Vaccine 32 (2014) 4324–4332

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Microstructured liposome subunit vaccines reduce lung inflammation and bacterial load after *Mycobacterium tuberculosis* infection

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ARTICLE INFO

Article history:
Received 16 December 2013
Received in revised form 4 May 2014
Accepted 6 June 2014
Available online 19 June 2014

Keywords:
Vaccine
Tuberculosis
Liposome
Adjuvants
Latent antigens
HspX

ABSTRACT

Background: Tuberculosis is a disease affecting millions of people throughout the world. One of the main problems in controlling the disease is the low efficacy of the Bacillus Calmette–Guérin (BCG) vaccine in protecting young adults. The development of new vaccines that induce a long-lasting immune response or that stimulate the immunity induced by BCG may improve the control of tuberculosis.
Methods: The use of microstructured liposomes containing HspX, with or without MPL or CpG DNA adjuvants, as vaccines for tuberculosis was evaluated. The HspX-specific humoral and cellular immune responses to the different vaccine formulations were compared.
Results: All vaccines containing liposome microparticles and HspX were immunogenic. Vaccines formulated with CpG DNA and HspX induced the strongest humoral and cellular immune responses, mainly by inducing interferon-γ and tumor necrosis factor-α expression by both $CD4^+$ and $CD8^+$ T cells. HspX and MPL mainly induced $CD8^+$ T-cell activation and specific humoral responses. When evaluated the protective efficacy of the formulations against *Mycobacterium tuberculosis* challenge, the microstructured liposome containing L-HspX and L-HspX-CPG DNA reduced both lung inflammatory lesions and the bacterial load.
Conclusion: We have thus demonstrated, for the first time, the use of microstructured liposomes as an adjuvant and delivery system for a vaccine formulation against tuberculosis.

1. Introduction

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (*Mtb*). It is a worldwide public health problem, and was responsible for 1.4 million deaths in 2011 [1]. It is estimated that one third of the world's population is infected with this bacillus and about 5–10% of infected people will develop active TB during their lives [1]. The Bacillus Calmette-Guérin (BCG) is a live attenuated vaccine in use for TB control, and although it effectively protects children from meningitis and milliary TB, its protection of young adults is highly variable [2]. Furthermore, BCG does not prevent the reactivation of latent TB [3]. Consequently, the development of improved vaccines that generate long-lasting protective immune responses or that boost BCG immunity is crucial [1,4,5]. Despite the absence of a consensual protective immune response desired by an ideal vaccine to TB, the most accepted parameters are those based on the control of active TB [6]. Consequently T cells producing IFN-γ and TNF-α have been evaluated in vaccination studies [7–9].

Several approaches have been used to generate subunit vaccines against TB, such as Mtb72F/AS02A, a fusion of the Mtb39a and Mtb32a *Mtb* antigens and the adjuvant AS02A that predominantly induces Th1 immune responses [10]. This candidate vaccine is in a phase II clinical trial and was developed to enhance the preexisting BCG immune response [11]. Another example is the vaccine Hybrid 1 (IC31), which combines *Mtb* antigens Ag85b and ESAT-6 with the adjuvant IC31 in CpG-DNA-containing cationic peptide [12,13]. Although they induce good immune responses, these vaccines

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http://dx.doi.org/10.1016/j.vaccine.2014.06.037
0264-410X/

target antigens that are not expressed by *Mtb* during its latent period [14].

In spite of the fact that no animal model for latency in TB is available, studies that address antigens produced during latency are of relevancy. Therefore, the incorporation of *Mtb* latent-phase-related antigens in new vaccines should generate vaccines that are directed against latent TB (LTB) [15–18], a condition that affects one third of the world's population [1]. HspX (α-crystallin or 16-kDa antigen) is a protein highly expressed by *Mtb* under stress conditions, including hypoxia, nutrient scarcity, and high levels of nitric oxide produced by macrophages [19]. This antigen has been shown to induce a strong immune response in mice and individuals with LTB [20–25].

Few subunit vaccines composed of HspX have been tested in animal models. Geluk et al. [23] showed that polyepitopes that induce $CD8^+$ T-lymphocytes responses were both immunogenic and antigenic. The association of HspX and other *Mtb* proteins has been studied intensively, and has shown that when HspX is combined or fused with other recombinant *Mtb* antigens, it retains its capacity to induce a specific immune response [7,26–28]. The main differences between those studies were that the vaccine formulations used various adjuvants to induce humoral [26] and cellular [26–28] immune response.

Subunit-protein-based vaccines include adjuvants, which are modulators of the immune response that play crucial roles in orchestrating the quality and type of immune responses induced [29]. Although several approaches have used HspX as the antigen, few studies have compared or evaluated the adjuvants or antigen carriers involved in the protective effects achieved with this protein. One of the adjuvants used is CpG DNA [26], which is recognized by the Toll-like receptor 9 (TLR9) pathway that activate B cell, dendritic cell (DC), and monocyte cell responses [30]. Another adjuvant in use is monophosphoryl lipid A (MPL), which is recognized by the TLR4 pathway and induces the production of Interleukin-12 sub-unit p70 (IL12p70), and consequently preferentially induces a cellular immune response with a Th1 phenotype [31]. Although these formulations have shown various degrees of protection against TB, improvement is still required so they can be moved on to clinical trials.

The use of liposomes in vaccine formulations against TB has shown good results [15,32]. They have the advantage of being easily formulated, with low levels of toxicity and immunogenicity [33–35]. However, the use of liposome formulations containing LTB-related antigens is still rare. The construction of liposomes into nanoparticles seems to be a promising strategy for vaccine development, and cationic liposomes, such as CAF01, have been used against TB [15,32]. The size of the particles directly influences the immune response induced, although this is still controversial [36–38]. Liposomes can vary in sizes ranging from 0.05 to 10 μm, as well as in the number of lipid layers (unilamellar or multilamellar vesicles). Liposomes used in vaccines shown to confer protection to TB were in the nanoscale (smaller than 1 μm) [36–38]. To our knowledge, liposomes with larger sizes such as the size of TB bacilli have not been used. These data, among others, prompted us to test whether microstructured liposomes with a size similar to that of the *Mtb* bacillus, associated with cellular-response-inducing adjuvants and HspX, would induce an effective and protective immune response against *Mtb*.

Therefore, the aim of this study was to evaluate whether microstructured liposomes can influence the humoral and cellular immune response in mice, using different vaccine formulations containing HspX, with or without the known adjuvants CpG DNA and MPL. We found that HspX-containing microstructured liposomes were immunogenic, and consequently directly influenced the immune response induced. The vaccine formulations containing the CpG DNA adjuvant predominantly induced specific humoral and cellular responses, including interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) production by both $CD4^+$ and $CD8^+$ T lymphocytes. The vaccine composed of HspX and MPL generated a humoral immune response and predominantly activated $CD8^+$ T lymphocytes. The vaccines that best reduced the pulmonary lesions and the pulmonary bacterial load induced by *Mtb* infection were composed of L-HspX and L-HspX-CpG DNA, the latter inducing stronger specific immune response. Therefore, microstructured liposomes composed of HspX and CpG DNA constitute a promising vaccine.

2. Materials and methods

2.1. Animals

Female specific-pathogen-free BALB/c mice aged 6–8 weeks, obtained from the Centro Multidisciplinar para investigação Biológica na Área da Ciência em Animais de Laboratório (CEMIB)—Unicamp—Campinas-Brazil, were maintained in micro isolators attached to HEPA-filtered racks for air intake and exhaustion. All animals were maintained according to the guidelines of Colégio Brasileiro Experimentação Animal (COBEA). The protocol was approved by the Comitê de Ética de Experimento Animal of Universidade Federal de Goiás (CEP-UFG; protocol number: 229/11).

2.2. Recombinant HspX protein production

The plasmid encoding the HspX (Rv2031c) antigen was provided by the Colorado State University (contract no. NO1-Al-75320) and the recombinant protein was expressed in *Escherichia coli* and purified according to CSU protocol SOP: RP021. Level of LPS contamination was confirmed to be lower than 10ηg endotoxin/mg of protein [39].

2.3. Preparation and characterization of microstructured liposomes

Microstructured liposomes containing HspX were prepared by the lipid-film hydration method [40]. In brief, phosphatidylcholine (PC) was dissolved in chloroform and placed in a round-bottomed glass tube. To obtain a thin dry lipid film, the organic solvent was removed in a nitrogen atmosphere. The flask was kept under vacuum for 24 h to ensure the complete removal of residual solvent. The dry lipid film was then hydrated for 2 h with different formulations of recombinant HspX (rHspX) and/or adjuvants in phosphate-buffered saline (PBS). Following hydration, the dispersion was vortexed for 5 min in order to promote the self-assembly of the phospholipids into bilayers and liposomal vesicles. The formulations were prepared to contain 30 mM PC. The mean diameter of the liposomes was assessed by dynamic light scattering in a Zetasizer Nano S instrument (Malvern) and the measurements were performed within 24 h of the preparation. All liposomes presented an average size of 4 μm (Fig. 1B and C).

2.4. Immunizations and Mtb challenge

The mice were distributed into 10 groups of 6 mice per group. Each group was vaccinated with a specific vaccine formulation: group 1: microstructured liposomes (Liposome); group 2: microstructured liposomes containing CpG DNA (ODN 1826; InvivoGen, L-CpG DNA); group 3: microstructured liposomes containing MPL (MPL from *Salmonella enterica* serotype Minnesota Re 595; Sigma–Aldrich, L-MPL); group 4: microstructured liposomes containing HspX (L-HspX); group 5: HspX–CpG DNA; group 6 microstructured liposomes containing HspX–CpG DNA (L-HspX–CpG DNA); group 7: HspX–MPL; group 8: microstructured

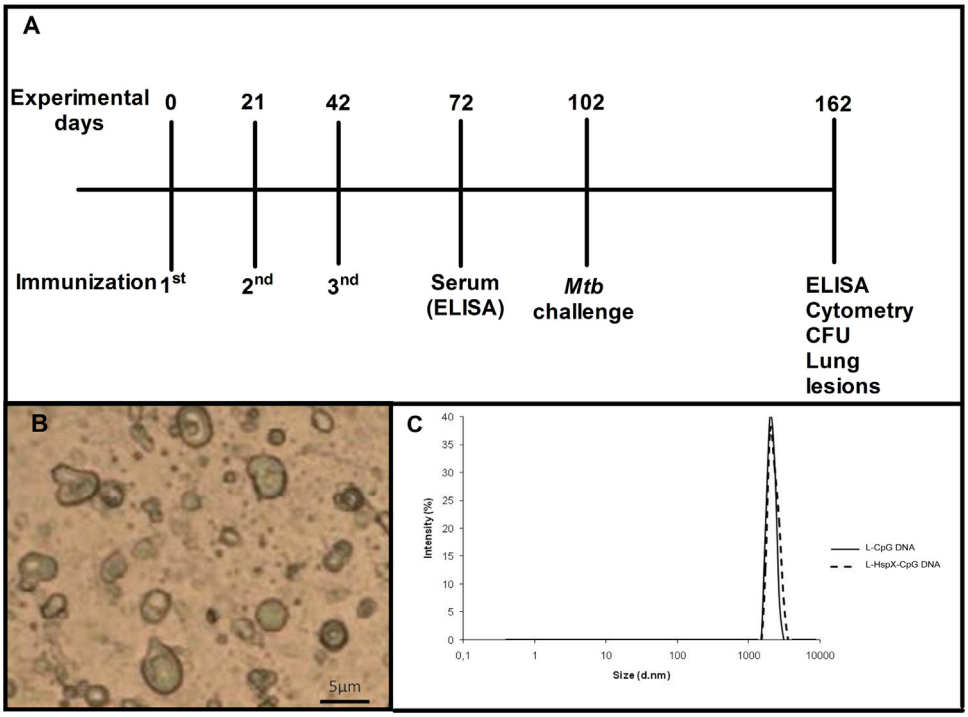

Fig. 1. Vaccination scheme and microstructured liposome characteristics. (A) Mice were subcutaneously immunized three times with formulations containing microstructured liposomes and HspX, associated or not with CpG DNA or MPL adjuvant. Thirty days after the last immunization, the mouse sera were collected for ELISA. Sixty days after the last immunization, the mice were challenged intravenously with 10^8 CFU/mL *Mtb*. Sixty days after challenge, the mice were euthanized and their spleens and lungs collected for CFU quantification, cytometry, and histopathological analysis. (B) Photomicrography representative of the vaccine formulations composed of microstructured liposomes (≈4 μm). (C) A representative graph showing the size distribution of representative liposomes as a function of the intensity of the scattered light.

liposomes containing HspX–MPL (L-HspX–MPL); group 9 received saline and was used for infection control (Infection); and group 10 received saline and was used for non infection control (Saline).

Vaccinations consisted of three subcutaneous doses of each formulation (100 μL) in the scruff of the neck with a 21-day interval. HspX, CpG DNA, and MPL were used at 20 μg/mL. As an additional control, a group of 6 animals were vaccinated with BCG strain Moreau.

Sixty days after the last immunization, the animals from all groups (except group 10) were intravenously infected with *Mtb* (strain H37Rv). On the following day, one animal from each group was euthanized to determine the initial infection dose. The protection and immune responses induced were evaluated 60 days after challenge.

2.5. Intravenous infection with Mtb and determination of lung bacterial load

M. tuberculosis (strain H37Rv) was prepared and described as in [41].

To evaluate the bacterial load on day 1 after infection, the animals were euthanized by cervical dislocation and the entire lung was homogenized in PBS with 0.05% Tween 80, and serial dilutions were plated on 7H11 agar supplemented with OADC (Oleic albumin dextrose catalase). The plates were cultured in a CO_2 incubator and 21 days later, the colony forming units (CFU) were determined. For protection evaluation, the lung bacterial load was evaluated at 60 days after infection.

2.6. Flow cytometry

The method of spleen and lung single cell preparation and staining for flow cytometry was described previously [41]. Aliquots (200 μL) of each cell suspension (1×10^6 cells/well) were distributed in a 96-well plate (Corning) and cultured without stimulus (medium alone), with concanavalin A (1 μg/mL), or with rHspX (10 μg/mL) for 4 h at 37 °C in a CO_2 incubator. The cells were acquired with a BD Biosciences FACSCanto II flow cytometer, and the data were analyzed using the FlowJo 8.7 software. One hundred thousand events were acquired from each sample.

2.7. Enzyme-linked immunosorbent assays (ELISA)

Blood was collected from retro-orbital plexus to obtain sera for ELISAs to detect IgG1 and IgG2a antibodies specific for rHspX. Recombinant HspX (10 μg/mL) was diluted with 0.05 M carbonate/sodium bicarbonate buffer (pH 9.6), adsorbed to polystyrene 96-well plates, and incubated for 18 h at 4 °C. Serial sera dilutions from 10^{-2} to 10^{-5} were performed and tested for specific IgG1 and IgG2a. All the following steps were described previously [7]. No difference in IgG titer was observed in all tested dilutions. Therefore the results shown are those obtained with 10^{-5} sera dilution.

2.8. Histopathological analysis

Sixty days after the intravenous infection of the mice, the right caudal lung lobes were removed and fixed in 10% buffered formaldehyde. Slices (5 μm thick) were cut and stained with hematoxylin and eosin (H&E) and analyzed with light microscopy (Axioscope A1; Carl Zeiss, Jena, Germany), and the images were processed with the AxioVision 4.7 software (Carl Zeiss). The histological scores were assessed after evaluating the following parameters: inflammatory infiltrate intensities, presence (or not) of foaming macrophages, and necrotic areas.

2.9. Statistical analysis

Data were analyzed with the Prism 4 version 4.0 software (GraphPad) and expressed as mean ± standard deviations (SD).

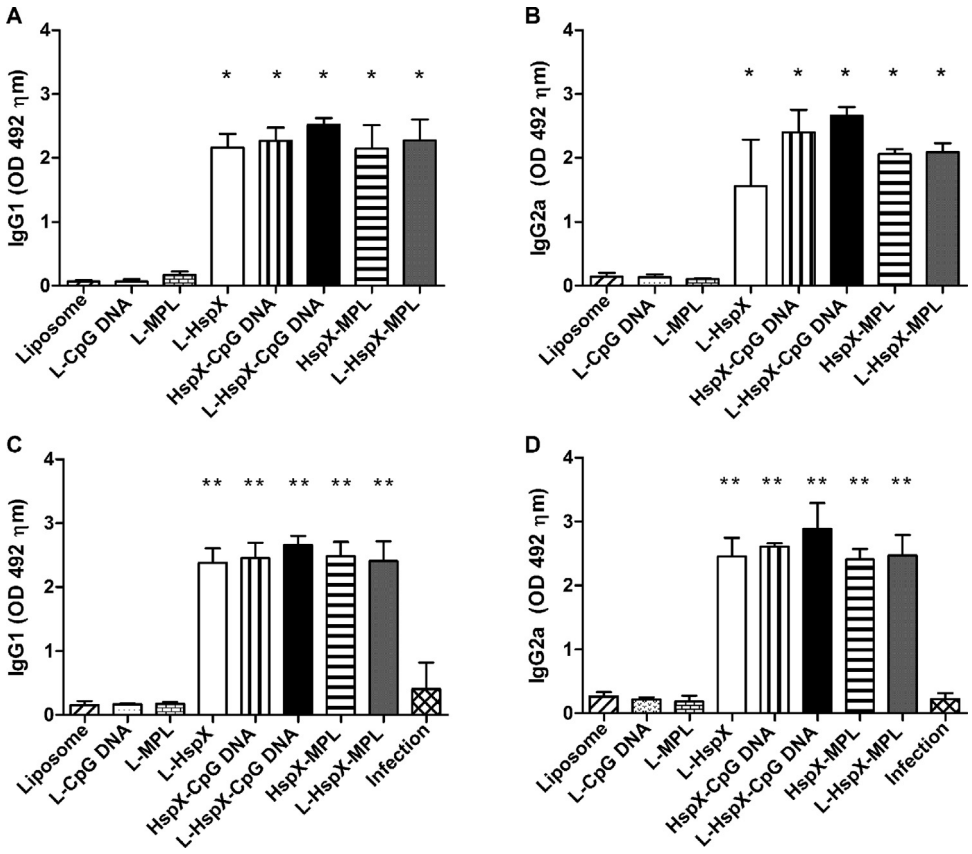

Fig. 2. Microstructured liposomes containing HspX induce IgG1- and IgG2a-class antibodies specific to HspX. BALB/c mice were immunized three times with microstructured liposomes containing HspX, associated or not with the adjuvant CpG DNA or MPL. Thirty days after the last immunization, the serum levels of IgG1 (A) and IgG2a (B) antibodies were evaluated. Statistically significant differences ($^{*}p < 0.05$) were observed between the HspX-vaccinated groups and the liposome controls. Immunized mice were challenged sixty days later with 10^7 CFU of *M. tuberculosis*. Sixty days after challenge, serum IgG1 (C) and IgG2a (D) levels were evaluated using indirect ELISA. Statistically significant differences ($^{**}p < 0.05$) were observed between the HspX-vaccinated groups and non-immunized infected animals. The graphs show the means and standard deviations of six animals per group. This is one representative experiment of two independent experiments performed.

Group comparisons were made with Kruskal–Wallis followed by Dunn's test. *T* test was used to compare CFU results between the paired groups. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Microstructured liposomes containing HspX induce specific IgG1 and IgG2a antibodies

The role of B cells in TB control is poorly understood and lesser is known about their role on TB vaccine development. Despite the controversy regarding humoral response to TB, the assessment of specific antibody levels is a predictor of the cellular type of response being induced; i.e. IgG2a associated with Th1 and IgG1 with Th2 sub populations [42,43]. To evaluate the effects of the different vaccine formulations containing microstructured liposomes, BALB/c mice were immunized with L-HspX, L-HspX–CpG DNA, or L-HspX–MPL, as described in the Methods (Fig. 1A). Regardless of the adjuvant, the vaccine formulations containing HspX induced high levels of specific IgG1 and IgG2a antibodies (Fig. 2A and B; $p < 0.05$). No significant differences of the levels of specific IgG1 or IgG2a were observed among the HspX vaccinated groups. Next, we questioned whether the Mtb infection would induce specific immune response to HspX. As shown in Fig. 2C and D, Mtb infection did not induce detectable levels of IgG1 or IgG2a directed against HspX. The animals immunized with the vaccine formulations containing HspX maintained the same levels of immunoglobulin after *Mtb* challenge (Fig. 2C and D). In contrast, the BALB/c mice vaccinated with liposome, L-CpG DNA or L-MPL alone showed no detectable levels of specific antibodies. These results show that these new vaccine formulations are immunogenic.

3.2. Microstructured liposomes containing HspX and CpG DNA induce specific CD4$^+$ T cells and CD8$^+$ T cells positive for IFN-γ or TNF-α

The vaccine formulated with L-HspX–CpG DNA generated more CD4$^+$IFN-γ^+ or CD4$^+$TNF-α^+-specific T cells in both the spleens (Suppl. Fig. 1, Fig. 3A and B, $p < 0.05$) and lungs (Fig. 3C and D, $p < 0.05$) than the other vaccine formulations. Higher levels of CD8$^+$IFN-γ^+ T cells were observed in the spleens of BALB/c mice vaccinated with L-HspX–CpG DNA or L-HspX–MPL (Fig. 4A, $p < 0.05$). The vaccine L-HspX–CpG DNA and HspX-CpG DNA induced higher CD8$^+$IFN-γ^+ T cells in the lungs (Fig. 4C).

As shown in Fig. 4, when the CD8$^+$TNF-α^+ T cells were evaluated, the vaccine formulation containing L-HspX–MPL induced them more effectively in both the spleen and lungs than the other formulations (Fig. 4B and D), although L-HspX–CpG DNA also generated these cells in the lungs (Fig. 4D). Mice vaccinated with L-CpG DNA or L-MPL alone did not induce these subpopulations of cells (Suppl. Fig. 2).

The vaccine L-HspX–CPG DNA was the best inducer of both CD4$^+$ and CD8$^+$ T cells in the spleens and lungs of infected animals (Figs. 3 and 4).

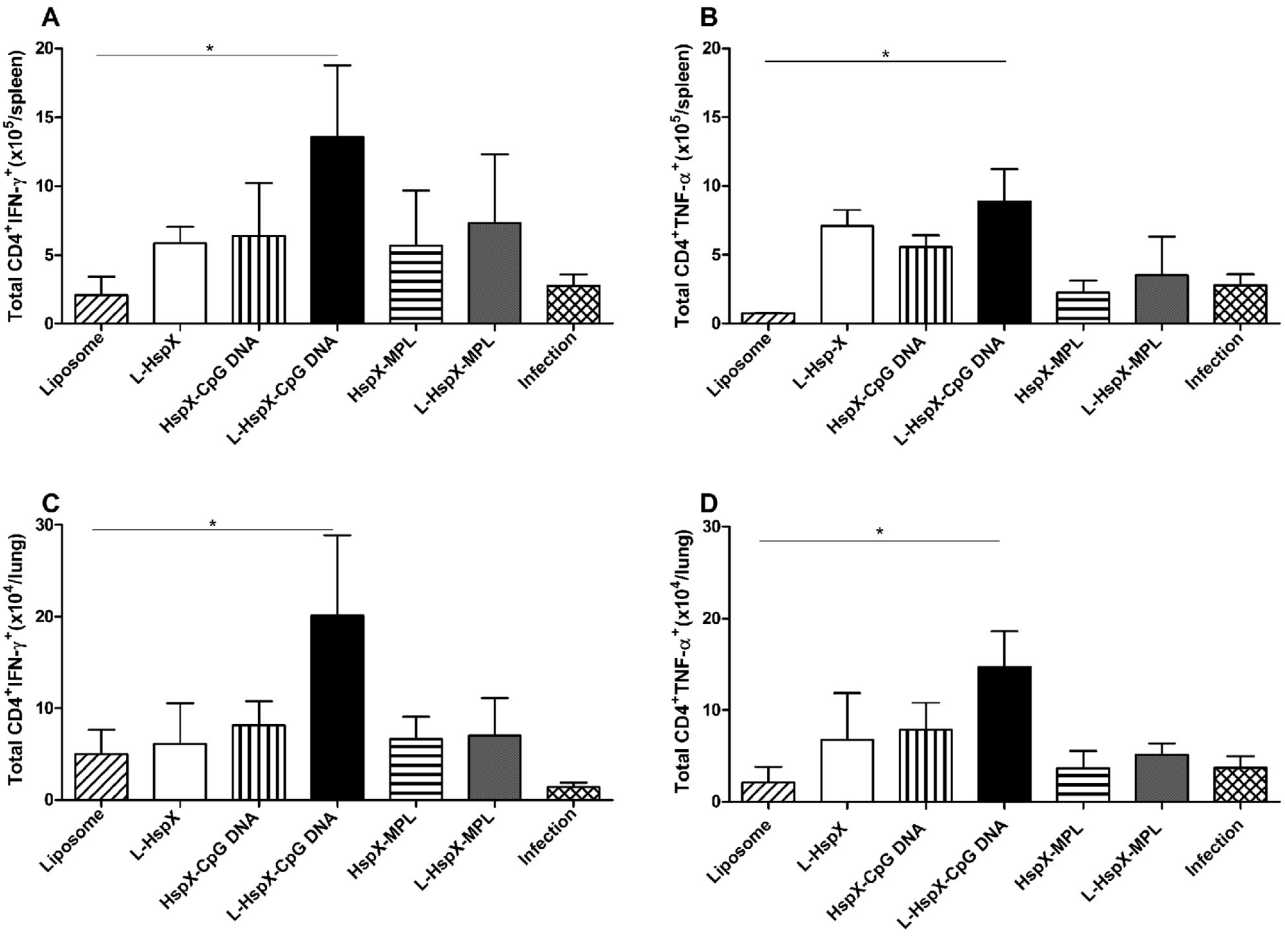

Fig. 3. Ex vivo CD4⁺-T-cell-specific response to HspX. Mice were immunized and challenged with *M. tuberculosis*. Sixty days after infection, their spleens and lungs were collected and the cells evaluated with flow cytometry. The cells were stimulated for 4 h with HspX. In A and B, the results for splenic cells are shown: (A) $CD4^+IFN\text{-}\gamma$ T cells, (B) $CD4^+TNF\text{-}\alpha^+$ T cells. C and D show the results for pulmonary cells: (C) $CD4^+IFN\text{-}\gamma$ T cells, (D) $CD4^+TNF\text{-}\alpha^+$ T cells. The graphs show the means and standard deviations of six animals per group. Kruskal–Wallis followed by Dunn's test was used to assess the existence of difference between HspX containing vaccine groups with liposome alone vaccination group ($^*p < 0.05$). This is one representative experiment of two independent experiments performed.

3.3. All vaccine formulations containing microstructured liposomes and HspX reduced lung inflammatory lesions and bacterial loads after Mtb challenge

Fig. 5 shows the lung bacterial loads 60 days after infection. Reductions in the CFU were observed in all groups vaccinated with HspX. The bacterial loads of BALB/c mice immunized with L-CpG DNA, L-MPL, or empty liposomes were similar to those of the animals infected with *Mtb*.

Intravenous infection with *Mtb* induced a strong and diffuse inflammatory response (Fig. 6A) compared to non-infected animals (Fig. 6B). Mice vaccinated with microstructured liposomes (Fig. 6C), L-CpG DNA (Fig. 6E), or L-MPL (Fig. 6G) displayed macrophages and lymphocyte foci. The pulmonary lesions in animals vaccinated with L-MPL showed foamy macrophages, which were not observed in the other vaccinated groups (Fig. 6G – insert). Inflammatory lesions similar to lymphoid follicles were observed in the L-MPL-vaccinated animals after *Mtb* infection (Fig. 6G).

All vaccines containing microstructured liposomes and HspX substantially reduced the lung inflammatory lesions, maintaining the lung structure after intravenous infection with *Mtb* (Fig. 6D, F and H). The lungs of animals vaccinated with L-HspX (Fig. 6D), L-HspX–CpG DNA (Fig. 6F), or L-HspX–MPL (Fig. 6H) showed only localized inflammatory lesions. Although L-HspX induced mononuclear periarteriolar cuff lesions (mainly attributable to macrophages; Fig. 6D), the inflammatory lesions observed in the lungs of mice vaccinated with L-HspX–CpG DNA were restricted to small bronchiolar lymphocyte infiltrates (Fig. 6F). As was observed for the L-MPL vaccine, immunization with L-HspX–MPL generated significant lymphocytic inflammatory infiltrates (Fig. 6G and H).

4. Discussion

This study shows, for the first time, the use of microstructured liposome formulations in a subunit vaccine for TB. Formulations containing microstructured liposomes and HspX were immunogenic generating high levels of specific IgG1 and IgG2a against HspX before and after infection with *Mtb*. Both $CD4^+$ and $CD8^+$ T cells positive for IFN-γ and TNF-α specific for HspX were also induced with these vaccines. The specific immune response achieved culminated in the reduction of the lung inflammatory lesions and reduced the bacterial loads.

The use of liposomes as an antigen carrier for subunit vaccines has become an alternative strategy for the development of new vaccines for TB. The liposome characteristics, which can be modified in terms of their composition, size, and charge, directly influence the immune response to an antigen [44]. Brewer et al. [36] showed that liposomes larger than 225 nm generated Th1-type immune responses, whereas Henriksen-Lacey et al. [38] demonstrated that the adjuvant type, rather than the liposome size, determined the immune response induced by vaccine formulations. Here, we have shown that liposomes with a size that approximates that of the

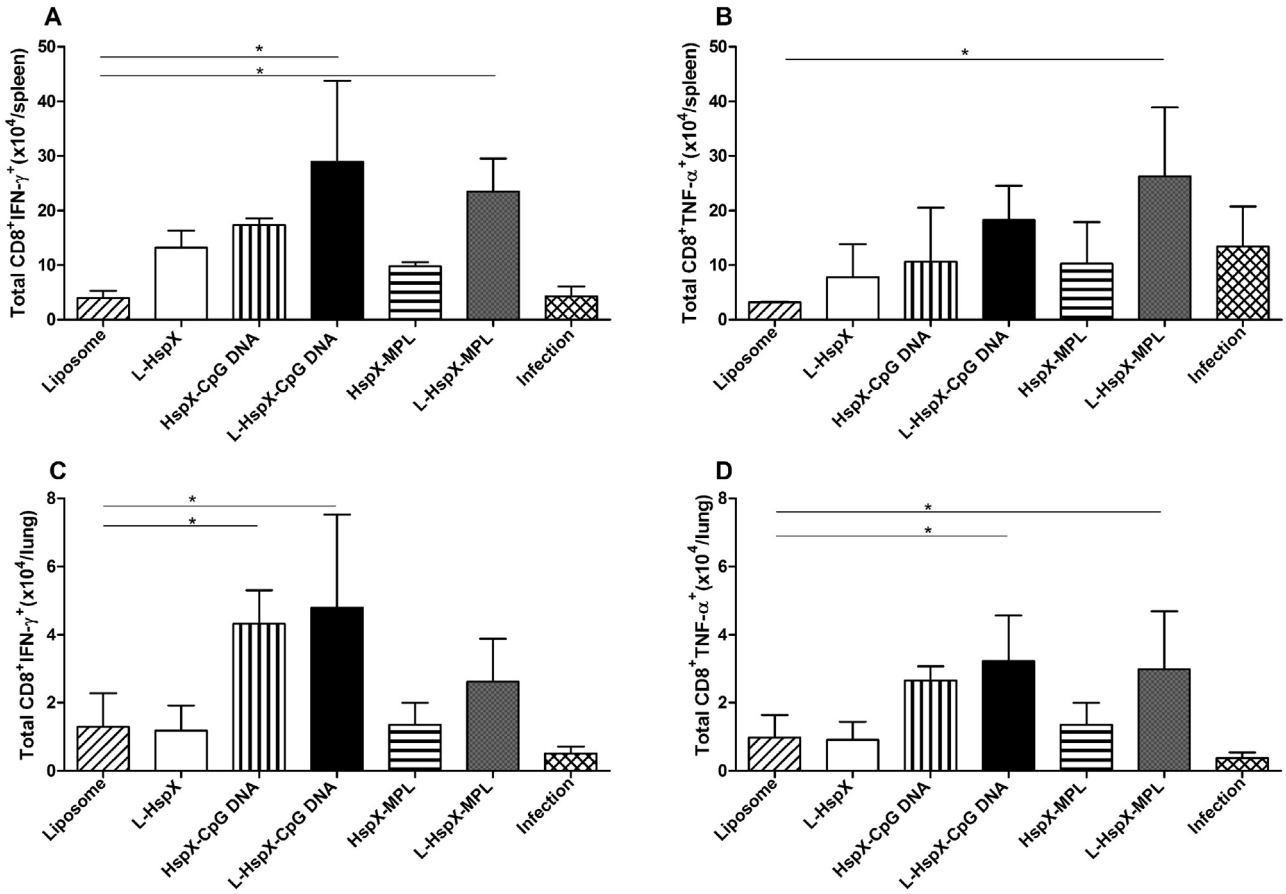

Fig. 4. Ex vivo $CD8^+$-T-cell-specific response to HspX. Mice were immunized and challenged with *M. tuberculosis*. Sixty days after infection, their spleens and lungs were collected and evaluated with flow cytometry. The cells were stimulated for 4 h with HspX. In (A and B), the results for splenic cells are shown: (A) $CD8^+IFN\text{-}\gamma^+$ T cells, (B) $CD8^+TNF\text{-}\alpha^+$ T cells. (C and D) show the results for pulmonary cells: (C) $CD8^+IFN\text{-}\gamma^+$ T cells, (D) $CD8^+TNF\text{-}\alpha^+$ T cells. The graphs show the means and standard deviations for six animals per group. Kruskal–Wallis followed by Dunn's test was used to assess the existence of difference between HspX containing vaccine groups with liposome alone vaccination group ($^*p < 0.05$). This is one representative experiment of two independent experiments performed.

microorganism combined with HspX are immunogenic, and induce a Th1-type immune response.

Several studies have shown the ability of HspX antigen vaccines to stimulate specific antibodies in BALB/c mice when CpG DNA, MPL, dimethyl dioctadecyl ammonium bromide, trehalose 6,6′-dimycolate, or aluminum hydroxide were used as the adjuvant [7,26–28,45], so HspX is a good antigen with which to test new vaccine formulations once the immune response is confirmed. The use of microstructured liposomes containing HspX generated both specific IgG1 and IgG2a antibodies in higher levels than those described previously [7,26–28,45]. Therefore, these microstructured liposomes can be used as an antigen carrier and also as an adjuvant, because L-HspX also induced specific antibody responses.

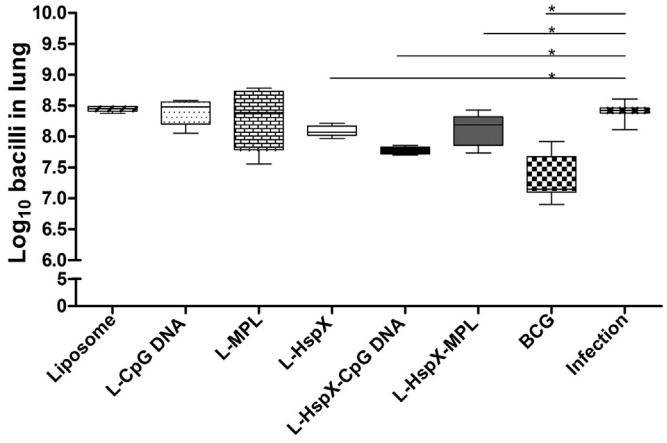

Fig. 5. Lung bacterial loads in BALB/c mice vaccinated and challenged with *M. tuberculosis*. Vaccine control animals were immunized subcutaneously with BCG. Sixty days after challenge, the lungs of the vaccinated and challenged animals were collected for CFU (colony forming units) assessment. A statistically significant difference ($^*p < 0.05$, *t* test) was observed between the HspX-vaccinated groups and infected controls ($n = 6$ mice per group). This is one representative experiment of two independent experiments performed.

As expected, mice infected with *Mtb* showed no specific antibodies against HspX, confirming the observations reported in the literature that low or no production of this protein occurs during the active phase of infection, so only a weak immune response is induced [27,46]. The role of B cells in *Mtb* infection has been neglected for decades, but their function in the pathogenesis of TB has become apparent. Torrado et al. [47] recently demonstrated that mice with insufficient B-cell maturation were more susceptible to *Mtb* aerosol infection than wild-type mice, which contradicts the old notion that B cells and their products (antibodies and cytokines) do not participate in the protective mechanisms against infection [48,49]. However, those authors could not infer a direct role for antibodies in this protection because treatment with sera from infected animals did not avert the susceptibility of mice with deficiency in antibody secretion by B cells [47]. Here, vaccination with microstructured liposomes clearly induced specific IgG1 and IgG2a antibodies. Even so, we cannot state that the antibody levels correlated directly with the protection induced because all the vaccine formulations induced specific antibodies and the best vaccine formulations, L-HspX–CpG DNA and L-HspX induced the same

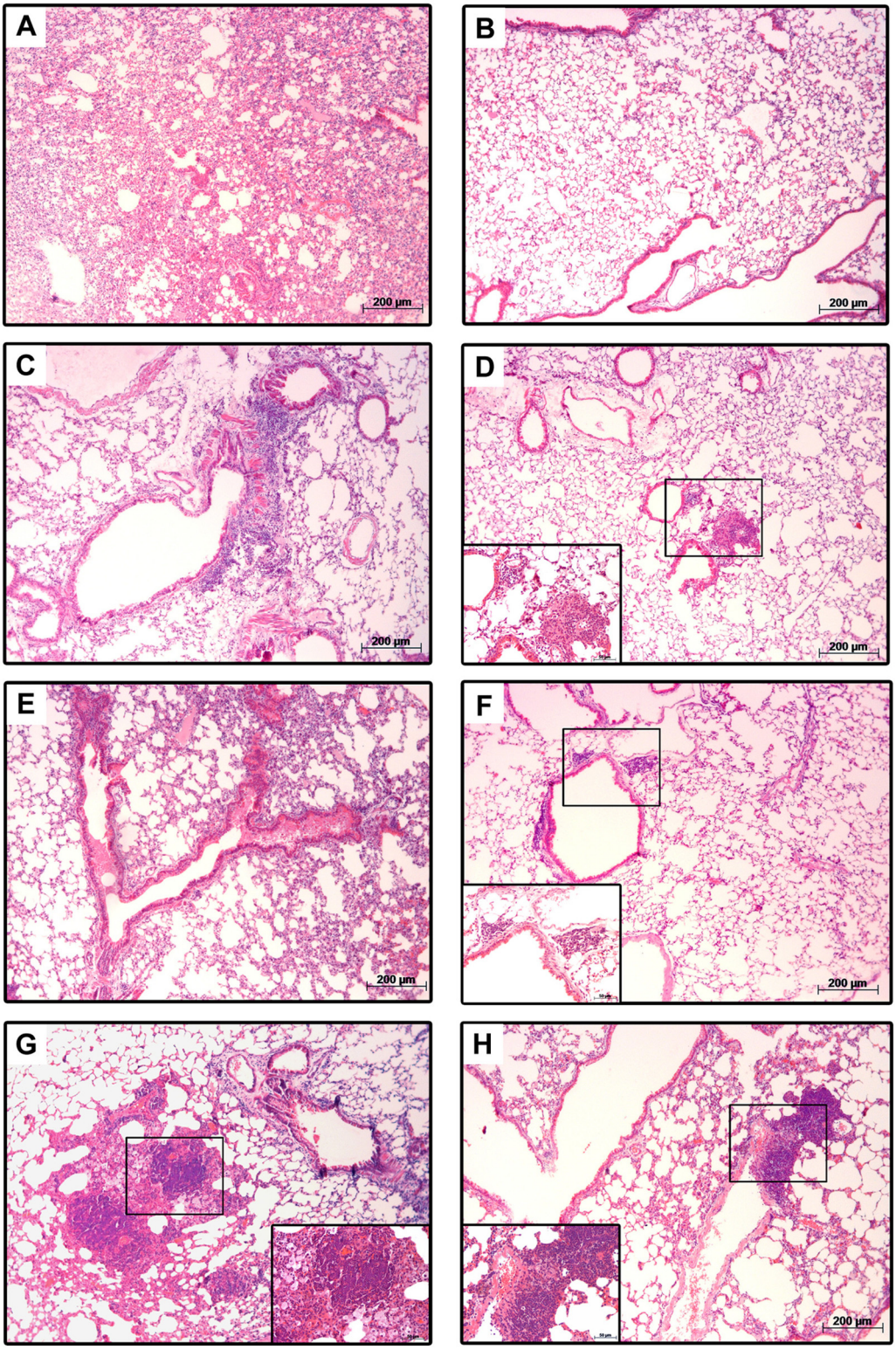

Fig. 6. Lung inflammatory lesions induced by *M. tuberculosis* infection compared with the lesions induced in mice vaccinated with microstructured liposomes and HspX. Sixty days after challenge, the lungs were processed, sectioned, and stained with H&E. Representative lesions in the infected groups: (A) infected control group; (B) non-infected control group; (C) liposomes (L) vaccinated group; (D) L–HspX vaccinated group; (E) L–CpG DNA vaccinated group; (F) L–HspX–CpG DNA vaccinated group; (G) L–MPL vaccinated group; (H) L–HspX–MPL vaccinated group. In (A), note the diffuse inflammatory lesions in the infected animals. All vaccine formulations induced small mononuclear-cell inflammatory infiltrates after *Mtb* challenge. Note the preservation of the lung parenchyma after *Mtb* challenge in all HspX vaccinated groups (D, F and H and inserts). (G) shows diffuse inflammatory lesions and the presence of foamy macrophages (G and insert).

antibody levels as the other formulations. However, we can speculate that the B-cell-based protective mechanisms involve helping T cells to differentiate adequately and memory cells to survive [49].

$CD4^+$ T cells are crucial in protecting mammalian hosts from *Mtb*, in particular because of its inflammatory cytokines that activate macrophage, and $CD8^+$ T cells among other cells [50]. Although we know that several $CD4^+$ and $CD8^+$ T-cell subtypes participate in the protection against TB, the main goal of this study was to investigate a new vaccine formulation. Therefore, we focused on the Th1-cell-type response because its involvement in this protection is very well documented [51]. Of all the vaccine formulations tested here, L-HspX–CpG DNA induced considerable $CD4^+$ and $CD8^+$ T-cell activation, with the production of IFN-γ and TNF-α. Similar results were observed when HspX was used together with Ag85 C and MPT51 with the same adjuvant [7]. It is tempting to believe that the response induced with this formulation was mainly attributable to the CpG DNA activation response (e.g., the TLR9 activation pathway). However, the HspX antigen may have contributed to both the humoral and cellular immune responses because when *Mtb* antigens, such as MPT51, were used with CpG DNA, only a discreet specific antibody was produced, although $CD4^+$ and $CD8^+$ T cells were activated [52].

Although many roles have been attributed to $CD4^+$ T cells, the roles of $CD8^+$ T cells in immune protection and therefore in the development of vaccines for TB are not completely clear, but their activation is associated with protection [53,54]. $CD8^+$ T cells can be cytotoxic to infected cells and $CD8^+$ T cells from infected mice and humans can produce cytokines (IFN-γ and TNF-α) [55]. Here, we have shown that microstructured liposomes containing HspX activate specific $CD8^+$ T cells with a capacity to produce type 1 cytokines. Coincidentally, this vaccine formulation reduced the inflammatory lesions induced by the infection. These results prompt us to ask whether an improved TB vaccine should favor the migration of specific cells to the same target organ of the pathogen.

The bacterial load is the best parameter with which to evaluate the protection conferred by a vaccine. In this study the intravenous route was used to challenge the animals and, although this is not the best route to evaluate efficacy of a vaccine for TB, it has been successfully used to compare vaccine formulation candidates [41,56,57]. All the vaccine formulations containing microstructured liposomes and HspX reduced the lung bacterial load, but the vaccine formulation containing L-HspX–CpG DNA was the one that achieved a reduction closest to BCG. Other researchers have also shown the potential utility of the antigen HspX and CpG DNA in vaccines for TB [26–28,52]. However, this is the first time that microstructured liposomes have been used.

Several vaccines that consistently reduce the bacterial load present a cost to the host, mainly because of the greater inflammatory response involved. This implies that a good vaccine should protect against the disease but also preserve the lung parenchyma [58]. The vaccine L-HspX–CpG DNA surpassed all the other formulations containing microstructured liposomes because it induced specific humoral and cellular immune responses, which reduced the bacterial load to the same level as the BCG vaccination, but with very little inflammatory reaction. Another interesting finding was that foamy macrophages only occurred in mice immunized with L-MPL and challenged with *Mtb*. One of the mechanisms that generate foamy macrophages is the activation of TLR4 and proinflammatory cytokines, such as TNF-α [59,60]. The adjuvant MPL appears to activate the immune response because it binds to TLR-4. Several authors have shown that in TB, foamy macrophages are reservoirs for TB bacilli because these cells lose their microbicide function [60]. Therefore, our results do not seem to favor MPL as an adjuvant for liposome-based subunit vaccines for TB.

In this study, we have shown that microstructured liposomes are good antigen carriers because they induce specific immune responses to the incorporated antigen. However, as postulated by Henriksen-Lacey et al. [38], the adjuvant–liposome combination is responsible for the immune response elicited rather than the liposomes alone. This is quite clear from our results, because only the combination of liposomes and CpG DNA induced both the humoral and cellular immune responses and migrated to the target organ.

Although our results are promising, TB protection might be improved even further if this vaccine formulation is used in a prime–boost strategy with BCG as the prime vaccine and the microstructured liposomes as the booster, because several studies have shown that an HspX subunit vaccine improves the protection induced by BCG when a prime–boost strategy is used [21,22,27,28].

This work reports, for the first time, the use of microstructured liposomes in a subunit vaccine formulation for TB. The vaccine L-HspX–CpG DNA showed the best results, reducing the bacterial load and the inflammatory response in the lung after an i.v. challenge.

Acknowledgments

The authors thank Laboratório de Farmacologia e Toxicologia cellular/Farmatec, UFG, for the use of the flow cytometer. We appreciate the technical assistance provided by Viviane Lopes Rocha, Bruna DS Silva and Erildo Ribeiro Silva.

This work was funded by grants from CNPq, CAPES and Fapeg.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.06.027.

References

[1] WHO. The global plan to stop TB 2011–2015. In: WHO report; 2012.
[2] Brennan MJ, Thole J. Tuberculosis vaccines: a strategic blueprint for the next decade. Tuberculosis 2012;92:S6–13.
[3] Rodrigues LC, Diwan VK, Wheeler JG. Protective effect of BCG against tuberculosis meningitis and military tuberculosis: a meta-analysis. Int J Epidemiol 1993;22:1154–8.
[4] Kaufmann SH. Tuberculosis vaccine development: strength lies in tenacity. Trends Immunol 2012;7:373–9.
[5] Doherty TM, Andersen P. Vaccines for tuberculosis: novel concepts and recent progress. Clin Microb Rev 2005;18:687–702.
[6] Cooper AM, Flynn JL. The protective immune response to *Mycobacterium tuberculosis*. Curr Opin Immunol 1995;4:512–6.
[7] de Sousa EM, da Costa AC, Trentini MM, de Araújo Filho JA, Kipnis A, Junqueira-Kipnis AP. Immunogenicity of a fusion protein containing immunodominant epitopes of Ag85C. MPT51, and HspX from *Mycobacterium tuberculosis* in mice and active TB infection. PLoS ONE 2012;7:e47781.
[8] Orr MT, Beebe EA, Hudson TE, Moon JJ, Fox CB, Reed SG, et al. A dual TLR agoinst adjuvant enhances the immunogenicity and protective efficacy of the tuberculosis vaccine antigen ID93. PLoS ONE 2014;1:e83884.
[9] da Costa AC, Nogueira SV, Kipnis A, Junqueira-Kipnis AP. Recombinant BCG: innovations on an old vaccine. Scope of BCG strains and strategies to improve long-lasting memory. Front Immunol 2014;5:152.
[10] Skeiky YA, Alderson MR, Ovendale PJ, Guderian JA, Brandt L, Dillon DC, et al. Differential immune responses and protective efficacy induced by components of a tuberculosis polyprotein vaccine. Mtb72F, delivered as naked DNA or recombinant protein. J Immunol 2004;12:7618–28.
[11] von Eschen K, Morrison K, Braun M, Ofori-Anyinam O, De Kock E, Pavithran P, et al. The candidate tuberculosis vaccine Mtb72F/AS02A: tolerability and immunogenicity in humans. Hum Vaccines 2009;7:475–82.
[12] Agger EM, Rosenkrands I, Olsen AW, Hatch G, Williams A, Kritsch C, et al. Protective immunity to tuberculosis with Ag85B-ESAT-6 in a synthetic cationic adjuvant system IC31. Vaccine 2006;26:5452–60.
[13] Schellack C, Prinz K, Egyed A, Fritz JH, Wittmann B, Ginzler M, et al. IC31, a novel adjuvant signaling via TLR9, induces potent cellular and humoral immune responses. Vaccine 2006;26:5461–72.
[14] van Dissel JT, Soonawalaa D, Joostena SA, Prinsa C, Arenda SM, Bangb P, et al. Ag85B–ESAT-6 adjuvanted with IC31 promotes strong and long-lived *Mycobacterium tuberculosis* specific T cell responses in volunteers with previous BCG vaccination or tuberculosis infection. Vaccine 2011;29:2100–9.
[15] Aagaard C, Hoang T, Dietrich J, Cardona PJ, Izzo A, Dolganov G, et al. A multistage tuberculosis vaccine that confers efficient protection before and after exposure. Nat Med 2011;17:189–95.

[16] Black GF, Thiel BA, Ota MO, Parida SK, Adegbola R, Boom WH, et al. Immunogenicity of novel DosR regulon-encoded candidate antigens of *Mycobacterium tuberculosis* in three high-burden populations in Africa. Clin Vaccine Immunol 2009;8:1203–12.
[17] Roupie V, Romano M, Zhang L, Korf H, Lin MY, Franken KL, et al. Immunogenicity of eight dormancy regulon-encoded proteins of *Mycobacterium tuberculosis* in DNA-vaccinated and tuberculosis-infected mice. Infect Immun 2007;2:941–9.
[18] Singh S, Saraav I, Sharma S. Immunogenic potencial of latency associated antigens against *Mycobacterium tuberculosis*. Vaccine 2014;6:712–6.
[19] Siddiqui KF, Amir M, Agrewala N. Understanding the biology of 16 kDa antigen of *Mycobcterium tuberculosis*: scope in diagnosis, vaccine design and therapy. Crit Rev Microb 2011;37:349–57.
[20] Spratt JM, Britton WJ, Triccas JA. In vivo persistence and protective efficacy of the bacilli Calmette Guerin vaccine overexpressing the HspX latency antigen. Bioeng Bugs 2010;1:61–5.
[21] Dey B, Jain R, Gupta UD, Katoch VM, Ramanathan VD, Tyagi AK. A booster vaccine expressing a latency-associated antigen augments BCG induced immunity and confers enhanced protection against Tuberculosis. PLoS ONE 2011;6:e23360.
[22] Taylor JL, Wieczorek A, Keyser AR, Grover A, Flinkstrom R, Karls RK, et al. HspX-mediated protection against tuberculosis depends on its chaperoning of a mycobacterial molecule. Immun Cell Biol 2012;00:01–10.
[23] Geluk A, Van den Eeden SJF, Van Meijgaarden KE, Dijkman K, Franken KLMC, Ottenhoff THM. A multistage-polyepitope vaccine protects against *Mycobacterium tuberculosis* infection in HLA-DR3 transgenic mice. Vaccine 2012;52:7513–21.
[24] Kaushik A, Singh UB, Porwal C, Venugopal SJ, Mohan A, Krishnan A, et al. Diagnostic potential of 16 kDa (HspX, α-crystalline) antigen for serodiagnosis of tuberculosis. Indian J Med Res 2012;5:771–7.
[25] Reis MC, Silva BD, Sousa EM, Junqueira-Kipnis AP. Role of antibodies reactive to HspX in discriminating pulmonary tuberculosis contacts with high risk of developing active disease. Braz J Infect Dis 2011;6:617–8.
[26] Chen L, Xu M, Wang ZY, Chen BW, Du WX, Su C, et al. The development and preliminary evaluation of a new *Mycobacterium tuberculosis* vaccine comprising Ag85b, HspX and CFP-10:ESAT-6 fusion protein with CpG DNA and aluminum hydroxide adjuvants. FEMS Immunol Med Microbiol 2010;59:42–52.
[27] Li Q, Yu H, Zhang Y, Wang B, Jiang W, Da Z, et al. Immunogenicity and protective efficacy of a fusion protein vaccine consisting of antigen Ag85B and HspX against *Mycobacterium tuberculosis* infection in mice. Scand J Immunol 2011;73:568–76.
[28] Niu H, Hu L, Li Q, Da Z, Wang B, Tang K, et al. Construction and evaluation of a multistage *Mycobacterium tuberculosis* subunit vaccine candidate Mtb10.4-HspX. Vaccine 2011;29:9451–8.
[29] MacLeod MKL, McKee AS, David A, et al. Vaccine adjuvants aluminum and monophosphoryl lipid A provide distinct signals to generate protective cytotoxic memory CD8T cells. Proc Natl Acad Sci USA 2011;108:7914–9.
[30] Bode C, Zhao G, Steinhagen F, Kinjo T, Klinman DM. CpG DNA as a vaccine adjuvant. Expert Rev Vaccines 2011;4:499–511.
[31] Casella CR, Mitchel TC. Putting endotoxin to work for us: monophosphoryl lipid A as a safe and effective vaccine adjuvant. Cell Mol Life Sci 2008;20:3231–40.
[32] Agger EM, Rosenkrands I, Hansen J, Brahimi K, Vandah BS, Aagaard C, et al. Cationic liposomes formulated with synthetic mycobacterial cord factor (CAF01): a versatile adjuvant for vaccines with different immunological requirements. PLoS ONE 2008;9:e3116.
[33] Tacken PJ, Torensma R, Figdor CG. Targeting antigens to dendritic cells in vivo. Immunobiology 2006;211:599–608.
[34] Moon JJ, Suh H, Bershteyn A, Stephan MT, Liu H, Huang B, et al. Interbilayer-crosslinked multilamellar vesicles as synthetic vaccines for potent humoral and cellular immune responses. Nat Mater 2011;10:243–51.
[35] McNeil SE, Rosenkrands I, Agger EM, Andersen P, Perrie Y. Subunit vaccines: distearoyl phosphatidyl choline-based liposomes entrapping antigen offer a neutral alternative to dimethyl dioctadecyl ammonium-based cationic liposomes as an adjuvant delivery system. J Pharm Sci 2011;100:1856–65.
[36] Brewer JM, Tetley L, Richmond J, Liew FY, Alexander J. Lipid vesicle size determines the Th1 or Th2 response to entrapped antigen. J Immunol 1998;8:4000–7.
[37] Brewer JM, Pollock KG, Tetley L, Russell DG. Vesicle size influences the trafficking, processing, and presentation of antigens in lipid vesicles. J Immunol 2004;10:6143–50.
[38] Henriksen-Lacey M, Devitt A, Perrie Y. The vesicle size of DDA:TDB liposomal adjuvants plays a role in the cell-mediated immune response but has no significant effect on antibody production. J Control Release 2011;154:131–7.
[39] Department of Microbiology, Immunology and Pathology, Mycobacteria Research Laboratory. http://www.cvmbs.colostate.edu/mip/tb/pdf/RP021.pdf
[40] Szoka FJ, Papahadjopoulos D. Comparative properties and methods of preparation of lipid vesicles (liposomes). Annu Rev Biophys Bioeng 1980;9:467–508.
[41] Junqueira-Kipnis AP, de Oliveira FM, Trentini MM, Twari S, Chen B, Resende DP, et al. Prime-Boost with Mycobacterium smegmatis recombinant vaccine improves protection in mice infected with *Mycobacterium tuberculosis*. PLoS ONE 2013;11:e78639.
[42] Maassen C, Boersma W, Holten-Neelen C, Claassen E, Laman J. Growth phase of orally administered Lactobacillus strains differentially affects IgG1/IgG2a ratio for soluble antigens: implications for vaccine development. Vaccine 2003;21:2751–7.
[43] Flynn JL, Chan J. Immunology of tuberculosis. Annu Rev Immunol 2001;19:93–129.
[44] Watson DS, Endsley AN, Huang L. Design considerations for liposomal vaccines: Influence of formulation parameters on antibody and cell-mediated immune responses to liposome associated antigens. Vaccine 2012;30:2256–72.
[45] Jeon BY, Kim SC, Eum S, Cho SN. The immunity and protective effects of antigen 85A and heat-shock protein X against progressive tuberculosis. Microbes Infect 2011;13:284–90.
[46] Cunningham AF, Spreadbury CI. Mycobacterial stationary phase induced by low oxygen tension: cell wall thickening and localization of the 16-kilodalton a-crystallin homolog. J Bacteriol 1998;180:801–8.
[47] Torrado E, Fountain JJ, Robinson RT, Martino CA, Pearl JE, Rangel-Moreno J, et al. Differential and site specific impact of B cells in the protective immune response to *Mycobacterium tuberculosis* in the mouse. PLoS ONE 2013;4:e61681.
[48] Turner J, Frank AA, Brooks JV, Gonzalez-Juarrero M, Orme IM. The progression of chronic tuberculosis in the mouse does not require the participation of B lymphocytes or interleukin-4. Exp Gerontol 2001;36:537–45.
[49] Maglione P, Xu J, Chan J. B cells moderate inflammatory progression and enhance bacterial containment upon pulmonary challenge with *Mycobacterium tuberculosis*. J Immunol 2007;178:7222–34.
[50] Bold TD, Ernst JD. CD4+ T cell-dependent IFN-γ production by CD8+ effector T cells in *Mycobacterium tuberculosis* infection. J Immunol 2012;5:2530–6.
[51] Cooper AM. Cell-mediated immune responses in tuberculosis. Annu Rev Immunol 2009;27:393–422.
[52] Silva BDS, Silva EB, Nascimento IP, Reis MCG, Kipnis A, Junqueira-Kipnis AP. MPT-51/CpG DNA vaccine protects mice against *Mycobacterium tuberculosis*. Vaccine 2009;27:4402–7.
[53] Ottenhoff THM. The knowns and unknowns of the immunopathogenesis of tuberculosis. Int J Tuberc Lung Dis 2012;11:1424–32.
[54] Behar SM. Antigen-Specific CD8+ T cells and protective immunity to tuberculosis. Adv Exp Med Biol 2013;783:141–63.
[55] Caccamo N, Meraviglia S, La Mendola C, Guggino G, Dieli F, Salerno A. memory and effector human CD8T cells phenotypical and functional analysis of specific for mycobacterial antigens. J Immunol 2006;177:1780–5.
[56] Orme IM, Roberts AD. Animal models of mycobacteria infection. Curr Protoc Immunol 2001;Chapter 19:Unit 19.5.
[57] You Q, Wu Y, Wu Y, Wei W, Wang C, Jiang D, et al. Immunogenicity and protective efficacy of heterologous prime-boost regimens with mycobacterial vaccines and recombinant adenovirus- and poxvirus-vectored vaccines against murine tuberculosis. Int J Infect Dis 2012;11:e816–25.
[58] Taylor JL, Ordway DJ, Troudt J, Gonzalez-Juarrero M, Basaraba RJ, Orme IM. Factors associated with severe granulomatous pneumonia in *Mycobacterium tuberculosis*-infected mice vaccinated therapeutically with hsp65 DNA. Infect Immun 2005;73:5189–93.
[59] Russell DG, Cardona PJ, Kim MJ, Allain S, Altare F. Foamy macrophages and the progression of the human TB granuloma. Nat Immunol 2009;9:943–8.
[60] Peyron P, Vaubourgeix J, Poquet Y, Levillain F, Botanch C, Bardou F, et al. Foamy macrophages from tuberculous patients granulomas constitute a nutrient rich reservoir for M. tuberculosis persistence. PLoS Pathog 2008;11:e1000204.