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Mucosal prime-boost vaccination for tuberculosis based on TLR triggering OprI lipoprotein from *Pseudomonas aeruginosa* fused to mycolyl-transferase Ag85A

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Abstract

Toll-like receptor (TLR) triggering is an important step in the induction of T helper (Th) type 1 T cells which are key players in protection against the intracellular pathogen *Mycobacterium* (*M.*) *tuberculosis*. Here we report on the construction of a fusion protein consisting of a tuberculosis vaccine candidate mycolyl-transferase antigen 85A (Ag85A, Rv3804c) coupled to the outer membrane lipoprotein I (OprI) from *Pseudomonas* (*P.*) *aeruginosa*, a documented TLR2/TLR4 trigger. Subcutaneous boosting with this fusion protein in the absence of adjuvant increased significantly the Ag85A-specific humoral but not cellular immune responses of Ag85A-DNA vaccinated mice. Intranasal priming of C57BL/6 mice with live, attenuated *Mycobacterium bovis* bacille Calmette-Guérin (BCG) vaccine, followed by intranasal boosting with OprI-Ag85A increased systemic and local antigen-specific interferon (IFN)-γ and interleukin (IL)-2 responses in spleen, draining cervical and mediastinal lymph nodes and particularly in lung tissue, as compared to responses in mice only vaccinated with BCG vaccine. Despite enhanced immune responses, boosting with OprI-Ag85A did not increase protective efficacy against *M. tuberculosis* of either plasmid DNA or BCG vaccine in this experimental setting.

Keywords: Tuberculosis; OprI; Ag85A

1. Introduction

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), remains the largest single bacterial cause of death globally, killing 2 million people each year [1]. It is estimated that about one-third of the world population is latently infected with *M. tuberculosis* and many of the adult TB cases result from the reactivation of this initially controlled latent TB. A defective immune system (caused by human immuno-deficiency virus (HIV) infection, malnutrition, stress, ageing and genetic factors) provides the basis of this reactivation. TB chemotherapy is expensive and long and the emergence of multi-drug resistant strains of *M. tuberculosis* can be attributed in the first place to poor compliancy [2]. Unfortunately, large-scale administration of the existing vaccine strain *Mycobacterium bovis* BCG, which in 2000 covered 86% of the world population, has so far been unable to eradicate tuberculosis [3].

The design of better tuberculosis vaccines has entered a new era and several novel vaccine candidates will pass phase 1 and phase 2 clinical trials during the coming years [3,4]. Yet, there are still many questions that remain unanswered and for one, the correlates of protection or – to use a more 'fashionable' term – the biomarkers of protection have not been fully defined as yet. In experimental mouse models the frequency of local Ag-specific, IFN-γ secreting T cells in the mediastinal lymph nodes has been reported to be a better indicator of protection against pulmonary tuberculosis than systemic responses [5]. Others state that the immune responses in lymphoid tissue outside the lungs are less indicative of protection from pulmonary disease than the lung responses at the surface of the respiratory tract [6,7].

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In a more general way there is no agreement on immune parameters that correlate with immune memory. It has been claimed that the size of the memory T cell pool is dependent on the size of the initial effector T cell pool. In this perspective vaccines should trigger an effector pool as large as possible without stimulating too long in order to avoid activation induced cell death [8]. However, it has been reported that even a Th1 inducing cytokine environment results in a population heterogeneous in its IFN-γ secretion, with an IFN-γ negative population developing into the protective memory population [9,10]. Hence, although IFN-γ is the most widely accepted indicator of protective immunity, this Th1 cytokine does not provide the whole answer and too high production levels might even be deleterious, possibly through induction of IL-17 [11].

TLR triggering is an important step in the induction of Th1 type T cells, which are key players in immune protection against intracellular pathogens such as *M. tuberculosis*. The development of effective Th1 promoting adjuvants, which may interact with these TLRs, is actually a major bottleneck in the formulation of protein subunit vaccines directed against intracellular pathogens such as HIV, the malaria parasite *Plasmodium* and *M. tuberculosis*. Lipidated outer membrane lipoprotein OprI from *Pseudomonas aeruginosa* is recognized by the innate immune system as a pathogen-associated molecular pattern and triggers antimicrobial effector mechanisms via the activation of TLR2 and TLR4 [12]. This results in a Th1 response, characterized by the production of the pro-inflammatory cytokines tumor necrosis factor (TNF)-α and IFN-γ. The Th1 inducing properties of OprI, which rely on its lipid tail, have previously been demonstrated in a mouse model of cutaneous leishmaniasis. Specifically, in BALB/c mice subcutaneous administration of a fusion of OprI and the carboxy-terminal part of the surface glycoprotein gp63 of *Leishmania major* triggers substantial Th1 cytokine production and biases antibody production to the immunoglobulin (Ig) G2a isotype [13]. In a model of classical swine fever, OprI can promote porcine monocyte derived dendritic cell activation and maturation, and act as a strong adjuvant for an E2/NS3-based subunit vaccine activating the T cell compartment towards a Th1 response [14]. Finally, in a mouse model of allergic asthma, intranasal instillation of OprI at the time of allergen challenge has been shown to divert the existing Th2 response to a Th1 response [12].

Here we describe the construction of a fusion-protein of OprI coupled to Ag85A of *M. tuberculosis*. The mycolyl-transferase Ag85A is a major secreted protein from *M. tuberculosis* involved in the biogenesis of cord factor [15,16]. In mice and guinea pigs, members of the Ag85 family have proven to be promising candidates for future TB vaccines, either as plasmid DNA or as protein subunit vaccine [17–20]. BCG vaccinated mice can be boosted with recombinant Modified Vaccinia Ankara virus (MVA) or adenovirus encoding Ag85A resulting in markedly increased Ag85A-specific T cell responses and the former approach has been tested successfully in humans [5,21,22]. Ag85A protein has also been used for boosting BCG and pDNA mediated immunity. Both protocols enhance the protective efficacy of the vaccine as compared to the homologous vaccine [23,24].

In this paper we report on similar attempts to increase immune responses and protective efficacy induced in Ag85A-DNA and *M. bovis* BCG vaccinated mice by booster immunizations with OprI-Ag85A.

2. Materials and methods

2.1. Construction of the OprI-Ag85A fusion protein

The DNA sequence encoding Ag85A was amplified by polymerase chain reaction (PCR) from pV1J.ns-Ag85A [19] with following primers: primer 1 (5′-cgcg*agATCT*TTT-CCCGGCCGGGCTT-3′) as a forward primer and primer 2 (5′-cgcg*gaatt****C*****TA**GGCGCCCTGGGGCG-3′) as a reverse primer. The sequence of Ag85A is indicated in capital letters, the restriction sites in italic, and the stop codon in bold. The Ag85A PCR product and the plasmid pVUB3 encoding OprI [25] were digested with BglII and EcoRI. Ligation resulted in the in frame fusion of the N-terminus of OprI (19 residues for the lipidation signal sequence and 59 residues of the mature protein) followed by Ag85A (295 residues). The plasmid pVUB3-Ag85A thus obtained was used to transform *Escherichia* (*E.*) *coli* TOP10F' and ampicillin resistant clones were verified by colony PCR and sequence analysis. The protein encoded by this fusion gene has a molecular mass of 41 kDa. During transport to the outer membrane, the lipidation signal sequence is cleaved off, resulting in a final molecular mass of 38 kDa, and the protein is triacylated.

2.2. Purification of OprI and OprI-Ag85A fusion protein

For the production of OprI and OprI-Ag85A overnight cultures of *E. coli* transformed with plasmid pVUB3 or pVUB3-Ag85A, respectively, were diluted 1:20 in fresh M9 medium (20 mM Na_2HPO_4, 10 mM KH_2PO_4, 10 mM NH_4Cl, 5 mM NaCl) supplemented with 0.2% casein amino acid hydrolysate, 0.2% glucose, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$ and 100 μg/ml ampicillin. Cultures were grown at 37 °C until they reached OD_{600} of 0.7–0.8, and then induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) during 2 h. The lipidated protein is targeted to the outer membrane and disrupts its integrity, hence longer induction times do not result in higher yields [26].

For the preparation of outer membranes the bacterial pellet was resuspended in GTE buffer (50 mM glucose, 25 mM Tris–HCl, pH 8.0, 10 mM ethylenediaminetetraacetic acid (EDTA)) with 4 mg/ml lysozyme. Cells were incubated on ice for 30 min, combined with 2% *N*-lauroyl-sarkosine and again incubated on ice. Intermittent sonication was performed until viscosity was lost. Outer membranes were pelleted at 92,000 × *g* for 2 h at 4 °C. The pellet was dissolved in sample buffer for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

OprI and OprI-Ag85A were purified from the outer membrane preparations by continuous elution SDS-PAGE electrophoresis using a BIORAD Prep-cell (11% gel for OprI-Ag85A, 15% gel for OprI). Fractions, checked by SDS-PAGE and Coomassie staining (11% gel for OprI-Ag85A, 15% gel for