Immunology 2004 **113** 130–138 doi:10.1046/j.1365-2567.2004.01931.x

Immune regulatory effect of pHSP65 DNA therapy in pulmonary tuberculosis: activation of CD8$^+$ cells, interferon-γ recovery and reduction of lung injury

V. L. D. BONATO,* E. D. C. GONÇALVES,* E. G. SOARES,† R. R. SANTOS JÚNIOR,* A. SARTORI,‡ A. A. M. COELHO-CASTELO* & C. L. SILVA* **TB Network, Department of Biochemistry and Immunology, †Department of Pathology, Ribeirão Preto School of Medicine, University of São Paulo, Brazil, and ‡Department of Microbiology and Immunology, Biosciences Institute, São Paulo State University, Brazil*

SUMMARY

A DNA vaccine based on the heat-shock protein 65 *Mycobacterium leprae* gene (pHSP65) presented a prophylactic and therapeutic effect in an experimental model of tuberculosis. In this paper, we addressed the question of which protective mechanisms are activated in *Mycobacterium tuberculosis*-infected mice after immune therapy with pHSP65. We evaluated activation of the cellular immune response in the lungs of infected mice 30 days after infection (initiation of immune therapy) and in those of uninfected mice. After 70 days (end of immune therapy), the immune responses of infected untreated mice, infected pHSP65-treated mice and infected pCDNA3-treated mice were also evaluated. Our results show that the most significant effect of pHSP65 was the stimulation of CD8$^+$ lung cell activation, interferon-γ recovery and reduction of lung injury. There was also partial restoration of the production of tumour necrosis factor-α. Treatment with pcDNA3 vector also induced an immune stimulatory effect. However, only infected pHSP65-treated mice were able to produce significant levels of interferon-γ and to restrict the growth of bacilli.

Keywords interferon-γ; *Mycobacterium tuberculosis*; pHSP65 DNA therapy; protection; T CD8$^+$ lymphocytes

INTRODUCTION

In developing countries, tuberculosis (TB) remains one of the three most common fatal infectious diseases, along with acquired immune deficiency syndrome and malaria.[1] The incidence of TB has always been high in these countries, and in industrialized countries it has re-emerged as a public health problem.[2] This has been accelerated by the advent of the human immunodeficiency virus epidemic and the increasing incidence of multidrug-resistant strains of *Mycobacterium tuberculosis*.[3] The current TB vaccine, a strain of *Mycobacterium bovis* called Calmette–Guérin bacillus (BCG), provides a relatively high degree of protection in experimental models of TB infection.[4] However, the efficacy of BCG in several field trials has been either minimal or undetectable.[5]

Progress in TB research has advanced as a result of the great amount of information obtained from the completion of the *M. tuberculosis* genome sequence.[6] This has led to the rapid characterization and expression of *M. tuberculosis* antigens with vaccine potential. In this context, a new form of vaccination, using DNA containing the gene for the antigen of interest, has been developed. This is a potent new method that can engender both humoral and cellular immune responses in a variety of murine and primate disease models.[7] In addition, DNA vaccines mimic the effects of live attenuated vaccines in their ability to induce major histocompatibility complex class I-restricted CD8$^+$ T-cell responses.[8] These DNA vaccines also contain specific nucleotide sequences that play an important role in their immunogenicity.[9–12]

Received 13 November 2003; revised 19 April 2004; accepted 25 May 2004.

Correspondence: Dr Vânia Luiza Deperon Bonato, Tuberculosis Research Center, Department of Biochemistry and Immunology, Ribeirão Preto School of Medicine, University of São Paulo, Avenida Bandeirantes, 3900, 14049–900, Ribeirão Preto, SP, Brazil. E-mail: vlbonato@fmrp.usp.br

A DNA vaccine based on the *M. leprae* heat-shock protein 65 gene (pHSP65) protected mice against subsequent *M. tuberculosis* challenge by establishing a cellular immune response characterized by antigen-specific $CD4^+$ and $CD8^+$ T lymphocytes, which both produce interferon-γ (IFN-γ) and are cytotoxic to infected cells.[13] Recently, a therapeutic effect of this vaccine in mice during the course of experimental TB was described.[14]

The development of a TB vaccine depends on knowledge of the protective immune response that follows infection. It is known that, once inhaled, *M. tuberculosis* bacilli are phagocytosed by alveolar macrophages that produce proinflammatory cytokines. These cytokines induce the recruitment and activation of T lymphocytes.[15] $CD4^+$ and $CD8^+$ T cells are essential to the containment of *M. tuberculosis* within granulomas.[16,17] The kinetics of production and the balance between the proinflammatory cytokines interleukin-6 (IL-6),[18] IL-12[19] and tumour necrosis factor-α (TNF-α)[20] and the inhibitory cytokines IL-10[21] and transforming growth factor-β[22] regulate the subsequent T-cell responses. Cytokines produced by $CD4^+$ and $CD8^+$ T cells, such as IFN-γ, play an essential role in this process because they activate macrophages that become microbicidal.[23,24] Therefore, the cross-talk between T cells and macrophages is essential for the final outcome of *M. tuberculosis* infection.[15] Recently, special attention has been given to dendritic cells, which undergo maturation following *M. tuberculosis* infection.[25]

In this study, we investigated the question of whether immune therapy with the DNA vaccine pHSP65 is able to modify T-cell priming during virulent *M. tuberculosis* infection without inducing tissue injury. In an attempt to characterize the immune mechanisms involved in the therapeutic effect, we initially counted lung $CD4^+$ and $CD8^+$ cells and analysed the cellular phenotype of these cells, cytokine production, and numbers of colony-forming units (CFUs) in the lungs of infected mice, either treated or untreated with pHSP65, and correlated these data with histopathological analysis.

MATERIALS AND METHODS

Mice

Female specific pathogen-free (SPF) BALB/c mice, were bred in the Animal Facility of the School of Medicine of Ribeirão Preto, University of São Paulo and used at 5–8 weeks old. They were kept under barrier conditions at a level III biohazard laboratory.

Bacteria

The H37Rv strain of *M. tuberculosis* (American Type Culture Collection, Rockville, MD) was grown in 7H9 Middlebrook broth (Difco Laboratories, Detroit, MI) for 7 days. The culture was harvested by centrifugation and the cell pellet was resuspended in sterile phosphate buffered saline (PBS) and vigorously agitated. The homogeneous suspension was filtered through 2-μm filters (Millipore, Bedford, MA). Viability of the *M. tuberculosis* suspension was pretested with fluorescein diacetate (Sigma, Saint Louis, MO) and ethidium bromide.[26]

Plasmid construction

The pcDNA3-hsp65 (pHSP65) vaccine was derived from the pcDNA3 vector (Invitrogen®, Carlsbad, CA), which was previously digested with *Bam*HI and *Not*I (Gibco BRL, Gaithersburg, MD), by inserting a 3·3-kb fragment corresponding to the *M. leprae* hsp65 gene and the cytomegalovirus intron A.[27] The vector pcDNA3 minus the hsp65 gene was used as a control. An LB liquid medium (Gibco BRL, Gaithersburg, MD) containing ampicillin (100 μg/ml) was used to culture DH5α *Escherichia coli*, transformed with either pcDNA3 plasmid or with the plasmid containing the hsp65 gene (pHSP65). Plasmids were purified using the Concert High Purity Maxiprep System (Gibco BRL, Gaithersburg, MD). Plasmid concentration was determined by spectrophotometry at $\lambda = 260$ and 280 nm using the Gene Quant II apparatus (Pharmacia Biotech, Cambridge, Buckinghamshire, UK).

Infection and immune therapy

Mice were anaesthetized by intraperitoneal injection of 2,2,2-tribromoethanol (Acros Organics, New Jersey, NJ) and tracheae were exposed. Infection was effected by intratracheal inoculation with 1×10^5 viable CFU of H37Rv *M. tuberculosis* strain diluted in PBS. Thirty days after infection, mice were given a series of four doses of plasmid DNA administered intramuscularly at 10-day intervals. Each mouse received 100 μg of pHSP65 or the pcDNA3 vector. Ten days after treatment, mice were killed by cervical dislocation under mild anaesthesia. At this time, the end of therapy, the infection had been allowed to develop for 70 days. An infected untreated group (RV-70 days) was used to compare the effect of pHSP65 therapy over the same period.

Monoclonal antibodies

To evaluate T-cell subsets, populations of macrophages and dendritic cells, and the expression of costimulatory and adhesion molecules, the following anti-mouse cell surface marker monoclonal antibodies (mAbs) were used: (i) phycoerythrin (PE) -labelled mAbs: anti-CD4 [clone H129.19, rat immunoglobulin G2a (IgG2a)], anti-CD8 (clone 53-6.7, rat IgG2a), anti-CD44 (clone IM7, rat IgG2b), anti-CD28 (clone 37.51, hamster IgG), anti-CD95 (clone Jo2, hamster IgG), and anti-CD95L (clone MFL3, hamster IgG); (ii) fluorescein isothiocyanate (FITC) -labelled mAbs: anti-CD4 (clone H129.19), anti-CD8 (clone 53-6.7), anti-CD18 (clone C71/16, rat IgG2a), anti-CD11b (CR3/Mac-1) (clone M1/70, rat IgG2b) and anti-CD11c (clone HL3, hamster IgG). Hamster IgG, rat IgG2a and rat IgG2b labelled with PE or FITC were used as isotype controls. All mAbs were purchased from PharMingen (San Diego, CA) and used according to the manufacturer's instructions.

Preparation of lung cells

Lungs were washed with sterile PBS and each was placed in a Petri dish containing incomplete RPMI-1640 medium

(Sigma). Lungs were fragmented and transferred to a conical tube containing digestion solution, prepared with Liberase Blendzyme 2 (Roche, Indianapolis, IN) diluted (0·5 μg/ml) in incomplete RPMI-1640. Samples were incubated at 37° under agitation for 30 min After incubation, the cells were dispersed by using a 10-ml syringe and pelleted by centrifugation for 10 min at 400 ***g***. Cells were then washed with complete RPMI-1640, passed through a Nytex screen (Sigma) and resuspended in complete RPMI-1640. Total cell counts were determined in a Neubauer chamber.

Fluorescence-activated cell sorter (FACS) analysis

Lung cells (1×10^7/ml) were initially incubated for 30 min at 4° with Fc Block (1 μg/10^6 cells) (PharMingen). Cells were then incubated with proper mAb (0·75 μg/10^6 cells) for 30 min at 4° in total darkness. Lymphocytes were analysed by flow cytometry using the CELL QUEST software FACSORT (Becton Dickinson, San Jose, CA). Ten thousand events per sample were collected and double-colour FACS analysis was performed as described elsewhere.[28]

Cytokine production

Levels of the cytokines IFN-γ, IL-4, IL-6, IL-12, TNF-α and IL-10 and of the chemokines monocyte chemotactic protein-1 (MCP-1) and inducible protein (IP)-10 in homogenized lung were measured by enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions (PharMingen). Purified monoclonal antimouse antibodies IFN-γ (R4-6A2), IL-4 (11B11), IL-6 (MP5-20F3), IL-12 (C15.6), IL-10 (JES5-2A5), MCP-1 (2H5) and IP-10 (A102-6) were used at 1 μg/ml. Purified anti-TNF-α monoclonal antibody (XT2211) and polyclonal anti-TNF-α were kindly provided by Dr João Santana da Silva (Department of Biochemistry and Immunology, School of Medicine of Ribeirão Preto, University of São Paulo, Brazil). Cytokine–antibody complexes were detected by addition of biotinylated anti-mouse IFN-γ (XMG1.2), IL-4 (BVD6-24G2), IL-6 (MP5-32C11), IL-12 (C17.8), IL-10 (SXC-1), MCP-1 (4E2/MCP) or IP-10 (CRG-2) at 1 μg/ml. Detection limits were: 20 pg/ml for IFN-γ, 10 pg/ml for IL-4, 5 pg/ml for IL-6, 20 pg/ml for IL-10, 40 pg/ml for IL-12 and 700 pg/ml for TNF-α. Detection limits for the chemokines were: 5 pg/ml for MCP-1 and 300 pg/ml for IP-10.

CFU determination

Aliquots of lungs harvested from infected untreated mice and from infected treated mice were incubated in digestion solution. Serial dilutions were plated on supplemented 7H10 agar media. Colonies were counted after 28 days of incubation at 37°.

Histology

The upper left lobe of each mouse lung was fixed in 10% formalin, embedded in paraffin blocks, prepared routinely, then sectioned for light microscopy. Sections (5 μm each) were stained either with haematoxylin & eosin or by the Ziehl–Neelsen method for detection of acid-fast bacilli.

Statistical analysis

All values are expressed as mean ± SEM. Data were investigated by analysis of variance (ANOVA) using GRAPHPAD INSTAT software. When the values indicated the presence of a significant difference, a Tukey–Kramer multiple comparisons test was used. Values of $P < 0{\cdot}05$ were considered significant.

RESULTS

Immune therapy sustained the influx of lymphocytes into the lungs

Previous results from our group showed that, 30 days post-infection with *M. tuberculosis*, the lungs of infected mice showed a significant influx of $CD8^+$ cells. Moreover, CD18 expression was up-regulated on $CD4^+$ and $CD8^+$ lung cells of infected mice compared to those of uninfected mice (PBS-injected mice).[28] In this context, to characterize the immune mechanisms activated after development of pulmonary TB in mice submitted to immune therapy with pHSP65, initially, counts were made of the $CD4^+$ and $CD8^+$ cells obtained from the lungs of control group (PBS-injected) mice; 30-day infected mice; 70-day infected untreated mice; infected pHSP65-treated mice; and from infected pcDNA3-treated mice.

The results presented in Fig. 1(a) show that, 30 and 70 days post-infection, there was an intense influx of $CD4^+$ and $CD8^+$ lymphocytes into the lungs in comparison with the control group. Infected mice submitted to immune therapy were able to recruit higher numbers of $CD8^+$ lymphocytes than those in the infected untreated group.

Figure 1(b) shows the influx of macrophages (Mac-1^+ cells) and dendritic cells ($CD11c^+$ cells) into the lungs of the different groups studied. There was a significant influx of macrophages and dendritic cells after 30 days of infection in comparison with the control group. At 70 days post-infection, $CD11c^+$ cell numbers were lower than the number detected in 30-day infected mice. Immune therapy did not affect the influx of Mac-1^+ and $CD11c^+$ cells into the lungs.

Therapy with pHSP65 up-regulated expression of CD18 and CD28 in cytotoxic lymphocytes

Since therapy with pHSP65 induced an increase in the number of $CD4^+$ and $CD8^+$ cells in the lungs in relation to hose found in infected untreated mice, the phenotype of these cells was investigated. Patterns of differentiation and activation of lymphocytes were analysed by comparison of CD18 and CD28 expression on the cell surface. Despite an increase in $CD4^+$ and $CD8^+$ cell numbers in the lungs of infected mice after 30 days of infection, the percentage of CD28 in these cells was down-regulated (Fig. 2a). After 70 days of infection, the percentage of CD28 in $CD8^+$ lymphocytes was also down-regulated in comparison to lung cells from PBS-injected mice (control group). Concurrently, infected mice submitted to immune therapy with

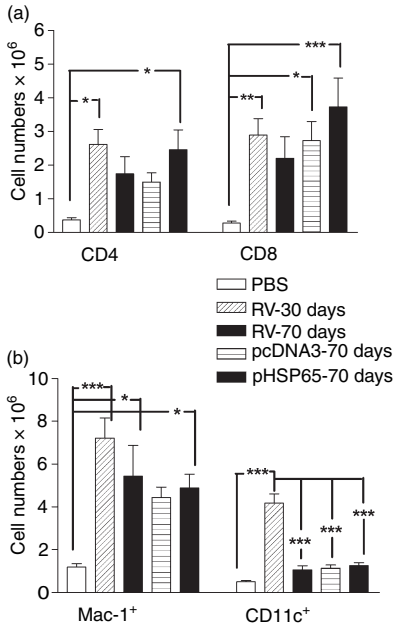

Figure 1. Lung lymphocyte, macrophage (Mac-1$^+$) and dendritic cell (CD11c$^+$) numbers obtained from uninfected mice (PBS), 30-day infected mice (RV-30 days), 70-day infected mice (RV-70 days), infected pcDNA-3-treated mice (pcDNA3-70 days) and infected pHSP65-treated mice (pHSP65-70 days). Results are expressed as the mean of CD4$^+$, CD8$^+$, (a), Mac-1$^+$, or CD11c$^+$, (b), cell numbers of 10–12 mice per group, evaluated individually, obtained from three independent experiments. Statistical significance: $*P < 0{\cdot}05$, $**P < 0{\cdot}01$, $***P < 0{\cdot}001$.

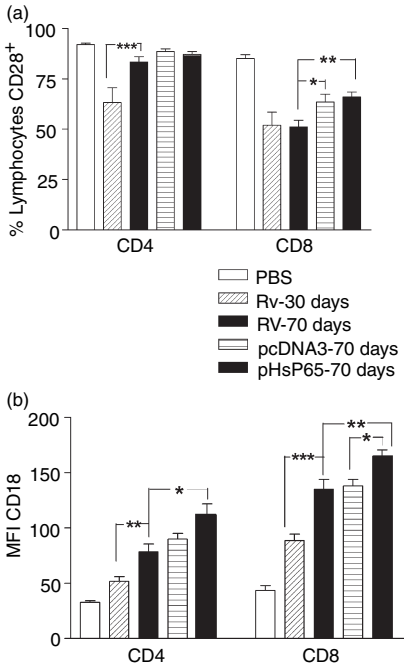

Figure 2. CD18 and CD28 expression in CD4$^+$ and CD8$^+$ lung lymphocytes obtained from uninfected mice (PBS), 30-day infected mice (RV-30 days), 70-day infected mice (RV-70 days), infected pcDNA-3-treated mice (pcDNA3-70 days) and infected pHSP65-treated mice (pHSP65-70 days). Results are expressed as the mean of the percentage of CD28$^+$ population, (a), or as the mean of the median of fluorescence intensity (MFI) for the CD18 receptor, (b), on CD4$^+$ and CD8$^+$ cells obtained from 10–12 mice per group, evaluated individually, from three independent experiments. Statistical significance: $*P < 0{\cdot}05$, $**P < 0{\cdot}01$, $***P < 0{\cdot}001$.

pHSP65 or with pcDNA3 vector presented a significant increase in the percentage of CD28 in CD8$^+$ lymphocytes. On the other hand, the percentage of CD28 expression in CD4$^+$ cells was up-regulated 70 days post-infection in relation to 30-day infected mice and was similar to the expression detected in the control group. Immune therapy with DNA vaccine had no effect on CD4$^+$ cells.

Expression of CD18 on CD4$^+$ and CD8$^+$ lymphocytes was also evaluated, and the results showed that mice treated with pHSP65 presented a significant up-regulation of CD18 expression in CD4$^+$ cells and CD8$^+$ cells when compared with the other experimental groups (Fig. 2b). At 70 days post-infection, we observed that CD4$^+$ and CD8$^+$ cells presented higher and more significant expression of CD18 in comparison with 30 days post-infection; infected pHSP65-treated mice presented greater up-regulation of CD18 expression in both CD4$^+$ and CD8$^+$ lymphocyte subsets than that of infected untreated mice (RV-70 days), and infected pcDNA3-treated mice. The expression of CD44^{hi}, a marker of effector and memory cells, was also evaluated on CD4$^+$ and CD8$^+$ lung cells. However, immune therapy with DNA had no significant effect on the expression of this receptor in relation to infected untreated mice (data not shown).

CD95 and CD95L were also up-regulated in lymphocytes after pHSP65 therapy

Fas (CD95) and FasL (CD95L) play important roles in the elimination of T cells that have been activated in response to foreign antigens. To investigate if these receptors were up- or down-regulated after immune therapy with pHSP65, we analysed their expression on CD4$^+$ and CD8$^+$ lung cells. The percentage of CD95L in CD4$^+$ cells was very similar among all groups: control group, 30-day infected mice, 70-day infected untreated mice and infected pcDNA3-treated mice. However, therapy with pHSP65 stimulated a significant up-regulation of CD95L in CD4$^+$ cells in comparison with infected untreated mice and with infected pcDNA3-treated mice. On the other hand, the infection induced down-regulation of CD95L in lung CD8$^+$ lymphocytes. This down-regulation was only partially counteracted by pHSP65 DNA therapy (Fig. 3a), because the CD95L expression in CD8$^+$ lung cells was not restored to the levels expressed by CD8$^+$ cells from control group.

Expression of CD95 in CD4$^+$ lymphocytes was very similar among all the analysed groups. Immune therapy with DNA induced up-regulation of CD95 on the surface of CD8$^+$ cells in relation to infected untreated mice.

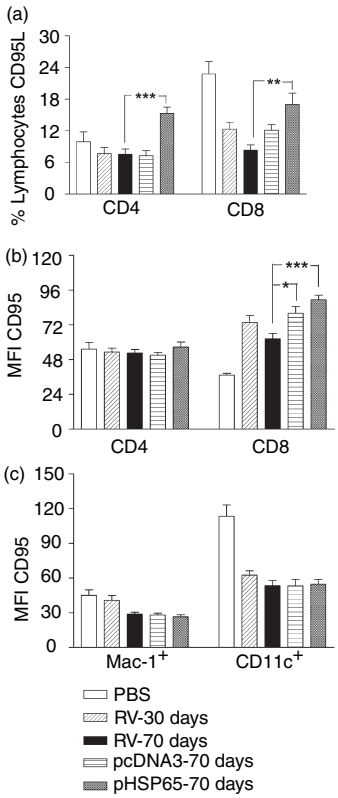

Figure 3. Percentage of $CD95L^+$ in $CD4^+$ and $CD8^+$ lung lymphocytes and CD95 expression in lung $CD4^+$, $CD8^+$, Mac-1^+ (macrophages) and $CD11c^+$ (dendritic cells) cells obtained from uninfected mice (PBS), 30-day infected mice (RV-30 days), 70-day infected mice (RV-70 days), infected pcDNA-3-treated mice (pcDNA3-70 days) and infected pHSP65-treated mice (pHSP65-70 days). Results are expressed as percentage of $CD95L^+$ population in $CD4^+$ and $CD8^+$ cells, (a), or as the mean of median of fluorescence intensity (MFI) for CD95 receptor on $CD4^+$, $CD8^+$, (b), Mac-1^+ and $CD11c^+$, (c), cells obtained from 10–12 mice per group, evaluated individually, from three independent experiments. Statistical significance: $*P < 0{\cdot}05$, $**P < 0{\cdot}01$, $***P < 0{\cdot}001$.

CD95 expression in lung macrophages (Mac-1^+) and dendritic cells ($CD11c^+$) was also evaluated. The CD95 receptor was down-regulated after *M. tuberculosis* infection and DNA treatment did not affect its expression. Down-regulation of CD95 was more pronounced in dendritic cells than in macrophages (Fig. 3c).

Therapy with pHSP65 restored production of IFN-γ

Quantitative levels of the cytokines IFN-γ, IL-4, IL-10 and IL-12, all of which regulate the immune response, were detected in lung homogenates. There was significant production of IFN-γ in the lungs of infected mice 30 days after infection. At 70 days post-infection, IFN-γ was significantly suppressed. However, pHSP65 therapy restored IFN-γ to levels near those detected in the lung homogenates of mice 30 days after infection (Fig. 4b). Treatment with the pcDNA3 vector also stimulated IFN-γ production but not in a significant way. Levels of IL-4 and IL-10 were very similar among the groups and about 20 and 10 times lower, respectively, than IFN-γ levels (Fig. 4c,d). There was a high level of IL-12 30 days after infection. However, 70 days after infection, all of these levels were lower and DNA therapy was unable to restore them (Fig. 4a).

DNA therapy partially restored TNF-α production, but had no affect on production of IL-6, IP-10, or MCP-1

Production of inflammatory cytokines was also detected in lung homogenates. TNF-α and IL-6 production was higher 30 days after infection than 70 days after infection. Therapy with pHSP65 or with pcDNA3 vector did not stimulate a significant increase in the levels of IL-6, but did up-regulate TNF-α levels (Fig. 5a,b).

Although adhesion receptors and inflammatory cytokines are co-ordinators of lymphocyte migration, chemokines also play a role. High levels of MCP-1 were also produced 30 days after infection. Production of this chemokine in the lungs diminished significantly after 70 days. Immune therapy with pHSP65 or with pcDNA3 was able to increase MCP-1 levels, although not significantly (Fig. 5c).

The results presented in Fig. 5(d) show that *M. tuberculosis*-infected mice produced high levels of IP-10 30 days following infection. By 70 days after infection, concentrations, although still elevated, were statistically lower and showed no change after immune therapy.

Therapy with pHSP65 decreased bacterial burden

By comparing the progression of infection among the groups, it was verified that, at 70 days postinfection, untreated mice had increased bacterial burdens in comparison with the group analysed at 30 days post-infection (Fig. 6a). This significant increase in bacterial burdens corresponded to 1·8 $\log_{10}$. Infected pHSP65-treated mice presented a significant decrease in the number of bacteria when compared with infected untreated mice. At 70 days post-infection, the difference in bacterial burden between infected untreated mice and infected pHSP65-treated mice was 1·0 $\log_{10}$. Treatment with pcDNA3 vector was not able to induce a significant decrease in bacterial burden. Bacterial counts in the lungs of infected untreated mice and infected pcDNA3-treated mice were not statistically different. Even when the progression of infection was evaluated 85 days after immune therapy with pHSP65, there was no increase in the number of bacilli. Bacterial burdens remained stationary (Fig. 6b).

Immune therapy with pHSP65 reduced lung injury

Histological sections of lungs from infected untreated and from infected DNA-treated mice were predominantly characterized by the presence of mononuclear cells. However, the examination of lungs of mice from different

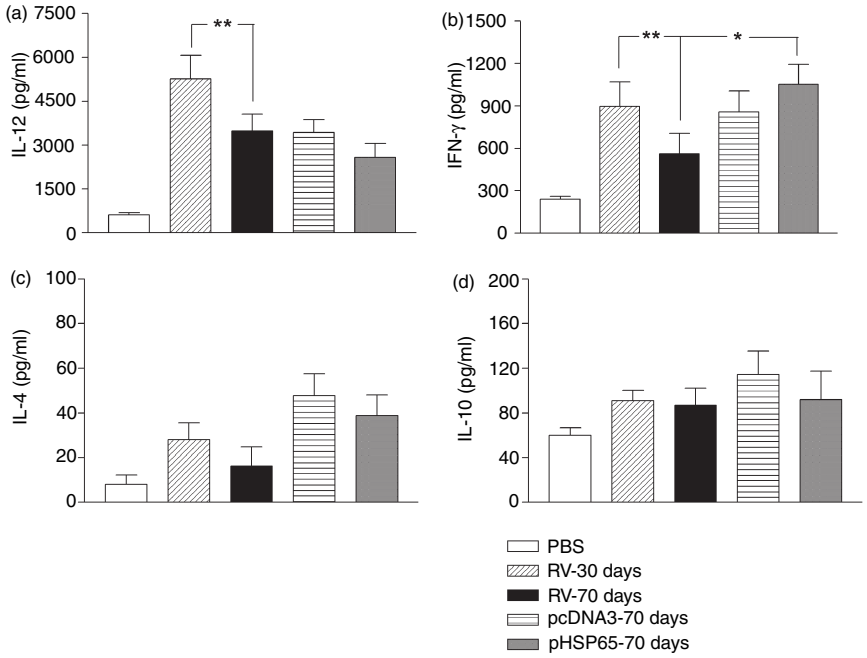

Figure 4. Production of regulatory cytokines IL-12, (a), IFN-γ, (b), IL-4, (c), and IL-10, (d), in the lungs of uninfected mice (PBS), 30-day infected mice (RV-30 days), 70-day infected mice (RV-70 days), infected pcDNA3-treated mice (pcDNA3-70 days) and infected pHSP65-treated mice (pHSP65-70 days). Results are expressed as the mean of cytokine concentration detected in lung homogenates obtained from 10 to 12 mice per group, evaluated individually, from three independent experiments. Statistical significance: $*P < 0{\cdot}05$, $**P < 0{\cdot}01$.

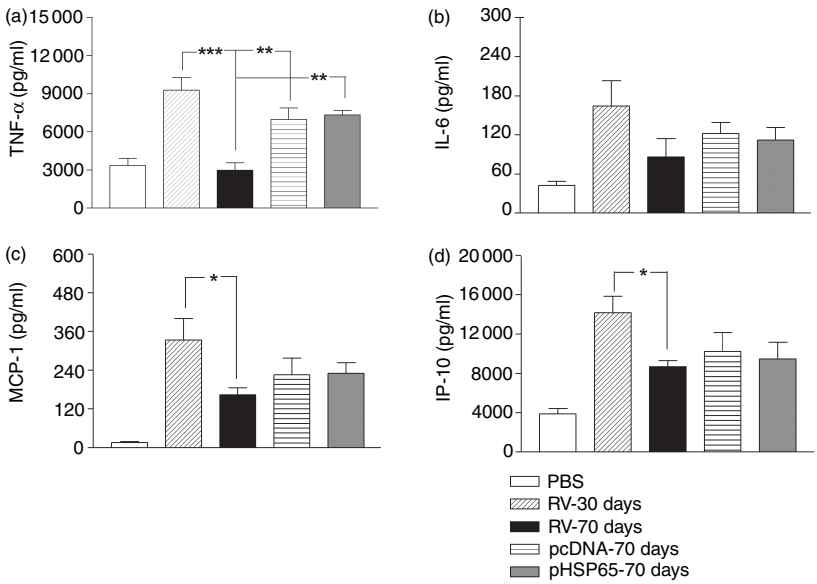

Figure 5. Production of inflammatory cytokines TNF-α, (a), and IL-6, (b), and chemokines MCP-1, (c), and IP-10, (d), in the lungs of uninfected mice (PBS), 30-day infected mice (RV-30 days), 70-day infected mice (RV-70 days), infected pcDNA-3-treated mice (pcDNA3-70 days) and infected pHSP65-treated mice (pHSP65-70 days). Results are expressed as the mean of cytokine concentration detected in lung homogenates obtained from 10 to 12 mice per group, evaluated individually, from three independent experiments. Statistical significance: $*P < 0{\cdot}05$, $**P < 0{\cdot}01$, $***P < 0{\cdot}001$.

groups revealed differences in the development and generation of a granulomatous response. Over the first 30 days of infection, small and medium-sized focal granulomas mainly consisted of perivascular and peribronchial lymphocytes, as well as macrophages (Fig. 7b). These macrophages were very large and

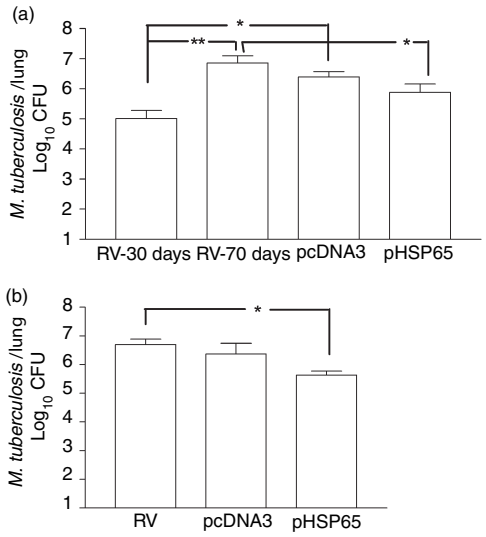

Figure 6. Progression of *Mycobacterium tuberculosis* infection in mice submitted to DNA therapy or no therapy. (a) Growth of *M. tuberculosis* in lungs of 30-day infected mice (RV-30 days), 70-day infected mice (RV-70 days), infected pcDNA3-treated mice (pcDNA3-70 days) and infected pHSP65-treated mice (pHSP65-70 days) was followed by a count of the colony-forming units (CFU). The data are expressed as the mean ± standard deviation of CFU of 10 mice per group, evaluated individually, from three independent experiments. (b) Growth of *M. tuberculosis* in the lungs of infected untreated mice, infected pcDNA3-treated mice and infected pHSP65-treated mice 85 days after the end of immune therapy. The data are expressed as the mean ± standard deviation of CFU of five mice per group. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$.

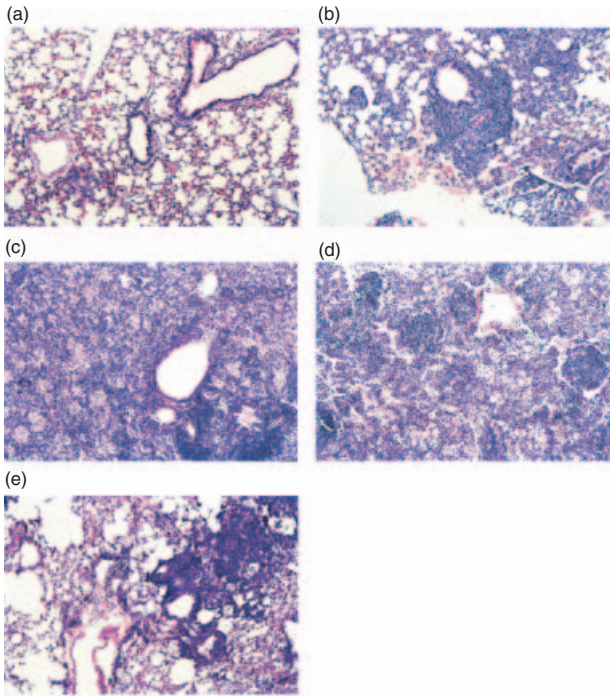

Figure 7. Histological representation of lung sections from uninfected mice (a), 30-day infected mice (b), 70-day infected mice (c), infected pcDNA-3-treated mice (d) and infected pHSP65-treated mice (e). Formalin-fixed lung tissue was prepared and sectioned for light microscopy. The tissue was stained with haematoxylin & eosin. Magnification is ×10. Images are representative of three independent experiments.

exhibited vacuolated cytoplasm, morphological features which are typical of activated cells. After 70 days of infection, there was intensified inflammation, characterized by a large quantity of foamy macrophages. Although lymphocytes could be seen within the lesions, most were present in lower numbers than in lungs harvested from infected mice after 30 days of infection (Fig. 7c). These results correlate with those presented in Fig. 1, in which $CD4^+$, $CD8^+$ and Mac-1^+ cell numbers in the lungs are shown. These mice also presented a higher number of granulomas, often exhibiting confluence. Mice treated with pHSP65 also presented an intense inflammatory infiltrate, containing perivascular lymphocytes surrounded by macrophages, which was distributed throughout the extent of the lung. However, they presented larger areas of lung parenchyma preservation than did infected untreated mice (RV-70 days) (Fig. 7e). Mice treated with pcDNA3 vector presented an intense inflammation throughout the lung that was similar to that found in infected untreated mice (Fig. 7d).

These results show that pHSP65 DNA therapy seems to induce an influx similar to that observed at 30 days of infection when therapy was initiated, and the mice presented a response related to the T helper type 1 (Th1) pattern.

DISCUSSION

The pHSP65 vaccine is known to have a therapeutic effect in mice infected intravenously.[14] However, it was uncertain which immune mechanisms were induced at the site of infection by therapy with this vaccine, allowing mice to control a pre-established *M. tuberculosis* infection. To investigate the mechanisms that drive the immune response towards a protective pattern in TB after DNA therapy, a model of intratracheal infection was used to evaluate *ex vivo* cellular activation in lungs. This strategy of evaluation was considered essential by our group because our previous reports focused on the characterization of spleen cell activation *in vitro*.[13,14]

According to our model of infection, after the first 30 days of infection, mice developed a response more related to the Th1 pattern, characterized by increased levels of IFN-γ and IL-12 in comparison with uninfected mice, which are crucial to the development of protective immunity.[19,23,24] Levels of IL-4 and IL-10 were similar to those of uninfected mice. However, in the chronic phase of infection (at 70 days), infected mice presented with down-regulation of the Th1 response, without exacerbation of the Th2 response.

The results presented in this study show that pHSP65 therapy was able to maintain a Th1 response at similar

levels to those detected in 30-day infected mice. The therapy restored normal IFN-γ production in infected pHSP65-treated mice when compared with infected untreated mice. DNA therapy, either with pHSP65 or pcDNA3 vector, was also able partially to restore production of inflammatory cytokines, such as TNF-α, which was down-regulated in infected untreated mice. These results suggest that IFN-γ production depends on antigen-specific stimulation exacerbated by treatment with pHSP65, because the pcDNA3 vector was not able to increase the levels of IFN-γ in a significant way. On the other hand, the innate immune response might be activated by the DNA backbone itself, such as pcDNA3 vector. DNA therapy was expected to improve production of IL-12 and chemokines. However, comparable levels of IL-12, MCP-1 and IP-10 were seen in both infected pHSP65-treated mice and infected untreated mice.

The influx of $CD4^+$ and $CD8^+$ cells into the lungs of infected, pHSP65-treated mice and the increased expression of CD18 and CD28 most in $CD8^+$ cells of pHSP65-treated mice confirm a more expressive pattern of antigen-specific cellular activation than that found in infected, untreated mice. However, DNA therapy with pcDNA3 vector also induced a stimulatory effect regarding the expression of CD28 receptor in lymphocytes. We suggest that the immune stimulatory effect of the pcDNA3 vector is associated with CpG motifs present on the ampicillin-resistance gene. An immune stimulatory effect of CpG motifs in an antigen-independent way was reported recently by Davila *et al*., who described the expansion of naïve and memory $CD4^+$ and $CD8^+$ T lymphocytes induced by CpG motifs.[29]

Up-regulation of CD28 and CD18 markers is expected after antigen priming. T-cell receptor engagement with CD28 costimulation is generally required for naïve T-cell activation, whereas activation of effector T cells and reactivation of memory cells are less dependent on CD28 costimulation. Fontenot *et al*. showed that in the lung, effector memory cells were independent of CD28 costimulation and presented low expression of this receptor. In contrast, memory $CD4^+$ T cells in the blood continued to require CD28 costimulation to play a functional activity.[30] In this context, up- or down-regulation on CD28 expression may be associated with transition in central to effector memory T cells. According to this, the up-regulation of CD28 on $CD8^+$ lung cells of infected DNA-treated mice may represent the influx of recently activated memory T lymphocytes.

In experimental TB, an apparently co-ordinated event involving the adhesion molecule CD18, cytokines and chemokines, establishes the pattern of T-cell migration into the lungs. However, in the chronic phase of infection (at 70 days), there was a significant decrease in the ability to recruit T cells. In spite of the decreased influx of T cells in the chronic phase of infection, this cell migration seems to be dependent on CD18. With this in mind, we suggest that the low influx of T cells may be related to the lower levels of IL-6, TNF-α, IP-10 and MCP-1 detected in the lungs of mice after 70 days of infection compared to those of mice evaluated after 30 days of infection. Histological analysis confirmed the cell number results (Fig. 1). Lung sections from mice evaluated after 30 days of infection and infected pHSP65-treated mice presented similar pattern of cellular infiltrate and similar levels of IFN-γ. On the other hand, lung sections of infected pcDNA3-treated mice presented low or moderate lymphocyte influx and predominant presence of macrophages. If both groups, infected pHSP65-treated mice and infected pcDNA3-treated mice, were able partially to restore TNF-α production and these mice presented a differential pattern of cellular infiltrate, it is probable that specific antigen stimulation and IFN-γ production, followed by pHSP65 therapy, play a differential function in the control of *M. tuberculosis* infection. In fact, despite the intense activation of a specific cellular immune response and the strong inflammatory response stimulated by DNA treatment, only pHSP65 therapy was able to restrict the growth of bacilli and to reduce the lung injury because infected untreated mice and infected pcDNA3-treated mice presented more severe destruction of lung parenchyma and increased bacterial burdens. We suggest that this effect may be correlated with activation-induced cell death (AICD), because we detected significant up-regulation of CD95 in $CD8^+$ lymphocytes of infected pHSP65-treated mice compared with those of infected untreated mice. It is possible that these recently activated lymphocytes, mainly $CD8^+$ $CD18^{hi}$ cells, migrate to the lungs, play their effector function and die by apoptosis (AICD). The up-regulation of CD95 and CD95L on T lymphocytes seems to be relevant to the control of experimental TB since Turner *et al*.[31] demonstrated that, in the chronic phase of experimental pulmonary TB, both $CD8^-$ and CD95/CD95L-deficient mice gradually lost their ability to limit bacterial growth. However, we did not perform experiments with CD95/CD95L-deficient mice to assure this hypothesis.

Results published by Taylor *et al*.[32] showed that immunization or treatment of infected mice with pHSP65 vaccine resulted in pulmonary necrosis. These authors used a model of pulmonary TB by low-dose aerosol exposure with *M. tuberculosis*. In fact, we do not understand how immunized or treated mice infected by using an aerosol generator calibrated to deliver 50–100 bacilli into the lungs developed a severe necrosis, while mice infected by the intratracheal route with 1×10^5 bacilli were protected by pHSP65 therapy. One possible explanation for these diverging results may be the different method (aerosol versus intratracheal) of antigen delivery and the differential experimental kinetics of both models. We are conscious that, with the intratracheal model of infection available in our laboratory, mice were unable completely to clear bacterial loads because they were infected with a high number of bacilli. However, even when infected with a high number of bacilli, infected pHSP65-treated mice were able to restrict bacterial loads in a significant way.

In conclusion, the data described here show that pHSP65 therapy presented an immune regulatory effect that drives the immune response to a cellular pattern. This cellular response, characterized by IFN-γ recovery and up-regulation of CD18, CD28 and CD95 in $CD8^+$ lung cells,

probably contributes to a decrease in bacterial loads and to a reduction in lung injury. Finally, our results suggest that the immune regulatory effect of this vaccine may be used in other experimental models, either to increase a cellular immune response or to down-regulate Th2 responses.

ACKNOWLEDGMENTS

We would like to thank Izaíra Tincani Brandão for general technical assistance, Alexandra Rosa Vieira Dias for technical assistance with flow cytometry experiments, Ana Maria da Rocha for technical assistance in histological sections and Márcia S. Z. Graeff for assistance in photomicroscopy. This study was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP).

REFERENCES

1 Collins HLK, Kaufmann SHE. The many faces of host responses to tuberculosis. Immunology 2001; **103:**1–9.
2 Raviglione MC. The TB epidemic from 1992 to 2002. Tuberculosis 2002; **83:**4–14.
3 Godfrey-Faussett P, Ayles H. Can we control tuberculosis in high HIV prevalence settings? Tuberculosis 2003; **83:**68–76.
4 Smith D. Protective efficacy of BCG in experimental tuberculosis. Adv Tuberc Res 1995; **22:**1–97.
5 Fine PE. The BCG story. Lessons from the past and implications for the future. Rev Infect Dis Supplement 1989; **2:**S353–9.
6 Cole ST, Brosch R, Parkhill J *et al*. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature 1998; **393:**537–44.
7 Gurunathan S, Wu CY, Freidag BL, Seder RADNA vaccines: immunology, application and optimization. Annu Rev Immunol 2000; **18:**927–74.
8 Ulmer JB, Donnelly JJ, Parker SE *et al*. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science 1993; **259:**17465–9.
9 Tascon RE, Soares CS, Ragno S, Stavropoulos E, Hirst EM, Colston MJ. *Mycobacterium tuberculosis*-activated dendritic cells induce protective immunity in mice. Immunology 2000; **99:**473–80.
10 Stacey KJ, Sweet MJ, Hume DA. Macrophages ingest and are activated by bacterial DNA. J Immunol 1996; **157:**2116–22.
11 Yamamoto S, Yamamoto T, Shimada S *et al*. DNA from bacteria, but not from vertebrates, induces interferons, activates natural killer cells and inhibits tumor growth. Microbiol Immunol 1992; **36:**983–97.
12 Krieg AM, Yi AK, Matson S *et al*. CpG motifs in bacterial DNA trigger direct B-cell activation. Nature 1995; **374:**546–9.
13 Bonato VL, Lima VM, Tascon RE, Lowrie DB, Silva CL. Identification and characterization of protective T cells in hsp65 DNA-vaccinated and *Mycobacterium tuberculosis*-infected mice. Infect Immun 1998; **66:**169–75.
14 Lowrie DB, Tascon RE, Bonato VL *et al*. Therapy of tuberculosis in mice by DNA vaccination. Nature 1999; **400:**269–71.
15 Hingley-Wilson SM, Sly LM, Reiner NE, MacMaster RW. The immunobiology of the mycobacterial infected macrophage. Mod Asp Immunobiol 2000; **1:**96–101.
16 Caruso AM, Serbina N, Klein E, Triebold K, Bloom BR, Flynn JL. Mice deficient in CD4 T cells have only transiently diminished levels of IFN-gamma, yet succumb to tuberculosis. J Immunol 1999; **162:**5407–16.
17 Flynn JL, Goldstein MM, Triebold KJ, Koller B, Bloom BR. Major histocompatibility complex class I-restricted T cells are required for resistance to *Mycobacterium tuberculosis* infection. Proc Natl Acad Sci USA 1992; **89:**12013–7.
18 Ladel CH, Blum C, Dreher A, Reifenberg K, Kopf M, Kaufmann HE. Lethal tuberculosis in interleukin-6-deficient mutant mice. Infect Immun 1997; **65:**4843–9.
19 Cooper AM, Magram J, Ferrante J, Orme IM. Interleukin 12 (IL-12) is crucial to development of protective immunity in mice intravenously infected with *Mycobacterium tuberculosis*. J Exp Med 1997; **186:**39–45.
20 Flynn JL, Goldstein MM, Chan J *et al*. Tumor necrosis factor-alpha is required in the protective immune response against *Mycobacterium tuberculosis* in mice. Immunity 1995; **2:**561–72.
21 Murray PJ, Young RA. Increased antimycobacterial immunity in interleukin-10-deficient mice. Infect Immun 1999; **67:**3087–95.
22 Hernandez-Pando R, Orozco H, Arriaga K, Sampieri A, Larriva-Sahd J, Marina-Madrid V. Analysis of the local kinetics and localization of interleukin-1-alpha, tumor necrosis factor-alpha and transforming growth factor-beta, during the course of experimental pulmonary tuberculosis. Immunology 1997; **90:**607–17.
23 Cooper AM, Dalton DK, Stewart TA, Griffin JP, Russel DG, Orme IM. Disseminated tuberculosis in interferon gamma gene-disrupted mice. J Exp Med 1993; **6:**2243–7.
24 Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA, Bloom BR. An essential role for interferon gamma in resistance to *Mycobacterium tuberculosis* infection. J Exp Med 1993; **178:**2249–54.
25 Gonzalez-Juarrero M, Orme IM. Characterization of murine lung dendritic cells infected with *Mycobacterium tuberculosis*. Infect Immun 2001; **69:**1127–33.
26 McDonough KA, Kress Y. Cytotoxicity for lung epithelial cells is a virulence associated phenotype of *Mycobacterium tuberculosis*. Infect Immun 1995; **63:**4802–11.
27 Lowrie DB, Silva CL, Colston MJ, Ragno S, Tascon RE. Protection against tuberculosis by a plasmid DNA vaccine. Vaccine 1997; **15:**834–8.
28 Bonato VL, Gonçalves EDC, Santos RR, Silva CL. Genetic aspects and microenvironment affect expression of CD18 and VLA-4 in experimental tuberculosis. Scand J Immunol 2002; **56:**185–94.
29 Davila E, Velez MG, Heppelmann CJ, Celis E. Creating space: an antigen-independent, CpG-induced peripheral expansion of naïve and memory T lymphocytes in a full T-cell compartment. Blood 2002; **100:**2537–45.
30 Fontenot AP, Gharavi L, Bennett SR, Canavera SJ, Newman LS, Kotzin BL. CD28 costimulation independence of target organ versus circulating memory antigen-specific CD4+ T cells. J Clin Invest 2003; **112:**776–84.
31 Turner J, D'Souza CD, Pearl JE *et al*. CD8 and CD95/95L-dependent mechanisms of resistance in mice with chronic pulmonary tuberculosis. Am J Respir Cell Mol Biol 2001; **24:**203–9.
32 Taylor JL, Turner OC, Basaraba RJ, Belishe JT, Huygen K, Orme IM. Pulmonary necrosis resulting from DNA vaccination against tuberculosis. Infect Immun 2003; **71:**2192–8.