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Immune Subdominant Antigens as Vaccine Candidates against *Mycobacterium tuberculosis*

Mark T. Orr,* Gregory C. Ireton,* Elyse A. Beebe,* Po-Wei D. Huang,* Valerie A. Reese,* David Argilla,* Rhea N. Coler,*† and Steven G. Reed*†

Unlike most pathogens, many of the immunodominant epitopes from *Mycobacterium tuberculosis* are under purifying selection. This startling finding suggests that *M. tuberculosis* may gain an evolutionary advantage by focusing the human immune response against selected proteins. Although the implications of this to vaccine development are incompletely understood, it has been suggested that inducing strong Th1 responses against Ags that are only weakly recognized during natural infection may circumvent this evasion strategy and increase vaccine efficacy. To test the hypothesis that subdominant and/or weak *M. tuberculosis* Ags are viable vaccine candidates and to avoid complications because of differential immunodominance hierarchies in humans and experimental animals, we defined the immunodominance hierarchy of 84 recombinant *M. tuberculosis* proteins in experimentally infected mice. We then combined a subset of these dominant or subdominant Ags with a Th1 augmenting adjuvant, glucopyranosyl lipid adjuvant in stable emulsion, to assess their immunogenicity in *M. tuberculosis*–naive animals and protective efficacy as measured by a reduction in lung *M. tuberculosis* burden of infected animals after prophylactic vaccination. We observed little correlation between immunodominance during primary *M. tuberculosis* infection and vaccine efficacy, confirming the hypothesis that subdominant and weakly antigenic *M. tuberculosis* proteins are viable vaccine candidates. Finally, we developed two fusion proteins based on strongly protective subdominant fusion proteins. When paired with the glucopyranosyl lipid adjuvant in stable emulsion, these fusion proteins elicited robust Th1 responses and limited pulmonary *M. tuberculosis* for at least 6 wk postinfection with a single immunization. These findings expand the potential pool of *M. tuberculosis* proteins that can be considered as vaccine Ag candidates. The Journal of Immunology, 2014, 193: 000–000.

* M. tuberculosis, the causative agent of tuberculosis (TB), is responsible for ∼9 million new cases of active TB and 1–1.5 million deaths annually (1). It is estimated that nearly 2 billion people are latently infected with *M. tuberculosis* worldwide, creating a large reservoir of carriers from which new cases of active TB disease may arise. The only approved vaccine for *M. tuberculosis*, bacillus Calmette–Guérin (BCG), developed nearly a century ago, is routinely given shortly after birth. However, the efficacy of BCG wanes in adolescents, and the vaccine does not consistently prevent the development of active pulmonary TB in adults (efficacy estimated between 0 and 80% with the lowest efficacy rates often found in countries with the highest burden of TB) (2–4). Thus, there is an urgent need for a new TB vaccine to either boost immunity primed by BCG or replace BCG (5, 6).

Development of an effective vaccine against TB requires optimization of target Ags capable of inducing immunity against a broad range of *M. tuberculosis* isolates, delivered in a manner capable of inducing durable and protective immune responses (5).

Most *M. tuberculosis*–infected individuals develop long-lived immunity, which can control and contain the bacilli in a T cell–dependent manner, with only 5–10% of latently infected individuals acquiring the disease over the course of their lifetime. CD4 T cells producing IFN-γ and TNF are essential for immunity to *M. tuberculosis*. Some studies have found that the frequency of TB-specific multifunctional CD4 Th1 cells, that is, cells that make a combination of IFN-γ, TNF, and/or IL-2 upon stimulation, correlates with vaccine efficacy, although this is not always the case. Under some conditions, CD8 T cells also contribute to *M. tuberculosis* control, although not to the same degree as CD4 T cells (7, 8).

The selection of optimal Ags for inclusion into a subunit vaccine continues to be an important research question. Much of the effort over the last 25 y has focused on T cell responses to immunodominant Ags of *M. tuberculosis*. Several of these defined Ags delivered as plasmid DNA, vectored DNA, or as recombinant protein in adjuvant have proved to be effective in animal models (9–19). Subunit TB vaccines are currently under development by multiple groups, including our own, and several have entered clinical trials. However, the selection of *M. tuberculosis* vaccine candidates has primarily focused on a small subset of immunodominant proteins, which may have limited the pipeline needed for development of a successful vaccine. Recent studies have found that T cell epitopes of known immunodominant Ags of *M. tuberculosis* are hyperconserved, implying that immune responses against them may be in some cases more beneficial to the bacilli than to the host (20, 21). In addition, T cell responses to TB Ags have been shown to be significantly higher in active TB than latent *M. tuberculosis* infection, suggesting that increased immunity may promote lung pathology and subsequently transmission (22–24). These observations have led to the hypothesis that *M. tuberculosis* has evolved to subvert the immune response against it by focusing
the response against Ags in ways that are beneficial for its survival (22, 25). In this article, we test the hypothesis that Ags that are weakly antigenic during primary \textit{M. tuberculosis} infection can be turned into protective vaccines when properly adjuvanted. If successful, this approach may circumvent the benefits of the T cell response to \textit{M. tuberculosis} by altering the antigenic focus.

Materials and Methods

Expression and purification of recombinant \textit{M. tuberculosis} proteins

DNA encoding selected \textit{M. tuberculosis} genes were PCR amplified from \textit{H.\textsubscript{fru}}:37 genomic DNA using \textit{Psa} DNA polymerase (Invitrogen). PCR primers were designed to incorporate specific restriction enzyme sites 5’ and 3’ of the gene of interest for directional cloning into the expression vector pET28a (Novagen). Purified PCR products were digested with restriction enzymes, ligated into pET28a using T4 DNA ligase (NEB), and transformed into XL10G cells (Stratagene). Recombinant pET28a plasmid DNA was recovered from individual colonies and sequenced to confirm the correctly cloned coding sequence. The recombinant clones contained an N-terminal six-histidine tag followed by a thrombin cleavage site and the \textit{M. tuberculosis} gene of interest.

Recombinant plasmids were transformed into the \textit{Escherichia coli} BL21 derivative Rosetta\textsuperscript{a} (DE3) pLysS (Novagen). Recombinant strains were cultured overnight at 37°C in 2% yeast tryptone containing appropriate antibiotic and transferred to fresh culture medium grown to midlog phase (OD at 600 nm of 0.5–0.7), and induced by the addition of 1 mM isopropyl \(\beta\)-D-thiogalactoside. Cultures were grown for an additional 3–4 h, cells were harvested by centrifugation, and bacterial pellets were stored at −20°C. Bacterial pellets were thawed and disrupted by sonication in 20 mM Tris (pH 8.0), 150 mM NaCl, and 1 mM PMSF, followed by centrifugation to fractionate the soluble and insoluble material. Recombinant His-tagged protein products were isolated under native (soluble recombinant proteins) or denaturing (8 M urea) conditions using Ni-nitrilotriacetic acid metal ion affinity chromatography according to the manufacturer’s instructions (Qiagen). Further purification by ion exchange and/or size exclusion chromatography was used as necessary to obtain >95% purity of \textit{M. tuberculosis} proteins, as determined by visualization using SDS-PAGE. Purified protein fractions were combined and dia lyzed against 20 mM Tris (pH 8.0), concentrated using Amicon Ultra 10-kDa molecular mass cutoff centrifugal filters (Millipore), and quantified using a bicinchoninic acid assay (Pierce). LPS contamination was evaluated by the \textit{Limulus} amebocyte lysate assay (Cambrex). All the recombinant proteins used in this study showed residual endotoxin levels <100 endotoxin units/mg protein.

The fusion proteins D91 and D97 were constructed in the same manner as we previously reported for D93 (9). In brief, the genes for \textit{Rv1886c}, \textit{Rv3478}, and \textit{Rv3619} were attached to the genes for either \textit{Rv2389} (D91) or \textit{Rv2875} (D97) using restriction site linkers and cloned into the pET28a vector. Fusion proteins were expressed in \textit{E. coli}, purified under denaturing conditions by chromatography on DEAE and Q Sepharose columns, and analyzed by SDS-PAGE on a 4–20% Tris glycine gel (Invitrogen). In the absence of \textit{E. coli} contamination was confirmed by immunoblotting with HRP-conjugated rabbit polyclonal anti-\textit{E. coli} Ab (1:1000; ViroStat). Residual LPS contamination was determined to be <15 endotoxin units/mg protein by the \textit{Limulus} amebocyte lysate assay (Cambrex).

Experimental animals and infection

Six- to 8-wk-old female CB\textsubscript{6}F1 mice were purchased from The Jackson Laboratory and maintained in specific pathogen-free conditions. Postinfection animals were maintained in BL3 containment according to the regulations and guidelines of the Infectious Disease Research Institute Institutional Animal Care and Use Committee. For vaccine efficacy studies, mice were immunized once, twice, or three times 3 wk apart by i.m. injection. Each immunization contained 5 pmol recombinant protein and 5 \(\mu\)g GLA-SE.

Four weeks after the last immunization, mice (\(n = 7–14\)-group) were aerogenically infected with \textit{M. tuberculosis} strain H37Rv (ATCC No. 35718; American Type Culture Collection) using a Glas-Col aerosol generator calibrated to deliver 50–100 bacteria into the lungs. An additional three unimmunized animals per infection were euthanized 1 d later to confirm the amount of bacteria delivered, and bacterial burden in the lungs was enumerated. Protection was determined 3–6 wk after challenge by harvesting the lungs from the infected mice, homogenizing the tissue in 0.1% PBS–Tween 80, and plating 5-fold serial dilutions on7H10 agar plates (Molecular Toxicology) for bacterial growth. Bacterial colonies were counted after incubation at 37°C for 14–21 d.

ELISPOTs

Four, 8, or 12 wk postinfection, splenocytes were isolated from four nonimmunized mice. RBCs were lysed using RBC Lysis Buffer (eBioscience) and resuspended in RPMI 1640 and 10% FBS. A MultiScreen 96-well filtration plate (Millipore) was coated with 10 \(\mu\)g/ml rat anti-mouse IFN-\(\gamma\) or TNF capture Ab (eBioscience and R&D Systems, respectively) and incubated overnight at 4°C. Plates were washed with PBS, blocked with 5% MTS, 10% FBS for at least 1 h at room temperature, and washed again. Splenocytes were plated at 2 \(\times\) 10\(^6\) cells/well and stimulated with media or recombinant protein (10 \(\mu\)g/ml) for 48 h at 37°C. Plates were developed according to the manufacturer’s protocol and fixed with 4% paraformaldehyde. Spots were counted on an automated ELISPOT reader (CTL Series 3A Analyzer; Cellular Technology) and analyzed with ImmunoSpot software (CTL Analyzer).

Intracellular cytokine staining

Four weeks after the final immunization, splenocytes were isolated from three to five animals per group. RBCs were lysed using RBC Lysis Buffer (eBioscience) and resuspended in RPMI 1640 and 10% FBS. Cells were plated at 2 \(\times\) 10\(^6\) cells/well in 96-well plates and were stimulated for 1 h with media or recombinant protein (10 \(\mu\)g/ml) at 37°C. GolgPlugg (BD Biosciences) was added and the cells were incubated for an additional 8 h at 37°C. Cells were washed and surface stained with fluorochrome-labeled Abs to CD4 (clone GK1.5) and CD8 (clone 53-6.7; Biolegend and eBioscience) in the presence of anti-CD16/32 (clone 2.4G2) for 20 min at 4°C. Cells were washed and permeabilized with Cytotox/Cytperm (BD Biosciences) for 20 min at room temperature. Cells were washed twice with Perm/Wash (BD Biosciences) and stained intracellularly with fluorochrome-labeled Abs to CD154 (clone MR1), IFN-\(\gamma\) (clone XMG-1.2), IL-2 (JES6-S3H4), and TNF (MPS-XT22; Biologend and eBioscience) for 20 min at room temperature. Cells were washed and resuspended in PBS. Up to 10\(^5\) events were collected on a four-laser LSRFortessa flow cytometer (BD Biosciences). Data were analyzed with FlowJo. Cells were gated as singlets > lymphocytes > CD4* CD8* > response positive. Analysis and presentation of distributions was performed using SPICE version 5.2, downloaded from http://exon.niaid.nih.gov/spice.

Statistical analysis

Bacterial burdens were normalized by \(\log_{10}\) transformation. Statistically significant differences in bacterial burden were determined using one-way ANOVA. Differences relative to saline-immunized animals were calculated using Dunn’s posttest using Prism 5 (GraphPad Software).

Results

Immunodominance hierarchy of \textit{M. tuberculosis} proteins in mice

To establish the immunodominance hierarchy of a large pool of recombinant \textit{M. tuberculosis} proteins (Fig. 1A) (26), we infected a cohort of CB\textsubscript{6}F1 mice with a low dose of aerosolized \textit{M. tuberculosis}. Four, 8, and 12 wk postinfection, we assessed the immune response to 84 recombinant \textit{M. tuberculosis} proteins by ELISPOT assay for IFN-\(\gamma\) and TNF (Supplemental Fig. 1). The Ags selected for analysis include a number incorporated in current vaccine and diagnostic candidates. They include proteins from several \textit{M. tuberculosis} protein families including PE/PPE, ESAT-like, membrane/secreted proteins, proteins associated with hypoxia and/or resuscitation, and virulence factors. There was a tight correlation between the number of TNF- and IFN-\(\gamma\)-producing cells specific for a given Ag, with TNF responses always being more frequent, except for \textit{Rv3875} (ESAT-6), to which the IFN-\(\gamma\) and TNF responses were similar (Supplemental Fig. 1). There was a wide spectrum of response frequencies in this mouse strain including a number of known dominant Ags such as \textit{Rv3875}, subdominant Ags such as \textit{Rv1886} (Ag85B), and poorly immunogenic Ags such as \textit{Rv3804} (Ag85A), all of which are components of candidate vaccines against TB (27, 28). The responses over time changed with different patterns, but in general, Ags that were...
recognized at 4 wk continued to be recognized at 8 and 12 wk postinfection. At 4 wk postinfection, the most frequent TNF responses were to Rv1738, Rv1253, Rv0054, Rv3619, and Rv1246. Currently, the most frequent IFN-\(\gamma\) responses were against Rv3875, Rv0125, Rv2389, Rv1738, and Rv0054 (Supplemental Fig. 1).

To assess how Ag dominance during primary infection may impact suitability of Ags for vaccine development, we chose Rv1738 and Rv0054 as Ags that were dominant by both TNF and IFN-\(\gamma\) production, Rv3619 that was dominant by TNF responses, Rv2389 that was dominant by IFN-\(\gamma\) responses, and Rv3875 that was unique in the equal IFN-\(\gamma\) and TNF response induction at 4 wk. Based on the response magnitude at 4 wk postinfection, we also selected four subdominant Ags (Rv1626, Rv1789, Rv1886, and Rv2875) and eight Ags that were weakly antigenic at this time (Rv0577, Rv1009, Rv1813, Rv2608, Rv3044, Rv3478, Rv3620, and Rv3804). The IFN-\(\gamma\) and TNF responses to these Ags at 4 wk after \(M.\) \(\text{tuberculosis}\) exposure are shown in Fig. 1B.

**Induction of CD4 T cell responses by vaccination with \(M.\) \(\text{tuberculosis}\) proteins**

To assess whether we could induce a Th1 response against the selected proteins, we immunized cohorts of mice with the recombinant proteins combined with the Th1 skewing adjuvant glucopyranosyl lipid adjuvant in stable emulsion (GLA-SE) (29, 30). We used the recombinant fusion protein ID93 (a fusion of Rv1813, Rv2608, Rv3619, and Rv320) as a positive control for elicitation of a robust Th1 response with GLA-SE (9). One month after the third immunization, we assessed IFN-\(\gamma\) and TNF responses, as well as upregulation of CD154 by CD4 T cells upon cognate Ag stimulation (Fig. 2). Four of the Ags, Rv1738, Rv1813, Rv2389, and Rv3620, did not elicit a detectable Th1 response. Rv1738 and Rv2389 were two of the dominant Ags during primary infection; thus, we hypothesize that cytokine responses to these proteins observed during primary infection (Fig. 1B) may be produced by CD8 T cells, which are only weakly induced with a recombinant protein immunization adjuvanted with GLA-SE. All of the other Ags elicited measurable Th1 responses, although there was considerable heterogeneity in the response magnitude. Importantly, a number of the subdominant and weak Ags induced robust Th1 responses including Rv0577, Rv3044, Rv3478, and Rv3804. Ag-specific CD8 T cell responses were not detected in any of the immunized groups. These data demonstrate that it is feasible to augment the Th1 response to weak Ags by vaccination.

**Protective efficacy of select \(M.\) \(\text{tuberculosis}\) proteins with adjuvant**

To determine whether the protective efficacy of vaccine Ags correlates to immune dominance during primary infection, we challenged cohorts of immunized mice with aerosolized \(M.\) \(\text{tuberculosis}\)
and determined pulmonary *M. tuberculosis* burden 3 wk later (Fig. 3). Of the dominant Ags, both Rv3875 and Rv3619 were strongly protective, whereas Rv1738 and Rv2389 elicited minimal protection as might be expected by the lack of vaccine-induced Th1 responses to these Ags (Fig. 2). Surprisingly, the dominant Ag Rv0054 induced a strong Th1 response to vaccination but produced little control of pulmonary *M. tuberculosis*. Of the subdominant Ags studied, Rv1886 was the most strongly protective, in line with many other studies of the protective efficacy of this Ag in animal challenge models. In addition, both Rv1789 and Rv2875 were highly protective, whereas Rv1626 elicited minimal protection despite robust Th1 responses after vaccination. Surprisingly, several of the Ags that were only weakly antigenic during primary infection were protective when included in a vaccine. Specifically, Rv3478, and to a lesser extent Rv2608 and Rv3804, limited pulmonary *M. tuberculosis* burden. Three of the weak Ags, Rv0577, Rv1009, and Rv3044, that elicited robust Th1 responses after vaccination failed to provide substantial protection. None of the Ags that failed to elicit Th1 response upon vaccination were protective (Rv1738, Rv1813, Rv2389, and Rv3620), suggesting that CD4 T cells were necessary for vaccine efficacy in this model. Taken together, these data demonstrate that there is little correlation between the magnitude of the TNF or IFN-γ response to an Ag during primary infection and the potential of that Ag to make an effective vaccine Ag (Fig. 4). Even when the four Ags that did not elicit a CD4 T cell response by vaccination were excluded from analysis there was little correlation between vaccine efficacy and IFN-γ or TNF ELISPOT magnitude during primary infection of unimmunized animals ($R^2 < 0.1$ in both cases).

**Fusion proteins of subdominant Ags are strongly protective against *M. tuberculosis***

Based on the protective efficacy of several of the subdominant Ags (defined by IFN-γ production during primary infection) demonstrated earlier, we developed two fusion protein Ags that included the three most protective subdominant Ags, Rv1886, Rv3478, and Rv3619, combined with either Rv2389 (also known as resuscitation factor D) or another subdominant Ag Rv2875, designated ID91 and ID97, respectively. Rv2875 demonstrated moderate levels of protection on its own (Fig. 3). Rv2389 was chosen because of its expression during reactivation from latency, which may be important for developing a multistage targeted vaccine against active TB (31). Immunization with ID91, ID93, or ID97

**FIGURE 2.** Immunogenicity of *M. tuberculosis* proteins adjuvanted with GLA-SE. Cohorts of mice were immunized three times with *M. tuberculosis* proteins adjuvanted with GLA-SE. One month after the final immunization, Ag-specific CD4 T cells were identified by intracellular IFN-γ, TNF, and/or CD154 expression after ex vivo stimulation with the cognate Ag as determined by flow cytometry. Data show results from one of three experiments with similar results ($n = 3$ animals/group).

**FIGURE 3.** Protective efficacy of *M. tuberculosis* proteins adjuvanted with GLA-SE. Cohorts of mice were immunized three times with *M. tuberculosis* proteins adjuvanted with GLA-SE or once with BCG. One month after the final immunization, animals were challenged with a low dose of aerosolized *M. tuberculosis*. Lung burdens were determined 3 wk postinfection. Data show results from one of five experiments with similar results ($n = 7$ animals/group). Dashed line indicates the mean burden in saline-immunized animals. *$p < 0.05$, **$p < 0.001$, ****$p < 0.0001$ relative to saline immunized controls, respectively.

**FIGURE 4.** Vaccine efficacy of *M. tuberculosis* proteins does not correlate with immunodominance. Protective efficacy against pulmonary *M. tuberculosis* burden for recombinant protein Ag vaccines (mean CFUimmunized − mean CFUsaline) is plotted against (A) IFN-γ or (B) TNF ELISPOT magnitude from 4 wk after primary infection. Data show results from one of two ELISPOT experiments (x-axis; $n = 4$ animals) and one of five protection experiments (y-axis; $n = 7$ animals/group) with similar results.
adjuvanted with GLA-SE elicited similar frequencies of Th1 cells after one or two immunizations. Surprisingly, both ID91 and ID97 showed a dramatic increase in the frequency of Th1 cells after a third immunization that was more substantial than for ID93 (Fig. 5A). Although ID91 and ID97 elicited a more extensive tertiary response than ID93, these Ags were at least as effective as ID93 in eliciting multifunctional Th1 cells that coproduced IFN-γ, TNF, and IL-2 or IFN-γ and TNF alone, suggesting we have not driven these cells to terminal exhaustion (Fig. 5B). For all three fusion proteins, the majority of the CD4 T cell response was contributed by only one of the component proteins, Rv1886 for ID91 and ID97 and Rv2608 for ID93 (Supplemental Fig. 2). Rv2875-, Rv3478-, and Rv3619-specific responses were raised with those fusion proteins that contained these Ags, although these responses were less frequent than those for Rv1886 or Rv2608. Not surprisingly, ID91 and ID93 elicited only very minor Rv2389- and Rv3620-specific Th1 responses and no response to Rv1813, respectively, as immunization with these individual components did not elicit robust Th1 responses (Supplemental Fig. 2).

Both ID91 and ID97 were at least as effective as ID93 in limiting pulmonary M. tuberculosis burden after an aerosol challenge. To our surprise, a single immunization with any of the three fusion proteins adjuvanted with GLA-SE was sufficient to reduce bacterial burden 3 wk after challenge (Fig. 5C). Typically, multiple immunizations with subunit vaccines are necessary to achieve vaccine efficacy in this model, indicating the potency of the ID91, ID93, and ID97 Ags. This vaccine efficacy was further enhanced with additional boosters ($p < 0.05$ and $p < 0.001$ for one versus three immunizations with ID93 and ID97, respectively), particularly for ID97, which reduced lung burden by $>90\%$ after three immunizations. Importantly, the reductions in bacterial burden were sustainable as two or three immunizations with ID93 or even a single immunization with ID91 or ID97 were sufficient to

**FIGURE 5.** Fusion proteins of subdominant Ags are highly immunogenic and limit pulmonary M. tuberculosis. Cohorts of 17 mice per group were immunized one, two, or three times with saline or the fusion proteins ID93, ID91, or ID97 adjuvanted with GLA-SE. (A) One month after the final immunization, Ag-specific CD4 T cells were identified by CD154, IFN-γ, TNF, and/or IL-2 expression after ex vivo stimulation with the cognate Ag. (B) The frequency of cells coexpressing IFN-γ, TNF, and/or IL-2 was analyzed using SPICE visualization software. One month after the final immunization, animals were challenged with a low dose of aerosolized M. tuberculosis. Lung burdens were determined at (C) 3 or (D) 6 wk postinfection. Data show results from one of two experiments with similar results [$n = 3$ animals/group for (A) and (B); $n = 7$ animals/group for (C) and (D)]. **$p < 0.01$, ***$p < 0.001$ relative to saline immunized controls, respectively.
maintain lower bacterial burden for at least 6 wk after challenge (Fig. 5D).

Discussion
To test the hypothesis that subdominant and/or weak \textit{M. tuberculosis} Ags are viable vaccine candidates and to avoid complications because of differential immunodominance hierarchies in humans and experimental animals, we defined the immunodominance hierarchy of 84 recombinant \textit{M. tuberculosis} proteins in experimentally infected mice. These responses were classified as dominant, subdominant, or weak according to the frequency of Ag-specific cells that produced IFN-$\gamma$ or TNF postinfection. When combined with the Th1 skewing GLA-SE adjuvant, the majority of these Ags were immunogenic in \textit{M. tuberculosis}-naive animals. A subset of these vaccines substantially reduced the \textit{M. tuberculosis} burden in the lungs of infected animals when given prophylactically. There was no correlation between immunodominance during primary \textit{M. tuberculosis} infection and vaccine efficacy, confirming the hypothesis that subdominant and weakly antigenic \textit{M. tuberculosis} proteins are viable vaccine candidates. The data suggest that induction of a Th1 response during vaccination is a prerequisite for vaccine efficacy, but vaccine efficacy cannot be predicted by the presence or magnitude of the Th1 response. That is, Ags that did not elicit Th1 responses uniformly failed to protect against pulmonary \textit{M. tuberculosis}, but not all Ags that induced Th1 responses made effective vaccine Ags. This discordance may be because of Ag load and/or presentation, immune subversion, Ag presentation by noninfected cells, or other causes.

In our development of the ID93 Ag, we have found that ~5 pmol Ag is optimal for induction of maximal Th1 responses and, similar to the findings of others, that a low dose of Ag may be beneficial for eliciting protective T cell responses (32). Based on this, we used equimolar amounts of each recombinant Ag. For Rv3875, this meant using only 0.05 $\mu$g protein for each immunization, which is at least two orders of magnitude lower than is typically used. Despite this very low dose of Ag, we were able to induce robust Th1 response that correlated with protection against pulmonary \textit{M. tuberculosis}. We have found previously that subtle alterations in the adjuvant formulation can modify the adjuvant capacity of the TLR4 agonist GLA molecule (30). In turn, this has a profound impact on vaccine efficacy. Some of the subdominant Ags that we found to be strongly protective with the GLA-SE adjuvant were much less protective when combined with a weaker adjuvant such as unformulated CpG ODN1826, which we reported previously (18). Thus, the choice of adjuvant or delivery system is critical to the evaluation of candidate vaccine Ags, at least for TB. The current TB vaccine pipeline is filled with candidates that vary not only in Ag composition, but also in adjuvant or delivery system, making direct comparisons between the merits of the different Ags complex (5, 6, 28).

One strategy to increase vaccine HLA coverage over a disparate patient population and to avoid immune evasion by epitope mutation is to include multiple Ags in the vaccine. We and others have taken this approach in developing ID93, H56, M72, and Aeras-402 (5, 6, 28). Using this approach, we combined the three most protective subdominant Ags into the ID91 and ID97 fusion proteins. In addition, ID91 contains the Rv2389 protein, which is involved in resuscitation of \textit{M. tuberculosis} from hypoxia (31). Inclusion of this Ag may more effectively target latent or reactivating bacteria, a condition that cannot be effectively modeled in mouse model of aerosolized \textit{M. tuberculosis} challenge (33). Both of these fusion proteins were at least as immunogenic and protective as the ID93 fusion. We were surprised to find that all three fusion proteins were effective in limiting pulmonary \textit{M. tuberculosis} burden after a single immunization, and this was further enhanced by subsequent boosters. For each fusion protein there was a clear Ag dominance hierarchy of the component proteins that was not predicted by vaccinating with each Ag individually (e.g., Rv1886 and Rv3478 were similarly immunogenic as individual proteins, but Rv1886 was the dominant response in ID91- and ID97-vaccinated animals). This dominance hierarchy may stem from MHC class II binding affinity, specific T cell precursor frequency, and/or other factors that may vary between mice and humans. Thus, the contribution of each fusion component to antigenicity in humans will need to be determined in potential clinical trials. We have previously found that all of the components of these fusions are antigenic in latently infected human volunteers, confirming their compatibility with one or more human MHC molecules (18).

Although the data presented in this article are limited to the immunodominance profiles in the mouse strain used, and thus do not directly point to ideal vaccine candidates (e.g., Rv1886 is a dominant Ag in humans with both active TB and latent infection), substantial efforts have been made to map the immunodominance hierarchy in latently infected volunteers. Using a large panel of peptides from the \textit{M. tuberculosis} proteome, Sette’s group (34) mapped a core of commonly recognized \textit{M. tuberculosis} peptides that bind to multiple HLA alleles. By implication, there was a large pool of these HLA-promiscuous \textit{M. tuberculosis} epitopes that do not induce T cell responses during natural latent infection. This peptide library could serve as a starting point for selecting subdominant epitopes to test as vaccine candidates under the hypothesis that increasing the \textit{M. tuberculosis}-specific T cell repertoire beyond that induced by infection is an effective vaccine strategy.

One caveat to this approach and indeed to the development of T cell–focused subunit vaccines against human diseases in general is the difficulty in translating a protein or peptide’s immunogenicity and efficacy from experimental animals to humans because of species differences in MHC alleles. Although it is not feasible to immunize human volunteers with a large array of \textit{M. tuberculosis} proteins, it may be reasonable to identify a panel of proteins with diverse immunodominance profiles as mapped by Sette’s group (34) or others to identify proteins that can be antigenic in a vaccine setting. Further, this approach to clinical experimentation could be augmented with the recently developed BCG challenge model in which immunized volunteers are intentionally exposed to BCG and followed for control and elimination of BCG from the injection site (35). This approach would allow human testing of this alternative approach to vaccine Ag selection.

An alternative or complementary approach would be to use one or more of the recently developed humanized mouse models (36). Although these models are imperfect in recreating the human immune system, recent advances including the humanized bone marrow, liver, and thymus transplantation mouse model in which APCs express human HLA and human T cells undergo positive and negative selection to develop a human T cell repertoire, as well as recently developed HLA class I and class II transgenic models, provide models for studying human T cell responses in a small-animal model (37–39). Importantly, several recent articles have described productive \textit{M. tuberculosis} and BCG infection in these animal models, although the human T cell response repertoire during infection was not assessed in these studies (40–42).

The data presented in this article do not in any way invalidate the approaches taken to identify the TB vaccine candidates developed to date. Rather, these data suggest that TB vaccine development should not be limited to proteins that are the most antigenic during natural infection. Although the first new phase II clinical trial of
a TB vaccine in 40 y, the modified Vaccinia Ankara virus expressing Rev3804 (MVA85A), failed to demonstrate protective efficacy in immunized infants, it provides important information both in clinical trial design and insight into the relationship between vaccine immunogenicity and efficacy (43). Specifically in the study population (infants given MVA85A shortly after BCG immunization), there was only a muted augmentation of the Ag85A-specific T cell response compared with the BCG-only control. The reasons for this muted response are unclear because previous phase I studies with this vaccine demonstrated substantial augmentation of Ag85A T cell responses in a variety of volunteer populations (44–46). These findings closely mirror our findings that induction of a vaccine-specific CD4 T cell response is a minimum that must be achieved if a vaccine has any chance of being effective. Our data support the concept that inducing robust Th1 responses to Ag85A can limit pulmonary M. tuberculosis growth, although other single Ags were more protective in the data presented in this article. Additional phase I and II data with other Ags and vaccine delivery systems will be crucial to understand what immunological parameters are important for vaccine efficacy. These findings will also be crucial to understand how effective various animal models of M. tuberculosis infection, be it mouse, rabbit, guinea pig, non-human primate, or other models, are in predicting vaccine efficacy. In regard to MVA85A, the preclinical animal data correctly predicted that in the absence of a strong boost of Ag85A-specific T cell responses, we could not expect the MVA85A vaccine to be efficacious (47).

In summary, the results presented in this article, wider than narrow, the pool of potential vaccine Ags and vaccine delivery systems will be crucial to understand what immunological parameters are important for vaccine efficacy. These findings will also be crucial to understand how effective various animal models of M. tuberculosis infection, be it mouse, rabbit, guinea pig, non-human primate, or other models, are in predicting vaccine efficacy. In regard to MVA85A, the preclinical animal data correctly predicted that in the absence of a strong boost of Ag85A-specific T cell responses, we could not expect the MVA85A vaccine to be efficacious (47).

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Disclosures

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References


Figure S1: Kinetics of T cell responses to select *Mtb* proteins

IFN-γ and TNF responses to recombinant *Mtb* proteins by splenocytes from mice four, eight, or twelve weeks after aerosolized *Mtb* infection. Data show results from one of two experiments with similar results (N=4 animals/timepoint).
Figure S2: CD4 T cell responses to fusion protein components in immunized mice

Cohorts of five mice per group were immunized three times with the fusion proteins (A) ID91, (B) ID93, or (C) ID97 adjuvanted with GLA-SE. One month after the final immunization antigen specific CD4 T cells were identified by CD154, IFN-γ, TNF or IL-2 expression by flow cytometry following ex-vivo stimulation with the cognate fusion antigen as well as each component protein of the fusion protein. Data show results from one of two experiments with similar results.