Increased levels of interferon-γ primed by culture filtrate proteins antigen and CpG-ODN immunization do not confer significant protection against *Mycobacterium tuberculosis* infection

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Summary

The results of various animal model studies of tuberculosis (TB) suggest that culture filtrate proteins (CFPs), which are antigens secreted by *Mycobacterium tuberculosis*, are largely responsible for improvements in TB vaccines. The great obstacle to developing protein subunit vaccines is that adjuvants are required in order to stimulate relevant protective immune responses. Acting as immune adjuvants, CpG-oligodeoxynucleotides (CpG-ODNs) promote the activation of Th1 cells and of pro-inflammatory cytokines. To evaluate the adjuvant role of CpG-ODNs in conferring enhanced immunogenic capacity and protection against *M. tuberculosis*, we immunized mice with CFP antigen combined with synthetic CpG-ODNs (CFP/CpG) or with incomplete Freund's adjuvant (IFA) (CFP/IFA). Immunization with CFP/CpG induced a T helper 1 (Th1)-biased response accompanied by a higher immunoglobulin G2a (IgG2a) antibody/IgG1 antibody ratio, elevated production of interferon-γ (IFN-γ) by spleen cells and in lungs. However, CFP/IFA-immunized mice presented higher levels of IgG1 antibodies, as well as increased production of IFN-γ, interleukin (IL)-5, and IL-10 by spleen cells, together with lower levels of IFN-γ in the lungs. Despite the stronger Th1 response seen in both groups, believed to be necessary for protection against TB, only mice immunized with CFP/IFA were protected after *M. tuberculosis* infection. Lung histology revealed that lung parenchyma were better preserved in CFP/IFA-immunized mice, which also presented intense lymphocyte recruitment to the lesion, whereas CFP/CpG-immunized mice presented severe pulmonary injury accompanied by necrosis. Based on the data presented, we discuss the widely accepted paradigm that high levels of IFN-γ are directly correlated with protection against experimental TB.

Keywords: tuberculosis; culture filtrate proteins; CpG oligodeoxynucleotides; vaccine, adjuvant

Introduction

The current tuberculosis (TB) vaccine, *Mycobacterium bovis* bacilli Calmette–Guérin (BCG), was developed nearly a century ago. Although this vaccine has proved efficient in preventing childhood TB, it does not consistently protect against pulmonary TB in adults.[1,2] Within this context, innovative strategies aimed at improving the BCG vaccine have shown promising results.[3–6] In addition, the characterization of the *M. tuberculosis* genome[7] as well as the efficacy of the protection seen in experimental models involving vaccines consisting of recombinant proteins formulated in adjuvants or vaccines with mycobacterial genes delivered via DNA vectors represent substantial progress toward the development of prophylactic strategies against TB.[8–11]

Among the different candidates for a protein to use in devising a subunit vaccine against TB, antigens actively secreted in *M. tuberculosis* cultures have generated particular interest.[12,13] Culture filtrate proteins (CFPs) are the principal targets of the T-cell response in mice, both at the height of infection[14] and in a state of memory

immunity,[15] as well as in humans with active TB.[16] It has been demonstrated that T cells responsible for the recall of protective immunity are directed to highly secreted protein fractions in a mouse model of TB.[17] Immunization with these antigens was shown to be as protective as that with live BCG vaccine in mice challenged with *M. tuberculosis*.[17] Studies performed in mouse and guinea-pig models have demonstrated the protective potential of the purified antigens contained in CFPs.[12,18] Recently, the combination of two constituent CFPs, corresponding to the 85B antigen and early secreted antigenic target-6000 MW (ESAT-6), was tested as a recombinant fusion-protein vaccine in experimental models of TB. This vaccine was found to be effective against experimental infection in mice,[11] guinea pigs[19] and non-human primates.[20] Since 2005, this vaccine has been in phase I clinical trial.[21]

The great obstacle to the development of protein subunit vaccines is the limited availability of new adjuvants capable of eliciting a suitable pattern of immune response. Various CFP antigens have been assayed in the presence of distinct adjuvants in experimental murine TB. These adjuvants include incomplete Freund's adjuvant (IFA), which was shown to induce protection comparable to that achieved through BCG immunization.[22] Recently, it was reported that bacterial CpG-containing oligodeoxynucleotide (CpG-ODN) motifs can be used as T helper 1 (Th1) adjuvants.[23] These CpG motifs are pathogen-associated molecular patterns (PAMPs) that are recognized by Toll-like receptor 9 and trigger an immunostimulatory cascade that culminates in the activation of B lymphocytes, T lymphocytes, natural killer cells, monocytes, macrophages and dendritic cells.[23–26] Together, these cells secrete cytokines and chemokines, such as interferon-γ (IFN-γ), interleukin (IL)-12, IL-1, IL-6, IL-18, and tumour necrosis factor-α (TNF-α).[23,25,26] It has been shown that mice immunized with BCG and injected with CpG-ODN plus recombinant IL-12 presented increased production of IFN-γ and were protected against *M. tuberculosis* challenge.[27] In addition, *M. tuberculosis*-infected mice treated with CpG-ODN have also been shown to present higher levels of IFN-γ.[28]

In the present study, we evaluated the ability of a CpG-ODN adjuvant to improve the immunogenic potential of CFP antigens (CFP/CpG). To that end, we compared the pattern of specific immune response induced and the protective efficacy of CFP/CpG with CFP emulsified in IFA (CFP/IFA).

Our results show that, in this murine model, despite the Th1-biased immune response elicited by immunization with CFP antigen and CpG-ODN, no protection against *M. tuberculosis* challenge was afforded. These data suggest that the currently accepted idea that increased levels of IFN-γ can be used as a singular marker of protection in TB should be re-evaluated.

Materials and methods

Animals

Specific pathogen-free (SPF) female BALB/c mice, 6–8 weeks old, were obtained from the local breeding facility of the University of São Paulo at Ribeirão Preto School of Medicine. Mice were housed under barrier conditions in a level III biohazard laboratory and provided with food and sterile water.

Culture filtrate proteins

The CFPs were kindly donated by Dr Gilles Marchal of the Institut Pasteur in Paris, France. Briefly, a virulent strain of *M. tuberculosis* H37Rv was cultured at 37° in round flasks containing 130 ml of Sauton medium. The culture medium was harvested after 14 days and filtered twice through a 0·22 μm-pore-size filter (Corning, New York, NY). The medium was intensively washed at 4° with deionized water containing butanol 4% on a YM-3 Amicon membrane (Millipore Corporation, Belford, MA) and concentrated around 100-fold. The concentrated media containing molecules with molecular masses above 3000 MW were freeze-dried and stored at −20°. The protein concentration was determined using a protein assay kit (Pierce, Rockford, IL).

Oligodeoxynucleotides

The CpG *Oligodeoxynucleotides (CpG-ODN)* were synthesised using custom primers (Invitrogen, San diego, CA) according to the following sequences:

Immunostimulatory CpG-ODN 1826: 5′-TCC ATG ACG TTC CTG ACG TT-3′

Non-stimulatory, control CpG 2041: 5′-CTG GTC TTT CTG GTT TTT TTC TGG-3′

Both ODNs had a nuclease-resistant phosphorothioate backbone. CpG 1826 has been well characterized for adjuvant activity with protein antigen.[29–32]

Immunizations and experimental procedure

Selected mice were immunized via subcutaneous injection of 50 μg of CFP plus 50 μg of CpG 1826 (CFP/CpG) in three doses given at 7 day-intervals. The remaining mice were divided into three groups: those receiving 50 μg of CFP emulsified in IFA (Gibco BRL, Gaithersburg, MD); those receiving 50 μg of CFP plus 50 μg of CpG 2041 (CFP/Control CpG); and those receiving only phosphate-buffered saline (PBS) (non-immunized group). All these groups also received three inoculations given at 7 day-intervals. Five animals per group were used for the immunogenicity assay. Mice were bled for the detection

of serum antibodies 15 days after the last immunization. The mice were then killed, and spleen cells (5×10^6/ml) were obtained as previously described.[33] The spleen cell samples were re-stimulated *in vitro* with 10 μg/ml of CFP for 48 hr at 37° and under an atmosphere of 5% CO_2. Negative and positive controls were performed with spleen cells cultured in the presence of complete medium (RPMI-1640, Sigma, St. Louis, MO), containing 10% fetal bovine serum (FBS), gentamicin, penicillin/streptomycin and 40 μg/ml of concanavalin A (Con A; Sigma), respectively. Supernatants were stored at −20°.

Bacteria and challenge

The H37Rv *M. tuberculosis* (American Type Culture Collection 27294, Rockville, MD) was grown in 7H9 Middlebrook Broth (Difco Laboratories, Detroit, MI) for 7 days. The culture was harvested by centrifugation, after which the cell pellet was resuspended in sterile PBS and vigorously agitated with glass spheres. The viability of the bacteria in suspension was evaluated using fluorescein diacetate (Sigma) and ethidium bromide, as previously described.[34] For the protection assay, immunized and non-immunized mice in the experimental groups were challenged with 1×10^5 of bacilli by the intratracheal route 60 days after the last immunization, as previously described.[8] Protection was assayed by counting colony-forming units (CFUs) 70 days after challenge.

The lower and medium right lobes of the lungs were washed with sterile PBS, placed in a Petri dish containing incomplete RPMI-1640 medium, and processed as previously reported.[8] Serial dilutions of digested lungs were plated on supplemented 7H11 agar media (Difco). The CFUs were counted 28 days after incubation at 37°.

Antibody detection

Levels of the anti-CFPs antibodies were determined in mouse sera using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer instructions (BD Biosciences – PharMingen, San Diego, CA). Plates (Maxisorp Nunc-Immuno; Nalge Nunc, Roskilde, Denmark) were coated with CFP (5 μg/ml) in a coating solution (14·3 mM Na_2CO_3, 10·3 mM $NaHCO_3$, 0·02% NaN_3, pH 9·6), incubated at 4° overnight and then blocked with 10% FBS in PBS for 60 min at 37°. Serum samples were applied in serial 10-fold dilutions from a starting dilution of 1 : 10. After incubating the plates for 2 hr at 37°, biotin-conjugated anti-mouse IgG1 (A85-1; Sigma) and IgG2a (R19-15; Sigma) were added for detection of specific antibodies. After washing, plates were incubated at room temperature for 30 min with StreptAB kit (Dako, Carpinteria, CA). To detect bound antibodies, the OPD substrate (Sigma) was added. The reaction was stopped by the addition of 50 μl of a 16% solution of sulphuric acid. The optical density (OD) was measured at 490 nm.

Cytokine production

Levels of the cytokines IFN-γ, IL-4, IL-5, IL-6, IL-10, IL-12, and transforming growth factor-β (TGF-β) were determined in spleen cell supernatants and in left lung lobe homogenates using ELISA according to the manufacturer instructions. The following purified monoclonal anti-mouse antibodies were used (1 μg/ml each): IFN-γ (R4–6A2); IL-4 (11B11); IL-5 (TRKF5); IL-6 (MP5–20F3); IL-10 (JES5-2A5); IL-12 (C15.6); and TGF-β (A75-2.1) (BD Pharmingen). Cytokine–antibody complexes were detected by addition of 0·5 μg/ml each of biotinylated anti-mouse IFN-γ (XMG1.2), IL-4 (BVD6-24G2), IL-5 (TRFK4), IL-6 (MP5-32C11), IL-10 (SXC-1), IL-12 (C17.8), and TGF-β (A75-3.1) (BD Pharmingen). For TNF-α detection, ELISA SET BD OptEIA™ (BD Pharmingen) was used. Detection limits were as follows: 78 pg/ml for IFN-γ and IL-12; 39 pg/ml for IL-4 and IL-5; 19 pg/ml for IL-6, IL-10, and TGF-β; and 15 pg/ml for TNF-α.

Histology

The upper right lobes of the lungs were fixed in 10% formalin, embedded in paraffin blocks, prepared routinely, and then sectioned for light microscopy. For the histopathological analyses and detection of acid-fast bacilli, sections (5 μm) were stained with haematoxylin & eosin or with Ziehl-Nielsen staining.

Statistical analysis

All values are expressed as mean ± SEM. Data were compared using analysis of variance (ANOVA) and PRISMA software. When the values indicated the presence of a significant difference, the Tukey test was used. Values of $P < 0{\cdot}05$ were considered significant.

Results

The CFP/CpG vaccine does not protect against *M. tuberculosis* challenge

Previous experiments were performed to determine the concentrations of CFP and CpG to be used in the current study. The following combinations were used in the various experimental groups: 25 μg of CFP plus 30 μg of CpG 1826; 50 μg of CFP plus 30 μg of CpG 1826; 100 μg of CFP plus 30 μg of CpG 1826; and 50 μg of CFP plus 50 μg of CpG 1826. We selected the 50 μg of CFP plus 50 μg of CpG 1826 combination based on the greater IFN-γ production and lower levels of IL-5/IL-10 in spleen

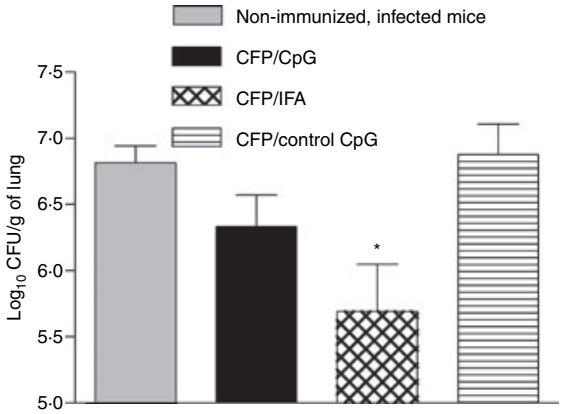

Figure 1. Protection efficacy evaluated in the lungs of mice. Mice were immunized subcutaneously (three doses given at 7 day intervals) with 50 μg of CFP plus 50 μg of CpG 1826 (CFP/CpG), 50 μg of CpG 2041 (CFP/Control CpG), CFP emulsified in IFA (CFP/IFA). Non-immunized mice received PBS by the same route. At 60 days after the last immunization, mice were challenged by intratracheal route with 1×10^5 bacilli of virulent *M. tuberculosis*. At 70 days after challenge, the lungs were processed for CFU assay. Results are expressed as $\log_{10}$ of number of CFU/g of lung obtained from the mean ± standard deviation of serial dilutions individually counted for each group. $^*P < 0{\cdot}05$ versus non-immunized control group. Results are representative of two identical experiments ($n = 5$).

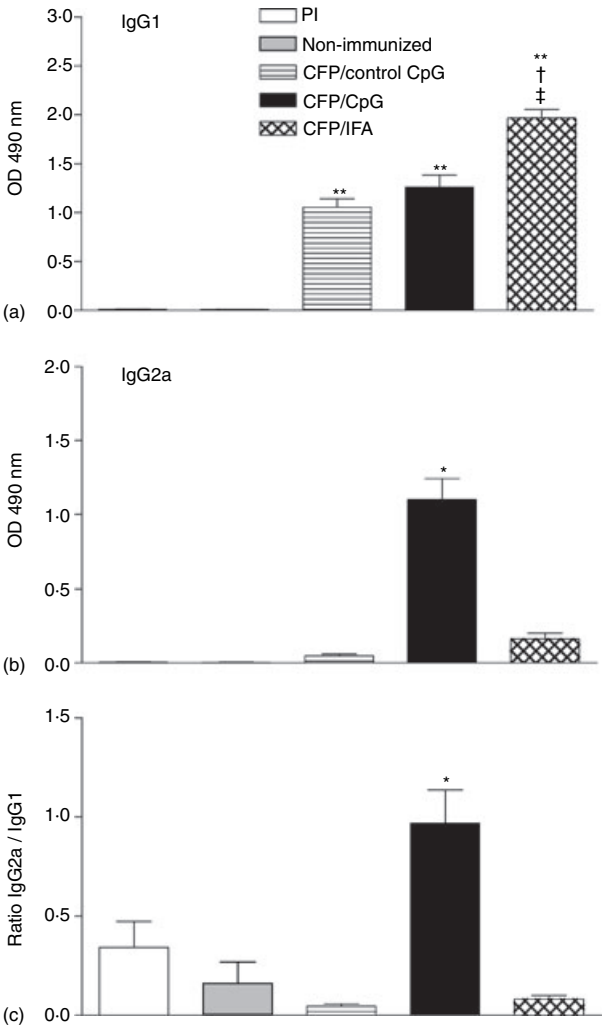

Figure 2. Detection of serum antibodies. Mice were immunized as described in Figure 1. At 15 days after the last immunization, mice were bled for detection of serum antigen-specific IgG1 (a) and IgG2a (b). The IgG2a/IgG1 ratio is also shown (c). Results are expressed as mean ± standard deviation of optical density individually analysed. PI = preimmune (sera). $^{**}P < 0{\cdot}05$ versus PI and the non-immunized group; $\dagger P < 0{\cdot}05$ versus the CFP/CpG group; $\ddagger P < 0{\cdot}05$ versus the CFP/Control CpG group; $^*P < 0{\cdot}05$ versus other groups. Results are representative of two identical experiments ($n = 5$).

cells, as well as the higher levels of anti-CFP IgG2a antibodies, obtained (data not shown).

After establishing the experimental protocol of immunization, we challenged the different groups of immunized mice to determine the protective efficacy of formulations. Surprisingly, we found that CFP/IFA immunization provided significantly greater protection than did CFP/CpG immunization (Fig. 1). This protection was evidenced by the fact that the numbers of CFUs in the lungs of CFP/IFA-immunized mice were approximately 1·2 $\log_{10}$ lower than those observed in the lungs of non-immunized, infected mice. In CFP/CpG-immunized mice, the same parameter was approximately 0·5 $\log_{10}$ lower than in non-immunized, infected mice and 0·8 $\log_{10}$ higher than in CFP/IFA-immunized mice.

The CFP/CpG vaccine generates a Th1 immune response, whereas the CFP/IFA vaccine generates a mixed Th1/Th2 immune response

In an attempt to understand the lack of protection observed in the CFP/CpG-immunized mice, we analysed the immune response against CFP induced by these two classes of adjuvant. First, we evaluated antibody production in serum from immunized mice. We found that all experimental combinations (CFP/CpG, CFP/IFA, and CFP antigen plus control CpG 2041, the last representing only protein, minus any adjuvant) stimulated significant secretion of IgG1 (Fig. 2a). However, the IgG1 levels resulting from CFP/IFA immunization were significantly higher than were those resulting from CFP/CpG immunization. The evaluation of the IgG2a isotype revealed that IgG2a levels were significantly higher in the sera of CFP/CpG-immunized mice than in CFP/IFA-immunized or CFP/Control CpG-immunized mice. These circulating IgG2a levels were also significantly higher than those seen in preimmune sera and in the sera of non-immunized mice (Fig. 2b). We also established the IgG2a/IgG1 ratio (Fig. 2c). Mice immunized with CFP/CpG presented higher IgG2a/IgG1 ratio than did those in the other groups. Next we evaluated cytokine secretion by spleen

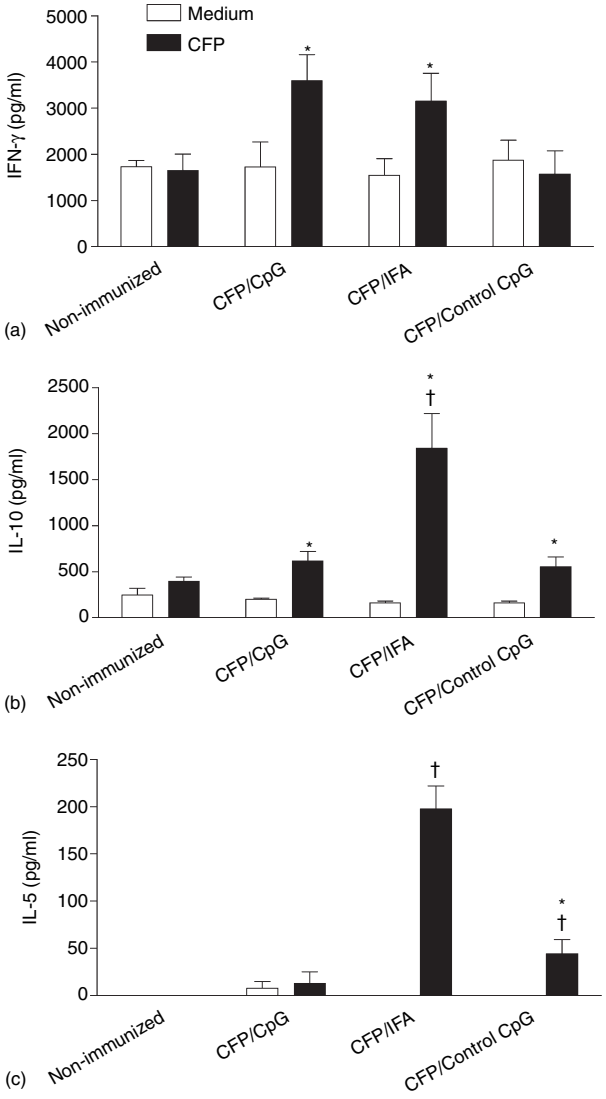

Figure 3. Cytokine production by spleen cells. Mice were immunized as described in Figure 1. At 15 days after the last immunization, the production of IFN-γ (a), IL-10 (b) and IL-5 (c) by spleen cells cultured with CFP (10 μg/ml) or Con A (40 μg/ml) was evaluated. Results are expressed as mean ± standard deviation of cytokine concentration individually analysed. ‡$P < 0{\cdot}05$ versus the other groups, †$P < 0{\cdot}05$ versus non-stimulated cells (medium); *$P < 0{\cdot}05$ versus the non-immunized group.

cells from immunized mice after *in vitro* stimulation with CFP antigens. Both classes of adjuvant (CpG and IFA) stimulated significant secretion of IFN-γ by spleen cells after specific re-stimulation *in vitro* when compared with cells from non-immunized mice stimulated *in vitro* with CFP. Spleen cells from mice immunized with CFP/Control CpG and from non-immunized mice produced similar quantities of IFN-γ (Fig. 3a). The spleen cells of mice immunized with CFP/CpG or with CFP/Control CpG presently significantly greater secretion of IL-10 than did those of non-immunized mice. However, after antigen-specific re-stimulation, IL-10 concentrations were nearly three times higher in spleen cells obtained from CFP/IFA-immunized mice than in those obtained from CFP/CpG-immunized mice (Fig. 3b). The CFP/IFA-immunized mice also presented significantly higher levels of IL-5 than did CFP/CpG-immunized mice (Fig. 3c). Spleen cells from all groups of mice presently significantly greater secretion of all cytokines evaluated when stimulated with Con A compared to non-stimulated cells (data not shown).

The CFP/CpG vaccine stimulates an increase in local IFN-γ production

Because the CFP/CpG vaccine stimulated significantly higher levels of IFN-γ, as well as lower concentrations of IL-5 and IL-10 by spleen cells, we analysed cytokine production at the site of infection. The infection stimulated the production of significant concentrations of IFN-γ, IL-10, IL-12, and TGF-β, as evidenced by the fact that levels of these cytokines were higher in all infected mice than in non-immunized, non-infected mice (Fig. 4). The *ex vivo* levels of IFN-γ were significantly higher in the lungs of CFP/CpG-immunized mice than in those of CFP/IFA-immunized mice (Fig. 4a). It is interesting to note that CFP/IFA-immunized mice produced quantities of IFN-γ that were even lower than those seen in non-immunized, infected mice. All of the groups presented low levels of IL-5 (Fig. 4b). However, the levels of IL-5 detected in the lungs of CFP/CpG-immunized mice were significantly lower than those detected in the lungs of CFP/IFA-immunized mice. Very low levels of IL-4 were also detected in the lungs of the various experimental groups (data not shown). Levels of IL-10 (Fig. 4c) and IL-12 (data not shown) were not statistically different among the experimental groups. However, we found that TGF-β levels were significantly higher in the lungs of CFP/IFA-immunized mice than in those of CFP/CpG-immunized mice or in those of non-immunized mice (Fig. 4d).

Local TNF-α secretion is not affected by immunization with CFP/CpG or CFP/IFA

In parallel with our studies of the cytokines that regulate the immune response (IFN-γ, IL-5, IL-12, IL-10, and TGF-β), we also determined the levels of the inflammatory cytokines TNF-α and IL-6. Concentrations of TNF-α were found to be similar in the lungs of mice immunized with CFP/CpG and in those of mice immunized with CFP/IFA (Fig. 5a). These concentrations were significantly lower than those detected in nonimmunized, infected mice. Despite the fact that levels of IL-6 were lower in the lungs of CFP/CpG-immunized mice than in those of non-immunized, infected mice, they were significantly

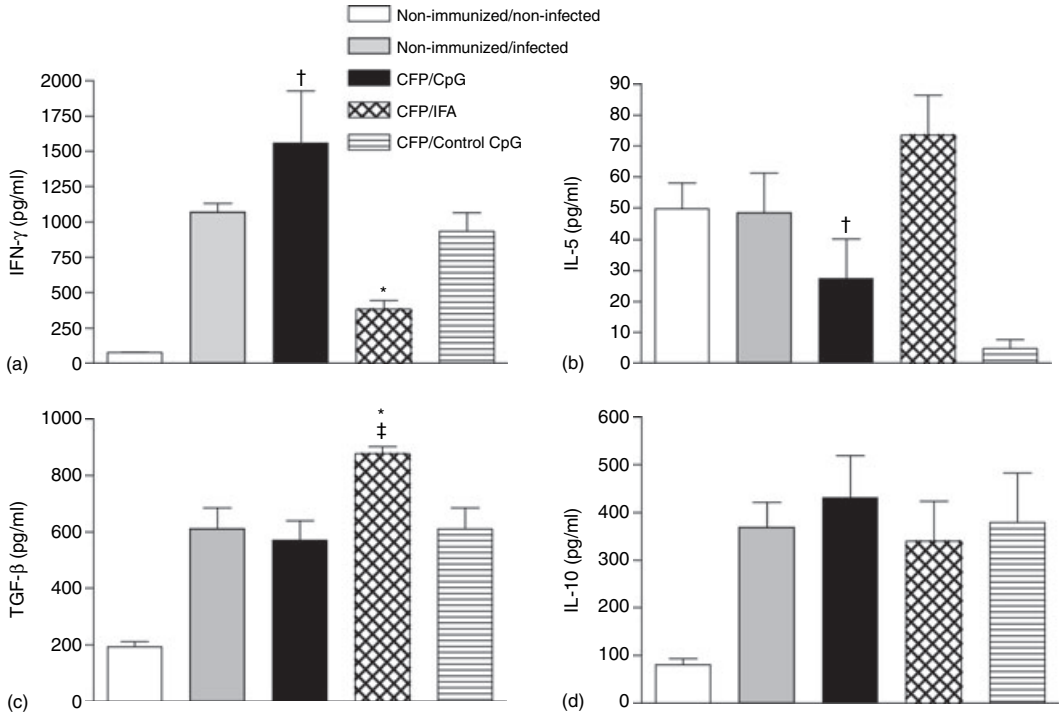

Figure 4. Cytokine detection in the lungs of mice. Animals were immunized and challenged as described in Fig. 1. At 70 days after challenge, local cytokine production was evaluated *ex vivo* in the lung homogenates. Results are expressed as mean ± standard deviation of cytokine concentrations individually analysed. *$P < 0{\cdot}05$ versus control non-immunized, infected mice; ‡$P < 0{\cdot}05$ versus the CFP/CpG group; †$P < 0{\cdot}05$ versus the CFP/IFA group. Results are representative of two identical experiments ($n = 5$).

higher than those found for CFP/IFA immunized mice (Fig. 5b). We also evaluated nitrite production in the lungs and found no significant differences among the groups studied (data not shown).

Mice immunized with CFP/CpG present extensive pulmonary injury

After 70 days of infection, non-immunized, infected mice presented pronounced pulmonary parenchyma involvement, characterized by isolated and confluent granulomas (Fig. 6b), whereas non-immunized, non-infected mice presented no such injury (Fig. 6a). The cell infiltrate consisted predominantly of macrophages, with minimal numbers of lymphocytes. The lungs of CFP/CpG-immunized mice presented extensive pulmonary injury characterized by the recruitment of neutrophils and areas of focal necrosis (Fig. 6c). However, in CFP/IFA-immunized mice, the pulmonary parenchyma was better preserved, the cell infiltrate was predominantly composed of peri-vascular/peri-bronchial lymphocytes, as well as containing a great number of macrophages, and there was no necrosis (Fig. 6d). Mice in the experimental control group (CFP/Control CpG) presented characteristics that were similar to those observed in non-immunized, infected mice (data not shown).

Discussion

The present study showed that the CFP/CpG subunit vaccine generated consistent Th1-biased cell immunity (significantly higher IFN-γ levels in the lung and spleen, together with significantly higher serum IgG2a levels). However, this was not accompanied by significantly efficacious protection. In addition, the lungs of CFP/CpG-immunized mice presented severe injury because of the extremely intense cell recruitment. The same antigen formulated in a different adjuvant (the CFP/IFA vaccine) induced a mixed response (significantly higher IL-5, IL-10 and IFN-γ levels in the spleen, increased TGF-β levels and lower levels of IFN-γ in the lungs, and significantly higher serum IgG1 levels), followed by a significant decrease in the bacterial load. The CFP/IFA-immunized mice exhibited extensive areas in which the pulmonary parenchyma was preserved.

It is relevant to mention that we delineated a vaccination protocol based on a large protection window (mice were challenged 60 days after the last immunization). Based on our previous finding that the numbers of $CD4^+$ and $CD8^+$ cells, IFN-γ, IL-12, TNF-α, monocyte chemoattractant-1, and IFN-inducible protein-10 are lower in the lungs of mice with advanced infection than in those of 30-day infected mice[8] we also choose to evaluate, in

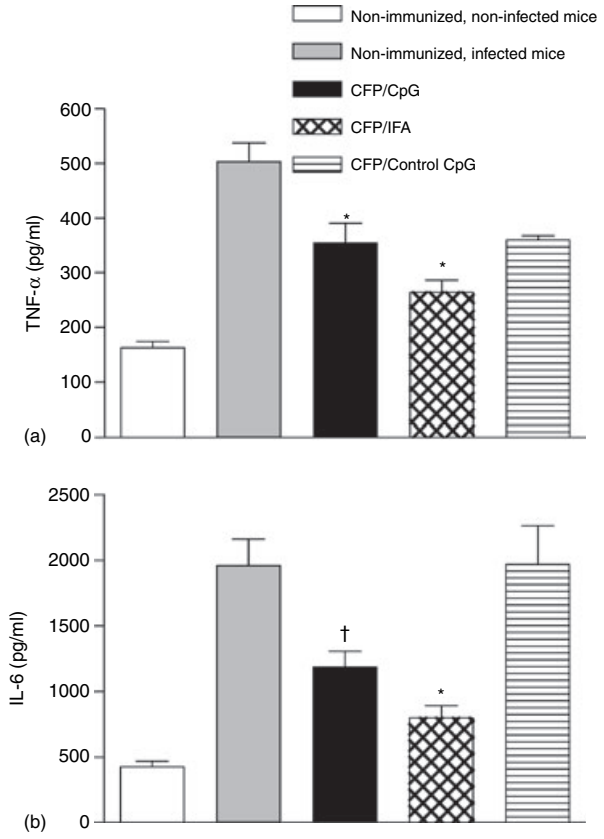

Figure 5. Inflammatory cytokines detection in the lungs of mice. Animals were immunized and challenged as described in Fig. 1. At 70 days after challenge, local cytokine production was evaluated *ex vivo* in the lung homogenates. Results are expressed as mean ± standard deviation of cytokine concentration individually analysed. *$P < 0{\cdot}05$ versus non-immunized, infected mice; †$P < 0{\cdot}05$ versus the CFP/IFA group. Results are representative of two identical experiments ($n = 5$).

the present study, the protective efficacy in the chronic phase of the experimental disease (70 days postinfection).

Because we found that CFP/CpG immunization did not provide significant protection against experimental disease, we reviewed the data in the literature and compared the various immunization protocols in terms of number of doses, CFP antigen concentration, adjuvant, interval between immunizations, protection window, route of challenge, and period chosen to evaluate the protective efficacy.[12,15,17,22,35,36] From our point of view, the most relevant variables were the protection window and the route of infection. The most common aspect was the evaluation of the efficacy of protection in the initial phase of the infection (2–3 weeks after challenge). Bearing this in mind, we believe that our experimental vaccination protocol might have generated protection if we had assayed protection in an earlier phase of the infection. However, the CFP/CpG vaccine was not found to be protective even when its efficacy was evaluated at 30 days after challenge (data not shown).

It is possible that the dose of CFP and CpG may influence the protective response. We determined the concentration of antigen and adjuvant based on the immune response induced by different concentrations of CFP and CpG (data not shown). In this experiment distinct concentrations of CFP and CpG modulated differently the immune response. We chose the dose that induced the strongest Th1 response. However, it might be possible that other concentrations used, which induced intermediate IFN-γ concentrations, may confer protection without tissue damage. Further studies are undergoing to evaluate this hypothesis.

Another possible explanation is that the short interval between the immunizations could induce the deletion of activated T-cell clones due to the excess antigen exposure. Although using a different antigen concentration (25 μg of CFP), adjuvant [dimethyl dioctadecyl ammonium bromide (DDA)] and route of infection (intravenous challenge), Lindblad *et al.* utilized the same interval used in the present study and also employed a large protection window.[22] The authors observed protection similar to that achieved with BCG immunization. We used 50 μg of CFP, 50 μg of CpG1826-ODN adjuvant and intratracheal challenge. Based on the results obtained by various authors using 20, 25, 30, 50 and 100 μg of CFP[12,15,22,35–37] we believe that the antigen concentration was not the differential in the efficacy of protection. Both adjuvants (DDA and CpG-ODN) induce a Th1 immune response.[23,38–40] Therefore, we suggest that the route of infection is a relevant aspect. Despite the high number of bacilli delivered via the intratracheal route of infection, they initially reach the lung, which is the site of primary infection. This route more closely mimics human infection than does the intravenous route, since the latter promotes primary infection in the spleen. We believe that the involvement of different cell populations and the compartmentalization of the immune response probably preclude the comparison of experimentally collected data.

Other mycobacterial antigens, such as the 27 000 MW and 19 000 MW lipoproteins, as well as the antigens 85 A and ESAT-6, harboured by adenylate cyclase *Bordetella pertussis* toxin[41–43] also induce responses similar to that reported here. Each of these vaccines had a deleterious effect on protection despite the induction of a strong Th1 response. Recently, Hsieh *et al.* reported that the addition of CpG-ODN to the CFP subunit vaccine formulated in DDA adjuvant failed to enhance the protective efficacy in a model of aerosol infection of C57BL/6 mice, despite increasing levels of IFN-γ.[44]

In an attempt to understand the lack of protection conferred by vaccine candidate antigens combined with adjuvants that are capable of inducing a favourable immune response, we analysed IL-4 expression in the lungs by immunohistochemistry (data not shown). Surprising, we found that the CFP/CpG vaccine also stimulated intense

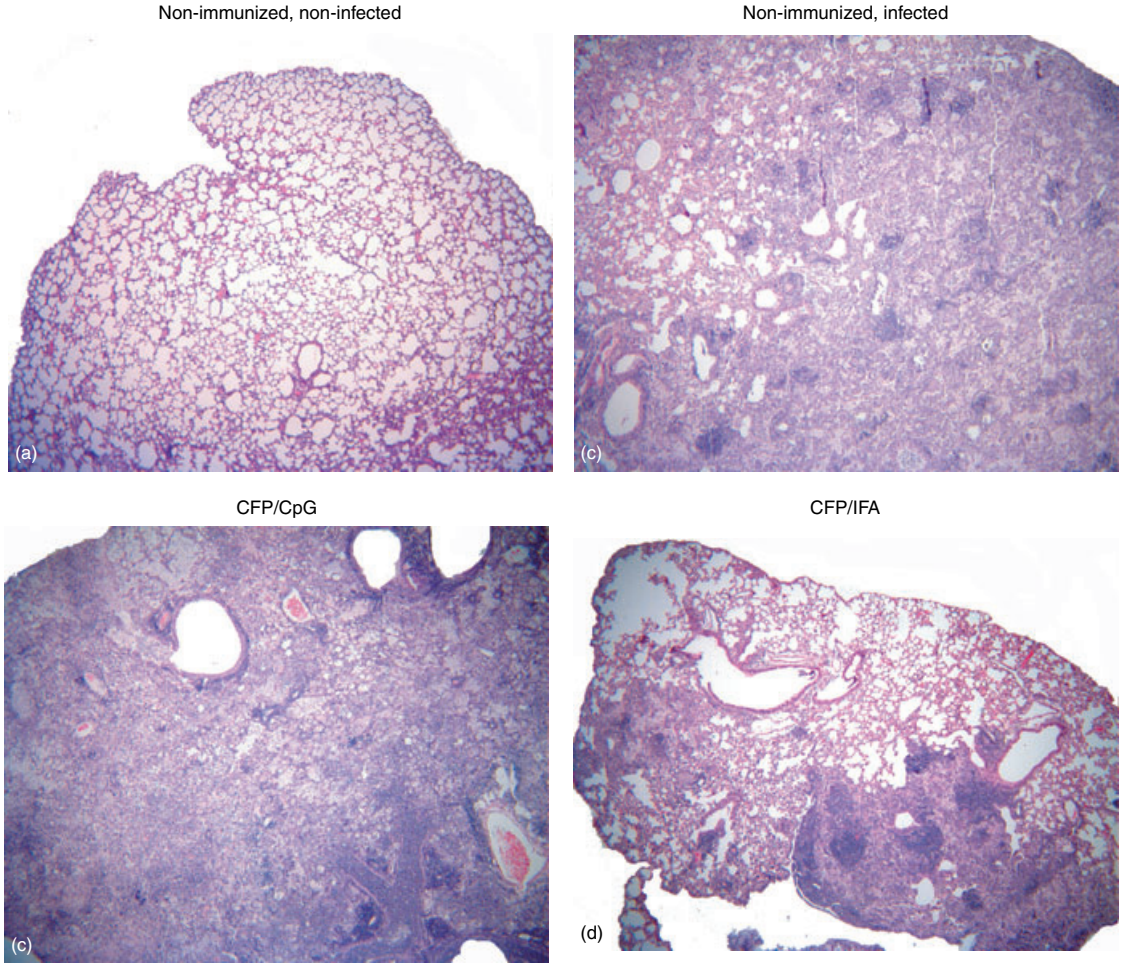

Figure 6. Histological representation of the lungs of mice. Lung sections from non-immunized (NI), non-infected mice (a), non-immunized, infected mice (b), CFP/CpG-immunized mice (c), and CFP/IFA-immunized mice (d). Magnification ×40.

local secretion of IL-4, more evidently after 70 days of infection than after 30 days of infection (data not shown). In the sections analysed, IL-4 levels increased in parallel with decreases in IFN-γ levels. Taken together, these findings indicate that the CFP/CpG vaccine indeed stimulates a Th1-biased immune response. However, it seems that antigens contained in the CFP fractions also strongly stimulate Th2 lymphocytes. Based on the results related to the IgG2a/IgG1 ratio and cytokine levels (in the spleen and lungs), we also suggest that the CFP/CpG vaccination protocol preferentially primes the cellular immune response. However, the magnitude of the Th1 response, characterized by IFN-γ levels in the spleen and lung, might not be the best or only parameter associated with protection against TB. According to Rook *et al. M. tuberculosis* is pathogenic, and progressive tuberculosis occurs because the potentially protective Th1 response is converted to an immunopathological response that fails to eliminate the bacteria.[45–48] Those authors proposed that the Th1 response is ineffective in controlling disease because it is corrupted by IL-4 production. Human TB is accompanied by increased expression of IL-4 by $CD4^+$ and $CD8^+$ T cells, and IL-4 production correlates significantly with cavitation and disease severity.[49,50] In experimental murine TB, the presence of at least 10% of IL-4-producing cells exacerbates the toxic effects of TNF-α, including fibrosis and necrosis.[47,51,52] Our results show that, despite the similar levels of TNF-α in the lungs of mice in all experimental groups, those immunized with CFP/CpG developed a deleterious response, which could be associated with intense production of IL-4 in lungs (data not shown). Further experiments are undergoing to evaluate the deleterious role of IL-4 production in CFP/CpG immunized mice.

The data presented here, initially obtained with the aim of studying an alternative prophylactic intervention against TB, could help us review the concepts previously established related to the protective immune response against *M. tuberculosis*. Immunological assays used to evaluate the response to *M. tuberculosis* after immunization and challenge should include the simultaneous analysis of various immunological parameters (effector $CD4^+$ T

cells, effector $CD8^+$ T cells, memory T cells, production of IL-12, production of antigen-specific IFN-γ, as well as production of cytokines that suppress Th1 response and of anti-inflammatory cytokines, such as IL-4, IL-10, and TGF-β). Taken together, simultaneous evaluation of diverse immunological parameters could provide a more realistic view of the *M. tuberculosis*–host relationship.

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