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Protective CD4 T Cells Targeting Cryptic Epitopes of *Mycobacterium tuberculosis* Resist Infection-Driven Terminal Differentiation

Joshua S. Woodworth,* Claus Sindbjerg Aagaard,† Paul R. Hansen,‡ Joseph P. Cassidy,‡ Else Marie Agger,* and Peter Andersen*

CD4 T cells are crucial to the control of *Mycobacterium tuberculosis* infection and are a key component of current vaccine strategies. Conversely, immune-mediated pathology drives disease, and recent evidence suggests that adaptive and innate responses are evolutionarily beneficial to *M. tuberculosis*. We compare the functionality of CD4 T cell responses mounted against dominant and cryptic epitopes of the *M. tuberculosis* 6-kDa early secreted Ag (ESAT-6) before and postinfection. Protective T cells against cryptic epitopes not targeted during natural infection were induced by vaccinating mice with a truncated ESAT-6 protein, lacking the dominant epitope. The ability to generate T cells that recognize multiple cryptic epitopes was MHC-haplotype dependent, including increased potential via heterologous MHC class II dimers. Before infection, cryptic epitope–specific T cells displayed enhanced proliferative capacity and delayed cytokine kinetics. After aerosol *M. tuberculosis* challenge, vaccine-elicited CD4 T cells expanded and recruited to the lung. In chronic infection, dominant epitope–specific T cells displayed a terminal differentiated KLRG1+/PD-1hi surface phenotype that was significantly reduced in the cryptic epitope–specific T cell populations. Dominant epitope-specific T cells in vaccinated animals developed into IFN-γ+ and IFN-γ,TNF-α–co-producing effector cells, characteristic of the endogenous response. In contrast, cryptic epitope–specific CD4 T cells maintained significantly greater IFN-γ+TNF-α+IL-2− and TNF-α+IL-2− memory-associated polyfunctionality and enhanced proliferative capacity. Vaccine-associated IL-17A production by cryptic CD4 T cells was also enhanced, but without increased neutrophilia/pathology. Direct comparison of dominant/cryptic epitope–specific CD4 T cells within covaccinated mice confirmed the superior ability of protective cryptic epitope–specific T cells to resist *M. tuberculosis* infection–driven T cell differentiation. *The Journal of Immunology*, 2014, 192: 3247–3258.

It is estimated that one third of all people are latently infected with *Mycobacterium tuberculosis*, resulting in nearly 9 million new cases of tuberculosis (TB) and 1.4 million deaths in 2011, a significant number of which were caused by multidrug and extremely drug-resistant strains (1). The only current TB vaccine, *Bacillus* Calmette–Guerin (BCG), an attenuated form of *Mycobacterium bovis*, is only partially effective, with an efficacy of 0–80% control of adult pulmonary TB in varied populations, and development of novel vaccines is a global health priority (2). CD4 T cell–mediated protection and pathology are closely linked and require a fine balance to control disease. The importance of CD4 T cell-mediated immunity against *M. tuberculosis* is highlighted by the increased incidence of primary and reactivated TB in HIV-infected individuals (1, 3). However, the endogenous T cell response to *M. tuberculosis* infection is only partially protective, such that ~10% of *M. tuberculosis*–infected HIV sera-negative individuals develop primary TB or reactivation of latent TB infection (LTBI) in their lifetime, and antibiotic treatment does not result in sterilizing memory immunity. Furthermore, mycobacterial infection directly influences the development of the innate immune response in a way that promotes bacterial growth, and recent evidence of hyper-conservation of dominant human T cell epitopes in the *M. tuberculosis* genome suggests that the natural T cell response to infection is evolutionarily advantageous for the bacteria (4, 5). Significantly, TB’s characteristic necrotizing immune tubercle is crucial for pulmonary bacterial dissemination between persons. Thus, innate and adaptive immunological responses to infection are responsible for disease control, as well as dissemination within and between individuals. A primary goal of next-generation TB vaccines has been the induction of an improved cellular immune response to better contain an initial bacterial insult and prevent disease development and spread. However, the optimal T cell response required to attain this goal is currently unknown. Decades of research in mice and other animal models have taught us the importance of Th1-associated IFN-γ in protection against *M. tuberculosis* (6, 7). However, although necessary, the strength of Th1 IFN-γ responses after BCG vaccination does not correlate with protection (8–10). Treated TB patients have a strong Th1 response to *M. tuberculosis*
production, and proliferative capacity at the site of infection. The T cell–mediated protection against TB, and contrast these cells with Th17 cells, which are not promoted during the natural infection or after vaccination with full-length ESAT-6. In this study, we investigate cryptic epitope–specific T cells better resist acquisition of the differentiation profiles of these cells and successful containment of disease is unclear, vaccine-induced multifunctional T cells have been associated with protection in mice (19–24).

Despite having >4000 genes, the endogenous cellular immune response against M. tuberculosis is directed against a relatively constrained set of dominant epitopes within a subset of protein targets. Therefore, in addition to T cell phenotype, directing a cellular immune response against the right, protective antigenic targets is also a major aim of vaccine design. One such Ag is the 6-kDa early secreted Ag (ESAT-6), present in virulent M. tuberculosis, but absent in BCG. ESAT-6 is a target of T cells in infected individuals, and dominant peptide epitopes have been mapped in various geographical populations (25, 26). In turn, several ESAT-6–containing vaccines have shown promising results in animal studies and are currently undergoing clinical trials (27–29).

Recently, vaccination using truncated ESAT-6 was shown to provide protection in mice via induction of CD4 T cells targeting cryptic epitopes within ESAT-6 (20). These cryptic epitopes are distinct from the single dominant epitope targeted by the endogenous M. tuberculosis infection, because they are not promoted during the natural infection or after vaccination with full-length ESAT-6. In this study, we investigate cryptic epitope–specific T cell–mediated protection against TB, and contrast these cells with dominant epitope–directed T cells for phenotype and functional development during infection. We find that, compared with dominant epitope–specific T cells, vaccine-elicted cryptic epitope–specific T cells better resist acquisition of the differentiation profiles associated with the endogenous M. tuberculosis–elicited T cell response as defined by surface marker expression, functional cytokine production, and proliferative capacity at the site of infection.

Materials and Methods

Mice

Studies were performed with 6- to 8-wk-old female BALB/c, C57BL/6, and CB6F1 (BALB/c × C57BL/6) from Harlan Netherlands and allowed a 1-wk rest period between delivery and initiation of experiments. Mice were housed in animal facilities at Statens Serum Institut, provided radiation-sterilized food (Harlan, Boxmeer, The Netherlands) and water ad libitum. Infected animals were housed in cages contained within laminar flow safety enclosures (Scantainer; Scanbur, Karlslunde, Denmark) in a separate biosafety level 3 facility. All handling and animal procedures were performed in compliance with the European Community Directive 86/609 for the care and use of laboratory animals.

Recombinant proteins

His-tagged dimeric ESAT-6 and Δ15ESAT6 were cloned from H37Rv chromosomal DNA, and expressed and purified from E. coli inclusion bodies by metal affinity as previously described (20).

Peptides

Ten 20-mer peptides, overlapping by 10 aa, spanning the ESAT-6 protein was synthesized by stepwise solid-phase peptide synthesis with a Teflon filter using the Fmoc/tbu protection scheme as previously described (30). The overlapping pool of TB10.4 peptides used was previously described (31). See Table I for peptide sequences.

Immunizations and infections

All mice were immunized s.c. at the base of the tail three times at 2-wk intervals. When given together with Δ15-E6, an additional ESAT-6 was given s.c. at the nape of neck. Cationic liposomes (CAFO1, 250 μg DNA/50 μg TDB) were mixed with 5 μg Ag in 0.1M Tris to a final volume of 200 μl for each injection. Negative control mice received an equivalent dose of CAFO1 alone. Mice were rested at least 10 wk before aerosol M. tuberculosis challenge. Virulent M. tuberculosis Erdman was grown to log-phase in Sauton medium (BD Pharmingen) stored at −80°C in Sauton growth media enriched with 0.5% glucose 0.2% Tween 80 at ~5 × 10^8 CFU/ml. M. tuberculosis was suspended in PBS Tween 20 (0.05%) and aerosolized for inhalation using a Glas-Col inhalation exposure system or Biaera exposure system controlled via AeroMP software. An average delivered dose of 50–150 CFU/animal was confirmed by CFU plating of total lung homogenates 1 d after challenge on Middlebrook 7H11 Bacto agar and enumerated after 3 wk at 37°C. Left lung lobes were homogenized from individual mice at 5–6 wk postchallenge, serially diluted, and CFU enumerated as described earlier.

Isolation of cells from tissue

Blood samples from 6–16 mice were pooled within immunization groups before PBMCs were isolated by density centrifugation over Lymphoprep (Cedarlane Laboratories, Burlington, ON, Canada) as per manufacturer’s protocol. Splenocytes were isolated from individual/pooled animals by forcing cells through a 70-μm nylon cell strainer (BD Pharmingen), following by hypotonic RBC lysis in 0.84% NH4Cl. Lung mononuclear cells (MNCs) from infected mice were isolated by collagenase type IV digestion (250 U/ml Sigma, St. Louis, MO) of lung tissue in RPMI 1640 containing 5% FCS at 37°C for 1–1.5 h, followed by two filtrations through a 70-μm cell strainer.

Cytokine secretion assays

A total of 2 × 10^6 PBMCs, splenocytes, or lung MNCs were cultured in round-bottom, 96-well microtiter plates in 200 μl complete RPMI media (RPMI 1640 supplemented with 1 mM l-glutamine, 50 μM 2-ME, 1% pyruvate, 1% penicillin-streptomycin, 1% HEPES, and 10% FCS (all Life TechnologiesInvitrogen, Carlsbad, Denmark)), and 2–5 μg Ag or 1 μg/ml Con A (to confirm T cell viability) at 37°C in a humidified incubator under 95% air, 5% CO2. For peptide mixtures, each peptide was present at 2 μg/ml. Splenocytes used as APCs were pulsed with 10 μg/ml peptide, washed three times, and added at 1 × 10^6 cells/well. Culture supernatants were harvested from lymphocyte cultures after 5 d of in vitro Ag stimulation and tested in triplicates, unless otherwise stated. For detection of secreted IFN-γ, 96-well Maxisorp microtiter plates (Nunc, Denmark) were coated with 1 μg/ml monoclonal rat anti-murine IFN-γ (clone R4-6A2; BD Pharmingen). Free binding sites were blocked with 2% (v/v) milk powder in PBS. IFN-γ was detected with a 0.1 μg/ml biotin-labeled rat anti-murine Ab (clone XMG1.2; BD Pharmingen) and 0.35 μg/ml HRP-conjugated streptavidin (Zymed, Invitrogen). The enzyme reaction was developed with 3,3′,5,5′-tetramethylbenzidine, hydrogen peroxide (MBL, Darmstadt, Germany), and stopped with 0.2 M H2SO4. rIFN-γ (BD Pharmingen) was used as a standard. Plates were read at 450 nm with an ELISA reader and analyzed with KC4 3.03 Rev 4 software.

Multiple cytokine analysis on culture supernatants was determined using a custom mouse MICRO ARRAY plate and analysis software (N505B-1; Meso Scale Discovery, Rockville, MD).

Proliferation assays

[3H]Thymidine incorporation assays to monitor Ag-specific cell proliferation were coperformed on cell cultures used for ELISA assays. A total of 125 μl supernatant was removed and replaced with 50 μl media containing 1 μCi methyl-[3H]thymidine (2.0 Ci/mmol; Perkin Elmer) per well. Cells were incubated overnight at 37°C in 5% CO2, for 18–20 h, harvested onto filters, and assayed for [3H]thymidine incorporation using a liquid scintillation counter (1205 Beta Counter, Pharmacia).

CFSE dilution assays to monitor cell division were performed on splenocytes or total lung MNCs isolated from M. tuberculosis–infected mice. Single-cell suspensions were labeled with 1 μM CFSE (CellTrace; Life Science Technologies) in PBS at room temperature for 8 min and washed extensively thereafter with media containing 10% FCS. A total of 10^5 cells was stimulated with 5 μg/ml peptide at 37°C in the dark and stained for surface markers CD4, CD8, and Fixable Viability Dye eFluor 780 (eBiosciences), before being analyzed by flow cytometry for dilution of CFSE signal. FlowJo software v 9.7.1 (Tree Star) proliferation analysis was used to fit data and calculate the percent divided precursor frequency of CD4 T cells for each sample.

Flow cytometry

A total of 1–2 × 10^6 lung MNCs was stimulated in vitro in V-bottom 96-well plates at 37°C in 200 μl complete media containing anti-CD49d (1 μg/ml) and anti-CD28 (1 μg/ml) Abs, in the presence of peptide (5 μg/ml) for 1 h,
Vaccination with the truncated ESAT-6 lacking the first 15 N-terminal specific CD4 T cells, defined as CD4+CD8- (I7B7; eBiosciences). KLRG1 and PD-1 expression were assessed on Ag-vaccinated with either full-length ESAT-6 or Δ15-E6 vaccination of C57BL/6 mice (C57BL/6 [H-2b], BALB/c [H-2d], and CB6F1 [H-2b/d]) were treated with 0.5 μg/ml anti-CD16/CD32 (2.4G.2; BD Pharmingen) before surface staining with anti–CD4-PE (GK1,5; BD Pharmingen), IL-2–allophycocyanin–Cy7 (JES6-5H4; BD Pharmingen), and IL-17A–PerCP–Cy5.5 (1B7; eBiosciences).

For KLRG1/PD-1 analysis, cells were incubated for 5 h in the presence of brefeldin A, because these markers began to downregulate with 12-h in vitro culture. Cells were treated with 0.5 μg/ml anti-CD4/CD8 (2.4G.2; BD Pharmingen) before anti–KLGR1-allophycocyanin (2F1; eBiosciences), and anti–PD1-PE-Cy7 (XMG1.2; BD Pharmingen), anti–CD8-allophycocyanin-Cy7 (53-6.7; BD Pharmingen), anti–IFN-γ-FITC (R4–6A2; BD Pharmingen), anti–CD4-allophycocyanin (clone RM4-5; BD Pharmingen) and IL-17A–PerCP–Cy5.5 (1B7; eBiosciences). KLRG1 and PD-1 expression were assessed on Ag-specific CD4 T cells, defined as CD4+CD8- and IFN-γ- and/or IL-17A-.

FlowJo v.8.8.7 (Tree Star) was used for flow cytometric analyses and cytokine Boolean gating within the CD4+CD44+ lymphocyte population. The relative proportions of cells producing different combinations were determined using PESTLE and SPICEv.5.22 software. Values for each combinatorial subpopulation from matched nonstimulated were subtracted as background from each sample before further analysis.

Statistical analysis

Prism 5 software was used to perform all statistical analyses (GraphPad, San Diego, CA). CFU data were log-transformed before analyses. One-way ANOVA with Tukey's multiple-comparison posttest was for all assay comparisons between multiple groups. Statistical differences are marked by asterisks in the figures and explained in the figure legends.

Results

Vaccine induction of protective T cells specific for cryptic epitopes is influenced by MHC diversity

Vaccination of BALB/c × C57BL/6 F1 mice (CB6F1) with full-length recombinant ESAT-6 protein adjuvanted with cationic liposomes (CAF01) generates a highly focused CD4 T cell response against the dominant N-terminal epitope (ESAT-61–20; Table I) that is also primed by M. tuberculosis infection (20). In contrast, vaccination with the truncated ESAT-6 lacking the first 15 N-terminal residues, rESAT-616–20, primes an alternative T cell response, targeting three cryptic epitopes in the C-terminal region not seen in nonvaccinated controls (20). In this study, three strains of mice (C57BL/6 [H-2b], BALB/c [H-2d], and CB6F1 [H-2bd]) were vaccinated with either full-length ESAT-6 or Δ15-E6 and challenged with aerosolized M. tuberculosis. Significant reduction of lung bacterial burden was seen in all strains that received ESAT-6 vaccination (Fig. 1A). In contrast, Δ15-E6 vaccination afforded protection only to BALB/c and CB6F1 mice (Fig. 1A). Epitope mapping of lung T cell responses during infection revealed that although BALB/c and CB6F1 mice generated protective T cell responses to cryptic ESAT-6 epitopes, in addition to the infection-driven endogenous P1 response, Δ15-E6 vaccination of C57BL/6 mice did not (Fig. 1B, see Table I for peptide sequences). Thus, reduction of CFUs by distinct cryptic epitope–directed T cells was confirmed in two separate mouse strains.

As expected, ESAT-6 vaccination elicited P1-specific dominant epitope responses in all three mouse strains (Fig. 1B) (20, 32). In contrast, Δ15-E6 vaccination elicited T cells specific for two cryptic epitopes (P4 and P9) in BALB/c mice, and three epitopes (P4, P6, and P9) in CB6F1 mice. To determine the MHC restriction of each epitope, lung MNCs from CB6F1 mice after Δ15-E6 vaccination and M. tuberculosis challenge were stimulated with C57BL/6, BALB/c, and CB6F1 splenocytes, pulsed with each immunogenic peptide epitope. Consistent with the epitope mapping in Fig. 1B, P1 epitopes are both H-2d-restricted and H-2d-restricted, whereas P4 and P9 epitopes are H-2b-restricted (Fig. 1C). Interestingly, P6-specific T cells were stimulated only by P6-pulsed CB6F1 splenocytes, indicating that this epitope is restricted by a heterodimeric class II MHC molecule that is H-2bd (Fig. 1C). Thus, higher MHC diversity allows for additional cryptic epitope–specific T cell responses in vivo.

Vaccine-induced cryptic epitope–specific T cells have distinct functional properties

We next wished to determine whether vaccine-induced dominant and cryptic epitope–specific T cells had inherent functional differences. Epitope mapping by thymidine incorporation assay after ESAT-6 or Δ15-E6 vaccination of CB6F1 mice confirmed that the Δ15-E6 vaccination elicited T cells specific for P4, P6, and P9, whereas the T cell response to full-length ESAT-6 was focused against the N-terminal P1 epitope with an additional proliferative response against the P9 peptide (Fig. 2A, 2B). Interestingly, comparison of proliferation versus IFN-γ revealed that P4-, P6-, and P9-specific T cells were more proliferative, but secreted less overall IFN-γ, than P1-specific T cells (compare Fig. 2A and 2B). Comparing P1- and P4-specific responses, a 4-fold lower P4-specific IFN-γ response was accompanied by a 4-fold higher proliferation stimulation index (SI). Although SIs varied somewhat between experiments, the ratio of SI/IFN-γ secretion for P4- and P6-specific responses was consistently higher than that of the dominant epitope P1-specific responses in all experiments performed, demonstrating an overall enhanced proliferative potential.

Increased cryptic epitope–specific T cell proliferation was not due to an obvious kinetic difference, because both P1- and P4-specific responses peaked 5 d poststimulation (Fig. 2C). In contrast, cytokine production kinetics differed. Dominant-epitope P1-specific cells reached maximal accumulation of secreted IFN-γ after 5 d of restimulation, whereas the P4-specific response was slower, not peaking until at least 9 d after stimulation (Fig. 2D).

CAF01-adjuvanted Ag can induce both Th1 and Th17 T cell responses, and we therefore also considered cytokine-specific differences (33). Decreased cytokine kinetics by P4-specific T cells was also observed for IL-17A, GM-CSF, IL-5, and IL-10 (data not shown). At peak time points after stimulation (days 5 and 9 for P1- and P4-specific T cells, respectively), similar overall relative cytokine profiles were observed, however with a higher IL-17A/IFN-γ ratio for P4-specific cells (Fig. 2E).

Thus, vaccine-elicited T cells specific for a cryptic ESAT-6 epitope have increased proliferation and a delayed Th1/Th17 response upon restimulation, which may indicate a distinct stage of differentiation and subsequent response to M. tuberculosis infection. Cryptic epitope–specific CD4 T cells respond to infection and resist terminal differentiation

We next investigated how the anti–ESAT-6 CD4 T cell response developed post aerosol M. tuberculosis infection. As previously

Table I. Overlapping peptides spanning ESAT-6 (20-mers overlapping by 10 aa)

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Residues</th>
<th>Sequence</th>
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<tr>
<td>P1</td>
<td>1–20</td>
<td>MTEQWQNFPIAEAAASAIQG</td>
</tr>
<tr>
<td>P2</td>
<td>11–30</td>
<td>IEAAASAIQGNTVSIIHLDL</td>
</tr>
<tr>
<td>P3</td>
<td>21–40</td>
<td>NVTSIIHSLLEDQGKQSLTCLKA</td>
</tr>
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<td>P4</td>
<td>31–50</td>
<td>EGKQSLTCLKAIAWGGGSGSEA</td>
</tr>
<tr>
<td>P5</td>
<td>41–60</td>
<td>AAWGGGSGSEAYQQVQQKWDA</td>
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<td>P6</td>
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<td>71–90</td>
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</tr>
<tr>
<td>P9</td>
<td>81–95</td>
<td>QAMASTEGNVTGMFA</td>
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</tbody>
</table>
described, the endogenous infection-promoted ESAT-6 T cell response is detectable in the lungs 2 wk post aerosol *M. tuberculosis* infection and is focused entirely on the P1 epitope in the adjuvant control mice (Fig. 3A) (20, 32). ESAT-6–vaccinated mice showed an enhanced P1-specific response in both the early and chronic stages of infection (Fig. 3A). In Δ15-E6–vaccinated mice, the P1 response was unchanged, whereas ESAT-6 cryptic epitope–specific T cells were recruited to the lungs by week 2 post *M. tuberculosis* infection, where they were maintained into chronic infection (Fig. 3A). Early in infection, the relative hierarchy of P4-, P6-, and P9-specific T cells mirrored that of preinfection (compare Figs. 2A, 2B, and 3A). However, by week 6, the P6 response increased relative to P4, perhaps indicating a differential availability of these epitopes in vivo as the infection progresses.

Intracellular cytokine staining (ICS) of restimulated lung MNCs was used to enumerate ESAT-6–specific lung cells throughout infection. Similar to the ELISA results, we found the endogenous P1 response was similar between the control and Δ15-E6 groups, but increased in ESAT-6–vaccinated mice 6 and 12 wk after *M. tuberculosis* challenge. Cryptic epitope-specific CD4 T cell responses, including P4, were found only in the Δ15-E6–vaccinated mice (Fig. 3B).

Intracellular cytokine staining (ICS) of restimulated lung MNCs was used to enumerate ESAT-6–specific lung cells throughout infection. Similar to the ELISA results, we found the endogenous P1 response was similar between the control and Δ15-E6 groups, but increased in ESAT-6–vaccinated mice 6 and 12 wk after *M. tuberculosis* challenge. Cryptic epitope-specific CD4 T cell responses, including P4, were found only in the Δ15-E6–vaccinated mice (Fig. 3B).

Surface marker phenotyping of T cells after *M. tuberculosis* challenge in humans and animal models has been instrumental in tracking the development of cellular immunity over time. In the mouse model of *M. tuberculosis* infection, careful TCR-transgenic T cell adoptive transfer studies have delineated lung CD4 T cell differentiation using the Killer cell Lectin-like Receptor subfamily G member 1 (KLRG1) and the B7 family member, PD-1, and mapped a progression from proliferative KLRG1<sup>hi</sup>/PD-1<sup>lo</sup> to nonproliferative, high-cytokine–producing terminally differentiated KLRG1<sup>lo</sup>/PD-1<sup>hi</sup> cells (34). We compared the KLRG1/PD-1...
phenotype of the dominant and cryptic epitope ESAT-6–specific CD4 T cells in the lungs of the vaccinated mice, 6 wk after 
*M. tuberculosis* challenge (Fig. 3C, 3D). P1-specific CD4 T cells in control mice exhibited a substantial proportion of KLRG1<sup>lo</sup>/PD-1<sup>hi</sup> cells (mean 30.3 ± 9.1%) terminally differentiated cells, which was significantly reduced in ESAT-6–vaccinated animals (12.6 ±

Specific combinatorial cytokine expression is associated with distinct differentiation status of CD4 T cells (35, 36). Therefore, in addition to surface phenotyping, we sought to compare the functional cytokine production profile of P1- and P4-specific CD4 T cells in the lungs of chronically infected mice. To investigate T cell polyfunctionality during infection, we used multiparameter ICS to determine the percent of lung resident P1- and P4-specific CD4<sup>+</sup> T cells secreting IFN-γ, TNF-α, IL-2, and IL-17A in *M. tuberculosis*-infected mice (Fig. 4A). Combinatorial Boolean gating analysis was then used to determine cytokine expression profiles. Six weeks after aerosol *M. tuberculosis* challenge, the endogenous ESAT-6 response (P1) in both control and Δ15-E6–vaccinated mice was dominated by Th1-skewed CD4 T cells producing either IFN-γ alone (IFN-γ<sup>+</sup>) or in combination with TNF-α (IFN-γ<sup>+</sup>TNF-α<sup>+</sup>), profiles that are associated with terminal differentiation (Fig. 4B). The P1 response in ESAT-6–vaccinated mice was composed of a more diverse and polyfunctional response, including IL-2–producing memory-associated double-cytokine (IL-2<sup>+</sup>TNF-α<sup>+</sup>) and triple-cytokine (IFN-γ<sup>+</sup>IL-2<sup>+</sup>TNF-α<sup>+</sup>) producers, as well as TNF-α<sup>+</sup> cells. However, similar to the endogenous response, IFN-γ<sup>+</sup>TNF-α<sup>+</sup> cells were the dominant population and, together with IFN-γ<sup>+</sup> single producers, comprised approximately half of the total ESAT-6–specific population (Fig. 4B, left). In contrast, the cryptic P4 response had a much more diverse phenotype, secreting various combinations of cytokines, dominated by IL-2–producing double-cytokine (IL-2<sup>+</sup>TNF-α<sup>+</sup>) and triple-cytokine (IFN-γ<sup>+</sup>IL-2<sup>+</sup>TNF-α<sup>+</sup>)–producing cells. In this group, IFN-γ<sup>+</sup> and IFN-γ<sup>+</sup>TNF-α<sup>+</sup> subsets accounted for only ∼25% (Fig. 4B, left). Similar analysis 12 wk after *M. tuberculosis* challenge revealed that, in the chronic stages of infection, both the endogenous and the vaccine-primed, P1-specific CD4 T cell populations acquired a dominant IFN-γ<sup>+</sup>TNF-α<sup>+</sup> subset (Fig. 4B, right). In contrast, IFN-γ<sup>+</sup>TNF-α<sup>+</sup> cells comprised <10% of the P4-specific T cells, which remained dominated by IL-2<sup>+</sup>TNF-α<sup>+</sup> and IFN-γ<sup>+</sup>IL-2<sup>+</sup>TNF-α<sup>+</sup> T cells (Fig. 4B, right).

Taken together with the PD-1<sup>hi</sup>/KLRG1<sup>+</sup> expression data (Fig. 3), these data suggest that KLRG1 expression on lung CD4 T cells associates with reduced polyfunctionality. Indeed, in a separate costaining analysis, we found that, compared with their KLRG1<sup>+</sup> counterparts, both vaccine-induced P1- and P4-specific KLRG1<sup>+</sup> cells are enriched for IL-2<sup>+</sup>TNF-α<sup>+</sup>, IFN-γ<sup>+</sup>IL-2<sup>+</sup>TNF-α<sup>+</sup>, and TNF-α<sup>+</sup> populations (Supplemental Fig. 1).

Given that vaccine-induced Th17 responses in *M. tuberculosis* infection can provide enhanced protection (12, 13), IL-17A production was also compared. Lung P1-specific T cells in control and Δ15-E6–vaccinated mice (i.e., endogenous infection-driven responses) were similar, with essentially no IL-17–producing cells post *M. tuberculosis* infection (Fig. 4C). In contrast, vaccine-elicited responses carried an IL-17A component. In ESAT-6–vaccinated mice, P1-specific cells produced IL-17A upon ex vivo restimulation (either alone or in combination with Th1 cytokines; Fig. 4C). Notably, more than half of all P4-specific T cells within
each cytokine subset produced IL-17A upon restimulation (Fig. 4C). This differed from the P1 response in ESAT-6–vaccinated mice, wherein the IFN-γ+TNF-α+ were relatively lacking in IL-17A coproduction (Fig. 4C).

Repeated BCG vaccination post-M. tuberculosis infection has been associated with IL-17–dependent lung neutrophilia and increased pathology (37). However, we found no indications of increased neutrophilia in vaccinated animals, where both groups had a similar decrease in total lung neutrophils and gross pathology versus controls (Supplemental Fig. 2 and data not shown).

Therefore, cryptic epitope–specific T cells maintain a memory-like cytokine profile and Th17 potential of their vaccination imprinting in chronic M. tuberculosis–infected lungs.

Decreased terminal differentiation and enhanced polyfunctionality of cryptic epitope–specific T cells in covaccinated mice

We sought to directly compare dominant and cryptic epitope–specific T cell responses within the same individual infected lungs via covaccination with ESAT-6 and Δ15-E6 before challenge. This strategy was chosen to avoid any indirect influence on T cell quality or differentiation as a result of a different M. tuberculosis growth rate in the ESAT-6–versus Δ15-E6–vaccinated groups. As expected, covaccinated mice had an enhanced P1 T cell response, presence of cryptic epitopes P4- and P6-specific T cells, and significantly reduced lung CFUs 6 wk after aerosol M. tuberculosis challenge, confirming the protective capacity of these cells (Fig. 5A, 5B). Curiously, covaccination resulted in a diminished P4 response that was not sufficient for further characterization by ICS. However, in congruence with results from our single Ag vaccinations, direct comparison of P1- and P6-specific cells within individual lung samples from animals where these vaccine responses coexist confirmed a significantly smaller proportion of KLRG1+ terminally differentiated cells among cryptic epitope–specific T cells during M. tuberculosis infection (Fig. 5C).

Parallel comparison of P1 and P6 CD4 T cell populations within each individual lung sample consistently revealed enhanced proportions of double-cytokine (IL-2+TNF-α+) and triple-cytokine (IFN-γ+IL-2+TNF-α+) positive memory-associated cells and a consistent reduction of infection-associated IFN-γ+ and IFN-γ−

FIGURE 3. Cryptic epitope-specific CD4 T cells expand postinfection and resist infection-driven terminal differentiation. CB6F1 mice were vaccinated three times before aerosol M. tuberculosis challenge. (A) IFN-γ ELISA of lung MNCs from mice vaccinated with CAF01 adjuvant alone (left), ESAT-6 (center), or Δ15-E6 (right) and cultured with ESAT-6 peptides 2 (top), 6 (middle), and 12 wk (bottom) postinfection. (B) Total number of P1- (filled circles) and P4-specific (open circles) CD4+ T cells in mice from (A) as determined by ICS of total CD4+CD44hi MNCs producing IFN-γ, TNF-α, IL-2, or IL-17. Bars represent the mean ± SEM of results of pooled lung MNCs from n = 6 mice. Lung MNCs specific for P1 or P4 by ICS were subgated for KLRG1/PD-1 expression. Representative plots (C) of P1-specific cells from CAF01 alone-vaccinated (top), ESAT-6 (middle), and P4-specific cells from Δ15-E6–vaccinated (bottom) mice are shown. Numbers depict gate frequency from total CD4+CD4+IFN-γ+/IL-17+ cells after P1 or P4 stimulation. (D) Compiled data from three independent experiments are shown. Bar represents mean ± SEM from n = 11 individual/paired samples from 18 mice. *p < 0.05, ***p < 0.001 versus CAF01 unless indicated.
TNFα+ cells within the P6-specific population compared with P1-specific cells (Fig. 5D). Thus, successful covaccination of mice to elicit both P1- and P6-specific T cells revealed that within a given individual, the vaccine-induced cryptic epitope–specific T cells were more resistant to acquisition of KLRG1 terminal differentiation marker expression and maintained greater IL-2–producing capacity associated with a memory-cell cytokine profile.

Cryptic epitope-specific T cells maintain proliferative capacity in the M. tuberculosis–infected lung

CD4 T cell terminal differentiation is often accompanied by decreased survival and decreased proliferation after stimulation. Therefore, we investigated the apoptosis and proliferation of P1- and P4-specific CD4 T cells from ESAT-6, Δ15-E6, or adjuvant control vaccinated mice 6 wk after M. tuberculosis challenge. Combining Annexin V staining with ICS, we found ∼5–10% of all ESAT-6–specific lung CD4 T cells in early apoptosis, with no significant difference between epitope specificity or vaccination groups (data not shown). We next looked at proliferative capacity of ESAT-6–specific lung CD4 T cells in chronic M. tuberculosis–infected animals revealed a reduced number of cell divisions by lung CD4 T cells, suggesting a general refractivity of differentiated effector CD4 T cells homed to the lung (Fig. 6A). P1 and P4 stimulation of lung MNCs from vaccinated mice revealed differences in proliferative potential of dominant and cryptic epitope–specific lung CD4 T cells 6 wk after M. tuberculosis challenge. Lung CD4 T cells isolated from all vaccine groups did not proliferate above background levels after P1 stimulation ex vivo (Fig. 6B). In contrast, cryptic epitope P4-specific CD4 T cells from Δ15-E6–vaccinated mice diluted CFSE label over the 5-d stimulation, indicating their ability to proliferate (Fig. 6B, right panel). A similar pattern of P1- and P4-specific proliferation was observed in the spleens of ESAT-6– and Δ15-E6–vaccinated mice, where there is also a significant bacterial burden 6 wk postaerosol M. tuberculosis infection (data not shown). In agreement with our previous results (Figs. 3, 4), ICS to determine the frequency of peptide-specific lung CD4 T cells at the start of the 5-d stimulation confirmed the presence of P1- and P4-specific T cells (Fig. 6C). Comparing this initial cytokine-based fre-
quency of peptide-specific cells with the “percent divided cells” determined from the CFSE dilution analysis (i.e., the background-corrected percent of cells in the initial CD4 population that divided at least once during 5 d of peptide stimulation), we found that a nearly equal percent of P4-specific cells was detected by both methods (Fig. 6C). In contrast, P1-specific cells were only detected by cytokine secretion, but not by proliferation ex vivo. These data demonstrate further functional differences between dominant and cryptic epitope–specific T cells at the site of \( \text{M. tuberculosis} \) infection consistent with a less differentiated state of cryptic epitope–specific T cells.

**Discussion**

\( \text{M. tuberculosis} \) reactivation and reinfection in humans and animal models highlight the ineffectiveness of the endogenous immune response against \( \text{M. tuberculosis} \) infection and the need for novel vaccine approaches. Moreover, hyperconservation of human T cell epitopes in the \( \text{M. tuberculosis} \) genome suggests that the \( \text{M. tuberculosis} \)–directed T cell response is evolutionarily advantageous to bacterial fitness (5). \( \text{M. tuberculosis} \) appears to actively direct cellular immune responses to induce an optimal granuloma structure for growth and, ultimately, disease immunopathology required for transmission between individuals. Thus, the endogenous bacterial-driven T cell response not only provides incomplete protection, but is likely required for pathogenesis. Therefore, in addition to providing an early response to \( \text{M. tuberculosis} \) infection via a pool of memory T cells, vaccination strategies must also consider modulating T cell function such that it does not ultimately develop to mimic the infection-driven response.

In this article, we report that vaccine induction of T cells against cryptic epitopes of the major \( \text{M. tuberculosis} \) Ag, ESAT-6, although T cells targeting these epitopes are never promoted during the natural infection, provide efficient protection against \( \text{M. tuberculosis} \). The ability to induce cryptic responses was dependent on MHC background. A more diverse MHC created a broader cryptic epitope repertoire, which may be relevant for translation of cryptic epitope vaccination into the diverse human population. After vaccine induction, we compared the functional properties of these cells. Cryptic epitope-specific T cells exhibited delayed onset of cytokine production and increased proliferation, indicating that these subdominant cells somehow may be in a different stage of differentiation (Fig. 2). Although we wished to also compare dominant/cryptic epitope–specific T cell cytokine profiles before \( \text{M. tuberculosis} \) infection, a limited number of P4- and P6-specific T cells detectable via ICS did not allow for an accurate comparison with the dominant epitope–specific population detected, and this was not resolved by increasing the \( \Delta 15\text{-E6} \) vaccine dose. Indeed, the much lower cryptic epitope response was also seen by IFN-\( \gamma \) secretion levels (Fig. 2B, 2D) and may explain why \( \Delta 15\text{-E6} \) was not significantly more protective than ESAT-6 in these studies. Nonetheless, \( \Delta 15\text{-E6} \) vaccine provides at least an equal reduction of CFUs as the ESAT-6 vaccine, which is consistent with an enhanced protective capacity of cryptic epitope T cells at the per-cell level.

**FIGURE 5.** Decreased terminal differentiation of cryptic epitope–specific CD4 T cells in covaccinated animals. CB6F1 mice were vaccinated three times with CAF01-alone or both ESAT-6 and \( \Delta 15\text{-E6} \), and challenged with aerosol \( \text{M. tuberculosis} \) for 6 wk. (A) Epitope mapping of the ESAT-6–specific responses by IFN-\( \gamma \) ELISA of supernatants from lung MNCs from CAF01 (gray bars) and ESAT-6 + \( \Delta 15\text{-E6} \) covaccinated (black bars) mice. Bar represents mean ± SEM of triplicate wells from pooled samples from \( n = 8 \) mice. (B) Total lung CFUs from individual mice in control and immunized groups, 6 wk after \( \text{M. tuberculosis} \) challenge. \(* * * p < 0.001 \) versus CAF01. (C) KLRG1/PD-1 expression on CD4+CD44+ lung MNCs from cells from ESAT-6 + \( \Delta 15\text{-E6} \) covaccinated mice specific for P1 or P6 (IFN-\( \gamma \)+ and/or IL-17+ by ICS) 6 wk after \( \text{M. tuberculosis} \) challenge. Bar represents mean ± SEM of percentage of cells with KLRG1+PD-1+ expression from 12 individual or pairwise-pooled mice from two independent experiments. Connected dots represent paired data points for the same lung sample. \(* * * p < 0.001 \) by paired t-test. (D) Background-subtracted Boolean gating analysis of ICS data, depicting cytokine expression of CD4+ CD44+ cells after P1 (gray bars) or P6 (black bars) restimulation of lung MNCs from ESAT-6 + \( \Delta 15\text{-E6} \) covaccinated mice 6 wk after \( \text{M. tuberculosis} \) challenge. Bar represents mean ± SEM percentage of Ag-specific cells with each cytokine profile from \( n = 8 \) mice pooled in pairs. Connected dots represent paired data points from the same lung sample. Corresponding pie charts for P1 (left) and P6 (right) are inset and significantly different (\( p < 0.0256 \)) by resampling method with 10,000 permutations using SPICE v.5.22.
After *M. tuberculosis* challenge, cryptic epitope–specific cells recognize Ag, expand, and home to the site of infection, similar to dominant epitope–specific cells demonstrating a bona-fide memory recall response to infection (Fig. 3). We first investigated whether these cells could better resist acquisition of bacterial-driven terminal differentiation and exhaustion using surface phe-

![Graphs showing proliferative capacity of CD4 T cells](image)

**FIGURE 6.** Proliferative capacity ESAT-6–specific CD4 T cells from *M. tuberculosis* lungs. CB6F1 mice were vaccinated three times with CAF01 alone or with ESAT-6 or Δ15-E6 and challenged with aerosol *M. tuberculosis* for 6 wk. (A) Live CD4+ cells from splenocytes (left) and lung MNCs (right) from CAF01-vaccinated mice *M. tuberculosis*–infected for 6 wk labeled with CFSE and stimulated with 1 μg/ml Con A (line) or left unstimulated (gray filled) for 5 d, and assessed for CFSE dilution by flow cytometry. (B) Live lung CD4 T cells after stimulation for 5 d with P1 (black line), P4 (dotted line), or vehicle control (gray filled) assessed for CFSE dilution. Representative plots from CAF01–only– (left), ESAT-6– (center), and Δ15-E6– (right)–vaccinated mice are shown. (C) Percent divided CD4+ cells after P1– or P4–specific stimulation based on CFSE dilution (gray bars) for each vaccine group from (B) was compiled and compared with the corresponding percent P1/P4–specific CD4 T cells (black bars) determined at the start of the 5-d proliferation assay by ICS and depicts percent CD4 cells that secrete “any cytokine” (IFN-γ/TNF-α/IL-2). Background values from nonstimulated controls were subtracted from percent divided and percent any cytokine-positive values. Bar represents mean ± SEM from n = 8 mice pooled pairwise. Data are representative of two experiments with similar results.
notype analysis. PD-1 is a B7-family protein expressed on exhausted T cells and is responsible for loss of effective control in chronic infection and cancer (38). In contrast, mice lacking PD-1 are more susceptible to M. tuberculosis infection (39–41). KLRGB expression is a surrogate marker of terminally differentiated, short-lived effector CD8 T cells in inflammation and viral infection models (42). Careful mapping of CD4 T cells in M. tuberculosis-infected murine lungs has shown that PD-1+ cells have a self-renewing capacity, whereas KLRGB PD-1hi cells are terminally differentiated Th1 cells with greater IFN-γ production (34). Therefore, M. tuberculosis infection appears to drive down PD-1 expression and promote pathology. We find that, during M. tuberculosis infection, significantly less cryptic epitope–specific CD4 T cells acquire a KLRGB PD-1hi phenotype, suggesting that these epitope responses somehow escape the strong driving power of the ongoing infection perhaps because of a limited availability of MHC-presented cryptic epitopes.

Although there is not yet a clear definition of what constitutes the optimal T cell response against M. tuberculosis, several studies have associated polyfunctional CD4 T cells, specifically IFN-γ+/IL-2+/TNF-α+ and IL-2+/TNF-α+ populations, with increased bacterial control, perhaps because of their larger memory and self-renewing capacity (19, 35, 43). Indeed, P1-specific T cells of ESAT-6-vaccinated mice reduced lung CFUs and had increased IFN-γ+/IL-2+/TNF-α+ and IL-2+/TNF-α+ cells over controls (Fig. 4) (20). In contrast, IFN-γ+/TNF-α+ and IFN-γ+/IL-2+ (single producers) CD4 T cells that dominate the M. tuberculosis-infected lung are found at more terminal stages of differentiation (35). Interestingly, we found that, after M. tuberculosis challenge, protective cryptic epitope–specific T cells primed by Δ15-E6 vaccination were far more dominated by the memory-associated T cell subsets, compared with the vaccine-induced P1 response in ESAT-6-immunized animals (Figs. 4, 5). These protective subsets were also maintained well into the chronic infection, suggesting they are more refractory to infection-induced differentiation over time. This is supported by the KLRGB PD-1 phenotype of these cells, showing that cryptic epitope–specific CD4 T cells resist infection-driven T cell differentiation (Figs. 3, 5), and our combinatorial analyses showed that KLRGB+ CD4 T cells contained much fewer IFN-γ+/IL-2+/TNF-α+ and IL-2+/TNF-α+ cells (Supplemental Fig. 1). Notably, a recent study in which BCG vaccination was boosted by the H1 (Ag85B+ESAT-6) vaccine showed similar results. Lindenstrom et al. (44) found that enhanced protection was associated with a reduced population of KLRGB M. tuberculosis–specific CD4 T cells in the boosted animals’ lungs, and that these cells showed a reduced capacity to make IL-2.

M. tuberculosis–specific Th17 cells have also been found to confer protection against infection, where early IL-17 recruitment of Th1 cells, as well as IFN-γ–independent mechanisms have been proposed (12, 13). Conversely, pathologic Th17 responses can be induced over time by repeated BCG vaccination of M. tuberculosis–infected mice (37). Ultimately, the chronic/latent nature of TB most likely requires a regulated Th17 response to maintain optimal control of the infection. Indeed, innate and adaptive sources of IFN-γ regulate IL-17–mediated protection and pathology in vivo (45, 46). We found that more than half of cryptic epitope–specific T cells expressed IL-17, often in conjunction with IFN-γ+/TNF-α+/IL-2-. Importantly, this was not associated with any increase in neutrophilia or lung pathology. IFN-γ+/IL-17+ coexpressing cells are not well studied; however, in Chlamydia muridarum infection, IFN-γ+ and IL-17+ synergize to upregulate inducible NO synthase, and IFN-γ+/IL-17+ coexpressing T cells have been associated with vaccine-induced protection (47, 48). In particular, Th1/Th17 plasticity of these cells in an inflammatory environment may be advantageous to maintaining a flexible T cell response capable of maintaining bacterial control without driving lung pathology and bacterial transmission in vivo.

KLRGB expression on both CD4 and CD8 T cells is associated with reduced proliferation in mice and humans (34, 49, 50), whereas IL-2 production is associated with increased autocrine proliferation in M. tuberculosis–challenged mice (44). Therefore, we hypothesized that cryptic epitope–specific T cells would have increased proliferative capacity during M. tuberculosis infection. Investigation of lung CD4 T cell proliferative capacity revealed that the P4-specific CD4 T cells were uniquely able to divide in an ex vivo proliferation assay. In contrast, P1-specific proliferation was not detected above background in any group, despite confirmation of the presence of P1-specific CD4 T cells and nonspecific CD4 T cell proliferation by Con A in all samples (Fig. 6C and data not shown). This was somewhat surprising in ESAT-6–vaccinated mice, in particular, where the P1-specific CD4 T cell population contained less terminally differentiated cells. One possible explanation is that an early robust IFN-γ production by activated IFN-γ+ and IFN-γ+TNF-α+ populations actively inhibited the CD4 T cell proliferation in vitro (51). In any case, the specific proliferation of the P4-specific cells is consistent with their very low KLRGB expression and cytokine production profiles, and further supports they are maintained as a population of less differentiated M. tuberculosis–specific T cells at the site of infection.

Importantly, because the lung CFUs were at similar levels in ESAT-6– and Δ15-E6–vaccinated groups in these studies, the CD4 T cell functional/phenotypic differences between dominant and cryptic epitope–specific T cells were inherent and not merely the result of a difference in bacterial burden/disease state. This was even more specifically shown by covaccination with ESAT-6 + Δ15-E6, where the same decrease in terminal differentiation and memory-associated cytokine production that was observed between vaccine-induced cryptic and dominant epitope–specific T cells was seen between T cell clones coexisting within the same animal (Fig. 5).

Our data support a preferential retention of polyfunctionality, proliferative capacity, and resistance to terminal differentiation by a protective population of cryptic epitope–specific T cells during M. tuberculosis infection. Alternatively, because P4- and P6-specific T cells are not primed by infection, we cannot exclude that their relatively improved T cell phenotype over P1 may be the result of the dilution of the P1-specific T cell population by T cells primed by M. tuberculosis over the course of infection (52). In this scenario, terminal effector differentiation can be seen as a hallmark of T cell imprinting of infection-primed naive T cells. Therefore, characterization of cryptic epitope–specific T cells allows specific monitoring of the vaccine response to infection and reveals that vaccine-primed CD4 T cells, per se, resist the bacterial-driven phenotype while responding to and controlling infection. Furthermore, in contrast with P1, all cryptic epitopes presented by APCs in vivo are engaged by the protective vaccine-primed cells and not diluted over time by M. tuberculosis–induced effectors, offering a unique and durable method to counteract M. tuberculosis–driven immune inadequacy.

Although the mouse model of TB cannot recapitulate all aspects of human disease, it has been an invaluable tool for understanding the cellular immune responses of M. tuberculosis infection. In this study, we use the mouse model of TB to address the realization that the M. tuberculosis–induced T cell response is important in both T cell protection and immunopathology. We find that cryptic epitope–specific T cells are more refractory to infection-mediated functional changes, and constitute a novel and unique vaccine
target that may add both longevity and the ability to modulate disease to conventional vaccines programs. Cryptic epitope–specific T cells can enhance the initial control of infection and maintain a less differentiated state during chronicity. In turn, enhanced maintenance of an effective pool of memory cells at the site of infection could enhance the response to repeated exposure to *M. tuberculosis* that is common to people in endemic regions. In addition, induction of cryptic epitope–specific T cell responses may be useful in reprogramming the T cell responses of latently infected individuals to reduce reactivation disease and contain transmission within the human population. Identifying and using cryptic epitopes in vaccines for a genetically heterogeneous human population is obviously challenging, but hopefully rationally engineered vaccine constructs that expose cryptic epitopes will in the future allow the clinical evaluation of this concept.

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### Disclosures

P.A. is a coinventor of patents relating to cationic liposomes as adjuvants and ESAT-6 as an Ag in vaccines. All rights have been assigned to the Statens Serum Institut. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial benefit from the subject matter or materials discussed in this article.

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