Rationalized design of a mucosal vaccine protects against *Mycobacterium tuberculosis* challenge in mice

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ABSTRACT

Pulmonary tuberculosis (TB) caused by *Mycobacterium tuberculosis* (*Mtb*) is a leading cause of global morbidity and mortality. The only licensed TB vaccine, *Mycobacterium bovis* bacillus Calmette-Guerin (BCG), has variable efficacy in protecting against pulmonary TB. Thus, the development of more effective TB vaccines is critical to control the TB epidemic. Specifically, vaccines delivered through the mucosal route are known to induce Th17 responses and provide superior protection against *Mtb* infection. However, already tested Th17-inducing mucosal adjuvants, such as heat-labile enterotoxins and cholera toxins, are not considered safe for use in humans. In the current study, we rationally screened adjuvants for their ability to induce Th17-polarizing cytokines in dendritic cells (DCs) and determined whether they could be used in a protective mucosal TB vaccine. Our new studies show that monophosphoryl lipid A (MPL), when used in combination with chitosan, potently induces Th17-polarizing cytokines in DCs and downstream Th17/Th1 mucosal responses and confers significant protection in mice challenged with a clinical *Mtb* strain. Additionally, we show that both TLRs and the inflammasome pathways are activated in DCs by MPL-chitosan to mediate induction of Th17-polarizing cytokines. Together, our studies put forward the potential of a new, protective mucosal TB vaccine candidate, which incorporates safe adjuvants already approved for use in humans. *J. Leukoc. Biol.* 101: 1373–1381; 2017.

Introduction

*Mtb*, the causative agent of TB, causes an estimated 1.1 million deaths and 9 million new cases each year [1]. Approximately one-third of the world’s population is latently infected with *Mtb* [1], with 5–10% of infected individuals developing active TB disease [1]. In addition, this public health threat has been confounded by the HIV/AIDS co-pandemic and the emergence of multidrug-resistant and extensively drug-resistant *Mtb* [2]. The only licensed TB vaccine currently in use, BCG, is a live attenuated vaccine that was passaged by Calmette and Guerin almost 100 yr ago. In most TB-endemic countries, BCG is administered intradermally shortly after birth [2]. Whereas BCG effectively protects against disseminated childhood TB, it has variable efficacy in protecting adolescents and adults against pulmonary TB [2]. Thus, the development of a more effective vaccine is a fundamental step in controlling the TB epidemic.

The transmission of *Mtb* occurs primarily through inhalation of fine droplets containing the bacilli from an infected individual. As the respiratory tract is the natural route of *Mtb* infection, mucosal vaccination is known to induce superior protection against *Mtb* challenge, when compared with parenteral routes of vaccination [3–6]. Enhanced protection by mucosal vaccination is thought to be a result of preferential trafficking of antigen-specific, vaccine-induced T cells back to the site of vaccination [7], possibly localizing within the airway lumen [6]. Thus, the development of a novel vaccine formulation, comprised of an immunodominant *Mtb* antigen(s) with an appropriate and safe mucosal adjuvant for use in humans, is crucial to improve current levels of vaccine-induced immunity against TB.

CD4+ Th cells producing IFN-γ have been conventionally thought to mediate protection against *Mtb* infection [8]. Accordingly, most of the TB vaccines in human clinical trials have evaluated IFN-γ production as a readout for vaccine efficacy [8]. However, despite induction of a strong IFN-γ response in humans [9], the first modern vaccine, modified

Abbreviations: "−/−" = deficient, ASC = adaptor protein apoptosis-associated speck-like protein containing caspase activation and recruitment domain, BCG = *Mycobacterium bovis* bacillus Calmette-Guerin, BMDC = bone marrow-derived dendritic cell, C57BL/6J = C57BL/6 J, CD = cluster of differentiation, cDMEM = DMEM with FBS, DC = dendritic cell, ESAT-6 = early secreted antigenic target 6 kDa protein, HLT = heat labile enterotoxin, IFN = interferon, Mtb = *Mycobacterium tuberculosis*, TLR = toll-like receptor

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vaccinia Ankara 85A, tested in humans did not improve vaccine efficacy conferred by BCG vaccination [10]. In recent times, we [7, 11–13] and others [14–16] have put forward the new concept that Th17 cells are critical for vaccine-induced immunity against TB. Importantly, mucosal vaccination with mucosal adjuvants, such as Escherichia coli HLTs [12] or cholera toxin [15], induces potent Th17 mucosal responses and mediates vaccine-induced protection. However, despite the ability of these mucosal adjuvants to induce protective Th17 responses [12, 15], there are serious concerns regarding the safety of using toxin subunits as mucosal adjuvants in humans. For example, Bell’s palsy has been observed following intranasal application of the vaccine Nasalalum, which contains E. coli HLTs as an adjuvant [17]. Therefore, we need to identify safe mucosal adjuvants that can induce Th17 mucosal responses and be incorporated into TB vaccines for mucosal delivery in humans.

Adjuvants can enhance the immunogenicity of vaccine antigens by eliciting a proinflammatory response by the recruitment of APCs to the mucosal site or exert their immunopotentiating effects by enhancing antigen presentation or inducing cytokine expression or by activation of mucosal APCs. In this study, we rationally screened adjuvants alone or in combination, for their ability to induce Th17-polarizing cytokines and function in a protective mucosal TB vaccine. Among these, adjuvants, such as chitosan [18], MPL [19], curdlan [20], poly I:C [21], R848 [22], CpG [23], and PGN [24, 25], have been shown to induce Th cell responses. MPL, curdlan, poly I:C, CpG, and R848 have also been tested for use in humans [26, 27]. Our new studies, described here, show that MPL (TLR-4 agonist), when used in combination with chitosan (inflammasmine stimulator), potently induces Th17-polarizing cytokines in DCs, downstream Th17 mucosal responses and confers significant protection upon challenge with a clinical HN878. Importantly, we have also identified that both TLRs and the inflammasmine pathways together are required for MPL-chitosan to induce Th17-polarizing cytokines to mediate adjuvant activity. Therefore, our studies have identified MPL-chitosan as a novel Th17-inducing mucosal adjuvant for use in a protective TB vaccine. As these individual adjuvant components have already been tested for human use, we propose the development of MPL-chitosan as a novel mucosal adjuvant amendable for future use in TB vaccines for humans.

MATERIALS AND METHODS

Mice

C57BL/6, IFN-γ−/− (The Jackson Laboratory, Bar Harbor, ME, USA), and IL-17−/−/− mice were bred under specific pathogen-free conditions at the Washington University in St. Louis. ESAT-6 TCR Tg mice on the recombinant-activating gene-deficient background [28] were obtained from the Trudeau Institute (Saranac Lake, NY, USA) and bred in-house. The NLRP3−/− and ASC−/− mice were provided by Dr. Uma Nagarajan from the University of North Carolina (Chapel Hill, NC, USA). The TLR4−/− and TLR2−/− mice were a generous gift from Drs. Kory Lavine and Laura Schuttpelz (Washington University in St. Louis, St. Louis, MO, USA), respectively. Mice maintained were used at 6–8 wk 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.

Generation and stimulation of BMDCs

BMDCs were generated from the bone marrow cells of mice, as we described previously [11]. In brief, cells were extracted from femurs and cultured in cDMEM containing 20 ng/ml rmGM-CSF (PeproTech, Rocky Hill, NJ, USA). Cells were cultured for 5 d, after which, an additional 10 ml fresh cDMEM containing 20 ng/ml rmGM-CSF was added. On d 7, the nonadherent cells (BMDCs) were harvested by vigorous pipetting and enriched by centrifugation. Cells were resuspended (1 × 10̇6 cells/ml) in cDMEM, and 500 μl aliquots were seeded into 24-well tissue-culture plates and rested overnight. DCs were subsequently cultured in the presence of various adjuvants (chitosan 25 μg/ml or/and MPL 25 μg/ml, curdlan 25 μg/ml, poly I:C 25 μg/ml, R848 25 μg/ml, CpG 25 μg/ml, PGN 20 μg/ml) or in medium alone. Culture supernatants were harvested for cytokine analysis at 48 h post-treatment.

Generation of ESAT-6-Tg T cells

A single-cell suspension from lymph nodes and spleens from Mtb-specific ESAT-6 T cell Tg (ESAT-6 TCR Tg) mice were provided as described [11]. The single-cell suspension was treated with Gey’s solution to remove any residual RBC, and CD4+ T cells were isolated using a CD4+ T Cell Isolation Kit (Miltenyi Biotec, San Diego, CA, USA). For response of resting T cells from ESAT-6 TCR Tg mice, the isolated naive T cells were subsequently cultured in complete IMDM containing IL-2 (10 U/ml) and ESAT-6–20 (10 μg/ml) and cocultured with BMDCs stimulated with MPL-chitosan for 6 d. Supernatants were harvested for cytokine analysis.

Detection of cytokines in culture supernatants

Culture supernatants were assayed for multiple cytokines, either using Milliplex (EMD Millipore, Billerica, MA, USA) or for single cytokine proteins, using Duoset ELISA (R&D Systems, Minneapolis, MN, USA), according to recommended standard protocols.

Vaccinations, Mtb infection, and determination of bacterial load

Adjuvants were obtained from commercial vendors: MPL (Avanti Polar Lipids, Alabaster, AL, USA); poly I:C (Sigma, St. Louis, MO, USA); chitosan, curdlan, R848, and CpG (InvivoGen, San Diego, CA, USA); and PGN (BEI Resources, Manassas, VA). ESAT-6–20 peptide was obtained from New England Peptide (Gardiner, MA, USA). For intranasal vaccinations, vaccine suspension was prepared with chitosan and MPL (50 μg each), mixed along with ESAT-6–20 peptide (133 μg), and delivered by the intranasal route. MPL-chitosan-ESAT-6 vaccine thus prepared was delivered to 6–8 wk old C57BL/6/J, IL-17−/−, or IFN-γ−/− mice, 3 times at 2 wk intervals, whereas mock-immunized mice received PBS as control. Four weeks after the last booster immunization, mice were challenged by aerosol with Mtb strain HN878 (BEI Resources). BCG (BCG Pasteur; Trudeau Institute) and Mtb strain HN878 (BEI Resources) were grown to mid-log phase in Proskauer Beck medium containing 0.05% Tween 80 and frozen in 1 ml aliquots at −80°C. Mice were vaccinated with 1 × 10̇6 CFUs BCG subcutaneously as relevant controls [11]. Four weeks after challenge, unvaccinated and vaccinated mice were euthanized by CO2 asphyxiation, and the lungs were aseptically excised and individually homogenized in physiologic saline solution. Serial dilutions of lung homogenates were plated on 7H11 agar for CFU and counted after 5 wk of incubation at 37°C, as described before [28].
**ELISPOT assay**
Antigen-specific IFN-γ and IL-17-producing cells in immunized lungs were detected by ELISPOT assay, as described [11]. In brief, 2 wk after the last immunization, lung single-cell suspension from immunized mice was seeded in antibody-coated plates at an initial density of 5 × 10⁶/well. Irradiated syngeneic spleen cells (2500 RADS), IL-2 (final concentration of 10 U/ml), in the presence or absence of ESAT-61 peptide (10 µg/ml), were added to the cultures. After 18 h, the cells secreting IFN-γ or IL-17 were detected using 5-bromo-4-chloro-3-indolyl phosphate/NBT chloride (Sigma), according to the manufacturer’s instructions. The frequency of responding cells was calculated using ImmunoSpot software, and the total number of cytokine-producing cells was determined (Cellular Technology Limited, Shaker Heights, OH, USA).

**Evaluation of inflammatory lesions and formation of B cell follicles in vaccinated mice by bright field and fluorescence microscopy**
Lungs from vaccinated and unvaccinated Mtb-infected mice were perfused with 10% neutral-buffered formalin and embedded in paraffin. Paraffin lung sections (5 µm) were stained with H&E, and percentage of area occupied by inflammatory cell infiltrates was calculated with an automated tool of the Zeiss Axioplan microscope. Serial sections of 5 µm paraffin-embedded lung tissues were also stained with primary antibodies specific for CD3 (clone M-20; Santa Cruz Biotechnology, Dallas, TX, USA) and biotinylated antibodies against CD45R/B220 (clone RA3-6B2; BD Biosciences, San Jose, CA, USA). To visualize the B cell follicles and T cells inside TB granulomas, we incubated lung sections with Alexa Fluor 568 donkey anti-goat IgG (A11057; Thermo Fischer Scientific), and representative pictures were taken with a Zeiss Axioplan microscope and recorded with a Hamamatsu camera. Morphometric analysis of B cell follicles was performed with the outline automated tool of the Zeiss Axioplan microscope.

**Statistical analysis**
Statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). For experiments with 2 groups, 2-tailed Student’s t-tests were performed. For 2 or more groups, a one-way ANOVA was used.

**RESULTS**

**MPL and chitosan synergistically induce Th17-polarizing cytokines, while inducing low levels of the anti-inflammatory cytokine IL-10**
We [7, 12] and others [16] have demonstrated that mucosal vaccine-induced protection against Mtb may be IL-17 dependent. However, as a result of safety concerns, no currently available mucosal adjuvants that potently induce Th17 responses in animal models are licensed for use in human TB vaccines. As mucosal DCs are likely the key APCs upon mucosal vaccination, we first rationally screened adjuvants known to induce Th17 cell-driving, polarizing cytokines, such as IL-1β, IL-6, and IL-23, in DCs in vitro. Among these, adjuvants, such as chitosan [18], MPL [19], curdlan [20], poly I:C [21], R848 [22], CpG [23], and PGN [24, 25], are thought to induce Th17-polarizing cytokines in DCs. Chitosan has both mucoadhesive and adjuvant properties and has been tested in human vaccine formulations [27]. Thus, we treated BMDCs with MPL, poly I:C, curdlan, R848, CpG, PGN, or chitosan individually and determined whether treatment induced the production of Th17-polarizing cytokines, such as IL-1β, IL-6, and IL-23. Our results show that treatment with MPL, poly I:C, curdlan, R848, CpG, and PGN alone did not consistently induce high levels of IL-1β. However, the adjuvants MPL, poly I:C, and curdlan induced high levels of IL-6 production in DCs (Fig. 1A and B). Incidentally, we found that treatment of DCs with chitosan by itself did not induce either high levels of IL-1β or IL-6. However, treating with either chitosan or MPL alone induced IL-23 levels in treated DCs (Fig. 1C). Thus, we next addressed whether cotreatment with chitosan, along with MPL, curdlan, or poly I:C, induced higher levels of Th17-polarizing cytokines than individually tested adjuvants. Whereas individual treatment with the above adjuvants induced low levels of IL-1β production, we found that cotreatment with chitosan induced significantly higher production of IL-1β in culture supernatants (Fig. 1A). In contrast, treatment with different adjuvants, along with chitosan, had no effect on IL-6 production, as individual adjuvants by themselves induced high levels of IL-6 production (Fig. 1B). However, whereas chitosan or MPL treatment alone induced IL-23 production in treated DCs, when poly I:C and R848 treatment was combined with chitosan, these treatments also induced IL-23 production in DCs (Fig. 1C). Although R848 treatment, in combination with chitosan, induced a significant increase in IL-1β and IL-23 levels in DCs, neither R848 treatment alone nor in combination with chitosan induced high IL-6 levels (Fig. 1A–C). IL-12p70 levels were below the threshold of detection for all samples tested. Together, our data suggest that cotreatment of TLR adjuvants, along with chitosan, may benefit the induction of Th17-polarizing cytokines, such as IL-1β and IL-23.

IL-10 is an anti-inflammatory cytokine that dampens Th17 responses in vaccine-induced immunity to Mtb infection [11, 30]. Thus, an ideal TB vaccine adjuvant should not induce high levels of IL-10. Therefore, we also measured IL-10 levels in culture supernatants of adjuvant-treated DCs. Curdlan treatment produced a significant increase in IL-10 levels in DCs, but this effect was dampened in combination with chitosan (Fig. 1D). Treatment of DCs with poly I:C, R848, CpG, and PGN resulted in the induction of low levels of IL-10. Combined treatment of DCs with chitosan and poly I:C produced a marginal increase of IL-10 (Fig. 1D). However, MPL, despite induction of strong Th17-polarizing cytokines (Fig. 1A–C), did not induce high IL-10 production in DCs alone or in combination with chitosan (Fig. 1D). Therefore, treatment with chitosan and MPL, in a synergistic manner, induced IL-1β and IL-23 production by DCs, whereas induction of IL-6 was solely dependent on MPL. Importantly, this treatment combination did not induce the anti-inflammatory cytokine IL-10 in DCs. Thus, we rationally selected MPL-chitosan as an adjuvant combination that may favor subsequent Mtb-specific Th17 responses in vitro and in vivo.

**MPL-chitosan treatment induces Th17-polarizing cytokines in DCs through a TLR-2/4-dependent and NLRP3-independent inflammasome pathway**
MPL is a potent TLR-4 agonist [31], whereas chitosan is thought to activate the inflammasome in LPS-stimulated macrophages by a mechanism dependent on phagocytosis [32]. To address mechanistically the signaling pathways in DCs that were involved...
in induction of Th17-polarizing cytokines upon MPL-chitosan treatment, we generated BMDCs from TLR-2/2 and TLR-4/2 mice, as well as mice deficient in inflammasome pathways, namely ASC/2 and NLRP3/2 [33]. We found that upon treatment with MPL-chitosan, NLRP3/2 BMDCs still produced substantial IL-1b, but IL-1b responses were substantially blunted in ASC/2 DCs (Fig. 2A). These results indicate that MPL-chitosan activates ASC inflammasome in an NLRP3-independent manner to induce IL-1b secretion. In addition, both TLR-2 and TLR-4 are critical for induction of IL-1b production following MPL-chitosan treatment, suggesting TLR-mediated activation of inflammasome (Fig. 2A). IL-6 production, induced following treatment with MPL-chitosan, was inflammasome independent, as both NLRP3/2 and ASC/2 DCs induced high levels of IL-6 upon treatment with MPL-chitosan (Fig. 2B). In sharp contrast, IL-6 production, following treatment with MPL-chitosan, was dependent on the presence of TLR-2 and TLR-4. In TLR-2/- as well as TLR-4/- BMDCs, IL-6 production was decreased significantly (Fig. 2B), suggesting that MPL-chitosan signals through both TLR-2 and TLR-4, and the loss of 1 receptor was sufficient to result in loss of IL-6 induction. Thus, our studies provide support that IL-6, induced upon MPL-chitosan treatment, is TLR-2/4 dependent and independent of the inflammasome, whereas IL-1b secretion is dependent on the ASC inflammasome activation, as well as through the TLR-2/4 pathways.

**MPL and chitosan treatment in DCs induces IL-17 and IFN-γ production in T cells**

As MPL-chitosan treatment induces Th17-polarizing cytokines, such as IL-1b, IL-6, and IL-23, in DCs, we next determined whether the treatment of DCs with the adjuvant combination will drive the differentiation of naive CD4+ T cell toward a Th17-differentiation pathway. Thus, DCs treated with MPL-chitosan were cocultured with naive CD4+ T cells isolated from Mtb-specific TCR Tg mice (ESAT-6 TCR Tg mice) [29], and ESAT-6 antigen in vitro. When naive CD4+ TCR Tg T cells were cultured with MPL-chitosan-primed DCs, we found that MPL-chitosan-treated DCs induced substantial IL-17 production, whereas IFN-γ levels were lower in T cell culture.
supernatants (Fig. 3A). However, when previously primed ESAT-6 TCR Tg T cells were rested in vitro and then restimulated with MPL-chitosan-treated DCs, we found that IFN-γ and IL-17 levels were substantially increased in restimulated *Mtb*-specific TCR Tg T cells (Fig. 3A). Thus, as MPL-chitosan treatment of DCs was able to drive the differentiation of naive T cells toward the Th17-differentiation pathway, along with induction of Th1 responses, we next determined whether MPL-chitosan, when used as an adjuvant, along with ESAT-61–20 antigen, will drive mucosal Th17 responses in vivo in vaccinated mice. C57BL/6J mice were mucosally vaccinated with the ESAT-61–20 MHC class II-restricted peptide, along with MPL-chitosan, and then boosted twice. We found that mucosal vaccination of ESAT-6 in MPL-chitosan potently induced a population of Th17 mucosal responses, whereas induction of ESAT-6-specific Th1 responses in the lung was limited (Fig. 3B). In contrast, both *Mtb*-specific Th1 and Th17 responses were induced in the spleen (Fig. 3B). Unvaccinated mice did not induce any responses (data not shown). Cells isolated from lungs and spleens and not stimulated with antigen in vitro did not induce cytokine responses (Fig. 3B). These in vivo results are consistent with our in vitro data showing that MPL-chitosan treatment of DCs can drive both Th1 and Th17 responses. Importantly, mucosal vaccination with ESAT-6 in MPL-chitosan preferentially induced Th17 responses, specifically in the lung, whereas inducing Th17/Th1 responses in the spleen.

MPL-chitosan mucosal TB vaccine protects upon challenge with *H. N. 878*

Our rationalized screening of adjuvants has identified MPL-chitosan as a potential mucosal adjuvant candidate for use in TB vaccines. Thus, we mucosally vaccinated C57BL/6J mice with the immunodominant *Mtb* antigen ESAT-61–20 in MPL-chitosan, followed by 2 boosts. In addition, a control group of mice was parenterally vaccinated with the gold standard TB vaccine, BCG. Vaccinated and unvaccinated mice were rested for 4 wk and then challenged with a low dose of aerosolized hypervirulent clinical *Mtb* strain HN878. As expected, BCG vaccination resulted in significant protective efficacy upon *Mtb* challenge (Fig. 4A). Importantly, we found that mucosal vaccination with ESAT-61–20 in MPL-chitosan also induced significant protection in the lung upon *Mtb* challenge compared with protection induced with BCG vaccination (Fig. 4A). In addition, mice mucosally vaccinated with ESAT-61–20 in MPL-chitosan, upon *Mtb* challenge, did not show increased lung inflammation compared with unvaccinated mice (Fig. 4B). In contrast, mice vaccinated with ESAT-61–20 in MPL-chitosan developed larger aggregates of B cell follicle-containing granulomas (Fig. 4C), a correlate associated with protective outcomes during vaccine responses following *Mtb* challenge [12, 34]. IL-17 has been shown to be important in mediating immunity against TB in mucosal vaccination models [12, 16]. To address if MPL-chitosan mucosal vaccine-induced protection was dependent on the production of IL-17 or IFN-γ, IL-17−/− and IFN-γ−/− mice were vaccinated with ESAT-61–20 in MPL-chitosan, rested, and challenged with *Mtb* HN878. Whereas vaccine-induced protection occurred in C57BL/6J–vaccinated mice, vaccine-induced protection was lost in the absence of IFN-γ or IL-17, suggesting an important role for both cytokines in MPL-chitosan TB vaccine-mediated, vaccine-induced protection (Fig. 4D). Thus, our study has identified use of MPL-chitosan as a new and safe mucosal adjuvant for use in a protective TB vaccine and mediates protection through the Th17/Th1 pathways.

**DISCUSSION**

TB continues to persist as a global health concern, and the emergence of drug-resistant strains of *Mtb* has further heightened the spectre of TB. The currently licensed BCG vaccine provides variable efficacy against adult pulmonary TB [35]. Thus, there have been concerted efforts to develop novel vaccines for TB that will provide improved protection upon *Mtb* challenge. Especially important in our search for novel TB vaccines is the development of potent mucosal TB vaccines, as mucosal routes of vaccination confer superior protection upon *Mtb* challenge [3–6]. Thus, the identification of safe mucosal adjuvants that can be incorporated into TB vaccines for use in humans is an
important focus area of vaccine research. In the current study, we show that the use of rationalized screening of Th17-inducing adjuvants resulted in the identification of MPL-chitosan as an adjuvant that can drive potent induction of Th17-polarizing cytokines in DCs, subsequent mucosal Mtb-specific Th17 responses in the lung, and mixed Th17/Th1 responses in the spleens of vaccinated mice. In addition, we show that vaccination with Mtb-specific antigen in MPL-chitosan confers protection in a mouse model of TB to levels of protection comparable with the gold standard vaccine, BCG. As both MPL and chitosan have already been individually tested safe for use in humans, our study has identified a novel use for MPL and chitosan as a combined mucosal adjuvant for use in TB vaccines, which can be developed for human use.

In an attempt to identify mucosal adjuvants that can induce T cell responses, we found that combined use of MPL and chitosan together can induce strong Th17/Th1 responses both in vitro and in vivo. Chitosan has both mucoadhesive and adjuvant properties [27], whereas MPL, a nontoxic derivative of LPS, exhibits adjuvant properties similar to those of the parent LPS molecule [36]. MPL functions as an adjuvant through the induction of chemokines, such as CCL-2 and CCL-3, resulting in the recruitment of APCs for antigen presentation [36]. In use by itself, MPL is considered a potent Th1-inducing adjuvant [37]. Furthermore, MPL-induced IL-6 in APCs is also thought to promote T cell differentiation into T follicular helper-like cells and drive antibody responses [38]. Our data show that combined use of MPL and chitosan together drives improved induction of IL-1β, IL-6, and IL-23, key cytokines involved in Th17 cell differentiation. In contrast, MPL and chitosan, individually and in combination, do not induce the anti-inflammatory cytokine IL-10, which can adversely limit Th17/Th1 responses [11]. Accordingly, DCs treated with MPL-chitosan are potent at driving naive T cells to differentiate into Th17/Th1 cells, both in vitro and in vivo, when formulated and delivered as a mucosal vaccine. Consistent with a role for MPL in driving Th1 responses [37], we observed IFN-γ induction in T cells cocultured with MPL-chitosan-treated DCs. Indeed, in
vivo, we found that mucosal delivery of Mtb antigen in MPL-chitosan also induced Th1 responses in the spleen but not in the lung. This differential localization of Th1 and Th17 cells may be associated with differential expression of chemokine receptors, such as CCR4, that allow homing of Th17 cells but not Th1 cells to mucosal sites [7]. Thus, our studies, in a rationalized manner, have identified that combined use of both MPL and chitosan together can serve as potent Th17/Th1-inducing adjuvants for use in TB vaccines. As IL-17A-/- and IFN-γ-/- vaccinated mice are not protected upon challenge with Mtb, our data suggest that Th17/Th1 responses are both required to mediate vaccine-induced protection against TB.

Chitosan has been reported to activate the inflammasome through an NLRP3-dependent pathway following phagocytosis in macrophages [32]. This activation of the inflammasome in macrophages was dependent on its acetylation and particle size, with smaller particles inducing a greater response [32]. However, we observe that in DCs, chitosan treatment by itself fails to induce IL-1β. However, combined treatment of chitosan with MPL induces substantial IL-1β induction in wild-type and NLRP3-/- DCs, whereas ASC-/- DCs showed reduced levels of IL-1β induction. These results could be a result of differential inflammasome activation in DCs by MPL-chitosan when compared with activation in macrophages by chitosan [32]. Thus, our data support a role for an NLRP3-independent, ASC-dependent inflammasome pathway in IL-1β induction when both TLR-2/4 engagement is activated through the combined use of MPL-chitosan. These effects could be a result of direct activation of the inflammasome pathway by TLR signaling without priming by IL-1R-associated kinase 1 [39] or through the involvement of another NLR protein. In contrast, induction of IL-6 by combined MPL-chitosan treatment does not depend on either NLRP3- or ASC-dependent inflammasome pathways. Instead, induction of IL-6 is mediated via the engagement of TLR-2 and TLR-4. Consistent with these results, although MPL is primarily considered a potent TLR-4 agonist, MPL can also engage TLR-2 to induce proinflammatory cytokines in human monocytes [40]. Thus, our studies project the involvement of both the inflammasome and TLR pathways in induction of Th17-polarizing cytokines upon MPL-chitosan treatment in DCs.

Our studies show that mucosal delivery of Mtb antigen in MPL-chitosan induces protective Th17/Th1 vaccine responses and upon challenge with a hypervirulent clinical Mtb strain, induces formation of protective TB granulomas, immune correlates that we have shown previously to be important for protective vaccine responses [12, 13]. MPL, used in this study, is a synthetic derivative but with a highly acceptable safety profile, making it suitable for use as a mucosal vaccine adjuvant in humans [41]. MPL has been approved as a component in an improved hepatitis B vaccine and other vaccines that are in various stages of clinical testing [41]. The intranasal administration of TLR-4 agonists has also shown that the mucosal route is safe and effective and may reduce possible safety concerns of systemic administration of TLR agonists. Chitosan has been designated as “generally recognized as safe” in the United States, Japan, and Italy, although it has not been used in any marketed vaccine formulations [42]. However, a few recent studies have elucidated upon the clinical applications of chitosan as an adjuvant. For example, the safety and immune-stimulatory capacity of a chitosan-glutamate intranasal delivery system for the diphtheria toxoid antigen CRM197, among healthy volunteers, has been reported [43]. Furthermore, a vaccine formulated with chitosan, Norovirus virus-like particle antigen and MPL as an immune enhancer, was administered intranasally in clinical trials, and no vaccine-related serious adverse effects were observed [44]. Based on these safety profiles, our studies project the combined use of MPL and chitosan as a potential adjuvant candidate for mucosal vaccine formulations against TB. Additionally, as Th17 vaccine responses are critical for protection against other significant pulmonary pathogens, such as Streptococcus pneumonia [45, 46], Bordetella pertussis [47, 48], Klebsiella pneumonia [49], and Pseudomonas aeruginosa [50], identification of safe and potent Th17-inducing adjuvants, such as MPL-chitosan, can be incorporated into effective mucosal vaccines against several important pulmonary diseases.

In summary, in the current study, we haverationally screened adjuvants and identified combined use of MPL and chitosan as a novel formulation for use as a mucosal adjuvant to generate protective immune responses against Mtb infection. The fact that this vaccine formulation is protective against emerging, clinically relevant Mtb strains, such as the HN878 strain, further supports the use of MPL-chitosan as a potent Th17-inducing adjuvant for mucosal use in human TB vaccines. Further investigations and trials with primate models will be very crucial toward achieving higher goals and translation of MPL-chitosan for use in a human TB vaccine.

AUTHORSHIP


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DISCLOSURES
The authors declare no conflicts of interest.

REFERENCES

KEY WORDS: tuberculosis • Th17/Th1 responses • mucosal immunity • adjuvants