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A novel nanoemulsion vaccine induces mucosal Interleukin-17 responses and confers protection upon *Mycobacterium tuberculosis* challenge in mice

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ABSTRACT

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*Mtb*) is contracted via aerosol infection, typically affecting the lungs. *Mycobacterium bovis* bacillus *Calmette-Guerin* (BCG) is the only licensed vaccine and has variable efficacy in protecting against pulmonary TB. Additionally, chemotherapy is associated with low compliance contributing to development of multidrug-resistant (MDR) and extensively drug-resistant (XDR) *Mtb*. Thus, there is an urgent need for the design of more effective vaccines against TB. Experimental vaccines delivered through the mucosal route induce robust T helper type 17 (Th17)/ Interleukin (IL) -17 responses and provide superior protection against *Mtb* infection. Thus, the development of safe mucosal adjuvants for human use is critical. In this study, we demonstrate that nanoemulsion (NE)-based adjuvants when delivered intranasally along with *Mtb* specific immunodominant antigens (NE-TB vaccine) induce potent mucosal IL-17 T-cell responses. Additionally, the NE-TB vaccine confers significant protection against *Mtb* infection, and when delivered along with BCG, is associated with decreased disease severity. These findings strongly support the development of a NE-TB vaccine as a novel, safe and effective, first-of-kind IL-17 inducing mucosal vaccine for potential use in humans.

1. Introduction

Mycobacterium tuberculosis (*Mtb*) latently infects one-third of the world's population, causing pulmonary tuberculosis in ~9 million people and resulting in ~1.4 million deaths each year [1]. The currently available TB vaccine, *Mycobacterium bovis* BCG (BCG), shows variable efficacy in protection against pulmonary tuberculosis. In addition, drug resistant *Mtb* strains have recently emerged. Thus, there is a great need for new TB vaccines [2]. TB vaccine development during the past decade has focused on targeting interferon-gamma (IFNγ) secretion from T helper 1 (Th1) cells to mediate early macrophage activation and bacterial killing [3]. A recombinant TB vaccine, MVA85A, was recently tested in human clinical trials. Despite inducing high levels of IFNγ production from T-cells [4,5], this vaccine failed to protect against TB disease [6,7]. These data highlight the importance of exploring new and more effective immune approaches to improve vaccine-induced immunity against TB.

Abbreviations: Ag85B, Antigen 85B; ANOVA, Analysis of variance; BCG, *Mycobacterium bovis* bacillus *Calmette-Guerin*; BCIP/NBT, 5-Bromo-4-chloro-3-indolyl phosphate/ nitro blue tetrazolium chloride; C, Centigrade; C57BL/6J, B6; CCL-5, Chemokine (C-C motif) ligand 5; CD, cluster of differentiation; CFU, colony forming unit; CXCL-13, Chemokine (C-X-C motif) ligand 13; CXCL-2, Chemokine (C-X-C motif) ligand 2; CXCL-9, Chemokine (C-X-C motif) ligand 9; DAPI, 4',6-Diamid ino-2-phenylindole; DC, Dendritic cells; ESAT-6, early secreted antigenic target 6 kDa protein; Gy, (gray) absorbed radiation dose; HLT, heat labile enterotoxin; I.N., intranasal; IFNγ, interferon gamma; IL, interleukin; Ml, milliliter; MPL, monophosphoryl lipid A; Mtb, Mycobacterium tuberculosis; MVA85A, modified vaccinia Ankara 85A; MyD88, Myeloid differentiation primary response gene 88; NE, nanoemulsion; Nm, nanometer; Ns, not significant; PBS, phosphate buffered saline; S.C, subcutaneous; S.D., standard deviation; TB, tuberculosis; TGF-β, Transforming growth factor beta; Th1, T helper type 1; Th17, T helper type 17; TLR, toll-like receptor; µg, microgram; µl, microliter; µm, micrometer.

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Our recent work has demonstrated that T helper type 17 (Th17) cells, which produce the cytokine interleukin-17 (IL-17), are primary effector cells mediating vaccine-induced protection against *Mtb* [8–11]. Additionally, intranasal (I.N.) vaccines induce better mucosal immunity and confer superior protection against mucosal infectious diseases, including TB [12–15], when compared to systemic routes of immunization [16]. Importantly, we and others have recently demonstrated that mucosal vaccination of *Mtb* antigens in Heat Labile enterotoxin (HLT) [8] or cholera toxin [17] induced mucosal Th17 responses, which confer protection upon *Mtb* challenge. Our mechanistic studies demonstrated that IL-17 induced chemokines localize cytokine-producing T-cells near *Mtb*-infected macrophages, forming lymphoid follicles within granulomas to mediate *Mtb* control [11,18]. In combination with DC transfer, a HLT-TB mucosal vaccine can provide superior near-sterilizing vaccine-induced protection against *Mtb* infection [19]. Despite the ability of these experimental mucosal adjuvants to induce protective Th17 responses and confer vaccine-induced protection, there are serious concerns regarding the safety of toxin subunits as mucosal adjuvants in human vaccines. For example, Bell's palsy has been observed following I.N. application of the vaccine Nasalflu, which contains *E. coli* HLT as an adjuvant [20]. Therefore, there is an urgent need to identify safe and effective mucosal TB vaccines that can induce lung mucosal IL-17 T-cell responses for use in humans. Nanoemulsions (NE) are oil-in-water emulsions formulated with antigen. NE adjuvant was safe and well-tolerated in human volunteers when used as a flu vaccine, and elicited both systemic and mucosal immunity following a single I.N. vaccination [21]. When delivered with antigen in mice by I.N. route, the NE-based vaccine induced Th17 responses [22]. In the current study, we tested whether NE-adjuvanted TB vaccine will confer protection against *Mtb* infection, and provide any advantage when compared to use of BCG vaccination. Our results show that mucosal delivery of NE along with *Mtb* immunodominant antigens (NE-TB vaccine) can confer *Mtb* control, to levels similar to BCG vaccination in mice. Importantly, our data also show that NE-TB vaccination either given concurrently or sequentially as a boost to initial priming using BCG, can significantly limit TB disease in infected mice. Thus our findings provide the basis for development of a NE-TB vaccine as a novel, safe and effective, first-of-kind IL-17 inducing mucosal vaccine for potential use in humans.

2. Materials and methods

2.1. Mice

C57BL/6J (B6), (Jackson Laboratories, Bar Harbor, ME) mice were bred under specific pathogen-free conditions at the Washington University in St. Louis. Mice maintained were used at 6 to 8 weeks of age and sex matched for all experiments. All animal experiments were performed in accordance with National and Institutional guidelines for animal care under approved protocols.

2.2. Vaccination and Mtb infection

The antigen proteins, ESAT-6 and Ag85B, were provided by BEI Resources (Manassas, VA) obtained under National Institutes of Health [NIH] contract AI-75320. The NE-TB vaccine was prepared by simple mixing of ESAT-6 and Ag85B antigens (1.5-fold concentrated) in PBS together with 60% W_{80}5EC nanoemulsion (NE) mucosal adjuvant (NanoBio Corporation, Ann Arbor, MI) at a 2:1 (volume : volume) ratio. NE is produced by high-speed emulsification of highly-refined soybean oil together with cetyl pyridinium chloride, Tween 80 and ethanol in water [22]. The nanoemulsion droplets have an average diameter of 450 nm. The final mucosal vaccine formulation consisted of 20% NE + ESAT-6 (2083 μg/ml) protein or NE + Ag85B (2083 μg/ml) protein, or a combination of both proteins with nanoemulsion (NE-TB vaccine) containing 20% NE + ESAT-6 (2083 μg/ml) + Ag85B (2083 μg/ml), such that I.N. administration of 12 μL total volume provided an antigen dose of 25 μg of each protein (ESAT-6/Ag85B) per animal as described below. As an adjuvant control, 20% NE also was prepared by mixing with PBS alone without addition of protein antigens.

Mycobacterium bovis Bacille *Calmette Guerin* (BCG Pasteur, Source: Trudeau Institute) and *Mycobacterium tuberculosis* strain HN878 (BEI Resources, Manassas, VA) were grown to mid-log phase in Proskauer Beck medium containing 0.05% Tween80 and frozen in 1 ml aliquots at −80 °C. BCG-vaccinated mice received 1×10^6 colony forming units (CFU) BCG subcutaneously (S.C.) [23]. The I.N. NE-TB vaccinations were carried out using a sterile pipette tip applied to the nares, and the mice were administered 12 μl (6 μl/nare) of the NE formulation containing 25 μg of antigen mixed with 20% NE. The NE-TB vaccine was delivered to 6–8-week-old B6 mice, three times at three week intervals, while mock-vaccinated mice received PBS as control. Some mice received S.C. BCG vaccination concurrently with NE-TB vaccine, or as a booster to BCG vaccination. Four weeks after the last booster immunization, mice were challenged by aerosol with a low dose (100 CFU) of *Mtb* strain HN878 (BEI Resources, Manassas, VA). Four weeks after challenge, unvaccinated and vaccinated mice were sacrificed by carbon dioxide (CO_2) asphyxiation, and the lungs were aseptically excised and individually homogenized in physiological saline solution. Serial dilutions of lung homogenates were plated on 7H11 agar for CFU and counted after 3 weeks of incubation at 37 °C as described before [24].

2.3. ELISpot assay

Antigen-specific IFNγ- and IL-17-producing cells in immunized lungs were detected by ELISpot assay as described [23]. Briefly, 2 weeks after the last immunization, single cell suspensions from lungs and spleens of immunized mice were seeded in antibody-coated plates at an initial density of 5×10^5 per well, n = 4–5 individual mice were used per group. Each well represented cells from an organ from each mouse. Irradiated syngeneic spleen cells (20 Gy), IL-2 (final concentration of 10 U/ml) in the presence of ESAT-6 or Ag85B proteins (10 μg/ml) were added to the cultures of ESAT-6 vaccinated and Ag85B vaccinated mice. Cells isolated from BCG vaccinated mice were restimulated with Ag85B protein, while cells from vaccinated mice receiving NE alone, NE + ESAT-6, or NE + Ag85B were stimulated with both Ag85B and ESAT-6 proteins. After 18 h, the cells secreting IFNγ or IL-17 were detected using BCIP/NBT (Sigma, St.Louis, MO) according to the manufacturer's instructions. The frequency of responding cells was calculated using ImmunoSpot software (Cellular Technology Limited, Shaker Heights, OH), and applied to the number of cells per sample to generate the total number of responding cells per organ. We have previously shown that neither cells cultured in the absence of peptide nor cells from uninfected mice produce detectable spots in ELISpot assays [10,25].

2.4. Evaluation of inflammatory lesions and formation of B cell follicles in vaccinated mice by bright field and fluorescent microscopy

Lungs from vaccinated and unvaccinated *Mtb*-infected mice were perfused with 10% neutral buffered formalin and embedded in paraffin. 5 μm paraffin lung sections were stained with hematoxylin and eosin, and percentage of area occupied by inflammatory cell infiltrates was calculated. Quantitation of inflammation was performed in a blinded fashion in individual upper right lobe

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