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Exposure to *Mycobacterium avium* Decreases the Protective Effect of the DNA Vaccine pVAXhsp65 Against *Mycobacterium tuberculosis*-Induced Inflammation of the Pulmonary Parenchyma

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Abstract

This work investigated the effect of previous *Mycobacterium avium* exposure on the protective ability of the DNA vaccine pVAXhsp65 against inflammation in the pulmonary parenchyma. BALB/c mice were presensitized with heat-killed *M. avium* and then immunized with three doses of pVAXhsp65 prior to challenge with *Mycobacterium tuberculosis*. *M. avium* sensitization induced high levels of spontaneous IL-5 production that were concomitant with a positive delayed-type hypersensitivity reaction; antigen-specific IFN-γ production was also observed upon splenocyte stimulation. Prior exposure to *M. avium* resulted in altered cytokine and antibody production induced by immunization with pVAXhsp65; instead of a Th1 response, vaccinated mice previously exposed to *M. avium* developed a strong Th2 response. This switch to a Th2 response coincided with the loss of the anti-inflammatory effect of pVAXhsp65 vaccination previously observed in the pulmonary parenchyma of mice infected with *M. tuberculosis*. These results suggest that exposure to environmental mycobacteria can modulate immune responses induced by mycobacterial vaccines other than bacillus Calmette–Guérin.

Introduction

Tuberculosis (TB), which is caused by *Mycobacterium tuberculosis*, is responsible for more than two million deaths each year [1, 2] and ranks among the 10 principal causes of death and disability worldwide [3]. The increasing prevalence of AIDS and TB coinfections combined with the emergence of TB strains resistant to all major chemotherapeutic drugs has emphasized the need for effective prophylactic agents. The only available TB vaccine, bacillus Calmette–Guérin (BCG), has been distributed since the 1920s, and more than three billion people have received this vaccine. Although large variation in vaccine efficacy exists, the major conclusions arising BCG vaccination studies are that BCG vaccination efficiently protects against leprosy [4] as well as against the most severe childhood manifestations of TB (milliary and meningeal forms) [5]. However, the vaccine's efficacy in preventing pulmonary TB is very limited. These conclusions are consistent with the 15-years follow-up trial of BCG vaccination comprising 360,000 individuals carried out in Cingleput, which found that BCG offered no protection against pulmonary TB in adults [6].

Many hypotheses have been proposed to explain the poor efficacy of BCG vaccination against pulmonary TB. Factors directly related to the vaccine, including inappropriate administration and storage have been suggested [7]. After the first successful vaccinations, BCG cultures were distributed worldwide and propagated under different conditions in various laboratories for several decades. This process resulted in genotypic and phenotypic differences between the original BCG strain and the various BCG daughter strains as well as differences among the daughter strains themselves [8]. Additionally, a number

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of hypotheses have suggested deficits in the immune response induced by BCG. As various T cell subsets participate in the immune response against mycobacteria, the lack of effective stimulation of the required T cell populations, especially CD8 T cells, may explain the insufficient levels of immunity induced by the BCG vaccine [9, 10]. Finally, the exposure of human populations in tropical regions to large quantities of environmental mycobacteria has been suggested to be the single most important factor for the low efficacy of BCG vaccination in these regions [11, 12]. The development of a new vaccine strategy is considered essential for the control of TB [13, 14]. However, vaccine development is challenging, as any successful vaccine must overcome the litany of issues and obstacles described earlier.

Our group has been working with a DNA vaccine constructed by inserting the gene encoding heat-shock protein 65 from *Mycobacterium leprae* (groEL/ML0317) into plasmid vectors. These constructs displayed both a prophylactic [15, 16] and therapeutic effect in a murine model of TB [17, 18]. This vaccine was also able to prime neonatal mice for a strong immune response at the adult stage [19].

The objective of this study was to evaluate whether previous contact with *Mycobacterium avium* could interfere with the protective effect of pVAXhsp65 vaccination against pulmonary parenchyma inflammation triggered by *M. tuberculosis*. BALB/c mice were intradermally sensitized with heat-killed *M. avium* and then immunized with pVAXhsp65 prior to a challenge with *M. tuberculosis*. *M. avium* presensitization modulated pVAXhsp65 vaccine efficacy in a deleterious way: the vaccine lost its protective anti-inflammatory effect against TB-induced inflammation of the pulmonary parenchyma.

Material and methods

Animals. Female BALB/c mice were bred in the Animal Facility of São Paulo State University (UNESP) at the Biosciences Institute and used between 4 and 6 weeks of age. The animals were fed a standard pellet diet and water *ad libitum*. All of the experimental animal protocols and animal manipulations were approved by the local ethics committee and carried out in compliance with the ethical guidelines adopted by Colégio Brasileiro de Experimentação Animal.

Plasmid DNA construction and purification. The pVAXhsp65 vaccine was derived from the pVAX vector that uses the CMV intron (Invitrogen®, Carlsbad, CA, USA). The pVAX vector was digested with BamH I and Not I (Gibco BRL, Gaithersburg, MD, USA), and a 3.3-kb fragment corresponding to the *M. leprae hsp65* gene was inserted. The empty pVAX vector was used as a control. DH5α *Escherichia coli* transformed with the pVAX plasmid or the plasmid carrying the *hsp65* gene (pVAXhsp65) were cultured in liquid LB medium (Gibco BRL) containing kanamycin (50 μg/ml). The plasmids were purified using the Concert High Purity Maxiprep System (Gibco BRL). Plasmid concentrations were determined by spectrophotometry at λ = 260 and 280 nm using the Gene Quant II apparatus (Pharmacia Biotech, Buckinghamshire, UK).

Immunization procedures. Groups of BALB/c mice were injected intradermally at the base of the tail with different concentrations of heat-killed *M. avium* (ATCC – 27294) (0.08×10^6, 4×10^6 and 200×10^6). Three weeks later, these animals were evaluated for specific anti-*M. avium* immunity or immunized with pVAXhsp65. The mice were immunized with three doses of pVAXhsp65 (100 μg/100 μl) plus 25% saccharose injected into the quadriceps muscle. The DNAhsp65 immunizations were administered at intervals of 15 days. Control animals received salt solution (the vaccine diluent) or pVAX (the empty vector).

Delayed-type hypersensitivity reaction. The delayed-type hypersensitivity (DTH) reaction was evaluated using the footpad-swelling test as previously described [20]. Briefly, control or immunized mice were inoculated in one of the back footpads with 1×10^7 heat-killed *M. avium* suspended in 50 μl PBS. Footpad thickness was measured just prior to and 72 h after antigen inoculation using a dial caliper (Mitutoyo, Tokyo, Japan). The results were expressed in mm.

Quantification of anti-hsp65 antibodies. Serum samples were collected by retro-orbital bleeding 3 weeks after heat-killed *M. avium* inoculation or 3 weeks after the last DNA vaccination, and hsp65-specific antibody levels were evaluated by enzyme-linked immunosorbent assay (ELISA). Maxisorp plates (Nunc, Roskilde, Denmark) were coated with 0.1 ml (0.5 μg/ml) of recombinant hsp65 (rhsp65) in coating solution (14.3 mM Na_2CO_3 and 10.3 mM $NaHCO_3$, pH 9.6), incubated at 4 °C overnight. The plates were then blocked with 10% foetal calf serum (FCS) in PBS for 1 h at 37 °C. Serum samples were diluted 1:10 and tested. After incubation for 2 h at 37 °C, anti-mouse IgG1 and IgG2a biotinylated conjugates (A85-1 and R19-15, respectively, from PharMingen, Fallbrook, CA, USA) were added for detection of specific isotype antibodies. After washing, the plates were incubated at room temperature for 30 min with the StreptAB kit (Dako, Carpinteria, CA, USA) and then developed by adding H_2O_2 and o-phenylenediamine dihydrochloride (OPD). Colour development was stopped with H_2SO_4, and the optical density was measured at 492 nm.

Evaluation of cytokine production. Spleen cells were obtained 3 weeks after heat-killed *M. avium* inoculation or 3 weeks after the last vaccination dose. The cells were adjusted to a concentration of 5×10^6 cells/ml in RPMI 1640 medium supplemented with 5% FCS, 20 mM glutamine and 40 IU/l of gentamicin. Next, the cells were

cultured in 48-well, flat-bottomed culture plates (Nunc, Life Tech. Inc., Maryland, MA, USA) in the presence of heat-killed *M. avium* (1.2×10^7/ml), rhsp65 (10 μg/ml) or concanavalin A (ConA) (10 μg/ml). Culture supernatants were harvested 48 h later, and cytokine levels were evaluated by ELISA. Cytokines were measured following manufacturer instructions (PharMingen). Purified monoclonal antibodies against IFN-γ (R4-6A2) and IL-5 (TRKF5) were used at 1 μg/ml as capture antibodies. The following biotinylated antibodies were used for detection: anti-IFN-γ (XMG1.2) and anti-IL-5 (TRFK4) at 0.5 μg/ml. After an incubation step followed by washing, plates were incubated with the StreptAB kit (Dako) at room temperature for 30 min and then developed by adding H_2O_2 and OPD. Colour development was stopped with H_2SO_4, and the optical density was measured at 492 nm. Cytokine concentrations were determined by comparison with standard curves obtained from recombinant cytokine samples run in duplicate.

Infection with M. tuberculosis and histopathological analysis. Groups of mice previously injected with heat-killed *M. avium*, vaccinated with pVAXhsp65 or mice that were exposed to *M. avium* and vaccinated were challenged 4 weeks later with *M. tuberculosis* (H37Rv – ATCC – 27294). The animals were first anaesthetized with tribromoethanol 2.5% (Acros Organics, Geel, Belgium) and then challenged through intratracheal instillation of a bacterial solution (10^4 bacilli/animal). Four weeks later, the animals were euthanized, and histopathological analysis was performed on lung sections. The upper left lung lobe of each mouse was fixed in 10% formalin, embedded in Paraplast plus (McCormick Scientific, St. Louis, MO, USA), prepared routinely and then sectioned for light microscopy. Sections (5 μm each) were stained with haematoxylin and eosin. Pneumonic areas were measured using the image analyzer Leica QWin software Version 3 for Windows™ (Microsoft Corporation, Redmond, WA, USA). Eight random fields for each experimental group were analysed at 200× magnification.

Statistical analysis. Results represent the mean ± SEM for each variable. Statistical analysis was performed using GraphPad InStat Version 1993 (San Diego, CA, USA). The nonparametric Mann–Whitney test was used to compare cytokine and antibody levels. Values of $P < 0.05$ were considered statistically significant.

Results

Inoculation with *M. avium* is associated with high levels of IL-5 production

Three weeks after injection with heat-killed *M. avium*, animals were challenged with particulate *M. avium* antigen, and the increase in the footpad thickness was measured 72 h later. A significant increase in footpad size was observed in the group inoculated with the higher dose of heat-killed *M. avium* compared to the non-immunized group (Fig. 1A).

Immediately following footpad measurements, the animals were euthanized, and their spleens were harvested for analysis. Splenocyte cell cultures were stimulated with *M. avium* or ConA, and IFN-γ and IL-5 concentrations were evaluated in the culture supernatants. Significant antigen-specific IFN-γ induction was detected in cell culture supernatants from mice previously inoculated with the intermediate and higher doses of heat-killed *M. avium*. Although all of the experimental groups exhibited significant IFN-γ production in response to ConA stimulation, IFN-γ levels were significantly lower in the three groups that were previously inoculated with *M. avium* (Fig. 1B). Stimulation with ConA-induced similar IL-5 levels in all of the experimental groups. Strikingly, splenic cells from mice exposed to *M. avium* spontaneously produced significant levels of IL-5; endogenous IL-5 production was most apparent in cell cultures from mice that received either the intermediate or high dose of heat-killed *M. avium* (Fig. 1C). Antigen-specific stimulation did not increase IL-5 production, and they were similar to the ones induced by polyclonal stimulation with ConA.

M. avium exposure modulates the immune response induced by DNAhsp65 vaccination

Three weeks after being injected with different *M. avium* doses, BALB/c mice were immunized with pVAXhsp65. Control groups received saline or empty vector (pVAX). Two weeks after the last dose, the mice were euthanized and splenic cytokine production and hsp65-specific antibody levels were examined. Prior exposure to heat-killed *M. avium* decreased both rhsp65-specific and ConA-induced IFN-γ production (Fig. 2A). Unstimulated, or endogenous, production of IL-5 was significantly higher in mice that were inoculated with the intermediate and the higher doses of *M. avium*. In response to ConA stimulation, pVAXhsp65-vaccinated mice produced less IL-5 than the control group. Interestingly, the previous contact with intermediate or high amounts of *M. avium* abolished the downmodulatory effect of pVAXhsp65 vaccination on IL-5 production (Fig. 2B). *M. avium* exposure also affected antibody production, increasing both IgG1- and IgG2a-specific antibodies: this increase was highly significant in animals inoculated with the higher dose of *M. avium* (Fig. 2C).

M. avium exposure decreases the protective effect of DNAhsp65 over the pulmonary parenchyma

Immunization with 3 doses of pVAXhsp65 resulted in protection of the pulmonary parenchyma against *M. tuberculosis*-induced inflammation. However, this effect was

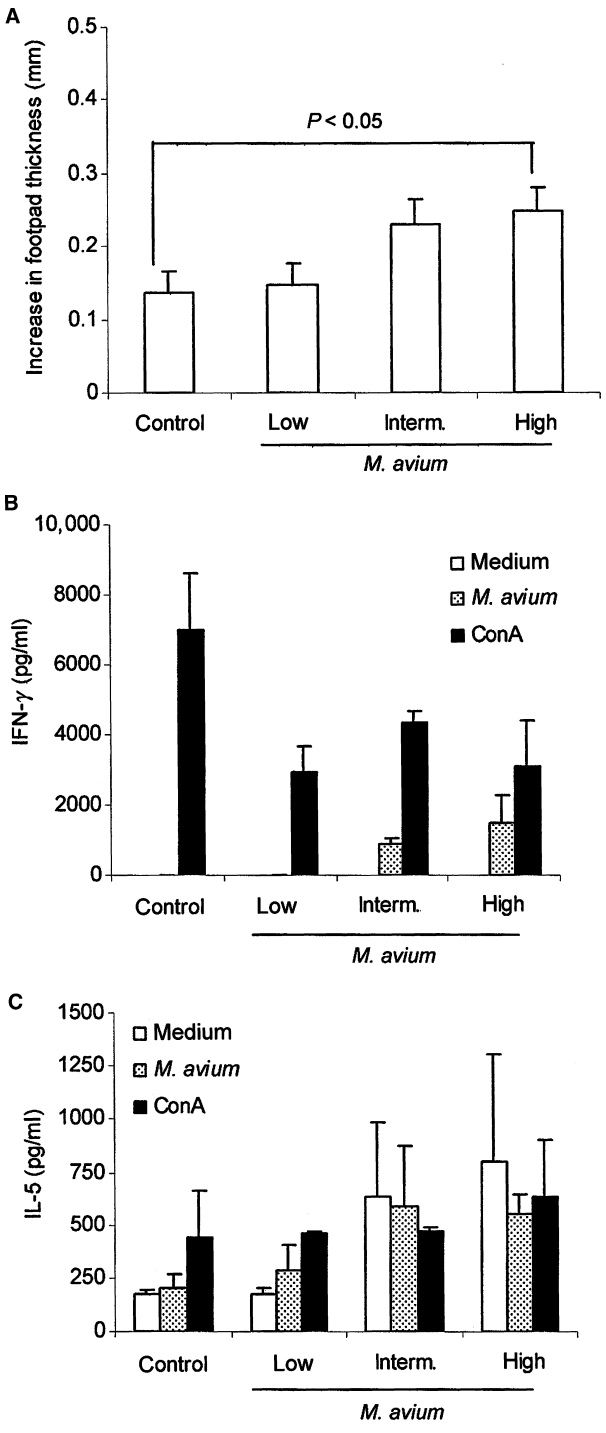

Figure 1 Immune response induced by injection with heat-killed *Mycobacterium avium*. BALB/c mice were injected with different concentrations of heat-killed *M. avium* (0.08×10^6, 4×10^6 and 200×10^6 CFU) by the subcutaneous route at the base of tail. Delayed-type hypersensitivity (DTH) (A) and *in vitro* cytokine production were evaluated 3 weeks later. DTH was tested by inoculation of *M. avium* (10^7 CFU/50 μl) in the left footpad. Footpad thickness was evaluated after 72 h using a caliper. IFN-γ (B) and IL-5 (C) were quantified by enzyme-linked immunosorbent assay in spleen cell cultures stimulated with *M. avium* (10^7 CFU/ml) or ConA (10 μg/ml) for 48 h. The results represent the geometric mean ± SEM of three animals.

blocked by previous exposure to *M. avium*. Lung sections from control mice showing a preserved pulmonary parenchyma can be observed in Fig. 3A. As expected, *M. tuberculosis* infection elicited the appearance of small- and medium-sized focal granulomas characterized by perivascular and peribronchial mononuclear cell infiltration. Necrotic areas were also observed throughout the lung tissue (Fig. 3B). Lung sections from mice immunized with pVAXhsp65 prior to infection with *M. tuberculosis* exhibited tissue architecture very similar to lung sections from control, untreated mice; only a few inflammatory foci were observed (Fig. 3C). However, lung sections from mice that were injected with *M. avium* before *M. tuberculosis* infection showed intense peribronchial and perivascular inflammatory infiltrates. Necrotic areas were also observed (Fig. 3D). Thus, *M. avium* exposure prior to pVAXhsp65 immunization completely abolished the protective effect of pVAXhsp65 over the pulmonary parenchyma (Fig. 3E). The loss of pVAXhsp65 protection was further confirmed by a quantitative comparison of the percentage of pneumonic areas in each of the different treatment groups (Fig. 3F).

Discussion

Mycobacterium bovis BCG, the current vaccine against infection with *M. tuberculosis*, offers variable protective efficacy in man. It has been suggested that exposure to environmental mycobacteria can interfere with the generation of BCG-specific immunity. pVAXhsp65 is a genetic construct containing the *hsp65* gene from *M. leprae* that has demonstrated prophylactic and therapeutic effects in experimental TB models; it is currently being optimized in numerous preclinical studies. As hsp65 is a ubiquitous antigen present in many bacteria, including all *Mycobacterium* sp., we asked whether previous exposure to these bacteria could affect the immunogenicity and protective efficacy of this vaccine, similar to the described effect of mycobacteria exposure on BCG vaccine efficacy.

Theoretically, subunit vaccines devised to substitute BCG would be less affected by environmental mycobacteria. This hypothesis has been based on the supposition that BCG vaccination failure is caused by cross-protective immunity, generated from exposure to environmental mycobacteria, that drives the early clearance of BCG. However, this report provides evidence that previous exposure to *M. avium* affects both the immunogenicity and protective effect of pVAXhsp65 vaccination.

We began our study by evaluating the immunogenicity of a preparation of heat-killed *M. avium*. Adult BALB/c mice were injected at the base of the tail following procedures already adopted by other authors [21]. Three different doses of heat-killed *M. avium* were tested: 0.08×10^6 (low), 4×10^6 (intermediate) and 200×10^6 (high). Three weeks later, mice exposed to heat-killed

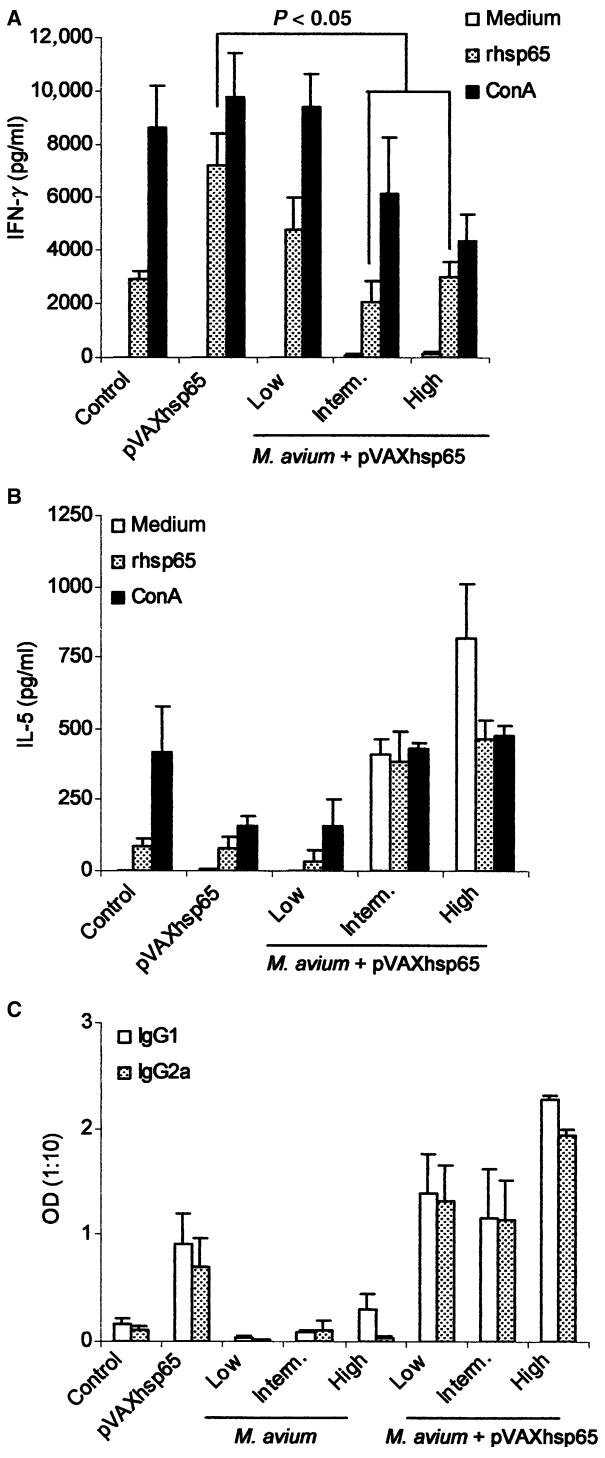

Figure 2 Effect of previous contact with *Mycobacterium avium* on the immune response induced by pVAXhsp65 immunization. BALB/c mice were injected with heat-killed *M. avium* (0.08×10^6, 4×10^6 and 200×10^6 CFU) by the subcutaneous route at the base of the tail. Three weeks later, they were immunized with three doses of pVAXhsp65 (100 μg each). The doses were administered 15 days apart. Two weeks after the last pVAXhsp65 dose, anti-hsp65 levels (A) were evaluated in blood samples and IFN-γ (B) and IL-5 (C) production were evaluated in spleen cell cultures by enzyme-linked immunosorbent assay. The results represent the geometric mean ± SEM of five animals.

M. avium exhibited a significant DTH reaction and stimulations of splenocyte cultures showed *M. avium*-specific production of IFN-γ induction. However, contrasting with these results, a significant decrease in IFN-γ production was observed in ConA-stimulated splenocyte cultures from mice immunized with heat-killed *M. avium*. Concomitantly, striking endogenous IL-5 production was detected in splenocyte cultures from mice exposed to *M. avium*. Levels of IL-5 production correlated to the dosage of *M. avium* injected; IL-5 levels increased with increased antigen exposure. A literature report supports our IL-5 finding. Coussens *et al.* [22], by using microarray analysis, suggested that T cells within the peripheral blood mononuclear cell (PBMC) population from *M. avium* subspecies *paratuberculosis* (*M. paratuberculosis*)–infected cows were skewed towards a Th2-like phenotype with the high expression of genes encoding IL-5 and GATA-3, a Th2-polarizing transcription factor. Therefore, we could attribute the decreased IFN-γ production in ConA-stimulated cultures to a Th2-polarized response.

However, this scenario seems to fit better with other characteristics of the *M. avium* complex. This group of mycobacteria is distinguished by the presence of a unique, highly antigenic group of lipids located on the cell surface known as glycopeptidolipids (GPLs) [23]. These molecules have been shown to be immunosuppressive and can induce a variety of immunomodulatory cytokines [24]. The total lipids in *M. avium* significantly suppressed the secretion of IL-12 and IFN-γ that are associated with a Th1 response [25, 26]. However, many other mechanisms must also be considered as the main or secondary causes of the decreased IFN-γ production. For example, during *M. avium* infection, T cells upregulate expression of CD95 on their surface and can be stimulated to undergo apoptosis by cross-linking of Fas [27, 28]. Additionally, it has been demonstrated that mycobacterial GPLs become inserted into phospholipid monolayers, disrupting in this way the responsiveness of different cell types, including mononuclear cells [29].

To test whether previous contact with *M. avium* could affect the immune response induced by pVAXhsp65 vaccination, mice were injected with heat-killed *M. avium*, and 3 weeks later, they were immunized with pVAXhsp65. Two parameters were evaluated, vaccine immunogenicity and protection against lung injury. Immunogenicity was ascertained by rhsp65-specific production of IFN-γ and IL-5 and by the levels of anti-hsp65 antibodies in the serum. Pulmonary injury was determined by the quantification of pneumonic areas in the lungs.

In terms of cytokine production, vaccination with pVAXhsp65 induced a predominantly Th1 profile characterized by high levels of IFN-γ and only basal IL-5 levels. These results are comparable with previous publications utilizing pVAXhsp65 or other DNA vaccines [30, 31]. A clear immunomodulatory effect was triggered by previous

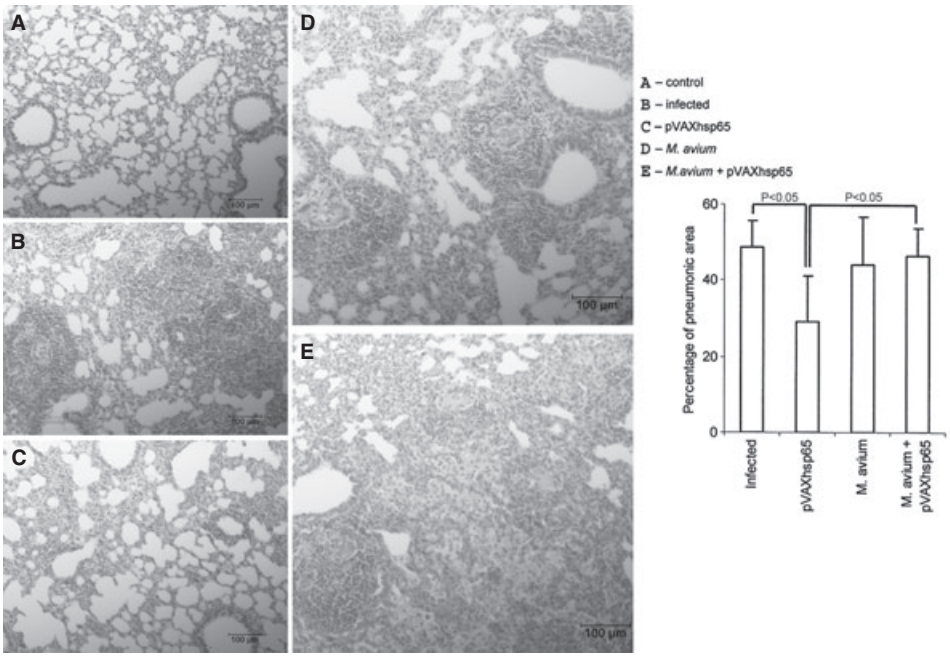

Figure 3 Effect of previous contact with *Mycobacterium avium* on the protective ability of pVAXhsp65 vaccination over the pulmonary parenchyma. BALB/c mice were injected with 200×10^6 CFU of heat-killed *M. avium* by an intradermal route at the base of the tail and then immunized with 3 doses of pVAXhsp65. Two weeks later, the mice were infected with *Mycobacterium tuberculosis* (10^4 bacilli/animal) by an intratracheal route. A quantitative histopathological analysis of the pneumonic areas was performed 30 days after infection. Lung samples from control (A) and mice infected with *M. tuberculosis* (B–E). Before infection, the animals were submitted to no treatment (B), to immunization with pVAXhsp65 (C), to inoculation with heat-killed *M. avium* (D) and to inoculation with *M. avium* and immunization with pVAXhsp65 (E). A–E are representative of five animals analysed. A quantitative comparison of the pneumonic areas among the four infected groups is shown in (F). This figure represents the mean of five animals analysed in each group.

contact with *M. avium*. Prior exposure to *M. avium* resulted in the pronounced reduction in rhsp65-specific and ConA-induced IFN-γ production; this result was most prominent with the high dose of *M. avium*. Interestingly, prior exposure to *M. avium* altered pVAXhsp65 vaccination-induced IL-5 responses; pVAXhsp65 vaccination was unable to downmodulate IL-5 production in mice previously exposed to *M. avium*. To obtain a clearer picture of this data, it was transformed into numerical values by calculating the ratio of IFN-γ to IL-5 levels in the pVAXhsp65 and *M. avium*/pVAXhsp65 experimental groups. This ratio dropped from 1685 in the first group to 6 in the second one, confirming that exposure to *M. avium* clearly changed the cytokine profile induced by pVAXhsp65 vaccination.

Even though *M. avium* exposure alone did not induce anti-hsp65 antibodies (data not shown), it significantly increased both hsp65-specific IgG1 and IgG2a antibody levels induced by pVAXhsp65 vaccination. The lack of anti-hsp65 antibodies in mice injected with heat-killed *M. avium* was a little unexpected because hsp65 has been described as an immunodominant antigen in mycobacterial infections [32]. However, in work by Nagabhushanam *et al.* [33], hsp65 was not considered a dominant T cell antigen in *M. avium* infections.

Thus, *M. avium* priming shaped pVAXhsp65-induced immunity such that a predominantly Th1 profile turned into a predominantly Th2 profile accompanied by a striking increase in antigen-specific antibody production. In the context of TB vaccines, a non-protective effect was already expected. Protection against TB appears to require not only a strong Th1 profile [34, 35] but also a concomitant immunoregulation that downmodulates Th2 response [36, 37].

Previous work from our laboratory has indicated a close relationship between the ability of pVAXhsp65 vaccination to decrease bacterial load and its ability to protect the pulmonary parenchyma from injury caused by *M. tuberculosis* [16, 18]. Here, we analysed the effect of previous exposure to *M. avium* on the ability of pVAXhsp65 vaccination to prevent lung injury by comparing the percentages of pneumonic areas. Lung sections from mice immunized with pVAXhsp65 prior to *M. tuberculosis* infection presented a structure very similar to control mice, with the presence of a limited number of inflammatory foci. Exposure to *M. avium* prior to infection with *M. tuberculosis* had no protective effect; further *M. avium* exposure prior to pVAXhsp65 vaccination completely abolished the protective effect pVAXhsp65 vaccination upon challenge with *M. tuberculosis*.

From a broad point of view, our results are supported by the literature that generally shows the deleterious interference of environmental mycobacteria in BCG vaccine efficacy [38–40]. Experiments performed using the *M. avium* complex have recently demonstrated that presensitization with species from this complex can modify BCG-induced immunity [41, 42]. In 2006, Flaherty *et al.* [43] published a well-conducted study examining exposure to environmental mycobacteria following BCG vaccination. These authors demonstrated that exposure to *M. avium* by the gastrointestinal route interfered with the established protective immune response generated by the previous BCG vaccination.

To explain our results in this context, we could imagine that previous contact with *M. avium*-primed the immune response against the hsp65 antigen. This hypothesis is plausible as hsp65 is highly conserved and present in all *Mycobacterium* spp. despite not being very immunogenic in *M. avium* [33]. The significant increase in IgG1 and IgG2a anti-hsp65 antibodies observed after pVAXhsp65 immunization in *M. avium*-primed mice supports this possibility. Additionally, it is possible that the high content of GPLs in the heat-killed *M. avium* preparation could be more available because of the heat-killing procedure, which would have a Th2-polarizing effect.

Possible priming with *M. avium* hsp65 could also be analysed from the perspective of the 'original sin' concept. This hypothesis was proposed by Francis [44] in the context of serological data from influenza infections. Later on, it was extended to cellular immunity and dengue infections [45]. According to the more encompassing theory, the immune response to a current infection may be dominated by T cells or antibodies that have higher avidity for epitopes encountered from a previous pathogen [46]. In this context, we could imagine that *M. avium* hsp65 primed the immune system, stamping an 'antigenic sin' that was later expanded by pVAXhsp65 vaccination. This enhanced anti-hsp65 response was not, however, protective in this model as it was highly polarized to a Th2-type response. If this reasoning is correct, we could hypothesize that a Th1-polarizing effect during the first encounter with hsp65 would allow a protective type of response to develop later.

In summary, we demonstrated that exposure to *M. avium* increased humoral responses but decreased cellular immunity induced by pVAXhsp65 vaccination. Also, these changes abolished the ability of pVAXhsp65 vaccination to protect the pulmonary parenchyma against *M. tuberculosis*-induced injury. These findings have particular relevance in the search for a new tuberculosis vaccine, as the areas where the vaccine is urgently required are the areas with a higher predominance of environmental mycobacteria.

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Conflict of interest

The authors declare no conflicts of interest.

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