Comparative Analysis of *Bacillus subtilis* Spores and Monophosphoryl Lipid A as Adjuvants of Protein-Based *Mycobacterium tuberculosis*-Based Vaccines: Partial Requirement for Interleukin-17A for Induction of Protective Immunity

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The development of adjuvants for vaccines has become an important area of research as the number of protein-based vaccines against infectious pathogens increases. Currently, there are a number of adjuvant-based *Mycobacterium tuberculosis* vaccines in clinical trials that have shown efficacy in animal models. Despite these novel adjuvants, there is still a need to design new and more versatile adjuvants that have minimal adverse side effects but produce robust long-lasting adaptive immune responses. To this end, we hypothesized that *Bacillus subtilis* spores may provide the appropriate innate signals that are required to generate such vaccine-mediated responses, which would be sufficient to reduce the mycobacterial burden after infection with *M. tuberculosis*. In addition, we compared the response generated by *B. subtilis* spores to that generated by monophosphoryl lipid A (MPL), which has been used extensively to test tuberculosis vaccines. The well-characterized, 6-kDa early secretory antigenic target of *M. tuberculosis* (ESAT-6; Rv3875) was used as a test antigen to determine the T cell activation potential of each adjuvant. Inoculated into mice, *B. subtilis* spores induced a strong proinflammatory response and Th1 immunity, similar to MPL; however, unlike MPL formulated with dimethyldioctadecylammonium (DDA) bromide, it failed to induce significant levels of interleukin-17A (IL-17A) and was unable to significantly reduce the mycobacterial burden after pulmonary infection with *M. tuberculosis*. Further analysis of the activity of MPL-DDA suggested that IL-17A was required for protective immunity. Taken together, the data emphasize the requirement for a network of cytokines that are essential for protective immunity.

*Mycobacterium tuberculosis* continues to be a worldwide public health problem, and there is good evidence to suggest that it will continue to affect human morbidity and mortality for many more years (1). The emergence of drug resistance has intensified the need to develop new vaccines, drugs, and diagnostics, and thus research in all of these areas has grown during the past decade. Currently, there is a pipeline of vaccines that are at various stages of preclinical and clinical development (2). These novel vaccines are intended to either replace or boost the existing antituberculosis vaccine, live attenuated *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), that has been in use since the early part of the 20th century (3). The use of BCG during this time has had limited effect on the spread of the disease, and its efficacy has been documented to vary from 0 to 80%, depending on the location and strain used by each country (4). However, BCG is still used as a neonatal vaccine in the vaccination programs of many countries as it affords a limited amount of protection, particularly for infants (5).

Of the vaccines that are currently under development, several are based on a polyprotein or fusion protein formulated with an adjuvant. Such adjuvants, including AS01/2 (6), IC31 (7), and GLA-SE (8), have been shown in animal models to induce a strong Th1 immune response that is required for the induction of protective immunity (9). Whether these adjuvants will prove to be capable of inducing the appropriate protective immunity will be determined only in clinical trials.

*Bacillus subtilis* is a Gram-positive endospore-forming bacterium, the spores of which have been used by other investigators as an adjuvant against viral (10), bacterial (11), and parasitic diseases (12), and therefore it was of interest to determine if it could be used as an adjuvant for tuberculosis vaccines. Based on previous studies, spores used as adjuvants were shown to increase antibody and T cell responses to a coadministered soluble antigen (Ag), including both antigen-specific CD4⁺ and CD8⁺ T cell responses, as well as complement- and non-complement-fixing antibody isotypes (13). Thus, we hypothesized that *B. subtilis* spores could function as an adjuvant for the development of a protective immune response to infection with *M. tuberculosis*. For comparison we were also interested in determining if spores could in fact perform as well as monophosphoryl lipid A (MPL), which has been used by us and other investigators to test novel vaccine antigens against tuberculosis (14, 15). In fact, MPL is a constituent of several adjuvants for vaccines currently in clinical trials, and thus it was also of interest to determine its mechanism of action in this vaccine formulation. For the current studies, *B. subtilis* spores were formulated with the *M. tuberculosis* antigen ESAT-6, and MPL was formulated with dimethyldioctadecylammonium bromide.
mide (DDA) and ESAT-6; both the spore and MPL formulations were administered via the subcutaneous (s.c.) route. ESAT-6 was chosen as an antigen as it is immunogenic and has been well characterized in the mouse model by multiple groups (16, 17) and therefore represents a good model antigen with which to compare the two adjuvants. When used to inoculate C57BL/6 mice, B. subtilis spores induced a strong proinflammatory response, characterized by significantly elevated gamma interferon (IFN-γ)-producing T cells, similar to the response observed with MPL. A significant difference between the two adjuvants was the ability of MPL to induce Th17 cells and the inability of the spores to reduce the mycobacterial burden in mice after pulmonary infection. Upon further analysis, we showed that interleukin 17A (IL-17A) plays a role in the response that is required to prime immune cells to enhance the protective capacity of a vaccine.

MATERIALS AND METHODS

Animals. Pathogen-free, female, 6- to 8-week-old C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained in an animal biosafety level 3 facility at Colorado State University (CSU) with sterile chow and water ad libitum. The Colorado State University Animal Care and Use Committee approved all experimental procedures.

Reagents. B. subtilis strain PA3 was obtained from the ATCC (catalog number 55567; Manassas, VA). Monophosphoryl lipid A (MLP) and di-methylolhexadecylammonium bromide (DDA) were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant ESAT-6 protein was obtained through the NIH/NIAID TB Vaccine Testing and Research Materials contract at CSU (HHSN266200400091c). Low-dose aerosol infection with M. tuberculosis. Mice were infected with virulent M. tuberculosis H37Rv (TMCC 102) via the aerosol route using a Glas-Col Airborne infection exposure system (Glas-Col, Terre Haute, IN), which delivered approximately 100 CFU of M. tuberculosis bacilli per mouse. Four weeks postinfection the CFU counts in the lung and spleen were evaluated by plating serial 10-fold dilutions of the lung homogenates on Middlebrook 7H11 agar plates. Plates were incubated at 37°C for 21 days, and colonies were counted.

Mouse inoculations. For the initial characterization of the effect of spores on the immune response, C57BL/6 mice (5 per group) were inoculated twice at a 15-day interval via the subcutaneous (s.c.) route with recombinant ESAT-6 protein (10 μg/mouse) alone or combined with 104 spores, 105 spores, or 106 spores or with phosphate-buffered saline (PBS). Mice were sacrificed on either day 15 or 30 after the final inoculation to determine the immune response. For studies in which the activity of B. subtilis spores was compared to that of MPL, C57BL/6 mice (5 per group) were inoculated twice at a 15-day interval via the s.c. route with PBS, MPL/DDA, 105 B. subtilis spores, or ESAT-6 protein alone (10 μg/mouse) as controls and ESAT-6 combined with either 25 μg of MPL plus 250 μg of DDA (14) or 105 B. subtilis spores. The mice were sacrificed on day 15 after the final inoculation to determine the immune response.

Lymphocyte isolation and in vitro antigen stimulation. Mice were humanely euthanized, and the spleens were removed and placed into RPMI 1640 medium (Life Technologies Corporation, Grand Island, NY). Single-cell suspensions were prepared as described previously and cultured (19), and cells were stimulated with or without antigen (ESAT-6 at 5 μg/ml or spore proteins at 5 μg/ml). The culture supernatants were collected after 24 h and stored at −80°C until used.

Analysis of ESAT-6-specific IFN-γ- and IL-17A-secreting T cells by enzyme-linked immunospot (ELISPOT) assay. Isolated splenocytes, at 1 × 106 or 2 × 105 cells per well, were seeded into a 96-well polyvinylidene difluoride (PVDF) microplate (EMD Millipore Corp.) that had been coated overnight with an anti-mouse IFN-γ or IL-17A capture antibody according to the manufacturer’s protocol (eBioscience, San Diego, CA). The cells were incubated at 37°C in 5% CO2 for 24 h with or without ESAT-6. The plate was then washed and incubated with cytokine-specific biotin-conjugated antibody and developed using avidin-conjugated horseradish peroxidase (HRP) followed by aminoethylidextran (AED) substrate solution (eBioscience). The number of IFN-γ or IL-17A spot-forming cells was determined using a Series 5 UV-Immunospot Analyzer from Cellular Technology, Ltd. (Shaker Heights, OH).

Ag-specific cytokine production. The levels of IL-1β, IL-2, IL-6, IL-10, and tumor necrosis factor alpha (TNF-α) in the supernatants were measured using a mouse-specific enzyme-linked immunosorbent assay (ELISA) kit (eBioscience). The stored samples were thawed, and the manufacturer’s protocol was used to determine the concentration. The sensitivity of cytokine detection was as follows: IL-1β, 8 pg/ml; IL-2, 2 pg/ml; IL-6, 4 pg/ml; IL-10, 30 pg/ml; and TNF-α, 8 pg/ml.

Low-dose aerosol infection with M. tuberculosis. Mice were infected with virulent M. tuberculosis H37Rv (TMCC 102) via the aerosol route using a Glas-Col Airborne inhalation exposure system (Glas-Col, Terre Haute, IN), which delivered approximately 100 CFU of M. tuberculosis bacilli per mouse. Four weeks postinfection the CFU counts in the lung and spleen were evaluated by plating serial 10-fold dilutions of the lung homogenates on Middlebrook 7H11 agar plates. Plates were incubated at 37°C for 21 days, and colonies were counted.

In vivo depletion of IL-17A. Mice inoculated twice with ESAT-6/ MPL/DDA as described above were treated at 1 day prior to and 2 days after each inoculation with anti-IL-17A antibody (clone 17F3) or an isotype control antibody (Bio X Cell, West Lebanon, NH). A cohort of mice was analyzed for T cell activation by ELISPOT assay, and the remaining mice were infected with M. tuberculosis as described above.

Data analysis. Data were initially analyzed for normality, after which one-way analysis of variance (ANOVA) was performed. If data met the normality assumption, then the one-way ANOVA was performed with a Bonferroni t test posttest. If data did not meet the normality assumption, a Kruskal-Wallis one-way ANOVA on ranks test was performed, with Dunn’s multiple comparison posttest for all pairwise comparisons. For comparison between two groups, a Student t test was used. SigmaStat, version 3.5 (Systat Software, Inc., Germany), was used for all analyses.

RESULTS

B. subtilis spores act as an adjuvant to induce Th1 cell immunity in vivo. In vitro analysis of the ability of B. subtilis spores to induce Th1 immunity was initially performed using bone marrow-derived dendritic cells. The data showed that when cells were stimulated with spores, there was an increase in the concentration of IL-12 within 6 h (see the supplemental material). To determine if
**FIG 1** *B. subtilis* spores induce a Th1 immune response to ESAT-6 protein at 15 days postinoculation. Mice were immunized s.c., twice with ESAT-6 alone or with ESAT-6 mixed with 10^5 or 10^6 spores (SP). Spleen cells were isolated and cultured with ESAT-6 protein to determine the number of spot-forming units (SFU) by ELISPOT assay. After 24 h supernatants were also collected and stored at −80°C until used. The number of IFN-γ-releasing cells was determined by ELISPOT assay (A), and IL-2 was measured by ELISA (B). Data are the median values + ranges of triplicate samples from five mice per group. *, P < 0.05.

*B. subtilis* spores were capable of inducing a Th1 immune response *in vivo*, C57BL/6 mice were inoculated with a formulation of either 10^5 or 10^6 spores plus the *M. tuberculosis* antigen ESAT-6. At day 15 postinfection, ELISPOT analysis for the production of IFN-γ was used as a measure of Th1 T cell activation in spleen cells of inoculated mice (Fig. 1A). Cells were cultured with ESAT-6 for 24 h. A Kruskal–Wallis one-way ANOVA on ranks test was used to determine if there were statistical differences between groups. Our data demonstrate that there was a significant difference between mice treated with ESAT-6 plus 10^5 spores and both PBS-treated mice (P < 0.05) and mice inoculated with ESAT-6 only (P < 0.05). In addition, inoculation with ESAT-6 plus 10^5 spores induced significantly greater numbers of IFN-γ-producing cells than PBS treatment (P < 0.05) but not ESAT-6-only treatment. There was no significant difference between groups receiving ESAT-6 with either 10^5 or 10^6 spores although there was a trend toward more spot-forming units in the group receiving 10^6 spores. To determine if inoculation of spores also enhanced T cell proliferation, cell culture supernatants were examined for IL-2 secretion, and cell culture supernatants were examined for IL-2 production by ELISA (Fig. 1B). There was no significant difference between the two groups inoculated with ESAT-6 plus spores; however, these groups produced significantly greater concentrations of IL-2 than the group vaccinated with ESAT-6 only or PBS (P < 0.05). Supernatants were also examined for TNF-α concentration (data not shown). Taken together, the data suggest that *B. subtilis* spores were capable of generating a potent and productive Th1 immune response.

To further analyze the ability of *B. subtilis* spores to induce Th1 immunity, we repeated the above study, but on this occasion, a group of mice that was inoculated with ESAT-6 plus 10^5 spores was included to determine if there was a dose effect. The immune response was then examined at day 30 postinoculation by taking spleen cells from mice and stimulating them with ESAT-6. Analysis of IFN-γ by ELISpot assay (Fig. 2A) indicated that there were significantly greater numbers of IFN-γ-secreting cells in the mice inoculated with ESAT-6 plus 10^5, 10^6, and 10^7 spores than in the mice inoculated with either PBS or ESAT-6 only (P < 0.05). There was no difference between groups inoculated with ESAT-6 and spores, suggesting that there was no dose effect in relation to the magnitude of the immune response. We next examined IL-2 concentrations from the supernatants of stimulated spleen cells (Fig. 2B). The concentration of IL-2 in mice inoculated with ESAT-6 plus either 10^5 or 10^6 spores and stimulated with ESAT-6 was significantly greater than in mice inoculated with either PBS or ESAT-6 alone (P < 0.05). No other differences were observed. When these supernatants were examined for TNF-α, there were no significant differences observed among groups when cells were stimulated with ESAT-6 alone (Fig. 2C). Taken together, the data suggest that spores act as an adjuvant that stimulates T cell responses by inducing IFN-γ- and TNF-α-producing cells.

**FIG 2** *B. subtilis* spores induce IFN-γ, IL-2, and TNF-α levels to ESAT-6 protein at 30 days postinoculation. Mice were inoculated s.c., twice with ESAT-6 alone or with ESAT-6 mixed with 10^5, 10^6, or 10^7 spores. Spleen cells were isolated and cultured with ESAT-6 protein. After 24 h, cells were developed for spot-forming units (SFU), and supernatants were collected and stored at −80°C until used. The number of IFN-γ-releasing cells was determined by ELISpot assay (A), and IL-2 (B) and TNF-α (C) were measured by ELISA. Data are the median values + ranges of triplicate samples from five mice per group. *, P < 0.05. ND, not detected.

*B. subtilis* spores fail to induce the production of IL-17A. Given that *B. subtilis* spores induced key Th1 cytokines that are required for resolution of *M. tuberculosis* infection, we next wanted to determine if *B. subtilis* spores induce immunity at a level similar to that of adjuvants that have already been shown to function in tuberculosis vaccines. One such adjuvant formulation is MPL-DDA, which has been used by us and others in the mouse model to investigate the vaccine potential on novel protein vac-

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April 2014 Volume 21 Number 4 cvi.asm.org 503

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significant antigen-specific T cells compared to MPL-DDA was then examined at the proinflammatory cytokine level. Specifically, cell culture supernatants were examined for the production of key cytokines such as IL-1β, IL-6, IL-10, and IL-17A that may be involved in the modulation of protective immunity (Fig. 3B). Mice inoculated with ESAT-6 plus MPL-DDA had significantly elevated concentrations of IL-1β when stimulated with ESAT-6 than all other groups (P < 0.05) except for mice inoculated with ESAT-6 plus 10⁶ spores. This last group also had significantly elevated levels of IL-1β compared to PBS-inoculated mice (P < 0.05). A similar outcome was observed with IL-6 when cells were stimulated with ESAT-6 alone. We analyzed IL-17A by ELISpot assay and found that IL-17A was significantly elevated under the culture conditions in mice inoculated with ESAT-6 plus MPL-DDA compared to all other groups (P < 0.05). Finally, we examined IL-10 production and found that this cytokine increased but that this increase was not statistically significant compared to all other groups in mice inoculated with ESAT-6 plus MPL-DDA.

Taken together, the data suggest that MPL-DDA is a much more potent stimulator of proinflammatory cytokines. Interestingly, although B. subtilis spores were able to induce IFN-γ production, we were not able to detect high levels under these culture conditions. The difference was more evident between the two adjuvants in regard to IL-17A production, which is important for protection against M. tuberculosis infection.

**Induction of an antibody response by B. subtilis spores.** Investigators using B. subtilis spores formulated with antigens from other pathogens have demonstrated the induction of significant antibody levels in inoculated mice (20–22). The role of antibody in protective immunity against tuberculosis remains controversial, and thus the ability of a vaccine adjuvant to induce antibodies may provide an extra dimension to its protective efficacy. To determine antibody responses induced by the adjuvants, mice were inoculated as described with ESAT-6 formulated with either 10⁴, 10⁵, 10⁶ spores or with MPL-DDA and sacrificed 15 days after the final inoculation. Antibody levels were determined in the sera of animals by ELISA for antibodies to ESAT-6 (Fig. 4). In general, mice inoculated with either 10⁵ or 10⁶ spores or MPL-DDA had elevated antibody levels to ESAT-6 and showed a significant increase in ESAT-6 antibodies compared to all other groups (P < 0.05). Thus, both B. subtilis spores and MPL-DDA induced significant antibody titers to ESAT-6 after inoculation.

**B. subtilis spores fail to cause a reduction in mycobacterial burden.** To determine if the immune response induced by B. subtilis spores was able to reduce the mycobacterial burden after low-dose aerosol infection, mice were inoculated as described with ESAT-6 plus 10⁶ spores and then infected with virulent M. tuberculosis, H37Rv, via the aerosol route 30 days after the final inoculation. Mice inoculated with ESAT-6 plus MPL-DDA were also included as a comparison. At day 30 postinfection, mice were sacrificed, and the number of CFU was determined in the lungs of mice (Fig. 5). There was a significant reduction in CFU counts in the lungs of mice inoculated with ESAT-6 plus MPL-DDA compared to mice inoculated with either PBS or MPL-DDA (P < 0.05); however, this reduction was not observed in mice inocu-
lated with ESAT-6 plus 10^6 spores compared to mice inoculated with 10^6 spores only.

**Partial requirement for IL-17A in protective immunity induced by MPL-DDA.** Given that one of the differences between the spore and MPL-DDA adjuvant formulations used was the production of IL-17A, we were interested to determine if this cytokine played a major role in conferring protective immunity by the MPL-DDA adjuvant. At the time of vaccination mice were treated with either anti-IL-17A monoclonal antibody (MAb) or an isotype control MAb and infected with a low-dose aerosol as described above. At day 30 postinfection, anti-IL-17A-treated mice had significantly greater CFU counts than the group treated with the isotype control (Fig. 6A). However, the anti-IL-17A-treated group also had significantly lower CFU counts than the saline-treated control group. Blocking IL-17 activity at the time of inoculation did not alter the induction of IFN-γ-producing cells (Fig. 6B). Taken together, the data suggest that protective immunity induced by the MPL/DDA formulation requires the contribution of IL-17A and that other cytokines such as IFN-γ would most certainly contribute.

**DISCUSSION**

Adjuvants that are required to induce a protective immune response against *M. tuberculosis* infection are urgently needed for formulations that consist of protein antigens. Currently, there is a concerted effort to develop such adjuvants although their mechanisms of action remain to be elucidated. The animal models of experimental tuberculosis and, in particular, the mouse model have proven to be effective in identifying novel candidates that can move forward for further testing. Novel vaccines, such as the MVA85A vaccine that recently completed phase II clinical trials, have shown us that the animal models may not provide all the answers, and thus the need for clinical trials is essential (23).

The current study examines two such adjuvants, one based on *B. subtilis* spores and one based on detoxified lipid A. Our studies have identified similarities and differences between the two adjuvants, and the fact that spores were unable to induce high levels of IL-17A, a key cytokine shown by others to be essential for protective immunity, indicates that spores may not be suitable as an adjuvant for tuberculosis vaccines. In relation to MPL, our studies concur with a previous report that showed that after subcutaneous inoculation of mice, MPL induced a high level of production of IL-2, TNF-α, and IFN-γ, in addition to IL-6, IL-17, and IL-10 (24). Although we found elevated levels of IL-10 in our culture system, they were not significantly different from those of the controls. Our current study is the first to identify IL-17A as an important cytokine required to be induced by an adjuvanted subunit vaccine for protective immunity to infection with *M. tuberculosis*. Further studies will be required to confirm these findings.

**FIG 4** *B. subtilis* spores and MPL induce antibodies against ESAT-6. Serum samples from inoculated mice were taken at 15 days postinoculation and tested by ELISA for ESAT-6-specific antibodies. Data are the median values ± ranges of triplicate samples from five mice per group. *, *P* < 0.05. OD$_{450}$, optical density at 450 nm.

**FIG 5** *B. subtilis* spores fail to induce protective immunity. Mice were inoculated with ESAT-6 plus spores or with ESAT-6 plus MPL-DDA twice via the s.c. route. At day 30 after the final inoculation, mice were infected with a low-dose aerosol of virulent *M. tuberculosis*. The CFU count was determined in the lungs at day 30 postinfection. Data are the mean values ± standard deviations (SD) of samples from five mice per group. *, *P* < 0.05.

**FIG 6** IL-17A is a partial requirement for protective immunity induced by MPL-DDA. Mice were treated with either anti-IL-17A or an isotype control at 1 day prior and 2 days after each of two inoculations of ESAT-6 plus MPL-DDA. Mice were infected with a low-dose aerosol of virulent *M. tuberculosis* 30 days after the final inoculation, and the number of CFU was determined at day 30 postinfection (A). A cohort of mice was also used at day 30 postinoculation to determine T cell activation by counting the spot-forming units of IFN-γ by ELISpot assay (B). Data are the mean values ± standard deviations (SD) (A) or mean values ± standard errors of the means (SEM) (B) of samples from five mice per group. *, *P* < 0.05. anti.
such as studies using IL-17A knockout (KO) mice or adoptive transfer studies. Interestingly, the requirement for IL-17A did not seem to be absolute as there was not a complete abolition of the reduction in the mycobacterial burden when mice were treated with anti-IL-17A. This may be due to several reasons, including the fact that in our study IL-17A was depleted at the time of inoculation of ESAT-6 and MPL-DDA and that therefore there may have been IL-17A present from other cell types at the time of infection that may have contributed to the partial protective effect. Second, the partial requirement for IL-17A for inducing protective immunity may have been due to the fact that IFN-γ was still produced in the absence of IL-17A and thus provided some protective immune response. The study was designed to assess the role of IL-17A during induction of protective immunity, and therefore an IL-17 knockout mouse was not chosen for this study. In addition, other mediators of immunity may also play a role in reducing the mycobacterial burden; one such mediator is known to be IFN-γ although mediators that have yet to be identified may also exist. Khader et al. (25) demonstrated that depletion of IL-17 during infection in vaccinated mice did indeed reduce the number of IFN-γ-producing cells, which they proposed was due to a reduction in levels of the chemokine that were responsible for recruitment of these cells. The two studies differ in that the current study suggests that IL-17- and IFN-γ-secreting cells develop independently, while the study of Khader et al. suggests that IL-17 is required for recruitment of IFN-γ-secreting cells during infection.

In general, B. subtilis spores have been used as a vaccine adjuvant for other infectious agents, such as highly pathogenic influenza virus, in a formulation for mucosal delivery (26). These studies reported that spores from the PY79 strain of B. subtilis induced high levels of IL-2, IL-1, and IL-6 in spleen cell cultures from immunized mice and a significant increase in antigen-specific antibodies, similar to results obtained with the current study although the strain used here was PA3. Interestingly these investigators also observed that spores alone could protect mice from infection with H5N1 virus in a dose-dependent manner that they attributed to the activation of natural killer cells and maturation of dendritic cells. Unfortunately, there were no data presented in that study regarding IL-17A production. Vaccines against rotavirus (10) and Clonorchis sinensis (12) have been developed, both of which were developed for mucosal delivery and were demonstrated to induce significant levels of antigen-specific antibodies. In the current study, B. subtilis spores were not used to inoculate mice via the mucosal route and may prove to be more efficacious, thus warranting further investigation.

B. subtilis spores have been shown to activate innate immune cells although it is not clear what mechanism is used; several studies have shown that although they are able to upregulate Toll-like receptor 2 (TLR2) and TLR4 mRNAs, they do not interact directly with these TLRs (27), while others have shown that action of Bacillus anthracis spores is TLR2 and MyD88 dependent (28). The activity of MPL is mediated via its interaction with TLR4 (29), which has been widely shown to induce a Th1 immune response (30, 31) and is a constituent of adjuvants that are currently in clinical trials (32). Therefore, it would be anticipated that the activity of spores and MPL would be similar, which our data suggest, although the activity of MPL in inducing a robust immune response was greater than that observed with spores. It is possible that B. subtilis spores also induced immune-modulatory signals that dampened proinflammatory signals, such as the production of IL-17A.

The addition of spore proteins to cultured spleen cells had the effect of increasing the IL-10 concentration even in cultures derived from mice inoculated with the MPL-DDA adjuvant. This may suggest that B. subtilis spores provide an inhibitory signal that may dampen IL-17 production sufficiently to prevent induction of protective immunity. Other investigators have shown that B. subtilis PB6 strain spores induced substantial levels of IL-10 but very low levels of IL-12, TNF-α, and IFN-γ on human peripheral blood mononuclear cells (33). Furthermore, others have demonstrated that blocking IL-10 in the mouse during vaccination with BCG resulted in enhanced Th1 and Th17 responses (34), and thus it will be of interest in future studies to determine if this is the mechanism responsible for preventing B. subtilis spores from inducing a robust protective immune response. Interestingly, blocking IL-17 activity during the time of inoculation with ESAT-6 plus MPL-DDA had no effect on IFN-γ-producing cells, indicating separate lineages for the induction of both cell types that are not dependent on each other. This may also help to explain why we observed a strong IFN-γ response in mice inoculated with ESAT-6 plus spores.

Both the spore and MPL formulations induced significantly elevated levels of anti-ESAT-6 serum antibodies. The role of antibodies in the protective immune response remains under debate (35), but it is interesting that the presence of antibodies to ESAT-6 did not affect infection outcome since the MPL-DDA formulation reduced the mycobacterial burden while the B. subtilis spores did not. It is possible that antibodies to ESAT-6 do not alter the course of infection or that the isotype of the induced antibody was not effective. Other investigators have shown that IgA may play a role in modulating infection (36), and thus mucosal administration of the B. subtilis vaccines may warrant further investigation. Killed spores have been shown to induce IgG2a (Th1 type antibody) when administered intranasally (26), but when given orally they induced IgG subclasses that were IgG1 and IgG2b, indicative of a Th2 type of immune response (37). MPL, when inoculated intratunously, resulted in IgG1, IgG2a, IgG2b, and IgG3 antibodies being produced (38). Mice vaccinated subcutaneously with MPL and trehalose dicorynomycolate (TDM) using M. tuberculosis-derived antigens had a higher ratio of IgG2b/IgG1 than mice inoculated with a formulation without adjuvant (39). When administered intranasally, an MPL adjuvanted formulation produced higher IgG2a and IgG2b responses than those of the group administered antigen alone, with a tendency toward a Th1-polarized response (40). Thus, the importance of the route of administration for vaccines to induce the appropriate immune response is demonstrated by these adjuvants.

Overall, the data presented identify a critical cytokine that is required for the induction of protective immunity to infection with M. tuberculosis by an adjuvant formulation. The antigen used in the current study is not intended to suggest that a single antigen is required for the protective effect of a vaccine in humans, and it is very likely that multipeptide or fusion protein vaccines would be more beneficial in providing a repertoire that will be recognized by the immune system in humans. The intention of the study was to compare adjuvant systems using a relatively simple M. tuberculosis antigen that has been well characterized in the C57BL/7 mouse model.
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