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Mucosal delivery switches the response to an adjuvanted tuberculosis vaccine from systemic TH1 to tissue-resident TH17 responses without impacting the protective efficacy

Mark T. Orr[a,b,*], Elyse A. Beebe[a], Thomas E. Hudson[a], David Argilla[a], Po-Wei D. Huang[a], Valerie A. Reese[a], Christopher B. Fox[a,b], Steven G. Reed[a,b], Rhea N. Coler[a,b]

[a] Infectious Disease Research Institute, Seattle, WA 98102, USA
[b] Department of Global Health, University of Washington, Seattle, WA 98105, USA

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ABSTRACT

Pulmonary tuberculosis (TB) remains one of the leading causes of infectious disease death despite widespread usage of the BCG vaccine. A number of new TB vaccines have moved into clinical evaluation to replace or boost the BCG vaccine including ID93+GLA-SE, an adjuvanted subunit vaccine. The vast majority of new TB vaccines in trials are delivered parenterally even though intranasal delivery can augment lung-resident immunity and protective efficacy in small animal models. Parenteral immunization with the adjuvanted subunit vaccine ID93+GLA-SE elicits robust TH1 immunity and protection against aerosolized *Mycobacterium tuberculosis* in mice and guinea pigs. Here we describe the immunogenicity and efficacy of this vaccine when delivered intranasally. Intranasal delivery switches the CD4 T cell response from a TH1 to a TH17 dominated tissue-resident response with increased frequencies of ID93-specific cells in both the lung tissue and at the lung surface. Surprisingly these changes do not affect the protective efficacy of ID93+GLA-SE. Unlike intramuscular immunization, ID93+GLA does not require the squalene-based oil-in-water emulsion SE to elicit protective CD4 T cells when delivered intranasally. Finally we demonstrate that TNF and the IL-17 receptor are dispensable for the efficacy of the intranasal vaccine suggesting an alternative mechanism of protection.

1. Introduction

Mycobacterium tuberculosis (*Mtb*) infects one-third of the world population and causes eight million cases of tuberculosis (TB) annually [1]. Several new vaccines have entered Phase 1 and 2 clinical testing to prevent infection or disease [2]. The first efficacy trial of one of these candidates, Modified Vaccinia virus Ankara expressing Ag85A (MVA85A), failed to reach either of these important benchmarks, reinforcing the need for new TB vaccine candidates [3]. We have developed a novel fusion protein antigen, designated ID93, consisting of four *Mtb* proteins, Rv1813, Rv2608, Rv3619, and Rv3620, and adjuvanted with the synthetic TLR4 agonist glucopyranosyl lipid adjuvant (GLA) formulated in a stable oil-in-water emulsion (SE). ID93+GLA-SE elicits a multifunctional TH1 response when delivered intramuscularly and limits both pulmonary and disseminated infection following exposure to aerosolized *Mtb* [4–6]. Intramuscular ID93+GLA-SE is currently undergoing Phase 2 immunogenicity and efficacy testing.

Immune responses to vaccine antigens can be shaped by the choice of vaccine adjuvant, the specific formulation of the antigen and adjuvant combination, and the route of vaccine delivery. Without the GLA-SE adjuvant ID93 elicits a TH2 response and does not protect against aerosolized *Mtb* in mice or guinea pigs [7]. When combined with the synthetic TLR4 agonist GLA in an oil-in-water stable nano-emulsion (SE) the vaccine drives robust TH1 responses and is protective. However, ID93+GLA formulated in an aqueous nanosuspension lacking an oil component (ID93+GLA-AF) fails to elicit protective TH1 responses, highlighting the importance of formulation effects on vaccine immunogenicity [6].

Mtb is primarily controlled by CD4 T cells, with both IFN-γ and TNF being implicated as critical effector cytokines [8]. Additionally IL-17 producing TH17 cells can contribute to vaccine efficacy

Abbreviations: GLA, glucopyranosyl lipid adjuvant; SE, stable emulsion; AF, aqueous formulation; Mtb, *Mycobacterium tuberculosis*; MVA85A, Modified Vaccinia virus Ankara expressing Ag85A; BALF, bronchoalveolar lavage fluid.

* Corresponding author at: 1616 Eastlake Ave E, Suite 400, Seattle, WA 98102, USA. Tel.: +1 206 858 6013; fax: +1 206 381 3678.
E-mail address: Mark.orr@idri.org (M.T. Orr).

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in some circumstances by recruiting TH1 cells to the site of infection [9]. *Mtb* is acquired following aerosol exposure; thus, inducing robust pulmonary immunity may augment vaccine efficacy. Therefore there is considerable interest in developing TB vaccines that preferentially induce lung-resident CD4 T cell responses where they can react quickly. Intranasal delivery of either virally vectored TB vaccines such as MVA85A or adjuvanted protein vaccines can enhance lung-resident immunity and efficacy against *Mtb* [10–12]. Besides enhancing lung-resident immunity, intranasally administered vaccines may facilitate various practical benefits of high importance in the developing world, such as eliminating the risk of disease transmission through improper needle use as well as the need for sharps waste containment, and increasing compliance among end users while reducing the need for trained medical personnel to administer the vaccine [13]. We previously reported that intramuscular immunization with ID93+GLA-SE elicits TH1 responses in both the spleen and lung which may account for its protective efficacy [6]. In the current paper we assess whether intranasal delivery of ID93+GLA-SE enhances lung-resident immunity and subsequent efficacy against aerosolized *Mtb* challenge.

2. Materials and methods

2.1. Mice and immunizations

Wild type C57Bl/6 and TNF$^{-/-}$ mice on the C57Bl/6 background were purchased from Jackson Laboratories (Bar Harbor, ME). IL-17R$^{-/-}$ mice on the C57Bl/6 background were a gift from Amgen, Seattle [14]. Mice were immunized with ID93 (0.5 μg) adjuvanted with GLA-SE or GLA-AF (5 μg) via intramuscular injection or intranasal delivery to both nostrils (25 μL total volume). Adjuvants were prepared as described previously [6]. Mice were anesthetized with ketamine and xylazine prior to intranasal immunization. Mice were immunized three times at 3 week intervals. All mice were maintained in specific pathogen-free conditions. After infection animals were maintained in BL3 containment. All procedures were approved by the IDRI institutional animal care and use committee.

2.2. Isolation of lymphocytes

Lymphocytes from BALF were isolated by lavaging 3 mL of cold PBS into the lungs via the trachea twice. Cells were concentrated by centrifugation and resuspended in RPMI1640 and 10% FCS for subsequent analysis. Mice were then perfused with 10 mL of cold PBS into the right ventricle. Perfused lungs were collected, digested for 30 min with Liberase TM (Roche), and dissociated using a GentleMACS system (Miltenyi) to isolate lung lymphocytes. Splenocytes were isolated by dissociation and red blood cells were lysed using Red Blood Cell Lysis Buffer (eBioscience).

2.3. In vivo labeling of T cells

In some experiments 5 μg of PE-Cy7 conjugated α-CD45.2 (clone 104) (BioLegend) was injected intravenously 3 min prior to euthanasia [15]. Lung lymphocytes were then isolated as described above and stained for CD4 (clone RM4-5), CD44 (clone IM7), CD69 (clone H1.2F3) and an I-A^b tetramer presenting the dominant epitope of Rv3619 (VIYEQANAHGQ), one of the components of ID93. APC labeled tetramers were provided by the NIH Tetramer Core Facility. Up to 10^6 events were collected on an LSR Fortessa flow cytometer (BD Biosciences). Data were analyzed with FlowJo v9. Cells were gated as singlets > lymphocytes > CD4$^+$ > CD44$^+$ tetramer+ > CD90.2.

2.4. Intracellular cytokine staining

Cells were plated at 2×10^6 cells/well and stimulated for 2 h at 37 °C with ID93 (10 μg/mL) or unstimulated. GolgiPlug (BD Biosciences) was added and the cells were incubated for an additional 8 h at 37 °C. Cells were washed and surface stained with fluorochrome-labeled antibodies to CD4 and CD8 (clone 53-6.7) (BioLegend and eBioscience) in the presence of anti-CD16/32 (clone 2.4G2) for 20 min. Cells were washed and permeabilized with Cytofix/Cytoperm (BD Biosciences) for 20 min. Cells were washed with Perm/Wash (BD Biosciences) and stained intracellularly with fluorochrome-labeled antibodies to CD154 (clone MR1), IFN-γ (clone XMG-1.2), IL-2 (clone JES6-5H4), TNF (clone MP6-XT22), GM-CSF (clone MP1-22E9), and IL-17A (clone TC11-18H10.1) (BioLegend and eBioscience) for 20 min. Cells were washed and resuspended in PBS. Up to 10^6 events were collected on an LSR-Fortessa flow cytometer (BD Biosciences). Data were analyzed with FlowJo v9. Cells were gated as singlets > lymphocytes > CD4$^+$ CD8$^-$ > cytokine positive. ID93 specific response frequencies were determined by subtracting the frequency of response positives of unstimulated cells from ID93 stimulated cells in matched samples.

2.5. Multiplex quantitation of secreted cytokines

Cells were plated at 2×10^5 cells/well and stimulated for 48 h at 37 °C with ID93 (10 μg/mL) or unstimulated. Supernatants were stored at −20 °C until analysis. Supernatants were analyzed in duplicate for secreted IFN-γ, IL,4, IL-5, IL-6, IL-10, IL-17A, IL-17F, IL-21, IL22, IL-23, GM-CSF, MIP-1α, and MIP1β using a custom ProcartaPlex Multiplex Immunoassay (eBioscience) according to the manufacturer's instructions. Alternatively supernatants were acid treated and analyzed for secreted active TGFβ1 using a custom ProcartaPlex Simplex Immunoassay (eBioscience) according to the manufacturer's instructions. Samples were read on a Luminex 200 (LuminexCorp) with MasterPlex Software (Hitachi Solutions America, Ltd.).

2.6. Mtb aerosol challenge and enumeration

Four weeks after the last immunization mice were aerogenically infected with *M. tuberculosis* H37Rv (ATCC No. 35718; American Type Culture Collection) using a GlasCol aerosol generator calibrated to deliver 50–100 bacteria into the lungs. Protection was determined at the indicated times after challenge by harvesting the lungs and spleens, homogenizing the tissue in 0.1% PBS–Tween 80, and plating fivefold serial dilutions on 7H10 agar plates (Molecular Toxicology) for bacterial growth. Bacterial colony forming units (CFU) were counted after incubation at 37 °C with 5% CO_2 for 14 days.

2.7. Statistics

Bacterial counts were log-transformed prior to analysis. ANOVA analysis with the Bonferroni correction for multiple comparisons was used to determine significant reductions in bacterial burden vs. the unimmunized, genotype-matched controls. Statistical analyses were performed using Prism software (GraphPad Software, Inc., La Jolla, CA).

3. Results

3.1. Vaccination route dictates durable TH1 or TH17 responses

Intramuscular immunization with ID93+GLA-SE elicits a robust TH1 response and limits *Mtb* in mice and guinea pigs. To determine

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