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

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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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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

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