**Myobacterium tuberculosis** Directs Immunofocusing of CD8^+^ T Cell Responses Despite Vaccination

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Mycobacterium tuberculosis Directs Immunofocusing of CD8+ T Cell Responses Despite Vaccination

Joshua S. Woodworth,*† Daniel Shin,* Mattij Volman,* Cláudio Nunes-Alves,* Sarah M. Fortune,‡ and Samuel M. Behar*‡

Vaccines that elicit T cell responses try to mimic protective memory T cell immunity after infection by increasing the frequency of Ag-specific T cells in the immune repertoire. However, the factors that determine immunodominance during infection and after vaccination and the relation between immunodominance and protection are incompletely understood. We previously identified TB10.4(20–28) as an immunodominant epitope recognized by H2-Kd–restricted CD8+ T cells after M. tuberculosis infection. Here we report a second epitope, EspA(150–158), that is recognized by a substantial number of pulmonary CD8+ T cells. The relative abundance of these T cells in the naive repertoire only partially predicts their relative frequency after M. tuberculosis infection. Furthermore, although vaccination with recombinant vaccinia virus expressing these epitopes changes their relative immunodominance in the preinfection T cell repertoire, this change is transient after challenge with M. tuberculosis. We speculate that factors intrinsic to the chronic nature of M. tuberculosis infection establishes the hierarchy of immunodominance and may explain the failure of some vaccines to provide protection. The Journal of Immunology, 2011, 186: 1627–1637.

For infectious diseases that can be prevented by humoral immunity, the goal of vaccination is to increase the amount of preexisting pathogen-specific Ab (e.g., neutralizing Abs) and increase the frequency of memory B cells (1, 2). The success of this strategy can be seen by the reduction and virtual elimination of several acute bacterial and viral diseases in human populations after widespread immunization (3). By analogy with this paradigm, the underlying strategy for vaccine development against pathogens subduced by T cell-mediated immunity has been to increase the frequency of preexisting pathogen-specific T cells (4). In the case of tuberculosis, there are no biomarkers of protective immunity, and consequently a better understanding of what constitutes a protective T cell response could more effectively guide vaccine development. In fact, the molecular requirements for T cell-mediated host resistance to tuberculosis have only been defined in broad terms. While CD4+ T cells are generally acknowledged to be crucial, the contribution of other T cell subsets defined in broad terms. While CD4+ T cells are generally acknowledged to be crucial, the contribution of other T cell subsets including γδ T cells, CD1-restricted and MR1-restricted T cells, and even conventional CD8+ T cells continues to be debated by many investigators (5, 6) (S. M. Behar, unpublished observation).

Measuring Mycobacterium tuberculosis-specific T cell responses after vaccination is difficult because relatively few T cell epitopes have been defined. This problem is compounded in people because of their MHC diversity, which selects and presents different peptide epitopes to T cells. Microbial proteins are cleaved by host proteolytic enzymes into peptide fragments that compete with endogenous host peptides for binding to MHC molecules. In the case of class I MHC presentation, cleavage by the proteasome and transport into the endoplasmic reticulum affects the spectrum of peptides that are loaded onto class I MHC molecules. The affinity of microbial peptides for MHC molecules is a major determinant of which epitopes are presented to T cells, and each MHC molecule and allelic variant has a different peptide sequence, or motif, that it preferentially binds (7). Although enumerating the frequency of Ag-specific T cells after immunization is an important indication of immunological efficacy of vaccination, not all T cells specific for M. tuberculosis Ags confer protection against infection. As the defining properties of Ags that elicit protective immunity have not been elucidated, distinguishing protective from nonprotective T cell epitopes can only be done experimentally.

The use of inbred mice as an experimental model to study T cell immunity eliminates the confounder of MHC diversity and has led to the identification of additional factors that determine the immunogenicity of microbial Ags. Both host and microbial factors influence processing and presentation of peptide epitopes. For example, activation of the APCs alters the spectrum of peptides generated during processing. IFN-γ activation of APCs induces immunoproteasome expression, which modulates processing of Ags. Technical improvements in the ability to quantify the naive T cell repertoire indicate that precursor frequency can affect immunodominance to microbial Ags (8). Microbial factors including whether proteins are secreted (9, 10), their abundance (11), and their elaboration of pathogen-associated molecular patterns (12) all affect immunogenicity of microbial proteins.

In this study, we have identified a second H2-Kd–restricted M. tuberculosis epitope, derived from EspA (Rv3616c), which is associated with the ESX-1 secretory apparatus and an important virulence factor. Armed with two distinct immunodominant epitopes recognized by pulmonary CD8+ T cells elicited by M. tuberculosis infection, we evaluated how establishment of immunodominance of CD8+ T cell responses occurs during M. tuberculosis infection. These experiments begin to define the relationship be-
tween chronic bacterial infection, immunodominance, and protection by CD8+ T cells.

Materials and Methods

Bacteria and aerosol infections

All infections were performed using virulent M. tuberculosis (Erdman strain). For each infection, a bacterial aliquot was thawed, sonicated twice for 10 s in a cup horn sonicator, and then diluted in 0.9% NaCl–0.02% Tween 80. A 15-ml suspension of M. tuberculosis was loaded into a nebulizer (MiniHEART nebulizer; Vortran Medical Technologies). Mice were infected via the aerosol route using a nose-only exposure unit (Intox Products) and received 100–200 CFU/mouse (13–15).

Ethics statement

All animal studies and procedures were approved by the Dana Farber Cancer Institute Animal Care and Use Committee (Animal Welfare Assurance no. A3023-01), under Public Health Service assurance of Office of Laboratory Animal Welfare guidelines.

Mice

Age-matched female BALB/c were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in a biosafety level 3 facility under specific pathogen-free conditions at the Animal Biocontainment Suite (Dana Farber Cancer Institute, Boston, MA).

Peptides

Peptides used in this study were Ag85A(103–111) (WYDQGSLGV) (16), Ag85A(113–121) (MPVGGQSSF) (19), Ag85A(142–161) (TFLTSELPG) (17), and Ag85A(187–195) (VYAGAMSG) (18), ESAT-6 (1–15) (MTEQWQAFGEEAAA) (19), EspA(150–158) (AYLVVKTLI), TB10.4(20–28) (VYAGTLQSL) (20), TB10.4(74–88) (SSTHEANTMMARDT) (21), and a library of EspA peptides (Table I) (Biosource International, Camarillo, CA, and/or CPC Scientific, San Jose, CA). The identity of each peptide was confirmed by mass spectrophotometry. The peptides were dissolved in DMSO to a concentration of 10 mM and stored at −20˚C until used. The peptides were used at a concentration of 10 μM for all in vitro assays. Peptides used for immunological assays were unpurified (>70% pure). The purity of peptides used for tetramer production was >95%.

Isolation of cells from infected tissue

Single-cell suspensions were prepared from spleens, lymph nodes, and lungs of infected mice as previously described (22, 23). Briefly, spleens and lymph nodes were prepared using pancreatin and collagenase type IV for 1–2 h at 37˚C (Sigma, St. Louis, MO) followed by filtration through a 70-μm mesh and enumerated by CFU assays (Remel). Colonies were counted after 3 wk of incubation at 37˚C.

Results

Flow cytometry

Spleen and lung cells were stained as previously described (14, 15) using Abs specific for mouse conjugated to Alexa 488, FITC, PE, PerCP, or allophycocyanin (BD Pharmingen) PE-Cy7, allophycocyanin-Cy7 (eBioscience, Biologeny) as indicated in the Results section. Tetramers were produced using EspA150–158-loaded H2-D1, TB10.420–28-loaded H2-K1, and TB10.474–88-loaded H2-L1 complexed to streptavidin (SA)-PE or SA-allophycocyanin (National Institutes of Health Tetramer Core Facility, Emory University Vaccine Center, Atlanta, GA). Cells were analyzed using a FACScanto (BD Biosciences) and FlowJo analysis software (Tree Star). Single-lymphocyte events were gated by forward scatter area versus height and side scatter for size and granularity. Tetramer positive staining from vaccinated and infected animals was determined by gating on CD8+ cells, and tetramer+ gates were established based on staining observed for uninfected mice and using SA-fluorochromes as a negative control.

Enumerating naive Ag-specific cells

Precursor frequency was performed essentially as previously described (24). Briefly, the spleen and axillary, mesenteric, cervical, inguinal, popliteal, and salivary lymph nodes were harvested from individual mice, dispersed, and filtered through a 70-μm mesh and enumerated for total and CD8+ T cell composition. The cell suspension was then co-stained with identical PE- and allophycocyanin-conjugated tetramers and then purified via anti-PE magnetic bead selection. Positive and negative fractions were then surface stained with anti-MHC II-CD11b, CD19, and CD4 as a “dumpy” channel, and anti-CD8a and CD11a or CD62L. Flow cytometry counting beads were added immediately before samples were collected by the cytometer to determine the fraction of tetramer+ cells collected (in most cases >90% of total sample was collected) and used to determine the total number and frequency of tetramer+ cells in each animal.

Generation of recombinant vaccinia virus

DNA fragments containing the entire TB10.4 and EspA genes was amplified from H37Rv M. tuberculosis cosmid DNA using 5’ and 3’ primers that included terminal Stul and SalI restriction sites, respectively. For epitope only viruses, 5’ and 3’ synthetic oligonucleotides encoding an ATG start codon, mammalian codon optimized sequences for the desired residues (Table III), and stop codons were annealed in vitro. The resultant products were cloned into pAS1008, a modified version of pSC11ss (25), and used to generate recombinant vaccinia viruses (rVV) using the protocol described previously (26). rVV.OVA, wild-type vaccinia, and pAS1008 were generous gifts from Michael Starnbach (Howard Medical School, Boston, MA). rVV.OVA preparations were treated with ammonium volume of 0.25 mg/ml trypsin for 30 min at 37˚C and diluted in PBS before immunization of mice.

CFU determination

After euthanasia by CO2 inhalation, infected tissue was aseptically removed. Bisected spleens and RPMI 1640-perfused left lung lobes were individually homogenized in 0.9% NaCl–0.02% Tween 80 with a Mini-Bead Beater 8 (Biospec Products, Bartlesville, OK). Viable bacteria were enumerated by plating 10-fold serial dilutions of organ homogenates onto 7H11 agar plates (Remel). Colonies were counted after 3 wk of incubation at 37˚C.

Intracellular and CD107 staining

Total lung mononuclear cells or splenocytes were prepared as above. Cells (0.5 × 10^6 to 1.0 × 10^7) were incubated with 10 μM peptide Ag in the presence of anti-CD107A–Alexa 488 and 100 U/ml IL-2 for 1 h. Brefeldin A (BD Pharmingen) was added for 4–5 h. Cells were then surface stained with anti-CD3ε, anti-CD8a, and anti-CD11b followed by intracellular staining for IFN-γ and IL-2 using BD PermWash Kit (as per manufacturer’s instructions). Samples were fixed in 1% paraformaldehyde and analyzed on a FACSCanTo.

Statistics

The Prism software program was used to perform all statistical analyses (GraphPad, San Diego, CA). CFU data were log10 transformed before analysis. Statistical significance was analyzed using one-way ANOVA and Bonferroni’s multiple comparison post-test.

Results

Identification of a novel CD8+ T cell epitope after infection

ESAT-6 and CFP10 are mycobacterial proteins encoded within the ESX-1 locus that are commonly recognized by circulating T cells in...
M. tuberculosis-exposed people and are immunodominant Ags in animal models (27). Protein secretion by the ESX-1 secretory apparatus is required for entry of these Ags into the class I MHC processing pathway (9). We performed experiments to determine whether the EspA protein, which is cosecreted with ESAT-6 and CFP10, is also a T cell Ag. The EspA C-terminal fragment induced T cell-dependent IFN-γ production by splenocytes obtained from M. tuberculosis-infected BALB/c and B6 mice, but not B10 BR mice, suggesting that EspA is recognized by T cells after infection (data not shown). To identify T cell epitopes of EspA, a library of 15-mer peptides overlapping by 9 aa and corresponding with the C-fragment was screened using highly purified CD4+ and CD8+ T cells from M. tuberculosis-infected mice (Fig. 1A, Table I). In addition, several candidate peptides from the N terminus that scored highly on class I MHC binding prediction algorithms were also tested. The region EspA(143–162) contained an epitope that was recognized by CD8+ T cells from infected BALB/c mice (Fig. 1A), and the nonamer EspA(150–158) was defined as a minimal epitope (Fig. 1B).

In addition to cytokine secretion assays, specific peptide-loaded MHC tetramers can be used to quantify Ag-specific CD8+ T cells in a sensitive and function-independent manner. EspA150–158 was highly predicted to bind to H2-Kd with a Rankpep score of 72.5% and a SYFPEITHI score of 33 (28, 29). An H2-Kd tetramer loaded with EspA150–158 identified CD8+ T cells in M. tuberculosis-infected BALB/c mice but not in H2 mismatched or uninfected

**FIGURE 1.** Identification of an EspA epitope that is recognized by CD8+ T cells. A, Highly purified pulmonary CD8+ T cells were used to screen an EspA peptide library (see Table I). The production of IFN-γ was measured by IFN-γ ELISA (displayed as OD405nm). Positive controls were the known epitope TB10.4(20–28) and negative controls were ESAT6(1–15) and CFP10(32–39). B, The minimal epitope of EspA(150–158) was defined using overlapping peptides. Production of IFN-γ was determined by ELISA. Peptide concentration is shown in log10 [M]. C, Identification of EspA- and TB10.4-specific CD8+ T cells. Lung cells from an M. tuberculosis-infected BALB/c mouse were gated first by size and then on CD3+CD8+ events. The frequency of EspA- and TB10.4-specific CD8+ T cells was determined by simultaneously staining with the H2-Kd/EspA(150–158) and H2-Kd/TB10.4(20–28) tetramers.
mice (data not shown). We previously described that after aerosol challenge, BALB/c mice generate a large CD8+ T cell response to the epitope TB10.420–28 (14, 15). By using the H2-Kd/EspA150–158 and H2-Kd/TB10.420–28 tetramers together, distinct CD8+ T cell populations were identified in M. tuberculosis-infected mice (Fig. 1C), which allowed the simultaneous comparison of the frequency of TB10.4- and EspA-specific CD8+ T cells during infection.

Relative immunodominance of T cell responses after M. tuberculosis infection

We next characterized the EspA150–158-specific CD8+ T cell response within the context of the overall cellular immune response by quantifying M. tuberculosis-specific T cells that recognize previously described H2restricted Ags in BALB/c mice after low-dose aerosol infection. Ag-specific T cell responses were detected using synthetic peptides corresponding with eight distinct epitopes from three Ags recognized by CD4+ T cells (Ag85A, TB10.4, and ESAT6) and three Ags recognized by CD8+ T cells (Ag85A, TB10.4, and EspA) (Table II). Purified T cells from the lung (Fig. 2) and spleen (not shown) of infected BALB/c mice obtained at different time points were stimulated using peptides in the presence of APCs (irradiated splenocytes). The CD4+ (Fig. 2A) and CD8+ (Fig. 2B) T cell responses were enumerated by IFN-γ ELISPOT.

Both CD4+ and CD8+ T cell responses were detected within 2 wk of infection. The CD4+ T cell response peaked at 4–5 wk postinfection and was dominated by Ag85A- and TB10.4-specific cells. In contrast, the peak pulmonary CD8+ T cell response occurred between 5 and 8 wk and was dominated by TB10.4-specific cells. EspA150–158-specific CD8+ T cells were a significant fraction of the IFN-γ-secreting cells and greater in frequency than CD8+ T cell responses to Ag85A epitopes. The frequency of Ag-specific T cells in the spleen was substantially lower although the relative

Table I. EspA peptides screened using M. tuberculosis-specific CD8+ T cells

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Location</th>
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<tbody>
<tr>
<td>GLYDLLGIGIPNQGGILYSSLEYFEKALELA</td>
<td>(16)</td>
</tr>
<tr>
<td>GLYDLLGIGIPNQGGI</td>
<td>(16)</td>
</tr>
<tr>
<td>LOIGIPNQGGILY</td>
<td>(13)</td>
</tr>
<tr>
<td>IGIPNQGGILYSSL</td>
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</tr>
<tr>
<td>PNQGGILYSSLEYF</td>
<td>(14)</td>
</tr>
<tr>
<td>GGIYSSLEYFEKAL</td>
<td>(15)</td>
</tr>
<tr>
<td>YSSLEYFEKALELA</td>
<td>(15)</td>
</tr>
<tr>
<td>AVDLTYIPVGVHLSAAFP</td>
<td>(14)</td>
</tr>
<tr>
<td>PVGVHLSAFAFP</td>
<td>(15)</td>
</tr>
<tr>
<td>HALSAAFQAP</td>
<td>(15)</td>
</tr>
<tr>
<td>AAFQPACAGAMV</td>
<td>(15)</td>
</tr>
<tr>
<td>APPCAGANAV</td>
<td>(15)</td>
</tr>
<tr>
<td>AGAMAVGA</td>
<td>(15)</td>
</tr>
<tr>
<td>AVGGAALYVX</td>
<td>(15)</td>
</tr>
<tr>
<td>GALAALVX</td>
<td>(15)</td>
</tr>
<tr>
<td>AYLAVX</td>
<td>(15)</td>
</tr>
<tr>
<td>VXTLNAVLL</td>
<td>(15)</td>
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</table>

The numbers in the left column correspond with the numbers in Fig. 1A. The numbers in parentheses represent the length of the respective synthetic peptide. Boldface numbers represent the number of the amino acid within the EspA protein sequence. The amino acid sequence of EspA is shown in boldface.
The hierarchy of immunodominance was similar (data not shown). This analysis was repeated twice, and a very similar hierarchy was evident. Using the frequency of Ag-specific T cells and the total number of pulmonary T cells, the number of IFN-γ–secreting CD4+ (Fig. 2C) and CD8+ (Fig. 2D) T cells per mouse lung was calculated. Between week 2 and 6, the dominant T cell responses increased in number between 125-fold and 410-fold. The CD4+ and CD8+ T cell responses closely parallel the pulmonary bacterial burden (Fig. 2E). Notably, the CD4+ and CD8+ T cell responses to TB10.4 follow similar kinetics, as previously described for the CD4+ and CD8+ T cell responses to CFP10 (Fig. 2E) (15).

The frequency of TB10.4- and EspA-specific CD8+ T cells was enumerated using peptide-loaded H2-tetramers. Similar to the results obtained with the ELISPOT, the frequency of tetramer+ cells peaked 6–8 wk postinfection, and late during infection TB10.4-specific CD8+ T cells reached a plateau (Fig. 2F, 2G). The TB10.4-specific CD8+ T cell response was nearly always dominant compared with the EspA-specific CD8+ T cell response; however, the degree of dominance varied, particularly late during infection. Of 45 mice analyzed in two experiments, the median ratio of TB10.4 to EspA-specific CD8+ T cells was 6.4 with a range of 0.5–642. Only one mouse had more EspA-specific CD8+ T cells than TB10.4-specific CD8+ T cells. Notably, the two extremes (0.5 and 642) were from a time point 33 wk postinfection, and in general, greater variability was observed late during infection.

Notably, TB10.4- and EspA-specific CD8+ T cell frequencies differ depending on whether they are measured by IFN-γ ELISPOT or by tetramers (compare Fig. 2B and 2F). We previously observed similar discrepancies for the CFP10-specific CD8+ T cell response (15) and TB10.4-specific CD8+ T cells (14), in which the frequency of IFN-γ–secreting T cells was ~10–15% of that detected by tetramers. It is unclear whether this is a technical issue or represents a change in function of the CD8+ T cells toward less cytokine production and more cytotoxic function (31). Thus, there is a hierarchy of Ag-specific CD4+ and CD8+ T cells that respond in a stereotypical way after M. tuberculosis infection.
Influence of naive T cell precursor frequency on immunodominance

Given the highly consistent hierarchy of T cell responses, we wished to investigate the factors that influence immunodominance after *M. tuberculosis* infection. Enumeration of naive Ag-specific T cells has suggested links between the precursor frequency, the kinetics of the cellular response, and the establishment of immunodominance after viral infections (32, 33). Whether such principles hold for immunodominance during a chronic infection such as tuberculosis is unknown.

Immunomagnetic selection of tetramer-bound cells with dual color tetramer staining is a technique that can sensitively and specifically detect naive Ag-specific T cells (24). To establish this method, we first applied this technique to identify H2-Ld–restricted naive T cells specific to the vaccinia virus F226–34 epitope, which is highly immunodominant after vaccinia infection of BALB/c mice (30). In vaccinia-infected mice, we identified an F226–34–specific CD8+ T cell population and confirmed our ability to enrich this population by magnetic bead selection (Fig. 3A, left panels). In parallel, we enumerated the naive F226–34–specific CD8+ T cells in uninfected animals (Fig. 3A, right panels). Lower surface expression of CD11a and CD44 on F226–34–specific CD8+ T cells obtained from uninfected versus memory mice confirmed the Ag inexperience of the naive tetramer-binding cells (data not shown). On average, ∼550 F226–34–specific CD8+ T cells were found in uninfected BALB/c mice, which is similar to that reported in DBA/2 mice (∼200/mouse) (34). Similarly, we used MHC class I tetramers to enumerate the precursor frequency of TB10.4 and EspA–specific CD8+ T cells in naive BALB/c mice (Fig. 3B). Relative to the frequency of F226–34–specific naive CD8+ T cells in uninfected mice (∼1:16,000), TB10.4 and EspA–specific naive CD8+ T cells were 25- to 50-fold less abundant (Fig. 3C). In addition, the frequency of naive TB10.4–specific CD8+ T cells was consistently 1.9-fold greater than EspA–specific CD8+ T cells (Fig. 3D). Thus, although the precursor frequency of naive TB10.4–specific CD8+ T cells was greater than EspA–specific CD8+ T cells, the 2-fold difference is not sufficient to explain the observed dominance of the TB10.4–specific response after *M. tuberculosis* infection.

Vaccination using rVV elicits *M. tuberculosis*-specific CD8+ T cells

The immunogenic EspA and TB10.4 epitopes were cloned into vaccinia virus (Table III). To confirm that the different epitopes were expressed and presented by vaccinia-infected cells, highly purified CD8+ T cells obtained from *M. tuberculosis*-infected BALB/c mice were cultured with vaccinia-infected syngeneic splenocytes. *M. tuberculosis*-specific CD8+ T cells recognized rVV.TB10, but not rVV.TB10CD4 or rVV.OVA (Fig. 4A), indicating that the TB1020–28 epitope was produced and presented by rVV.TB10-infected cells. Similarly, CD8+ T cells recognized splenocytes infected with rVV.EspAFL and rVV.EspA, or P815 cells infected with rVV.EspA (Fig. 4B, 4C). Although these data indicate that the EspA150–158 epitope was expressed, it appeared to be presented less efficiently than the TB10.4 epitope. This is deduced from the finding that whereas rVV.TB10.4 maximally acti-
vated CD8+ T cells (maximum activation indicated by the response to the TB10.4-28 peptide presented by rVV.OVA-infected cells), rVV.EspA-infected APCs stimulated only ~5% of the maximal response to EspA150-158 peptide epitope (Fig. 4A, 4B).

The ability of rVV.TB10 and rVV.EspA to elicit CD8+ T cells specific for the TB10.20-28 and EspA150-158 epitopes was measured in vivo. Acute peritoneal infection with rVV.TB10 and rVV.EspA induced CD8+ T cells specific for TB10.20-28 and EspA150-158, respectively (Fig. 4D). Vaccine-elicited TB10.20-28 and EspA150-158-specific CD8+ T cells secreted cytokines associated with host protection against M. tuberculosis including both IFN-γ (Fig. 4E) and TNF (data not shown). The frequency of cytokine-producing CD8+ T cells correlated with the frequency of tetramer+ CD8+ T cells.

In some experiments, only the TB10.20-28-specific CD8+ T cell response was statistically significant. We also observed that the response to the vaccinia epitope F2 was consistently significantly lower after in vivo infection with rVV.EspA compared with rVV. OVA or rVV.TB10 (Fig. 4F). Therefore, the low frequency of elicited EspA150-158-specific CD8+ T cells may be due to toxicity of the construct or competition between different epitopes for presentation. We found the frequency of M. tuberculosis-specific T cells in the spleen was relatively low after recombinant vaccinia infection compared with what we observe during chronic M. tuberculosis infection (compare with Fig. 1). However, an 0.3% frequency of TB10.20-28-specific CD8+ T cells in the spleen represents ~300,000 cells, which is >8,000-fold expansion based on the precursor frequency in naive mice (Fig. 3).

Single CD8+ T cell epitope vaccination failed to protect mice from M. tuberculosis infection

Two hypotheses concerning the ability of TB10.4- and EspA-specific CD8+ T cells to provide protection against M. tuberculosis were considered. Because TB10.4 represents a dominant CD8+ T cell response in BALB/c mice, we tested whether eliciting TB10.20-28-specific CD8+ T cells would protect mice against challenge with virulent M. tuberculosis. Additionally, because TB10.4 is already such a dominant response in BALB/c mice, we considered the possibility that priming mice against EspA, ordinarily a less dominant response, would provide better protection. Unvaccinated mice or mice vaccinated with bacillus Calmette-Guérin (BCG), rVV.OVA, rVV.TB10, or rVV.EspA were challenged with M. tuberculosis, and the lung CFU was measured 4 and 14 wk later (Fig. 5). As expected, BCG provided significant protection (measured 4 wk postinfection only). In contrast, neither vaccination with rVV.TB10 nor vaccination with rVV.EspA provided protection compared with unvaccinated mice (Fig. 5).

Vaccination only transiently alters CD8+ T cell immunodominance postinfection

The inability of rVV constructs to protect mice against M. tuberculosis infection was surprising because we demonstrated that vaccination elicited Ag-specific CD8+ T cells with many of the features of protective T cells. The dynamics of the CD8+ T cell response after aerosol challenge with M. tuberculosis was investigated in detail. Twelve days after challenge with aerosolized M. tuberculosis, a time point when Ag-specific T cells are just beginning to be recruited to the lung, an increased frequency of pulmonary TB10.20-28- and EspA150-158-specific CD8+ T cells was detected in mice vaccinated with rVV.TB10 and rVV.EspA, respectively (Fig. 6A). However, these CD8+ T cell expansions were relatively small compared with the ultimate primary CD8+ T cell response to M. tuberculosis (compare with Fig. 2).

In an independent experiment, mice vaccinated with rVV.EspA had an increased frequency of CD8+ T cells that produced IFN-γ when stimulated with EspA in vitro 2 wk after M. tuberculosis infection (Fig. 6B), and a significant proportion of these cells also expressed CD107A on their surfaces, indicating their cytotoxic potential (Fig. 6B). A similar trend was seen for CD8+ T cells specific for TB10.20-28 in rVV.TB10-vaccinated mice, although the difference was not statistically significant. Indeed, we detected a significant increase in both the frequency and absolute number of TB10.20-28- and EspA150-158-specific CD8+ T cells in the lungs 2 wk after infection of mice vaccinated with rVV.TB10 and rVV.EspA, respectively (Fig. 6C). However, between 4 and 14 wk, a time during which the CD8+ T cell response to TB10.20-28 and EspA150-158 is near maximum, there were no differences in the frequency of TB10.4- or EspA-specific CD8+ T cells in the lungs (Fig. 6D) and spleens (not shown) of vaccinated and unvaccinated infected mice.

Discussion

We have identified an epitope within the virulence factor EspA as a novel target of CD8+ T cells in the mouse model of tuberculosis. Our longitudinal analysis revealed that EspA-specific CD8+ T cell response sits within a consistent hierarchy of immunodominance, which is subdominant to TB10.4-specific CD8+ T cells but more frequent than Ag85A-specific CD8+ T cells. We find a similar regularity of immunodominance in the CD4+ T cell response to M. tuberculosis infection. The CD4+ and CD8+ T cell responses have comparable peak and plateau kinetics after infection in this model, contrary to a recent report (35) but consistent with our and others’ previous findings (14, 15, 36–38). These data show a consistent orchestrated adaptive immune response to aerosol M. tuberculosis infection.

To understand further the factors governing immunodominance during M. tuberculosis infection, we enumerated the precursor frequency of the dominant TB10.4- and subdominant EspA-specific T cells in naive animals. Similar to reports from viral systems, the relative naive precursor frequency predicted the relative immunodominance after infection (8, 33). However, the relatively small difference in precursor numbers (<2-fold) we observed translated into a much greater difference during M. tuberculosis.

<table>
<thead>
<tr>
<th>Table III. Recombinant vaccinia virus constructs</th>
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<tr>
<td>Virus</td>
</tr>
<tr>
<td>rVV.TB10.4</td>
</tr>
<tr>
<td>rVV.TB10.4CD4</td>
</tr>
<tr>
<td>rVV.TB10.4FL</td>
</tr>
<tr>
<td>rVV.EspA</td>
</tr>
<tr>
<td>rVV.EspAFL</td>
</tr>
<tr>
<td>rVV.OVA</td>
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</table>

The first methionine (nonbold) was introduced as a start signal for the translation of the epitope and is not part of the original mycobacterial protein sequence.

<sup>a</sup>Underlined residues represent defined H2<sup>b</sup> restricted epitopes.

CD4, contains epitope recognized by CD4+ T cells; FL, full length.
tuberculosis infection (>6-fold) (Figs. 2, 3). In addition, we did not observe that, compared with subdominant responses, the dominant (TB10.4) specific CD8+ T cell response had accelerated kinetics toward peak abundance, as has been reported in acute viral infection (8). Factors such as bacterial immune subversion and a relatively delayed adaptive response may influence T cell immunodominance during *M. tuberculosis* infection.

Vaccination to enhance a subdominant CD4+ T cell in mice can protect from subsequent *M. tuberculosis* infection, and subdominant CD8+ epitope vaccination has been shown to alter immunodominance to Sendai virus infection (39, 40). To investigate whether CD8+ T cell immunodominance can be altered in *M. tuberculosis* infection, and to determine the relative protective capacity of epitopes at different positions within the hierarchy, we created rVVs that expressed and presented TB10.4 and EspA epitopes. The viruses elicited significant TB10.4- and EspA-specific CD8+ T cell populations that, although smaller than those directed against the dominant viral Ag, were able to increase effectively their precursor frequency in immune animals and transiently alter the relative frequency of TB10- and EspA-specific CD8+ T cells during the very early part of the immune response to *M. tuberculosis*. However, by the time that the peak bacterial load was reached, the infection-driven hierarchy of epitope responses was restored and maintained during the remainder of the infection.

Previously, we showed that DNA vaccination inducing a similarly small *M. tuberculosis*-specific CD8+ T cell response to a single CFP10 epitope resulted in early recruitment of *M. tuberculosis*-specific CD8+ T cells to the lung and reduced the bacterial burden in susceptible C3H mice. Therefore, we were initially surprised to find that our rVV-based vaccination against a single EspA or TB10.4 epitope did not protect BALB/c mice against *M. tuberculosis*. This was despite a vaccine-elicited response that responded with enhanced kinetics to infection including early expansion in lung tissue by day 12–14 (Fig. 4B), similar in magnitude to that seen in antibiotic-induced memory immune mice (14). These data appear to contrast with both our previous findings that a CD8+ T cell response to a single dominant epitope (41) provides protection and the findings of others that *M. tuberculosis*-Ag vaccination of CD4+ T cell-deficient mice provides protection (42, 43). However, these previous studies were
done in highly susceptible mouse strains (C3H and CD4-depleted) that may constitute particular cases where CD8+ T cells can mediate protection. Specifically, animals unable to generate an endogenous CD4+ T cell response to infection may benefit from an enhanced CD8+ T cell response. Similarly, the genetic defect in C3H mice associated with susceptibility may be partially compensated by early CD8+ T cell responses (44, 45). In contrast, eliciting TB10.420–28-specific CTLs was unable to protect BALB/c mice from aerosol M. tuberculosis challenge (21). Thus, in more resistant backgrounds, the role of CD8+ T cells in protection may be particularly stage-specific, with CD8+ T cells most important during the latent stage of infection in resistant mice (46).

We have previously shown that perforin activity is required for CD8+ T cell protection in M. tuberculosis infection (47). However, the first studies in perforin-deficient mice only detected differences in long-term survival (44, 45). In contrast, eliciting TB10.420–28-specific CTLs was unable to protect BALB/c mice from aerosol M. tuberculosis challenge (21). Thus, in more resistant backgrounds, the role of CD8+ T cells in protection may be particularly stage-specific, with CD8+ T cells most important during the latent stage of infection in resistant mice (46).

A

B

C

D

FIGURE 6. rVV transiently alters immunodominance of CD8+ T cell response to M. tuberculosis. A, Twelve days after aerosol M. tuberculosis challenge, the frequency of lung CD8+ T cells that bound H2-Kd/TB10.420–28 (left) and H2-Kd/EspA150–158 (right) tetramers was determined in rVV-vaccinated mice. B, Two weeks after M. tuberculosis challenge, the frequency of IFN-γ-producing cells (left) and subset of these cells with surface CD107A (right) was determined in rVV-EspA–vaccinated mice by peptide stimulation and intracellular cytokine staining of lung T cells. Bar, mean ± SEM. *p < 0.05 versus media control. C, Two weeks after M. tuberculosis infection, the frequency (left) and absolute number (right) of TB10.420–28 (top) and EspA150–158 (bottom) specific lung resident CD8+ T cells was determined by tetramer staining in nonvaccinated or rVV-vaccinated mice. D, Four weeks and 14 weeks after M. tuberculosis challenge, the frequency of TB10.420–28 (top) and EspA150–158 (bottom) specific lung resident CD8+ T cells was determined by tetramer staining. *p < 0.05 (determined by one-way ANOVA with Bonferroni post-test).

duced M. tuberculosis-infected mice using tetramers versus IFN-γ ELISPOT could be interpreted as a change in function during chronic infection (Fig. 2). Distinct populations of IFN-γ-producing and cytolytic CD8+ T cells are associated with persistent infection, whereas cells with both capacities were most protective (34). In the early response to M. tuberculosis infection, CD8+ T cells from rVV-vaccinated mice were both cytotoxic and IFN-γ-producing. After peak infection, this enhanced population was undetectable within the infection-driven response. Although not examined in this study, vaccination that can maintain polyfunctional CD8+ T cells in the latest stages of infection may improve survival.

Another explanation for the transient nature of the alteration in immunodominance stimulated by vaccination with rVV is that different T cell clones were primed and expanded. Although M. tuberculosis-specific CD8+ T cells elicited by rVV vaccination recognized the same epitopes that are recognized by CD8+ T cells after M. tuberculosis infection, the TCRs used by CD8+ T cells elicited by rVV versus M. tuberculosis could differ. As such, the avidity of alternate TCRs elicited after rVV vaccination may be reduced compared with that of those naturally elicited by
M. tuberculosis infection. This difference could affect the expansion of certain T cell clonotypes as well as affect their function and ability to provide protection against M. tuberculosis (50, 51).

The realization that the bacterially induced immunodominance superseded the vaccine-driven response poses question about the current vaccine strategies in development. Recent studies have suggested that the granulomatous response to mycobacterial infection, once thought to be a host-determined protective reaction to infection, may in effect represent bacterial-induced manipulation of immunity to enhance bacterial spread and survival (52). Furthermore, M. tuberculosis may have specifically evolved to retain epitopes recognized by the human T cell response, suggesting that it derives benefit from a directed immune response (53).

Conversely, a recombinant adeno virus expressing M. tuberculosis Ags ESAT6 and Ag85A elicits CD8+ T cells specific for the ESAT615-29 epitope, but these CD8+ T cells did not expand during infection. The failure of this vaccine to protect against M. tuberculosis challenge could stem from the lack of ESAT615-29 epitope presentation by M. tuberculosis-infected macrophages (54, 55). Given that most subunit vaccines deliver known immunodominant and a subdominant CD8+ T cell epitope, TB10.4- and EspA-specific CD8+ T cells readily expanded and homed to the lung. However, in both cases, the infection-driven immunodominance prevailed, and the immunological control of bacterial burden was unaltered.

In this study, we report to our knowledge the first demonstration of the ESX-1 functionally associated EspA as a T cell Ag in M. tuberculosis infection and have used it to probe the host and bacterial determinant of immunodominance of potential vaccine targets. EspA is an important bacterial virulence factor of M. tuberculosis and is required for the secretion of ESX-1 Ags CFP10 and ESAT-6. The identification of EspA as a bona fide T cell Ag has strong potential for both vaccine and diagnostic applications.

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Disclosures

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