CD8+ T cells are essential for host defense to intracellular bacterial pathogens such as Mycobacterium tuberculosis (Mtb), Salmonella species, and Listeria monocytogenes, yet the repertoire and dominance pattern of human CD8+ antigens for these pathogens remains poorly characterized. Tuberculosis (TB), the disease caused by Mtb infection, remains one of the leading causes of infectious morbidity and mortality worldwide and is the most frequent opportunistic infection in individuals with HIV/AIDS [2]. Therefore, we undertook this study to define immunodominant CD8 Mtb antigens. First, using IFN-γ ELISPOT and synthetic peptide arrays as a source of antigen, we measured ex vivo frequencies of CD8+ T cells recognizing known immunodominant CD4+ T cell antigens in persons with latent tuberculosis infection. In addition, limiting dilution was used to generate panels of Mtb-specific T cell clones. Using the peptide arrays, we identified the antigenic specificity of the majority of T cell clones, defining several new epitopes. In all cases, peptide representing the minimal epitope bound to the major histocompatibility complex (MHC)-restricting allele with high affinity, and in all but one case the restricting allele was an HLA-B allele. Furthermore, individuals from whom the T cell clone was isolated harbored high ex vivo frequency CD8+ T cell responses specific for the epitope, and in individuals tested, the epitope represented the single immunodominant response within the CD8 antigen. We conclude that Mtb-specific CD8+ T cells are found in high frequency in infected individuals and are restricted predominantly by HLA-B alleles, and that synthetic peptide arrays can be used to define epitope specificities without prior bias as to MHC binding affinity. These findings provide an improved understanding of immunodominance in humans and may contribute to a development of an effective TB vaccine and improved immunodiagnostics.

Introduction

Infection with Mycobacterium tuberculosis (Mtb) remains an important cause of infectious disease, morbidity, and mortality worldwide [1] and has emerged as a major opportunistic infection in individuals with HIV/AIDS [2]. Control of infection with Mtb relies heavily on the cellular immune system, that is, the interaction of lymphocytes and Mtb-infected macrophages and dendritic cells (DCs) [3,4]. CD8+ T cells are associated with strong CD4+ T cell responses, and are not only essential for effective immunity to viral pathogens, but also for immunity to some intracellular bacteria, such as Listeria monocytogenes and Salmonella species [5]. Increasing experimental evidence in the mouse tuberculosis (TB) model has suggested a protective role for CD8+ T cells in the host response. For example, adoptive transfer or in vivo depletion of CD8+ cells showed that this subset could confer protection against subsequent challenge [6–8]. β2-microglobulin-deficient mice, deficient in expression of major histocompatibility complex (MHC) class I, are more susceptible to Mtb [9] and to large doses of Bacille Calmette Guérin [10] infection than their wild-type littermates. This finding has been corroborated in CD8-deficient mice [11] and other mice deficient in class I processing and presentation [11–13]. However, mice lacking class Ia-restricted CD8+ T cells demonstrate more moderate susceptibility to Mtb infection [14,15]. In humans, Mtb-specific CD8+ T cells have been identified in Mtb-infected individuals and include CD8+ T cells that are classically, MHC-Ia, restricted [16–22], and non-classically, MHC-Ih, restricted by HLA-E [18,23], and by CD1 [24–26]. Taken together, studies of mice and humans support an important role for CD8+ T cells in TB immunity.

For most infections, the repertoire of the CD8 response is shaped by the entry of antigen into the MHC-I processing pathway, binding of peptides and/or non-peptide antigens to MHC-I molecules, and recognition of these structures by T cells. Ultimately, a relatively limited subset of pathogen-specific T cells emerge, a process that has been termed immunodominance [27]. While substantial effort has focused on defining immunodominant CD8 antigens for important human viral pathogens such as HIV and cytomegalovirus (CMV), little is known about the antigens recognized by

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Abbreviations: APC, antigen-presenting cell; CMV, cytomegalovirus; DC, dendritic cell; LCL, lymphoblastoid cell line; LTBI, latent tuberculosis infection; MHC, major histocompatibility complex; Mtb, Mycobacterium tuberculosis; PBMC, peripheral blood mononuclear cell; TB, tuberculosis

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CD8+ T cells are essential for host defense to intracellular bacterial pathogens such as Mycobacterium tuberculosis (Mtb), Salmonella species, and Listeria monocytogenes, yet little is known about the antigens recognized by human CD8+ T cells in response to tuberculosis (TB). TB, the disease caused by Mtb infection, remains one of the leading causes of illness and death worldwide and is a frequent complicating infection in individuals with HIV/AIDS. Therefore, we undertook this study to define commonly recognized CD8 Mtb antigens. First, we measured the frequencies of CD8+ T cells recognizing Mtb antigens known to be recognized by CD4+ T cell antigens in persons infected with Mtb. In addition, we identified the Mtb antigen and epitope recognized by several CD8+ T cell clones isolated from infected individuals. The epitope was presented to the T cell clones by an HLA-B allele in all but one case. We conclude that Mtb-specific CD8+ T cells are found in high frequency in infected individuals and are restricted predominantly by HLA-B alleles. These findings provide an improved understanding of how the human immune system recognizes intracellular pathogens and may contribute to the development of an effective TB vaccine and improved immunodiagnostics.

**Author Summary**

CD8+ T cells are essential for host defense to intracellular bacterial infections. Furthermore, although a number of commonly recognized CD4 Mtb antigens have been described [28,29] (ESAT-6, CFP10, Ag85, etc.), surprisingly little is known about common Mtb antigens recognized by human CD8+ T cells. The majority of CD8+ epitopes that have been identified were defined by testing of Mtb peptides selected for high-affinity binding to MHC class Ia molecules (HLA-A2 in most cases; [19,20,30–34]). In almost all of these examples, however, the ex vivo frequency of these T cells in Mtb-infected individuals is low or undetectable, suggesting that these specificities may not represent immunodominant responses. In contrast, in the limited cases in which T cells have been used to define epitopes contained in selected Mtb antigens, high ex vivo frequencies have been demonstrated [17,35], suggesting that a T cell–centered approach can identify immunodominant epitopes. Moreover, CD8+ T cell responses to some Mtb antigens that represent good CD4 antigens (CFP10, ESAT-6, Ag85, and Mtb39) have been detected at high frequency in persons infected with Mtb [17–19,34]. Therefore, we used a limited library of overlapping synthetic peptides representing several known CD4 Mtb antigens to determine the magnitude of the CD8 response to these antigens in persons with active TB and latent TB infection (LTBI), as well as uninfected individuals. Furthermore, we utilized a panel of Mtb-specific CD8+ T cell clones to define minimal epitopes recognized within these antigens and determined the contribution of these novel epitopes to the ex vivo Mtb-specific CD8 response.

**Results**

**Definition of Immunodominant Mtb-Specific CD8 Antigens**

To define immunodominant Mtb-specific CD8 antigens, and to determine whether or not these responses result from infection with Mtb, we have used CD8+ T cells from uninfected donors, those with LTBI, or those actively infected with Mtb. Responses were determined either directly ex vivo, or using CD8+ T cell clones obtained by limiting dilution cloning on Mtb-infected autologous DCs [36]. As much is known about dominant CD4 Mtb antigens, a panel of these commonly recognized antigens was selected for further evaluation. These were Mtb39, CFP10, Mtb84, Mtb9.9A, ESAT-6, Ag85b, 19KDa, and EsxG. To avoid bias introduced by using peptides of predicted HLA binding specificity, we synthesized overlapping peptides (15 aa, overlap 11 aa) to represent the proteins of interest [17].

To accurately determine the ex vivo effector cell frequencies of CD8+ T cells, linear regression analysis was used. As shown in Figure 1, using D466 as an example, magnetic bead–purified CD8+ T cells were tested against peptide-pulsed DCs over a range of CD8+ T cell numbers in an IFN-γ ELISPOT assay. A positive assay was determined as described below and if positive, the antigen-specific frequency was determined using linear regression.

Uninfected individuals (n = 14), those with LTBI (n = 20), and those with active TB (n = 12) were evaluated for CD8 responses to a panel of Mtb CD4+ T cell antigens, as well as to

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**Figure 1.** Determination of Human Effector Cell Frequencies Ex Vivo Using the IFN-γ ELISPOT Assay

Magnetic bead–purified CD8+ T cells from a single donor (D466) were cultured with DCs (20,000/well) either infected with Mtb (H37Rv, multiplicity of infection = 50) or pulsed with peptide pool representing CFP10 (5 μg/ml each peptide; 15-mer overlap by 11 aa) in an IFN-γ ELISPOT assay. Each responding T cell population was tested in duplicate at four different cell concentrations. To determine the effector cell frequency of antigen-specific T cells, the average number of spot forming units per well for each duplicate was plotted against the number of responder cells per well. Linear regression analysis was used to determine the slope of the line, which represents the frequency of antigen-specific T cells. The assay was considered positive, reflecting the presence of a primed T cell response, if the binomial probability for the number of spots was significantly different by experimental and control assays, i.e., if the experimental line is statistically significantly different from the control line. The frequency of Mtb-specific and CFP10-specific T cells demonstrated in this figure was 1/307 and 1/1676, respectively.

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Mtbc-infected DCs. All individuals tested had robust CD8+ T cell responses to Mtbc-infected DCs and were of greater magnitude in individuals with active TB than in those with LTBI (p = 0.01; Figure 2; Table 1). However, CD8+ T cell responses to the panel of Mtbc antigens were found almost exclusively in those infected with Mtbc in that statistically significant differences between uninfected and Mtbc-infected individuals were noted for seven of ten antigens for both the magnitude of the response (Figure 2) and the proportion of positive assays (Table 1). However, differences in CD8+ T cell responses between individuals with active TB and LTBI were not statistically different. While strong CD8+ T cell responses were observed against many of the antigens tested, it is equally notable that several individuals with strong Mtbc-directed CD8+ T cell responses did not have demonstrable responses to many of the antigens tested (unpublished data).

Definition of the Minimal Epitope Recognized by Mtbc-Specific CD8+ T Cell Clones

These ex vivo frequency data demonstrated the presence of high-frequency responses to a number of known Mtbc antigens, but did not shed light on the restricting allele, minimal epitope, or dominance hierarchy within the gene of interest. To address this question, we performed limiting dilution cloning of human CD8+ T cells using Mtbc-infected DCs [36], and generated panels of both classically and non-classically HLA-restricted CD8+ T cell clones. Using peptide pools representing known CD4 antigens, the antigenic specificity of the HLA-Ia-restricted clones was defined in detail for a single representative clone, D466T, derived from an individual with active TB. As shown in Figure 3A, testing the clone against autologous DCs pulsed with a panel of peptide pools unambiguously defined the antigenic specificity of CFP10. The clone was then tested with a panel of peptide pools representing known Mtbc CD4 antigens, and was found to be specific for CFP10. The clone was then tested with a panel of peptide pools representing known Mtbc CD4 antigens, and was found to be specific for CFP10.
B4501 and C1601, presented the epitope to the clone, identifying both B4501 and C1601 as possible restricting alleles. However, C1601\textsuperscript{+} D433 LCL did not present the epitope, eliminating C1601 as a candidate-restricting allele. Therefore, D466 D6 was restricted by HLA-B4501. As demonstrated in Figure 4, by testing each plausible epitope over a broad range of concentrations, the minimal epitope was defined as CFP102–12 for D466 D6. Experimental data supporting the assignment of the minimal epitope is provided for each clone in Figure 5, and a summary of the antigenic specificity, minimal epitope, and HLA-restricting allele is presented in Table 3. Unexpectedly, all but one of the T cell clones were restricted by HLA-B alleles. Furthermore, a minority of those observed were 9 aa in length.

Immunodominance of MTb CD8 Epitopes in Infected Individuals

Because each of the individual CD8\textsuperscript{+} T cell clones was derived based on growth in the presence of Mtb-infected DCs, we sought to determine whether or not the antigen and epitopes identified reflected immunodominant epitopes ex vivo. Two independent approaches were pursued, the first to determine if the response was present at high frequency, and the second to determine what proportion of the total response to the antigen was constituted by the epitope. To determine the ex vivo effector cell frequency, as described in Figure 1, each epitope was tested using autologous DCs and magnetic bead–purified CD8\textsuperscript{+} T cells derived from the donor from whom the T cell clones was isolated. A summary of the ex vivo epitope-specific effector cell frequencies is presented in Table 3. For comparison, effector cell frequencies using autologous DCs infected with Mtb as the antigen-presenting cells (APCs) are shown as well. For 11 CD8\textsuperscript{+} T cell clones recognizing distinct epitopes, the epitope-specific frequency exceeded 50% of the total Mtb-specific CD8\textsuperscript{+} T cell response. For six of these clones, the epitope-specific frequency actually exceeded the total frequency of Mtb-specific CD8\textsuperscript{+} T cells. Conversely, for two clones, the epitope-specific T cell frequency constituted the minority of the total Mtb-specific CD8\textsuperscript{+} T cell response. Thus, overall, the epitopes reflected high-frequency responses, and could be considered a response that has been primed by exposure to Mtb. Notably, T cell clones isolated from four donors recognized CFP10. To determine if the epitopes defined reflected a substantial

### Table 1. CD8\textsuperscript{+} T Cell Responses to Known TB Antigens

| Antigen         | Mtb-Infected Individuals | Mtb-Uninfected Individuals | p-Value (2-Tail Fisher’s) |
|-----------------|-------------------------|-----------------------------|--------------------------|
|                 | Number Positive\textsuperscript{a} / Number Tested (%) | Number Positive\textsuperscript{a} / Number Tested (%) |                          |
| Mtb DC          | 17/17 (100)             | 11/11 (100)                 |                          |
| Mtb39 Pool A    | 13/30 (43)              | 0/14 (0)                    | 0.003                    |
| Mtb39 Pool B    | 10/30 (33)              | 0/14 (0)                    | 0.01                     |
| CFP10           | 14/30 (47)              | 1/14 (7)                    | 0.02                     |
| Mtb8.4          | 13/30 (43)              | 0/14 (0)                    | 0.003                    |
| Mtb9.9A         | 10/25 (40)              | 1/14 (7)                    | 0.06                     |
| ESAT 8          | 12/25 (48)              | 0/14 (0)                    | 0.003                    |
| Ag85b Pool A    | 5/22 (23)               | 0/14 (0)                    | 0.37                     |
| Ag85b Pool B    | 4/22 (18)               | 0/14 (0)                    | 0.14                     |
| 19kd            | 6/22 (27)               | 1/12 (8)                    | 0.38                     |
| EsxG            | 9/22 (41)               | 0/14 (0)                    | 0.006                    |

\textsuperscript{a}Positive assay defined in text.

### Table 2. Many CD8\textsuperscript{+} T Cell Clones Recognize Known CD4 Antigens

| Donor | TB Status | HLA-Ia Antigens (Number)\textsuperscript{a} | Antigen Identified (Number)\textsuperscript{b} | Number Distinct Antigens\textsuperscript{c} | Number Distinct Epitopes\textsuperscript{d} |
|-------|-----------|-------------------------------------------|---------------------------------------------|--------------------------------|--------------------------------|
| D431  | Active TB | 1                                         | 0                                          | 0                                            | 0                                             |
| D432  | Active TB | 14                                        | 4                                          | 2                                            | 2                                             |
| D466  | Active TB | 11                                        | 10                                         | 1                                            | 2                                             |
| D571  | Active TB | 7                                         | 7                                          | 1                                            | 1                                             |
| D480  | Active TB | 6                                         | 6                                          | 1                                            | 1                                             |
| D481  | Active TB | 11                                        | 11                                         | 1                                            | 1                                             |
| D426  | LTBI      | 1                                         | 0                                          | 0                                            | 0                                             |
| D443  | LTBI      | 1                                         | 1                                          | 1                                            | 1                                             |
| D454  | LTBI      | 2                                         | 2                                          | 2                                            | 2                                             |
| D504  | LTBI      | 7                                         | 7                                          | 1                                            | 1                                             |
| Totals|           | 61                                        | 42                                         | 10                                           | 11                                            |

\textsuperscript{a}Number of clones derived from donor.

\textsuperscript{b}Number of clones for which cognate antigen was identified.

\textsuperscript{c}Total number of distinct antigens identified from the clone set.

\textsuperscript{d}Total number of distinct epitopes identified from the clone set.

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proportion of the total response to the antigen of interest, magnetic bead–purified CD8\textsuperscript{+} T cells from three donors with sufficient available peripheral blood mononuclear cells (PBMCs) were tested for reactivity to each individual 15-mer peptide, the peptide pool, and peptide representing the minimal epitope. As is demonstrated in Figure 6, the ex vivo frequencies to the minimal epitope, 15-mer peptide(s) containing the minimal epitope, and peptide pool were remarkably concordant. These data, then, suggest that for each donor a dominance hierarchy has been clearly established, and was reflected in the original clones. Finally, as is noted in Table 3, daughter clones of identical specificity were frequently identified, a result that would be predicted based on an immundominance hierarchy. TCR V beta staining was used to confirm the clonal relationship between daughter clones. Interestingly, in two cases, the identical minimal epitope and HLA restriction was represented by two distinct clones (Table 3).

**HLA Binding Affinity to CD8\textsuperscript{+} T Cell Epitopes**

Because much work on human CD8\textsuperscript{+} T cell responses to Mtb has relied upon the use of HLA prediction algorithms, as each epitope was defined we asked whether or not the epitopes would have been predicted by these approaches. Given the prevalence of HLA-B alleles and 10-mer and 11-mer epitopes, it is perhaps not surprising that many of these epitopes were not ranked strongly (unpublished data). This left open the possibility that either HLA binding was indeed not predictive of antigenicity, or simply highlighted the limitations of those algorithms at the time they were used. To address this question experimentally, the IC\textsubscript{50} for each peptide that had been synthesized in the course of definition of the minimal epitope was determined against a panel of human HLA molecules (Table S1). Shown in Table 3 is the IC\textsubscript{50} for the minimal epitope with the cognate restricting allele. These data demonstrate that the T cell epitopes bound avidly to HLA, and show a high degree of concordance between the T cell epitope data and HLA binding data (Figure 5; Table S1).

**Discussion**

Although the complete repertoire of CD8\textsuperscript{+} T cell responses in Mtb remains incompletely characterized, the following conclusions can be drawn. First, CD8\textsuperscript{+} T cell responses are
present in persons infected with Mtb at frequencies that are comparable to that seen following many common viral infections such as vaccinia, influenza, and CMV [37,38]. This conclusion is based both on the pooled peptide experiments described above, and on the observation that when defined, dominant epitopes are present at high \textit{ex vivo} frequencies. Conversely, we have not observed high-frequency responses to CD4$^+$ T cell antigens in those without evidence of infection

Table 3. Summary of CD8$^+$ T Cell Epitopes Identified

| Donor | Mtb-Specific T Cells | Clone$^b$ | Gene$^c$ | HLA-Restricting Allele | Epitope Location | Epitope Sequence | Epitope-Specific T Cells$^a$ | MHC Binding Affinity (IC$_{50}$ nm) | V Beta Region |
|-------|----------------------|----------|---------|------------------------|----------------|----------------|-----------------------------|----------------------------------|-------------|
| 160   | 61$^d$               | D160 1–18 (0) | CFP10  | B44   | 2–11 | AEMKTDAATL | 360 | 38 |
| 160   | 61$^d$               | D160 1–6 (0) | CFP10  | B14   | 85–94 | RADEEOQDAL | 120 | NA |
| 432   | 216                  | D432 H12 (1) | CFP10  | B3514 | 49–58 | TAAQAADVRF | 258 | 2.011$^e$ | 5.3 |
| 466   | 473                  | D466 A10 (9) | CFP10  | B4501 | 2–9  | AEMKTDAATL | 2,458 | 48 | IND |
| 466   | 814                  | D466 D6 (0) | CFP10  | B4501 | 2–12 | AEMKTDAATL | 1,993$^d$ | 6.2 | 22 |
| 481   | 405                  | D481 C10 (9) | CFP10  | B1502 | 75–83 | NIIQAQVQY | 1,715 | 14$^g$ | 9 |
| 481   | 405                  | D481 C11 (0) | CFP10  | B1502 | 75–83 | NIIQAQVQY | 1,715 | 14$^g$ | 13.6 |
| 480   | 598                  | D480 F6 (5) | CFP10  | B0801 | 3–11 | EEMKTDAATL | 387 | 79 | 13.1 |
| 571   | 49                   | D571 B12 (2) | CFP10  | B4402 | 2–11 | AEMKTDAATL | 31 | 38 | IND |
| 571   | 49                   | D571 E9 (3) | CFP10  | B4402 | 2–11 | AEMKTDAATL | 31 | 38 | 14 |
| 504   | 126                  | D504 E4 (0) | EssG   | A0201 | 3–11 | LLDAHIPOL | 72 | 0.39 | 8 |
| 454   | 138                  | D454 B10 (0) | EssG   | B0801 | 53–61 | AAHMFVAA | 88 | 0.22 | IND |
| 454   | 138                  | D454 H1–2 (0) | Mtb8.4 | B1501 | 33–43 | AVINTCNQYQ | 24 | 10 | 7.1 |
| 432   | 216                  | D432 A3 (1) | Mtb8.4 | B3514 | 61–69 | ASPVQQSYL | 210 | 127$^f$ | 14 |
| 443   | 53                   | D443 H9 (0) | AgB8B  | B4102 | 144–153 | ELPQWLSNKR | <10 | NA | 22 |

\textit{a}IFN-$\gamma$ spot forming units per 250,000 CD8$^+$ T cells.

\textit{b}Number of sister clones is in parentheses.

\textit{c}TubercuList accession numbers are CFP10 (Rv3874) and EssG (Rv0287).

\textit{d}Published previously in [17].

\textit{e}Measured binding affinity to B3501 is shown.

\textit{f}Given that D466A10 also recognized this longer peptide, this \textit{ex vivo} frequency may reflect the response to the shorter peptide CFP102–9 as well.

\textit{g}Measured binding affinity to B1501 is shown.

IND, indeterminate; NA, not available; TBD, to be done.

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with Mtb. This observation strongly supports the hypothesis that these responses reflect an adaptively acquired response to infection with Mtb, rather than an innate response. By contrast, CD8$^+$ T cell responses to Mtb-infected DCs were equivalent in infected and uninfected individuals. Using limiting dilution analysis, we have studied the proportions of Ia- versus Ib-restricted CD8$^+$ T cell responses in Mtb-infected compared to uninfected individuals and have noted that Ib-restricted CD8$^+$ T cell responses predominate in uninfected individuals (D. Lewinsohn, unpublished data). Therefore, we speculate that the response detected to Mtb-infected DCs in uninfected individuals may reflect chiefly an Ib-restricted response. While we did not observe an association of responses to specific antigens with disease status, a larger study might reveal more subtle differences.

All but one of the epitopes that have been mapped to date are restricted by HLA-B molecules. The reasons for this possible skewing are not yet clear. It is a formal possibility that the cloning methods we used biased isolation of HLA-B-over HLA-A-restricted T cell clones. However, using identical cloning methodology, we have isolated T cell clones specific for vaccinia, CMV, and influenza that did not display a preference for HLA-B restriction (D. Lewinsohn, unpublished data). Furthermore, in all three cases where we tested ex vivo the entire set of peptides representing CFP10, the entire response mapped to the HLA-B-restricted epitope (Figure 6). Therefore, we speculate that Mtb antigens may preferentially bind to HLA-B molecules, that Mtb preferentially interferes with HLA-A processing and presentation, that infection with Mtb leads to selective upregulation of HLA-B, or that HLA-B is preferentially delivered to the Mtb phagosome. Nonetheless, these data are consistent with those reported by Kiepiela et al. [39], in which HIV-specific T cell responses were found to be 2.5-fold more likely to be HLA-B- than HLA-A-restricted, and observed that viral load was more closely linked to HLA-B than HLA-A alleles. Human infection with mycobacteria has been demonstrated in pre-urban Egypt [40] and in pre-Columbian Peru [41], observations consistent with the hypothesis that mycobacterial infection has driven the diversity and peptide-binding repertoire of the HLA-B locus. Furthermore, our data is consistent with the hypothesis that the diversity of HLA-B alleles is related to the containment of both viral and bacterial pathogens. Hence, delineation of immunodominant epitopes and antigens within the context of important human pathogens will likely require careful evaluation of those epitopes presented by HLA-B.

While the immune response within an individual to a given antigen is narrowly focused, dominant epitopes that would be useful for population-based analysis have yet to be defined. This conclusion is based on the fact that few of the HLA-A2 epitopes described to date have proved to be widely recognized, and, most importantly, on the observation that a wide variety of HLA alleles appear to be used in the recognition of Mtb antigens. The antigen CFP10 is an excellent case in point. As is demonstrated in Table 3, T cell clones have been used to define high-frequency epitopes restricted by a variety of HLA alleles. While the N-terminal 15 aa could reasonably be considered immunogenic, in all but one case (CFP102-11; HLA-B44) the minimal epitope defined has been unique to the individual from whom the T cells were derived.

By using a T cell–driven approach to epitope identification, it is possible to define dominant epitopes in humans infected with Mtb. While it is striking that for several of the T cell clones the ex vivo frequency of epitope-specific T cells was equal to or exceeded the total ex vivo frequency of Mtb-

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**Figure 6. Profiling of Immunodominance Pattern for CFP10**

To determine the effector cell frequencies, autologous DCs (20,000/well) were pulsed either with each individual 15-mer peptide (5 μg/ml), the peptide pool (PP; 5 μg/each peptide), or the minimal epitope (ME) determined from T cell clones derived from each donor (D466:CFP102-11; D480:CFP103-11; D481:CFP1075-83; 5 μg/ml), and tested against 250,000 magnetic bead–purified CD8$^+$ T cells. IFN-γ release was assessed by ELISPOT after 18 h of co-culture. Each point represents the mean of duplicate determinations.

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specific T cells, we acknowledge that the use of peptide-loaded DCs to determine T cell frequency likely overrepresents the proportion of the total Mtb-specific CD8\(^+\) T cell responses. Possible reasons are that Mtb infection is likely to produce relatively less cognate peptide compared to peptide loading, and that Mtb infection could possibly interfere with class I processing and presentation. Although the current observations are limited to a small panel of known CD4 antigens, current work is underway to perform a genome-wide survey of dominant CD8 antigens in Mtb. Definition of this panel will likely prove useful in the further study of the natural history of infection with Mtb and in the design of novel vaccines and diagnostics.

In TB, evaluation of epitopes based strictly on HLA binding algorithms has focused on HLA-A2, and has often failed to define dominant epitopes. Our observation that many of the epitopes are HLA-B restricted, and often longer than 9 aa, for which these algorithms are less robust, may help explain these findings. However, when evaluated experimentally, all of the minimal epitopes exhibit high-affinity binding to the cognate-restricting allele. In this regard, substantial progress in HLA prediction algorithms is evident \([42,43]\), and could facilitate more efficient identification of dominant epitopes. Here, more information with regard to longer peptides will be of benefit. Finally, we speculate that these caveats are likely to apply to other intracellular pathogens as well.

One limitation to current knowledge is that the responses in humans have been made at a single time point. In this regard, a feature of Mtb is the chronic exposure to antigen that may persist for many years. How this chronic infection influences the shaping of the immune response and dominance repertoire is an important question that remains unresolved. The advent of new reagents for immunologic studies in the mouse model \([44]\) will likely be useful in this regard. For example, chronic antigenic exposure seems likely to alter the affinity of the T cell response over time. Furthermore, it is possible that such long-term infection might lead to clonal exhaustion or dysfunction as has been described for chronic viral infection \([38]\). Finally, given the very high-frequency responses that we and others have observed to Mtb-infected DCs and to single antigens, it appears that the immune response to Mtb occupies a sizable fraction of the host’s immunological activity, similar to that previously observed for infection with CMV \([45]\). If so, then this may have important implications for the aging immune system, and potentially for the requirements for the long-term containment of intracellular infection. For example, it is possible that threshold Mtb-specific CD8 frequencies are necessary for the maintenance of a state of chronic persistence. Conversely, the substantial immunological effort directed at Mtb may limit the host’s ability to effectively combat novel infections. As a result, this static picture leaves open important questions as to the evolution of the Mtb-specific responses, and its relationship with chronic infection with Mtb.

It seems likely that Mtb has evolved potent mechanisms to modulate the immune response. At present, specific mechanisms for MHC-I immune modulation have not been described. However, it appears that TLR II stimulation via the Mtb-derived 19-kDa lipoprotein can modulate both MHC-I and MHC-II antigen processing, and can interfere with IFN-\(\gamma\) signaling \([46–49]\). Further work on the T cell subsets important in Mtb, including the immunodominant epitopes, will extend our understanding of the immunology of TB and potentially contribute to the development of a vaccine against this major killer.

How the Mtb-specific CD8\(^+\) T cell response fits into the natural history of infection with Mtb remains poorly characterized. For example, the ontogeny of the CD8\(^+\) T cell response relative to infection with Mtb remains unknown, as does the relationship of CD8\(^+\) T cell frequencies with regard to bacterial burden. However, by defining commonly recognized CD8\(^+\) T cell antigens and epitopes, it will become increasingly possible to employ direct ex vivo analysis to more precisely define Mtb-specific T cell responses in various subject groups of particular interest.
for γδ T cell receptor expression and CD8 expression by FACS and expanded as described below. V beta usage was determined using the IOTest Beta Mark Kit from Beckman Coulter, catalog #IM3497 (http://www.beckmancoulter.com).

**Expansion of T cell clones.** To expand the CD8⁺ T cell clones, a rapid expansion protocol using anti-CD3 mAb stimulation was used as described previously [18].

**Generation and infection of peripheral blood DCs.** Monocyte-derived DCs were prepared according to a modified method of Romani et al. [18,59]. To generate Mtbi-infected DCs, cells (1 x 10⁷) were cultured overnight in the presence of Mtbi at a multiplicity of infection = 50:1. We have determined that this multiplicity of infection is optimal for detection of Mtbi-specific CD8⁺ T cells, as heavy infection is required to optimize entry of antigen into the class I processing pathway [60]. After 18 h, the cells were harvested and reseeded in R10% human serum.

**MHC binding assays.** The MHC peptide binding assay utilized measures the ability of peptide ligands to inhibit the binding of a radiolabeled peptide to purified MHC molecules, and has been described in detail elsewhere [61]. Briefly, purified MHC molecules, test peptides, and a radiolabeled probe peptide are incubated at room temperature in the presence of human β2-microglobulin and a cocktail of protease inhibitors. After a 2-d incubation, binding of the radiolabeled peptide to the corresponding MHC class I molecule is determined by capturing MHC/peptide complexes on W6/32 antibody (anti-H-2 B, C, or anti-H-2 A) coated plates, and measuring bound cpm using a microscintillation counter. For competition assays, peptide yielding 50% inhibition of the binding of the radiolabeled peptide is calculated. Peptides are typically tested at six different concentrations covering a 100,000-fold dose range, and in three or more independent assays.

Under the conditions utilized, where [label] < [MHC] and IC₅₀ > [MHC], the measured IC₅₀ values are reasonable approximations of the true Kd values.

**IFN-γ ELISPOT assay.** The IFN-γ ELISPOT assay was performed as described previously [18]. For determination of ex vivo frequencies of CD8⁺ T cells responding to Mtbi infection or Mtbi antigens, CD8⁺ T cells were positively selected from PBMCs by using magnetic beads described previously [18]. For determination of IFN-γ ELISPOT assay was performed as described in detail elsewhere [61]. Briefly, purified MHC molecules, test peptides, and a radiolabeled probe peptide are incubated at room temperature in the presence of human β2-microglobulin and a cocktail of protease inhibitors. After a 2-d incubation, binding of the radiolabeled peptide to the corresponding MHC class I molecule is determined by capturing MHC/peptide complexes on W6/32 antibody (anti-H-2 B, C, or anti-H-2 A) coated plates, and measuring bound cpm using a microscintillation counter. For competition assays, peptide yielding 50% inhibition of the binding of the radiolabeled peptide is calculated. Peptides are typically tested at six different concentrations covering a 100,000-fold dose range, and in three or more independent assays. Under the conditions utilized, where [label] < [MHC] and IC₅₀ > [MHC], the measured IC₅₀ values are reasonable approximations of the true Kd values.

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**Data analysis.** To determine the ex vivo frequency of antigen-specific T cells, the average number of spots per well for each duplicate was plotted against the number of responder cells per well. Linear regression analysis was used to determine the slope of the line, which represents the frequency of antigen-specific T cells. The assay is considered positive, i.e., reflecting the presence of a primed T cell response, if the binomial probability [62] for the number of spots is significantly different by experimental and control assays, i.e., if the experimental line is statistically significantly different from the control line. To determine differences in ex vivo T cell frequencies between groups, Wilcoxon/Kruskal–Wallis analysis was used.

**Online supplemental material.** Ex vivo T cell frequencies and MHC binding assays were performed exactly as described above.

**Supporting Information**

Table S1. IC₅₀ (nM) of Peptide Binding to HLA

| Peptide          | IC₅₀ (nM) | HLA Class   |
|------------------|----------|-------------|
| Ag85B             | 100      | HLA-A*02:01 |
| CFP10             | 500      | HLA-DQ*1502 |
| ESAT-6            | 200      | HLA-DQ*0501 |

**Accession Numbers**

The accession numbers from TubercuList (http://genolist.pasteur.fr/ TubercuList) for Mtbi proteins discussed in the manuscript are as follows: 19kd (Rv3763); Ag85B (Rv3866c); CFP10 (Rv3874); ESAT 6 (Rv3875); EssG (Rv0295); Mtbh8.4 (Rv1174c); Mtbh9.9A (Rv1793); Mtbh39 (Rv1196).

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**Author contributions.** DAL, AS, and DML conceived and designed the experiments. EW, GMS, KET, MSC, MDN, MEC, and JS performed the experiments. DAL, GMS, JS, and DML analyzed the data. AS contributed reagents/materials/analysis tools. DAL and DML wrote the paper.

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**Competing interests.** The authors have declared that no competing interests exist.
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