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Protective efficacy of a lipid antigen vaccine in a guinea pig model of tuberculosis

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ABSTRACT

The bacillus Calmette Guérin (BCG) vaccine, the only licensed vaccine against TB, displays partial and variable efficacy, thus making the exploitation of novel vaccination strategies a major priority. Most of the current vaccines in pre-clinical or clinical development are based on the induction of T cells recognizing protein antigens. However, a large number of T cells specific for mycobacterial lipids are induced during infection, suggesting that lipid-based vaccines might represent an important component of novel subunit vaccines. Here, we investigated whether immunization with defined mycobacterial lipid antigens induces protection in guinea pigs challenged with *M. tuberculosis*. Two purified mycobacterial lipid antigens, the diacylated sulfoglycolipids (Ac_2SGL) and the phosphatidyl-*myo*-inositol dimannosides (PIM_2) were formulated in biophysically characterized liposomes made of dimethyl-dioctadecyl-ammonium (DDA) and synthetic trehalose 6,6′-dibehenate (TDB). In three protection trials, a reduction of bacterial load in the spleen of inoculated animals was consistently observed compared to the unvaccinated group. Moreover, a reduction in the number of lesions and severity of pathology was detected in the lungs and spleen of the lipid vaccine group compared to unvaccinated controls. As the degree of protection achieved is similar to that observed using protein antigens in the same guinea pig model, these promising results pave the way to future investigations of lipid antigens as subunit vaccines.

1. Introduction

The only tuberculosis (TB) vaccine currently available is BCG (bacillus Calmette-Guerin), an attenuated *Mycobacterium bovis* strain, given soon after birth to protect against severe forms of TB in childhood. However, BCG's efficacy against pulmonary TB in adults is highly variable and depends on, amongst other factors, ethnicity and geographical location [1]. Therefore, a more efficient vaccine is needed, particularly one offering greater protection to adults. Among novel strategies under investigation, one aims at replacing the BCG with an improved whole-organism prime vaccine, which could be either a recombinant BCG or an attenuated strain of *Mycobacterium tuberculosis*. An alternative strategy consists of developing a subunit post-exposure boosting vaccine, designed to enhance and prolong the protection already provided by BCG [2,3].

What constitutes an essential and sufficient immune response for vaccination-induced protection against TB infection is still unclear [2]. Many vaccine candidates are based on the induction of conventional MHC-restricted Th1 cytokine producing T cells, although there is no clear proof that such vaccines are efficacious in humans. Whilst some studies have questioned the long-held belief that IFN-γ suffices as biomarker of protection [4], recent data have re-affirmed the role of antigen specific IFN- γ secreting cells in reducing the risk of developing TB disease in BCG vaccinated infants [5]. There is a need to develop new vaccine concepts that exploit immunological diversity, and which target unconventional immunity, such as immunity mediated by antibodies, CD1-restricted αβ T cells, γδ T cells and MAIT cells [2].

CD1-restricted αβ T cells are stimulated by lipids, which are presented by the non-polymorphic group 1 CD1 proteins (CD1a, CD1b and CD1c isoforms) expressed by dendritic cells, B lymphocytes and epithelial cells [6–8]. These T cells have essential effector functions, including cytolytic capacity, secretion of pro-inflammatory cytokines such as IFN- γ and bactericidal activity. The *M. tuberculosis* cell wall is very rich in lipids and many of these

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have been characterized as antigens of CD1b-restricted T cells [9–13]. The analysis of the T cell response during human *M. tuberculosis* infection clearly indicates that infected patients have increased CD1-restricted T cell responses to lipid antigens [10,11,14]. Moreover, a study described the existence of lipid-recognizing T cells displaying memory phenotype over one year after curative treatment [15].

The important role of lipids in the adaptive immune response to *M. tuberculosis* infection was also provided using animal models. Vaccination of cattle, which naturally express CD1b, with an adjuvanted (DDA) mycobacterial glycolipid resulted in a specific T cell response comparable in strength to that mounted against a model protein adjuvanted in the same way [16]. Immunization of guinea pigs with mycobacterial lipid mixtures was shown to induce a lipid-specific CD1-restricted response [17]. Moreover, vaccination of guinea pigs with *M. tuberculosis* total lipids before a challenge infection with *M. tuberculosis* led to the reduction of the bacterial load and improved pathology [18]. Mice cannot be used as animal models to evaluate lipid antigen vaccines because they only express the group 2 CD1 isoform (CD1d), which so far has not been found to present mycobacterial glycolipid antigens. In contrast, the guinea pig is a suitable animal model as they express homologs of human CD1 proteins, including four CD1b (*i.e.*, guinea pig CD1B1 to B4), three CD1c (CD1C1 to C3) and one CD1e orthologs [8]. Moreover, guinea pigs are sensitive to *M. tuberculosis* infection and their patterns of granulomatous inflammation are similar to humans [19].

In this study, we evaluated in *M. tuberculosis* challenged guinea pigs a combination of purified mycobacterial lipid antigens, diacylated sulfoglycolipids (Ac_2SGL) and phosphatidyl-*myo*-inositol dimannosides (PIM_2), formulated in biophysically characterized liposomes. We selected these lipids because of their important properties. They are respectively able to activate CD8 and CD4 αβ T cells [10,13,20]. Ac_2SGL is specific to *M. tuberculosis*, while PIM_2 is ubiquitous to all mycobacterial species. PIM_2 also have adjuvant properties [21,22].

2. Materials and methods

2.1. Animals – Compliance with regulations on animal welfare and ethics

Dunkin Hartley guinea pigs free from pathogen-specific infection were used according to UK Home Office Legislation for animal experimentation and studies were approved by a local ethical committee at Public Health England. Individual animals were randomly assigned to vaccine groups and identified using subcutaneously implanted microchips (PLEXX BV, The Netherlands). Group sizes were determined by statistical power calculations (Minitab version 16) with the aim to reliably detect a difference between the median colony forming units (CFU) per ml of 1.0 $\log_{10}$.

2.2. Purification of lipid antigens

Ac_2SGL was purified as previously described [10,23] from a MmpL8::hyg mutant of *M. tuberculosis* H37Rv Pasteur strain [24]. PIM_2 was prepared from *M. bovis* BCG and corresponds to "fraction A" described in [25]. Purity of molecules was assessed by TLC and MALDI-Tof-MS, as previously described [26].

2.3. Liposomes preparation

The liposomes were prepared by the thin film method as previously described [27]. Briefly, the four components, DDA (Avanti polar lipids), TDB (Cayla-Invivogen), PIM_2, natural or synthetic sulfoglycolipids (Ac_2SGL and SL37, respectively) were added in the ratio 1250 μg/250 μg/250 μg/250 μg. DDA and TDB were solubilized in chloroform/methanol 8:2 (by vol.), PIM_2 in chloroform/methanol/water 60:35:8 (by vol.) and sulfoglycolipids (SGL) in chloroform/methanol 9:1 (by vol.). The solvents were next removed under N_2, allowing the formation of a lipid film. This film was dried overnight under vacuum and hydrated in 10 mM Tris HCl pH 7.4 to a final concentration of 1.25 mg/ml/dose of DDA by heating at 60 °C and vortexing 30 s every 5 min for 30 min. LUV were prepared at 60 °C using the mini-extruder set (Avanti Polar Lipids) using polycarbonate membranes (0.1 μm). Size and zeta potential measurements were performed on samples diluted 1:200 (N = 3) at 25 °C using a DynaPro Nanostar (Wyatt Technology) and a NanoZS (Malvern Instruments) respectively. For viscosity and refractive index, the value of pure water, *i.e.* 1.0, was used.

2.4. Phase transition temperature determination by steady-state fluorescence polarization

1,6-Diphenyl hexatriene (DPH) (Molecular probes) dissolved in dimethylformamide (12.5 μM) was incorporated to the liposomes preparation without exceeding 0.5% of the total volume and 200:1 lipid/probe ratio. Fluorescence anisotropy measurements were performed using the protocol described by Carayon et al. [28].

2.5. T Cell activation assays

Dendritic cells (3×10^4/well) were incubated for 2 h at 37 °C with different concentrations of liposomes before addition of T cells (10^5/well in triplicate). Supernatants were harvested after 36 h of incubation, and GM-CSF release was measured by using enzyme-linked immunosorbent assay kits. Data are expressed as mean ng/mL ± standard deviation (SD) of triplicates.

2.6. Immunization of guinea pigs and challenge

Groups of eight female Dunkin-Hartley guinea-pigs (250 g) were intramuscularly inoculated three times at 3 weeks intervals with PIM_2/SGL in DDA:TDB (1 mL/guinea-pig divided between the two hind quadriceps muscles). A positive control group of animals was given 250 μl of BCG Danish 1331 (Statens Serum Institut, Denmark) (5×10^4 CFU per dose) sub-cutaneously at week 0. A negative control group consisted of unvaccinated animals. Animals were aerosol-challenged, 6 weeks after the final inoculations with a low dose (10–50 CFU/animal) of *M. tuberculosis* H37Rv (NCTC 7416), generated from a suspension at 3×10^6 CFU/ml using a modified Henderson apparatus and AeroMP control unit, as previously described [29].

2.7. Bacterial load and histopathology analysis

Four weeks post-challenge, animals were euthanized by intraperitoneal injection of sodium pentobarbital (Dolethal, Vetoquinol UK Ltd) and lungs and spleen were removed aseptically. The spleen minus a small apical section and the combined left apical, cardiac, right cardiac and right diaphragmatic lung lobes were homogenized in 5 and 10 ml sterile water, respectively. Serial dilutions were plated (0.1 ml per plate, in duplicate) on Middlebrook 7H11 selective agar (bioMerieux UK Ltd). After 3–4 weeks incubation at 37 °C, colonies were counted to measure CFU/ml of homogenate. Total CFU was calculated by multiplying CFU/ml by the homogenate volume. Where no colonies were observed, a minimum detection limit was set by assigning a count of 0.5 colonies, equating to 5 CFU/ml. Samples for histopathology were processed and analysed as described in [30].

2.8. Statistical analysis

Pair-wise analysis of the log transformed CFU values was performed using the Mann-Whitney non-parametric test to compare

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