The Journal of Immunology



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*J Immunol* 2013; 191:2514-2525; Prepublished online 31 July 2013;
doi: 10.4049/jimmunol.1301161
http://www.jimmunol.org/content/191/5/2514



*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

# Protection against Tuberculosis with Homologous or Heterologous Protein/Vector Vaccine Approaches Is Not Dependent on CD8$^+$ T Cells

**Susan L. Baldwin,*[,1] Lance K. Ching,*[,1] Samuel O. Pine,[†] Magdalini Moutaftsi,* Elyse Lucas,* Aarthy Vallur,* Mark T. Orr,* Sylvie Bertholet,[‡] Steven G. Reed,*[,§,¶] and Rhea N. Coler*[,§]**

**Considerable effort has been directed to develop *Mycobacterium tuberculosis* vaccines to boost bacille Calmette-Guérin or for those who cannot be immunized with bacille Calmette-Guérin. We hypothesized that CD4$^+$ and CD8$^+$ T cell responses with a heterologous prime/boost vaccine approach could induce long-lived vaccine efficacy against *M. tuberculosis* in C57BL/6 mice. We produced an adenovirus vector expressing ID93 (Ad5-ID93) for induction of CD8 T cells to use with our candidate tuberculosis vaccine, ID93/glucopyranosyl lipid adjuvant (GLA)-stable emulsion (SE), which induces potent Th1 CD4 T cells. Ad5-ID93 generates ID93-specific CD8$^+$ T cell responses and induces protection against *M. tuberculosis.* When Ad5-ID93 is administered in a prime-boost strategy with ID93/GLA-SE, both CD4$^+$ and CD8$^+$ T cells are generated and provide protection against *M. tuberculosis*. In a MHC class I–deficient mouse model, all groups including the Ad5-ID93 group elicited an Ag-specific CD4$^+$ T cell response and significantly fewer Ag-specific CD8$^+$ T cells, but were still protected against *M. tuberculosis*, suggesting that CD4$^+$ Th1 T cells could compensate for the loss of CD8$^+$ T cells. Lastly, the order of the heterologous immunizations was critical. Long-lived vaccine protection was observed only when Ad5-ID93 was given as the boost following an ID93/GLA-SE prime. The homologous ID93/GLA-SE prime/boost regimen also induced long-lived protection. One of the correlates of protection between these two approaches was an increase in the total number of ID93-specific IFN-γ–producing CD4$^+$ T cells 6 mo following the last immunization. Our findings provide insight into the development of vaccines not only for tuberculosis, but other diseases requiring T cell immunity.** ***The Journal of Immunology*****, 2013, 191: 2514–2525.**

Tuberculosis (TB) is the most common cause of infectious disease-related mortality worldwide. One in three people are currently infected with *Mycobacterium tuberculosis* (1). Although the incidence rate of TB has declined by 2.2% between 2010 and 2011, there were ∼8.7 million new cases in 2011, and 1.4 million deaths associated with TB during this time (2). In addition, there has been an increase in multiple drug-resistant TB in 2011 (630,000 cases), and 84 countries have reported extensively drug-resistant TB cases (2). Bacille Calmette-Guérin (BCG) is the only approved vaccine against *M. tuberculosis* and has been shown to reduce the risk of serious childhood forms of TB. BCG efficacy, however, wanes over time and is not effective against pulmonary disease in adults (3). The absence of a robust, protective vaccine underscores the need to develop a long-lasting and effective vaccine for the prevention of TB.

Our laboratory has developed a novel TB vaccine known as ID93/glucopyranosyl lipid adjuvant (GLA)-stable emulsion (SE), which is currently in Phase I human clinical trials. ID93 is a fusion protein comprising four *M. tuberculosis* proteins, as follows: Rv2608 (PE/PPE family), Rv3619, Rv3620 (EsX family of virulence factors), and Rv1813 (upregulated during latency) (4, 5). When combined with the synthetic TLR4 adjuvant, GLA-SE, ID93 boosts the BCG vaccine and prolongs survival in guinea pigs (5). GLA-SE is a hexa-acylated lipid A derivative shown previously to have multifunctional immunomodulatory activity similar to monophosphoryl lipid A (MPL) on murine cells, but is more potent on a molar basis on human dendritic cells and PBMCs (6). In the absence of a BCG prime, ID93/GLA-SE is also effective as a prophylactic vaccine, capable of reducing lung bacterial burden in the mouse model and reducing lung pathology and improving survival in the guinea pig model of *M. tuberculosis* infection (7).

Correlates of cellular immune protection against *M. tuberculosis* are poorly defined. The prevalent belief is that Th1 CD4$^+$ T lymphocytes are critical, but a role for mouse, nonhuman primate, and human CD8$^+$ T cell responses in defense against *M. tuberculosis* has also been reported (8–14). Viral vaccine vectors such as adenoviral or modified vaccinia Ankara (MVA) expression vectors have been widely used to generate both CD4$^+$ and CD8$^+$ T cell responses against *M. tuberculosis* (15–25) and other pathogens (26–28). Human clinical trials have been completed with MVA85A, a vaccine developed against TB, in several age groups, including adults and infants (19, 20, 29). The first Phase IIb safety and efficacy trial to use a TB vaccine made to boost

*Infectious Disease Research Institute, Seattle, WA 98102; [†]Allergan, Irvine, CA 92612; [‡]Novartis Vaccines and Diagnostics, I-53100 Siena, Italy; [§]Department of Global Health, University of Washington, Seattle, WA 98195; and [¶]Immune Design, Seattle, WA 98102

[1]S.L.B. and L.K.C. contributed equally to this work.

Received for publication April 30, 2013. Accepted for publication June 27, 2013.

This work was supported by National Institutes of Health Grants AI-044373 and AI-078054 and National Institutes of Health Contracts AI-25479 and HHSN272200800045C.

Address correspondence and reprint requests to Dr. Susan L. Baldwin, Infectious Disease Research Institute, 1616 Eastlake Avenue East, Suite 400, Seattle, WA 98102. E-mail address: susan.baldwin@idri.org

The online version of this article contains supplemental material.

Abbreviations used in this article: BCG, bacille Calmette-Guérin; GLA, glucopyranosyl lipid adjuvant; ICS, intracellular cytokine staining; i.d., intradermal; IDRI, Infectious Disease Research Institute; MPL, monophosphoryl lipid A; MVA, modified vaccinia Ankara; SE, stable emulsion; TB, tuberculosis.



www.jimmunol.org/cgi/doi/10.4049/jimmunol.1301161

BCG was recently described, in which BCG-immunized infants were boosted with MVA85A (30). Although the outcome of the trial resulted in lack of significant efficacy against *M. tuberculosis* with this vaccine strategy in BCG-primed infants, it may still prove to be effective in adults, against pulmonary TB (30). Other human clinical trials that have been completed with adenoviral vector vaccines include vaccines against both HIV (31) and malaria (32, 33), in addition to those using MVA vaccines against several infectious diseases such as HIV (34, 35), malaria (36), influenza (37), and smallpox (38). The Ad5-vectored *Plasmodium falciparum* vaccine encoding the 3D7 circumsporozite protein (NMRC-MV. AD-PfC) approach resulted in primarily $CD8^+$ T cell responses, but did not induce sterile protection in human volunteers (33). Some HIV adenoviral vaccine approaches have involved a DNA prime/adenoviral boost regimen that augments both HIV-1 $CD4^+$ and $CD8^+$ T cells and humoral immune responses (39–41).

We hypothesize that, by inducing both $CD4^+$ and $CD8^+$ T cell responses with a heterologous prime/boost vaccine approach through use of the ID93/GLA-SE subunit vaccine and an adenoviral vector expressing the ID93 protein, we can provide protection against primary *M. tuberculosis* infection and long-lived memory capable of clearing *M. tuberculosis*. To test this hypothesis, we developed an adenovirus type 5 vector expressing ID93 (Ad5-ID93), produced as a proof-of-principle vaccine for the generation of Ag-specific $CD8^+$ T cells, which could be used alone or as part of a heterologous prime/boost strategy in combination with ID93/GLA-SE. We previously demonstrated that the ID93/GLA-SE subunit vaccine induces a potent $CD4^+$ Th1 cell response that effectively provides protection against *M. tuberculosis* (5, 7). In this work, we show the outcome of Ad5-ID93 immunization when given as a homologous prime/boost regimen or when administered as part of a heterologous prime-boost strategy with ID93/GLA-SE. We also look mechanistically at the requirement of ID93-specific $CD8^+$ T cells in a MHC class I–deficient mouse model that has diminished levels of $CD8^+$ T cells. This work sheds additional insight into the development of new TB vaccines and will foster a new appreciation for novel prime/boost strategies against *M. tuberculosis* and similar pathogens that require the generation of potent Ag-specific Th1 $CD4^+$ T cells.

## Materials and Methods

### *ID93*

ID93 is a recombinant fusion protein incorporating four *M. tuberculosis* Ags, as follows: Rv2608, Rv3620, Rv1813, and Rv3619 (5).

### *Recombinant adenovirus serotype 5 expressing ID93 (Ad5-ID93)*

DNA encoding ID93 was inserted into an adenoviral type 5 vector with a CMV promoter (Viraquest, North Liberty, IA). Replication-deficient recombinant adenovirus clones expressing either no Ag (Ad5-null) or full-length ID93 (Ad5-ID93) were constructed. Adenoviral stocks were amplified, purified by CsCl gradient, and dialyzed to remove the cesium. Virus preps were quantitated by plaque assay.

### *Cell culture*

AD-293 cells are human embryonic kidney cells transformed with sheared adenovirus type 5 DNA (E1 region) with an increased adherence phenotype. Cells were grown in DMEM (Invitrogen) with 10% heat-inactivated FBS and penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C and 5% $CO_2$.

### *Transgene expression of Ad5-ID93 (Western blot) transgene expression verification*

AD-293 cells were infected with Ad5-ID93. Cells were harvested 72 h later and pelleted by centrifugation (1500 relative centrifugal force, 5 min). The washed pellet was resuspended in 1 ml PBS, and 100 μl cell suspension was combined with 20 μl 6× buffer (0.375 M Tris, 12% $NaDodSO_4$, 0.45 M DTT, 60% glycerol, 0.03% bromophenol blue) and then boiled for 5 min prior to gel electrophoresis (Tris-glycine gel; Invitrogen, Grand Island, NY). After separation, proteins were transferred to nitrocellulose and probed with a purified mAb raised against recombinant ID93 (1:50,000 dilution), followed by HRP-conjugated goat anti-mouse Ab (Jackson ImmunoResearch Laboratories; 1:10,000 dilution). Blots were developed using ECL detection reagent (GE Healthcare, Piscataway, NJ) and imaged by exposing to Kodak Blue XB-1 film (Perkin Elmer, Waltham, MA).

### *Glucopyranosyl lipid adjuvant*

The synthetic TLR-4 agonist, GLA, was bulk manufactured by Avanti Polar Lipids (Alabaster, AL) and formulated at the Infectious Disease Research Institute (IDRI) in an oil-in-water SE.

### *Identification of MHC I– and MHC II–restricted ID93 epitopes*

Splenocytes from mice immunized with either ID93/GLA-SE or Ad5-ID93 were stimulated with a 15-mer peptide library spanning ID93. To identify minimal $CD8^+$ T cell epitopes, sequences of positive 15-mer peptides were used to predict the MHC I–restricted T cell epitopes using a consensus prediction algorithm on the Immune Epitope Database (http://www.immuneepitope.org/). The sequence of the MHC class I–restricted 8-mer epitope, included in Rv2608, is VTNLHTAIM, whereas the two MHC class II 15-mer–restricted epitopes from the Rv2608 and Rv3619 proteins (in the ID93 fusion protein) are NFAVLPPEVNSARIF and FQVIYEQANAHGQKV, respectively.

### *Animals*

Female C57BL/6 mice (5–7 wk old) were purchased from Charles River Laboratories (Wilmington, MA). For studies using MHC class I–deficient mice, female C57BL/6 mice (7–9 wk old) and age-matched $H2K^{b-/-}D^{b-/-}$ double-knockout mice (42, 43) were purchased from Taconic (Hudson, NY). Animals were housed at the IDRI animal facility under specific pathogen-free conditions. All procedures were performed in accordance with the regulations and guidelines of the IDRI institutional animal care and use committee.

### *Immunization and murine protection model*

Mice ($n$ = 10/group) were immunized two to three times, 3 wk apart. A recombinant replication-deficient adenovirus serotype 5 containing either no Ag (Ad5-null) or the ID93 Ag (Ad5-ID93) was administered intradermally (i.d.) in the right hind footpad at a dose of $5 \times 10^8$ virus particles in 30 μl 20 μM HEPES plus 3% sucrose, and in the left hind footpad at the same dose and volume for the second immunization. Groups also receiving one or more injections of recombinant ID93 (0.5 μg) in the presence or absence of 5 μg GLA-SE were inoculated i.m. Saline controls were immunized i.m. two or three times, 3 wk apart. Vaccine control mice immunized with the BCG (Pasteur strain; Sanofi Pasteur) vaccine were given a single i.d. dose of $5 \times 10^4$ CFU at the base of the tail. We have previously shown that mice given adjuvant alone (GLA-SE) provide similar results to the saline control group, neither of which are protective against *M. tuberculosis*; thus, we have not included an adjuvant-alone group in these experiments (5).

### *ID93 Ab ELISA*

Serum was collected from immunized mice on day 56 (2 wk after the last immunization), and ID93-specific endpoint titers for IgG1, IgG2c, and total IgG were performed, as previously described (5, 7). Plates were read at 450 nm with a reference filter set at 570 nm using a Versamaxx microplate reader (Molecular Devices, Sunnyvale, CA). Reciprocal dilutions corresponding to endpoint titers were determined utilizing Soft Max Pro5 software (San Diego, CA) with a 0.1 absorbance cutoff.

### *Cytokine ELISPOTs*

IFN-γ and IL-5 ELISPOTs were performed on fresh splenocytes harvested 1 wk following the final immunization ($n$ = 3 mice/group), as previously described (7). Splenocytes ($2 \times 10^5$ cells/well) were stimulated with either media, Con A (0.75 μg/ml), or ID93 protein (10 μg/ml). To determine whether cytokine-producing cells were induced from $CD4^+$ or $CD8^+$ T cells, stimulations were also performed with ID93-specific 15-mer $CD4^+$ peptides or an ID93-specific 8-mer $CD8^+$ peptide. Spots were counted on an automated ELISPOT reader (C.T.L. Seri3A Analyzer; Cellular Technology, Cleveland, OH) and analyzed with ImmunSpot software (C.T.L. Analyzer).

### *Flow cytometry (intracellular cytokine staining)*

Splenocytes from three mice per group were isolated 1 wk following the final immunization for evaluation of effector T cell responses. Splenocytes

were also harvested 6 mo following the final immunization for evaluation of memory T cell responses. Cells were plated at $2 \times 10^6$ cells/well in 96-well U-bottom plates and restimulated with either media (negative control), PMA plus ionomycin (1 μg/ml each; as a positive control), ID93 (10 μg/ml), or a pool of the two 15-mer peptides containing I-$A^b$–restricted epitopes (1 μg/ml each) and a H-$2^b$–restricted 8-mer peptide (1 μg/ml). In some cases, fluorochrome-conjugated anti-CD107a (clone ebio1d4b) was added (1:200) at the time of stimulation. GolgiPlug (eBioscience, San Diego, CA) was added after 2 h at 1 μg/ml and incubated with cells for 8 h at 37°C. Cells were surface stained with fluorochrome-conjugated mAbs to CD4 (clone RM4-5), CD8 (clone 53-6.7), and CD44 (clone IM7) (eBioscience) in PBS with 20% normal mouse serum (Phenix Research Products, Candler, NC). Cells were fixed and permeabilized for 20 min at room temperature using Cytofix/Cytoperm (BD Biosciences, San Jose, CA), according to the manufacturer's instructions, and stained intracellularly for 15 min at room temperature with fluorochrome-conjugated mAbs to IFN-γ (clone XMG1.2), TNF (clone MP6-XT22), and IL-2 (clone JES6-5H4) (eBioscience). Cells were washed twice with BD Perm/Wash buffer 1× (BD Biosciences) and resuspended in PBS with 1% BSA prior to collection on a modified 3-laser BD LSRII or Fortessa flow cytometer (BD Biosciences) and analysis using FlowJo software (Tree Star). Lymphocytes were gated by forward and side scatter, and 20,000 $CD4^+$ events were acquired for each sample. Analysis and presentation of distributions were performed using SPICE version 5.2, downloaded from the National Institutes of Health site (http://exon.niaid.nih.gov/spice). Postexposure intracellular cytokine staining (ICS; similar to the procedure described for splenocytes) was performed on the lungs of immunized mice challenged with *M. tuberculosis* 6 mo after the second immunization. Prior to staining, as mentioned above, lung cells were stained for viability using a fixable Live/Dead stain kit at 4°C for 30 min (Invitrogen, Carlsbad, CA). Lungs were taken at the time of the harvest for CFUs (6 wk after challenge).

### *Aerosol* M. tuberculosis *infection*

Mice were aerogenically challenged with a low-dose aerosol of *M. tuberculosis* H37Rv (ATCC 35718; American Type Culture Collection, Manassas, VA) 6 wk, or 6 mo, after the final immunization ($n = 7$ mice/group), as previously described (7). All operations were performed under biosafety level-3 conditions.

### *Bacterial burden (CFUs)*

To assess bacterial burden, tissues were homogenized through a 45 μM cell strainer and serially diluted 5-fold in 0.1% Tween 80, PBS. Bacterial burden was assessed by plating these dilutions onto Middlebrook 7H11 agar (Molecular Toxicology, Boone, NC) and counting colonies after 2–3 wk of incubation at 37°C with 5% $CO_2$. Protective efficacy promoted by the vaccine was compared with the protective levels afforded when only saline was administered. Reductions in bacterial burden in the lungs are calculated as follows: $\text{MeanLog}_{10}\ \text{CFU}_{\text{saline}} - \text{MeanLog}_{10}\ \text{CFU}_{\text{vaccine}}$.

### *Statistical analysis*

Statistical analyses were performed using GraphPad Prism software (La Jolla, CA). Standard one-way ANOVA, followed by a Dunnett's multiple comparison test, was used for analyses involving more than two groups of mice, unless otherwise indicated. A Student's *t* test was used when comparisons of two groups were performed. The *p* values ≤0.05 were considered significant. Data reported in this work are representative of at least two experiments, except the long-term vaccine efficacy study, which is representative of a single study.

## Results

### *ID93 expression by the Ad5-ID93 expression vector*

Ad5-ID93 was constructed to determine whether ID93-specific $CD8^+$ T cells generated from this vaccine alone, or in a heterologous prime/boost regimen with our candidate subunit vaccine (ID93/GLA-SE), could effectively induce protective immune responses against *M. tuberculosis*. The Ad5-ID93 expression vector was constructed by inserting DNA for the ID93 fusion protein into an adenoviral type 5 vector with a CMV promoter. A recombinant adenovirus clone expressing no Ag (Ad5-null) was also constructed.

We first tested the Ad5-ID93 vector to ensure that the vector properly expressed the ID93 fusion protein. Expression of ID93 in Ad5-ID93–infected 293 cells was verified by Western blot (Fig. 1A). Purified ID93 was included at 1 and 2 ng as a control for the lysates (loaded with 1.25, 2.5, or 5 μl lysate) from Ad5-ID93–infected cells.

Next, we determined whether the expressed ID93 protein from the Ad5-ID93 vaccine elicited immune responses in vivo, by measuring Ab responses following immunization with either Ad5-ID93, ID93/GLA-SE, or heterologous prime/boost immunizations with both the subunit and adenoviral vector vaccines. Two weeks after the final immunization, significant levels of anti-ID93 IgG

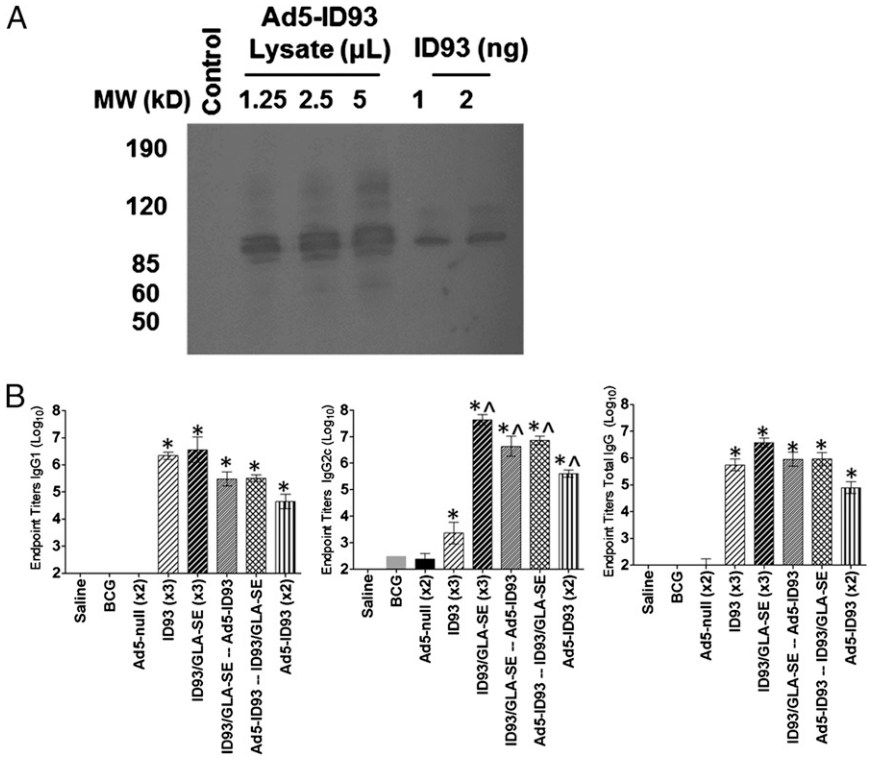


**FIGURE 1.** (**A**) Expression of ID93 by Western blot from an adenoviral vector (subtype 5) with an ID93 fusion protein gene insert. Different amounts of lysates (1.25, 2.5, and 5 μl) from Ad5-ID93–infected AD-293 cells were added to the wells, as indicated. Purified ID93 protein was also included as a positive control at 1 and 2 ng. (**B**) ID93-specific endpoint Ab titers (5 mice/group), including IgG1, IgG2c, and total IgG 2 wk after the last immunization. Results are shown as averages ± SD and are representative of two separate experiments. *$p < 0.05$ compared with saline, ^$p < 0.05$ compared with ID93 immunization.

Abs were detected in the serum of all Ad5-ID93– and ID93/GLA-SE–immunized but not saline-, BCG-, or Ad5-null–injected animals (Fig. 1B). ID93-specific IgG1 and IgG2c Ab titers were also measured (Fig. 1B). As with ID93/GLA-SE, all Ad5-ID93 vaccine regimens induced greater ID93-specific IgG2c responses compared with either the saline or unadjuvanted ID93-injected groups, indicative of a Th1-biased response.

### *Immunogenicity: Ad5-ID93, ID93/GLA-SE, and heterologous prime/boost regimens*

Mice were inoculated twice with Ad5-ID93 (×2), 3 wk apart. A separate group of animals received a single injection of Ad5-ID93 in a prime-boost regimen with recombinant ID93 protein plus GLA-SE, formulated in an oil-in-water emulsion (ID93/GLA-SE). Ad5-ID93 was tested either as the priming agent (Ad5-ID93→ID93/GLA-SE) or as a boost (ID93/GLA-SE→Ad5-ID93). Responses were compared with a subunit vaccine regimen with ID93/GLA-SE, administered i.m. either two or three times, 3 wk apart, as indicated.

Ag-specific T cell cytokine responses to ID93, CD4 peptide pools, or the CD8 peptide were examined by ELISPOT assay (Supplemental Fig. 1). Splenocytes ($n$ = 3 mice/group) were harvested 1 wk following the last immunization and stimulated ex vivo with either media, ID93, a CD4 peptide pool (CD4pp), or CD8 peptide (CD8pp). These ID93 peptides comprise dominant MHC I–restricted and MHC II–restricted epitopes identified in IFN-γ ELISPOT and ICS assays.

All vaccines including ID93/GLA-SE and/or Ad5-ID93 generated higher frequencies of ID93-specific cells producing IFN-γ compared with saline-treated controls or to unadjuvanted ID93 (Supplemental Fig. 1). Only regimens incorporating the ID93/GLA-SE vaccine elicited strong IFN-γ $CD4^+$ T cell responses by ELISPOT following stimulation with the MHC II–restricted epitope (CD4pp), whereas only ID93/GLA-SE, followed by Ad5-ID93, or two injections of Ad5-ID93 elicited a significant IFN-γ response following stimulation with the MHC I–restricted epitope (CD8pp) (Supplemental Fig. 1). Without adjuvant, ID93 elicited an IL-5 response following stimulation with either the ID93 protein or the MHC II–restricted epitope (Supplemental Fig. 1).

### *Intracellular cytokine staining*

Expression of IFN-γ, TNF, and IL-2 cytokines by splenic CD4/$CD44^{high}$ and CD8/$CD44^{high}$ T cells from C57BL/6 mice was measured 1 wk after the last immunization and determined by ICS following restimulation with ID93 (Fig. 2A–D). A high proportion of Ag-experienced ($CD44^{high}$) $CD4^+$ T cells secreting IFN-γ (>10%), TNF (>10%), or IL-2 (>10%) was observed for all mice receiving a regimen with an ID93/GLA-SE arm compared with saline-injected mice (Fig. 2A). Consistent with our ELISPOT data, all Ad5-ID93 containing immunization strategies generated $CD8^+$ T cells producing IFN-γ (>10%), TNF (>10%), in addition to IL-2 (>5% for Ad5-ID93 prime/ID93/GLA-SE boost and Ad5-ID93 [2×]) (Fig. 2B). CD107a, which is found on the surface of degranulating cells, adds a functional parameter of Ag-stimulated $CD8^+$ T cells; anti-CD107a–stained $CD8^+$ T cells represent Ag-specific cytotoxic effector cells (44). Significant percentages of CD107a-stained $CD8^+$ T cells were observed in animals immunized at least once with Ad5-ID93 (Fig. 2B). Polyfunctional $CD4^+$ T cells producing IFN-γ, IL-2, and TNF (all >5%) were detected in groups that received at least one injection of ID93/GLA-SE, but were not detected to significant levels for mice inoculated with unadjuvanted ID93, or BCG (Fig. 2C). The greatest proportion of $3^+$ and $2^+$ cytokines (pie charts; Fig. 2C) secreted from $CD4^+$ T cells was observed in the ID93/GLA-SE group, followed by equal responses in the heterologous prime/boost groups, and lastly the Ad5-ID93 group; the order of the prime/boost regimen did not matter with respect to the proportion of triple, double, or single cytokines. Polyfunctional $CD107a^+CD8^+$ T cells, producing IFN-γ, IL-2, and TNF (~5% frequency), were observed only in the Ad5-ID93 (2×) group and the group that received a prime with Ad5-ID93, followed by a boost with ID93-GLA-SE (Fig. 2D), although all groups that were given at least one Ad5-ID93 immunization had significant levels of polyfunctional $C107a^+CD8^+$ T cells producing IFN-γ and TNF (>5% frequencies) (Fig. 2D), including the ID93/GLA-SE prime/Ad5-ID93 boost regimen. No significant percentages of single cytokines from CD8/$CD44^{high}$ splenocytes were observed except in the group given Ad5-ID93 (2×), where ~1.4% single IFN-γ–producing $CD8^+$ T cells were induced (data not shown). When absolute numbers of ID93-specific IFN-γ–producing T cells were determined, the ID93/GLA-SE–immunized group induced significantly higher IFN-γ–secreting $CD4^+$ T cells (Fig. 2E). Alternatively, all of the Ad5-ID93–containing immunization regimens induced significantly higher numbers of IFN-γ–secreting $CD8^+$ T cells compared with saline (Fig. 2E). Similar numbers of IFN-γ–producing effector $CD8^+$ T cells 1 wk after the last immunization were observed regardless of the order of immunization. Ad5-ID93 (2×) induced statistically greater numbers of IFN-γ–secreting $CD8^+$ T cells compared with the heterologous prime/boost approaches (Fig. 2E).

### *Short-term protection against* M. tuberculosis *with ID93/GLA-SE, Ad5-ID93, or heterologous prime/boost approaches with ID93/GLA-SE and Ad5-ID93*

Protective efficacy of the Ad5-ID93 vaccine was assessed by enumerating bacterial burden in the lungs 6 wk following a low-dose aerosol challenge with *M. tuberculosis* H37Rv in four independent studies (Fig. 3 shows the results of one experiment; Table I shows the results of three additional protection studies). As expected, bacterial burden in the lungs of BCG-immunized mice was reduced significantly compared with saline-treated controls ($p < 0.05$). Reduction of *M. tuberculosis* burden in the lung was observed in C57BL/6 mice that were immunized with two (Table I) or three doses of recombinant ID93/GLA-SE ($p < 0.05$), compared with the saline control group (Table I, Fig. 3). Protection against *M. tuberculosis* was also observed in the lungs of mice following either a homologous Ad5-ID93 prime/boost (Table I, Fig. 3) or a heterologous prime/boost regimen with ID93/GLA-SE and Ad5-ID93 compared with saline, regardless of whether the subunit vaccine was given as the prime or the boost (Fig. 3). Mice immunized twice with the Ad5-null vector (Table I, Fig. 3), or immunized with the ID93 protein alone (Fig. 3), did not provide statistically significant protection against challenge with *M. tuberculosis*.

### *Ag-specific $CD8^+$ T lymphocytes in either ID93/GLA-SE or Ad5-ID93–containing immunization strategies are not required for protection against* M. tuberculosis

*MHC class I–deficient mouse model.* We next measured responses to immunization in a MHC class I–deficient mouse model to determine whether the Ad5-ID93 vaccine strategies required a $CD8^+$ T cell response to elicit protection against *M. tuberculosis*. Homozygous H-$2K^b$ and H-$2D^b$ double-targeted mutation mice are devoid of class Iα cell surface molecules and exhibit a reduction in the total number of $CD8\alpha\beta^+$ peripheral lymphocytes (43). We first measured the total number of $CD4^+$ and $CD8^+$ T cells in the wild-type C57BL/6 mice versus the MHC class I–deficient mice to confirm previous findings. As expected, there were significantly fewer $CD8^+$ T cells in the MHC class I–deficient mice (Supplemental Fig. 2). In addition, the $H2K^{b-/-}D^{b-/-}$ mice compensated

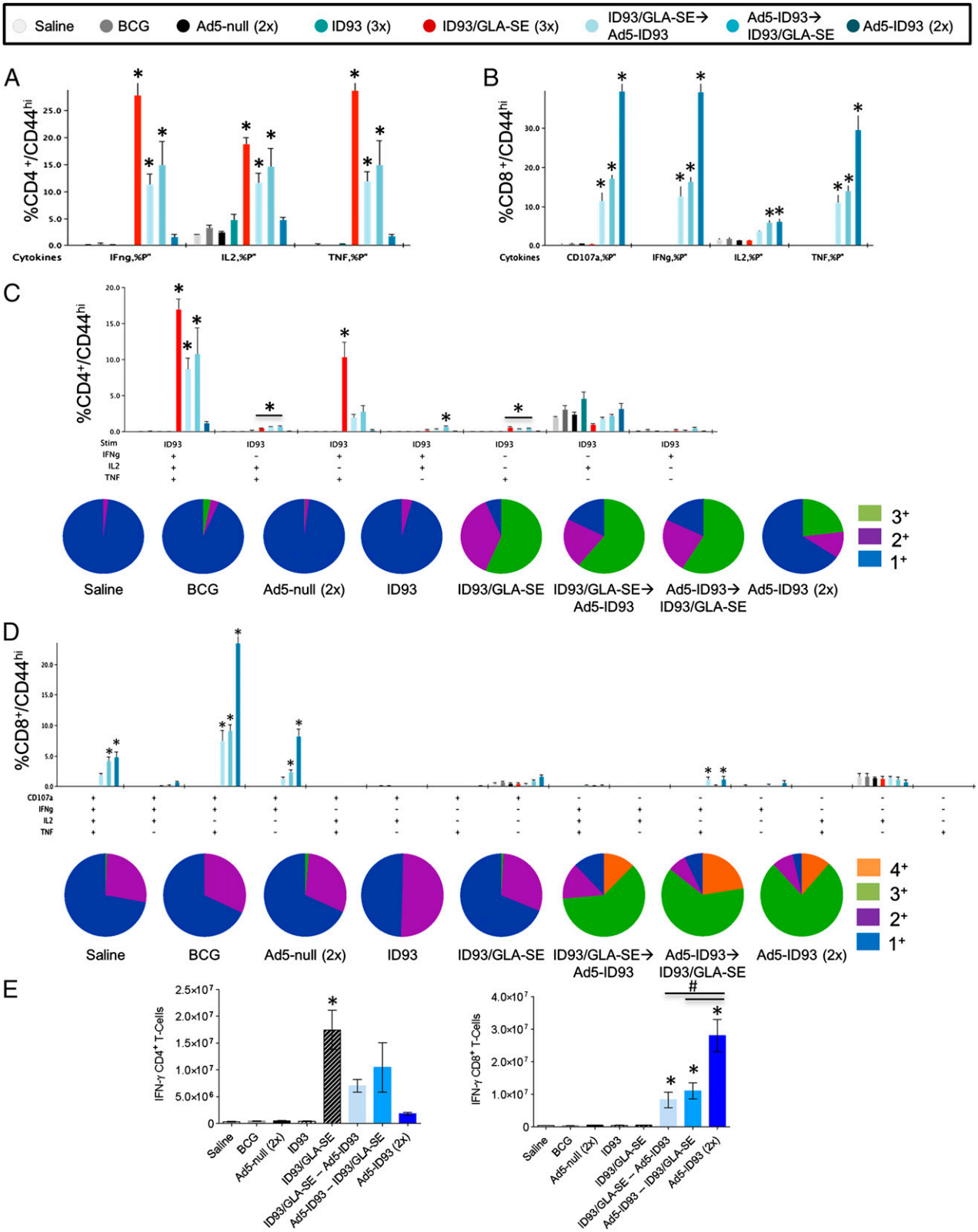


**FIGURE 2.** ID93-specific Th1 and/or CD8 cytokine responses from C57BL/6 mice immunized with ID93/GLA-SE and/or Ad5-ID93. Splenocytes were harvested from three mice/group, 1 wk following the final immunization. Cells were stimulated with ID93 for 12 h in the presence of GolgiStop. ID93-specific cells were identified by CD3 and either CD4 or CD8, and were further gated on $CD44^{high}$-expressing cells. The average percent frequency of each cell type ± SEM is shown, and results are representative of two independent studies. (**A**) ID93-specific $CD4^+/CD44^{high}$ T cells expressing IFN-γ, TNF, or IL-2; (**B**) $CD107a^+/CD8^+$ T cells and $CD8^+/CD44^{high}$ T cells expressing IFN-γ, TNF, IL-2; (**C**) polyfunctional cytokine-expressing $CD4^+/CD44^{high}$ T cells plus pie charts representing total triple, double, and single cytokines; (**D**) polyfunctional cytokine-expressing $CD8^+/CD44^{high}/CD107a^+$ T cells and pie charts representing quadruple-, triple-, double-, and single-stained cells; and (**E**) absolute numbers of IFN-γ–producing $CD4^+$ or $CD8^+$ T cells ± SD ($n = 3$ mice/group). *$p < 0.05$ compared with saline, #$p < 0.05$ versus Ad5-ID93 (2×).

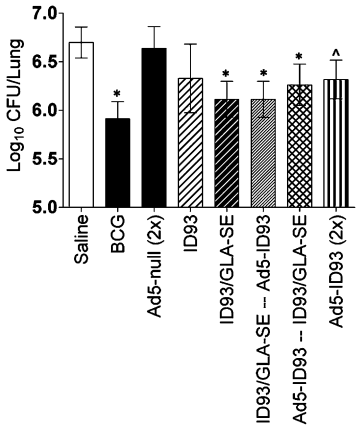


**FIGURE 3.** Bacterial load within the lungs in immunized C57BL/6 mice, 4 wk following challenge with *M. tuberculosis* H37Rv. Results are represented as $Log_{10}$ CFU within the lung. Results are representative of two separate experiments. $*p < 0.05$ compared with saline.

for the loss of $CD8^+$ T cells with approximately twice as many $CD4^+$ T cells in the spleen (Supplemental Fig. 2).

To determine the outcome of the immune response in the class I–deficient mice following immunization with the Ad5-ID93 vaccine candidates, we characterized the recall response to either ID93 or ID93 peptides. Similar to responses observed in C57BL/6 mice, the class I–deficient mice elicited robust $CD4^+$ T cell responses directed to ID93 by ELISPOT in all mice that were immunized at least once with ID93/GLA-SE (Supplemental Fig. 3). In contrast to the wild-type mice, there were no significant ID93-specific $CD8^+$ T cell responses in any of the Ad5-ID93–immunized groups, as indicated by the lack of responses to the CD8-specific peptide stimulation (Supplemental Fig. 3), whereas IL-5 responses were unchanged compared with the responses seen in wild-type mice (Supplemental Fig. 3). By flow cytometric analysis, significant frequencies of $CD4^+$/$CD44^+$ T cell responses expressing IFN-γ, TNF, or IL-2, and polyfunctional responses in which all three cytokines are expressed, were observed in mice immunized with ID93/GLA-SE (Fig. 4A, 4C). Significant levels of IL-2 from $CD4^+$ T cells were produced from mice given a heterologous prime/boost with ID93/GLA-SE, followed by Ad5-ID93 (Fig. 4A). Polyfunctional $CD4^+$ T cell responses were also observed in the regimens that included Ad5-ID93 (Fig. 4C). ID93-specific TNF, and TNF/IL-2 double-cytokine–producing cells, were observed in all mice immunized with at least one arm of the ID93/GLA-SE vaccine regimen (Fig. 4C). An increase in IFN-γ/IL-2 double-positive $CD4^+$ T cells was observed in the groups given Ad5-ID93 (2×), Ad5-ID93 followed by ID93/GLA-SE and ID93/GLA-SE (Fig. 4C). Even though $CD8^+$ T cells were significantly reduced in the MHC class I–deficient mice, there were still enhanced levels of $CD8^+$ T cell cytokine responses (including IFN-γ and TNF) observed 1 wk after immunization in the group given Ad5-ID93 (Fig. 4B). Negligible frequencies of polyfunctional $CD8^+$ T cell responses were observed in the MHC class I–deficient mice, although there was a significant frequency (~1%) of CD8/$CD107a^+$ T cells that expressed TNF in animals immunized with Ad5-ID93 (data not shown). Absolute numbers of ID93-specific IFN-γ–producing $CD4^+$ or $CD8^+$ T cells were also determined in the MHC class I–deficient mice. Animals immunized with the ID93/GLA-SE regimen and those that received an ID93/GLA-SE prime followed by an Ad5-ID93 boost induced significant numbers of IFN-γ–secreting $CD4^+$ T cells compared with the saline control (Fig. 4D). A more dramatic decrease in IFN-γ–producing $CD8^+$ T cells in the MHC class I–deficient mice was observed in the groups given Ad5-ID93 (mice given Ad5-ID93 twice had an 11.4-fold decrease in ID93-specific IFN-γ–secreting $CD8^+$ T cells compared with wild-type mice; ID93/GLA-SE followed by an Ad5-ID93 boost had an 8.68-fold reduction in these $CD8^+$ T cells, and a 12.35-fold decrease was observed in mice immunized with Ad5-ID93, followed by ID93/GLA-SE) (Fig. 4D compared with Fig. 2D).

Next, we examined the contribution of $CD8^+$ T lymphocytes to Ad5-ID93–induced vaccine protection against *M. tuberculosis* challenge. Protective efficacy of each immunization strategy was evaluated 6 wk after challenge in H-$2K^{b-/-}D^{b-/-}$ knockout mice. Immunization of H-$2K^{b-/-}D^{b-/-}$ mice with Ad5-ID93 (either homologous or heterologous immunization with ID93/GLA-SE) and ID93/GLA-SE immunization results in significant bacterial burden reduction in the lungs of the *M. tuberculosis*-infected mice (Fig. 5). Protection in the H-$2K^{b-/-}D^{b-/-}$ knockout mice was not impaired by the significant reduction of Ag-specific $CD8^+$ T cells, suggesting that the $CD4^+$ T cell responses in these immunized animals may compensate for the lack of Ag-specific CD8 T cells.

Table I. Protection with ID93/GLA-SE or Ad5-ID93 in C57BL/6 mice against infection with *M. tuberculosis*

| Groups | Treatment | $Log_{10}$ | SD | SE | $Log_{10}$ Protection in the Lungs versus Saline | *p* Value versus Saline |
|---|---|---|---|---|---|---|
| Experiment 1 | | | | | | |
| 1 | Saline | 6.64 | 0.15 | 0.06 | – | – |
| 2 | BCG | 5.81 | 0.20 | 0.08 | 0.84 | < 0.05 |
| 3 | Ad5-null ($5 \times 10^8$ vp) i.d. footpad (2×) | 6.59 | 0.18 | 0.07 | 0.05 | > 0.05 |
| 4 | ID93/GLA-SE (5 μg), i.m. (2×) | 6.02 | 0.23 | 0.09 | 0.62 | < 0.05 |
| 5 | ID93/GLA-SE (5 μg), i.m. (3×) | 6.06 | 0.24 | 0.09 | 0.58 | < 0.05 |
| 6 | ID93/GLA-SE, i.m.–Ad5-ID93 i.d. footpad ($5 \times 10^8$ vp) | 6.24 | 0.16 | 0.06 | 0.40 | < 0.05 |
| 7 | Ad5-ID93 i.d. footpad ($5 \times 10^8$ vp)–ID93/GLA-SE, i.m. | 6.02 | 0.21 | 0.08 | 0.62 | < 0.05 |
| 8 | Ad5-ID93 ($5 \times 10^8$ vp) i.d. footpad (2×) | 6.30 | 0.21 | 0.08 | 0.34 | < 0.05 |
| Experiment 2 | | | | | | |
| 1 | Saline | 6.62 | 0.29 | 0.11 | – | |
| 2 | BCG | 5.50 | 0.3 | 0.11 | 1.12 | < 0.01 |
| 3 | Ad5-null ($5 \times 10^8$ vp) i.d. footpad (2×) | 6.47 | 0.18 | 0.07 | 0.15 | > 0.05 |
| 4 | Ad5-ID93 ($5 \times 10^8$ vp) i.d. footpad (2×) | 6.16 | 0.3 | 0.12 | 0.46 | < 0.05 |
| Experiment 3 | | | | | | |
| 1 | Saline | 5.90 | 0.23 | 0.09 | – | – |
| 2 | BCG | 5.19 | 0.19 | 0.07 | 0.71 | < 0.01 |
| 3 | Ad5-ID93 ($5 \times 10^8$ vp) i.d. footpad (2×) | 5.46 | 0.12 | 0.05 | 0.44 | < 0.01 |

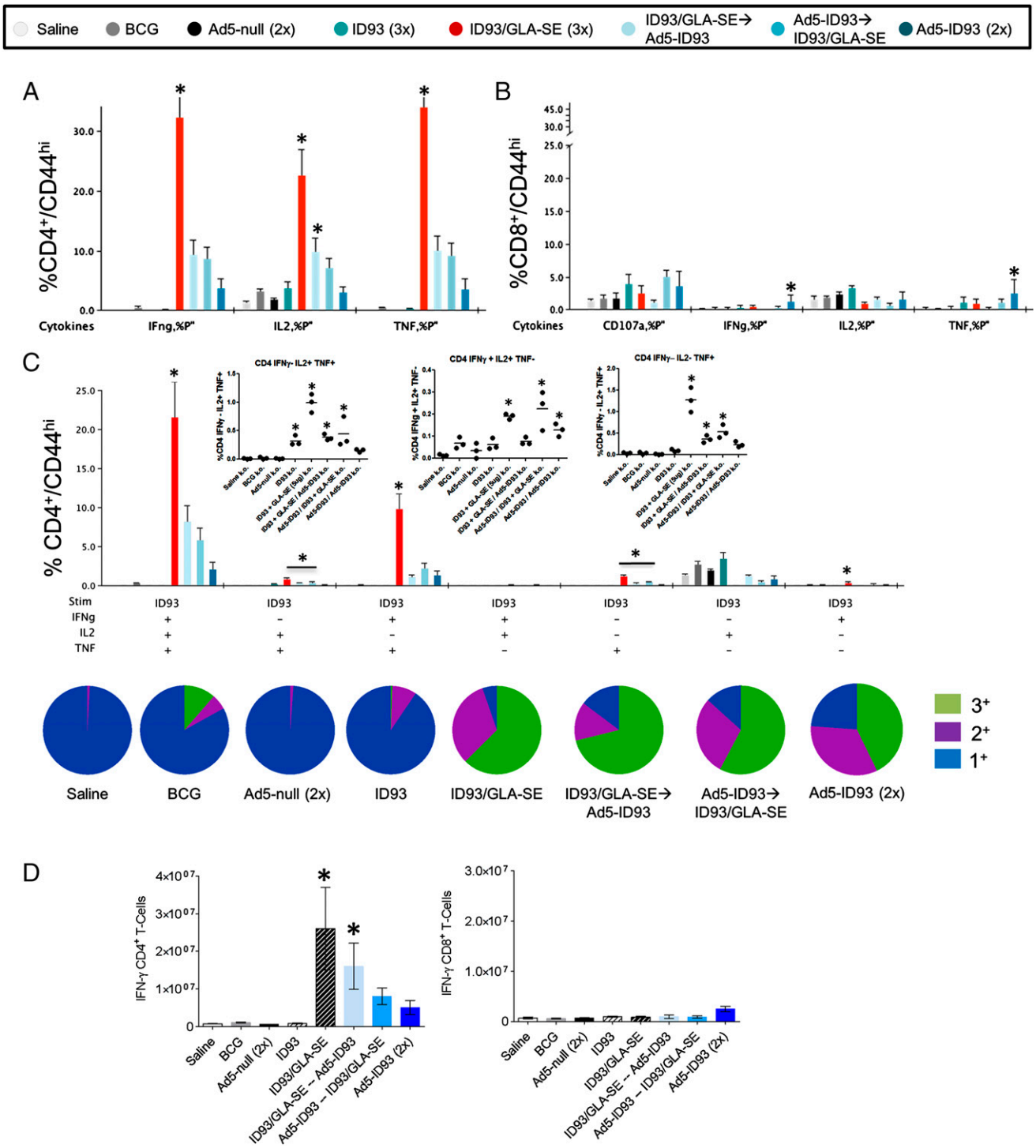


**FIGURE 4.** ID93-specific Th1 cytokine responses from $H2K^{b-/-}D^{b-/-}$ (MHC class I–deficient) mice immunized with ID93/GLA-SE and/or Ad5-ID93. Splenocytes were harvested from three mice/group, 1 wk following the final immunization. Cells were stimulated with ID93 for 12 h in the presence of GolgiStop. ID93-specific cells were identified by CD3 and either CD4 or CD8, and were further gated on $CD44^{high}$-expressing cells. The average percent frequency of each cell type ± SEM is shown, and results are representative of two independent studies. The average percent frequency of each cell type ± SEM is shown, and results are representative of two independent studies. (**A**) ID93-specific $CD4^+/CD44^{high}$ T cells expressing IFN-γ, TNF, or IL-2; (**B**) $CD107a^+/CD8^+$ T cells and $CD8^+/CD44^{high}$ T cells expressing IFN-γ, TNF, IL-2; (**C**) polyfunctional cytokine-expressing $CD4^+/CD44^{high}$ T cells and pie charts representing total triple, double, and single cytokines; and (**D**) absolute numbers of IFN-γ–producing $CD4^+$ or $CD8^+$ T cells ± SD ($n$ = 3 mice/group). *$p < 0.05$ compared with saline.

*Long-lived protection with ID93/GLA-SE or heterologous prime/boost approach with ID93/GLA-SE prime and Ad5-ID93 boost*

We next determined which immunization approach(es) would provide durable, long-lived vaccine efficacy, defined as protection against *M. tuberculosis* 6 mo following the last immunization. Immune responses were also measured 6 mo following immunization to determine the profile of immune responses required for long-term vaccine protection against *M. tuberculosis*. The importance of the order of the heterologous prime/boost immuni-

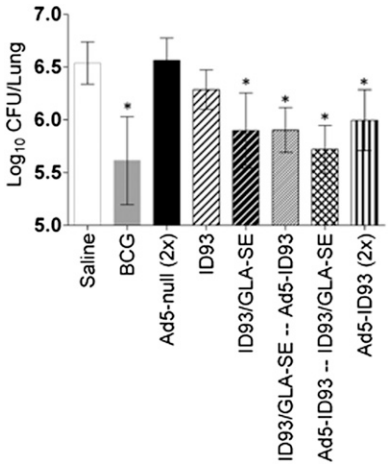


**FIGURE 5.** Bacterial load within the lungs of immunized $H2K^{b-/-}D^{b-/-}$ mice (MHC class I deficient) 4 wk following challenge with *M. tuberculosis* H37Rv. Results are represented as $Log_{10}$ CFU within the lung. Results are representative of two separate experiments. $*p < 0.05$ compared with saline.

zation strategy was apparent in the immune responses measured 6 mo following immunization. The subunit vaccine mainly induced a $CD4^+$ T cell response and no significant levels of $CD8^+$ T cells (Fig. 6A, 6B). An increased frequency of long-lived IFN-γ–secreting T cells was observed only from the splenocytes of animals previously immunized with the ID93/GLA-SE subunit vaccine (Fig. 6A). Significant frequencies of polyfunctional $CD4^+$ T cells (>0.5%) were also observed within the ID93/GLA-SE group compared with saline, including cells secreting triple IFN-γ, TNF, and IL-2, or double IFN-γ and TNF cytokines (Fig. 6C). Although not significant, there were greater proportions of $3^+$ and $2^+$ cytokine-producing cells (indicated in the pie charts) in the protein prime/vector boost group compared with the other two Ad5-ID93 vaccine strategies. No significant levels of IFN-γ, IL-2, or TNF-secreting $CD44^{high}/CD8^+$ T cells were observed in any of the immunized groups 6 mo postimmunization (Fig. 6B) compared with saline. However, there were slightly enhanced polyfunctional $CD8^+$ T cell responses (>0.1%) expressing IFN-γ and TNF observed in the group given the subunit ID93/GLA-SE vaccine, followed by a heterologous Ad5-ID93 boost, with more proportions of double, triple, and quadruple cytokine-expressing cells in this group (shown in the Fig. 6D pie charts) compared with the other groups. For single cytokine-expressing $CD8^+$ T cells, only the Ad5-ID93 prime followed by ID93/GLA-SE boost induced significant levels of single IFN-γ–producing $CD8^+$ T cells (~0.6%, data not shown); none of the groups induced significant levels of single cytokine $CD8^+$ T cells producing either TNF or IL-2 (data not shown). Absolute numbers of IFN-γ–secreting $CD4^+$ and $CD8^+$ T cells were also analyzed. Significant numbers of IFN-γ–secreting $CD4^+$ T cells were elicited in groups given the ID93/GLA-SE subunit vaccine, or when mice were immunized with the protein prime/adenovirus boost strategy (Fig. 6E). None of the vaccine strategies elicited significant absolute numbers of IFN-γ–secreting $CD8^+$ T cells (Fig. 6E), although the total $CD8^+$ T cells were elevated in the protein prime/vector boost-immunized mice. T cell responses within the lungs were also assessed in mice that were challenged with *M. tuberculosis* 6 mo after the last immunization. ICS was performed on lungs harvested 6 wk after *M. tuberculosis* challenge (Supplemental Fig. 4). Similar to responses seen in the spleen, the lungs of animals immunized with ID93/GLA-SE showed significant frequencies of IFN-γ–secreting $CD4^+$ T cells. Increased levels of TNF from $CD4^+$ T cells were also seen in the ID93/GLA-SE group, and in the two heterologous prime/boost groups (Supplemental Fig. 4A). The highest frequency of IFN-γ/TNF polyfunctional $CD4^+$ T cells was observed in the ID93/GLA-SE and in the ID93/GLA-SE prime/Ad5-ID93 boost groups (Supplemental Fig. 4C). There were no significant Ag-specific $CD8^+$ T cell responses in the lungs with any of the immunized groups, although all Ad5-ID93–containing vaccines had increased CD107a responses (Supplemental Fig. 4B, 4D).

Next, we measured the efficacy of the vaccine strategies against *M. tuberculosis* 6 mo after immunization in C57BL/6 mice. Mice administered with either two injections of the subunit vaccine (ID93/GLA-SE) or a prime/boost with ID93/GLA-SE followed by Ad5-ID93 significantly reduced the *M. tuberculosis* bacterial burden in the lung compared with mice injected with saline (Fig. 7). There was a clear benefit, therefore, of the protein prime followed by a vector boost, compared with the reciprocal approach in which the vector was given first followed by a protein boost.

## Discussion

Our previous work has demonstrated that IDRI's lead vaccine candidate ID93 combined with GLA-SE protects mice and guinea pigs against *M. tuberculosis* infection when administered as a prophylactic vaccine in the presence or absence of BCG priming (5, 7). Protection in these studies is mediated in large part by the induction of polyfunctional, Ag-specific $CD4^+$ T cell responses (5, 7). In this study, we constructed a recombinant adenovirus vector expressing the ID93 Ag to determine whether the generation of ID93-specific $CD8^+$ T cells elicited by the Ad5-ID93 vaccine (using either a homologous or heterologous prime/boost approach with the ID93 subunit vaccine) could provide short- or long-lived protection against *M. tuberculosis*. We also wanted to address whether the addition of an Ag-specific $CD8^+$ T cell response elicited by the Ad5-ID93 vaccine, combined with $CD4^+$ T cell–mediated protection elicited by ID93/GLA, could further boost and enhance protective responses against *M. tuberculosis*. The current vaccine against TB, BCG, loses its efficacy over time and fails to protect against adult pulmonary TB. A recent hypothesis regarding the reasons for lack of BCG protection in adults is the failure to generate significant numbers of long-lasting central memory T cells, including CD8 central memory T cells (45). Strategies to increase BCG efficacy have included ways to increase CD8 T cell responses, such as designing *Mycobacterium bovis* BCG mutants that secrete listeriolysin to promote increased MHC class I presentation (46, 47). Generating additional BCG mutants (48, 49), overexpressing *M. tuberculosis* proteins from recombinant *M. bovis* BCG (50, 51), or delivering BCG to different target tissues, such as the lymph node (52), have also shown promise for increasing BCG potency.

We show that the recombinant adenovirus vector expressing the ID93 Ag (Ad5-ID93) generated ID93-specific $CD8^+$ T cells. When Ad5-ID93 is given twice in a prime/boost regimen, or when it is given as a heterologous prime/boost with an ID93/GLA-SE vaccine, all of these strategies provided short-lived vaccine protection following challenge with *M. tuberculosis* in the murine model. In MHC class I–deficient mice, lacking significant levels of Ag-specific $CD8^+$ T cells, the Ad5-ID93–containing vaccines, as well as the ID93/GLA-SE subunit vaccine, still provided significant levels of short-lived vaccine protection against *M. tuberculosis*. $CD8^+$ cytotoxic T responses play key protective roles by killing virally infected cells within the lung following infection with viruses such as influenza (53, 54); however, the protective role of $CD8^+$ T cells in *M. tuberculosis* is less understood. Our

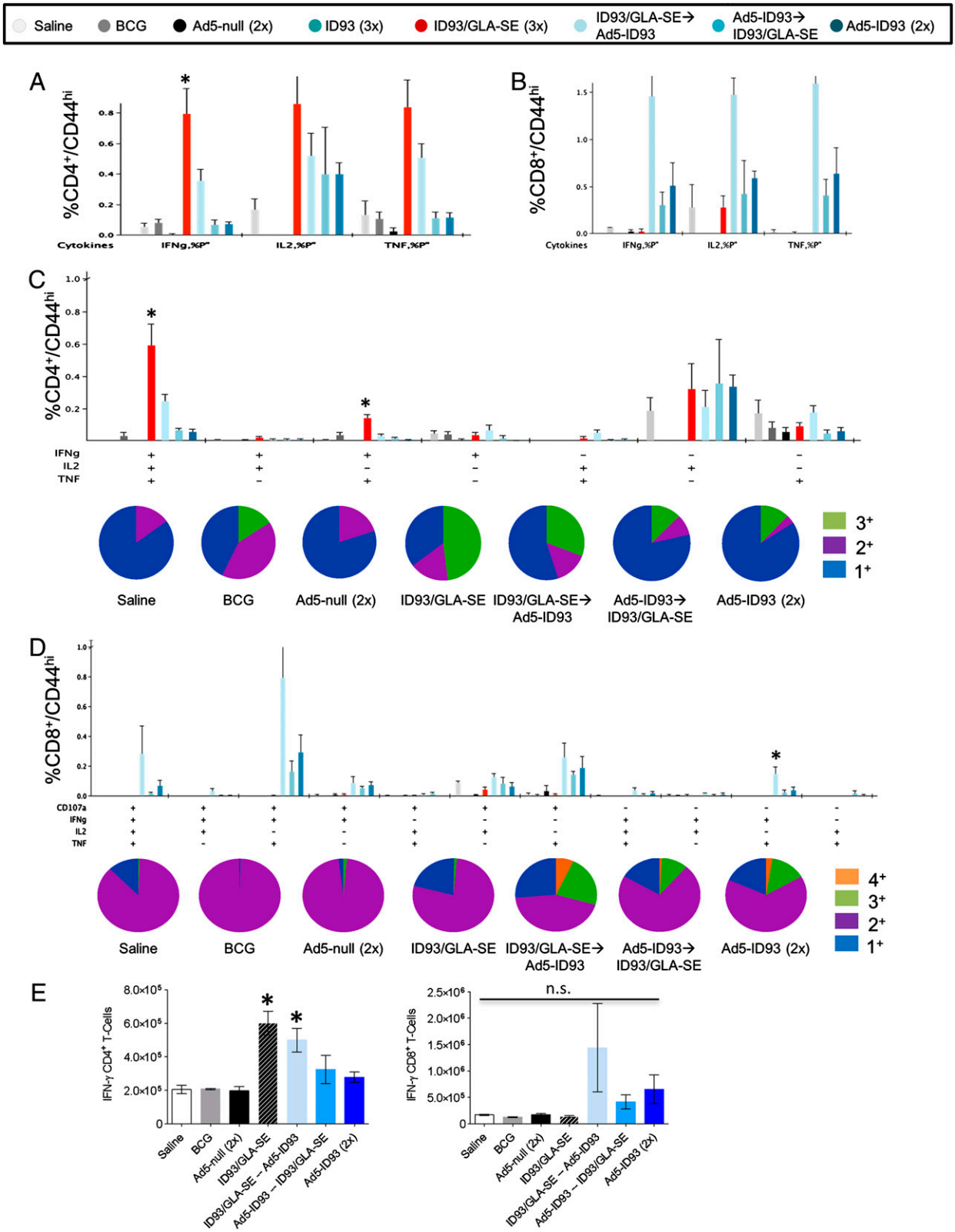


**FIGURE 6.** Enhanced $CD4^+$ or $CD8^+$ T cell cytokine responses from C57BL/6 mice 6 mo after the last immunization. Mice were immunized twice, 3 wk apart. ICS was performed, as described in the legend for Fig. 2. (**A**) Percent frequency of $CD4^+/CD44^{high}$ T cells producing IFN-γ, TNF, or IL-2; (**B**) percent frequency of $CD8^+/CD44^{high}$ T cells expressing IFN-γ, TNF, or IL-2; (**C**) percent frequency of polyfunctional cytokine-expressing ID93-specific $CD4^+/CD44^+$ T cells and pie charts representing $CD4^+$ T cells expressing $3^+$, $2^+$, or $1^+$ (triple, double, or single) cytokines; (**D**) percent frequency of polyfunctional cytokine-expressing $CD8^+/CD44^+$ T cells and pie charts representing $CD8^+$ T cells expressing $4^+$, $3^+$, $2^+$, or $1^+$ stained cells. Quadruple-stained cells are represented by CD107a-positive, triple cytokine–expressing cells; (**E**) the absolute numbers of IFN-γ–producing $CD4^+$ or $CD8^+$ T cells ± SD 6 mo following immunization ($n = 3$ mice/group). This long-lived vaccine immunogenicity study was performed once. *$p < 0.05$ compared with the saline control group.

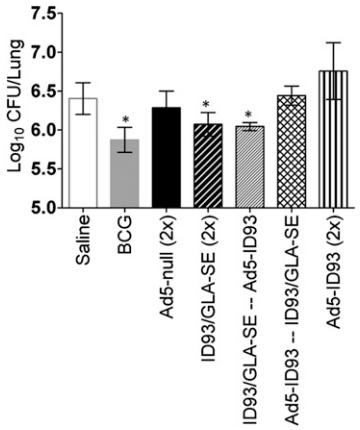


**FIGURE 7.** Long-lived vaccine efficacy 6 mo following immunization with both ID93/GLA-SE and a heterologous approach: ID93/GLA-SE prime/Ad5-ID93 boost. Bacterial load within the lungs of C57BL/6 mice, 6 wk following challenge with *M. tuberculosis* H37Rv. Results are represented as $Log_{10}$ CFU within the lung. $*p < 0.05$ compared with saline. One experiment is represented.

data suggest that the $CD4^+$ T cell responses may have compensated for the absence of Ag-specific $CD8^+$ T cells in the MHC class I–deficient mice. Bennekov et al. (15) showed that their adenoviral vector vaccine expressing Ag85B and ESAT-6 induced a strong ESAT-6–specific $CD8^+$ T cell response, but failed to provide protection. When the authors adjuvanted the adenovirus vaccine with MPL and dimethyl dioctadecyl ammonium, a dominant Ag85B-specific $CD4^+$ T cell response was elicited, which led to a protective response against *M. tuberculosis*. We show that in MHC class I–deficient mice, reduced numbers of $CD8^+$ T cells are elicited compared with wild-type mice as expected; however, a small, but significant number of effector $CD8^+$ T cells expressing IFN-γ was still induced in Ad5-ID93–immunized mice. In addition, a significant percentage of $CD4^+$ T cells producing IFN-γ and IL-2 was induced with the Ad5-ID93 vaccine. Polyfunctional $CD4^+$ T cell responses were also observed in the heterologous prime/boost approaches. The ID93/GLA-SE and Ad5-ID93 approaches provided protection in the lungs in both the wild-type and MHC class I–deficient mice, showing the importance of $CD4^+$ T cell responses with these ID93 vaccines.

The homologous Ad5-ID93 vaccine was able to provide short-lived protection (6 wk after the last immunization) in C57BL/6 mice, but the predominant Ag-specific $CD8^+$ T lymphocytes generated in these mice were inadequate for providing long-lived vaccine-mediated protection against *M. tuberculosis*. The order of the heterologous prime and boost was critical; the only effective heterologous regimen that provided long-lived protection against *M. tuberculosis* was the protein prime/vector boost approach. Bouillet et al. (55) have also shown that the order of heterologous immunizations can lead to enhanced immunity over other regimens; memory T cells and both IgG1 and IgG2a Ab responses are increased in BALB/c mice when a recombinant malaria protein combined with Montanide ISA720 adjuvant was included as a prime, and an adenoviral vector vaccine given as a boost. In our study, the ID93/GLA-SE subunit vaccine was also capable of providing long-lasting protection, 6 mo following immunization. Our data also show that durable $CD4^+$ Th1 cells secreting IFN-γ were generated in the groups that provided long-lived protective immunity. Six months after the boost immunization, both the ID93/GLA-SE subunit vaccine and the subunit prime/adenovirus boost vaccine strategies elicited significant frequencies of ID93-specific polyfunctional $CD4^+$ T cells, including IFN-γ and TNF double-positive $CD4^+$ T cells. At this timepoint, the ID93/GLA-SE prime/Ad5-ID93 boost approach also elicited polyfunctional $CD8^+$ T cells producing double cytokines: IFN-γ/TNF and IFN-γ/IL-2, which were enhanced compared with what was observed with the homologous Ad5-ID93–immunized group, which failed to provide long-lived protection. Although we showed that Ag-specific $CD4^+$ T cells can compensate for $CD8^+$ T cell generation in the MHC class I–deficient mice, the combined $CD4^+$ and $CD8^+$ T cell responses elicited by the heterologous protein prime/vector boost approach could have contributed to the long-lived vaccine protection in the wild-type mice. Interestingly, both of the protective vaccines induced significant numbers of Ag-specific IFN-γ–producing $CD4^+$ T cells, providing a clue into a potential correlate of long-lived protection. The importance of the order in which heterologous TB vaccines are given has recently been reported (56). A recombinant TB protein (rMT1721) combined with GLA, followed by a plasmid DNA vaccine expressing the same Ag, induced a more robust immune response, including the generation of both Ag-specific $CD4^+$ and $CD8^+$ T cells, compared with the opposite immunization strategy in which $CD8^+$ T cells were undetectable; however, the authors did not measure protection as a measure of efficacy following this regimen (56). Many others, however, have included the vector first, followed by a protein boost in heterologous prime/boost approaches. One such notable example is the HIV Thai trial (RV144), in which the vaccine strategy elicited 31% protective efficacy against HIV. In this case, a recombinant canarypox vector vaccine was given as a prime, followed by an adjuvanted subunit vaccine boost (57). In rhesus macaques, a promising combination of an adenovirus serotype 35 (Ad35) vector encoding circumsporozoite protein (Ad35.CS), followed by boosting with the RTS,S/AS02A (an MPL/TLR4 ligand-containing adjuvant) subunit vaccine, induced long-lived Th1 T cell responses and Ab responses (58). Unlike these prime/boost strategies, our results show enhanced protection when the subunit vaccine is given first, followed by the adenoviral vector boost.

We believe that immune responses measured 6 mo after immunization, just prior to infection, could shed light into which memory T cell responses are important for the generation of long-lived vaccine efficacy. Notably, the ID93/GLA-SE group was the only group that had a significant polyfunctional $CD4^+$ T cell response at this timepoint, whereas the heterologous ID93/GLA-SE prime/Ad5-ID93 boost approach, which also resulted in long-lived protection against *M. tuberculosis*, induced significant polyfunctional $CD8^+$ T cells. Both successful vaccine strategies, however, maintained significant numbers of IFN-γ–producing ID93-specific $CD4^+$ T cells, suggesting that this may represent at least one of the factors that led to long-lived, durable vaccine efficacy. These Th1 memory $CD4^+$ T cells, seen 6 mo after the last immunization with the protein prime/vector boost strategy, may have provided help for the maintenance of the double cytokine (IFN-γ and TNF)–secreting Ag-specific $CD8^+$ T cells, a hypothesis that warrants further exploration. Others have shown the importance of generating Ag-specific $CD4^+$ T cells to positively influence $CD8^+$ T cell responses directed against *M. tuberculosis* (59). In our study, long-lived vaccine responses in wild-type C57BL/6 mice occurred with two different vaccine strategies, one that included predominantly Ag-specific $CD4^+$ T cell responses (ID93/GLA-SE), and the other that induced both Ag-specific $CD4^+$ and $CD8^+$ T cell responses (ID93/GLA-SE prime, followed by an Ad5/ID93 boost). Adenoviral delivery of ID93 was therefore successful only when given as a boost to the ID93/GLA-SE subunit vaccine. Although we saw long-lived protection with the heterologous prime/boost approach, this did not offer better protection than our current TB vaccine,

ID93/GLA-SE. This suggests that the additional viral-vectored vaccine approach, designed to elicit $CD8^+$ T cell responses, is not a suitable replacement for our subunit approach. It is unclear why the Ad5/ID93 vaccine did not work when given as a prime. We are currently pursuing many different reasons for the failure of the Ad5-ID93 prime approach. One such possibility is that the lowered frequency of ID93-specific $CD8^+$ T cells may not have effectively recognized *M. tuberculosis*-infected cells in the lung. Another potential factor for the failed responses in Ad5-ID93–primed mice may have been due to vector immunity, in which immune responses may have been directed to the vaccine after the boost. Importantly, both of the successful vaccine strategies elicited significant numbers of IFN-γ–secreting $CD4^+$ T cells 6 mo following immunization within the spleen after immunization. Increased frequencies of ID93-specific IFN-γ– and TNF-expressing $CD4^+$ T cells were also observed within the lungs after *M. tuberculosis* infection in the mice given either ID93/GLA-SE or ID93/GLA-SE prime/Ad5-ID93 boost, which most likely contributed to the protection observed. Even though we show that lack of Ag-specific $CD8^+$ T cells in MHC class I–deficient mice did not adversely affect any of the ID93 immunization strategies (including the subunit prime/adenoviral boost approach) when tested in short-term vaccine efficacy studies, we do not know whether this would be the case for long-term vaccine efficacy, and so this remains to be tested. The data presented in this study show that, whereas the Ad5-ID93 vaccine, which induced predominantly $CD8^+$ T cell responses, was sufficient to elicit short-lived protection against *M. tuberculosis*, as measured by the ability to reduce the bacterial load in the lungs of *M. tuberculosis*-infected mice, this approach was not sufficient for long-lived vaccine efficacy. This goes against the current dogma, in which $CD8^+$ T cells are thought to play a role during the chronic stage of infection with *M. tuberculosis*. Our studies included the footpad/i.d. route of administration for our Ad5-ID93 studies, whereas others have shown that immunization via the mucosal route (intranasal) with recombinant adenoviral-based TB vaccines expressing Ag85A lead to enhanced protection against TB compared with other routes of administration (21, 23–25). We plan to test the mucosal route of the Ad5-ID93 in future studies to determine whether this could influence the long-lived protective efficacy of Ad5-ID93.

In summary, we show that ID93-specific $CD8^+$ T cell responses generated with either the Ad5-ID93 vaccine or the heterologous Ad5-ID93 vaccine combined with a prime or boost with ID93/GLA-SE are not required for short-lived protective immune responses against *M. tuberculosis* in mice. We also show that long-lived immunity to ID93 vaccines can be elicited with either an ID93/GLA-SE subunit approach, or by priming with the subunit vaccine (ID93/GLA-SE) and boosting with an Ad5-ID93 vaccine (using a heterologous vaccine approach). Both approaches led to a significant number of ID93-specific IFN-γ–producing $CD4^+$ T cells, suggesting that the generation of a robust Th1 $CD4^+$ T cell response may be required for the durability of each of these vaccine strategies and for induction of protective immunity against TB. These types of approaches will most likely be needed to provide long-lasting vaccine responses that prevent the continuing spread of TB.

## Acknowledgments

We acknowledge Ian Bishop, Tara Evers, Valerie Reese, and David Argilla for technical expertise, and Garrett Poshusta who worked on a prior version of another adenoviral vector expressing ID83. We also thank Dr. Christopher Fox, Dr. Thomas Vedvick, and members of the IDRI Process Science and Formulations team who provided formulated reagents.

## Disclosures

The authors have no financial conflicts of interest.

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