta razon y por la necesidad de acortar el largo tratamiento de la tuberculosis drogosensible existe interès en desarrollar nuevas formas de tratamiento basadas en potenciar la respuesta inmune protectora o abatir la respuesta inmune deletèrea, a lo cual se le denomina genericamente inmunoterapia. El objetivo de este proyecto es diseñar y probar nuevos esquemas inmunoterapeuticos basados sobre todo en el conocimiento generado por nuestro grupo sobre la inmunopatología de la tuberculosis utilizando modelos experimentales murinos.

6a. HIPÓTESIS

Definido como un enunciado comprobable acerca de la relación entre una variable dependiente y una variable independiente.

La estimulaciòn de la respuesta Th-1 y de la activaciòn macrofàgica, asi como la inhibición de la respuesta Th-2 y de otros factores inmunosupresores tendra un efecto terapeutico significativo en la tuberculosis producida por bacterias drogosensibles y multidrogoresistentes y permitira ademas acortar el tiempo de tratamiento con antibioticos convencionales.

7. METODOLOGÍA: DISEÑO GENERAL

Describir el diseño general del estudio y, si es pertinente, especificar los siguientes puntos:

a) Diseño del estudio: describir si es aleatorio/no aleatorio, controlado, de cohorte, tipo de cegamiento(doble-ciego,simple), tipo de controles(placebo, medicamento activo), periodo de lavado.

b) Descripción de la maniobra o intervención

c) Tamaño de la muestra (# de pacientes a incluir; justificar el cálculo)

d) Mecanismos de asignación del tratamiento

e) Grupos de tratamiento

f) Duración del seguimiento individual

Reactivar respuesta protectora (TH1/macrofagos activados): **Administración de micobacterias sapròfitas.** La micobacteria saprofita *M.vaccae* puede ser un eficiente agente inmunoterapeutico porque activa eficientemente a las cèlulas protectoras Th1 y CD-8+ y suprime la activaciòn de linfocitos Th-2. Estudiaremos el efecto de este sapròfito en ratones infectados con una cepa MDR y la cepa drogosensible H37Rv, asi como su eficiencia como agente coadyuvante a la quimioterapia. Para esto la cepas de *M. tuberculosis* H37Rv y MDR seràn cultivadas en el medio lìquido PBY, después de un mes, las micobacterias son separadas y ajustadas a 2.5×10^5 en 100 µl de amortiguador PBS y mantenidas a –70°C hasta su uso. Ratones machos BALB/c de 6-8 semanas de edad son anestesiados con 56 mg/kg de pentotal sòdico administrado por via peritoneal, la traquea se expone quirúrgicamente y 2.5×10^5 bacterias viables suspendidas en 100 µl de PBS se inyectan usando una jeringa de insulina. Posteriormente la insiciòn quirùrgica es suturada con seda esteril. Los ratones así infectados se almacenan en cajas con microaisladores conectados a un sistema de presiòn negativa. Todo este procedimiento y los sacrificios posteriores se realizan en campanas de seguridad biològica. Al dia 60 postinfecciòn, los ratones sobrevivientes se distribuyen al azar en seis grupos experimentales. Grupos de 20 ratones tuberculosos en dos experimentos diferentes, se trataràn con una suspensión de *M. vaccae* a dosis de 100 µg en 100µl administrado por sonda nasogastrica. Para evaluar la capacidad de M.vaccae para acortar el tiempo de tratamiento con antibioticoterapia convencional, un grupo de 20 animales se tratara con antibioticoterapia convencional, constituida por: rifampicina (10 mg/kg), isoniazida (10 mg/kg), y pirazinamida (30 mg/kg), administrados diariamente a travez de sonda intragástrica, el segundo grupo se tratara con antibioticos mas M. vaccae administrado por sonda intragastrica una vez por semana. El tercer grupo se tratarà solamente con M. vaccae y el grupo control exclusivamente recibirà la soluciòn vehiculo. El quinto grupo corresponde a ratones infectados con el aislado clinico MDR, el cual se tratara con M. vaccae y ultimo grupo es el grupo control infectado con el aislado MDR que recibe solamente el vehículo. Cinco ratones por cada grupo se sacrificaran los dias 7, 15, 30 y 60 dias posteriores al inicio del tratamiento. Se tendran 10 animales mas de cada grupo que se se dejaran sin sacrificarlos con el fin de determinar sobreviva. Se realizaran dos experimentos completos por separado para determinar reproducibilidad. Para evaluar la eficiencia del regimen inmunoterapeutico ademas de curvas de sobrevida, se realizara estudio histològico/morfomètrico (porcentaje de area pulmonar afectada por neumonía), mediciòn de la carga bacilar pulmonar por determinación de unidades formadoras de colonia (UFC) y la expresión genètica de las citocinas IFN, TNF, IL-4 y la enzima iNOS por RT-PCR en tiempo real, de acuerdo a las tècnicas ya estandarizadas en nuestro laboratorio.

Uso y caracterizaciòn molecular del Factor de Transferencia (FT): Los FT son productos dializables de bajo peso molecular obtenidos de cèlulas inmunològicas, los cuales son capaces de transferir inmunidad mediada por cèlulas (IMC) de donadores inmunizados a sujetos receptores no sensibilizados. Actualmente no se conoce la estructura quìmica ni los mecanismos moleculares de acciòn del FT, varios estudios han mostrado que le FT es un conjunto de proteinas de bajo peso molecular. Nuestro objetivo es la caracterizaciòn molecular del FT y determinar su efecto terapéutico en el modelo murino de tuberculosis, para el primer objetivo se usara la cromatografía de líquidos de alta resolución acoplado a la espectrometría de masas en tiempo real; pare evaluar su efecto terapeutico, grupos de animales se trataran con 1µg de cada una de las proteinas aisladas suspendida en 50µl de sol salina por via intraperitoneal, una vez por semana, los grupos experimentales y los puntos de sacrifico seran iguales a los descritos en el apartado anterior.

Activaciòn de cèlulas dendriticas: En nuestro modelo experimental una característica inmunopatogenica importante es el retraso en la activaciòn de las cèlulas dendriticas. Por lo tanto, la activacion temprana de estas cèlulas es una estrategia potencialmente util de inmunoterapia. Para lograr esto se utilizarà terapia gènica utilizando adenovirus recombinantes que expresan factor estimulante de colonias granulocitico-macrofagico (SCF-GM). La tècnica para la producción de los adenovirus recombinantes la tenemos estandarizada y publicada. Se administrara una sola dosis de rAD-CSF un dia antes de la infecciòn y después en los dias 3, 7, 14, 21, 28 y 60 se sacrificaran grupos de 8 animales y se compararàn con ratones control que reciben por via intratraqueal el adenovirus desnudo. Los parámetros por evaluar seràn la sobrevida, carga bacilar (UFC), histopatologia/morfometrìa, expresión de citocinas por RT-PCR en tiempo real y citometrìa de flujo en suspensiones celulares obtenidas de los pulmones determinando la cantidad y porcentaje de celulas dendriticas activadas (CD-11c, MHC-II, CD-80). Debido a que después del dia 21 de infecciòn las cèlulas dendriticas activadas disminuyen progresivamente, un segundo experimento serà la administración del rAd-CSF por via intratraqueal a partir del segundo mes postinfecciòn y evaluando su eficiencia como se describiò anteriormente. Otra estrategia sera la utilización de anticuerpos fusiòn que reconocen por su porciòn Fab al receptor de membrana DEC-205 y que en su porciòn FC tienen unida la proteina ESAT-6 de *M. tuberculosis*. DEC-205 se expresa en células dendríticas y participa en la presentación antigénica El antìgeno ESAT-6 induce gran parte de la respuesta inmune celular contra *M. tuberculosis*. Para determinar la eficiencia in-vivo del anticuerpo, se usaran ratones BALB/c y C57BL/6 a los cuales se les administran 5µg de DEC-ESAT con 10µg de polyI:C por vía subcutánea o intranasal. Los grupos control recibiran polyI:C, PBS, y DEC-ESAT. Los ratones se sacrificaran en las semanas 2 y 5 después de la administración del anticuerpo fusión, de cada ratón se haran suspensiones celulares del bazo, los ganglios poplíteos y mediastinales y el pulmón (sitio de infección). Las cèlulas se estimularan in-vitro con antigenos totales, la proteína ESAT-6 completa o una librería de péptidos traslapados de esta. Después se realizara citometrìa de flujo para cèlulas CD4 y CD8 con tinciòn intracelular para detectar IFN.

Estimulaciòn de la producción de interleucina 12 (IL-12). La IL-12 es una citocina crucial en la diferenciación de células Th1 productoras de IFN. Para el tratamiento en modelos experimentales y pacientes es necesaria la administración de IL-12 recombinante tres veces por semana, lo cual es muy costoso. Nuestra estrategia sera la administracion de adenovirus recombinantes que expresàn IL-12 en una sola dosis intranasal e intratraqueal, tanto de forma profilactica (un dia antes de la infecciòn intratraqueal, como de forma terapeutica (dos meses después de la administración de *M. tuberculosis* drogosensible y MDR por via intratraqueal). La eficiencia terapeutica serà

determinada por curvas de sobrevida, cuantificaciòn de UFC, histologìa/morfometrìa y expresión de citocinas.

Inducción de la producción de oxido nitrico. Los macrofagos fagocitan y destruyen a la bacteria, seleccionan y presentan antigenos para activar a los linfocitos T y regulan la respuesta inmunològica a traves de la producción de diversas citocinas. Los macròfagos activados destruyen a las micobacterias por la producción de radicales libres de oxigeno y de nitrógeno. Otra forma potencialmente util de inmunoterapia es el inducir alta producción de oxido nitrico. El grupo de la Universidad de Salamanca ha producido mas de 200 compuestos derivados de amino-alcoholes y diaminas, el compuesto diamino 8b incrementa significativamente la producción de nitritos en macrofagos no activados y los compuestos 6f y 7a lo hacen en macròfagos activados. Estos compuestos se probaran como agentes inmunoterapeuticos en la fase avanzada de nuestro modelo murino de tuberculosis progresiva, administrandolos por via intraperitoneal en dos dosis diferentes cada tercer dia, sacrificando ratones infectados con las cepas H37Rv y MDR en los dias 7, 15, 30 y 60 post-tratamiento y evaluando su capacidad terapeutica con los ensayos descritos.

Nuestra segunda estrategia es suprimir los efectos inmunes deletereos que contribuyen a la progresión de la tuberculosis: **Supresión de la actividad del TGF.** Esta citocina se produce mucho durante la fase progresiva de la enfermedad y recientemente informamos que su receptor soluble tipo III recombinante (betaglicano) fue muy eficiente terapéuticamente pero indujo inflamación excesiva, cuando ademàs del betaglicano se administrò un antinflamatorio (ac niflumico), se indujo una eficiente actividad inmunoterapeutica, En este proyecto estudiaremos su efecto en la tuberculosis producida por bacterias MDR

Promoción de apoptosis macrofàgica. La infección in-vitro de macròfagos con *M. tuberculosis* induce apoptosis y esta provoca la muerte de la bacteria. Durante la fase avanzada de la enfermedad hay muchos macrofagos resistentes a morir por apoptosis, permitiendo asi a la bacteria tener un habitat de larga vida. Para inducir apoptosis macrofagica se probaran bloqueadores de factores de transcripcion que previenen a esta, como el factor relacionado a hipoxia (HIF), que entre otras funciones induce la expresión de Bcl-2 previniendo la apoptosis de celulas inflamatorias. La molécula 2MD es un eficiente inactivador de HIF y se determinara su eficiencia terapeutica en ratones infectados con bacterias MDR, asi como su eficiencia de acortar la antibioticoterapia convencional, usando las tecnicas ya mencionadas.

Uso de anticuerpos especificos en contra de antigenos inmunodominantes en tuberculosis progresiva y latente. En modelos experimentales de nocardiosis se ha mostrado que la administración de anticuerpos de clase IgM producidos en contra de antigenos inmunodominantes son eficientes agentes inmunoterapeuticos. Hemos producido anticuerpos IgM policlonales en contra de antigenos inmunodominantes de M. tuberculosis, los cuales se administraran por via i.p en 3 dosis, una por semana, sacrificando grupos de animales después de 7, 14, 30 y 60 dias, al inicio de la infección o dos meses después, determinando su eficiencia con el mismo esquema descrito en los apartados anteriores.

Se ha publicado que la administración de anticuerpos monoclonales de clase IgA en contra de la proteina micobacteriana alfa cristalina y anticuerpos bloqueadores anti-IL4 en ratones Balb/c con tuberculosis activa tiene un significativo efecto terapeutico. La proteina alfa cristalina se sobrexpresa en infecciòn latente, por lo tanto es posible que la administración de los anticuerpos bloqueadores puedan prevenir la reactivaciòn, para estudiar esto usaremos nuestro modelo murino de infecciòn latente, el cual se basa en ratones hibridos F1 C57Bl y DBA, infectados por via intrataqueal con una dosis muy

baja (1000 bacterias) de M. tuberculosis H37Rv. La administración de corticosterona (3mg/lt) en el agua de bebida produce rapida reactivaciòn. Cuando la infecciòn latente sea estable (7 meses), administraremos a grupos de 20 animales la combinación del anticuerpo monoclonal IgA anti alfa cristalina, mas anticuerpos bloqueadores de IL-4. Otro grupo recibira solo los anticuerpos anti alfa-cristalina. El tercer grupo solo recibira los anticuerpos anti IL-4 y el grupo control recibira el vehículo. Después de un mes se administrara la corticosterona y se sacrificaran grupos de 5 ratones después de 7, 15, 30 y 60 dias de la ultima administración de los anticuerpos. Un pulmon, derecho o izquerdo, se destinara para estudio histològico y el otro para la cuantificaciòn de UFC.

8. METODOLOGÍA: CRITERIOS DE SELECCIÓN

a) Criterios de inclusión (considerar que no participen en otras investigaciones y anticoncepción en caso necesario)
b) Criterios de exclusión:
c) Criterios de eliminación (considerar embarazo en caso necesario

Criterios de inclusión: ratones machos Balb/c de 6 a 8 semanas de edad, SPF
Criterios de exclusión: Animales evidentemente enfermos
Criterios de eliminación: se sacrificaran por razones humanitarias a los animales que muestren signos evidentes de enfermedad muy avanzada (perdida excesiva de peso, tiraje intercostal, piloereccion generalizada, inmovilidad)

9. METODOLOGÍA: DESENLACES Y VARIABLES

a) Variable/desenlace principal a medir principal
b) Variables/desenlaces secundarias a medir
c) Frecuencia de las mediciones
d) Criterios de éxito y falla en caso necesario
e) Estrategia de análisis estadístico.
Cuando corresponda deben especificarse y fundamentarse las técnicas, aparatos y/o instrumentos (esto incluye equipos mecánicos/electrónicos/cibernéticos especiales, formatos de evaluación, cuestionarios, tablas de cotejo, etc.) que se utilizarán en la medición, señalando los criterios de validez, reproducibilidad y controles de calidad que se tengan de los mismos

Desenlace principal a medir: el nivel de eficacia terapeutica conferido por los diversos tratamientos en animales que se sacrifican por exsanguinacion previa anestesia con la administración peritoneal de pentobarbital sodico. Se determinara la carga bacilar pulmonar por cuantificacion de UFC, daño tisular por morfometria automatizada (porcentaje de neumonía) y expresión de citocinas por RT-PCR en tiempo real. La frecuencia de los sacrificios se describe en la metodología general
Criterio de éxito: Disminución significativa de la carga bacteriana y daño pulmonar en los pulmones de los animales tratados cuando se comparen con los animales controles, asi como expresión de citocinas predominantemente de tipo Th1. El analisis estadistico se realizará con la prueba de Kaplan Meier para la sobrevida y pruebas de anova y T para la carga bacteriana, daño histologico y expresión de citocinas comparando los grupos tratados con los controles
La tecnica de infeccion por inyeccion intratraqueal esta perfectamente estandarizada en nuestro laboratorio, es rapida y permite un excelente control de la dosis de bacterias.
La Cromatografía de líquidos de alta resolución se ha convertido en la mas popular y versátil de las técnicas analíticas modernas cuando esta acoplada a la espectrometría de

masas en tiempo real. Los sistemas cromatograficos de líquidos de alta resolución se utilizan actualmente en una amplia variedad de campos. Cada día se aumentan la confiabilidad de los datos analíticos. Nuestro protocolo de abordaje de caracterización molecular de biomoléculas del FT será inicialmente a través de la separación cromátográfica de componentes mediante la técnica de cromatografía líquida de alta resolución en fase reversa (RP-HPLC). Para la alta resolución de separación y aislamiento molecular a homogenidad total se usara un cromatógrafo bidimensional de líquidos de alta resolución para continuar con el desarrollo de procesamientos de muestras en grado preparativo, semipreparativo y analítico del FT, también este equipo nos permitirá demostrar controles de calidad de compuestos diversos, analizando impurezas y contaminantes. El espectrómetro de masa es un instrumento que permite analizar con gran precisión la composición de diferentes elementos químicos e isótopos atómicos, separando los núcleos atómicos en función de su relación masa-carga. Con frecuencia se acopla a un cromatógrafo de líquidos. El espectrómetro de masas mide razones carga-masa de iones, calentando un haz de luz de material del compuesto a analizar hasta vaporizarlo y ionizar los diferentes átomos. El haz de iones produce un patrón especifico en el detector que permite analizar el compuesto químico. Este instrumento se utiliza en investigación para el análisis de sustancias y mezclas complejas (FT).

10. RIESGOS Y BENEFICIOS DEL ESTUDIO

a) Molestias generadas por el estudio (en caso de tomas de sangre, anotar el número total de punciones, la cantidad de sangre por punción y/o total y la frecuencia de las punciones.)
b) Riesgos potenciales(presencia de complicaciones o efectos adversos, considerar interacciones medicamentosas, considerar efectos psicológicos de los métodos de evaluación, v.gr.:encuestas sobre temas sensibles)
c) Métodos de detección de los riesgos anticipados
d) Medidad de seguridad para el diagnóstico oportuno y prevención de dichos eventos
e) Procedimientos a seguir para resolverlos en caso de que se presenten
f) Beneficios directos esperados
g) Beneficios indirectos esperados
h) Ponderación general de riesgos contra beneficios del estudio propuesto

Los animales son anestesiados cuando se infectan y cuando son sacrificados, ya se menciono el criterio para sacrificarlos por razones humanitarias. Los animales se almacenan en grupos de 5 ratones por microaislador y se hace la limpieza cambiando la cama, alimento y agua dos veces por semana. Los riezgos potenciales para el personal que trabaja con estos animales son minimos pues contamos con campanas de seguridad biologica, sistema de aisladores con presion negativa y flujo de aire filtrado constante, uniformes quirurgicos y cubrebocas de alta seguridad, a todo los participantes se les entrena y los procedimientos de infeccion, sacrifico y cambio de los animales se realiza dentro de la campana. Los restos de los animales y desechos potencialmente contaminados se almacenan en bolsas especiales para ser posteriormente incinerados, con estas medidas no ha existido ningun accidente en mas de 15 años de trabajo, Los beneficios esperados son la caracterizacion de cuando menos 3 regimenes inmunoterapeuticos eficientes en su fase preclinica, la publicación de cuando menos 10 trabajos en revistas internacionales y la formación de al menos 3 estudiantes de postgrado. Los beneficios son mucho mayores que los riezgos, pues los modelos experimentales estan muy bien estandarizados y tenemos experimentos preliminares con resultados satisfactorios en la mayoria de las estrategias propuestas

a) Especificar costos (directos/indirectos, monetarios, en tiempo de participación, visitas/traslados) que la investigación genere para los sujetos del estudio (Especificar si las consultas, exámenes de laboratorio/gabinete y tratamientos médicos/quirúrgicos, generados con motivo del estudio serán o no cubiertos por el paciente/sujeto de investigación
b) Especificar las compensaciones que se ofrecerán (reposición de gastos incurridos por la participación en el estudio;v.gr.: pago de transporte, alimentación, estancia, etc).
c) Especificar los incentivos que se ofrecerán en caso que corresponda (se entiende incentivo como uin ofrecimiento o influencia que compete a realizar una acción sin que implique una desviación importante con nuestro plan general de vida; v.gr.:dar un libro por haber participado)
Nota: Una compensación/incentivo fuera de proporción se considera una actitud coercitiva.

Todos los costos seran cubiertos por el CONACYT, este es un proyecto multigrupal de ciencia básica que contempla la compra de dos equipos grandes, uno de estos es para el Instituto Nacional de Psiquiatria. No existen compensaciones economicas para ninguno de los participantes.

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FIRMAS DE AGOSTO

VERONICA GONZALEZ — DEPOSITOS E INFORMES FINANCIEROS DEL FONDO SECTORIAL DE INVESTIGACION EN LA EDUCACION.

rdar) Volver a Buscar) Siguiente en Lista) Anterior en Lista) Notificar)

11 DIC/2014 f"

1. **TÍTULO DEL PROYECTO: Diseño y prueba de nuevos regimenes inmunoterapeuticos para tratar a la tuberculosis**

2. INVESTIGADORES

2a. IDENTIFICACIÓN DE INVESTIGADORES

Nombre, firma y adscripción de cada uno de los investigadores participantes. El investigador principal deberá ser un profesional adscrito a la Institución(médico de base o investigador y no un alumno de curso, residente, pasante, interno,etc).

INVESTIGADOR	POSICIÓN INSTITUCIONAL	POSICIÓN EN EL PROYECTO	EXTENSIÓN	CORREO-E
Rogelio Hdez I	Inv CM-F	Invest principa	2194	rhpando@quetz
Diana Aguilar	Inv CM-D	Invest Asocia	2194	aguilarleon@hoi
Hector Orozc	Inv CM-D	Invest Asocia	2194	horozcoe@yah
Leonardo Ort	Tecnico	Tec bioterio	2194	

2b. PERTINENCIA DEL GRUPO DE INVESTIGADORES CON RESPECTO DEL PROYECTO

Brevemente describa las calificaciones del grupo investigador con respecto del proceso de investigación científica en general y con respecto del proyecto presentado (v.gr.:grado académico, experiencia laboral, miembro del sistema de investigadores de los INS, del SNI, etc).

El Dr Hernández Pando es patologo y doctor en inmunologia, durante 20 años ha realizado investigación en inmunopatologia experimental de la tuberculosis, es investigador F y SNI-3. La Dra Aguilar Leon es QFB y doctora en biología experimental, experta en el manejo de animales de experimentación y diversas tecnicas inmunologicas y moleculares, es investigadora D y SNI-1. El Dr Hector Orozco es médico, experto en manejo de animales de experimentación y diversas tecnicas morfologicas, es investigador C. El Sr L Ortiz es el unico tecnico de biotero en el pais con experiencia en el cuidado de animales de experimentación infectados con germenes patogenos. Los Dres Aguilar y Orozco realizaran el trabajo experimental supervisando a los estudiantes asociados al proyecto

3. INSTITUCIONES PARTICIPANTES

Nombre y dirección de la o las instituciones participantes. Para estudios multicéntricos añadir los datos del centro

Instituto Nacional de Ciencias Medicas y Nutrición. Departamento de Patología, Seccion de Patología Experimental. Direccion: Vasco de Quiroga 15, Tlalpan. Telefono 54870900 ext2194. Investigador responsable y coordinador del proyecto: Dr Rogelio Hernandez Pando

Instituto Nacional de Psiquiatria. Periferico, Investigador responsable Dr Benito Antón

Universidad de Salamanca, Facultad de Farmacia Departamento de Quimica Farmaceutica. Direccion: Campus Miguel de Unamuno, 37007 Salamanca España.

PATROCINIO

4b. ORGANISMOS PATROCINADORES

Nombre, dirección y teléfono de la o las organizaciones, instituciones o laboratorios que aportarán recursos

Consejo Nacional de Ciencia y Tecnología. Av Insurgentes Sur 1582. Col Credito Constructor. Benito Juarez 03940, Mexico DF

MARCO TEÓRICO

Explicar detalladamente los fundamentos disponibles a la fecha en los que se basa el estudio que se propone(sentido biológico, datos de experimentos en animales o en humanos):

a) Antecedentes:

b) Definición del problema:

c) Justificación:

La tuberculosis es una importante enfermedad infecto-contagiosa que afecta principalmente a los pulmones y produce profundas alteraciones en el sistema inmunològico. El agente causal *Mycobacterium tuberculosis* es un microorganismo patógeno intracelular facultativo que puede producir tanto enfermedad progresiva como infección latente asintomática. Generalmente la primoinfección tuberculosa se lleva a cabo en los pulmones durante la niñez y en la mayoría de los casos es controlada por el sistema inmunológico, solo el 10 % de estos casos sufrirán la enfermedad progresiva En esta primoinfección tuberculosa, aun en aquellos casos que son controlados por el sistema inmunológico, no todas las bacterias son eliminadas, algunos bacilos permanecen en los tejidos en un estado quiescente con poca o nula actividad reproductiva por el resto de la vida del individuo infectado, lo cual se denomina infección latente. Actualmente se calcula que anualmente esta enfermedad produce 2 millones de defunciones a nivel mundial, con 8 millones de nuevos casos y un tercio de la humanidad tiene infecciòn latente, lo cual la convierte en la enfermedad infecto-contagiosa más relevante a nivel mundial. Un aspecto de gran importancia es que a pesar de que actualmente es curable, se necesita de una terapia combinada de 4 antibióticos por 6 a 9 meses, lo que redunda en una alta tasa de abandono del tratamiento, lo que promueve recaidas y el surgimiento de cepas resistentes a múltiples antibióticos (MDR, XDR), lo cual complica aun mas el tratamiento al incrementar su costo y generar mas efectos nocivos para el paciente, mientras que para las cepas XDR no existe actualmente forma de curarlas, por esta razon y por la necesidad de acortar el largo tratamiento de la tuberculosis drogosensible existe interès en desarrollar nuevas formas de tratamiento basadas en potenciar la respuesta inmune protectora o abatir la respuesta inmune deletèrea, a lo cual se le denomina genericamente inmunoterapia. El objetivo de este proyecto es diseñar y probar nuevos esquemas inmunoterapeuticos basados sobre todo en el conocimiento generado por nuestro grupo sobre la inmunopatología de la tuberculosis utilizando modelos experimentales murinos.

6a. HIPÓTESIS

Definido como un enunciado comprobable acerca de la relación entre una variable dependiente y una variable independiente.

La estimulaciòn de la respuesta Th-1 y de la activaciòn macrofàgica, asi como la inhibición de la respuesta Th-2 y de otros factores inmunosupresores tendra un efecto terapeutico significativo en la tuberculosis producida por bacterias drogosensibles y multidrogoresistentes y permitira ademas acortar el tiempo de tratamiento con antibioticos convencionales.

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7. METODOLOGÍA: DISEÑO GENERAL

Describir el diseño general del estudio y, si es pertinente, especificar los siguientes puntos:

a) Diseño del estudio: describir si es aleatorio/no aleatorio, controlado, de cohorte, tipo de cegamiento(doble-ciego,simple), tipo de controles(placebo, medicamento activo), periodo de lavado.

b) Descripción de la maniobra o intervención

c) Tamaño de la muestra (# de pacientes a incluir; justificar el cálculo)

d) Mecanismos de asignación del tratamiento

e) Grupos de tratamiento

f) Duración del seguimiento individual

Reactivar respuesta protectora (TH1/macrofagos activados): **Administración de micobacterias sapròfitas.** La micobacteria saprofita *M.vaccae* puede ser un eficiente agente inmunoterapeutico porque activa eficientemente a las cèlulas protectoras Th1 y CD-8+ y suprime la activaciòn de linfocitos Th-2. Estudiaremos el efecto de este sapròfito en ratones infectados con una cepa MDR y la cepa drogosensible H37Rv, asi como su eficiencia como agente coadyuvante a la quimioterapia. Para esto la cepas de *M. tuberculosis* H37Rv y MDR seràn cultivadas en el medio lìquido PBY, después de un mes, las micobacterias son separadas y ajustadas a 2.5×10^5 en 100 µl de amortiguador PBS y mantenidas a –70°C hasta su uso. Ratones machos BALB/c de 6-8 semanas de edad son anestesiados con 56 mg/kg de pentotal sòdico administrado por via peritoneal, la traquea se expone quirúrgicamente y 2.5×10^5 bacterias viables suspendidas en 100 µl de PBS se inyectan usando una jeringa de insulina. Posteriormente la insiciòn quirùrgica es suturada con seda esteril. Los ratones así infectados se almacenan en cajas con microaisladores conectados a un sistema de presiòn negativa. Todo este procedimiento y los sacrificios posteriores se realizan en campanas de seguridad biològica. Al dia 60 postinfecciòn, los ratones sobrevivientes se distribuyen al azar en seis grupos experimentales. Grupos de 20 ratones tuberculosos en dos experimentos diferentes, se tratarán con una suspensión de *M. vaccae* a dosis de 100 µg en 100µl administrado por sonda nasogastrica. Para evaluar la capacidad de M.vaccae para acortar el tiempo de tratamiento con antibioticoterapia convencional, un grupo de 20 animales se tratara con antibioticoterapia convencional, constituida por: rifampicina (10 mg/kg), isoniazida (10 mg/kg), y pirazinamida (30 mg/kg), administrados diariamente a travez de sonda intragástrica, el segundo grupo se tratara con antibioticos mas M. vaccae administrado por sonda intragastrica una vez por semana. El tercer grupo se tratarà solamente con M. vaccae y el grupo control exclusivamente recibirà la soluciòn vehiculo. El quinto grupo corresponde a ratones infectados con el aislado clinico MDR, el cual se tratara con M. vaccae y ultimo grupo es el grupo control infectado con el aislado MDR que recibe solamente el vehículo. Cinco ratones por cada grupo se sacrificaran los dias 7, 15, 30 y 60 dias posteriores al inicio del tratamiento. Se tendran 10 animales mas de cada grupo que se se dejaran sin sacrificarlos con el fin de determinar sobreviva. Se realizaran dos experimentos completos por separado para determinar reproducibilidad. Para evaluar la eficiencia del regimen inmunoterapeutico ademas de curvas de sobrevida, se realizara estudio histològico/morfomètrico (porcentaje de area pulmonar afectada por neumonía), mediciòn de la carga bacilar pulmonar por determinación de unidades formadoras de colonia (UFC) y la expresión genètica de las citocinas IFN, TNF, IL-4 y la enzima iNOS por RT-PCR en tiempo real, de acuerdo a las tècnicas ya estandarizadas en nuestro laboratorio.

Uso y caracterizaciòn molecular del Factor de Transferencia (FT): Los FT son productos dializables de bajo peso molecular obtenidos de cèlulas inmunològicas, los cuales son capaces de transferir inmunidad mediada por cèlulas (IMC) de donadores inmunizados a sujetos receptores no sensibilizados. Actualmente no se conoce la estructura quìmica ni los mecanismos moleculares de acciòn del FT, varios estudios han mostrado que le FT es un conjunto de proteinas de bajo peso molecular. Nuestro objetivo es la caracterizaciòn molecular del FT y determinar su efecto terapéutico en el modelo murino de tuberculosis, para el primer objetivo se usara la cromatografía de líquidos de alta resolución acoplado a la espectrometría de masas en tiempo real; pare evaluar su efecto terapeutico, grupos de animales se trataran con 1µg de cada una de las proteinas aisladas suspendida en 50µl de sol salina por via intraperitoneal, una vez por semana, los grupos experimentales y los puntos de sacrifico seran iguales a los descritos en el apartado anterior.

Activaciòn de cèlulas dendriticas: En nuestro modelo experimental una característica inmunopatogenica importante es el retraso en la activaciòn de las cèlulas dendriticas. Por lo tanto, la activacion temprana de estas cèlulas es una estrategia potencialmente util de inmunoterapia. Para lograr esto se utilizarà terapia gènica utilizando adenovirus recombinantes que expresan factor estimulante de colonias granulocitico-macrofagico (SCF-GM). La tècnica para la producción de los adenovirus recombinantes la tenemos estandarizada y publicada. Se administrara una sola dosis de rAD-CSF un dia antes de la infecciòn y después en los dias 3, 7, 14, 21, 28 y 60 se sacrificaran grupos de 8 animales y se compararàn con ratones control que reciben por via intratraqueal el adenovirus desnudo. Los parámetros por evaluar seràn la sobrevida, carga bacilar (UFC), histopatologia/morfometrìa, expresión de citocinas por RT-PCR en tiempo real y citometrìa de flujo en suspensiones celulares obtenidas de los pulmones determinando la cantidad y porcentaje de celulas dendriticas activadas (CD-11c, MHC-II, CD-80). Debido a que después del dia 21 de infecciòn las cèlulas dendriticas activadas disminuyen progresivamente, un segundo experimento serà la administración del rAd-CSF por via intratraqueal a partir del segundo mes postinfecciòn y evaluando su eficiencia como se describiò anteriormente. Otra estrategia sera la utilización de anticuerpos fusiòn que reconocen por su porciòn Fab al receptor de membrana DEC-205 y que en su porciòn FC tienen unida la proteina ESAT-6 de *M. tuberculosis*. DEC-205 se expresa en células dendríticas y participa en la presentación antigénica El antìgeno ESAT-6 induce gran parte de la respuesta inmune celular contra *M. tuberculosis*. Para determinar la eficiencia in-vivo del anticuerpo, se usaran ratones BALB/c y C57BL/6 a los cuales se les administran 5µg de DEC-ESAT con 10µg de polyI:C por vía subcutánea o intranasal. Los grupos control recibiran polyI:C, PBS, y DEC-ESAT. Los ratones se sacrificaran en las semanas 2 y 5 después de la administración del anticuerpo fusión, de cada ratón se haran suspensiones celulares del bazo, los ganglios poplíteos y mediastinales y el pulmón (sitio de infección). Las cèlulas se estimularan in-vitro con antigenos totales, la proteína ESAT-6 completa o una librería de péptidos traslapados de esta. Después se realizara citometrìa de flujo para cèlulas CD4 y CD8 con tinciòn intracelular para detectar IFN.

Estimulaciòn de la producción de interleucina 12 (IL-12). La IL-12 es una citocina crucial en la diferenciación de células Th1 productoras de IFN. Para el tratamiento en modelos experimentales y pacientes es necesaria la administración de IL-12 recombinante tres veces por semana, lo cual es muy costoso. Nuestra estrategia sera la administracion de adenovirus recombinantes que expresàn IL-12 en una sola dosis intranasal e intratraqueal, tanto de forma profilactica (un dia antes de la infecciòn intratraqueal, como de forma terapeutica (dos meses después de la administración de *M. tuberculosis* drogosensible y MDR por via intratraqueal). La eficiencia terapeutica serà

determinada por curvas de sobrevida, cuantificaciòn de UFC, histologìa/morfometrìa y expresión de citocinas.

Inducción de la producción de oxido nitrico. Los macrofagos fagocitan y destruyen a la bacteria, seleccionan y presentan antigenos para activar a los linfocitos T y regulan la respuesta inmunològica a traves de la producción de diversas citocinas. Los macròfagos activados destruyen a las micobacterias por la producción de radicales libres de oxigeno y de nitrógeno. Otra forma potencialmente util de inmunoterapia es el inducir alta producción de oxido nitrico. El grupo de la Universidad de Salamanca ha producido mas de 200 compuestos derivados de amino-alcoholes y diaminas, el compuesto diamino 8b incrementa significativamente la producción de nitritos en macrofagos no activados y los compuestos 6f y 7a lo hacen en macròfagos activados. Estos compuestos se probaran como agentes inmunoterapeuticos en la fase avanzada de nuestro modelo murino de tuberculosis progresiva, administrandolos por via intraperitoneal en dos dosis diferentes cada tercer dia, sacrificando ratones infectados con las cepas H37Rv y MDR en los dias 7, 15, 30 y 60 post-tratamiento y evaluando su capacidad terapeutica con los ensayos descritos.

Nuestra segunda estrategia es suprimir los efectos inmunes deletereos que contribuyen a la progresión de la tuberculosis: **Supresión de la actividad del TGF.** Esta citocina se produce mucho durante la fase progresiva de la enfermedad y recientemente informamos que su receptor soluble tipo III recombinante (betaglicano) fue muy eficiente terapéuticamente pero indujo inflamación excesiva, cuando ademàs del betaglicano se administrò un antinflamatorio (ac niflumico), se indujo una eficiente actividad inmunoterapeutica, En este proyecto estudiaremos su efecto en la tuberculosis producida por bacterias MDR

Promoción de apoptosis macrofàgica. La infección in-vitro de macròfagos con *M. tuberculosis* induce apoptosis y esta provoca la muerte de la bacteria. Durante la fase avanzada de la enfermedad hay muchos macrofagos resistentes a morir por apoptosis, permitiendo asi a la bacteria tener un habitat de larga vida. Para inducir apoptosis macrofagica se probaran bloqueadores de factores de transcripcion que previenen a esta, como el factor relacionado a hipoxia (HIF), que entre otras funciones induce la expresión de Bcl-2 previniendo la apoptosis de celulas inflamatorias. La molécula 2MD es un eficiente inactivador de HIF y se determinara su eficiencia terapeutica en ratones infectados con bacterias MDR, asi como su eficiencia de acortar la antibioticoterapia convencional, usando las tecnicas ya mencionadas.

Uso de anticuerpos especificos en contra de antigenos inmunodominantes en tuberculosis progresiva y latente. En modelos experimentales de nocardiosis se ha mostrado que la administración de anticuerpos de clase IgM producidos en contra de antigenos inmunodominantes son eficientes agentes inmunoterapeuticos. Hemos producido anticuerpos IgM policlonales en contra de antigenos inmunodominantes de M. tuberculosis, los cuales se administraran por via i.p en 3 dosis, una por semana, sacrificando grupos de animales después de 7, 14, 30 y 60 dias, al inicio de la infección o dos meses después, determinando su eficiencia con el mismo esquema descrito en los apartados anteriores.

Se ha publicado que la administración de anticuerpos monoclonales de clase IgA en contra de la proteina micobacteriana alfa cristalina y anticuerpos bloqueadores anti-IL4 en ratones Balb/c con tuberculosis activa tiene un significativo efecto terapeutico. La proteina alfa cristalina se sobrexpresa en infecciòn latente, por lo tanto es posible que la administración de los anticuerpos bloqueadores puedan prevenir la reactivaciòn, para estudiar esto usaremos nuestro modelo murino de infecciòn latente, el cual se basa en ratones hibridos F1 C57Bl y DBA, infectados por via intrataqueal con una dosis muy

baja (1000 bacterias) de M. tuberculosis H37Rv. La administración de corticosterona (3mg/lt) en el agua de bebida produce rapida reactivaciòn. Cuando la infecciòn latente sea estable (7 meses), administraremos a grupos de 20 animales la combinación del anticuerpo monoclonal IgA anti alfa cristalina, mas anticuerpos bloqueadores de IL-4. Otro grupo recibira solo los anticuerpos anti alfa-cristalina. El tercer grupo solo recibira los anticuerpos anti IL-4 y el grupo control recibira el vehículo. Después de un mes se administrara la corticosterona y se sacrificaran grupos de 5 ratones después de 7, 15, 30 y 60 dias de la ultima administración de los anticuerpos. Un pulmon, derecho o izquerdo, se destinara para estudio histològico y el otro para la cuantificaciòn de UFC.

8. METODOLOGÍA: CRITERIOS DE SELECCIÓN

a) Criterios de inclusión (considerar que no participen en otras investigaciones y anticoncepción en caso necesario)
b) Criterios de exclusión:
c) Criterios de eliminación (considerar embarazo en caso necesario

Criterios de inclusión: ratones machos Balb/c de 6 a 8 semanas de edad, SPF
Criterios de exclusión: Animales evidentemente enfermos
Criterios de eliminación: se sacrificaran por razones humanitarias a los animales que muestren signos evidentes de enfermedad muy avanzada (perdida excesiva de peso, tiraje intercostal, piloereccion generalizada, inmovilidad)

9. METODOLOGÍA: DESENLACES Y VARIABLES

a) Variable/desenlace principal a medir principal
b) Variables/desenlaces secundarias a medir
c) Frecuencia de las mediciones
d) Criterios de éxito y falla en caso necesario
e) Estrategia de análisis estadístico.
Cuando corresponda deben especificarse y fundamentarse las técnicas, aparatos y/o instrumentos (esto incluye equipos mecánicos/electrónicos/cibernéticos especiales, formatos de evaluación, cuestionarios, tablas de cotejo, etc.) que se utilizarán en la medición, señalando los criterios de validez, reproducibilidad y controles de calidad que se tengan de los mismos

Desenlace principal a medir: el nivel de eficacia terapeutica conferido por los diversos tratamientos en animales que se sacrifican por exsanguinacion previa anestesia con la administración peritoneal de pentobarbital sodico. Se determinara la carga bacilar pulmonar por cuantificacion de UFC, daño tisular por morfometria automatizada (porcentaje de neumonía) y expresión de citocinas por RT-PCR en tiempo real. La frecuencia de los sacrificios se describe en la metodología general
Criterio de éxito: Disminución significativa de la carga bacteriana y daño pulmonar en los pulmones de los animales tratados cuando se comparen con los animales controles, asi como expresión de citocinas predominantemente de tipo Th1. El analisis estadistico se realizará con la prueba de Kaplan Meier para la sobrevida y pruebas de anova y T para la carga bacteriana, daño histologico y expresión de citocinas comparando los grupos tratados con los controles
La tecnica de infeccion por inyeccion intratraqueal esta perfectamente estandarizada en nuestro laboratorio, es rapida y permite un excelente control de la dosis de bacterias.
La Cromatografía de líquidos de alta resolución se ha convertido en la mas popular y versátil de las técnicas analíticas modernas cuando esta acoplada a la espectrometría de

masas en tiempo real. Los sistemas cromatograficos de líquidos de alta resolución se utilizan actualmente en una amplia variedad de campos. Cada día se aumentan la confiabilidad de los datos analíticos. Nuestro protocolo de abordaje de caracterización molecular de biomoléculas del FT será inicialmente a través de la separación cromátográfica de componentes mediante la técnica de cromatografía líquida de alta resolución en fase reversa (RP-HPLC). Para la alta resolución de separación y aislamiento molecular a homogenidad total se usara un cromatógrafo bidimensional de líquidos de alta resolución para continuar con el desarrollo de procesamientos de muestras en grado preparativo, semipreparativo y analítico del FT, también este equipo nos permitirá demostrar controles de calidad de compuestos diversos, analizando impurezas y contaminantes. El espectrómetro de masa es un instrumento que permite analizar con gran precisión la composición de diferentes elementos químicos e isótopos atómicos, separando los núcleos atómicos en función de su relación masa-carga. Con frecuencia se acopla a un cromatógrafo de líquidos. El espectrómetro de masas mide razones carga-masa de iones, calentando un haz de luz de material del compuesto a analizar hasta vaporizarlo y ionizar los diferentes átomos. El haz de iones produce un patrón especifico en el detector que permite analizar el compuesto químico. Este instrumento se utiliza en investigación para el análisis de sustancias y mezclas complejas (FT).

10. RIESGOS Y BENEFICIOS DEL ESTUDIO

a) Molestias generadas por el estudio (en caso de tomas de sangre, anotar el número total de punciones, la cantidad de sangre por punción y/o total y la frecuencia de las punciones.)
b) Riesgos potenciales(presencia de complicaciones o efectos adversos, considerar interacciones medicamentosas, considerar efectos psicológicos de los métodos de evaluación, v.gr.:encuestas sobre temas sensibles)
c) Métodos de detección de los riesgos anticipados
d) Medidad de seguridad para el diagnóstico oportuno y prevención de dichos eventos
e) Procedimientos a seguir para resolverlos en caso de que se presenten
f) Beneficios directos esperados
g) Beneficios indirectos esperados
h) Ponderación general de riesgos contra beneficios del estudio propuesto

Los animales son anestesiados cuando se infectan y cuando son sacrificados, ya se menciono el criterio para sacrificarlos por razones humanitarias. Los animales se almacenan en grupos de 5 ratones por microaislador y se hace la limpieza cambiando la cama, alimento y agua dos veces por semana. Los riezgos potenciales para el personal que trabaja con estos animales son minimos pues contamos con campanas de seguridad biologica, sistema de aisladores con presion negativa y flujo de aire filtrado constante, uniformes quirurgicos y cubrebocas de alta seguridad, a todo los participantes se les entrena y los procedimientos de infeccion, sacrifico y cambio de los animales se realiza dentro de la campana. Los restos de los animales y desechos potencialmente contaminados se almacenan en bolsas especiales para ser posteriormente incinerados, con estas medidas no ha existido ningun accidente en mas de 15 años de trabajo, Los beneficios esperados son la caracterizacion de cuando menos 3 regimenes inmunoterapeuticos eficientes en su fase preclinica, la publicación de cuando menos 10 trabajos en revistas internacionales y la formación de al menos 3 estudiantes de postgrado. Los beneficios son mucho mayores que los riezgos, pues los modelos experimentales estan muy bien estandarizados y tenemos experimentos preliminares con resultados satisfactorios en la mayoria de las estrategias propuestas

a) Especificar costos (directos/indirectos, monetarios, en tiempo de participación, visitas/traslados) que la investigación genere para los sujetos del estudio (Especificar si las consultas, exámenes de laboratorio/gabinete y tratamientos médicos/quirúrgicos, generados con motivo del estudio serán o no cubiertos por el paciente/sujeto de investigación
b) Especificar las compensaciones que se ofrecerán (reposición de gastos incurridos por la participación en el estudio;v.gr.: pago de transporte, alimentación, estancia, etc).
c) Especificar los incentivos que se ofrecerán en caso que corresponda (se entiende incentivo como uin ofrecimiento o influencia que compete a realizar una acción sin que implique una desviación importante con nuestro plan general de vida; v.gr.:dar un libro por haber participado)
Nota: Una compensación/incentivo fuera de proporción se considera una actitud coercitiva.

Todos los costos seran cubiertos por el CONACYT, este es un proyecto multigrupal de ciencia básica que contempla la compra de dos equipos grandes, uno de estos es para el Instituto Nacional de Psiquiatria. No existen compensaciones economicas para ninguno de los participantes.

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"010/2014"

Titulo: Diseño y prueba de nuevos regimenes inmunoterapeuticos para tratar a la tuberculosis

Resumen

La tuberculosis es una enfermedad infectocontagiosa de gran importancia clinica y epidemiológica, la cual ha sido declarada por la OMS como una emergencia mundial debido a que causa dos millones de muertes al año y un tercio de la inmunidad se encuentra en estado de infección latente. Debido a que su tratamiento requiere del uso de cuando menos 3 antibióticos por un largo periodo de tiempo, existen importantes problemas de abandono y en consecuencia recaídas o emergencia de bacterias multidrogoresistentes, es por esto que es necesario acortar el tiempo de tratamiento o diseñar nuevas formas de terapia para la enfermedad producida por cepas multidrogoresistentes. Una alternativa potencial es la inmunoterapia. En este proyecto se proponen diferentes estrategias inmunoterapeuticas que están fundamentadas en los resultados obtenidos por nuestro grupo con el uso de modelos experimentales de tuberculosis progresiva y latente en murinos. De hecho, la prueba de la eficacia de los nuevos regimenes inmunoterapeuticos se basa también en el uso de ambos modelos. Nuestra hipótesis es que la estimulación de la respuesta inmunológica protectora (actividad de linfocitos Th-1 y macrófagos) durante la fase avanzada de la tuberculosis progresiva o la supresión de la actividad inmunológica que contribuye con la progresión de la enfermedad activa (actividad de linfocitos Th2, sobreproducción de moléculas supresoras de la inmunidad celular), coadyuvara con mejorar significativamente el tratamiento convencional con antibióticos y también permitirá un mejor control de la enfermedad por bacterias multidrogoresistentes. Nuestro objetivo principal es aportar nuevos tratamientos fundamentados en la regulación del sistema inmune que permitan un mejor control de esta importante enfermedad infecciosa, para lo cual se reúnen 3 grupos de investigación totalmente consolidados, con funciones, experiencia y actividades diferentes pero totalmente complementarias y bien integradas. El grupo del Dr. San Feliciano de la Universidad de Salamanca España tiene un largo historial científico en el diseño y producción de nuevos fármacos con diversas actividades, entre los cuales se encuentran medicamentos inmunomoduladores con actividad estimulante de linfocitos T, promotores de producción de oxido nítrico y activación de macrófagos y supresores de la síntesis de prostaglandinas. El grupo del Dr. Antón del Instituto Nacional de Psiquiatría tiene una amplia experiencia en la caracterización bioquímica de moléculas con gran potencialidad biotecnológica y el grupo del Dr. Hernández Pando del Instituto Nacional de Ciencias Medicas y Nutrición, que tiene una larga experiencia en el desarrollo de modelos experimentales para estudiar los mecanismos inmunológicos que participan en la protección y en la progresión y daño tisular producido por M. tuberculosis en el pulmón. El objetivo final es aportar cuando menos 3 regimenes inmunoterapeuticos que sean patentables y de gran interés para continuar con ensayos clínicos, además de la publicación de cuando menos 10 trabajos en revistas internacionales y la preparación de al menos 5 maestros o doctores en investigación biomédica básica.

Antecedentes

La tuberculosis es una importante enfermedad infecto-contagiosa que afecta principalmente a los pulmones y produce profundas alteraciones en el sistema inmunológico (1). El agente causal *Mycobacterium tuberculosis* es un microorganismo patógeno intracelular facultativo que puede producir tanto enfermedad progresiva como infección latente asintomático (1). Generalmente la infección inicial o primoinfección tuberculosa se lleva a cabo en los pulmones durante la niñez y en la mayoría de los casos es controlada por el sistema inmunológico, solo el 10 % de estos casos sufrirán la enfermedad progresiva (2). Es interesante que en esta primoinfección tuberculosa, aun en aquellos casos que son controlados por el sistema inmunológico, no todas las bacterias son eliminadas, algunos bacilos permanecen en los tejidos en estado quiescente con poca o nula actividad reproductiva por el resto de la vida del individuo infectado (2). Este estado infeccioso se denomina infección latente, es clínicamente asintomático y solo se manifiesta por los resultados positivos de la prueba de hipersensibilidad cutánea a la tuberculina (PPD) o por un estudio radiográfico del tórax que muestre nódulos fibróticos o cicatrices calcificadas indicativas de primo-infección tuberculosa resuelta (2).

Actualmente se calcula que anualmente esta enfermedad produce 2 millones de defunciones a nivel mundial, con 8 millones de nuevos casos y un tercio de la humanidad tiene infección latente, lo cual la convierte en la enfermedad infecto-contagiosa más relevante a nivel mundial (3). Un aspecto de gran importancia es que a pesar de que actualmente es curable, se necesita de una terapia combinada de 4 antibióticos por 6 a 9 meses, lo que redunda en una alta tasa de abandono del tratamiento, por falta de adhesión correcta al régimen o debido a efectos secundarios de los medicamentos. Esto situación ha promovido recaídas y el surgimiento de cepas resistentes a múltiples antibióticos (MDR) o incluso extensamente resistentes a antibióticos (XDR-TB), lo cual complica aun mas el tratamiento al incrementar su costo y generando mas efectos nocivos para el paciente, mientras que para las cepas XDR no existe actualmente forma de curarlas (4). Es precisamente por la emergencia creciente de cepas MDR y XDR y por la necesidad de acortar el largo tratamiento de la tuberculosis drogosensible, que existe interés en desarrollar nuevas formas de tratamiento basadas en potenciar la respuesta inmune protectora o abatir la respuesta inmune deletérea, a lo cual se le denomina genéricamente inmunoterapia [5].

Después del descubrimiento del agente causal de la tuberculosis, Roberto Koch intentó curar la enfermedad por medio de inmunoterapia, administrando filtrados de cultivo de la bacteria. Otros investigadores como Spahlinger [6] y Jousset [7], también lo intentaron administrando antisueros producidos en animales. La administración de filtrados de cultivo produjo necrosis extensa de los tejidos afectados (fenómeno de Koch) y tuvo que abandonarse [8]. Los resultados obtenidos por Spahlinger o Jousset son difíciles de interpretar retrospectivamente y debido al advenimiento de antibióticos eficaces que rápidamente generaron confianza en su uso, los esfuerzos para desarrollar la inmunoterapia fueran abandonados. Sin embargo en los últimos 12 años varios grupos de investigación, incluido el nuestro, han desarrollado novedosos esquemas inmunoterapeuticos con muy buenos resultados en animales de experimentación, ya sea coadyuvando el tratamiento con antibióticos [5] o manipulando la respuesta de inmunidad celular. El objetivo de este proyecto es diseñar y probar nuevos esquemas inmunoterapeuticos basados en lo que se conoce sobre la respuesta inmune en la tuberculosis y sobre todo en el conocimiento generado por nuestro grupo sobre la inmunopatología de la tuberculosis utilizando modelos experimentales murinos.

Mecanismos inmunològicos en la tuberculosis

Respuestas protectoras

La inmunidad protectora en contra de *M. tuberculosis* depende de la participaciòn de linfocitos T cooperadores de tipo 1 (CD4+ Th1) los cuales secretan interferon gamma (IFN) y de esta forma activan a los macròfagos [9]. Mutaciones genèticas que afectan al sistema IL-12/IL-23/IFN-γ producen incremento en la suceptibilidad a desarrollar tuberculosis, lo cual confirma que la respuesta Th-1 es muy importante. Sin embargo, estas anormalidades sobre todo incrementan la suceptibilidad para desarrollar tuberculosis por bacterias poco virulentas [9], lo cual sugiere que otros mecanismos protectores deben de participar. De hecho, normalmente existe producción de IFN-γ en los pulmones de los pacientes con tuberculosis, lo mismo se observa en modelos experimentales en ratones en los que parece que esta citocina se puede también asociar al desarrollo de inmunopatología [10].

Existen también evidencias que señalan a las células T-CD8 citotoxicas como elementos importantes en la inmunidad protectora en tuberculosis. Ratones con anulación functional (knock-out) de los genes relacionados con presentación antigénica como los HLA clase 1 (TAP-1, CD8, β2m, MHC clase 1 cadena pesada), además de los experimentos de transfecciòn celular y de eliminación de linfocitos CD8+ han mostrado que sobre todo durante la fase avanzadas de la enfermedad, estas cèlulas son tambièn importantes en el control de la tuberculosis (11, 12). Los linfocitos CD8+ y otras cèlulas citoxicas (CTL) pudieran ser aun mas importantes en la enfermedad humana, pues estos tienen moleculas que los ratones no tienen como las granulosinas, las cuales son eficientes para destruir a la micobacteria [13]. Existen ademas otras subclases de linfocitos que reconocen antìgenos presentados por HLA-E o por el grupo de moleculas CD1 (CD1a, b & c) [11]. La granulisina se produce en linfocitos CD8+ humanos (y algunas CD4+), asi como en cèlulas NK, NKT y linfocitos γ/δ. Con frecuencia hay ademas perforinas, las cuales permiten el acceso de las granulisinas a las bacterias intracelulares [13].

Otro participante importante es la respuesta inmunològica inata, como lo son los peptidos antimicrobianos naturales tales como las defensinas y catelicidinas, las cuales son eficientes para destruir a la bacteria y son producidas por cèlulas epiteliales y macròfagos activados por el receptor TLR-2 y la vitamina D3 [14].

Respuesta suversiva

Ademàs de identificar los elementos celulares y moleculares que participan en la protecciòn inmunològica contra *M. tuberculosis*, las cuales pueden ser estimuladas para inducir una mayor eliminaciòn de la bacteria y asi constituirse en una modalidad de inmunoterapia. Tambièn es necesario identificar los mecanismos inmunològicos que suprimen la actividad Th-1 y/o citotoxica, facilitando de esta manera la proliferaciòn bacteriana y progresiòn de la enfermedad, pues su eventual supresión puede constituir otra modalidad de inmunoterapia. Algunas de estas son:

Respuesta Th2

Las citocinas IL4 e IL-13 producidas por los linfocitos Th-2 pueden suprimir la respuesta protectora Th-1 y ademas contribuir al desarrollo de necrosis y fibrosis [15, 16]. En efecto, la expresiòn de IL-4 esta aumentada en la tuberculosis activa y correlaciona con la gravedad de la enfermedad [17-19]. Es interesante que la expresiòn de IL-4 es particularmente elevada en pacientes tuberculosos de paises subdesarrollados, en donde ademàs la actividad protectora de la vacuna BCG no es

buena [20]. Aparentemente existen factores ambientales (por ejemplo la infestacion parasitaria [21]) y genèticos [22] que pueden potencialmente explicar esto. El polimorfismo de la quimiocina CCL2 que polariza la respuesta Th-2 es la responsable del incremento en la suceptibilidad a la tuberculosis en el 50% de los casos en nuestro pais y en Corea [22], y probablemente tambièn en otros paises. El mismo polimorfismo se asocial a asma en Europa [23]. La IL-4 tiene numerosos efectos potencialmente deletereos en tuberculosis, tales como inhibiciòn de la apoptosis de macròfagos infectados con micobacterias, incremento en la disponibilidad de hierro intracellular [24], e inhibiciòn de la expansion de celulas citotoxicas [25].

TGF-β

La producciòn excesiva del factor de transformacion tumoral beta (TGF-β) ha sido demostrada en tuberculosis humana y experimental y parece que tambièn tiene una gran importancia patogènica. Las cèlulas monucleares perifericas de pacientes tuberculosos liberan grandes cantidades de TGF-β [26, 27], y estas son aun mas abundantes en las lesiones pulmonares [26]. Los niveles sèricos de TGF-β estan sobre todo muy elevados en los pacientes con enfermedad avanzada [28], lo cual es particularmente caracteriztico en pacientes brasileños con tuberculosis producida por cepas MDR [29]. El lipido de la pared micobacteriana asociado a manosa lipoarabinomanan (ManLAM) es un potente inductor de TGF-β [30]. Los monocitos humanos y macròfagos alveolares producen TGF-β bioactivo al estimularlos con la micobacteria, de manera que el microrganismo no solo induce la producciòn sino tambien la activaciòn del TGF-β [31]. Celulas obtenidas por lavado bronquial de pacientes con tuberculosis producen espontaneamente y al mismo tiempo TGF-β y sus receptores TGF-βRI y TGF-βRII [32]. El uso de inhibidores naturales de esta citocina restaura la respuesta de celulas T de los pacientes *in vitro*, incrementando las funciones efectoras de los macròfagos en contra de la micobacteria (33). El TGF-β es importante en la tuberculosis porque incrementa la apoptosis de las cèlulas T activadas por antigenos de la micobacteria [34-36], promueve el crecimiento de la bacteria en macròfagos [37] y porque ademàs es un eficiente inhibidor de la funciòn citotoxica de las cèlulas CD8+. [38].

Existe una fuerte interacciòn entre IL-4 y TGF-β. Las cèlulas de sangre perifèrica de los pacientes con las formas mas avanzadas de tuberculosis tienen la tasa mas alta de producciòn y secreción de IL-4 y TGF-β [28]. Las citocinas Th2, incluidas la IL-4 e IL-13, incrementan la liberaciòn y activaciòn de TGF-β, ademàs la fibrosis pulmonar se asocia a la respuesta Th-2 [39]. Nuestro grupo ha demostrado que la tuberculosis pulmonar progresiva desarrollada en ratones Balb/c knock-out del gen de IL-4 tienen menos fibrosis [40]. En estos animales tambièn se observò nula expression de TGF-β durante la fase progresiva de la enfermedad, mientras que en los ratones "wild type" el TGF-β se produjo abundantemente durante la enfermedad avanzada [40]. Por lo tanto, en la tuberculosis como en asma, la IL-4 es un factor fundamental en el control de la expresión del TGF-β.

Tuberculosis pulmonar progresiva, el modelo experimental.

El objetivo principal de nuestra línea de investigación ha sido la caracterización de los mecanismos inmunológicos que contribuyen a la protección en contra del bacilo tuberculoso y los mecanismos inmunopatológicos que participan en el proceso de la progresión de la enfermedad y del daño tisular. Consideramos teóricamente que la caracterización de estos procesos inmunológicos podrá permitir la intervención inmunoterapeutica que contribuya a promover la actividad protectora y/o abatir los

mecanismos que faciliten la progresión de la enfermedad. De esta manera pensamos que se puede mejorar el control y la prevención (vacunación) de esta enfermedad.

La tuberculosis es una enfermedad muy compleja, en la que participan factores del huésped (constitución genética, respuesta inmunológica), de la bacteria (genes y factores de virulencia) y del medio ambiente (pobreza, desnutrición, hacinamiento). Es por la participación activa de todos estos factores que resulta muy difícil estudiar la respuesta inmunológica en pacientes y la alternativa es desarrollar modelos experimentales que semejen lo más posible a la enfermedad humana. Nuestro grupo de investigación ha desarrollado un modelo experimental de tuberculosis pulmonar progresiva, que se fundamenta en el uso de animales genéticamente idénticos (cepa singénica de ratòn Balb/c), los cuales se infectan por la vía natural (inyección intratraqueal) con bacterias vivas y virulentas (cepa prototipo H37Rv). La dosis empleada de bacterias para infectar a los ratones es elevada ($2.5x10^5$), pues nuestro objetivo es desarrollar un modelo de enfermedad progresiva y los ratones no son huéspedes naturales de las micobacterias (41). La inyección intratraqueal permite un mejor control de la dosis y garantiza que la mayoría, sino es que todas las bacterias realmente se depositen en el pulmón. Estas son ventajas sobre el uso de nebulizadores como instrumento de infección, pues con estos instrumentos se puede permitir la deglusión de las bacterias y en consecuencia la estimulación del tejido linfoide asociado a la mucosa digestiva, lo que seguramente modifica significativamente la respuesta inmunológica sistémica.

Nuestro modelo experimental de tuberculosis pulmonar progresiva se caracteriza por el establecimiento de dos fases durante el desarrollo de la enfermedad (42). La primera fase es la etapa temprana, la cual corresponde al primer mes de infección y se caracteriza histológicamente por la presencia de infiltrado inflamatorio constituido por linfocitos y macrófagos en el intersticio alveolo-capilar, alrededor de vénulas y bronquios. Durante la segunda semana postinfección los granulomas empiezan a formarse y una semana después alcanzan su máxima madurez (42). Estos granulomas de fase temprana y el infiltrado inflamatorio intersticial y perivenular coexistente están constituidos principalmente por linfocitos T CD-4 de tipo 1 productores de IFN e IL-2 y macrófagos activados productores de TNF e IL-1 (42, 43). Como se menciono anteriormente, la protección en tuberculosis depende de la inmunidad celular representada por linfocitos T y macrófagos (1). Nuestro modelo experimental corrobora esta información y otras observaciones realizadas en humanos y en animales de experimentación en donde se ha demostrado que los macrófagos activados y los linfocitos Th-1 son los principales elementos inmunológicos que protegen en contra del bacilo tuberculoso (1). La elevada producción de IFN activa a los macrófagos, estimulándolos a producir gran cantidad de la enzima oxido nítrico sintetasa inducible (iNOS) (44). La iNOS genera la producción de oxido nítrico (ON), el cual reacciona con radicales libres de oxigeno que también son producidos en gran cantidad y al mismo tiempo generando así la producción de peroxinitrilos, compuestos muy inestables que se asocian rápidamente a diferentes constituyentes bioquímicos celulares, entre estos uno de los blancos principales son las proteínas (en particular el aminoácido tirosina). Las proteínas nitrosiladas experimentan cambios conformacionales con pérdida irreversible de sus funciones lo que contribuyen a la muerte celular. Por otro lado, los macrófagos activados también secretan gran cantidad de IL-1, citocina que al igual que el TNF contribuye a activar a los macrófagos y además estimula la producción de IL-2 y su receptor (43). En nuestro modelo experimental el acmé de la respuesta protectora es en el día 21, cuando los granulomas alcanzan su plena madurez, lo cual se corrobora porque coexiste con la máxima respuesta de hipersensibilidad tardía cutánea (DTH) en

contra de antígenos micobacterianos (41-44). De esta manera es durante la fase temprana de la infección y debido al predominio de linfocitos Th-1 y macrófagos activados que se controla temporalmente la infección.

La segunda etapa de la enfermedad en este modelo experimental corresponde a la fase avanzada o progresiva, la cual se caracteriza por gran incremento en el número de bacterias vivas en los pulmones, así como áreas progresivas de consolidación neumónica con focos de necrosis y extensa fibrosis intersticial, que en conjunto conduce a la muerte (41-47). Desde el punto de vista inmunológico, durante esta fase se incrementa significativamente la presencia y actividad de los linfocitos T cooperadores de tipo 2 (Th-2) (42), los cuales se caracterizan por producir las interleucinas 4, 5, 6, 10 y 13. En particular, la IL-4 es una citocina fundamental en la inducción de la diferenciación de los linfocitos B productores de anticuerpos. Además la IL-4 al igual que la IL-10 y la IL-13 son eficientes antagonistas de las células Th-1. En consecuencia, consideramos que la emergencia de los linfocitos Th-2 durante la fase progresiva de la enfermedad puede contrarrestar la actividad protectora de las células Th-1, contribuyendo así a favorecer la progresión de la enfermedad (1, 42). De manera interesante, también los macrófagos experimentan modificaciones morfo-funcionales muy importantes, pues el citoplasma de estas células se llena de bacterias y de numerosas vacuolas que contienen lípidos bacterianos, en particular lipoarabinomanan (43), un lípido de la pared bacteriana que es muy eficiente para desactivar a los macrófagos. Además, estos macrófagos vacuolados disminuyen significativamente su producción de TNF, IL-1 e iNOS, e incrementan notablemente su capacidad productora de potentes citocinas antinflamatorias y supresoras de la inmunidad celular, como el factor de transformación tumoral (TGF) y la IL-10 (43, 44). El TGF es también una eficiente citocina inductora de la proliferación fibroblastica y de la síntesis de colágena, contribuyendo así a generar fibrosis. Además estas cèlulas vacuoladas son tambien eficientes productoras de lipidos bioactivos como la prostaglandina E2, la cual comparte varias actividades inmunosupresoras con el TGF, como son la desactivaciòn macrofàgica, supresión de la expresión de MHC-II y la producción de TNF e IFN (45). Es tambièn interesante que estas cèlulas expresan elevadas concentraciones de la molécula anti-apoptosica Bcl-2, con lo cual evitan morir por apoptosis y asi permiten una mayor sobrevida lo cual garantiza un habitat de largo plazo para las bacterias (46). Además, estos macròfagos vacuolados sobrexpresan la molécula Fas e inducen apoptosis de linfocitos Th-1 que expresan ligando de Fas (46), permitiendo asi la eliminación especifica de la fuente celular mas importante de IFN.

En conclusión, nuestro modelo experimental de tuberculosis muestra que en la fase progresiva de la enfermedad existen importantes anormalidades inmunológicas que permiten la sobrevida y proliferación bacteriana. Dicho modelo ha sido utilizado exitosamente para evaluar diversas estrategias inmunoterapeuticas, como hormonas esteroideas androgènicas naturales y sintèticas (47, 48), inhibidores naturales del TGF (49) o productos celulares como el factor de transferencia (50). En general, dichos tratamientos se inician cuando la enfermedad se encuentra en fase progresiva (dos meses), usando bacterias drogosensibles (cepa H37Rv) o un aislado clinico multi-drogo resistente (cepa MDR). Otro aspecto experimentalmente muy informativo que esta plenamente estandarizado en este modelo experimental, es agregar el agente inmunoterapeutico a la antibioticoterapia convencional, con el objetivo de evaluar si es posible acortar el tiempo de tratamiento con antibioticos, usando asi al agente inmunoterapeutico como un coadyuvante de los antibioticos (48-50).

Hipótesis

La estimulaciòn de la respuesta Th-1 y de la activaciòn macrofàgica, asi como la inhibición de la respuesta Th-2 y de otros factores inmunosupresores tendra un efecto terapeutico significativo en la tuberculosis producida por bacterias drogosensibles y multidrogoresistentes y permitira ademas acortar el tiempo de tratamiento con antibioticos convencionales.

Objetivo

Diseñar y probar nuevos regimenes inmunoterapeuticos que tengan efecto significativo en el control del crecimiento de bacterias multidrogoresistentes y en acortar el tratamiento convencional con antibioticos para un mejor control de la tuberculosis drogo-sensible.

Estrategia experimental

Nuestra primera estrategia serà reactivar la respuesta protectora Th-1 y la activaciòn macrofàgica que se encuentran muy disminuidas durante la enfermedad progresiva, tanto en pacientes como en nuestro modelo experimental. Dicha reactivaciòn la induciremos a traves de las siguientes estrategias:

1.- Administración de micobacterias sapròfitas y actinomicetos. La carga antigénica es un factor importante que participa en la estimulación preferencial de la respuesta Th1 o Th2. En varios modelos experimentales se ha demostrado que una carga antigénica pequeña normalmente es muy eficiente para activar a las células Th-1, mientras que una elevada carga antigénica activa la respuesta Th-2 (51). Por lo tanto, durante la fase inicial de la tuberculosis al existir un número relativamente pequeño de bacterias, hay activación preferencial de la respuesta protectora Th-1, mientras que durante la fase avanzada de la enfermedad cuando existe elevada cantidad de bacterias, hay predominio de la estimulación de células Th-2. Experimentalmente nosotros hemos comprobado este factor, al presensibilizar a los ratones con una cantidad pequeña de bacterias saprofitas muy inmunogénicas muertas por calor ($1x10^4$ células de *Mycobacterium vaccae*), se produce una eficiente activación de las células Th-1. En consecuencia, cuando se retan a estos ratones dos meses después por vía intratraqueal con un elevado número de micobacterias vivas y virulentas se produce un control muy eficiente de la enfermedad (52). En comparación, cuando se inmunizan ratones con una gran cantidad de *M. vaccae* ($1x10^5$), se induce fuertemente la respuesta Th-2, lo cual permite que la enfermedad rápidamente progrese y produzca la muerte unos días después de inocular bacterias vivas y virulentas de la cepa H37Rv por vía intratraqueal (52). Esta observación tiene una importancia significativa en la enfermedad humana, si se considera que las micobacterias saprofitas, que en paises tropicales son muy abundantes en el medio ambiente, no son parte de la flora intestinal normal, por lo tanto la cantidad y calidad de micobacterias saprofitas con que confrontemos en nuestro medio ambiente serán fundamentales en conferir protección o susceptibilidad a la enfermedad.

Mycobacterium. vaccae, puede ademàs ser un eficiente agente inmunoterapeutico porque tambièn activa eficientemente a las cèlulas citotoxicas CD-8+ y suprime la activaciòn de linfocitos Th-2 productores de IL-4. Ademas antìgenos expresados en esta bacteria de forma recombinante les confiere una gran actividad adyuvante [53, 54]. La inmunización con *M. vaccae* muerta por calor (autoclave) produce activaciòn de cèlulas CD8+ las cuales sinergizan la destrucciòn de macròfagos infectados con M. tuberculosis [55]. *M. vaccae* induce la respuesta CD8+ en contra de antìgenos que esta bacteria contiene [56], o en contra de antìgenos que se mezclan con ella [57], entre los cuales

esta el antìgeno hsp65 [56]. La mayoria de los antìgenos de *M. vaccae* cruzan con los de *M. tuberculosis*, es por esto que la administraciòn de esta bacteria sapròfita induce protecciòn en contra de *M. tuberculosis*. Ademàs *M. vaccae* puede suprimir la respuesta Th-2 aun cuando esta respuesta ya se haya establecido [57-62]. El mecanismo de esta suppression es atravès de la induccìon de cèlulas T reguladoras CD25+ $CD45RB^{low}$ [63]. Estas cèlulas fueron especificas para antìgenos presentes durante su induccìon, pero una vez activadas por sus antìgenos especificos, estas pudieron suprimir linfocitos Th-2 vecinos que reconocian antìgenos no relacionados [63]. Por lo tanto, la supresión de cèlulas Th-2 por celulas T reguladoras que reconocen cruzadamente antigenos de *M. vaccae* con *M. tuberculosis* debe ser un efecto inmunoterapeutico importante inducido por esta micobacteria sapròfita.

El efecto terapeutico de *M. vaccae* en nuestro modelo experimental de tuberculosis pulmonar en ratones Balb/c fue inicialmente publicado en 1996 [64]. Cuando se administra *M. vaccae* muerto por autoclave en los dias 60 y 90 despuès de la infeccion, se disminuyo en 1- 2 logaritmos el numero de bacterias vivas en el pulmon y tambièn se redujo significativamente la expresión de IL-4 [64]. *M. vaccae* administrado por via subcutanea u oral inhibe eficientemente la actividad Th-2 en modelos experimentales de alergia [61]. Nuestro objetivo ahora es usar *M. vaccae* administrandolo oralmente, pues en experimentos preliminares hemos observado que es eficiente para tratar la tuberculosis experimental, ya que al igual que como se ha documentado con la administración subcutanea (64), *M.vaccae* administrado oralmente eincrementa la expresión de IFN, TNF e iNOS, e inhibe la IL-4 y el TGF-β. (Fig 1).

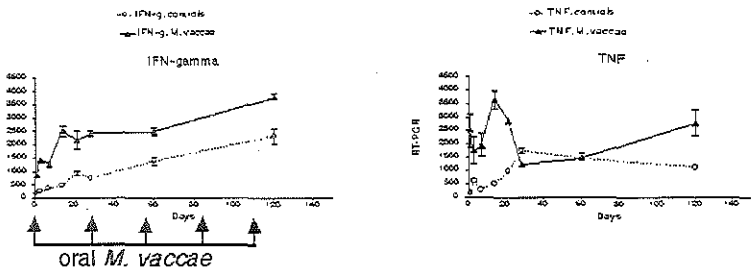

Fig 1.- Efecto de M.vaccae administrado oralmente en los dias señalados, en comparación con el control (circulos vacios), los ratones tratados con M. vaccae produjeron mas IFN y TNF.

Estudiaremos tambièn el efecto de este sapròfito en ratones infectados con una cepa MDR y su eficiencia como agente coadyuvante a la quimioterapia. Para esto la cepa virulenta de *M. tuberculosis* H37Rv o el aislado clinico MDR seràn cultivadas en el medio lìquido de Proskauer y Beck modificado por Youmans. Después de cultivarse por un mes, las micobacterias son separadas y ajustadas a 2.5×10^5 bacterias en 100 µl de amortiguador de fosfatos salino (PBS), alicuoteadas y mantenidas a –70°C hasta su uso. Antes de usarlas las bacterias son recontadas y su viablilidad se corrobora. Ratones machos BALB/c de 6-8 semanas de edad son anestesiados con 56 mg/kg de pentotal sòdico administrado por via peritoneal, la traquea se expone quirúrgicamente y 2.5×10^5 bacterias viables suspendidas en 100 µl de PBS se inyectan usando una jeringa de insulina. Posteriormente la insiciòn quirùrgica es suturada con seda esteril. Los ratones así infectados se almacenan en cajas con microaisladores conectados a un sistema de

presiòn negativa. Todo este procedimiento y los sacrificios posteriores se realizan en campanas de seguridad biològica P3. Este protocolo ha sido aprobado por el Comité de Investigaciòn en Animales de Experimentaciòn del Instituto Nacional de Ciencias Mèdicas y Nutriciòn "Salvador Zubirán".
Al dia 60 postinfecciòn, los ratones sobrevivientes se distribuyen al azar en seis grupos experimentales. Grupos de 20 ratones tuberculosos en dos experimentos diferentes, se trataràn con una suspensión de *M. vaccae* a dosis de 100 microgramos. Para evaluar la capacidad de *M.vaccae* para acortar el tiempo de tratamiento con antibioticoterapia convencional, el segundo grupo será tratado con la antibioticoterapia convencional, constituida por: rifampicina (10 mg/kg), isoniazida (10 mg/kg), y pirazinamida (30 mg/kg), administrados diariamente a travez de sonda intragástrica, mas *M. vaccae* administrado por sonda intragastrica una vez por semana. El tercer grupo se tratarà solamente con antibióticos y el grupo control que exclusivamente recibirà la soluciòn vehiculo. El quinto grupo corresponde a ratones infectados con el aislado clinico MDR, el cual se trata bajo el mismo esquema con *M. vaccae* y el sexto y ultimo grupo es el grupo control infectado con el aislado MDR que recibe solamente el vehículo. Cinco ratones por cada grupo se sacrificaran los dias 7, 15, 30 y 60 dias posteriores al inicio del tratamiento. Se tendran 10 animales mas de cada grupo que se se dejaran sin sacrificarlos con el fin de determinar sobreviva. Se realizaran dos experimentos completos por separado para checar reproducibilidad.
Para evaluar la eficiencia del regimen inmunoterapeutico ademas de construir curvas de sobrevida, se realizara estudio histològico/morfomètrico para medir la extensión del daño tisular (porcentaje de area pulmonar afectada por neumonía detrminado por morfometria automatizada), mediciòn de la carga bacilar pulmonar por determinación de unidades formadoras de colonia y la cuantificaciòn de la expresión genètica de las citocinas IFN, TNF, IL-4 y la enzima iNOS por RT-PCR en tiempo real, de acuerdo a las tècnicas ya estandarizadas en nuestro laboratorio (41-50).

2.- **Uso y caracterizaciòn molecular del Factor de Transferencia en tuberculosis.**
Los factores de transferencia (FT) o dializados leucocitarios son productos dializables de bajo peso molecular obtenidos de cèlulas del sistema inmunològico, los cuales son capaces de transferir respuestas de hipersensibilidad tardìa cutanea (DTH) o de inmunidad mediada por cèlulas (IMC) de donadores inmunizados a sujetos receptores no sensibilizados inmunologicamente vírgenes (65). Actualmente no se conoce la estructura quìmica ni los mecanismos moleculares de acciòn del FT. Sin embargo, varios estudios han mostrado que el FT es un conjunto de proteinas de bajo peso molecular (<5000 Da) que pueden ser purificados en un alto grado de homogenidad (66). En general, todos los FT hasta ahora probados son eficientes en corregir o incrementar las respuestas de IMC y son antìgeno especìficos (66-68). Desde su descubrimiento por Sherwood Lawrence, hace mas de 50 años (65), las aplicaciones terapeuticas y profilàcticas han sido el aspecto màs importante e interesante del FT (68). En efecto, se ha demostrado que el FT es muy efectivo como tratamiento en aquellas enfermedades en las que la IMC tiene una participaciòn fundamental en su protecciòn o control, tales como infecciones virales (herpes simplex, varicella zoster), enfermedades por bacterias intracelulares (tuberculosis, lepra) e infestaciones parasitarias (leishmaniasis, toxoplasmosis) (69-73), asi como inmunodeficiencias primarias (enfermedad granulomatosa crònica de la infancia, síndrome de Wiskott Aldrich) (74) y algunos tipos de cancer (75). En nuestro modelo experimental de tuberculosis progresiva, la obtención de factor de transferencia a partir de linfocitos aislados del bazo de animales con 21 dias postinfecciòn, tiempo en el cual se presenta la maxima

respuesta protectora, y su posterior administración semanal por via intraperitoneal en ratones Balb/c con enfermedad avanzada produjo un notable efecto inmunoterapeutico, con incremento en la producción de IFN, TNF e iNOS, asi como acentuada disminución de IL-4, lo cual permitio reducir significativamente la carga bacilar pulmonar y la extensión del daño pulmonar (50). Este tratamiento tambièn sinergizo con la quimioterapia convencional reduciendo el tiempo de administración de los antibioticos. Este efecto fue estrictamente dosis dependiente, incluso dosis mayores a la terapeutica produjeron extensa necrosis y mayor mortalidad como consecuencia de un exceso en la producción de TNF e inmunopatología asociada (50).

En el presente proyecto uno de nuestros principales objetivos serà la caracterizaciòn molecular del FT.

Como ya se ha mencionado anteriormente, el Factor de Transferencia (FT) es uno de los descubrimientos mas excitantes en el campo de la terapéutica realizado en décadas recientes. Aunque no se ha logrado su caracterización molecular desde hace mas de 50 años, se ha propuesto que el FT esta compuesto por una mezcla muy compleja de moléculas mensajeras inmunológicas de baja masa molecular (v.g., debajo de los 10 kDa) que producen los organismos inmunocompetentes. Su papel funcional general propuesto es transferir señales de reconocimiento inmunológico entre células inmunológicas. Tamvién como se ha menicionado con anterioridad, ya que se ha demostrado la acción terapéutica preclínica del Factor de Transferencia en la tuberculosis, en el presente proyecto proponemos abordar su caracterización estructural y determinar el papel terapéutica de sus componentes en el moodelo murino de infección pulmonar in vivo con M. Tuberculosis.

La Cromatografia de líquidos de alta resolución se ha convertido, sin lugar a dudas, en la mas popular y versátil de las técnicas analíticas modernas en los laboratorios de hoy, acoplado a la espectrometría de masas en tiempo real. Los sistemas cromatográficos de líquidos de alta resolución se utilizan actualmente en una amplia variedad de campos. Día a día aumentas los requerimientos de confiabilidad de los datos analíticos y de eficiencia en el flujo del trabajo del laboratorio de investigación. Nuestro protocolo de abordaje de caracterización molecular de biomoléculas del FT será inicialmente a través de lña separación cromátográfica de componentes mediante la técnica de cromatografia líquida de alta resolución en fase reversa (RP-HPLC).

Para llevar a cabo este trabajo en forma óptima, con alta resolución de separación y asislamiento molecular a homogenidad total, tenemos la necesidad de adquirir un cromatógrafo bidimensional de líquidos de alta resolución para continuar con el desarrollo de procesamientos de muestras en grado preparativo, semipreparativo y analítico en Factor de Transferencia . Este instrumento estará enfocado en la provisión de soluciones integradas para el desarrollo de nuevas estrategias de identificación y separación de nuevos blancos para Factor de Transferencia, así como su diseño para nuevas estrategias para el diagnostico de enfermedades por la separación, purificación, caracterización y identificación de proteínas y de otras macromoléculas. Al igual que nos permitirá demostrar controles de calidad de compuestos involucrados en el desarrollo básico, clínico y farmacéutico de protocolos de investigación que están en curso el la actualidad, deducir, analizar impurezas y contaminantes para determinar su composición, y buscar metabolitos y biomarcadores para la evaluación de rutas metabólicas.

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Aunque tenemos la carencia de este equipamiento y metodología de abordaje de separación cromatográfica optimas, ya hemos iniciado y desarrollado trabajo experimental poreliminar exitoso dirigido a la separación, purificación, caracterización e identificación de los extractos dializables de leucocitos (Factor de Transferencia). Estos resultados se fueron obtenidos con un equipamiento cromatpográfico de RP-HPLC muy viejo, (modelo de hace mas de 25 años), el cual consiste en un cromatógrafo de líquidos de alta resolución de un marca Waters, modelo 717 de automuestreador, bombas de flujo M-45 y M 510, un integrador modelo 24-2 así como un detector dual de absorbancia modelo 2487 y una liofilizadora para solventes polares y no polares marca thermo electrón, para concentración y preservación de las muestras. Cabe resaltar que la separación y purificación cromatográfica semepreparativa del factor de transferencia no ha sido logrado con alta resolución y (ver abajo Fig. "a", ""b" y "c"), un facto experimental indispensable para su posterior análisis *ad hoc* para la identificación y determinación estructural de componentes biomoleculares del FT por metodologías de espectrometría de masas.

Se separo el extracto de leucocitos por cromatografía en fase reversa en un gradiente de composiciones de solventes polares y no polares, además, el mismo extracto se corre en el mismo gradiente de composiciones en el mismo tiempo, esto, para concentrar la muestra.

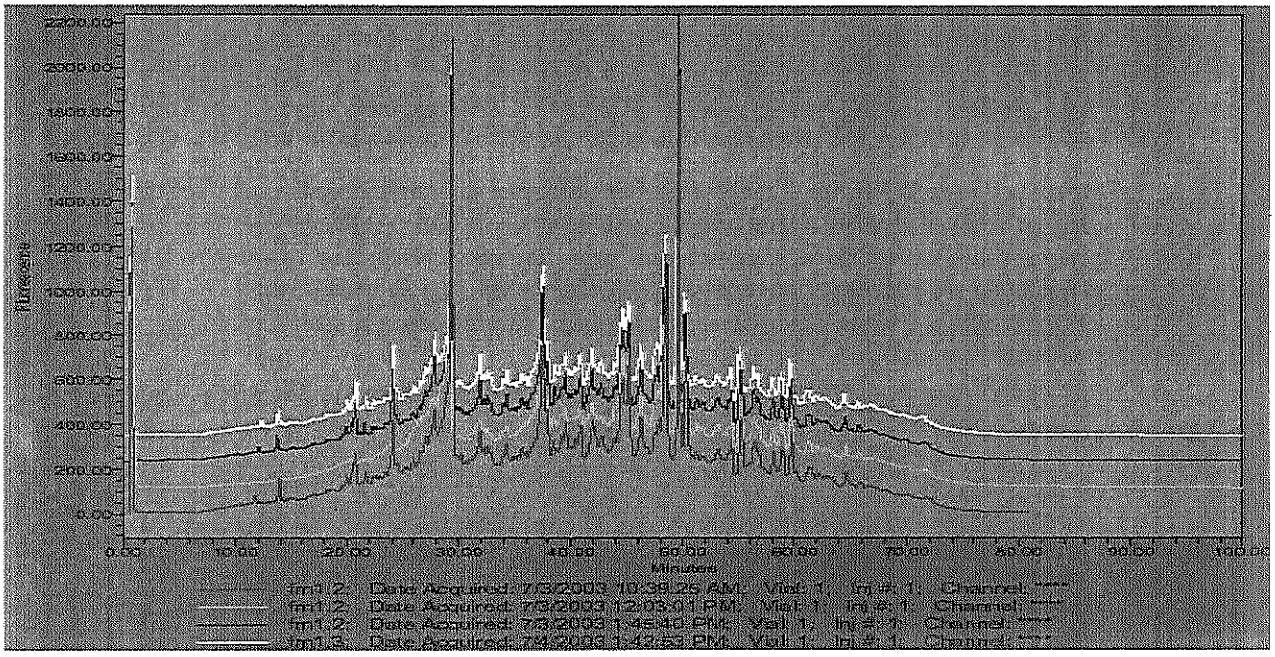
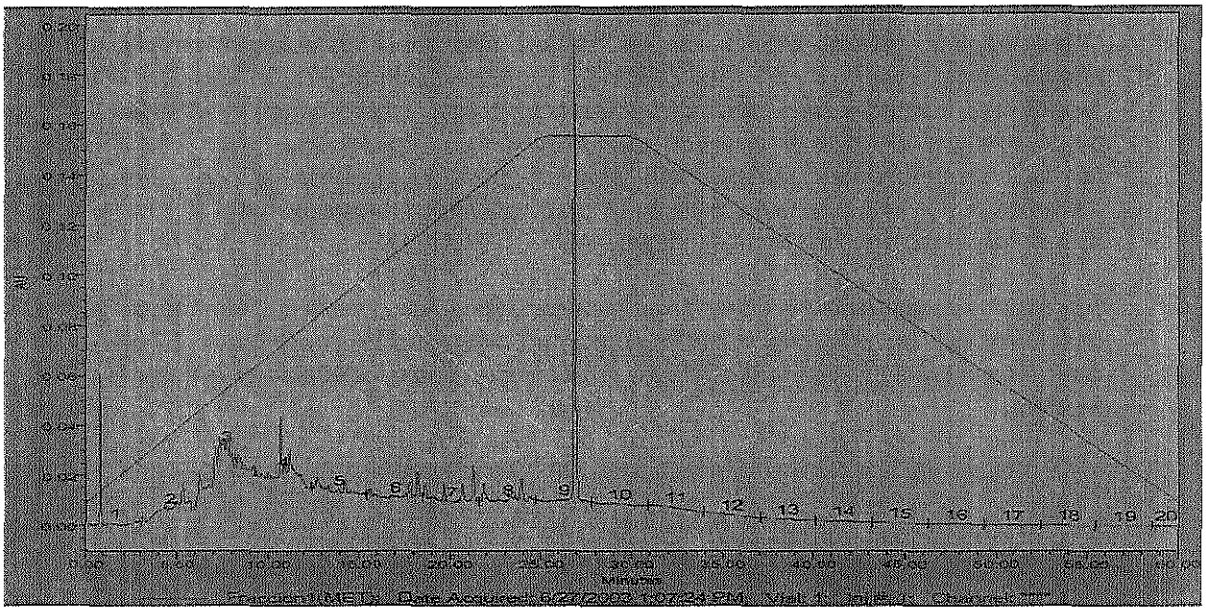


Figura (a)., Extracto dializable de leucocitos concentrados al cromatógrafo de líquidos de alta resolución. Cada uno de los números significa la cantidad de subfracciones que se colectan del factor de transferencia total.

Al mismo tiempo que se concentra el factor de transferencia, se colecta el mismo en porciones del extracto al mismo tiempo, en un colector de fracciones que esta integrado



Una vez que se tienen las 20 subfracciones del factor de transferencia total, cada una de ellas se vuelven a meter a cromatografía de líquidos de alta resolución para tratar de separar cada uno de los componentes del factor de transferencia con una resolución aceptable, esto permitirá, a la espectrometría de masas una identificación y caracterización con un índice de error muy bajo, para un análisis bioinformática posterior.

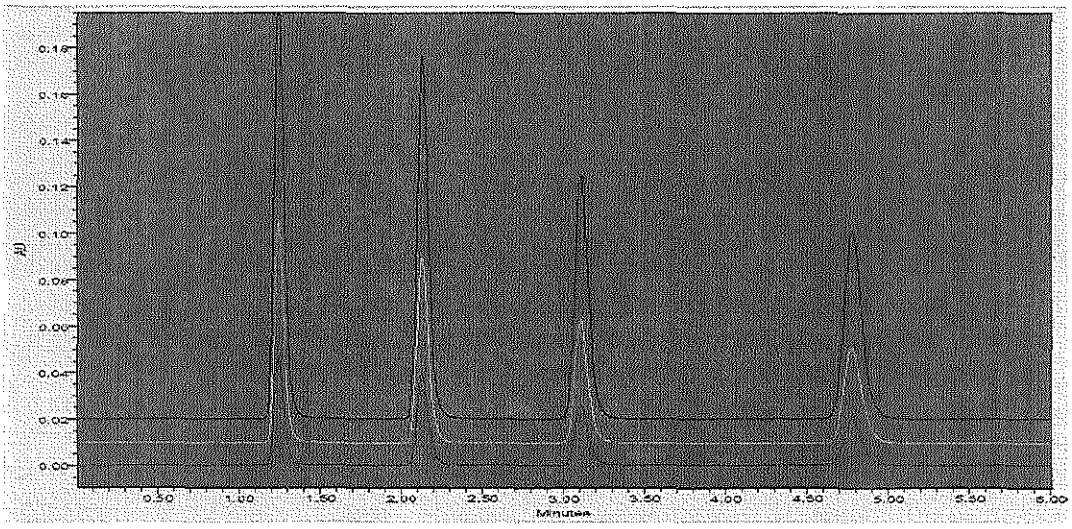


Figura (c)., Separación individual de componentes del factor de transferencia.

Para esto es necesario que el cromatógrafo bidimensional de líquidos de alta resolución cuente con los componentes mínimos necesarios para desarrollar protocolos de investigación en Factor de Transferencia.

Los componente necesarios que debe contar el cromatógrafo bidimensional de líquidos de alta resolución son los siguientes:

- Sistema de bombeo para operación en gradiente binario, ternario y cuaternario
- Sistema de bombeo para operación en gradiente binario, ternario y cuaternario.
- Automuestreador
- Sistema de control de temperatura para columnas
- Detector de arreglo de diodos con intervalo de longitud de onda de 195 a 650nm
- Válvula inerte de alta presión

- Colector de fracciones con modos de operación Simple, Pico, Ventana, Manual y Muestreo.

- Monitoreador de conductividad y pH
- Software cromatografico para el control de una amplia variedad de instrumentos de diferentes fabricantes.
- Opción de adecuabilidad de sistema para evaluar el desempeño de el sistema y separaciones cromatograficas.
- Estación de trabajo PC e Impresora láser blanco/negro.

Este Cromatógrafo bidimensional de líquidos de alta resolución se trabajara en serie con un espectrómetro de masas, para la identificación segura de los diferentes elementos químicos que provienen del Factor de Transferencia

El espectrómetro de masa es un instrumento que permite analizar con una gran precisión la composición de diferentes elementos químicos e isótopos atómicos, separando los núcleos atómicos en función de su relación masa-carga. Puede utilizarse para identificar los diferentes elementos químicos que forman un compuesto o determinar el contenido isotópico de diferentes elementos en un mismo compuesto. Con frecuencia se acopla a un cromatógrafo de líquidos, en una técnica híbrida conocida por sus iniciales en inglés, LC-MS

El espectrómetro de masas mide razones carga-masa de iones, calentando un haz de luz de material del compuesto a analizar hasta vaporizarlo e ionizar los diferentes átomos. El haz de iones produce un patrón especifico en el detector que permite analizar el compuesto químico. Este instrumento se utiliza en investigación para el análisis de sustancias y mezclas complejas (Factor de Transferencia). En términos generales, moléculas diversas tienen masas diversas, así se determina que moléculas están presentes en una muestra. Otra característica de estos quipos es el nivel de certeza para separar los iones de una muestra, estos iones se envían a un compartimiento de aceleración, se aplica un campo magnético a un lado del compartimiento que atrae a cada uno de los iones con la misma fuerza (suponiendo carga idéntica) y se los desvía a un detector., los iones más ligueros se desviarán más que los iones pesados porque la fuerza aplicada a cada ion es igual pero los iones ligeros tienen menos masa. El detector mide exactamente cuán lejos se ha desviado cada ion y, apartir de ese dato se calcula el cociente masa por unidad de carga. Con estas ventajas funcionales, hemos hechos

ensayos preliminares de determinación estructural de componentes moleculares de subfracciones cromatográficas de RP-HPLC del FT total. Nuestros resultados preliminares consisten en la identificación estructural de al menos 10 componentes de muy baja masa molecular distintos a partir de una de estas subfracciones (no revelamos identidad estructural por motivos de tramite de registro de patente actual). Un resultado representativo de estos hallazgos preliminares se muestra abajo en la figura "d". Estas moléculas ya se han sintetizado y se han evaluado su acción inmunológica y terapéutica en el modelo murino de infección pulmonar in vivo de M. Tuberculosis. En la Figura "E", se muestra un ensayo representativo de la evaluación de la capacidad de inducción de la producción de interferon-gamma de algunas de estas biomoléculas sintetizadas. En la Figura "F", en el panel (A) se muestra un resultado representativo de las curvas de sobreviva de ratones infectados con M. Tuberculosis a las 4 y 8 semanas post-infección, los cuales fueron inmunizados una vez por semana, durante cuatro semanas u 8 semanas consecutivas, con una inyección intraperitoneal de 1 ug de cada biomolécula identificada y sintetizada del FT. En el panel (B) de la misma figura, se muestra en forma paralela, los resultados de cuenta bacilar del M- Tuberculosis expresada en millones de unidades formadoras de colonias (UFCs) de estas bacilo/pulmón/animal infectado/tratado con biomoléculas de FTs .

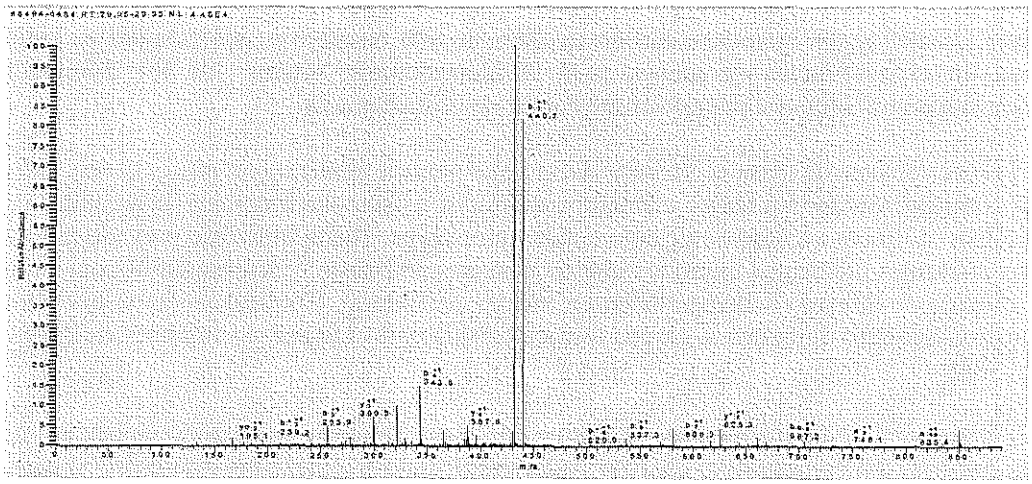


Figura (d)., Espectro de fracciones del factor de transferencia.

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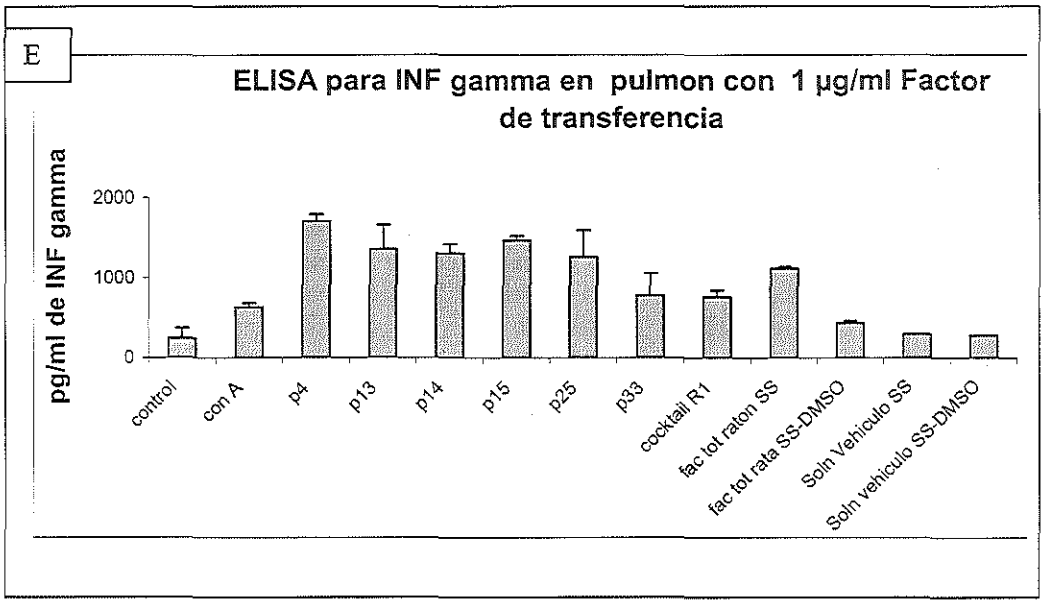
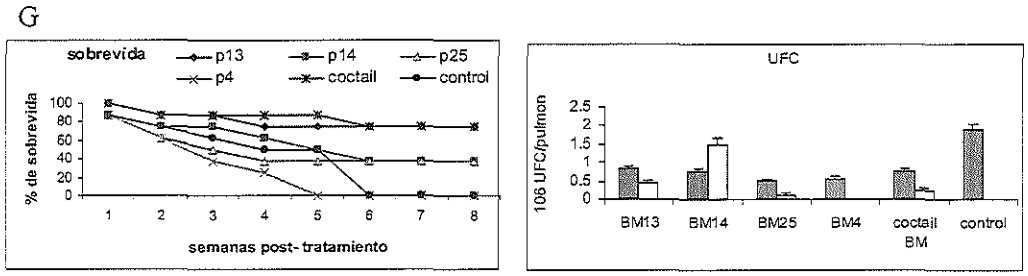


Figura E: expresión de IFN gamma de celulas estimuladas con biomoleculas de factor de transferencia por ELISA



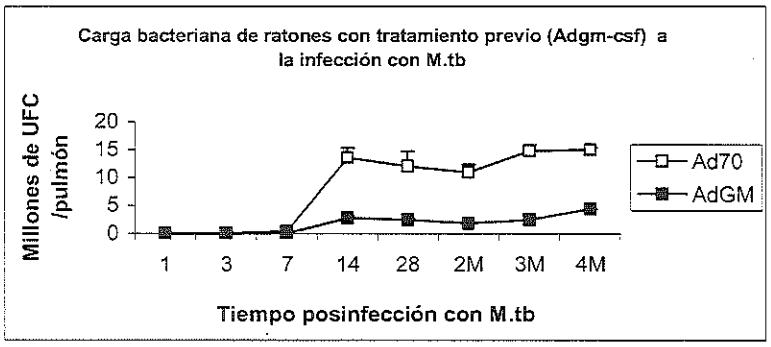
(A) (B)

Figura F . (A) grafico de curva de sobreviva y (B) Unidades formadoras de colonias (UFCs) obtenidas de ratones tuberculosis post tratamiento con biomóleculas de Factor de Transferencia.

3.- Activaciòn de cèlulas dendriticas: En nuestro modelo experimental de tuberculosis progresiva, hemos demostrado que una característica importante desde el punto de vista inmunopatogènico es el retraso en la activaciòn de las cèlulas dendriticas, tanto en el pulmòn como en los ganglios linfàticos mediastinales (76, 77). En efecto, la cinètica de las células dendriticas durante el curso de la enfermedad utilizando lavados bronquiales y citometrìa de flujo, mostraron que estas cèlulas aparecen una semana después de la infecciòn, alcanzan su màximo numero en el dia 21 y después declinan paulatinamente durante la fase progresiva de la enfermedad. Esto contrasta con la instilacion via intratraqueal de peptidos como los contenidos en el derivado proteico purificado (PPD) de cultivo de *M. tuberculosis*, el cual induce en horas la aparición de un gran nùmero de células dendriticas activadas. Por lo tanto, el retraso en la activaciòn de cèlulas dendriticas es un mecanismo de evasión inmunològica de *M. tuberculosis* y la posibilidad de activar a estas cèlulas de manera mas temprana y eficiente es una estrategia potencialmente util de inmunoterapia. Para lograr esto planteamos dos tipos

de estrategia, la primera es el uso de citocinas altamente eficientes en la activaciòn de cèlulas dendriticas, como el factor estimulante de colonias granulocitico y macrofàgico (SCF-GM por sus iniciales en ingles) y la segunda es la administración de anticuerpos dirigidos a receptores distintivos de cèlulas dendriticas que porten moléculas altamente inmunogènicas de la micobacteria.

Para la primera estrategia se utilizarà terapia gènica, utilizando adenovirus recombinantes que expresan SCF-GM. Durante los ultimos 5 años hemos estado utilizando terapia gènica para acortar el tratamiento convencional con antibioticos y controlar la tuberculosis MDR, utilizando específicamente adenovirus recombinantes que expresan IFN (rAd-IFN). La tècnica para la producción de los adenovirus recombinantes la tenemos estandarizada y nuestros resultados mostraron que ratones tratados con una sola dosis de rAD-IFN, administrada por instilaciòn intratraqueal fue capaz de tener un efecto terapeutico significativo en ratones con tuberculosis avanzada producida por bacterias drogosensibles (H37Rv) y MDR, este tratamiento tambièn fue capaz de acortar significativamente el tratamiento con antibioticos (Mata D, Hernandez Pando R, manuscrito enviado a publicación). Nuestra estrategia para activar mas rapidamente a las cèlulas dendriticas se fundamentarà en la administración de una sola dosis de rAD-CSF un dia antes de la infecciòn y después en los dias 3, 7, 14, 21, 28 y 60 se sacrificaran grupos de 8 animales y se compararàn con ratones control que reciben por via intratraqueal el adenovirus que expresa la proteina verde fluorescente (rAD-GFP). Los parámetros por evaluar seràn la sobrevida, carga bacilar (UFC), histopatologìa/morfometrìa, expresión de citocinas por RT-PCR en tiempo real y citometrìa de flujo en suspensiones celulares obtenidas de los pulmones determinando la cantidad y porcentaje de celulas dendriticas activadas (CD-11c, MHC-II, CD-80)(76, 77). De hecho, en un experimento preliminar en el que se siguió este diseño se pudo observar una significativa reducción de la carga bacilar pulmonar en los ratones que recibieron el adenovirus recombinante que expresa CSF.



Debido a que nuestros estudios previos mostraron que después del dia 21 de infecciòn las cèlulas dendriticas activadas disminuyen progresivamente durante la enfermedad avanzada, un segundo experimento serà la administración del rAd-CSF por via intratraqueal a partir del segundo mes postinfecciòn y evaluando su eficiencia como se describiò anteriormente. En esta modalidad de tratamiento contamos con la colaboración del Dr Zou Xing, director de la Unidad de Terapia Gènica de la Universidad de MacMaster en Ontario Canada, quien es un experto mundial en el area.

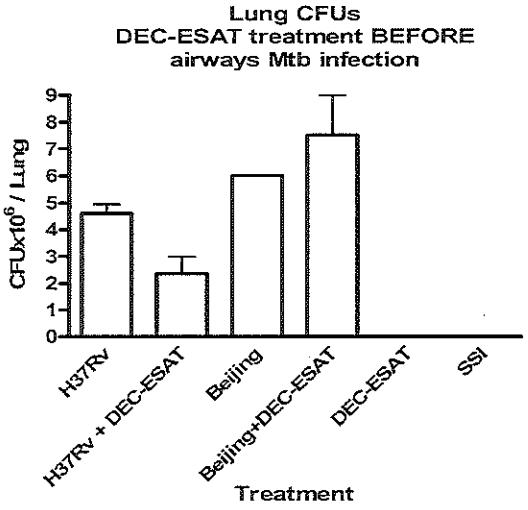
En cuanto a la segunda estrategia, se utilizaràn anticuerpos fusiòn que reconocen por su porciòn Fab al receptor de membrana DEC-205 y que en su porciòn FC tienen unida la proteina ESAT-6 de *M. tuberculosis*. La molécula DEC-205 es homóloga al receptor de manosa de macrófagos, esta molécula cuenta con 10 dominios tipo lectina (205KDa)

cuyo ligando natural se desconoce hasta el momento, sin embargo se han utilizado anticuerpos monoclonales como ligandos surrogados. DEC-205 se expresa en células dendríticas (DC) en tejidos periféricos no linfoides, como las células de Langerhans (LC). Esta claramente establecido que DEC-205 participa en la presentación antigénica tanto en el contexto de MHC-II (fenómeno mediado por el dominio citosólico de la molécula) y MHC-I. El antìgeno ESAT-6 (early secretory antigenic 6KDa) es una proteína que se produce en las primeras horas de cultivo de *M. tuberculosis* y es secretada en forma de heterodímero junto con otra proteína llamada CFP-10 (culture filtrate protein 10) ambas codificadas en la región de diferenciación 1 (RD1)33-35 (78). Se ha demostrado que gran parte de la respuesta inmune celular que induce la infección con *M. tuberculosis* está dirigida a ESAT-6, en particular al péptido formado por los primeros 20 aminoácidos en el extremo amino46-48. Cabe señalar que este péptido es reconocido en primates, ratones y humanos, lo que indica que es ampliamente conservada su inmunogenicidad.

Debido a la funciòn de DEC-205 en presentaciòn antigènica, recientemente se ha propuesto que usando anticuerpos contra esta molécula con proteínas exógenas acopladas como ESAT-6 puede ser una estrategia muy eficiente para inducir una gran respuesta de IFN en contra de ESAT-6. Por lo tanto, nuestro objetivo serà administrar este anticuerpo por via subcutanea y determinar si tiene algún efecto terapeutico en nuestro modelo experimental de tuberculosis progresiva. El anticuerpo fusión anti-DEC205-ESAT6 ya se ha producido, clonando y fusionando la proteína ESAT6 de *Mycobaterium bovis* en el extremo carboxilo de las cadenas pesadas del anticuerpo de ratón anti-DEC205. Para obtener este anticuerpo se realizò mutagénesis dirigida que eliminó del anticuerpo fusión el sitio de reconocimiento de los receptores Fc, mientras que las cadenas ligeras no sufrieron variación. Los plásmidos portadores de la cadena pesada modificada y la cadena ligera se transfectaron en la línea celular 293T, que es un mieloma murino de células adherentes el cual es ampliamente utilizado para la producción de proteínas recombinantes de origen eucarionte. Del sobrenadante de cultivo se purificò el anticuerpo fusión anti-DEC205-ESAT6 utilizando columnas de SEFADEX-Proteína A. Posteriormente se comprobò que el anticuerpo producido correspondio al anticuerpo fusión realizando cromatografía SDS-PAGE, en la que se observò que la proteína producida tiene un peso molecular 6KDa mayor al control que contiene el anticuerpo de ratón anti-DEC205. Para determinar si el anticuerpo fusión producido conservaba la capacidad de unión a DEC-205 se utilizaron células CHO transfectadas con la proteína DEC-205 de ratón, las cuales se incubaron con el anticuerpo fusión y se detectó la unión del anticuerpo tiñéndolo con un anticuerpo anti-ratón acoplado a FITC. Para determinar la eficiencia in-vivo del anticuerpo, se usaron ratones BALB/c y C57BL/6 a los cuales se les administró 5μg de DEC-ESAT con 10μg de polyI:C por vía subcutánea o intranasal. También se inyectaron grupos control con polyI:C, PBS, y DEC-ESAT. Los ratones se sacrificaron en las semanas 2 y 5 después de la administración del anticuerpo fusión, de cada ratón se hicieron suspensiones celulares del bazo (respuesta sistémica), los ganglios poplíteos e inguinales (órganos linfoides dístales al sitio de administración), los ganglios mediastinales (órganos linfoides locales al sitio de administración), y el pulmón (sitio de infección). Las cèlulas obtenidas se estimularon in-vitro con antigenos del sonicado de *Mycobacterium tuberculosis*, la proteína ESAT-6 completa, o una librería de péptidos traslapados de la proteína ESAT-6. Después de 12h de incubaciòn, se realizò citometria de flujo para cèlulas CD4 y CD8 con tinciòn intracelular para detectar IFN. Los resultados mostraron una significativa cantidad de celulas CD4 y CD8 productoras de IFN en ganglios linfàticos y pulmòn. La producción de este anticuerpo fusiòn fue realizada por Aaron

Silva, como parte de su tesis Doctoral, en el laboratorio del Prof R Steinmann descubridor y lider mundial en la investigación de las cèlulas dendriticas, bajo la supervisión del Dr Leopoldo Flores, colaborador del presente proyecto y experto en cèlulas dendriticas.

Los experimentos por realizar con este anticuerpo fusiòn seràn para evaluar su capacidad profiláctica, administrando dos dosis del anticuerpo fusión, 60 y 30 días antes de la infección. En un experimento preliminar de este tipo se observo que dicha estrategia tiene un efecto prometedor, pues los ratones asi tratados mostraron menos carga bacilar que los no tartados.



El otro experimento informarà sobre el efecto terapéutico, en el que los ratones recibiran dos dosis de anti-DEC205-ESAT6, 1 y 4 días después de la infección. En ambos casos se usarà nuestro modelo experimental de tuberculosis progresiva en ratones Balb/c y la eficiencia terapeutica se determinarà por curvas de sobrevida, carga bacteriana pulmonar, expresión de citocinas por RT-PCR en tiempo real, citometrìa de flujo para determinar cinètica de cèlulas dendriticas activadas en suspensiones celulares y lavados pulmonares e histologìa/morfometrìa, como ya se ha descrito anteriormente.

4.- Estimulaciòn de la producción de interleucina 12 (IL-12). La IL-12 (compuesta por las subunidades p35 y p40) es una citocina crucial en la diferenciación de células Th1 productoras de IFN (79). Se ha reportado que pacientes con defectos genéticos en la ruta de señalización de la IL-12 desarrollan mayor susceptibilidad a infección con micobacterias ambientales y al bacilo BCG (79). El papel que desempeña la IL-12 en la resistencia a la micobacteria quedó demostrado cuando dichos pacientes fueron tratados con la citocina recombinante en combinación con antifímicos, mostrando una mejoría clínica importante. En estudios recientes, se ha demostrado que la administración temprana de la IL-12 recombinante a ratones Balb/c infectados con *M. tuberculosis* resultó en una reducción significativa en la carga bacteriana, y el mejoramiento de las alteraciones histopatológicas, con un incremento concomitante de la sobrevida de dichos animales. Así mismo, la neutralización de la IL-12 al inicio de la infección, dio como resultado un incremento en la carga bacilar (80, 81). Finalmente, ratones "knock-out" IL-12p40$^{-/-}$ se mostraron altamente susceptibles a la infección por *M. tuberculosis,* un defecto asociado con la reducción en la producción de IFN (82). Estos experimentos establecen claramente la importancia de la IL-12 exógena (IL-12e)

en la inducción de respuesta Th1 protectora. Además, en dichos trabajos se establece que para el control del crecimiento de las micobaterias es conveniente su administración en intervalos (tres veces por semana). Sin embargo, estos trabajos utilizan a la citocina recombinante, lo cual es muy costoso y por lo tanto poco pràctico, ademàs no se ha estudiado el efecto que este tratamiento tiene en tuberculosis producido por bacterias MDR. Nuestra estrategia inmunoterapeutica en este caso sera la utilización de adenovirus recombinantes que expresàn IL-12 (rAd-IL12), en una sola dosis administrada intranasalmente e intratraquealmente, tanto de forma profilactica (un dia antes de la infecciòn intratraqueal con una dosis alta de M. tuberculosis H37Rv), como de forma terapeutica (dos meses después de la administración de *M. tuberculosis* drogosensible y MDR por via intratraqueal) cuando la respuesta Th1 ha declinado acentuadamente, esto es de interés en tuberculosis pues se ha postulado que la acción de la IL-12 es solo importante para el inicio de la inmunidad antimicrobiana (80). Los rAd-IL12 ya los hemos producido en colaboración con el Dr Xing y su dosis y eficiencia en la inducción de la producción de IFN ya la hemos determinado en ratones Balb/c no tuberculosos. La eficiencia terapeutica serà determinada por curvas de sobrevida, cuantificaciòn de unidades formadoras de colonia bacterianas en los pulmones, histologia/morfometrìa y expresión de citocinas determinada por RT-PCR en tiempo real.

5.- Inducción de la producción de oxido nitrico. Como fue mencionado en los antecedentes, los macrofagos son cèlulas esenciales en el control de la tuberculosis, pues fagocitan y destruyen a la bacteria, ademas de que seleccionan y presentan antigenos para activar a los linfocitos T y regulan la respuesta inmunològica a traves de la producción de diversas citocinas (1). Los macròfagos activados destruyen eficientemente a las micobacterias por la producción de radicales libres de oxigeno y de nitrógeno. Nuestro modelo experimental claramente ilustra la importancia del oxido nitrico, al mostrar el maximo de expresión genètica de la enzima iNOS en el dia 21 postinfecciòn en coexistencia con una gran cantidad de proteinas bacterianas nitrosiladas (nitrotirosina) en el citoplasma y pared celular, mientras que durante la fase progresiva de la enfermedad existe una acentuada disminución de la expresión de iNOS (44). A diferencia de los murinos, en la tuberculosis humana la participación del oxido nitrico ha sido controversial pues al parecer esta molécula no participa de forma tan directa en la destrucción de las micobacterias, sin embargo nosotros hemos mostrado por inmunohistoquimica una gran expresión de iNOS y nitrotirosina en granulomas de pacientes tuberculosos (83) y lepromatosos (84). Por lo tanto, consideramos que otra forma potencialmente util de inmunoterapia es el inducir incremento en la producción de oxido nitrico durante la tuberculosis progresiva.

El grupo del Dr. Arturo SanFeliciano de la Facultad de Farmacia de la Universidad de Salamanca, ha estado trabajando activamente desde hace varios años en la producción de inmunomoduladores para el tratamiento del cancer y rechazo de transplantes. Recientemente en dicha Universidad se ha creado el Instituto de Medicina Tropical y uno de sus objetivos es la producción de nuevos antibioticos e inmunomoduladores, para el tratamiento de enfermedades infecciosas de gran importancia mèdica como la tuberculosis. El grupo del Dr SanFeliciano ha producido mas de 200 compuestos derivados de amino-alcoholes y diaminas y los han probado en ensayos in-vitro en cuanto sus actividades inmunomoduladoras (85, 86). De manera interesante se ha demostrado en ensayos in-vitro utilizando macrofagos alveolares, que el compuesto diamino 8b incrementa significativamente la producción de nitritos en cèlulas no activadas, mientras que los compuestos 6f y 7a lo hacen en macròfagos activados con lipopolisacaridos y ademas incrementan la cantidad de linfocitos CD4 y

CD8 cuando se administran a ratones Balb/c. Estos compuestos son por lo tanto candidatos muy prometedores para probarlos como agentes inmunoterapeuticos en la fase avanzada de nuestro modelo murino de tuberculosis progresiva, administrandolos por via intraperitoneal en dos dosis diferentes cada tercer dia, sacrificando ratones infectados con las cepas H37Rv y MDR en los dias 7, 15, 30 y 60 post-tratamiento y evaluando su capacidad terapeutica con los ensayos descritos. Es importante mencionar que la producción por parte del grupo español de agentes inmunomoduladores es constante y después de ensayarlos in-vitro con macròfagos alveolares y determinar que sean de baja toxicidad se probaran en nuestro modelo experimental. Por lo tanto, la participación del grupo de la Universidad de Salamanca es determinante, y su actividad y contribuciòn no la puede realizar otro grupo mexicano.

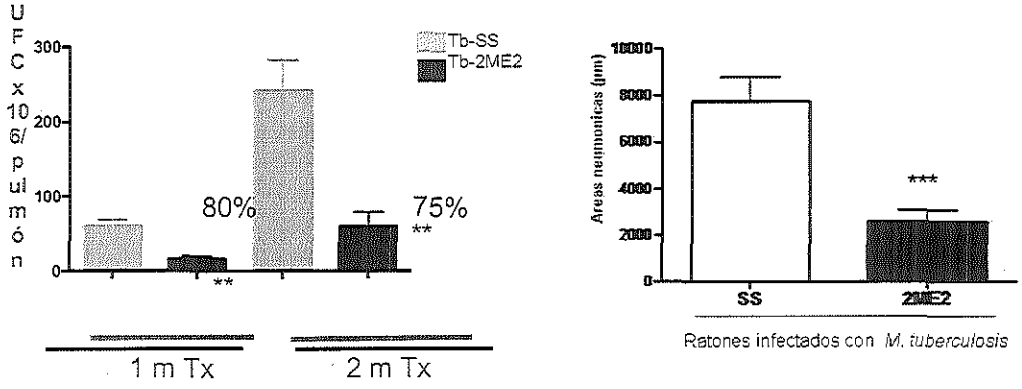
La segunda estrategia general de nuestro proyecto es suprimir los efectos deletereos que tiene la respuesta inmune sobre el control del crecimiento bacteriano durante la enfermedad avanzada y que contribuyen a la progresión de la misma, para lo cual se proponen las siguientes estrategias:

1.- Supresión de la actividad del TGF. En parrafos anteriores se menciono la destacada participación inmunomoduladora y anti-inflamatoria que tiene el TGF en la tuberculosis humana y experimental. En nuestro modelo experimental esta citocina se produce en gran cantidad durante la fase progresiva de la enfermedad (43). Recientemente informamos que ratones con tuberculosis avanzada producida por la cepa drogosensible H37Rv tratados con el receptor soluble tipo III recombinante (betaglicano), un potente bloqueador de TGF-β [49], fue muy eficiente terapeuticamente, pues dicho tratamiento indujo mayor producciòn de IFN-γ, IL-2, TNF e iNOS, lo cual produjo significativa disminución de la carga bacilar pero con inflamación excesiva y mayor consolidación pulmonar, lo cual indica que el TGF-β tiene un papel muy importante como protector del efecto destructor que tiene la inflamación excesiva. Interesantemente, cuando ademàs del betaglicano soluble se administrò un antinflamatorio (ac niflumico un eficiente bloqueador de la enzima cicloxigenasa 2 productora de prostaglandinas), se indujo una eficiente actividad Th-1 con minima inflamaciòn [49]. La administracion conjunta de antibioticos con betaglicano y acido niflumico redujo eficientemente el tiempo de tratamiento y la fibrosis al provocar tambièn una reducciòn significativa de la IL-4 [49]. Por lo tanto esta es una forma muy eficiente de inmunoterapia, pero no hemos estudiado su efecto en la tuberculosis producida por bacterias MDR y tampoco lo hemos comparado con otros inhibidores del TGF como inhibina o peptido latente de TGF. Nuestro objetivo en el presente proyecto serà probar la eficiencia del betaglicano en ratones infectados con cepas MDR administrado por via intraperitoneal dos veces a la semana a partir del mes de infecciòn, asi como su efecto al combinarlo con acido niflumico. Ademas probaremos siguiendo el mismo esquema de tratamiento a otros inhibidores del TGF. Para este objetivo contamos con la colaboración del Dr Fernando Lopez Casillas, experto en TGF y sus receptores con quien hemos tenido una larga y fructifera colaboración estudiando la participación del TGF en la tuberculosis pulmonar experimental.

2.- Promoción de apoptosis macrofàgica. Estudios in-vitro han demostrado que la infecciòn de macròfagos por *M. tuberculosis* induce apoptosis y que esta produce no solo la muerte del macròfago, sino tambien de la bacteria (87, 88), lo cual es influido por el nivel de virulencia del bacilo, pues cepas virulentas inducen mas apoptosis que las no virulentas (46). Nosotros hemos informado recientemente, que en nuestro modelo experimental existe una gran cantidad de macròfagos activados apoptosicos después del primer y tercer dia de la infecciòn, lo cual se relaciona con una producción elevada de

TNF e iNOS. Interesantemente, durante la fase progresiva de la enfermedad los macrofagos vacuolados expresan mucho la molécula anti-apoptòsica Bcl-2, lo cual los hace significativamente resistentes a morir por apoptosis, permitiendo asi a la bacteria tener un habitat celular de larga vida (46), que ademas es una fuente muy importante de moléculas inmunosupresoras como TGF, IL-10 y PGE-2 entre otras. Por lo tanto, una estrategia inmunoterapeutica potencial es el uso de moléculas o fármacos que promuevan la apoptosis macròfagica y asi destruyan el nicho de la bacteria y la fuente de moléculas inmunosupresoras.

Una estrategia util para cumplir con este objetivo es el uso de bloqueadores de factores de transcripcion que previenen la apoptosis inducida por la hipoxia. La presencia de abundante infiltrado inflamatorio intralveolar consecuencia del crecimiento de la bacteria es un aspecto distintivo de nuestro modelo experimental, lo cual produce insuficiencia respiratoria e hipoxia. Experimentos en curso han demostrado que en nuestro modelo la neumonía induce intensamente la expresión del factor relacionado a hipoxia (HIF), específicamente en los macròfagos vacuolados. El factor HIF entre otras funciones, induce la expresión de Bcl-2 previniendo la apoptosis de celulas inflamatorias (89). Por lo tanto, el bloqueo farmacologico del HIF puede promover eficientemente la apoptosis macrofàgica. La molécula 2MD es un eficiente y selectivo inactivador de HIF (89) y en un experimento preliminar observamos que la administración intratraqueal de este durante la fase progresiva en nuestro modelo, disminuyó significativamente la carga bacilar, en coexistencia con abundantes macròfagos apoptòsicos en las areas neumònicas.



Nuestro objetivo ahora es reproducir este experimento y determinar la eficiencia terapeutica del 2M2 en ratones infectados con bacterias MDR y su capacidad de acortar la antibioticoterapia convencional en ratones infectados con la cepa H37Rv.

Ademas tambièn realizaremos experimentos con fármacos promotores de apoptosis que bloquean el efecto antiapoptosico de la molécula Bcl2, como el obatoclax GX-15, la cual es capaz de unirse a una pequeña concavidad dentro de la molécula Bcl-2 lo cual suprime su actividad (90). Esta molécula experimental ya contamos con ella, nuestros colaboradores en este objetivo son la Dra Sara Huerta y el Dr Mario Vega, del Centro Mèdico La Raza IMSS, quienes las han usado exitosamente para eliminar celulas cancerosas. Ademàs nuestros colegas de la Universidad de Salamanca han sintetizado aminoalcoholes de cadena larga y derivados deamina que en cèlulas Jurkatt in-vitro inducen acentuada apoptosis, a traves de la activaciòn de caspasa 3 y activacion mitocondrial (86). Por lo tanto, otro grupo numeroso de nuevas moléculas inductoras de

apoptosis existen y estan siendo producidas por el grupo español, algunas de las cuales pueden ser eficientes agentes inmunoterapeuticos en tuberculosis.

3.- Supresión de la síntesis de prostaglandinas. El mediador inflamatorio prostaglandina E-2 (PGE-2) tiene un notable efecto inmunosupresor sobre la respuesta de inmunidad celular (74). En concentraciones altas la PGE-2 tiene funciones muy similares a las del TGFβ, dado que suprime la producción de IFNγ, IL-2 e IL-12 (75, 76, 77) y también inhibe la activación macrofágica (78) disminuyendo la producción de TNFα e iNOS (79). En nuestro modelo de tuberculosis pulmonar progresiva hemos informado que la producción de PGE-2 durante la fase progresiva de la enfermedad es muy alta y que al suprimirla con un bloqueador farmacològico como el àcido niflùmico se reconstituye significativamente la actividad protectora Th-1 (52). En consecuencia, el bloqueo de la síntesis de prostaglandinas es potencialmente una nueva y quizas eficiente forma de inmunoterapia.

Para la producción de prostaglandinas es fundamental la participación de las enzimas fosfolipasas, las cuales liberan al acido araquidònico de los fosfolipidos membranales, permitiendo que las cicloxigenasas actuen sobre el ac araquidònico convirtiendolo en prostaglandinas. Por lo tanto, se puede suprimir la síntesis de prostaglandinas a traves de inactivar las ciclooxigenasas o las fosfolipasasa. El grupo del Dr SanFeliciano de la Universidad de Salamanca ha producido diversos aminoalcoholes de cadena larga y lipidos diamina que suprimen eficientemente in-vitro la actividad de fosfolipasas citosolicas y de secreción, los cuales son muy poco tòxicos (85). Actualmente ya disponemos de dos de estos fármacos para empezar a tratar a ratones durante la fase progresiva de la enfermedad (2 meses postinfecciòn), que es cuando existe una gran producción de PGE-2 lo cual suprime la inmunidad protectora Th-1. Es importante mencionar que el grupo español esta produciendo mas derivados sintèticos de esta familia de moléculas y las esta ensayando in-vitro. Por lo tanto nuevas y mas eficientes supresores farmacologicos de estas enzimas seran producidas, algunas de las cuales pueden ser eficientes agentes inmunoterapeuticos en la tuberculosis.

4.- Supresión de la glicoproteina acida alfa 1 (AGP). La AGP es una de las mas de 30 proteinas de fase aguda, que normalmente se sintetizan en el higado como parte de la reacción de fase aguda (RFA). La RFA es una serie de mecanismos moleculares y celulares encaminados a proteger y aislar agentes agresores inductores de inflamación (91). Las proteinas de fase aguda son una familia diversa de moléculas con funciones diversas, específicamente la AGP ha demostrado que tiene actividades anti-inflamatorias e inmunosupresoras, como la inactivaciòn de neutròfilos y macròfagos, asi como la inducción en la secreción del receptores solubles de TNF e IL-1.
Recientemente hemos demostrado que en nuestro modelo experimental de tuberculosis progresiva existe una gran producción por los macròfagos vacuolados de AGP durante la fase progresiva de la infecciòn. Es interesante que cuando esta proteina se bloquea su actividad con anticuerpos policlonales especìficos (Dr Erasmo Martinez Cordero) se indujo una alta producción de IFN, TNF e iNOS, lo cual disminuyò significativamente la carga bacilar pulmonar y la extensión de la neumonia (92). Otra funciòn de la AGP es la uniòn e inactivaciòn de medicamentos y recientemente se reportò que la AGP es muy eficiente para unir y bloquear a los antibioticos primarios y algunos de los de segunda linea que se usan para tratar la tuberculosis (93). Es por lo tanto posible que al bloquear esta proteina durante la fase progresiva de la infecciòn, en conjunto con la antibioticoterapia convencional se pueda mejorar la eliminación bacilar, no solo por potenciar la respuesta inmune protectora, sino tambièn por facilitar la biodisponibilidad de los medicamentos. Tambièn probaremos la eficiencia del tratamiento inactivador de AGP en animales infectados con bacterias MDR, administrando los anticuerpos

bloqueadores a partir del segundo mes de infecciòn y siguiendo el mismo esquema de tiempos de sacrificio y analisis de la eficiencia terapeutica que se ha descrito en los apartados anteriores.

La inmunidad humoral en tuberculosis, el uso de anticuerpos especificos en contra de antigenos inmunodominantes como una nueva modalidad inmunoterapeutica en tuberculosis progresiva y latente

Siempre se ha considerado que la inmunidad celular es la unica que confiere protecciòn en las infecciones por organismos intracelulares facultativos como *M. tuberculosis*. Sin embargo, publicaciones recientes en modelos experimentales de tuberculosis (94) o nocardiosis han mostrado que la administración de anticuerpos, sobre todo de clase IgM, producidos en contra de antigenos inmunodominantes del microorganismo (nocardia, en un modelo murino de micetoma) son eficientes agentes inmunoterapeuticos. El grupo del Dr Mario Cesar Salinas de la Universidad de Nuevo Leon ha publicado estos interesantes resultados y en colaboración con el se han producido anticuerpos policlonales de clase IgM en contra de un extracto antigenico proteico total de *M. tuberculosis* y de los antigenos micobacterianos inmunodominantes 85b y P-38. El grupo del Dr Salinas ya nos ha enviado los anticuerpos y estamos en condiciones de empezar los experimentos terapeuticos en nuestro modelo experimental de tuberculosis progresiva, administrandolos por via intraperitoneal en 3 dosis, una por semana, sacrificando grupos de animales después de 7, 14, 30 y 60 dias después de la ultima administración de los anticuerpos y determinando su eficiencia con el mismo esquema descrito en los apartados anteriores.

Recientemente, el grupo del Prof Jaruj Ivanyi del Guy Hospital en Londres ha publicado que la administración de anticuerpos de clase IgA en contra de la proteina micobacteriana alfa cristalina y anticuerpos bloqueadores anti-IL4 en ratones Balb/c con tuberculosis activa tiene un significativo efecto terapeutico, manifestado por una acentuada disminución de la carga bacilar (96). Se ha reportado que la alfa cristalina es una proteina de stress que sobre todo se expresa en infecciòn latente, por lo tanto es posible que la misma estrategia terapeutica tenga un eficiente efecto previniendo la reactivaciòn de la infeccion latente. Como se menciono en los antecedentes, un tercio de la humanidad (1700 millones de personas) tienen tuberculosis latente y se calcula que el 10% de estos sujetos sufriran reactivaciòn de la enfermedad. Es por lo tanto necesario desarrollar tambièn estrategias inmunoterapeuticas dirigidas a prevenir la reactivaciòn de la tuberculosis latente, sobre todo en sujetos inmunodeprimidos como pacientes con SIDA o diabeticos descompensados. Este es precisamente nuestro objetivo, para lo cual usaremos nuestro modelo murino de infecciòn latente, el cual se basa en el uso de ratones hibridos F1 de la cruza entre ratones C57Bl y DBA, infectados por via intrataqueal con una dosis muy baja (1000 bacterias) de la cepa de *M. tuberculosis* H37Rv por via intratraqueal (97). En este modelo se produce una baja y estable carga bacilar pulmonar con lesiones granulomatosas sin neumonía, respuesta inmunològica totalmente sesgada de tipo Th-1 y sobrevida total (97), pero la administración de corticosterona (3mg/lt) en el agua de bebida produce rapida reactivaciòn con elevada carga bacilar y mortalidad (98). Nuestra estrategia serà entonces usar este modelo y cuando la infecciòn latente sea estable (7 meses), administraremos a grupos de 20 animales la combinación del anticuerpo monoclonal IgA anti alfa cristalina, mas anticuerpos bloqueadores de IL-4. Otro grupo con el mismo numero de animales recibira solo los anticuerpos anti alfa-cristalina. El tercer grupo solo recibira los anticuerpos anti IL-4 y el grupo control recibira el vehículo. Después de un mes se administrara la corticosterona y se sacrificaran grupos de 5 ratones después de 7, 15, 30

y 60 dias de la ultima administración de los anticuerpos. Un pulmon, derecho o izquerdo, se destinara para estudio histològico y el otro para la cuantificaciòn de la carga bacilar por la determinación de unidades formadoras de colonia, como se ha descrito previamente (96). Para este objetivo contamos con la colaboración del Prof Ivanyi y el Dr Gustavo Falero del Instituto Finley de Biotecnología en la Habana Cuba, quien es el productor de los anticuerpos monoclonales anti-alfa cristalina. De hecho este reactivo ya lo tenemos para empezar el experimento.

Inmunidad inata en tuberculosis, el uso de peptidos antimicrobianos del tipo de las beta-defensinas como agentes terapeuticos en la tuberculosis.

Un aspecto de gran importancia en la respuesta inmunològica en contra de la tuberculosis la cual ha empezado a estudiarse recientemente es la participación de la inmunidad inata. En este aspecto el grupo del Dr Eduardo Sada del INER y nosotros hemos sido de los primeros en estudiar específicamente la participación de peptidos antimicrobianos en el control del crecimiento bacilar. Usando nuestros modelos experimentales de tuberculosis progresiva y latente hemos informado recientemente que el epitelio bronquial produce activamente y en gran cantidad defensinas beta 3 y 4 (98). De manera interesante, ambos peptidos se producen durante la fase temprana de nuestro modelo de tuberculosis progresiva y después durante la enfermedad progresiva disminuyen significativamente. En el modelo de infecciòn latente, ambos peptidos se producen 5 veces mas que en la fase temprana del modelo de tuberculosis progresiva y su expresión desaparece cuando se reactiva la enfermedad con la administración de corticosterona (98). Usando inmunoelectronmicroscopìa hemos demostrado recientemente, que en nuestro modelo de infecciòn latente existen micobacterias en el citoplasma de celulas bronquiales y que estas se encuentran asociadas a una abundante cantidad de defensinas beta-3 (99), lo cual sugiere que este tipo de peptidos antimicrobianos participan en el control del crecimiento bacilar tanto en enfermedad progresiva como en infecciòn latente. Nuestro proposiciòn por lo tanto, es que el uso de defensinas puede tambien ser util como una nueva forma de inmunoterapia en tuberculosis.

Las defensinas beta recombinantes estan disponibles comercialmente pero son muy caras, lo cual hace muy poco practico su uso como tratamiento. Por este motivo nuestra proposiciòn es usar inductores de la expresión de defensinas beta como el aminoácido isoleucina (100) y el uso de adenovirus recombinantes que expresan beta defensinas 3 y 4. Utilizando una linea celular de neumocitos tipo II la cual es susceptible a la infecciòn por M. tuberculosis y en consecuencia produce una gran cantidad de defensinas beta, ha sido posible confirmar y establecer la cantidad de isoleucina capaz de inducir una eficiente producción de defensinas beta 3 y 4. Mas aun, la administración intratraqueal en ratones Balb/c no infectados nos permitio confirmar que este aminoácido es tambien capaz de inducir eficientemente la producción de defensinas in-vivo. Por lo tanto, nuestra estrategia serà administrar cada 72 hr isoleucina en gotas nasales o intratraquealmente en ratones Balb/c con tuberculosis avanzada (dos meses post-infeccion) y sacrificar grupos de 8 ratones después de 15, 30 y 60 dias para determinar su eficiencia terapeutica siguiendo el esquema antes descrito. Para la segunda estrategia que es el uso de adenovirus recombinantes que expresan defensina beta 3 y 4, contamos con la colaboración de la Dra Marcela del Rio, de la Universidad Complutense de Madrid. La Dra del Rio ha trabajo con inmunoterapia usando este tipo de vectores adenovirales en un modelo de quemadura cutanea en cerdo, y ha demostrado que la aplicación topica de estos virus en el area quemada previene significativamente infecciones. Nuestra estrategia serà administrar este tipo de adenovirus por via

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intratraqueal en una sola dosis en animales con tuberculosis progresiva, utilizando nuestra experiencia utilizando rAd-IFN descrita en parrafos anteriores.

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