Neonatal BCG Immunization Followed by DNAhsp65 Boosters: Highly Immunogenic but not Protective Against Tuberculosis – a Paradoxical Effect of the Vector?

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Abstract

A new tuberculosis vaccine is urgently needed. Prime-boost strategies are considered very promising and the inclusion of BCG is highly desirable. In this investigation, we tested the protective efficacy of BCG delivered in the neonatal period followed by boosters in the adult phase with a DNA vaccine containing the hsp65 gene from *Mycobacterium leprae* (pVAXhsp65). Immune responses were characterized by serum anti-hsp65 antibody levels and IFN-γ and IL-5 production by the spleen. Amounts of these cytokines were also determined in lung homogenates. Protective efficacy was established by the number of colony-forming units (CFU) and histopathological analysis of the lungs after challenge with *Mycobacterium tuberculosis*. Immunization with BCG alone triggered a significant reduction of CFU in the lungs and also clearly preserved the pulmonary parenchyma. BCG priming also increased the immunogenicity of pVAXhsp65. However, boosters with pVAXhsp65 or the empty vector abolished the protective efficacy of BCG. Also, higher IL-5 levels were produced by spleen and lungs after DNA boosters. These results demonstrated that neonatal BCG immunization followed by DNAhsp65 boosters is highly immunogenic but is not protective against tuberculosis.

Introduction

One-third of the world's population is infected with *Mycobacterium tuberculosis* and the disease caused by this pathogen is the leading cause of death from an infectious disease [1]. Factors such as co-infection with human immunodeficiency virus and emergence of drug resistance in *M. tuberculosis* strains added more burden to tuberculosis (TB) treatment [2]. The only licensed vaccine against TB is the attenuated *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) that is recommended by the World Health Organization for all infants under 1 year of age. Around 100 million newborn children are given this vaccine and the global vaccine coverage is estimated to be 80% [3]. In spite of this extensive use, large numbers of well-documented trials showed extensive variation, from 0 to 80%, in BCG protective efficacy [4]. This has been attributed to variability in BCG vaccine strains, to expo-

sure to environmental mycobacteria as well as host genetic characteristics [5, 6]. Although the overall efficacy is low, most studies agree that BCG protects against disseminated disease in newborns and children but that this immunity wanes with age, resulting in no or insufficient protection against adult pulmonary TB [7, 8]. In this context, there is great interest in the development of new vaccines against tuberculosis. A number of alternative living and non-living putative TB vaccines are being studied and discussed by many authors [9, 10]. Experimental evidences have indicated that DNA vaccines, because of their ability to induce a strong Th1 type of response, could contribute to TB control. DNA constructs encoding mycobacterial antigens such as the 65-kDa heat shock protein (hsp65), Ag85A, Ag85B and PstS3 induced significant protective immunity [11, 12]. In addition, attempts to improve BCG by administering lower doses, by enhancing viability of BCG through

improved lyophilization procedures, oral delivery and prime-boost protocols are being explored [13–15]. Safe and effective heterologous prime-boost regimens, which boosts or augments BCG or rBCG, is being considered the most realistic strategy for future TB control through immunization [8].

As a new TB vaccine will be administered to human neonates, it must be realized that neonates and young infants, as well as newborns from numerous animal species including mice, show particular limitations in generating protective immune responses. Neonatal murine immunization models using conventional vaccine antigens (measles, tetanus toxoid) in BALB/c mice, gave responses similar to those found early in human infants. Early life B cell responses generally resulted in a slower and weaker increase of vaccine antibodies compared with adult mice. Furthermore, analyses of T-cell responses to these conventional vaccines indicated that early life T cell differentiation was preferentially polarized towards a Th2 pattern [16]. As a consequence of this Th2 bias, there is a deficiency in the induction of IFN-γ, TNF-α and cytotoxic T-cell responses that are essential for protection against many intracellular pathogens, including *M. tuberculosis*.

Researchers from our group previously demonstrated that a DNA plasmid encoding the *Mycobacterium leprae* 65-kDa heat shock protein displayed both prophylactic and therapeutic effects in a murine model of tuberculosis [17, 18]. As these experiments were all performed with adult BALB/c mice, we investigated the protective efficacy of a prime-boost strategy initiated at the neonatal period. Neonate mice were primed with BCG and boosted in the adult phase with a DNA vaccine containing the hsp65 gene from *M. leprae* (pVAXhsp65). Even though priming with BCG determined an increased immune response to pVAXhsp65, this strategy decreased the protective effect of BCG.

Materials and methods

Animals. BALB/c mice were bred in the Animal Facility of São Paulo State University (UNESP), at the Biosciences Institute, and were used at 5 days (neonates) or 4 weeks of age (young adults). The animals were fed on a standard pellet diet and water *ad libitum*. The animal protocols used in this work were approved by the local ethical committee according to the guidelines adopted by the Brazilian College of Animal Experimentation.

Experimental design. Neonate BALB/c mice were immunized with BCG and boosted in the adult phase with two doses of pVAXhsp65 (BCG/vaccine group). The following control groups were included: BCG (neonate mice immunized with BCG only), BCG-vector (neonate mice immunized with BCG and boosted later with the empty vector), PBS/vector and PBS/vaccine (mice injected in the adult phase with two doses of the vector

or the vaccine, respectively). Two weeks after the last DNA dose, the animals were either sacrificed and used for immune response analysis or challenged with 10^5 CFU of *M. tuberculosis* H37Rv by the intratracheal route to analyse the protective efficacy of the vaccination strategy. Anti-hsp65 antibodies were evaluated in individual sera and cytokines (IFN-γ and IL-5) were quantified in spleen cell culture supernatants and also in lung homogenates. Vaccination efficacy was evaluated by CFU determination and histopathological lung analysis.

Genetic vaccine construction. The vaccine pVAXhsp65 was derived from the pVAX vector (Invitrogen®, Carlsbad, CA, USA), previously digested with *Bam*HI and *Not*I (Gibco BRL, Gaithersburg, MD, USA) by inserting a 3.3-kb fragment corresponding to the *M. leprae* hsp65 gene and the CMV intron A. The empty pVAX vector was used for control. DH5α *Escherichia coli* transformed with plasmid pVAX or the plasmid carrying the hsp65 gene (pVAXhsp65) was cultured in LB liquid medium (Gibco BRL) containing ampicillin (100 μg/ml). The plasmids were purified using the Concert High Purity Maxiprep System (Gibco BRL). Plasmid concentrations were determined by spectrophotometry at $\lambda = 260$ and 280 nm by using the Gene Quant II apparatus (Pharmacia Biotech, Buckinghamshire, UK).

Immunization procedures. Neonate mice were injected with BCG of the Moreau strain (10^5 bacilli) administered by the intradermal route at the base of the tail. Boosters in the adult phase were administered with pVAXhsp65 (100 μg/100 μl) in 25% saccharose delivered in the quadriceps muscle. Two pVAXhsp65 doses were administered at 15 days interval. Control animals received BCG during the neonatal period or two DNA doses (pVAXhsp65 or pVAX) in the adult phase.

Anti-hsp65 antibody levels. Sera were collected by retro-orbital bleeding 2 weeks after the last DNA dose and anti-hsp65 specific antibody levels were evaluated by enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well plates (Maxisorp Nunc-Immuno plates) were coated with 0.1 ml of purified recombinant hsp65 (5 μg/ml) in coating solution (14.3 mM Na_2CO_3, 10.3 mM $NaHCO_3$, pH 9.6), incubated at 4 °C overnight and then blocked with 10% fetal calf serum (FCS) in PBS for 60 min at 37 °C. Diluted serum samples (1:25) were tested. After incubation for 2 h at 37 °C, monoclonal antibody rat anti-mouse IgG1 and IgG2a biotinylated conjugates (A85-1 and R19-15, respectively, PharMingen), were added for detection of specific antibodies. After washing, plates were incubated at room temperature for 30 min with StreptAB kit (Dako, Carpinteria, CA, USA) and then revealed by adding H_2O_2 + OPD. Colour development was stopped by using H_2SO_4, and optical density was measured at 492 nm.

CFU determination. Four weeks after infection with *M. tuberculosis* the bacterial load in the lungs was

evaluated. Briefly, the organs were washed with sterile PBS and each one 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 °C 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. Serial dilutions were plated on supplemented 7H10 agar medium. Colonies were counted 28 days after incubation at 37 °C.

Histopathological analysis. The upper left lobe of each mouse lung was fixed in 10% formalin, washed in PBS for 24 h, dehydrated in a graded ethanol series, embedded in glycol metacrylate resin (Historesin Embedding Kit; Leica, Heidelberg, Germany) and then sectioned for light microscopy. Sections (3 μm each) were stained with haematoxylin & eosin. The sections were analysed using a Leica DMLB 80 microscope connected to a Leica DC300FX camera.

Cytokine evaluation. Two weeks after the last DNA dose, animals were sacrificed and splenic cells were collected and adjusted to 5×10^6 cells/ml in RPMI 1640 medium supplemented with 5% FCS, 20 mM glutamine and 40 IU/l of gentamicin. The cells were cultured in 48-well flat-bottomed culture plates (Nunc, Life Tech. Inc., Maryland, MA, US) in the presence of 40 μg/ml of Concanavalin A (ConA). Cytokine levels in culture supernatants were evaluated 48 h later by ELISA. Cytokines were measured according to the manufacturer's instructions (PharMingen). Briefly, purified monoclonal antibodies, anti-IFN-γ (R4-6A2) and anti-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. Cytokines were also evaluated in the lung homogenates 4 weeks after challenge with *M. tuberculosis*, i.e, at the same time as CFU were determined in the lungs.

Statistical analysis. All figures express the mean ± SEM for each variable. Statistical analysis was performed using unpaired *t*-test. Values of $P < 0.05$ were considered statistically significant.

Results

Neonatal BCG immunization primes for a strong immune response to pVAXhsp65 in the adult phase

Immunization of neonate BALB/c mice (5 days old) with BCG did not induce anti-hsp65 antibody production (not shown). Administration of two pVAXhsp65 doses to adult mice by the intramuscular route induced significant levels of antigen-specific IgG1 and IgG2a. Both isotype levels were significantly increased if the animals were previously primed with BCG in the neonatal period. Increased levels of IgG1 and IgG2a can be observed in Fig. 1A and B, respectively. Inoculation of the empty vector (control) did not induce anti-hsp65 antibodies in animals previously primed or not with BCG.

Neonatal BCG immunization followed by DNAhsp65 boosters in the adult phase is not protective in experimental tuberculosis

Immunization with BCG in the neonatal period induced protective immunity that remained during adult life. This was clearly demonstrated by a significant decrease in the number of CFU in the lungs of these animals, 30 days after challenge with *M. tuberculosis*. However, the animals that received two boosters with pVAXhsp65 in
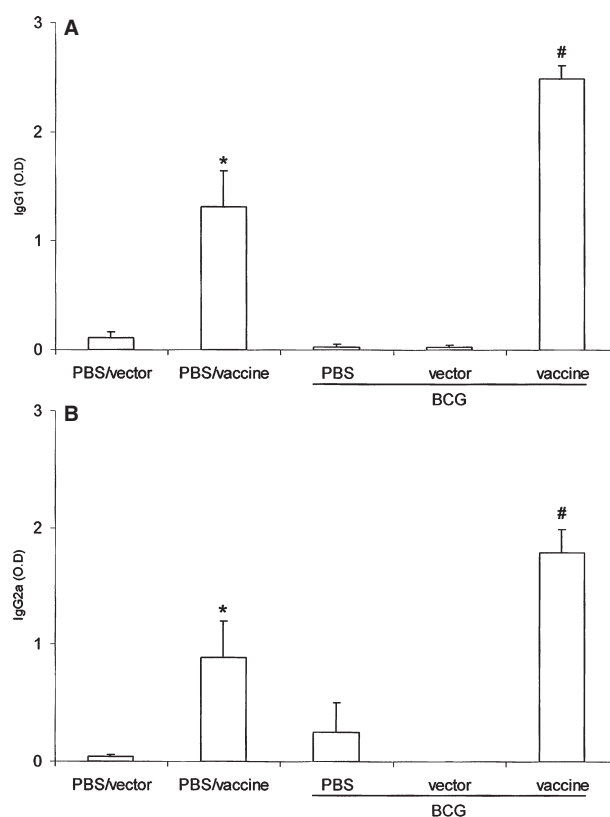

Figure 1 Effect of BCG priming on antibody induction by pVAXhsp65 immunization. Neonate mice (5 days old) were immunized in the base of the tail with BCG (10^5 bacilli). Four weeks later, in the adult phase, they received two pVAXhsp65 doses, delivered 2 weeks apart. Two weeks after last DNA dose, IgG1 (A) and IgG2a (B) anti-hsp65 antibody levels were determined by ELISA in blood samples diluted 1:25. Results are presented as mean ± SEM from 5 to 8 mice per group. *$P < 0.05$ in comparison with corresponding control (PBS/Vector); #$P < 0.05$ in comparison with PBS/Vaccine.

the adult phase lost this protective immunity. Interestingly, the empty vector (pVAX) triggered a similar deleterious effect, abolishing the protective ability of BCG. These results are shown in Fig. 2A. The control group that was inoculated with two DNAhsp65 doses in the adult phase showed no protection (non-immunized control: 5.88 log CFU per lung; two DNAhsp65 doses: 6.14 log CFU per lung). Lung histopathological analysis showed a very well preserved parenchyma in BCG-vaccinated mice (Fig. 2D), very similar to the structure found in normal animals (Fig. 2B). As expected, infected non-immunized control animals showed a striking alteration of the parenchyma characterized by intense inflammatory infiltrates dominated by mononuclear cells (Fig. 2C). In animals that received DNAhsp65 boosters, the parenchyma was similar to the control group (infected and without previous immunization) i.e., presented a clear and abundant inflammatory process (Fig. 2E).

Cytokine modulation by DNA in mice primed with BCG in the neonatal period

This aspect was initially evaluated by production of IFN-γ and IL-5 in spleen cell cultures from immunized animals stimulated with ConA and later, in mice challenged with mycobacteria, by quantification of these two cytokines in lung homogenates. Administration of pVAXhsp65, or the control vector, to mice previously immunized with BCG did not affect their ability to produce IFN-γ in response to ConA stimulation. However, this procedure clearly increased IL-5 production of spleen cells stimulated with ConA. These results can be
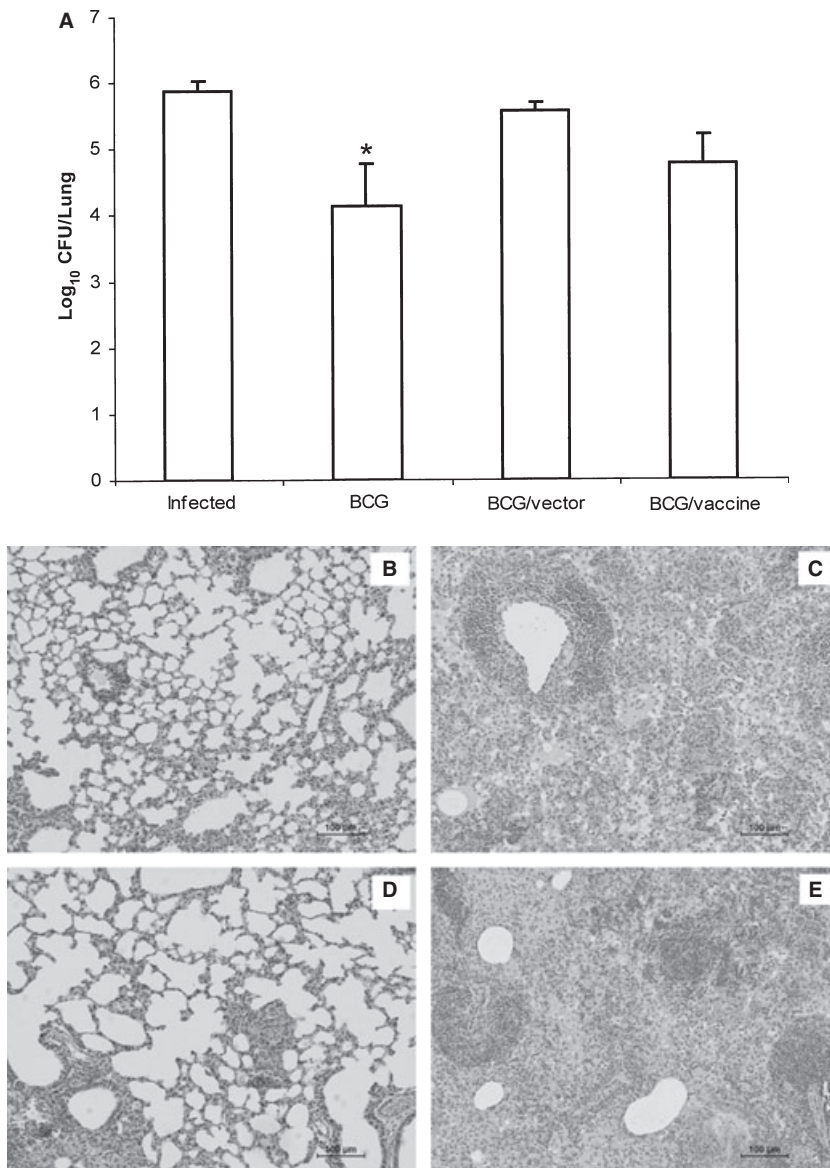
Figure 2 Effect of pVAXhsp65 booster on the protective efficacy of BCG. Neonate mice (5 days old) were immunized in the base of the tail with BCG (10^5 bacilli). Four weeks later, in the adult phase, they received two pVAXhsp65 doses, delivered 2 weeks apart. Two weeks after last DNAhsp65 dose the animals were infected with 10^5 CFU of *M tuberculosis* H37Rv by intratracheal route. Four weeks later, the number of live bacteria in the lungs was determined and expressed as Log10 CFU/lung (A) and the lung parenchyma structure was analysed in H&E stained lung sections: normal mice (B); non-immunized infected mice (C); BCG immunized infected mice (D); BCG/pVAXhsp65 immunized infected mice (E). CFU results are presented as mean ± SEM from 4 to 7 mice. *$P < 0.05$ in comparison with non-immunized control.

observed in Fig. 3A and C, respectively. No differences were observed in the levels of IFN-γ in the lung homogenates (Fig. 3B). However, similar to the results found in the spleen, higher IL-5 levels were found in lung homogenates from groups that received DNA boosters (pVAXhsp65 and pVAX) after BCG immunization in comparison with BCG alone (Fig. 3D). Levels of IFN-γ and IL-5 produced by spleen cell cultures (3850 and 240 pg/ml, respectively) and present in lung homogenates (5500 and 245 pg/ml, respectively) in mice immunized with two DNAhsp65 doses were similar to their respective controls.

Discussion

In the current study, by using an experimental tuberculosis model, we investigated the immunogenicity and the protective efficacy of a prime-boost strategy employing BCG and pVAXhsp65. Neonate mice were initially primed with BCG and then, in the adult phase, boosted twice with a DNA vaccine containing the hsp65 gene from *M. leprae* (pVAXhsp65). Animals immunized only with pVAXhsp65 produced both, IgG2a and IgG1 anti-hsp65 antibodies, suggesting that this vaccine was inducing a mixed pattern (Th1/Th2) of response. This assumption was based on literature reports that correlate IgG2a and IgG1 with the predominance of Th1 and Th2 responses, respectively. Priming with BCG in the neonatal period, before administration of the DNA vaccine, did not change this mixed profile but clearly improved immunogenicity, determining a significant increase in the production of both isotypes. In the light of these observations, our results suggested that BCG priming in the neonatal period was not skewing the immune response to Th1 or Th2 but instead, it was simply priming for a robust immune response later in the adult phase.

From previous experience with this genetic vaccine, we know that the immunity induced by two doses delivered by the intramuscular route is not protective in experimental tuberculosis. Based on this and the fact that BCG increased pVAXhsp65 immunogenicity, we then tested the protective efficacy of BCG / pVAXhsp65 association. Neonatal BCG vaccination, as was expected from the literature reports, was able to protect the mice challenged with *M. tuberculosis* by the intratracheal route. This protection was characterized by a significant reduction in the number of CFU in animals that were previously vaccinated with BCG. Histopathological analysis also sustained BCG protective ability by showing a very well-preserved lung parenchyma with no signs of inflammation. However, animals that received boosters with pVAXhsp65 or with the empty vector itself in the adult phase lost the protective immunity determined by BCG against *M. tuberculosis*. This was established by the recovery of similar CFU numbers in the lungs of these animals and the non-immunized ones. Also, the lung parenchyma in both, vector and vaccine-boosted animals, was similarly affected, i.e., they presented clear inflammatory infiltrates.
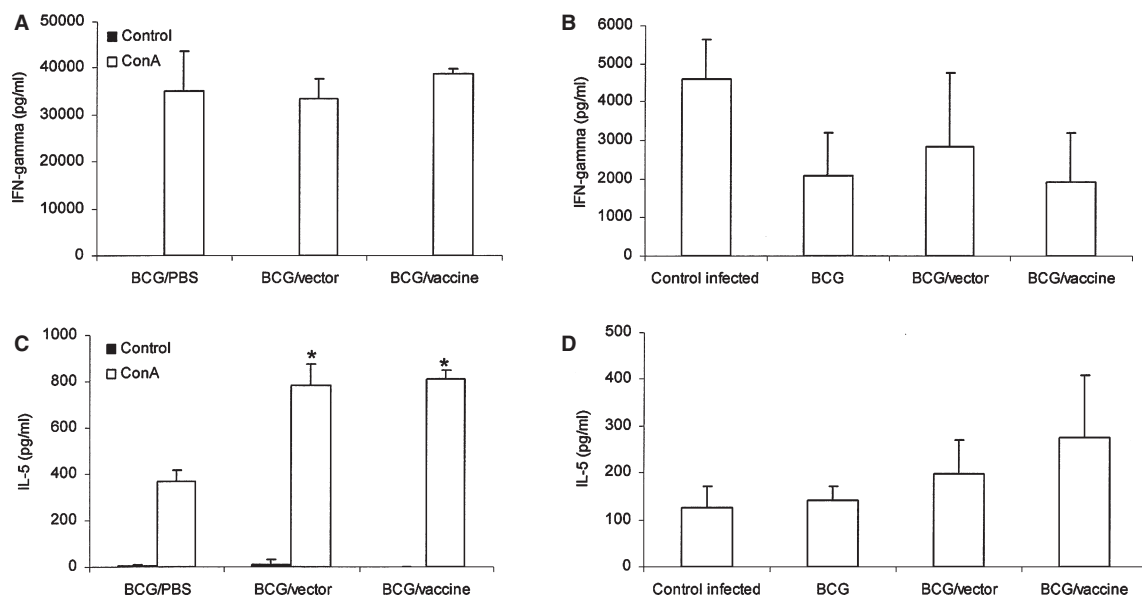

Figure 3 Boosters with DNA (pVAX or pVAXhsp65) increase IL-5 production in mice neonatally primed with BCG. Neonate mice were immunized with BCG and boosted, in the adult phase, with two DNA doses (pVAX or pVAXhsp65). Two weeks after last DNA dose, the animals were tested for IFN-γ (3A) and IL-5 (3C) production by splenic cells or infected with *M. tuberculosis*. Four weeks after infection, the animals were sacrificed and concentration of IFN-γ (3B) and IL-5 (3D) was determined in lung homogenates. Results are presented as mean ± SEM from 4 to 8 mice. *$P < 0.05$ in comparison with BCG/PBS group.

A direct comparison of our results and other publications is hampered by the diversity of prime-boost protocols employed to evaluate protection in tuberculosis. The work of Skinner et al. [19], for example, differently from our findings, showed that neonatal calves primed at birth with BCG vaccine and later boosted with DNA vaccines containing hsp65, hsp70 or Apa, showed enhanced protection against bovine tuberculosis. Even though some authors alerted for the relevance of the immunization sequence [20], Skinner et al. [19], observed that this aspect was not critical. Similarly to our results, Majlessi et al. [21] described increased immunogenicity but not increased protection by using a prime-boost protocol.

An attractive possibility to explain the failure of this prime-boost protocol is that BCG is determining both, priming for hsp immune response and also providing a mixed (Th1 + Th2) microenvironment for the ensuing immune response. In this scenario, the immune response induced by pVAXhsp65 vaccination would be a mixed and strong immune response against hsp65. The antibody results observed in this work point to this possibility and it is also highly supported by various reports. Qazi et al. [22] recently published that BCG primed the immune system for the induction of immunity against a variety of heat shock proteins (hsp) including hsp65. Interestingly, analysis of immunoglobulin isotype profiles and IFN-γ production induced against hsps revealed induction of a mixed Th1 / Th2 response. The literature also supports the view that BCG immunization equally establishes mixed Th1/ Th2 microenvironments in adult and neonate mice [23]. Interestingly, although BCG induced a potent Th1-type response to mycobacterial antigens, it promoted the production of both Th1- and Th2-type cytokines in response to unrelated vaccines [24]. Another possibility that needs to be considered relates to the BCG dose used to prime the neonate mice. As BCG dose defines the Th1 / Th2 nature of the immune response [25], it would be very convenient to evaluate the effect of lower BCG doses in this protocol. As prime-boost strategies represent a relatively new approach in vaccinology, factors as interval between priming and boosting must also be considered, as has been recently discussed [26, 27]. Another interesting explanation for this failure is the possible induction of T regulatory cells by a combination of BCG with DNAhsp65. Both BCG and hsp65 have been described as potent immunomodulators to control inflammation associated with allergy and autoimmune diseases [28, 29]. In support of this hypothesis, we recently observed that the genetic vaccine containing the hsp65 gene from M. leprae was able to control inflammation in experimental diabetes [30, 31].

One of the most intriguing findings of this investigation was the fact that both, vector and vaccine, equally decreased BCG protective ability. This strongly indicates that non-coding sequences of the vector were somehow interfering with the immunity established by BCG vaccination. To try to explain the mechanism involved in this deleterious effect triggered by DNA inoculation, we compared the cytokine profile in BCG and BCG / DNA experimental groups. Splenic cells from BCG, BCG / vector and BCG / vaccine groups produced similar IFN-γ levels after ConA stimulation. However, groups boosted with pVAX or pVAXhsp65 produced significantly higher IL-5 amounts. Interestingly, lung homogenates from BCG/vector and BCG/vaccine groups also showed higher IL-5 levels. Even though these results are not completely enlightening, they suggest that the vector itself is deleteriously modulating the protective immunity established by BCG. It is well accepted that tuberculosis control requires a Th1 type of response which can be compromised by a Th2 response [32, 33]. We suggest, therefore, that the vector is somehow accentuating the Th2 profile. This possibility is, at least partially, supported by literature reports. For example, unmethylated CpG motifs that are prevalent in bacteria, including in bacterial plasmids used as vectors for genetic vaccines, are able to enhance both innate and specific (humoral and cellular) immunity via TLRs [34]. It is also described that oligodeoxynucleotides lacking CpG dinucleotides mediate Toll-like receptor 9 dependent Th2 biased immuno stimulation [35]. Even though the CpG content of plasmid vectors is not completely described, we and other authors showed evidences that non-coding plasmid DNA was able to inhibit autoimmunity processes mediated by Th1 cells in experimental diabetes and encephalomyelitis [31, 36–38].

In conclusion, our results demonstrated that priming of newborn mice with BCG followed by pVAXhsp65 boosters later in adult life significantly increased immunogenicity of this genetic vaccine. However, this kind of strategy did not determine protection in experimental tuberculosis. The results also showed that boosters with both, pVAXhsp65 and pVAX (empty vector) abrogated the BCG protective ability. Further studies are necessary to elucidate this negative effect mediated by the vector itself. This investigation would allow elimination of this negative effect and probably to reveal the possible positive role of vaccine in a similar prime-boost strategy.

Acknowledgment

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