Dissecting Mechanisms of Immunodominance to the Common Tuberculosis Antigens ESAT-6, CFP10, Rv2031c (hspX), Rv2654c (TB7.7), and Rv1038c (EsxJ)

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Dissecting Mechanisms of Immunodominance to the Common Tuberculosis Antigens ESAT-6, CFP10, Rv2031c (hspX), Rv2654c (TB7.7), and Rv1038c (EsxJ)

Cecilia S. Lindestam Arlehamn,* John Sidney,* Ryan Henderson,* Jason A. Greenbaum,* Eddie A. James,† Magdalini Moutaftsi,‡ Rhea Coler,‡ Denise M. McKinney,* Daniel Park,§ Randy Taplitz,§ William W. Kwok,† Howard Grey,* Bjoern Peters,* and Alessandro Sette*

Diagnosis of tuberculosis often relies on the ex vivo IFN-γ release assays QuantiFERON-TB Gold In-Tube and T-SPOT.TB. However, understanding of the immunological mechanisms underlying their diagnostic use is still incomplete. Accordingly, we investigated T cell responses for the TB Ags included in the these assays and other commonly studied Ags: early secreted antigenic target 6 kDa, culture filtrate protein 10 kDa, Rv2031c, Rv2654c, and Rv1038c. PBMC from latently infected individuals were tested in ex vivo ELISPOT assays with overlapping peptides spanning the entirety of these Ags. We found striking variations in prevalence and magnitude of ex vivo reactivity, with culture filtrate protein 10 kDa being most dominant, followed by early secreted antigenic target 6 kDa and Rv2654c being virtually inactive. Rv2031c and Rv1038c were associated with intermediate patterns of reactivity. Further studies showed that low reactivity was not due to lack of HLA binding peptides, and high reactivity was associated with recognition of a few discrete dominant antigenic regions. Different donors recognized the same core sequence in a given epitope. In some cases, the identified epitopes were restricted by a single specific common HLA molecule (selective restriction), whereas in other cases, promiscuous restriction of the same epitope by multiple HLA molecules was apparent. Definition of the specific restricting HLA allowed to produce tetrameric reagents and showed that epitope-specific T cells recognizing either selectively or promiscuously restricted epitopes were predominantly T effector memory. In conclusion, these results highlight the feasibility of more clearly defined TB diagnostic reagent.


Tuberculosis (TB) is one of the major causes of death from infectious disease worldwide, claiming 1.4 million lives in 2010. In addition, ~9 million new cases of TB were reported in 2010. The vast majority (90–98%) of infected individuals are able to contain the infection asymptomatically, resulting in latent infection. However, 2–10% will develop active TB in their lifetime, resulting in further spread of the disease (1).

Current diagnosis of TB relies on the conventional immunologic assay, the tuberculin skin test (TST). This test is primarily useful in ruling out TB infection, but it cannot reliably distinguish active TB infection from recovered TB, latent TB, Mycobacterium bovis bacillus Calmette-Guérin (BCG) vaccination, or infection with nontuberculous mycobacteria. A major advance in distinguishing prior or current infection with Mycobacterium tuberculosis from BCG vaccination and some of the nontuberculous mycobacterial diseases occurred with the introduction of ex vivo analysis of peripheral blood cells for responses to two M. tuberculosis-specific Ags, early secreted antigenic target 6 kDa (ESAT-6) (Rv3875) and culture filtrate protein 10 kDa (CFP10) (Rv3874). These Ags are located in the region of difference 1, which is absent from M. bovis BCG and the majority of environmental mycobacteria, resulting in their diagnostic specificity (2–4). The IFN-γ release assays (IGRAs) T-SPOT.TB and QuantiFERON-TB Gold In-Tube both use ESAT-6 and CFP10. These Ags have been studied extensively (3, 5–16) and are often used as tools to examine M. tuberculosis-specific immune responses. In addition to ESAT-6 and CFP10, QuantiFERON-TB Gold In-Tube also uses the Rv2654c (TB7.7) Ag.

The goal of this study was to examine responses by latently infected individuals to a set of TB Ags commonly used for diagnostic purposes. Importantly, we were also interested in determining whether the responses to these Ags were uniformly positive. If not, we wanted to examine the concept that inclusion of defined epitopes derived from Ags present in IGRAs or other Ags might be an avenue to enhance the performance of diagnostic tests reliant on the use of these same common Ags. As representative Ags, we chose Rv2031c (hspX, 16 kDa) and Rv1038c (EsxJ). On the basis of the results of this initial study, we expected to provide a platform for a much broader analysis with multiple Ags and even perform a genome-wide screen. Several Ags encoded by the DosR regulon have been described as preferentially recognized by individuals with latent infection (17–20). One of these Ags, Rv2031c, has been studied in detail, and several T cell epitopes have been described previously (20–23). The Rv2031c Ag has been shown to be predominantly expressed when M. tuberculosis is subjected to
oxygen deprivation in vitro (24, 25), conditions thought to be encountered by *M. tuberculosis* in vivo when persisting in an immunocompetent host (26).

Several other proteins from TB have been identified as Ags, including members of the ESAT-6 protein family (27). One of these Ags, Rv1038c (EsxJ), has been previously identified as an immunodominant Ag (28, 29) and exhibits a high degree of homology with other members of the ESAT-6 protein family such as Rv3620c (EsxW), Rv2347c (EsxP), Rv1197 (EsxK), and Rv1792 (EsxM).

The availability of peptides and sensitive ELISPOT assays makes it possible to investigate responses ex vivo, precluding the need for in vitro stimulation and expansion of the cells. The capacity to study responses to Ags ex vivo affords probing and an understanding of the immunological mechanisms and specificity underlying the diagnostic use of the IGRAs.

In the current study, we report a side-by-side comparison of ESAT-6, CFP10, Rv2031c, Rv2654c, and Rv1038c in latently infected individuals. By combining an ex vivo T cell response approach with HLA peptide binding assays and subjecting HLA typing, we were able to identify and characterize the molecular mechanisms influencing their recognition by human T cells. There are striking differences in recognition when comparing these Ags, with CFP10 and ESAT-6 being the most dominantly recognized. Further characterization of dominant antigenic regions revealed that the same minimal epitope core sequence is recognized by multiple donors. Furthermore, depending on the specific epitope, the mechanism of dominance could be attributed to either the prevalence of a single specific HLA molecule or promiscuous recognition. These data provide new insights into T cell responses with CFP10 and ESAT-6 being the most dominantly recognized.

**Materials and Methods**

**Study subjects**

Leukapheresis samples were obtained from 40 adults, 22 with latent TB infection (LTBI) and 18 control donors from the University of California, San Diego, Antiviral Research Center clinic (age range 20–65 y). Ethnicity of subjects studied can be found in Supplemental Table II. Subjects were initially identified by having a history of a positive TST. LTB T cells were confirmed by a positive QuantiFERON-TB Gold In- Tube (Cellestis, Victoria, Australia) as well as a history, physical examination, and/or chest x-ray that was not consistent with active TB. None of the study subjects had been vaccinated with BCG or had laboratory evidence of HIV or hepatitis B. The control donors had a negative TST as well as a negative QuantiFERON-TB Gold In-Tube. Approval for all procedures was obtained from the Institutional Review Board (FWA#0000032).

**Peptides**

Sets of peptides of 15 aa in length, overlapping by 10 residues, were synthesized to cover the entire length of Rv3784 (CFP10), Rv3875 (ESAT-6), Rv2031c (hspX), Rv1038c (EsxJ), and Rv2654c (TB7.7). These peptides were combined into pools of up to 10 peptides with any given pool containing peptides from only one Ag. For mapping of minimal epitope core sequences, sets of peptides of 15 aa in length, overlapping by 14 residues, were synthesized to cover the antigenic region sequences. These peptides were tested individually.

Peptides were purchased from Mimotopes (Clayton, Victoria, Australia) and/or A and A (San Diego, CA) as crude material on a small (1 mg) scale. Peptides used for tetramers were synthesized on a larger scale and purified (>95%) by reversed-phase HPLC. The Immune Epitope Database (IEDB) submission identification number for the peptides is IEDB000031.

**MHC purification**

MHC molecules were purified from EBV-transformed homoygous cell lines by mAb-based affinity chromatography, as described in detail elsewhere (30). HLA-DR, -DQ, and -DP molecules were captured by a repeated passage of lysates over LB3.1 (anti–HLA-DR), SPV-L3 (anti–HLA-DQ), and B7/21 (anti–HLA-DP) columns.

**MHC–peptide binding assays**

Assays to quantitatively measure peptide binding to MHC class II molecules are based on the inhibition of binding of a high-affinity radiolabeled peptide to purified MHC molecules and have been described in detail elsewhere (30). Briefly, 0.1–1 nM radiolabeled peptide was incubated at room temperature or 37°C with 1 μM to 1 nM purified MHC in the presence of a mixture of protease inhibitors. Following a 2- to 4-d incubation, the percentage of MHC-bound radioactivity was determined by capturing MHC/peptide complexes on LB3.1 (DR), L243 (DR), HB180 (DR/DQ/DQ), SPV-L3 (DQ), or B7/21 (DP) Ab-coated Optiplates (Packard Instrument, Meriden, CT), and bound counts per minute was measured using the TopCount (Packard Instrument) microscintillation counter. Under the conditions used, where [label]–[MHC] and IC50 ∼ [MHC], the measured IC50 values are reasonable approximations of the true Kd values (31, 32).

**PBMC isolation and HLA typing**

PBMC were obtained by density gradient centrifugation (Ficoll-Hypaque; Amershams Biosciences, Uppsala, Sweden) from 100 ml leukapheresis sample, according to the manufacturer’s instructions. Cells were suspended in FBS (Gemini Bio-products, Sacramento, CA) containing 10% DMSO, and cryopreserved in liquid nitrogen for further analysis.

Genomic DNA isolated from PBMC of the study subjects by standard techniques (QiAmp; Qiagen, Valencia, CA) was used for HLA typing. High-resolution Luminex-based typing for HLA class I and class II was used, according to the manufacturer’s instructions (Sequence-Specific Oligonucleotides typing; One Lambda, Canoga Park, CA). Where needed, PCR-based methods were used to provide high-resolution subtyping. (Sequence-Specific Primer typing; One Lambda).

**Ex vivo IFN-γ ELISPOT assay**

PBMC were incubated at a density of 2 × 10^6 cells/well and were stimulated with either peptide pools (1 μg/ml) or individual peptides (10 μg/ml), PHA (10 μg/ml), or medium containing 0.25% DMSO (corresponding to percent DMSO in the pools/peptides) as a control in 96-well flat-bottom plates (Immmobilon-P; Millipore, Bedford, MA) coated with 10 μg/ml anti–IFN-γ mAb (clone AN18; Mabtech, Stockholm, Sweden). Each peptide was tested in triplicates. Following a 20-h incubation at 37°C, the wells were washed with PBS/0.05% Tween 20 and then incubated with 2 μg/ml biotinylated IFN-γ mAb (clone R4-6A2; Mabtech) for 2 h. The spots were developed using Vectastain avidin/biotin complex peroxidase (Vector Laboratories, Burlingame, CA) and 3-amino-9-ethylcarbazole (Sigma-Aldrich, St. Louis, MO) and counted by computer-assisted image analysis (KS-ELISPOT reader; Zeiss, Munich, Germany). The level of statistical significance was determined with a Student t test using the mean of triplicate values of the response against relevant pools or individual peptides versus the response against the DMSO control. Responses against peptides were considered positive if the net spot-forming cells (SFC) per 10^5 were ≥20, the stimulation index ≥ 2, and p < 0.05.

**Magnetic bead separation**

For experiments that use depletion of CD4 or CD8 T cells, these cells were isolated by magnetic bead positive selection (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer’s instructions, and effluent cells (depleted cells) were used for experiments.

For all experiments that used purified CD4^+ T cells, the cells were isolated by magnetic bead positive selection (Miltenyi Biotec), according to the manufacturer’s instructions.

**HLA restriction**

To determine the HLA locus restriction of identified epitopes, Ab inhibition assays were performed. CD4^+ T cells purified by positive selection together with effluent cells (at a 2:1 ratio) were incubated with 10 μg/ml Abs (Strategic Biosolutions, Windham, ME) against HLA-DR (LB3.1), -DP (B7/21), or -DQ (SVLP3) 30 min prior to peptide addition. IFN-γ cytokine production against positive peptides was then measured in an ELISPOT assay as described above. The pan-MHC class I Ab (W6/32) was used as a control.

**Tetramer staining**

MHC class II tetramers conjugated using PE-labeled streptavidin were provided by the Tetramer Core Laboratory at the Benaroya Research Institute at Virginia Mason (Seattle, WA). CD4^+ T cells were purified using the Miltenyi T cell isolation kit II, according to the manufacturer’s instructions. Purified cells (~10 × 10^6) were incubated in 0.5 ml PBS containing 0.5% BSA and 2 mM EDTA (pH 8) (MACS buffer) with a
1:50 dilution of class II tetramer for 2 h at room temperature. Cells were then stained for cell surface Ags using anti-CD4-FITC, anti-CCR7-APC-EFluor780, anti-CD45RA-EFluor450, and anti-CD8, -CD14, -CD19-PerCP-Cy5.5 (all from eBioscience) to exclude non-T cell containing populations of cells that bound tetramer. Tetramer-specific T cell populations were positively enriched by incubating cells with 50 μl anti-PE microbeads (Miltenyi Biotec) for 20 min at 4°C. After washing, cells were resuspended in 5 ml MACS buffer and passed through a magnetized LS column (Miltenyi Biotec) for 20 min at 4˚C. After washing, cells were resuspended on an LSR II flow cytometer (BD Immunocytometry Systems) and analyzed using FlowJo software.

Results

Ex vivo T cell responses to common TB Ags vary dramatically in frequency and magnitude

In a first series of experiments, we analyzed T cell reactivity against the CFP10 (Rv3874), ESAT-6 (Rv3875), Rv2031c (hspX), Rv2654c (TB7.7), and Rv1038c (EsxJ) Ags. For this purpose, we measured ex vivo production of IFN-γ by PBMCs from 18 LTBI donors, using ELISPOT assays and 15-mer peptides overlapping by 10 aa spanning the entire length of the proteins. The peptides were arranged in 11 Ag-specific pools (two per Ag, except Rv2031c which had three) of ~10 peptides (9 ± 0.7) each. The most frequently recognized proteins were CFP10 and ESAT-6, which elicited responses in 67 and 56% of the LTBI donors, respectively. Less frequent responses were detected for Rv2031c (28%) and Rv1038c (11%). None of the LTBI donors studied responded to Rv2654c, which was somewhat surprising because this Ag is included in the QuantiFERON-TB Gold In-Tube test. When the data were scrutinized from the standpoint of magnitude of responses, a superimposable hierarchy of responses was observed (Fig. 1). As expected, no responses were observed in PBMCs from 18 TB uninfected control donors (Fig. 1). As a criterion of positivity, consistent positive responses in three out of four or independent experiments was required, which, in our experience, is important for defining the most robust, and thereby most relevant, responses. Accordingly, in the case of one pool, where positive responses were noted in only two of four independent experiments, the Ag/donor combination was conservatively deemed negative. For all other cases, the criterion of positivity was met. In conclusion, these results highlighted that, when responses are evaluated side-by-side using ex vivo analysis to avoid potential biases introduced by in vitro restimulation, there are large variations in terms of the frequency and magnitude of responses against common TB Ags.

The number of epitopes contained within each Ag influences but does not fully explain immunodominance

Although the data shown above clearly establishes an immunodominance hierarchy within the set of Ags analyzed, it was unclear whether this dominance might be reflective of a large(r) number of different epitopes being recognized in the more dominant Ags or, alternatively, whether dominance at the Ag level might reflect the presence of a limited number of more dominant epitopes. To address this issue, each positive peptide pool was next deconvoluted, and individual reactive peptides were identified. The patterns of reactivity derived on the basis of these experiments are shown in Figs. 2A, 3A, 4A, and 4B. The recognition of overlapping peptides defines several antigenic regions (one or adjacent overlapping peptides recognized, based on response frequency and/or magnitude). The number of donors recognizing each of the regions of the various Ags is indicated as well as the magnitude of responses measured in the ELISpot assay (SFC sum).

Two different patterns became apparent. For Rv1038c (Fig. 4B), only one antigenic region could be discerned. In the case of the three remaining Ags, CFP10, ESAT-6, and Rv2031c, four distinct regions were apparent in each. For ESAT-6, some reactivity over the entire protein sequence was observed, but discrete dominant regions could be discerned. The weakest Ag of the four recognized Ags had the fewest antigenic regions; however, the two immunodominant Ags (ESAT-6 and CFP10) had the same number of antigenic regions as the less antigenic protein Rv2031c, indicating that the number of antigenic regions is not the sole determinant of immunogenicity for these Ags.

Epitope paucity in Rv2654c and Rv1038c is not due to lack of peptides binding to HLA class II molecules

One potential explanation for the paucity of epitopes/antigenic regions observed in the case of Rv2654c and Rv1038c is that these Ags might contain fewer or no HLA class II binding peptides as compared with the more dominant CFP10, ESAT-6, and Rv2031c Ags, which contain at least four different epitopes each.

To examine this issue, a panel of 27 HLA class II binding assays was assembled, representative of common HLA class II DR, DP, and DQ molecules expressed in the general population. The panel of assays, together with their genotypic frequencies and phenotypic frequencies, is shown in Supplemental Table I. Next, the LTBI donors analyzed were HLA typed by a high-resolution Luminex-based method (Supplemental Table II), and the coverage of this specific donor population afforded by the panel of 27 HLA binding assays was calculated. Each individual usually expresses a total of eight HLA class II genes (one copy of HLA DRB1, B3/4/5, DP, and DQ genes on each chromosome). All (100%) of the individuals in
the donor cohort expressed at least one of the 27 HLA molecules in our assay panel, and for 83%, the assay panel provided coverage of six or more donor HLA class II types (Supplemental Fig. 1). Closely related alleles that have an identical peptide binding region were considered a match when HLA typing was compared with the 27 HLA molecules in the assay panel. These results demonstrate that the selected panel of HLA molecules affords high coverage of the specific donor population investigated.

**FIGURE 2.** Determination and characterization of antigenic regions from ESAT-6. PBMC from LTBI donors were incubated with 10 μg/ml peptides from ESAT-6, and the number of IFN-γ–producing cells was enumerated in an ELISPOT assay. ESAT-6 has four antigenic regions, indicated by capped lines. The numbers of donors tested for each region were n = 7 for Regions 1 and 2 and n = 5 for Regions 3 and 4. Shown are total SFC (black line and closed circles) and number of donors responding to each peptide (dashed line and open circles) versus peptides tested (15-mer overlapping by 10) (A). Minimal epitope core sequence within each antigenic region for ESAT-6. Shown are the average net SFC/10^6 PBMC for each donor in response to the tested peptides (15-mer overlapping by 14) from at least two independent experiments. Boxes indicate selected epitopes containing the minimal epitope core sequence. For each individual peptide, samples were tested in triplicate. Error bars indicate SD. The dotted line indicates the 20 net SFC/10^6 cells threshold used to define positivity (B, C). Selected epitopes identified from Regions 1, 2, and 4, showing donors responding, position in protein, and sequence. Minimal epitope core sequence is highlighted in bold (D, E).
Each of the overlapping peptides previously tested for recognition by the donor PBMCs was tested for binding to each of 27 purified HLA molecules (IEDB Submission number 100492). On average, each peptide bound 6.7 (± 5.6) molecules at the 1000 nM threshold previously reported to be associated with immunogenicity for HLA class II responses (33–35) and that each Ag contained a minimum of nine peptides binding at least 20% of the alleles tested (Table I). Importantly, the binding distribution did not differ appreciably when more dominant (CFP10, ESAT-6, and Rv2031c) and less dominant (Rv1038c and Rv2654c) Ags were compared, thus indicating that the incidence of HLA binding peptides did not account for the immunodominance hierarchy observed. In conclusion, the subdominance and the paucity of epitopes observed in the case of Rv2654c and Rv1038c cannot be
ascribed to these Ags simply containing no or fewer HLA class II binding peptides as compared with the more dominant Ags, CFP10, ESAT-6, and Rv2031c.

Further characterization of antigenic regions

We next sought to determine whether the immunodominant epitopes/antigenic regions corresponded to a single epitope similarly recognized by different donors or rather the presence of overlapping but distinct epitopes. For ESAT-6 and CFP10 three antigenic regions each and for Rv2031c and Rv1038c one antigenic region each were analyzed in more detail.

To determine the epitope core sequence from each antigenic region, recognition of every possible 15-mer spanning the identified antigenic region (i.e., 15-mer overlapping by 14 aa) was analyzed. In general, the same minimal epitope core sequence was recognized by the different donors responding to a given antigenic region (Figs. 2B, 2C, 3B, 3C, 4C, 4D) and defined by the pattern of reactivity to the peptides overlapping by 14 aa. The minimal epitope sequence was defined by the epitope core region necessary for optimal reactivity. For example, there is a loss of reactivity when ESAT-614–28 is compared with ESAT-6 15–29, which suggests that residue 29 is the C-terminal boundary of the core region. Similarly, there is loss of reactivity when ESAT-623–37 is compared with ESAT-6 22–36, suggesting that residue 22 is the N-terminal of the crucial core region. Thus, the minimal epitope core region needed for optimal reactivity corresponds to ESAT-622–29 (VTSIHSLL). Accordingly, for each antigenic region, a representative 15-mer epitope containing the minimal epitope core sequence and additional flanking residues was chosen for further investigation. A summary of selected epitopes for each donor tested is shown in Figs. 2D, E, 3D, 3E, 4E, and 4F.

Table I. Summary of number of HLA molecules bound by 15-mers overlapping by 10 aa covering ESAT-6, CFP10, Rv2031c, Rv2654c, and Rv1038c

<table>
<thead>
<tr>
<th>Ag</th>
<th>Total No. of Peptides</th>
<th>No. of Peptides Binding ≥20% of Alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFP10</td>
<td>18</td>
<td>9</td>
</tr>
<tr>
<td>ESAT-6</td>
<td>17</td>
<td>9</td>
</tr>
<tr>
<td>Rv2031c</td>
<td>27</td>
<td>14</td>
</tr>
<tr>
<td>Rv1038c</td>
<td>18</td>
<td>9</td>
</tr>
<tr>
<td>Rv2654c</td>
<td>15</td>
<td>11</td>
</tr>
</tbody>
</table>

FIGURE 4. Determination of minimal epitopes from Rv2031c and Rv1038c. For a description of experimental conditions, see the legend for Fig. 2. Rv2031c has four antigenic regions (n = 1 for Region 1, n = 3 for Regions 2 and 3, and n = 5 for Region 4), and Rv1038c has one antigenic region (n = 2), indicated by the capped lines labeled by region numbers (A, B). Minimal epitope core sequence within each antigenic region for Rv2031c and Rv1038c (C, D). Selected epitopes identified from Region 4 Rv2031c and Region 1 Rv1038c, showing donors responding, position in protein, and sequence. Minimal epitope core sequence is highlighted in bold (E, F).
Having defined the minimal core sequences recognized allowed us to further characterize the phenotype of epitope-reactive cells and their restriction. First, we examined responses in PBMC depleted of either CD4\(^+\) or CD8\(^+\) T cells to establish the major T cell subset responsible for the responses. As shown in Fig. 5A, a total loss of peptide reactivity was observed in all of the CD4\(^+\) T cell-depleted PBMC fractions, whereas CD8\(^+\) depletion had no effect. These results demonstrate that the epitopes identified are CD4, and thus also likely HLA class II, restricted.

Next, to verify this assumption and we determined the particular HLA class II loci used. For this purpose, the capacity of Abs specific for HLA-DR, -DP, or -DQ to inhibit the epitope-specific response was tested (representative data; Fig. 5B). Locus restriction could be determined for 30 of the 35 (86%) recognition events analyzed (donor/epitope combinations) (Table II). For the remainder, either lack of a sufficiently strong response or an inconclusive inhibition pattern precluded a definitive locus assignment. DR restriction was noted for 18 responses and DP and DQ restriction for 11 each; 8 responses were restricted by multiple loci in the same donor (Table II). These data confirmed the CD4/CD8 depletion data and formally demonstrate class II restriction and also illustrate how a diverse set of alleles and loci are involved in restricting these responses. Interestingly, several antigenic regions/epitopes were restricted by multiple loci, as discussed in more detail below.

Selective versus promiscuous restriction of selected epitopes

The HLA typing data for each donor responding to a given epitope was compared with the locus determination data presented above and the in vitro HLA binding data (Table II). Each of the epitopes was tested for binding to purified HLA molecules (IEDB Submission number 1000492). Furthermore, for each donor/epitope combination for which a restricting locus could be assigned, we noted the corresponding alleles expressed by the donor for which the epitope was shown to bind the epitope with either strong (<100 nM) or intermediate (<1000 nM) affinities. These allelic molecules are highlighted by green and yellow shading, respectively, in Table II. In approximately half of the cases, the epitope was restricted by multiple HLA class II loci (ESAT-6\(_{3-17}\), CFP10\(_{40-54}\), CFP10\(_{72-86}\), Rv2031\(_{c106-120}\), and Rv1038\(_{c28-42}\) or was indeed restricted by a single HLA class II locus, but the...
individuals responding to the epitope did not share a common HLA binding molecule that could be considered as a candidate for a single restriction element (ESAT-670–84 and Rv2031c111–125). These data suggest that promiscuous recognition in the context of multiple HLA class II molecules may be a mechanism significantly contributing to epitope immunodominance.

Conversely, in the case of three other epitopes (ESAT-6 18–32, ESAT-673–87, and CFP10 52–66), all responses were restricted by a single locus, and all responders shared an allelic variant shown to bind the epitope with high affinity. In these cases, we were able to infer specific restriction, as listed in the “Inferred Restricting HLA Allele” column (Table II). A large majority of the donors expressing that particular HLA class II allele also responded to the given epitope. Specifically, four of five donors expressing DRB5*01:01 responded to CFP10 52–66, and three of five donors expressing DQB1*03:01 responded to ESAT-6 72–87, as listed in the “No. of Donors Responding” column (Table II), thus further strengthening the proposed restrictions.

On average each of these three single locus restricted peptides bound 6.3 \( \pm \) 1.2 alleles. By contrast, the promiscuously restricted peptides bound 12.3 \( \pm \) 5.3 alleles with high binding affinity (100 nM). These results suggest that certain epitopes are immunodominant on the basis of an alternative mechanism, namely that although they are selectively restricted by one HLA.

### Table II. Summary of epitope characteristics

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Donor</th>
<th>SFC*</th>
<th>Restricting HLA Locus</th>
<th>DRB1/3/4/5, DQB1, DPB1</th>
<th>Inferred Restricting HLA Allele</th>
<th>No. of Donors Expressing Allele Responses</th>
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</thead>
<tbody>
<tr>
<td>Promiscuous ESAT-670–84</td>
<td>TU25</td>
<td>85</td>
<td>DQ, DR</td>
<td>DRB1<em>08:04, DQB1</em>02:01, DQB1*03:19 ( ^a )</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TU9</td>
<td>53</td>
<td>DR</td>
<td>DRB1<em>01:01, DRB1</em>08:04</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TU22</td>
<td>82</td>
<td>DQ</td>
<td>DQB1*06:02</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TU8</td>
<td>25</td>
<td>Undetectable</td>
<td>DRB1*01:01</td>
<td>DRB1<em>08:02, DQB1</em>04:02 ( ^a )</td>
<td></td>
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<tr>
<td>CF10p24</td>
<td>TU23</td>
<td>236</td>
<td>DR</td>
<td>DRB1<em>01:01, DRB1</em>11:01, DRB1*02:02</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>TU7</td>
<td>103</td>
<td>DP, DQ, DR</td>
<td>DRB1<em>01:01, DRB1</em>08:03, DQB1*04:02 ( ^a )</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>TU22</td>
<td>70</td>
<td>DP</td>
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<td>DR</td>
<td>DRB1<em>01:01, DRB1</em>08:04</td>
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<td></td>
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**Bold**, restricting HLA locus; green, strong binding (<100 nM); yellow, intermediate binding (100–999 nM); gray, not available.

*Mean SFC/10^6 PBMC, average of two independent experiments.

*Variant alleles that have an identical peptide binding region to an allele in the assay panel and the inferred restricting allele.
Phenotypic characterization of selected epitopes

To further characterize some of the identified epitopes, we selected one representative selectively restricted and one representative promiscuously restricted epitope for further experiments. In the case of CFP1052-66, the experiments above indicated DRB5*01:01 as the restriction element. In the case of the promiscuous epitope CFP1040-54, DRB1*01:01 was indicated as one of the likely restricting elements, because three of four donors that responded express DRB1*01:01, and CFP1040-54 binds this allele with high affinity. Accordingly, respective MHC–peptide tetramer reagents were prepared for these CFP10 epitopes. To increase sensitivity, tetramer staining of CD4+ purified cells followed by a magnetic bead enrichment technique was used (36).

The tetramer staining experiments confirmed the HLA restriction of the two CD4+ T cell responses (Fig. 6). Epitope-specific T cell responses were detected in three donors at frequencies 0.1–0.5% (mean of 0.33 ± 0.23 SD) above background for DRB1*01:01 CFP1040-54 and in six donors at frequencies 0.1–10% (mean of 2.54 ± 3.97 SD) above background for DRB5*01:01 CFP1053-66. Only a small number of background tetramer+ cells were detected with the epitope-specific tetramers in the HLA-matched control donors and in LTBI donors with an HLA mismatch for DRB5*01:01 CFP1053-66 (Fig. 6A), which confirmed that tetramer specificity was derived from the epitope and HLA molecule combination.

The memory subset phenotype was addressed using Abs to CD45RA and CCR7 (37–39). As shown in Fig. 6B and 6C, DRB1*01:01 CFP1040-54 epitope-specific tetramer+CD4+ T cells predominantly consisted of CD45RA+CCR7− effector memory cells in all three donors analyzed, followed by central memory T cells (CCR7+CD45RA−). Percentages ranged between 68.3 and 77.5% (SD ± 4.6) for effector memory T cells and 12.6–23.2% (SD ± 5.5) for central memory T cells. Only a minor fraction of the tetramer+CD4+ T cells appeared to be naive (CCR7+CD45RA−) or effector T cells (CCR7+CD45RA+). Similarly, for the DRB5*01:01-CFP1052-66 response, epitope-specific tetramer+ CD4+ T cells predominantly consisted of CCR7+CD45RA− effector memory cells in all six donors analyzed, followed by central memory T cells (CCR7+CD45RA−), percentages ranging between 60.2 and 91.6% (SD ± 12.9) for effector memory T cells and 1.5–20.4% (SD ± 13.0) for central memory T cells. Again, only a minor fraction of the tetramer+CD4+ T cells appeared to be naive (CCR7+CD45RA+) or effector T cells (CCR7+CD45RA+) (Fig. 6C).

Discussion

Although the QuantiFERON-TB Gold In-Tube and T-SPOT.TB IGRAs are highly successful as TB diagnostics, they are also not without problems. Both tests are an empirical mixture of peptides, and the epitopes have not been characterized ex vivo, even though the assays are performed in ex vivo settings. This results in a fundamental lack of understanding of the immunological mechanisms and specificity underlying the diagnostic utility. Several fundamental questions remain to be answered. Namely, 1) are all Ags equally recognized with similar prevalence (response frequency) and magnitude of responses or does significant variation exist from one Ag to the next? 2) If significant variability exists, is it possible to identify additional Ags that might enhance the antigenic composition of the IGRAs? 3) What are the molecular features of Ag recognition at the population level (i.e., what determines which

![FIGURE 6. HLA restriction and memory phenotype of CFP10-specific CD4+ T cells using MHC class II tetramers. CD4-purified cells from LTBI donors, HLA-matched control donors, or HLA-mismatched LTBI donors (for DRB5*01:01) were stained with MHC class II tetramers, and tetramer+ cells were isolated following magnetic bead enrichment. Plots are gated on CD4+ T cells, and the numbers indicate the percentages of tetramer+ cells isolated from each donor’s CD4+ population. DRB1*01:01 CFP1040-54, n = 3 LTBI donors and DRB5*01:01 CFP1053-66, n = 6 LTBI donors (A). Memory phenotype of tetramer+ cells for two representative donors per tetramer. Plots are gated on total CD4+ T cells (black background) or epitope-specific CD4+ T cells (red dots). The numbers represent the percentages of tetramer+CD4+ T cells in the gate (B). Pie chart representation of the proportion of CCR7−CD45RA−, CCR7+CD45RA−, CCR7+CD45RA+, and CCR7−CD45RA+CD4+ T cells for each tetramer, DRB1*01:01 CFP1040-54 (n = 3) and DRB5*01:01 CFP1053-66 (n = 6). Effector memory T cells are CCR7−CD45RA−, central memory T cells are CCR7+CD45RA−, naive T cells are CCR7+CD45RA+, and effector T cells are CCR7+CD45RA+ (C).]
Ags and epitopes are most dominant? 4) Are a myriad of epitopes recognized, differing from donor to donor, or are a limited number of epitopes recognized in the context of multiple donors (prevalence) or even across different HLAs (promiscuity)? 5) If dominant epitopes can be identified, could tetramer reagents be used to more thoroughly characterize immune responses, either diagnostically or prognostically?

This study represents, to the best of our knowledge, the first in-depth direct ex vivo side-by-side comparison of the responses to the commonly used TB Ags CFP10, ESAT-6, Rv2031c, Rv1038c, and Rv2654c, as well as an in-depth investigation of the mechanisms influencing their recognition by human T cells. The direct ex vivo approach avoids introducing potential bias as a result of in vitro restimulation and expansion of T cells, and thereby delineates a more physiologically relevant immune response. Although direct ex vivo responses to ESAT-6 and/or CFP10 have been investigated previously (9, 11, 12, 40, 41), most studies used less physiological techniques such as short-term T cell lines (13–16, 20, 21, 42).

In our side-by-side comparison, we found striking levels of variation in terms of prevalence of Ag recognition and magnitude of responses. Interestingly, we could not identify any responses to Rv2654c, which has been shown to be recognized by T cells during TB infection and is included in the QuantiFERON In-Tube Gold diagnostic test for TB (13, 14). Previous studies used short-term T cell cultures (13, 14), which could explain the discrepancy between this study and earlier work and highlights the necessity of revisiting Ag reactivity with approaches more closely reflective of the techniques used in the assays (i.e., ex vivo detection of IFN-γ).

Regarding whether it is possible to identify additional Ags that might enhance the antigenic composition of IGRA s, we observed high reactivity to Rv2031c, with 28% of the LTBI donors responding. This demonstrates that other prevalently recognized Ags can be identified and potentially included in diagnostic tools for TB, although this study was not aimed at identifying new Ags with diagnostic potential. We are conducting a genome-wide screen that will address this issue in more detail and may identify many additional Ags (manuscript in preparation).

This study provides, to the best of our knowledge, the first actual molecular HLA binding affinity data for these commonly used TB Ags using a panel of HLA class II molecules representative of the most common alleles worldwide. Previous studies have reported bioinformatics predictions of ESAT-6 epitopes (43, 44).

The low frequency or lack of recognition of Rv1038c and Rv2654c could not be explained by the lack of HLA class II binding peptides. It has been shown for infectious diseases such as malaria and HIV, and more recently also for allergy, that peptide binding to a HLA molecule is vital, but not by itself sufficient, for recognition by epitope-specific T cells (45–47). Other factors that might play a role in Ag recognition are the function of the protein, expression levels, stage of expression, Ag presentation, and so on, factors that could also be influenced by the disease state. These factors are also interesting but beyond the scope of the current investigation and best suited for a genome-wide analysis.

The identified antigenic regions from CFP10, ESAT-6, Rv2031c, and Rv1038c correlated well with data previously available in the literature (7, 9, 10, 12, 13, 41, 42). However, the side-by-side comparison provided additional insights into the frequency and magnitude of responses directed against these Ags. Most importantly, the recognition of dominant antigenic regions/epitopes in multiple donors was mapped to the recognition of the same minimal epitope core sequence jointly recognized by multiple donors. This could be explained by either restriction by a single specific HLA molecule or recognition of the same peptide in the context of multiple HLA types recognizing largely overlapping epitopes. Our results indicated that both selective and promiscuous restriction contribute to immunodominance. The molecular mechanisms underlying promiscuous recognition are not clear, and one hypothesis is that the processing of Ags preferentially generates certain peptide fragments. Furthermore, it is known that HLA class II molecules share peptide binding repertoires (33–35, 48), which could also contribute to promiscuous restriction of a particular epitope. Regardless of the mechanism, these results indicate that it is possible to identify epitopes prevalently recognized by LTBI subjects and therefore suggest that further studies identifying the specific epitopes recognized in TB infection could lead to better molecularly defined diagnostic assays. The reduction in complexity afforded by the definition of the dominant epitopes could in turn allow elimination of poorly or unrecognized Ags and epitopes to make room for highly prevalent epitopes derived from additional Ags. A substantial number of LTBI patients are negative for the IGRA Ags. This is consistent with the fact that IGRA is by necessity a mixture of different Ags. Our results further emphasize that an approach based on inclusion of only one of the IGRA Ags is not feasible and rather suggest that inclusion of additional epitopes and Ags might be beneficial. Future studies should include a larger study population from different ethnicities and geographic locations as well as include patients with different disease states. This would provide answers for different HLA phenotypes as well as whether patients with different disease states show a different recognition pattern.

The question of whether dominant epitopes can be used in conjunction with tetrameric reagents or ICS assays to more thoroughly characterize immune responses, either diagnostically or prognostically, was addressed. We found that all ex vivo-detected epitopes are CD4 restricted. This demonstrates that CD8-restricted epitopes for these Ags are a minor component of the ex vivo IFN-γ response measured by IGRA s and is consistent with CD8-restricted epitopes for ESAT-6 and CFP10 having been defined using T cell lines (5, 11, 49). Ag-specific T cell phenotypes have been well described in human viral infections, as well as in some bacterial infections (40, 50, 51), and these analyses provide important information regarding effector function. We found the phenotype of CFP10 epitope-specific CD4+ cells to be predominately effector memory cells, CD45RA−CCR7− (37–39), which is consistent with previous studies in HIV+ LTBI individuals and infected mice (40, 52, 53). This observation was true for both promiscuously and selectively restricted epitopes. It is not known whether there is some degree of bacterial replication ongoing in the individuals tested in this study, even though they have no symptoms of active TB (chest X-ray negative), because there is no accurate test for Ag levels associated with MTB infection in humans. Some bacterial replication could potentially drive the cells to maintain an effector memory phenotype, but further studies are needed to determine whether it is due to persistence of Ags or whole bacteria or if it represents the longevity of the immune response even in the absence of antigenic stimuli. Furthermore, it has previously been shown that the majority of cells responding to Rv2031c are effector memory T cells and that effector memory cells dominate the immune responses in TB (54, 55). Moreover, long-term persistence of TB-reactive cells has been described previously (54). It would perhaps be unlikely to detect central memory cells in PBMC samples, because they mostly reside in lymphoid organs and few circulate. In addition, it should be noted that there might be a weakness in testing blood samples for LTBI in that the T cells that are cognizant of infection in LTBI are probably localized in the lymphoid organs and are not accessible through peripheral blood. These cells would be expected to be recruited to the lungs after exposure or after reac-
tivation. In conclusion, these data demonstrate how the epitopes identified following the approaches described in this paper can be used to develop tetrameric reagents and suggest that definition and production of mixtures of highly defined tetrameric reagents could represent the next generation of diagnostic reagents, allowing for quantitation and characterization of responses to an unprecedented level of detail.

Finally, a side-by-side comparison of commonly used TB Ags shows that different Ags are recognized with drastically different prevalence (response frequency) and magnitude of responses. The recognition of Rv2031c also suggests that it is possible to identify epitopes in the context of multiple donors (prevalent) or even across different HLAs (promiscuous), suggesting that eliminating poorly or not recognized Ags and epitopes to make room for highly prevalent epitopes derived from additional Ags might represent a powerful avenue to generate more molecularly defined diagnostic reagents. Likewise, tetrameric reagents could be used to more thoroughly characterize immune responses, either diagnostically or prognostically.

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Disclosures
The authors have no financial conflicts of interest.

References


