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IMMUNOLOGICAL ASPECTS

# Efficacy of gene-therapy based on adenovirus encoding granulocyte-macrophage colony-stimulating factor in drug-sensitive and drug-resistant experimental pulmonary tuberculosis

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SUMMARY

Tuberculosis (TB), although a curable disease, remains a major cause of morbidity and mortality worldwide. It is necessary to develop a short-term therapy with reduced drug toxicity in order to improve adherence rate and control disease burden. Granulocyte-macrophage colony-stimulating factor (GM-CSF) may be a key cytokine in the treatment of pulmonary TB since it primes the activation and differentiation of myeloid and non-myeloid precursor cells, inducing the release of protective Th1 cytokines. In this work, we administrated by intratracheal route recombinant adenoviruses encoding GM-CSF (AdGM-CSF). This treatment produced significant bacterial elimination when administered in a single dose at 60 days of infection with drug sensitive or drug resistant Mtb strains in a murine model of progressive disease. Moreover, AdGM-CSF combined with primary antibiotics produced more rapid elimination of pulmonary bacterial burdens than conventional chemotherapy suggesting that this form of treatment could shorten the conventional treatment.



## 1. Introduction

Tuberculosis (TB), although a curable disease, still remains a major cause of morbidity and mortality worldwide with 9.6 million new active cases and 1.5 million deaths during 2014 [1]. TB treatment is long and has many side effects with the consequence of treatment abandonment and the development and spread of multi drug-resistant (MDR) and extensively drug-resistant (XDR) TB. Therefore, it is necessary to develop short-term therapy with reduced drug toxicity improving adherence rate and control of the disease burden [2].

First-line anti-TB agents that form the core of treatment regimen include isoniazid (INH), rifampin (RIF), ethambutol (EMB) and pyrazinamide (PZA). This combination of antibiotics is necessary for killing slow- and fast-growing mycobacteria [3]. The emergence of drug-resistant strains of *M. tuberculosis* (Mtb) has been related to immunodeficiency and pathological features such as lung cavities with the consequence of poor antibiotic penetration and bacterial overgrowth [3,4]. Adjunct immunotherapy to the standard antibiotics with recombinant cytokines such as interferon gamma (IFNγ) has been evaluated in clinical trials. It has been demonstrated that IFNγ therapy, in subcutaneous, intramuscular, and aerosolized formulations, may increase the bacterial clearance and improve the symptoms [5–7]. In addition, patients with MDR-TB treated with nebulized IFNγ in combination with antibiotic treatment, showed a decrease of constitutional symptoms and an increased clearance of Mtb in sputum, suggesting that improving the immune response with adjunct immunotherapy may be more effective than the administration of only antibiotics [7]. Nevertheless, treatment with recombinant proteins has several disadvantages, such as multiple dose administration with a wide range of effects depending on glycosylation-motifs and inhibitors development [8,9]. Thus, it is necessary to introduce more efficient

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methods of cytokine delivery or new approaches for inducing the expression of cytokines locally in the affected organ.

IFNγ and granulocyte-macrophage colony-stimulating factor (GM-CSF) are significant participants in the immune protection against pulmonary and extra-pulmonary TB. It has been demonstrated in human and experimental models, that genetic deficiency or blocking of GM-CSF leads to high susceptibility, fast-progression and death by Mtb infection [10–12]. Thus, GM-CSF may be a key cytokine in the immunotherapeutic treatment of pulmonary TB. Indeed, GM-CSF primes the activation and differentiation of myeloid and non-myeloid precursor cells, inducing the release of protective Th1 cytokines such as IL-12, IFNγ and TNFα. GM-CSF is necessary for the activation of macrophages (MØ) and dendritic cells (DC) [13]. Activated MØ are related to the expression of the transcription factor IRF-5, which is induced by GM-CSF stimulation [14]. It is known that resident alveolar MØ (AM) are one of the target cells most susceptible to and permissive of Mtb infection [15], and it has been demonstrated that GM-CSF plays a critical role for AM development and function [16,17]. Thus, enhancing the bactericidal function of AM should be an effective target for immunotherapy in progressive pulmonary TB.

We have previously demonstrated that gene therapy based on adenoviruses encoding GM-CSF (AdGM-CSF) increased protective immunity when administered in a single dose one day before Mtb infection in BALB/c mice. Recombinant adenoviruses infected the airways epithelium and AM, increased the production of GM-CSF which induced rapid and efficient activation of DC that induced the production of IFNγ, TNFα and iNOS, permitting efficient control of mycobacterial growth. One single dose of AdGM-CSF administration was also effective in preventing experimental latent TB reactivation and transmission [17]. The aim of the present study was to determine the therapeutic efficacy of AdGM-CSF administered during late progressive disease, used alone or in conjunction with the conventional chemotherapy in mice infected with drug-sensitive strain H37Rv or with MDR Mtb clinical isolate.

## 2. Materials and methods

### 2.1. Ethics statement

The Institutional Ethics Committee of the National Institute of Medical Sciences and Nutrition 'Salvador Zubirán' approved animal studies, in accordance with the guidelines of the Mexican regulations on Animal Care and Experimentation NOM 062-ZOO-1999.

### 2.2. Mycobacterium tuberculosis strains growth

Drug-sensitive Mtb strain H37Rv (ATCC no.25618) and MDR strain (CIB-99 clinical isolate, resistant to streptomycin, INH, RIF, EMB and PZA) [18], were grown in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI, USA) supplemented with 0·2% (v/v) glycerol, 10% oleic acid, albumin, dextrose and catalase (OADC enrichment media, BBL, Becton Dickinson, Franklin Lakes, New Jersey) and 0.02% (v/v) Tween-80 at 37 °C. Mid log-phase cultures were used for all experiments. For *in vivo* studies, mycobacteria were counted by colony-forming unit (CFU) and stored at −70 °C until use. Before use, mycobacteria aliquots were thawed and pulse-sonicated to remove the clumps.

### 2.3. Progressive pulmonary TB in BALB/c mice

We used the murine model of intra-tracheal (i.t.) infection described previously [19–21]. Briefly, 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 containing $2.5 \times 10^5$ CFU Mtb drug sensitive H37Rv or MDR clinical isolate were inoculated intratracheally (i.t.) using a stainless steel cannula (Thomas Scientific, Swedesboro, NJ) connected to an insulin syringe. Mice were maintained in vertical position until they spontaneously recovered. Infected mice were divided into groups of five in cages fitted with micro-isolators in biosafety level 3 facilities.

### 2.4. Treatment of infected mice with AdGM-CSF or Addl70-3

Construction, expression, biological effect and titration of adenovirus encoding GM-CSF (AdGM-CSF) or empty-control adenovirus (Addl70-3) have been reported previously [22]. After 60 days of infection, when the chronic or progressive phase of the infection is well established [19–21], animals were anaesthetized (sevoflurane; Abbott Laboratories, Abbott Park, IL, USA) and 100 μl of isotonic sterile endotoxin-free saline solution with $1 \times 10^9$ plaque-forming units (PFU) of AdGM-CSF or empty adenovirus control Addl70-3 were i.t. inoculated using a stainless steel cannula. Mice were maintained in vertical position until they spontaneously recovered. Groups of six mice were sacrificed by exsanguination under anaesthesia at 7, 15, 30 and 60 days of post-treatment. Four right lungs, per each time point, were obtained to quantify lung mycobacterial loads by colony forming units (CFU), four left lungs were used to assess tissue damage (pneumonia) by automated morphometry; and four (two right lungs plus two left lungs, each one from different mice) were obtained to assess the immunological effect by the quantification of activated DC using flow cytometry and cytokines expression by RT-PCR as described below.

In order to study the efficacy of adenovirus-based cytokine gene transfer adjunct to conventional chemotherapy, two groups of mice infected with Mtb H37Rv strain at two month post-infection were treated with a single dose of AdGM-CSF or as a control Addl70-3 ($1 \times 10^9$ pfu) i.t., plus conventional antibiotics: RIF (10 mg/kg), INH (10 mg/kg), and PZA (30 mg/kg), administered intragastrically (i.g.), once every day during two months. A third group of mice received only conventional chemotherapy administered i.g. Briefly, for conventional antibiotics administration, the antibiotics were suspended at the desired concentrations in 200 μL distilled water per mouse using 1 mL syringe. The rigid stainless steel cannula (Thomas Scientific, Swedesboro, NJ) was inserted through the mouth into the stomach and mice were maintained vertically until complete administration of the antibiotic was done. Following the treatment, mice were euthanized by exsanguination under anaesthesia at 7, 15, 30 and 60 days of post-treatment. The efficacy of each treatment in each time point of sacrifice was determined by quantifying the mycobacterial load in five right lungs (CFU) and by assessing the extent of tissue damage using histopathology/automated morphometry in five left lungs. All data points represent mean values ± SD of five animals for a representative experiment, and two independent experiments were performed.

### 2.5. Determination of CFU in infected lungs

Right lungs from each of the four mice at each time-point were used to determine CFU. The lungs were homogenized with a polytron device (Kinematica, Lucerne, Switzerland) in sterile tubes containing 1 mL 0.05% Tween-80 in PBS. Four consecutive logarithmic dilutions of each homogenate were spread onto duplicate plates containing Bacto Middlebrook 7H10 agar (Difco, Detroit, MI, USA) enriched with oleic acid, albumin, catalase and dextrose-enriched medium (Becton Dickinson, Sparks, MD, USA). The number of colonies was counted after 15 and 21 days of incubation at 37 °C with 5% $CO_2$. Data are reported as the mean values ± SD from



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