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# Antigen-presenting cells transfected with Hsp65 messenger RNA fail to treat experimental tuberculosis

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## Abstract

In the last several years, the use of dendritic cells has been studied as a therapeutic strategy against tumors. Dendritic cells can be pulsed with peptides or full-length protein, or they can be transfected with DNA or RNA. However, comparative studies suggest that transfecting dendritic cells with messenger RNA (mRNA) is superior to other antigen-loading techniques in generating immunocompetent dendritic cells. In the present study, we evaluated a new therapeutic strategy to fight tuberculosis using dendritic cells and macrophages transfected with Hsp65 mRNA. First, we demonstrated that antigen-presenting cells transfected with Hsp65 mRNA exhibit a higher level of expression of co-stimulatory molecules, suggesting that Hsp65 mRNA has immunostimulatory properties. We also demonstrated that spleen cells obtained from animals immunized with mock and Hsp65 mRNA-transfected dendritic cells were able to generate a mixed Th1/Th2 response with production not only of IFN- $\gamma$  but also of IL-5 and IL-10. In contrast, cells recovered from mice immunized with Hsp65 mRNA-transfected macrophages were able to produce only IL-5. When mice were infected with *Mycobacterium tuberculosis* and treated with antigen-presenting cells transfected with Hsp65 mRNA (therapeutic immunization), we did not detect any decrease in the lung bacterial load or any preservation of the lung parenchyma, indicating the inability of transfected cells to confer curative effects against tuberculosis. In spite of the lack of therapeutic efficacy, this study reports for the first time the use of antigen-presenting cells transfected with mRNA in experimental tuberculosis.

Key words: Tuberculosis; Therapeutic immunization; CD; mHSP65; DNA vaccine; APCs

## Introduction

Tuberculosis (TB) is a major public health problem and remains one of the major causes of mortality from an infectious disease worldwide. About one-third of the world's population has been infected with *Mycobacterium tuberculosis*. Today, the greatest difficulty in the treatment of TB is the necessity of continuous medication over a long period of time with multiple drugs (1). Most patients are averse or unable to complete a full course of medication, and patients who do not consistently undergo their therapies are at risk of relapsing and acquiring multidrug-resistant TB (1,2). Additionally, a sizable increase in the emergence of multidrug-resistant TB and the appearance of extremely drug-resistant TB, which is particularly dangerous for

people with HIV/AIDS, has been found in all regions of the world. Thus, immunotherapy has been proposed as an alternative to treat TB, especially drug-sensitive TB. In addition, immunotherapy that enhances the efficiency of the immune response in *M. tuberculosis*-infected patients could be of additional value as a strategy to complement anti-bacterial chemotherapy. In the last decade, various nonspecific or antigen-specific immunological agents, such as DNA plasmids (3,4), cytokines (5), immunoglobulins (6), and mycobacterial antigens (7), have been used either alone or as adjuncts to chemotherapeutic regimens with varying success rates.

Dendritic cells (DCs) are the most powerful antigen-

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presenting cells (APCs) that induce and maintain immune responses. These cells are notably efficient in the uptake and processing of antigen, express high levels of major histocompatibility complex class I and class II, as well as costimulatory molecules, and migrate from the site of antigen uptake toward the lymph node, where they present major histocompatibility complex (MHC) class I- and class II-restricted peptides to prime naive T cells (8). The combination of these properties makes DCs particularly suitable as vehicles for antigen presentation in immunotherapy. DC-based vaccines have therapeutic potential against tumors as demonstrated in murine models and several human clinical trials (9,10). DCs can be pulsed with a synthetic peptide (11) or full-length protein (12), transfected with DNA or RNA (13,14) or transduced with recombinant viruses (15). However, comparative studies suggest that messenger RNA (mRNA) transfection is superior to other antigen-loading techniques in generating immunocompetent DCs (16,17). In addition to DCs, several studies also mention the use of macrophages as alternative vehicles for antigen presentation (18,19).

Experimental data collected over the last few years have shown that a DNA vaccine encoding the *M. leprae* 65-kDa heat shock protein (DNA-Hsp65) has prophylactic and therapeutic efficacy in a murine model of TB (4,20). The therapeutic effect of this vaccine was associated with the presence of CD8+/CD44hi IFN- $\gamma$ -producing cytotoxic cells (21,22). In addition, it was demonstrated that the DNA-Hsp65 vaccine could be taken up by CD11b+ (macrophages) and CD11c+ (DCs) cells after its administration in mice (23).

Based on the results obtained from studies with DNA-Hsp65 and on recent data about the use of mRNA-loaded DCs in cancer treatment, we examined the therapeutic effect of immunizing TB-infected mice with DCs and macrophages transfected with Hsp65 mRNA. DCs generated from murine bone marrow cells and macrophages obtained from a peritoneal lavage were transfected with Hsp65 mRNA using electroporation or passive pulsing. We demonstrated that the therapeutic immunization with Hsp65 mRNA-transfected DCs or macrophages was not able to reduce the bacterial load in the lungs of infected animals. The mice received two doses of transfected cells intravenously or subcutaneously, and neither strategy was efficient in inducing therapeutic effects against TB. Additionally, the production of cytokines in the lung and the histopathology of infected mice were not altered by the immunization with transfected cells. In spite of the lack of therapeutic effects, this study represents the first time that APCs transfected with mRNA were used during an infection with *M. tuberculosis*.

## Material and Methods

### Ethics statement

BALB/c (WT) mice, 6- to 8-week-old males, were purchased from the Central Animal House of the Ribeirão Preto Campus, USP. The mice were maintained under standard

conditions in the Animal House of the Departamento de Imunologia, Faculdade de Medicina de Ribeirão Preto, USP, Ribeirão Preto, SP, Brazil. All experiments with animals were approved and conducted in accordance with the guidelines of the Animal Care Committee of Faculdade de Medicina de Ribeirão Preto, USP (No. 053/2006).

### Plasmid and *in vitro* transcription of Hsp65 mRNA

The pcDNA<sub>3A</sub>-Hsp65 construct was derived from the pcDNA<sub>3</sub> vector (Invitrogen, USA). The vector was previously digested with *Bam*HI and *Apal* (Invitrogen), and a 1.8-kb fragment corresponding to the *M. leprae* Hsp65 gene was inserted. For *in vitro* transcription, the pcDNA<sub>3A</sub>-Hsp65 plasmid was linearized with *Apal*, and synthetic Hsp65 mRNA was generated using the mMMESSAGE mMACHINE<sup>®</sup> T7 Ultra Reaction system (Ambion, USA) according to manufacturer recommendations. The concentration and quality of the pcDNA<sub>3A</sub>-Hsp65 plasmid and the Hsp65 mRNA were assessed by spectrophotometry and agarose gel electrophoresis.

### DC generation from bone marrow

Murine DCs were generated according to the protocol described by Lutz et al. (24), with some modifications. Briefly, bone marrow cells prepared from the femora and tibiae of naive BALB/c mice were depleted of red blood cells with ACK lysis buffer (150 mM NH<sub>4</sub>Cl, 10 mM KCO<sub>3</sub>, 0.1 mM EDTA, pH 7.3) and cultured in RPMI 1640 medium (Gibco-BRL, USA) supplemented with 10% fetal bovine serum (Gibco-BRL), 100 mM Na pyruvate, 100 mM non-essential amino acids, 200 mM L-glutamine, 100 IU/mL penicillin, 100  $\mu$ g/mL streptomycin, 10  $\mu$ g/mL gentamycin, 10 mM 2-ME, 10 ng/mL murine GM-CSF (BD Biosciences, USA) and 5 ng/mL murine IL-4 (BD Biosciences) at 37°C and 5% CO<sub>2</sub>. On day 3, the non-adherent granulocytes and T and B cells were gently removed, and fresh DC medium was added. On days 5, 7, and 9, 80% of the medium was replaced with fresh DC medium. On day 10, non-adherent cells were harvested for phenotypic analysis (flow cytometry) and mRNA transfection.

### Transfection of DCs and macrophages

The mRNA transfection of DCs (from the bone marrow) and peritoneal macrophages (obtained from a peritoneal lavage with cool PBS) was performed by electroporation (for intravenous immunization) or passive pulsing (for subcutaneous immunization). For electroporation, immediately before transfection, DCs and macrophages were washed twice in PBS, pH 7.2, and the cells (1 x 10<sup>6</sup> cells) were resuspended in 400  $\mu$ L Opti-MEM (Gibco-BRL). The cells were mixed with 5  $\mu$ g mRNA in a 0.4-cm sterile disposable electroporation cuvette and transfected in a Gene Pulser Xcell<sup>™</sup> (Bio-Rad, USA) with a voltage pulse of 300 V in combination with a capacitance of 150  $\mu$ F. After electroporation, the cells were immediately resuspended in RPMI 1640 medium and further incubated for 1 h at 37°C and 5% CO<sub>2</sub>. For contact transfection, the cells were incubated with 5  $\mu$ g mRNA for

1 h in Opti-MEM (Gibco-BRL) at 37°C and 5% CO<sub>2</sub>. These transfected cells were used for *in vitro* flow cytometry analysis and *in vivo* immunization.

### Flow cytometry analysis

Dendritic cells derived from the differentiation of bone marrow cells and macrophages obtained from peritoneal lavage were pre-incubated with 2.4 G2 monoclonal antibodies (mAb) to block FcγR (Pharmingen, USA) and were then incubated with the relevant mAb for 30 min at 4°C. DCs were labeled with anti-CD11c-FITC (clone HL3), and macrophages were stained with anti-CD11b-FITC. A biparametric gate was drawn around the cell population in the forward and side scatter dot plot. The gated populations were subsequently selected according to CD11b<sup>+</sup> or CD11c<sup>+</sup> staining. Anti-CD80, anti-CD86, anti-CD40, and anti-IA<sup>d</sup> (MHC II) were used as phycoerythrin-conjugates (all antibodies were purchased from Pharmingen). Analytical flow cytometry was carried out using a FACScan instrument (Becton Dickinson, USA), and the data were processed using the WinMDI software.

### Animals and immunization procedures

Female 8-week-old BALB/c mice were obtained from the animal facility at Faculdade de Medicina de Ribeirão Preto, USP. Infected animals were kept in the biohazard facility at Laboratory Biosafety Level 3 and were housed in cages within a laminar flow safety enclosure under standard conditions. For the immunogenicity assay, mice were intravenously injected in the retro-orbital venous sinus with one dose of Hsp65 mRNA transfected DCs (DCs/Hsp65 mRNA, 1 × 10<sup>6</sup> cells/dose), Hsp65 mRNA transfected macrophages (macrophages/Hsp65 mRNA, 1 × 10<sup>6</sup> cells/dose), DCs only (mock DCs, 1 × 10<sup>6</sup> cells/dose), or macrophages only (mock macrophages, 1 × 10<sup>6</sup> cells/dose). For the BCG immunization, one dose of the Moreau strain was delivered in the dorsum by the subcutaneous injection of 10<sup>5</sup> live bacteria in 100 μL saline. For the therapeutic immunization assays, mice were injected intravenously in the retro-orbital venous sinus or subcutaneously in the dorsum with two doses (at 2-week intervals) of DC/Hsp65 mRNA (1 × 10<sup>6</sup> cells/dose), macrophages/Hsp65 mRNA (1 × 10<sup>6</sup> cells/dose), mock DCs (1 × 10<sup>6</sup> cells/dose), or mock macrophages (1 × 10<sup>6</sup> cells/dose). The immunogenicity and therapeutic immunization assays were performed using 5 animals per group.

### Proliferation assay

Splenocytes were isolated from the immunized mice (immunogenicity assay) and labeled with CFSE (Invitrogen). The 5 mM stock solution of CFSE was diluted to 5 μM in a volume of PBS equal to the volume in which spleen cells (2.5 × 10<sup>6</sup>/400 μL) were suspended, and the cells were subsequently incubated at 37°C for 5 min. The labeling process was quenched by adding 1/20 of the volume of heat-inactivated fetal bovine serum (FBS) (Gibco-BRL) to the sample. After 1 min, CFSE-labeled cells were washed twice, recounted,

and adjusted to a concentration of 2.5 × 10<sup>6</sup> cells/mL in RPMI 1640 culture medium (Gibco-BRL), which contained 10% FBS (Gibco-BRL), gentamycin, and penicillin/streptomycin. Next, the cells were plated onto 48-well tissue culture plates (2.5 × 10<sup>6</sup> cells/well). The cells were stimulated with 10 μg BCG or 20 μg Hsp65 recombinant protein plus 30 μg/mL Polymixin B (Sigma, USA) for 72 h at 37°C and 5% CO<sub>2</sub>. Negative and positive controls were performed using spleen cells cultured in the presence of culture medium only or with 20 μg concanavalin A (Sigma), respectively. The cells were harvested and analyzed by flow cytometry.

### Cytokine evaluation

For the immunogenicity assay, splenocytes from mice killed 15 days after immunization were purified by lysing the erythrocytes. The cells (2.5 × 10<sup>6</sup>) were stimulated *in vitro* with 10 μg/mL Hsp65 recombinant protein or with 20 μg/mL concanavalin A. A negative control was performed using spleen cells cultured at 37°C and 5% CO<sub>2</sub> in the presence of complete medium (RPMI-1640, Gibco-BRL) containing 10% FBS (Gibco-BRL), gentamycin, and penicillin/streptomycin. The level of cytokines in the culture supernatant was evaluated 48 h later. Supernatants were stored at -20°C. For cytokine measurements of the lung, the entire left lobe of the lung was removed 30 days after therapeutic immunization with the transfected cells. The tissue was homogenized in 2 mL RPMI 1640 and centrifuged at 450 g, and the supernatant was stored at -70°C. The levels of the cytokines IFN-γ, IL-12, IL-5, and IL-10 were determined in the spleen cell supernatants, and IFN-γ and IL-12 were measured in the left lung lobe homogenates by ELISA according to manufacturer instructions. The capture antibodies and biotinylated monoclonal antibodies for IFN-γ (R4-6A2, XMG1.2), IL-12 (C15.6, C17.8), IL-5 (TRKF5, TRFK4), and IL-10 (JES5-2A5, SXC-1) as well as the recombinant cytokines, were purchased from Pharmingen.

### Experimental infection with *M. tuberculosis*

The H37Rv *M. tuberculosis* strain (American Type Culture Collection 27294, USA) was grown in 7H9 Middlebrook Broth (Difco Laboratories, USA) for 7 days. The culture was harvested by centrifugation and the cell pellet was then resuspended in sterile PBS and vigorously shaken with glass spheres. The viability of the bacteria in suspension was evaluated using fluorescein diacetate (Sigma) and ethidium bromide. For the therapeutic immunization assays, the mice were first infected by the intranasal route with 10<sup>5</sup> viable colony-forming units (CFU) of *M. tuberculosis* H37Rv in 100 μL PBS. Thirty days after *M. tuberculosis* infection, the mice were treated with the transfected cells. Fifteen days after treatment, the mice from all groups were euthanized.

### Determination of *M. tuberculosis* CFU in lungs

The determination of *M. tuberculosis* CFU was performed as described previously (4). Briefly, the number of live bacteria

was determined by extracting the lower and medium right lobes of the lung, washing the lobes with sterile PBS, and subsequently plating 10-fold serial dilutions of the homogenized tissue onto Middlebrook 7H11 agar medium (Difco) supplemented with 0.2% (v/v) glycerol and 10% (v/v) FBS. Next, the colonies were counted after culturing for 28 days at 37°C. The CFU are reported as log<sub>10</sub> CFU/g lung.

### Histology

Fifteen days after the therapeutic immunization, the upper right lobes of the lungs were fixed in 10% formalin, embedded in paraffin blocks, prepared routinely, and subsequently sectioned for light microscopy. For the histopathological analyses, sections (5 µm) were stained with hematoxylin and eosin. The slides were evaluated using a Leitz Model Aristoplan microscope (Germany) connected to a Leica Model DFC280 color camera (Germany) linked to a PC computer. To perform a morphometric analysis of the lung parenchyma, an integrating eyepiece with a coherent system made of a 100-point grid consisting of 50 lines of known length was coupled to the slides and evaluated by light microscopy. The fractions of collapsed and normal pulmonary areas were determined by the point-counting technique at a magnification of 400X across 10 random, non-coincident microscopic fields. The points falling on a tissue area were counted and divided by the total number of points in each microscopic field. Thus, the data are reported as the fractional area of pulmonary tissue.

### Statistical analysis

Data are reported as means ± SD and were analyzed using GraphPad Prism version 5.0 for Windows (GraphPad Software, USA). The significance of the difference among groups was calculated by ANOVA followed by the Tukey multiple comparison test. Values of  $P < 0.05$  were considered to be significant.

## Results

### Phenotype of macrophages and dendritic cells transfected *in vitro* with Hsp65 mRNA

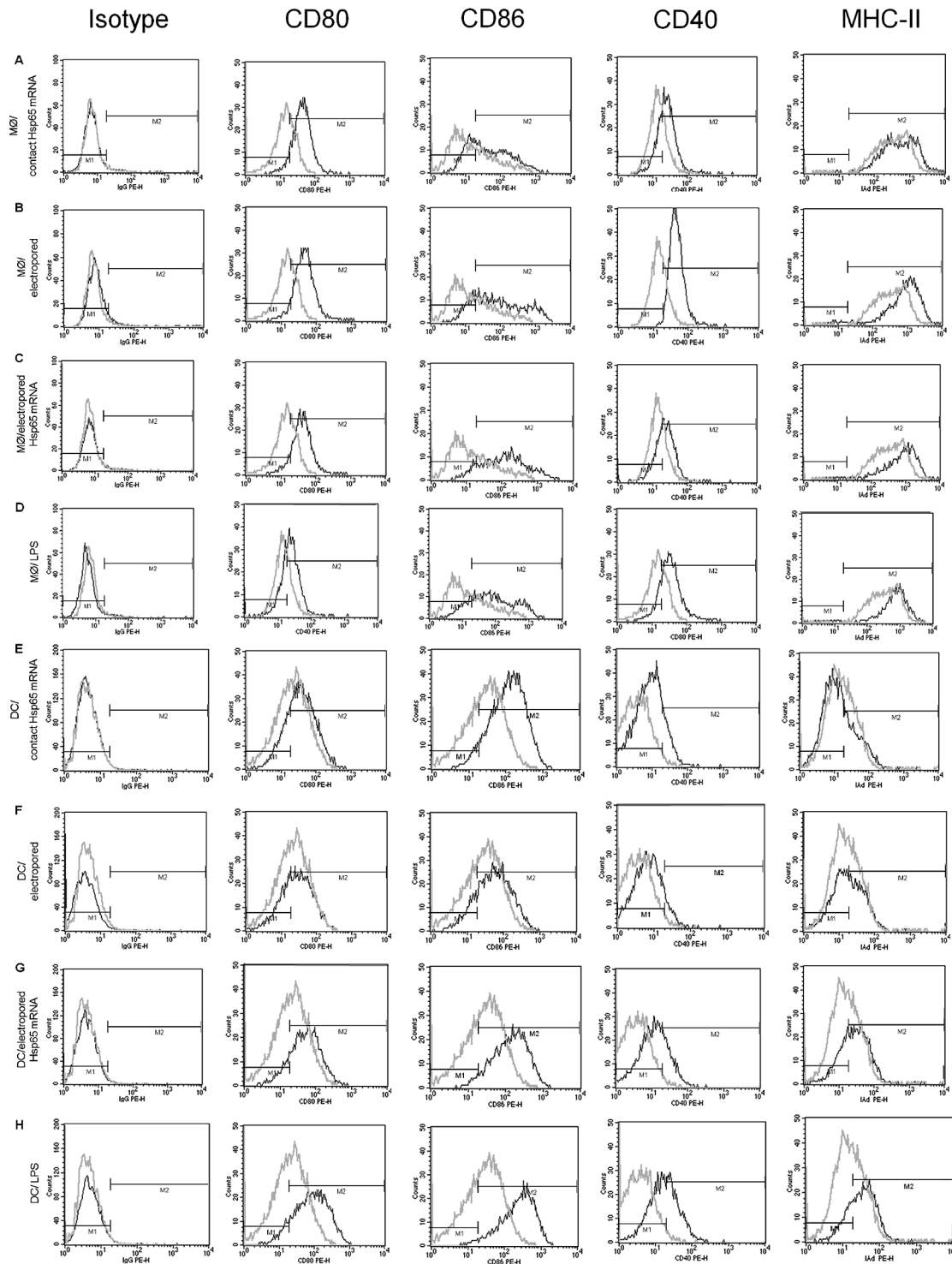
For the phenotypic characterization of the APCs that were pulsed with Hsp65 mRNA, electroporated cells (mock) or APCs that were electroporated with Hsp65 mRNA, we evaluated the expression of cell surface markers. Untreated APCs or cells treated with lipopolysaccharide were used for negative and positive controls, respectively (Figure 1D and H). Twenty-four hours after the stimuli, the expression of CD80, CD86, CD40, and MHC class II (MHC-II) was analyzed. The analysis of the histograms in Figure 1 reveals a different expression profile of surface molecules between the macrophages and DCs. This difference is most evident in the histograms representing the expression of MHC-II; the macrophages obtained from the peritoneal lavage express higher levels of MHC-II compared

to DCs differentiated from bone marrow cells. In addition, the expression profile of MHC-II barely changes when the cells are transfected (pulsed or electroporated) with Hsp65 mRNA for both APC populations. Macrophages pulsed with mRNA, electroporated only (mock) and electroporated with mRNA showed increased expression of CD80, CD40 and especially CD86 (Figure 1A-C). The surface expression pattern of the DCs is represented in Figure 1E-G. The cells transfected with Hsp65 mRNA (pulsed or electroporated) showed an up-regulation of CD80, CD86, and CD40. The mock transfected DCs had a slightly increased expression of CD86 and CD40. Taken together, these results indicate that macrophages and also DCs acquired surface markers, specifically CD86, of partially mature APCs when submitted to Hsp65 mRNA transfection. Additionally, in our analysis, the process of electroporation caused an increase in the expression of some co-stimulatory molecules on macrophages and DCs (Figure 1B and F). Moreover, Hsp65 mRNA also showed to some extent the ability to induce the maturation of APCs.

The percentage of CD11c+ cells (derived from the differentiation of bone marrow cells) was always checked and an average of 72% ( $72.03 \pm 7.8$ ) of the cells showed positive labeling. The remaining cells in the culture were CD11b+. The viability of APCs following transfection (electroporation) was 80 to 90% (data not shown), quite similar to the viability of the cells prior to transfection. Viability was checked with Trypan blue.

### Immune responses induced by immunization with Hsp65 mRNA-transfected macrophages or Hsp65 mRNA-transfected DCs

For the immunogenicity assay, mice received one dose of Hsp65 mRNA-electroporated DCs (DC/Hsp65 mRNA,  $1 \times 10^6$  cells/dose), Hsp65 mRNA-electroporated macrophages (macrophages/Hsp65 mRNA,  $1 \times 10^6$  cells/dose), electroporated DCs (mock DCs,  $1 \times 10^6$  cells/dose), or electroporated macrophages (mock macrophages,  $1 \times 10^6$  cells/dose) by intravenous injection. The control groups were immunized subcutaneously with BCG or intravenously with saline. Fifteen days after immunization, the animals were sacrificed, and cell proliferation and cytokine production were evaluated after *in vitro* stimulation of the spleen cells with recombinant Hsp65 protein or BCG lysate. When we assessed the lymphoproliferation induced in the spleen cells recovered from the immunized animals, only the splenocytes derived from mice immunized with Hsp65 mRNA-transfected DCs induced T-cell proliferation. Moreover, the proliferative capacity of T cells did not depend on secondary stimulation with the Hsp65 antigen *in vitro* (Figure 2). This result suggests that the DCs that developed in this system are capable of activating T cells *in vivo* following adoptive transfer but that the response seems to have multiple specificities. It is probable that during the *in vitro* culture, the exposure of DCs to the foreign proteins contained in



**Figure 1.** The phenotype of macrophages (A-D) and dendritic cells (E-H) transfected *in vitro* with Hsp65 mRNA. Macrophages and dendritic cells (DCs) were transfected by contact with Hsp65 mRNA (A and E), electroporated (B and F), electroporated with 5  $\mu$ g Hsp65 mRNA (C and G) or stimulated with lipopolysaccharide (LPS; D and H). The phenotype of the cells was analyzed by flow cytometry, and the histograms show the expression of the CD80, CD86, CD40, and MHC-II molecules. The gray lines show cells that were not stimulated, and the black lines show stimulated cells. Data are representative of 3 experiments yielding similar results. M $\phi$  = macrophages.

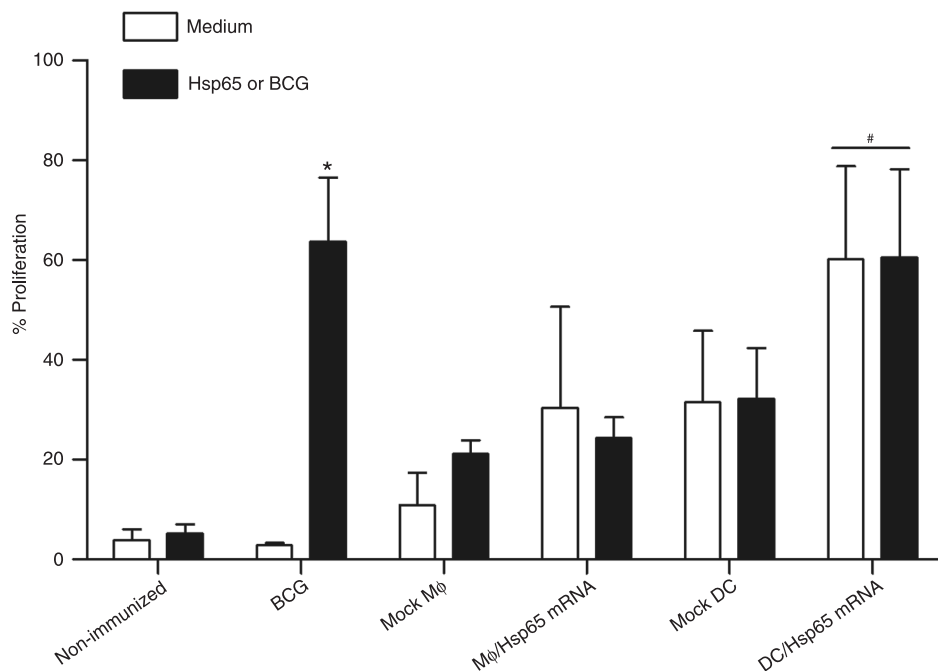
FBS, combined with the fact that the proliferation assay was conducted in the presence of FBS, was responsible for the T-cell proliferation. The splenocytes isolated from mice immunized with Hsp65 mRNA-transfected macrophages were not able to proliferate *in vitro* (Figure 2). The cytokine assays revealed that spleen cells recovered from both mock and Hsp65 mRNA-transfected DC-immunized mice produced high levels of IFN- $\gamma$ , IL-10 and IL-5 (Figure 3B-D). It is probable that cytokine production occurred due to the expression of co-stimulatory molecules (Figure 1). The cells recovered from mice immunized with Hsp65 mRNA-transfected macrophages were able to produce only IL-5 (Figure 3D). We hypothesized that this result might be an intrinsic characteristic of the immune response induced by this type of APC. Although only IL-12 production could be detected from the cells recovered from the animals vaccinated with BCG, we believe that IFN- $\gamma$  production can be attributed to an indirect action of other cells *in vivo*, such as natural killer cells (Figure 3A). The analysis of cytokine production induced by immunization with DCs reflects the ability of this subtype of professional APC to be potentially capable of inducing a mixed Th1/Th2 profile with high levels of IFN- $\gamma$ , as well as IL-5 and IL-10. In contrast, the

immunization with macrophages seemed to evoke only a Th2 response.

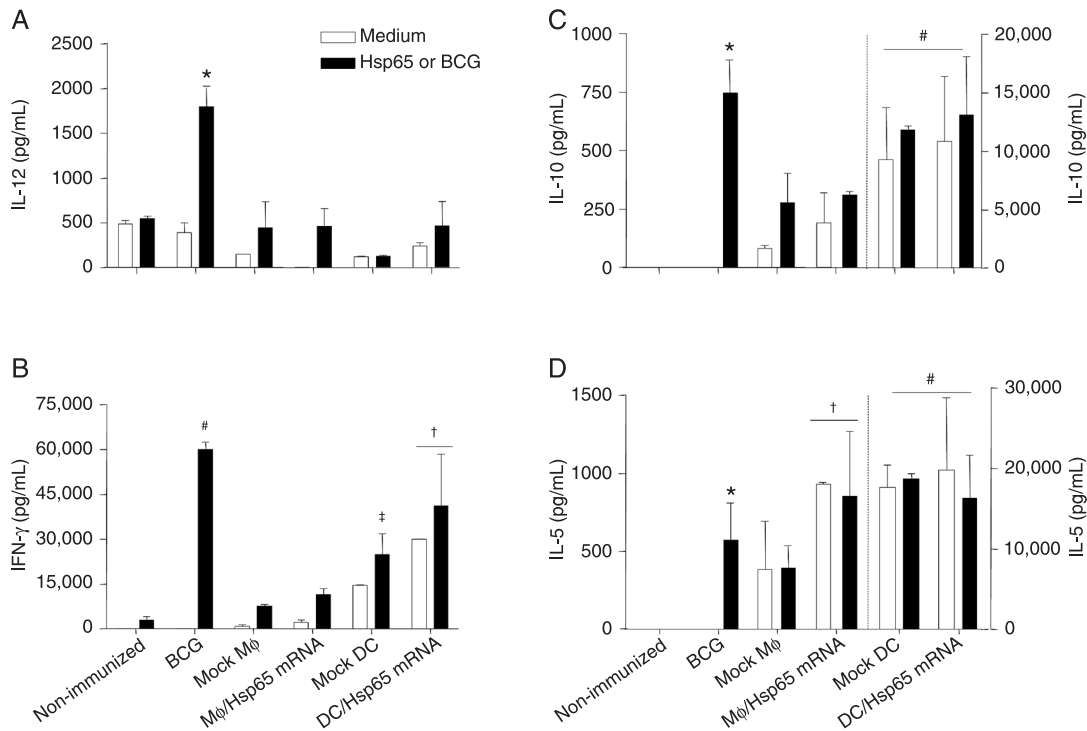
#### Mice infected with *M. tuberculosis* and treated with Hsp65 mRNA-transfected APCs

For the therapy tests, BALB/c mice were infected intranasally with  $1 \times 10^5$  bacilli from the H37Rv virulent strain of *M. tuberculosis*. Thirty days after infection, the mice were treated with 2 doses of different APC formulations: mock macrophages, mock DCs, macrophage/Hsp65 mRNA, or DC/Hsp65 mRNA by intravenous or subcutaneous injection (therapeutic immunization). Fifteen days after the second dose of transfected cells, the lungs were extracted, and the therapeutic effect was evaluated according to the number of CFU, lung tissue preservation and cytokine production.

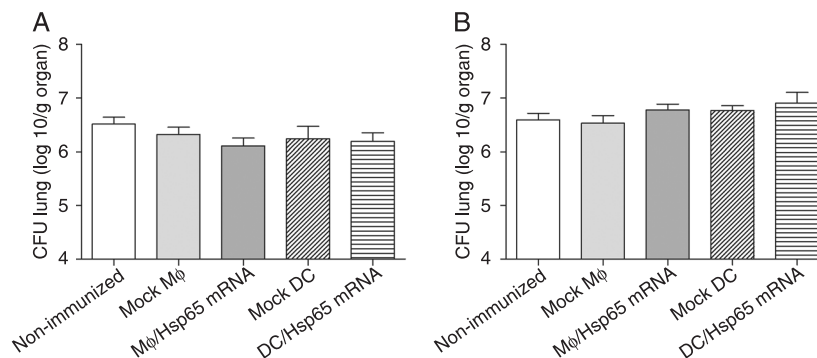
First, we evaluated the number of bacteria in the lungs of infected and treated mice, and no therapeutic effect was detected (Figure 4A). A change in the immunization route also did not reduce bacterial loads (Figure 4B). The preservation of the lung parenchyma, in addition to a reduction of CFU, is essential to assess the effectiveness of new treatments against TB. Thus, the lungs of infected and treated animals were collected and submitted to histo-



**Figure 2.** Evaluation of Hsp65-specific immune responses by immunization with Hsp65 mRNA-transfected macrophages or Hsp65 mRNA-transfected dendritic cells (DCs). Mice were immunized (one dose, intravenous) as described in Material and Methods (Immunogenicity assay section). Fifteen days after immunization, the splenocytes were collected for proliferation assays. Data are reported as means  $\pm$  SD of 5 mice. Results are representative of 2 experiments. \* $P < 0.05$  versus the non-immunized group, medium/BCG, mock macrophage group, macrophage/Hsp65 mRNA group, and mock DC group; # $P < 0.05$  versus the non-immunized group, medium/BCG, mock macrophage group, stimulated-macrophage/Hsp65 mRNA group, and mock DC group (ANOVA followed by the Tukey multiple comparison test). M $\phi$  = macrophages.



**Figure 3.** Cytokine production by spleen cells from immunized mice. Mice were immunized as described in Material and Methods (Immunogenicity assay section). Fifteen days after immunization, the production of IL-12 (A), IFN- $\gamma$  (B), IL-10 (C), and IL-5 (D) by spleen cells cultured with Hsp65 (10  $\mu$ g/mL), BCG (10  $\mu$ g/mL) or concanavalin A (20  $\mu$ g/mL) was evaluated. Data are reported as means  $\pm$  SD absorbance of 5 mice. Each result is representative of 2 experiments. A, \*P < 0.05 versus the medium-BCG and other groups; B, #P < 0.05 versus the medium-BCG and other groups; †P < 0.05 versus the non-immunized group, medium-BCG, mock macrophage group, medium-macrophage/Hsp65 mRNA; †P < 0.05 versus the non-immunized group, medium-BCG, mock macrophage group, macrophage/Hsp65 mRNA group; C, \*P < 0.05 versus the non-immunized group, medium-BCG, mock macrophage group, macrophage/Hsp65 mRNA group; #P < 0.05 versus the other groups; D, \*P < 0.05 versus the non-immunized group; †P < 0.05 versus the other groups (ANOVA followed by the Tukey multiple comparison test). M $\phi$  = macrophages; DC = dendritic cells.

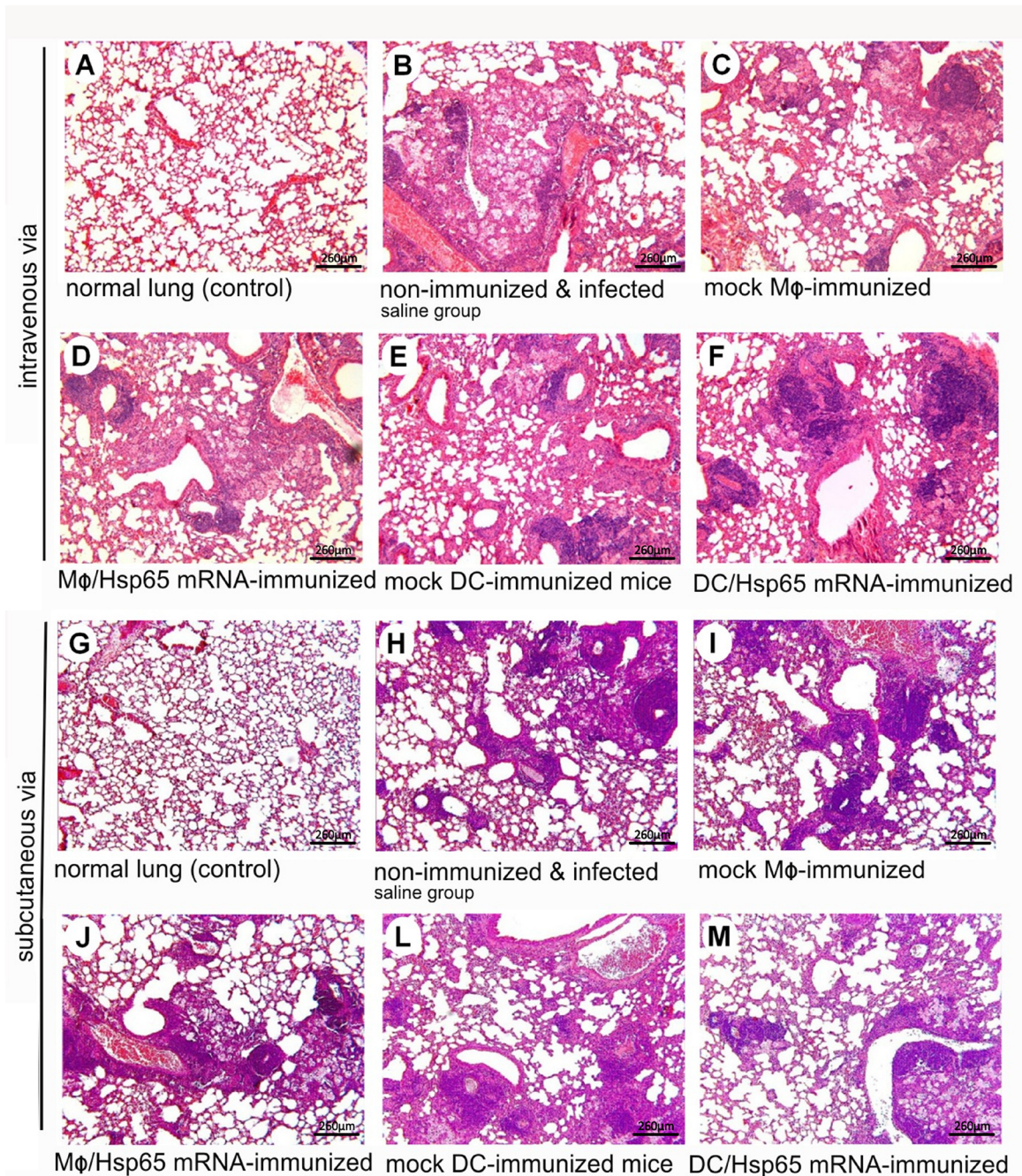


**Figure 4.** Calculation of the numbers of *Mycobacterium tuberculosis* in the lungs from BALB/c mice immunized by intravenous (A) or subcutaneous (B) injection. Mice were infected with *M. tuberculosis* and treated with transfected cells as described in Material and Methods. Data are reported as log<sub>10</sub> of the number of colony-forming units (CFU)/g per lung obtained from means  $\pm$  SD of the serial dilutions individually counted for each group. Results are representative of 2 experiments. M $\phi$  = macrophages; DC = dendritic cells.



pathological (Figure 5) and morphometric analysis (Figure 6). As shown in Figure 5 (B-F,H-M), the lungs revealed a tuberculous pneumonia with a milder impairment of tissue

parenchyma that showed an intense inflammatory infiltrate characterized by the presence of mononuclear cells and of bronchus-associated lymphoid tissue. There were also



**Figure 5.** Histological representation of the lungs of mice immunized by intravenous (A-F) or subcutaneous (G-M) injection. Mice were infected with *Mycobacterium tuberculosis* and treated with transfected cells as described in Material and Methods. Lung sections from normal lung (control, A and G), non-immunized and infected mice (saline group, B and H), mock macrophage-immunized mice (C and I), macrophage/Hsp65 mRNA-immunized mice (D and J), mock dendritic cell (DC)-immunized mice (E and L), and DC/Hsp65 mRNA-immunized mice (F and M) are shown. Representative sections of HE staining are shown. Mφ = macrophages.

numerous xantomatous macrophages, which suggested the presence of bacilli. No differences were found between the saline group (Figure 5B and H) and the other experimental groups (Figure 5C-F,I-M). The noninfected groups correspond to the normal lung tissue (Figure 5A and G). Morphometric analysis indicated that there was partial damage to the lung with 40% of the tissue area inflamed, but there were no differences between the various experimental groups (Figure 6A and B). No differences were found in the histopathological (Figure 5 H-M) and morphometric analysis (Figure 6B) between the experimental groups and the respective infected and untreated control group when the cells were administered by the subcutaneous route. Finally, when we assessed the cytokine levels directly from the lung homogenate from infected and treated animals (Figure 7A-D), no significant differences were found in the IL-12 and IFN- $\gamma$  levels between the experimental groups (mock macrophages, mock DCs, macrophage/Hsp65 mRNA, or DC/Hsp65 mRNA) and the saline groups irrespective of whether the immunization route was intravenous (Figure 7A and B) or subcutaneous (Figure 7C and D).

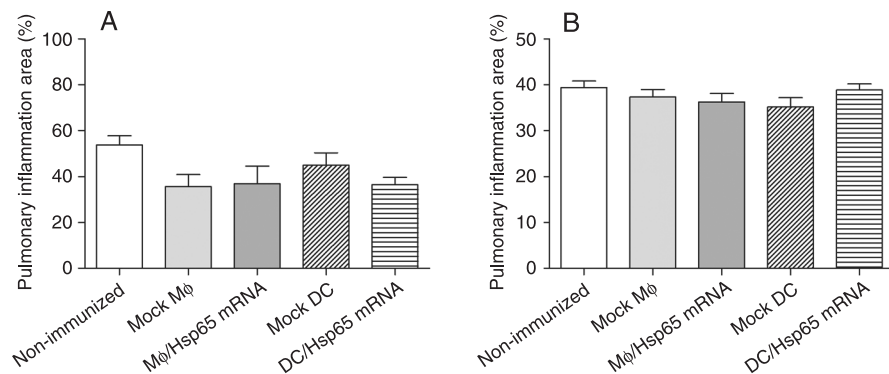
## Discussion

In the present study, we assessed the ability of macrophages and DCs transfected with the messenger RNA of the Hsp65 protein from *M. leprae* to induce an immune response and to exert a therapeutic effect against *M. tuberculosis* infection in an adoptive transfer system.

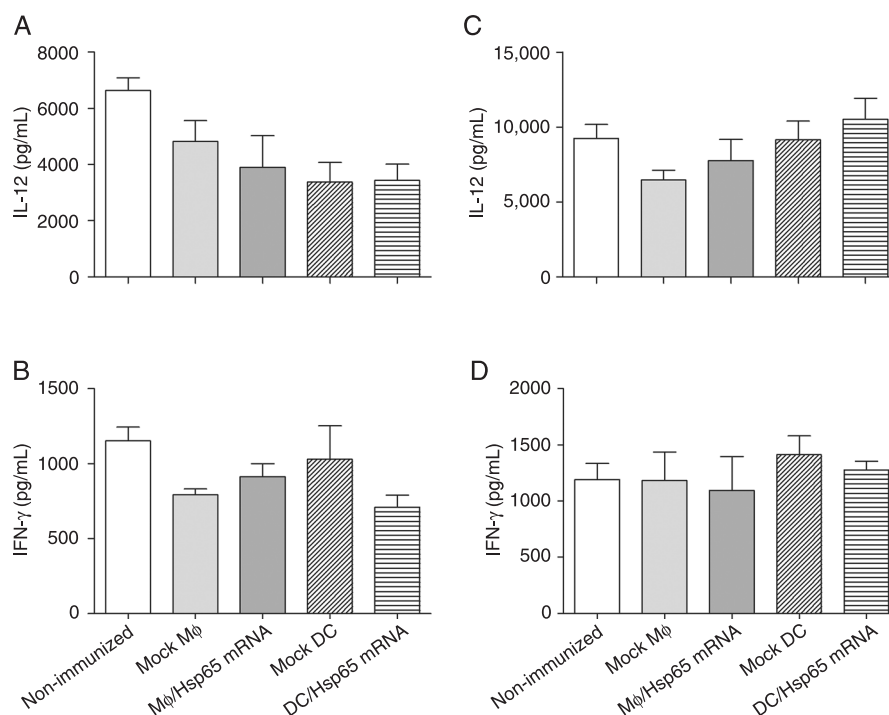
Our results showed that immunization with APCs transfected with Hsp65 mRNA presented no curative effects against TB infection because we could not detect any decrease in lung

bacterial load or the preservation of the lung parenchyma in infected mice.

Effective antimycobacterial immunity is presumed to



**Figure 6.** Morphometric analysis of lung parenchyma from BALB/c mice immunized by intravenous (A) or subcutaneous (B) injection. Mice were infected with *Mycobacterium tuberculosis* and treated with transfected cells as described in Material and Methods. We performed morphometric analysis of lung histology sections. Data represent means  $\pm$  SD percent of the pulmonary area affected by inflammation of 5 mice per group. Results are representative of 2 experiments. M $\phi$  = macrophages.



**Figure 7.** Cytokine detection in the lungs of BALB/c mice immunized by intravenous (A and B) or subcutaneous (C and D) injection. Mice were infected with *Mycobacterium tuberculosis* and treated with transfected cells as described in Material and Methods. Local cytokine production was evaluated *ex vivo* in the lung homogenates. Data are reported as means  $\pm$  SD absorbance of 5 mice. The results are representative of 2 experiments. M $\phi$  = macrophages; DC = dendritic cells.

be due to a Th1 response, which is dominated by antigen-specific T lymphocytes that produce IFN- $\gamma$  and are cytotoxic to infected cells (22,25). A Th2 response is characterized by IL-4 production, which is predominant during an infection with *M. tuberculosis*; Th2 responses have been reported to be non-protective in TB (22,26,27). A shift in the balance toward a Th1 response may be beneficial, providing a criterion for the selection of protective antigens against tuberculosis. The Hsp65 antigen has been shown to be effective in treating TB when administrated as a DNA plasmid, and its effect was related to the CD8<sup>+</sup>/CD44<sup>hi</sup> IFN- $\gamma$ -producing cytotoxic cells (21,22). Moreover, it has been demonstrated that one 10- $\mu$ g dose of naked mRNA encoding Hsp65 was enough to protect mice from *M. tuberculosis* infection. It was also demonstrated that this naked Hsp65 mRNA could stimulate Th1-specific cytokine production. In addition, Western blot analysis confirmed the mRNA Hsp65 stability and the Hsp65 protein expression in transfected cells at various time points after the mRNA uptake (28). However, our approach, which used the same antigen in a different formulation (using APCs as vehicles for Hsp65 mRNA transference), did not show the same therapeutic effects. We believe that the failure of this new model lies either in the inability of transfected APCs to promote a shift to a protective Th1 response or in the fact that transfected APCs may create a modulation of the immune response. In agreement with this hypothesis, DC-activated T cells proliferated vigorously *in vitro* without obvious signs of antigenic specificity upon adoptive transfer into naive recipients (Figure 2). When we assessed the cytokine production, we observed a similar result from cells recovered from mice immunized with transfected DCs (Figure 3). We speculate that the same effect could not be observed from cells harvested from macrophage-immunized mice due to the intrinsic properties of these APCs in our experimental model. In fact, the low effectiveness of macrophages when compared with DCs in an APC-based immunization model has already been reported (19). Another explanation is based on nonspecific T-cell activation due to bystander effects provided by APCs stimulated via cytokine networks. As shown in Figure 3, DCs and, to a lesser extent, macrophages produced significant quantities of different cytokines *in vitro*. It has been demonstrated that, following the vaccination of mice with exogenous DCs, the animal's endogenous DCs can activate T-cells in a bystander nonspecific manner (29). Moreover, in a study with cancer patients, it has been demonstrated that T-cell reactivity to a mock DC immunization reflects a possible T-cell stimulation through co-stimulatory molecules and cytokines (30). The analysis of cytokine production induced by immunization with DCs reflects the ability of this subtype of APCs to be potentially capable of inducing a mixed Th1/Th2 response with high levels of IFN- $\gamma$ , as well as of IL-5 and IL-10 (Figure 3). This phenomenon may have direct implications for a therapeutic approach because protective effects against TB are strongly associated with

a predominantly Th1 response.

Independent of the mechanism of action of the mock cells, we believe that there are details concerning the immunization protocols that should be considered. Another possibility that could explain the absence of therapeutic effects in our model might be related to the maturation status of APCs. Immature DCs might not be ignored by the immune system but might instead lead to tolerance (31,32). Moreover, the type of stimulatory signals that DCs and possibly other APCs emit could also be a critical factor for the induction of a protective response. In an experimental model of leishmaniasis, DCs pulsed with antigens from *Leishmania major* were observed to be activated with CpG oligonucleotides (CPG ODN), TNF- $\alpha$  or anti-CD40 Ab, but only those treated with CPG ODN were able to induce protection. This phenomenon was associated with the induction of a potent Th1 response *in vivo* (33). Other models draw attention to the degree of activation of these APCs. A specific case report showed that the incomplete activation of the DCs in a standard immunization against leishmaniasis led to an undesirable Th2 response, resulting in lack of protection (34). In contrast, there are also cases in which a protective response is observed even in the absence of DC stimulation prior to immunization (35). Curiously, in an experimental model against TB, immunization with a DC-pulsed peptide was able to induce protection without any requirement for DC activation (36).

Another element that seems to be involved in the generation of protection in DC-based vaccines is the route of administration. Although it was demonstrated that antigen-loaded DCs may prime the T-cell response regardless of the route of injection, the quality of the response may be affected. An intravenous immunization seems to favor the migration of DCs initially to the lung with subsequent accumulation in the spleen. In contrast, after subcutaneous administration, DCs preferentially accumulate in the T-cell areas of the draining lymph nodes (37). Regardless of the information provided by those studies, a standard protocol of the proper inoculation route has yet to be established. In the present study, we tested the effectiveness of two immunization routes, i.e., intravenous and subcutaneous and we did not find any differences between these two strategies.

The attempt to induce protection by transferring DC-pulsed peptides or proteins from *M. tuberculosis* has been described in some experimental models with diverse results. Some studies have shown a protective ability of DCs infected with BCG or *M. tuberculosis*. In these models, it is clear that the pathogens have the ability to promote the maturation of DCs and that DCs are important in protecting against the disease. Moreover, this effect was linked to the induction of a strong T-cell response with the production of IFN- $\gamma$  (38). However, variability between these models, such as differences in the infection model and the immunization route, make it difficult to establish clearly the

parameters that led to the protection. Studies performed by other groups also obtained protection when DCs were pulsed with *M. tuberculosis* peptides; again, the results were quite variable with differences in the necessity for the maturation of DCs, the number of doses, the amount of transferred cells, and the route of immunization (36,39). In contrast, other immunization studies with DCs were not able to induce protection against TB. Rubakova et al. (35), in addition to showing the influence of FBS, noted a lack of protection with intravenous immunization. Furthermore, a lack of protection was also observed in conjunction with increased inflammation of the lung parenchyma when DCs pulsed with Ag85 from *M. tuberculosis* were administered by the intranasal route (40).

It is evident from the results reported here that further manipulation of APCs might be required to regulate their

capacity to trigger Th1-like immunological properties *in vivo* to achieve an optimal outcome of immunization. Additionally, manipulations of the mRNA sequences may be useful to increase antigen expression and to contribute to the induction of a robust effector and memory immune response. Moreover, the data presented in this study primarily reflect the difficulty of inducing protection against *M. tuberculosis* and raise questions concerning the best parameters to generate an effective response in an APC-based immunization.

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