Highly Persistent and Effective Prime/Boost Regimens against Tuberculosis That Use a Multivalent Modified Vaccine Virus Ankara-Based Tuberculosis Vaccine with Interleukin-15 as a Molecular Adjuvant

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Novel immunization strategies are needed to enhance the global control of tuberculosis (TB). In this study, we assessed the immunizing activity of a recombinant modified vaccinia Ankara (MVA) construct (MVA/IL-15/5MtB) which overexpresses five Mycobacterium tuberculosis antigens (antigen 85A, antigen 85B, ESAT6, HSP60, and Mtb39), as well as the molecular adjuvant interleukin-15 (IL-15). Homologous prime/boost studies showed that the MVA/IL-15/5MtB vaccine induced moderate but highly persistent protective immune responses for at least 16 months after the initial vaccination and that the interval between the prime and boost did not significantly alter vaccine-induced antituberculosis protective immunity. At 16 months, when the Mycobacterium bovis BCG and MVA/IL-15/5MtB vaccine-induced protection was essentially equivalent, the protective responses after a tuberculous challenge were associated with elevated levels of gamma interferon (IFN-γ), IL-17F, Cxcl9, and Cxcl10. To amplify the immunizing potential of the MVA/IL-15/5MtB vaccine, a heterologous prime/boost regimen was tested using an ESAT6-antigen 85B (E6-85) fusion protein formulated in dimethyldiotacylammonium bromide/monophosphoryl lipid A (DDA/MPL) adjuvant as the priming vaccine and the MVA/IL-15/5MtB recombinant virus as the boosting agent. When MVA/IL-15/5MtB vaccine boosting was done at 2 or 6 months following the final fusion protein injections, the prime/boost regimen evoked protective responses against an aerogenic M. tuberculosis challenge which was equivalent to that induced by BCG immunization. Long-term memory after immunization with the E6-85-MVA/IL-15/5MtB combination regimen was associated with the induction of monofunctional CD4 and CD8 IFN-γ-producing T cells and multifunctional CD4 and CD8 T cells expressing IFN-γ/tumor necrosis factor alpha (TNF-α), TNF-α/IL-2, and IFN-γ/TNF-α/IL-2. In contrast, BCG-induced protection was characterized by fewer CD4 and CD8 monofunctional T cells expressing IFN-γ and only IFN-γ/TNF-α and IFN-γ/TNF-α/IL-2 expressing multifunctional T (MFT) cells. Taken together, these results suggest that a heterologous prime/boost protocol using an MVA-based tuberculosis vaccines to boost after priming with TB protein/adjuvant preparations should be considered when designing long-lived TB immunization strategies.

Despite being an ancient disease, tuberculosis (TB) remains one of the most devastating causes of morbidity and mortality worldwide. Each year about 9 million new cases of TB are detected and 2 million people die from this disease (12, 28). The magnitude of the disease reservoir is immense, with about 2 billion people or one-third of the world’s population thought to be infected with Mycobacterium tuberculosis. In recent years, control of TB has been exacerbated by the deadly intersection of the HIV and TB epidemics and the emergence of multiple-drug-resistant tuberculosis (15, 29).

The failure of current TB immunization procedures to adequately protect against M. tuberculosis infections is largely responsible for the unsuccessful global control of this disease. Although the current TB vaccine, Mycobacterium bovis BCG, has been widely used for at least 50 years, its efficacy has been shown to be highly variable in well-controlled clinical trials (3, 5). While BCG is moderately protective against disseminated TB in children, BCG is ineffective in protecting against the most prevalent form of the disease, adult pulmonary TB. The inadequacy of BCG immunization to control the TB epidemic has stimulated a worldwide effort to develop more-effective TB vaccination strategies. These immunization strategies have included live attenuated strains, viral vectored vaccine constructs, and subunit formulations. These new approaches have potential advantages relative to BCG immunization, such as increased safety, improved stability, and decreased interference by exposure to environmental mycobacteria. However, developing a vaccination strategy based on genetic or subunit vaccines that induces strong and sustainable antituberculosis
cellular immunity is a significant scientific challenge. Although immunizations with single antigen preparations have usually been inadequate for controlling intracellular infections, recent studies have shown that levels of pathogen-specific cellular immunity can be substantially enhanced by combining different vaccines in a heterologous prime/boost regimen (2, 11, 13, 18, 23). These heterologous prime/boost vaccination strategies often improve the magnitude and quality of T-cell responses and can broaden epitope coverage. Clearly, the magnitude, quality, and breadth of vaccine-induced cellular immunity are critical for the establishment of effective antituberculosis T-cell memory.

Recombinant modified vaccinia virus Ankara (MVA) constructs are among the strongest boosting agents used in prime/boost protocols. MVA vaccines have consistently been shown to boost both primed CD4 and CD8 T-cell responses against intracellular pathogens. In cattle and rhesus macaques, an MVA recombinant virus expressing the M. tuberculosis antigen 85A boosted BCG-induced protection against an M. tuberculosis challenge (26, 27). In humans, immunization with the MVA85A vaccine induced high levels of antigen-specific gamma interferon (IFN-γ)-secreting T cells in BCG-naive healthy volunteers and boosted preexisting antmycobacterial immune responses elicited by previous BCG vaccination (2, 18). In earlier studies from our laboratory, we showed that a novel recombinant MVA vaccine which coexpressed five TB antigens and interleukin-15 (IL-15) (incorporated as a molecular adjuvant) induced robust antituberculosis immune responses in mice (19, 21). IL-15 is a promising adjuvant because it plays a role in a wide range of relevant biological activities, including the activation and proliferation of CD8 T cells and NK T cells, the maintenance of CD8 memory cells, and the differentiation and maturation of B cells (24). In this study, we extended our studies of the MVA/IL-15/5Mtb vaccine by showing that immunization with this viral vectored vaccine induces long-lasting antituberculosis protective immunity. Importantly, we demonstrated that priming with a TB fusion protein/adjuvant formulation followed by heterologous boosting with the MVA/IL-15/5Mtb recombinant virus evoked robust protective responses that were associated with the induction of high frequencies of CD4 and CD8 multifunctional T (MFT) cells.

MATERIALS AND METHODS

Animals. C57BL/6 female mice were obtained from Jackson Laboratories (Bar Harbor, ME). All mice used in this study were maintained under sterile conditions at the Center for Biologies Evaluation and Research, Bethesda, MD. The mice were vaccinated with selected vaccine preparations at 6 to 8 weeks old.

Vaccine preparations. MVA recombinant virus expressing ESA16, Ag85A, Ag85B, HSP65, Mtb39A, and IL-15 was constructed as described previously (21). The E6-85 fusion protein is encoded by the ESA16 gene fused to the region of the Ag85B gene corresponding to the N terminus without the Ag85B signal sequence. These genes were cloned by PCR using the following primers with encoded restriction enzyme sites and two alanines between the ESA16 and Ag85B genes: ESA16-forward primer, AGCATATGGTACCCGACGAGCATTTGAG; ESA16-reverse primer, GAGAAGTTTCGCGAATACCTCCAGGACGACGACGTTG; and Ag85B-forward primer, AGCAAGTCTCGGCTTCTCGGCCGCGGGTTTCGAGT; Ag85B-reverse primer, GCAGGCGCGCGCGGGCTTACGAACATCGGAG. The PCR products were ligated into the pET-23b plasmid (Novagen). The recombinant plasmid was transformed and expressed in BL21 cells. The fusion protein was purified using nickel chelation and immunoprecipitation.

Immunization schedules. C57BL/6 female mice were vaccinated once subcutaneously with 10^7 CFU of BCG Pasteur 6 weeks before challenge. For the wild-type MVA vector (as a control) or the MVA/IL-15/5Mtb vaccine, mice were administered two doses of 5 × 10^7 PFU subcutaneously at 1 month apart. The E6-85 protein/adjuvant preparation was made by mixing 1.5 mg/ml dimethyldioctadecylammonium bromide (DDA) (Kodak), 0.25 mg/ml monophosphoryl lipid A (MPL) (Avanti Polar Lipids, Alabaster, AL), and 25 mg/ml of the E6-85 protein. For this study, 0.2 ml of the E6-85 protein/DDA/MPL adjuvant vaccine and the adjuvant alone were given to mice three times at 2 weeks apart with 5 μg of protein per vaccination by the subcutaneous route. For the E6-85 protein/MVA/IL-15/5Mtb heterologous prime/boost immunizations, three doses of the E6-85 protein/adjuvant formulation were given 2 weeks apart, followed at either 2 or 6 months by two doses of the MVA/IL-15/5Mtb vaccine given 1 month apart. For the MVA/IL-15/5Mtb/E6-85 protein prime boost, the 2 doses of MVA/IL-15/5Mtb vaccine were given 2 or 6 months prior to 3 E6-85 protein/adjuvant vaccinations. As a control, 3 doses of the DDA/MPL adjuvant were injected 2 weeks apart, followed 6 months later by 2 doses of the MVA/IL-15/5Mtb vaccine.

Figure 1 gives an abbreviated timeline of the studies.

Murine aerogenic M. tuberculosis infection model. Five mice from each group were aerogenically challenged with M. tuberculosis Erdman K1 (Trudeau Mycobacterial Culture Collection) suspended in phosphate-buffered saline (PBS) at a concentration known to deliver 200 CFU in the lungs over a 30-min exposure in a Middlebrook chamber (Glas Col, Terre Haute, IN) (7). To confirm the infection dose, mice were sacrificed 4 h after exposure and organs were harvested to determine CFU numbers. The harvested lungs and spleens were homogenized separately in PBS-0.04% Tween 80 using a Seward Stomacher 80 blender (Tecmar, Cincinnati, OH). The homogenates were serially diluted in PBS-0.04% Tween 80 and plated on Middlebrook 7H11 agar (Difco) plates containing 10% OADC enrichment (Becton Dickinson, Sparks, MD) medium, 10 μg/ml ampicillin, 50 μg/ml cycloheximide, and 2 μg/ml 2-thiophenacarboxylic acid hydride (TCH) (Sigma). The addition of TCH to the agar plates inhibits BCG growth but has no affect on M. tuberculosis growth. Plates were incubated at 37°C for 2-3 weeks, and then CFU were counted to determine the organ bacterial burden.

RNA isolation and PCR array analysis of cytokine responses from immune cells. At 2 and 16 months postvaccination, lungs were harvested from 5 mice from each experimental group 10 days after an aerogenic challenge with M. tuberculosis. Sterile PBS was added, and the lungs were sliced and shredded with a scalpel to obtain a suspension. A single-cell suspension of lung lymphocytes was obtained by passing the shredded lung suspension through a 70-μm cell strainer (BD Biosciences, Franklin Lakes, NJ) followed by a 5-min centrifugation at 1,000 rpm. The supernatant was discarded, and the remaining solution was treated with ACK lysis buffer (Lonza, Walkersville, MD) for 5 min, followed by 2 washes with PBS, and stored in RNA later (Qiagen, Valencia, CA). The RNasey minikit (Qiagen) was used to isolate the total RNA from the cell suspension. Equal amounts of RNA from these samples were reverse transcribed to cDNA using a Superscript First-Strand cDNA synthesis kit (Invitrogen, San Diego, CA). Oligoribonucleotide desoxynucleotide (GGPDH) primers were used to determine the quality of the cDNA conversion of each sample by PCR. The cytokine/chemokine lung transcriptional responses were determined using the cDNA as the template for RT^2 qPCR primer assays (SA Biosciences, Frederick, MD) and an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). Mouse cytokine/chemokine primers were obtained from SA Biosciences (Frederick, MD). The mRNA expression levels for each cytokine were then normalized according to the manufacturer’s instructions using the formula described in the RT^2 qPCR primer assay user manual. The relative cytokine expression values in immune cell cultures were determined by dividing the cytokine expression levels in vaccine candidate samples by the expression values for naive controls (16). This value represents the mean increase or decrease of RNA expression compared to that for naive controls.

Characterization of cytokine production using flow cytometry. Splenic cells from naive and vaccinated mice were isolated by disrupting the spleens in complete Dulbecco’s modified Eagle medium (dMEM) consisting of 10 mM HEPES, 2.0 mM L-glutamine, and 0.1 mM MEM nonessential amino acids with 10% fetal bovine serum (FBS) using a 3-ml syringe barrel. After passage of the spleen homogenate through a cell strainer, the resulting single-cell suspension was washed with cDMEM-FBS and treated for 1 min with 5.0 ml ACK lysis buffer (Lonza). After cells were washed with an equal volume of medium, the cells were resuspended in cDMEM-FBS, counted, and added to wells of a 24-well plate at a density of 2.5 × 10^5 cells per well in 1.0 ml cDMEM-FBS. For measurement of antigen-specific responses, BCG was added to the wells at a multiplicity of infection (MOI) of 0.5 bacilli per spleen cell. Wells which contained only spleen cells served as unstimulated controls. Infections were allowed to proceed overnight, followed by the addition of GolgiPlug (BD Biosciences, 1 μl per well). After 5 h of incubation, the unbound cells were removed from the wells and transferred to fluorescence-activated cell sorter (FACS) tubes, washed with PBS, and resuspended in ~50 μl PBS. Live-Dead stain (Invitrogen) (10 μl of a 1:100 dilution) was added to each tube, followed by incubation for 30 min at...
RESULTS

Long-term protection against *M. tuberculosis* challenge after vaccination with a recombinant MVA virus coexpressing five *M. tuberculosis* antigens and IL-15. In an earlier report, we described the creation and characterization of a multivalent MVA vectored vaccine which expresses five *M. tuberculosis* antigens as well as the immunostimulatory cytokine IL-15 (21). We demonstrated that this multivalent MVA vaccine induced robust antituberculosis protective responses and conferred protection against an aerogenic *M. tuberculosis* challenge. In subsequent studies, we focused on evaluating the persistence of the immune response induced by this MVA/IL-15/5Mtb vaccine candidate. For these experiments, two doses of the MVA/IL-15/5Mtb vaccine were administered because our initial studies with this vaccine and work with related MVA vectors have shown that two MVA doses were needed to evoke optimal cellular responses (8, 9). The data summarized in Table 1 compare the protective responses detected at 2 and 12 months after two subcutaneous immunizations (1 month apart) with the MVA/IL-15/5Mtb vaccine candidate. As controls, 2 doses of wild-type MVA virus or one injection of live BCG vaccine were given. At 2 months postvaccination, modest protection against an *M. tuberculosis* aerogenic challenge was seen in the lungs of mice immunized with the MVA/IL-15/5Mtb construct (−0.74 log_{10} CFU) relative to the stronger BCG-induced protective responses (−1.37 log_{10}). In contrast, protection against dissemination to the spleen was not significantly different for the two vaccine groups at the 2-month time point. At 12 months postvaccination, highly persistent protective responses were seen in the lungs of mice immunized with BCG vaccine or our MVA/IL-15/5Mtb construct. The pulmonary protective responses seen at 1 year after the immunizations were essentially unchanged for both of these vaccine groups. Importantly, the MVA virus used as a control did not induce antituberculosis protective immunity. The organ bacterial burdens at 2 and 12 months postvaccination were not significantly different for animals given the wild-type MVA virus relative to those for naive controls.

To compare the persistence following a tuberculous challenge of the protective responses induced by immunization with BCG or MVA/IL-15/5Mtb vaccine, mice were vaccinated twice with MVA/IL-15/5Mtb 1 month apart and then challenged by the aerosol route with *M. tuberculosis* 1 month later.
TABLE 1. Organ bacterial burdens after an aerogenic *M. tuberculosis* challenge at 2 and 12 months postvaccination and 1 month postchallenge

| Vaccination group | 2 months postvaccination | 12 months postvaccination |
|-------------------|--------------------------|---------------------------|
|                   | Lung | Spleen | Lung | Spleen |
| Naive             | 6.37 ± 0.13 | 5.37 ± 0.18 | 6.34 ± 0.07 | 5.55 ± 0.06 |
| BCG               | 5.00 ± 0.07* (−1.37)* | 4.17 ± 0.12* (−1.20) | 5.01 ± 0.05* (−1.33)* | 3.94 ± 0.22* (−1.61)* |
| MVA               | 6.10 ± 0.07 | 5.29 ± 0.10 | 5.96 ± 0.17 | 5.42 ± 0.39 |
| MVA/IL-15/5Mtb    | 5.63 ± 0.02* (−0.74) | 4.47 ± 0.06* (−0.90) | 5.64 ± 0.04* (−0.70) | 5.15 ± 0.14* (−0.40) |

* Mice were vaccinated twice with the MVA constructs 1 month apart or once with the BCG vaccine and then challenged by the aerosol route with *M. tuberculosis* 2 or 12 months after the primary immunization. Mean difference between naive and experimental CFU are given in parentheses.

Organ bacterial burdens were evaluated at 1 and 4 months postchallenge (Fig. 2). In this study, mice were immunized with our MVA/IL-15/5Mtb vaccine by either the subcutaneous route or the intranasal route. As a control, mice were injected with BCG subcutaneously 2 months before the challenge. At 1 month postchallenge, significant (compared to that for naive animals; *P* < 0.05) pulmonary protection was again seen in animals vaccinated subcutaneously with MVA/IL-15/5Mtb (−0.74 log_{10} CFU), while stronger protective responses were detected in mice immunized with BCG (−1.20 log_{10}). The level of protection in the lungs seen after intranasal administration of the MVA/IL-15/5Mtb vaccine (−0.91 log_{10}) was also significantly elevated relative to naive levels but was not different from the responses seen after the subcutaneous injections. More importantly, at 4 months postchallenge, the protection induced by our MVA/IL-15/5Mtb vaccine remained unchanged (relative to that at 1 month) while the BCG-induced protective responses decreased. At this time point, no significant differences in the protective responses between the three immunization groups were detected. Clearly, the postchallenge protective immune responses elicited by mice vaccinated with the MVA/IL-15/5Mtb vaccine were again extremely persistent.

Recent studies have suggested that increasing the time intervals between immunizations during a prime/boost protocol may permit increased differentiation of effector memory cells to central memory cells before boosting and thereby enhance the effectiveness of the immunizations (4, 10, 22). To investigate the impact of expanding the time intervals between immunizations, mice were boosted subcutaneously with the MVA/IL-15/5Mtb vaccine at 1 or 7 months after a homologous priming vaccination and then were aerogenically challenged with *M. tuberculosis* 3 months later. As seen in Table 2, the longer immunization schedule did not have a significant impact on the pulmonary protective responses generated by the prime/boost immunization (−0.62 log_{10} CFU for 1 month versus −0.74 log_{10} for 7 months). For both MVA/IL-15/5Mtb vaccination regimens, the protective responses in the lung were significantly elevated compared to those for naive mice but were significantly less than the BCG-induced responses at 4 and 10 months after BCG vaccination (*P* < 0.05).

In the same study, the persistence of the MVA/IL-15/5Mtb vaccine-induced protective responses was confirmed when the vaccinated mice were challenged 9 months after a 7-month MVA/IL-15/5Mtb booster immunization. Despite the long 16-month interval between the initial vaccination and challenge, a reduction of 0.81 log_{10} CFU in lung bacterial burdens was seen.

**FIG. 2.** Persistence of the MVA/IL-15/5Mtb vaccine-induced protective immune responses in the lung after an aerosol infection with *M. tuberculosis*. Mice were vaccinated subcutaneously (SC) with MVA/IL-15/5Mtb recombinant virus or wild-type MVA virus and intranasally (IN) with MVA/IL-15/5Mtb recombinant virus. As controls, other groups of mice remained nonvaccinated (naive) or were vaccinated subcutaneously with BCG. Two months after the initial vaccination, the mice were challenged with a low dose of *M. tuberculosis* and then sacrificed at 1 or 4 months postchallenge. Lung homogenates were plated on Middlebrook 7H11 plates, and mycobacterial CFU were counted 2 to 3 weeks later.

**TABLE 2.** Persistence of antituberculosis protective immune responses induced by MVA/IL-15/5Mtb in lungs with different prime/boost schedules

| Vaccine          | 1-month boost, challenge 4 months after primary vaccination | 7-month boost, challenge 10 months after primary vaccination | 7-month boost, challenge 16 months after primary vaccination |
|------------------|----------------------------------------------------------|----------------------------------------------------------|----------------------------------------------------------------|
| MVA/IL-15/5Mtb   | −0.62 ± 0.05* | −0.47 ± 0.07* | −0.81 ± 0.06* |
| BCG              | −1.15 ± 0.13* | −1.22 ± 0.08*# | −0.70 ± 0.10* |

* Mice were boosted with the MVA/IL-15/5Mtb construct at 1 or 7 months after the initial immunization. The control mice were given only a single dose of 10^6 CFU of BCG. At 4, 10, or 16 months after the primary immunization, the vaccinated mice were challenged by the aerosol route with *M. tuberculosis* Erdman. The pulmonary bacterial burdens were evaluated at 4 weeks after the aerogenic challenge. * Significant reduction in organ bacterial burdens relative to those for vaccination with MVA/IL-15/5Mtb construct.

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*a* Significant reduction in CFUs compared to naive controls (*p* < 0.05).

*b* Significant reduction in CFUs compared to mice vaccinated SC with MVA construct.
for the MVA/IL-15/5Mtb-immunized mice relative to results for naive controls. Importantly, this response was no different from the protection detected at 5 and 10 months after the initial vaccination. However, while robust protective responses were detected at 5 and 10 months after the MVA/IL-15/5Mtb immunizations given 7 months apart (16 months after the initial vaccination) or at 9 months after 2 immunizations were given at either 2 or 6 months following the BCG vaccination, significantly lower protection was detected at 16 months post-immunization with BCG (−0.70 log$_{10}$). As seen previously, the protective immunity induced by BCG in the mouse model clearly wanes after 16 months in the mouse model of pulmonary tuberculosis (7). At the longest time interval, the protection seen for both the MVA/IL-15/5Mtb and BCG vaccine groups was essentially equivalent.

To identify immune molecules associated with the MVA/IL-15/5Mtb vaccine-induced protective immunity, mice were primed by immunization with BCG or wild-type MVA using real-time PCR. For these studies, mice were aerogenically challenged with a low dose of $M$. tuberculosis either at 1 month following the second MVA/IL-15/5Mtb vaccination or wild-type MVA control vaccination (2 months after the initial vaccination) or at 9 months after 2 MVA/IL-15/5Mtb immunizations given 7 months apart (16 months postvaccination). The single BCG vaccination was administered at the time of the initial MVA injection. At 10 days postchallenge, the mice were sacrificed, the lungs were removed, and RNA was purified from lung cells. Subsequently, the levels of expression of 13 cytokine or chemokine genes previously shown to be up- or downregulated in vaccinated animals were assessed (16). As seen in Table 3, at 2 months postimmunization and at 10 days postchallenge, the expression of 7 of these immune mediators was increased in the lungs of mice vaccinated with either BCG or MVA/IL-15/5Mtb relative to that for naive controls (IFN-γ, IL-12p40 subunit, IL-17F, IL-27β, Cxcl9, Cxcl10, and Cxcl11). In contrast, the expression of IL-1, IL-2, IL-4, IL-21, transforming growth factor β (TGF-β), and TNF-α was not elevated relative to that for naive controls. For the upregulated genes, only the pulmonary expression of IFN-γ and IL-17F was significantly increased ($P < 0.05$) for BCG-vaccinated animals relative to that for those immunized with MVA/IL-15/5Mtb vaccine. Interestingly, none of the cytokine genes that were evaluated were differentially regulated, compared to results for naive mice, in mice vaccinated with wild-type MVA virus.

At 16 months postvaccination, similar pulmonary cytokine and chemokine responses were detected in BCG-vaccinated mice at 10 days postchallenge. Again, elevated expression of IFN-γ, IL-17F, IL-27β, Cxcl9, Cxcl10, and Cxcl11 transcripts were detected. Although postchallenge IL-12p40, IL-27β, and Cxcl11 expression decreased in MVA/IL-15/5Mtb-immunized mice at 16 months postvaccination, the expression of IFN-γ, IL-17F, Cxcl9, and Cxcl11 were again significantly elevated compared to that for naive controls at this extended time point. Similar to the 2-month time point, the relative expression of the test genes at this later time was not significantly enhanced in mice vaccinated with wild-type MVA virus.

| TABLE 3. Cytokine and chemokine responses at 2 and 16 months after initial vaccination |
|---------------------------------------------|---------------------------------------------|---------------------------------------------|
| Cytokine or chemokine | 2 months post-vaccination with: | 16 months post-vaccination with: |
|                          | BCG | MVA-VC | MVA/IL-15/5Mtb | BCG | MVA-VC | MVA/IL-15/5Mtb |
| IFN-γ | $^{*}12.9 ± 4.3^{*}$ | 1.7 ± 0.5 | 6.2 ± 0.9$^{*}$ | 6.8 ± 1.7$^{*}$ | 0.8 ± 0.2 | 4.2 ± 1.8$^{*}$ |
| IL-12 p40 | 2.7 ± 1.2 | 1.8 ± 0.5 | 3.2 ± 1.2$^{*}$ | 1.7 ± 0.2 | 0.5 ± 0.2 | 1.0 ± 0.2 |
| IL-17f | $^{*}22.7 ± 7.6^{*}$ | 1.4 ± 0.6 | 3.9 ± 0.7$^{*}$ | 25.6 ± 7.8$^{*}$ | 1.2 ± 0.2 | 11.8 ± 4.2$^{*}$ |
| IL-27β | 4.2 ± 0.8 | 1.1 ± 0.1 | 3.9 ± 0.7$^{*}$ | $^{*}2.7 ± 0.4^{*}$ | 0.9 ± 0.1 | 1.3 ± 0.3 |
| Cxcl9 | 6.4 ± 0.3$^{*}$ | 1.0 ± 0.1 | 6.4 ± 0.3$^{*}$ | $^{*}30.1 ± 4.0^{*}$ | 1.6 ± 0.5 | 4.5 ± 0.8$^{*}$ |
| Cxcl10 | 8.3 ± 2.1$^{*}$ | 0.8 ± 0.1 | 4.9 ± 0.5$^{*}$ | $^{*}11.8 ± 3.8^{*}$ | 1.3 ± 0.4 | 2.5 ± 1.2$^{*}$ |
| Cxcl11 | 8.9 ± 3.5$^{*}$ | 1.1 ± 0.1 | 10.2 ± 4.8$^{*}$ | $^{*}20.5 ± 8.5^{*}$ | 2.9 ± 2.1 | 1.5 ± 0.5 |

$^{*}$, significant increase in expression levels compared to results for naive animals ($P < 0.05$); #, significant increase in expression levels compared to results with MVA/IL-15/5Mtb ($P < 0.05$).
the E6-85/MVA/IL-15/5Mtb (−1.15 log₁₀) and BCG (−1.06 log₁₀) vaccine groups. Significantly less protection in the lung was seen for mice vaccinated with the E6-85 protein preparation (−0.52 log₁₀; P < 0.05) than for the MVA/IL-15/5Mtb-boosted animals, while the differences for the MVA/IL-15/5Mtb/E6-85 (−0.93 log₁₀) and adjuvant/MVA/IL-15/5Mtb (−0.87 log₁₀) experimental groups approached statistical significance (P < 0.10). Importantly, at both prime/boost intervals, the levels of antituberculosis protective immunity generated by immunization with the E6-85/MVA/IL-15/5Mtb combination or BCG vaccine were essentially equivalent. Also, at each time interval, the order of the prime/boost immunizations seemed to have an impact on the outcome. Our results suggested that the MVA/IL-15/5Mtb vaccine was more effective as a boosting agent because the E6-85 prime/MVA/IL-15/5Mtb boost regimen elicited higher levels of protective immunity in the lung than the MVA/IL-15/5Mtb prime/E6-85 boost schedule at both the 2- and 6-month prime/boost vaccination intervals.

Recent reports have suggested that the induction of multifunctional T (MFT) cells is associated with enhanced immunity against intracellular pathogens (1, 6, 17). To determine whether immunization with the various vaccine regimens induced elevated MFT responses, spleen cells were removed from vaccinated mice, stimulated with BCG overnight, and then analyzed by multiparameter flow cytometry. The frequencies of CD4 and CD8 cells expressing IFN-γ, TNF-α, and IL-2 were evaluated by intracellular cytokine staining (Fig. 3). Using these procedures, CD4 T cells expressing only IFN-γ were the most frequent; IFN-γ-expressing CD4⁺ splenocytes from mice vaccinated with the E6-85/MVA/IL-15/5Mtb combination (6.75%), BCG vaccine (3.49%), or the adjuvant/MVA/IL-15/5Mtb regimen (2.5%) were significantly elevated relative to naive controls. Among the splenocytes expressing multiple cytokines, substantially elevated levels of CD4 T cells expressing IFN-γ and TNF-α were detected in all vaccine groups relative to levels for naive controls. In particular, 27- and 14-fold-increased frequencies (compared to those for naive controls) of CD4 T cells expressing IFN-γ and TNF-α were seen in the E6-85/MVA/IL-15/5Mtb prime/boost and BCG experimental groups, respectively. Additionally, the relative frequencies of splenic CD4 T cells expressing IFN-γ, TNF-α, and IL-2 were significantly elevated in all vaccination groups. While only 0.01% of naive CD4 T cells in the spleen expressed all three cytokines, 7- to 35-fold-increased frequencies of triple-positive CD4 T cells were detected for the E6-85/MVA/IL-15/5Mtb (0.35%), BCG (0.16%), and E6-85/adjuvant (0.07%) groups. It is of interest that significant relative increases in splenic CD4 T cells expressing TNF-α and IL-2 were seen only for the E6-85/MVA/IL-15/5Mtb (0.24%, compared to 0.03% for naive controls) and adjuvant/MVA/IL-15/5Mtb (0.13%) immunization groups (Fig. 3A). These TNF-α/IL-2-positive CD4 cells have been classified as possible central memory cells, and a role in the long-term memory response has been suggested for these double-positive cells (17).

Although the results were generally more variable, similar MFT results were seen with splenic CD8 T cells (Fig. 3B). Monofunctional IFN-γ-expressing cells were the most frequent CD8 T cells detected. Significantly higher frequencies of CD8 T cells expressing only IFN-γ were seen in the E6-85/MVA/IL-15/5Mtb (9.20%), adjuvant/MVA/IL-15/5Mtb (6.80%), and BCG (5.68%) vaccination groups than for naive mice (1.16%). Sevenfold-increased levels of IFN-γ/TNF-α (+) CD8 T cells compared to results for naive mice were detected in the highly protective E6-85/MVA/IL-15/5Mtb and BCG vaccine groups, and elevated relative concentrations of TNF-α/IL-2 CD8 T cells were seen after the E6-85/MVA/IL-15/5Mtb heterologous prime/boost. Furthermore, the frequencies of triple-positive splenic CD8 T cells were significantly elevated in the E6-85/MVA/IL-15/5Mtb (0.23%) and BCG (0.21%) vaccine groups relative to results for naive controls (0.04%).

In previous studies, MFT cells have been shown to make severalfold more IFN-γ than monofunctional T cells (24, 25). To evaluate the levels of IFN-γ produced by monofunctional and MFT CD4 cells, the median fluorescence intensity (MFI) was determined for IFN-γ-secreting cells within experimental groups by reviewing the flow cytometric plots. The median fluorescence for the splenocytes expressing only IFN-γ recovered from animals vaccinated with the E6-85/MVA/IL-15/5Mtb combination or BCG was about 6,000 units. Interestingly, the median fluorescence intensities for the IFN-γ/TNF-α (+) CD4 cells from the BCG-vaccinated (27,000) and E6-85/MVA/IL-15/5Mtb-vaccinated (24,000) mice were 4- to 5-fold higher than those for the monofunctional cells. Additionally, for the triple-positive splenic CD4 T cells from BCG- and E6-85/MVA/IL-15/5Mtb-vaccinated mice, the median fluorescence intensities were increased about 3-fold (18,000) and 4-fold (23,000), respectively, compared to results for the monofunctional IFN-γ⁺ splenic CD4 T cells induced by the same

TABLE 4. Impact of time interval between priming and boosting on the bacterial burdens assessed 1 month after an aerosol M. tuberculosis challenge

| Exptl group                  | 2 months postprime | 6 months postprime |
|------------------------------|--------------------|--------------------|
|                              | Lung               | Spleen             | Lung               | Spleen             |
| Naive                        | 4.13 ± 0.31        | 3.47 ± 0.13        | 5.60 ± 0.05        | 4.57 ± 0.1         |
| BCG                          | 2.90 ± 0.24 (-1.23)| 2.35 ± 0.24 (-1.13)| 4.54 ± 0.08 (-1.06)| 3.48 ± 0.1 (-1.14)|
| E6-85                        | 3.19 ± 0.23 (-0.94)| 3.33 ± 0.18        | 5.08 ± 0.94 (-0.52)*| 4.38 ± 0.21*       |
| E6-85 + MVA/IL-15/5Mtb       | 2.96 ± 0.18 (-1.15)| 3.09 ± 0.20        | 4.45 ± 0.19 (-1.15)| 3.43 ± 0.08 (-1.14)|
| MVA/IL-15/5Mtb + E6-85       | 3.27 ± 0.16 (-0.86)| 3.25 ± 0.20        | 4.67 ± 0.06 (-0.93)| 4.26 ± 0.12*       |
| Adj + MVA/IL-15/5Mtb         | ND                 | ND                 | 4.73 ± 0.27 (-0.87)| 4.04 ± 0.17*       |

* Parenthetical values are given when P < 0.05 compared with results for naive control. *+ vaccine groups with organ bacterial burdens significantly increased (P < 0.05) over those for the heterologous E6-85 + MVA/IL-15/5Mtb vaccine group.

b ND, not done.
vaccines. Taken together, these data strongly suggest that the MFT cells identified in this study make at least 3- to 5-fold more IFN-γ than monofunctional IFN-γ-producing cells.

**DISCUSSION**

To date, classical vaccination approaches have not been sufficient to develop highly effective vaccines against tuberculosis. Consequently, many recent experimental immunization strategies against this disease have focused on combining partially effective vaccines to achieve superior levels of efficacy relative to individual vaccination regimens. In particular, heterologous prime/boost regimens have been shown to induce strong and broad cellular immunity and an enhanced quality of T-cell responses (2, 11, 13, 18, 23, 26, 27). In this study, we showed that a heterologous prime/boost regimen using a TB fusion protein (E6-85) formulated in DDA/MPL adjuvant as a prime and an MVA recombinant virus expressing ESAT6, antigen 85B, antigen 85A, Hsp60, and Mtb39 with IL-15 as an adjuvant (MVA/IL-15/5Mtb) as the boosting agent elicited antituberculosis immunity in the mouse model which was equivalent to the protective responses induced by BCG vaccine. Moreover, using multiparameter flow cytometry, we showed that the E6-85/MVA/IL-15/5Mtb prime/boost regimen evoked diverse CD4 and CD8 T-cell responses. The most protective vaccines in this study, the E6-85/MVA/IL-15/5Mtb combination and live BCG vaccine, induced significantly elevated levels of multifunctional CD4 and CD8 T-cell responses. The less-protective E6-85/adjuvant formulation generally elicited a more modest MFT-cell and monofunctional response than the more protective vaccination regimens. Interestingly, the median IFN-γ response for the MFT cells detected in these experiments was at least 3-fold higher than the median levels of IFN-γ in cells making only this cytokine.
IFN-γ, TNF-α, and IL-2, are functionally superior to monofunctional cells (14). In mice, the induction of MFT cells by immunization correlated with the extent of protection against *Leishmania major* (6). In humans, the presence of MFT cells has been associated with the control of viremia in nonprogressive HIV infections (1). Several immune mechanisms have been suggested to explain the presumed superiority of MFT cells (25). First, MFT cells seem to secrete more IFN-γ than monofunctional cells. Since IFN-γ is a critical cytokine in the defense against mycobacterial infections, elevated IFN-γ concentrations should enhance infection control. Second, the secretion of IFN-γ and TNF-α, another important antimycobacterial immune modulator, from the same cell may increase local intracellular killing. Finally, IL-2 secretion from MFT cells should promote T-cell expansion and enhance long-term survival of memory CD4 cells in vivo.

A clear difference in the T-cell response profile evoked by prime/boost immunization with the E6-85/MVA/IL-15/5Mtb vaccine regimen compared to BCG vaccination was the significantly elevated percentages of splenic CD4 T cells making both TNF-α and IL-2 (Fig. 3A). Lindenstrøm et al. have also reported that a TB subunit vaccine preparation induces long-lived TNF-α/IL-2 double-positive cells, while fewer cells with the same phenotype were identified in the spleens of BCG-vaccinated animals (17). These data strongly suggest that the type of vaccine administered can impact the quality and magnitude of the vaccine-induced T-cell memory response. The induction of TNF-α/IL-2-positive MFT cells and cells that make only IL-2 in response to MVA/IL-15/5Mtb vaccinations may explain the persistence of the MVA response seen in the 16-month study with the MVA/IL-15/5Mtb vaccine. The failure of BCG to induce this double–positive phenotype may contribute to the waning BCG protective response detected at 16 months postvaccination in the same study. This intriguing observation suggests that further studies should explore the role of the vaccine-induced TNF-α/IL-2–positive T-cell subset in the persistence of antituberculosis protective immunity.

Recent vaccination studies have indicated that increasing the intervals between immunizations may permit enhanced maturation of effector memory cells to more-potent central memory cells prior to boosting, which ultimately should lead to improved vaccine efficacy (4, 10, 22). In our studies using the MVA/IL-15/5Mtb vaccine candidate as a boosting agent, no impact on the antituberculosis protective responses was observed when the time interval between priming and boosting was increased. With both homologous (1-month versus 7-month boosts) and heterologous (2-month versus 6-month boosts) regimens, the MVA/IL-15/5Mtb vaccine-induced antituberculosis protection was not dependent on the immunization schedule. Even extending the postboosting vaccination-challenge interval to 11 months (Table 1) did not reduce the protective effectiveness of the MVA/IL-15/5Mtb vaccine. Interestingly, in humans, the interval between an initial BCG immunization and an MVA85 booster vaccination did not influence the magnitude of the antituberculosis immune response (20). Since MVA does not replicate in mammalian cells, viral persistence cannot explain the longevity of MVA/IL-15/5Mtb-induced immunity. It is more likely that the capacity of the MVA/IL-15/5Mtb vaccine to induce strong and broad cellular immunity (including IFN-γ, IL-17, and IL-27 expression) (Table 3) and to effectively boost memory cell populations diminished any impact increasing (or decreasing) immunization time intervals may have on the induction of protective immunity.

In sum, we have shown that vaccination with our MVA/IL-15/5Mtb vaccine induces highly persistent antituberculosis protective responses. Furthermore, immunization with a heterologous prime/boost regimen using a TB fusion protein formulated with DDA/MPL adjuvant followed by boosting with MVA/IL-15/5Mtb induces substantial protective immunity in a mouse model of pulmonary tuberculosis. Most clinical efforts related to TB vaccines are currently focusing on the development of vaccines to boost BCG responses. While this approach sidesteps concerns about discontinuing BCG use, it also does not resolve safety issues related to BCG immunization in areas to which HIV is endemic and the continued uncertainty about the overall effectiveness of BCG-induced immunity. This heterologous E6-85 protein/MVA/IL-15/5Mtb prime/boost regimen provides an alternative strategy for combating the devastating TB epidemic.

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