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Dissecting Mechanisms of Immunodominance to the Common TB Antigens ESAT-6, CFP10, Rv2031c (hspX), Rv2654c (TB7.7) and Rv1038c (EsxJ)[1]

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**[*,2]

[*]Division of Vaccine Discovery, La Jolla Institute for Allergy and Immunology, 9420 Athena Circle, La Jolla, California, 92037 USA

[†]Benaroya Research Institute at Virginia Mason, 1201 Ninth Ave, Seattle, Washington, 98101 USA

[‡]Infectious Disease Research Institute, 1124 Columbia Street, Suite 400, Seattle, Washington, 98104 USA

[§]Antiviral Research Centre, University of California, San Diego, 220 Dickinson Street, Suite A, San Diego, California, 92103 USA

Abstract

Diagnosis of tuberculosis often relies on the *ex vivo* interferon gamma release assays QuantiFERON-TB Gold In-Tube and T-SPOT.TB. However, understanding of the immunological mechanisms underlying their diagnostic utility is still incomplete. Accordingly, we investigated T cell responses for the TB antigens included in the these assays and other commonly studied antigens; ESAT-6, CFP10, Rv2031c, Rv2654c, and Rv1038c. PBMC from latently infected individuals were tested in *ex vivo* ELISPOT assays with overlapping peptides spanning the entirety of these antigens. We found striking variations in prevalence and magnitude of *ex vivo* reactivity, with CFP10 being most dominant, followed by ESAT-6 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), while 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 show that epitope-specific T cells recognizing either selectively or promiscuously restricted epitopes were predominantly T effector memory (T_{EM}). In conclusion, these results highlight the feasibility of more clearly defined TB diagnostic reagent.

Introduction

Tuberculosis (TB) is one of the major causes of death from infectious disease worldwide, claiming 1.4 million lives in 2010. In addition, almost 9 million new cases of TB were reported in 2010. The vast majority (90–98%) of infected individuals are able to contain the

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[2]Corresponding author: Dr. Alessandro Sette, Phone: (858) 752-6916, Fax: (858) 752-6985, alex@liai.org.

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, *M. bovis* Bacillus Calmette-Guérin (BCG) vaccination, or infection with non-tuberculous mycobacteria. A major advance in distinguishing prior or current infection with *M. tuberculosis* from BCG vaccination and some of the non-tuberculous mycobacterial diseases occurred with the introduction of *ex vivo* analysis of peripheral blood cells for responses to two *M. tuberculosis*-specific antigens, ESAT-6 (Rv3875, early secreted antigenic target, 6kDa) and CFP10 (Rv3874, culture filtrate antigen, 10kDa). These antigens are located in the region of difference 1 (RD1), which is absent from *M. bovis* BCG and the majority of environmental mycobacteria resulting in their diagnostic specificity (2–4). The interferon-gamma release assays (IGRAs) T-SPOT.TB and QuantiFERON-TB Gold In-Tube both utilize ESAT-6 and CFP10. These antigens 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 utilizes the Rv2654c (TB7.7) antigen.

The goal of this study was to examine responses by latently infected individuals to a set of TB antigens commonly utilized for diagnostic purposes. Importantly, we were also, interested in determining if the responses to these antigens were uniformly positive. If not, we wanted to examine the concept that inclusion of defined epitopes derived from antigens present in IGRAs or other antigens might be an avenue to enhance the performance of diagnostic tests reliant on the use of these same common antigens. As representative antigens we chose Rv2031c (hspX, 16kDa) and Rv1038c (EsxJ). Based on the results of this initial study we expected to provide a platform for a much broader analysis with multiple antigens, and even perform a genome-wide screen. Several antigens encoded by the DosR regulon have been described as preferentially recognized by individuals with latent infection (17–20). One of these antigens, Rv2031c has been studied in detail and several T cell epitopes have been described (20–23). The Rv2031c antigen 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 antigens, including members of the ESAT-6 protein family (27). One of these antigens, Rv1038c (EsxJ), has been previously identified as an immunodominant antigen (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 antigens *ex vivo* affords probing and an understanding of the immunological mechanisms and specificity underlying the diagnostic utility of the IGRAs.

In the present 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 subject 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 antigens, 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 to commonly utilized TB antigens, and highlight novel differences in immunodominance patterns and restrictions.

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 (UCSD) Antiviral Research Center (AVRC) clinic (age range 20–65 years). Ethnicity of subjects studied can be found in Supplementary table 2. Subjects were initially identified by having a history of a positive TST. Latent TB was confirmed by a positive QuantiFERON-TB Gold In-Tube (Cellestis, Victoria, Australia), as well as a history, physical exam and/or chest X-ray that was not consistent with active tuberculosis. 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#00000032).

Peptides

Sets of peptides of 15 amino acids in length, overlapping by 10 residues, were synthesized to cover the entire length of Rv3874 (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 antigen. For mapping of minimal epitope core sequences, sets of peptides of 15 amino acids 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 (1mg) scale. Peptides utilized for tetramers were synthesized on larger scale, and purified (>95%) by reversed phase HPLC. The Immune Epitope Database (IEDB) submission identification number for the peptides is 1000489.

MHC purification

MHC molecules were purified from EBV transformed homozygous cell lines by monoclonal antibody-based affinity chromatography, as described in detail elsewhere (30). HLA-DR, DQ and DP molecules were captured by 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 of radiolabeled peptide was coincubated at room temperature or 37°C with 1 μM to 1 nM of purified MHC in the presence of a cocktail of protease inhibitors. Following a 2 to 4 day incubation, the percent of MHC bound radioactivity was determined by capturing MHC/peptide complexes on LB3.1 (DR), L243 (DR), HB180 (DR/DP/DQ), SPV-L3 (DQ) or B7/21 (DP) antibody coated Optiplates (Packard Instrument Co., Meriden, CT), and bound cpm measured using the TopCount (Packard Instrument Co.) microscintillation counter. Under the conditions

utilized, where [label]<[MHC] and $IC_{50} \geq$ [MHC], the measured IC_{50} values are reasonable approximations of the true K_d values (31, 32).

PBMC isolation and HLA typing

PBMC were obtained by density gradient centrifugation (Ficoll-Hypaque, Amersham Biosciences, Uppsala, Sweden) from 100 ml of leukapheresis sample, according to the manufacturer's instructions. Cell were suspended in fetal bovine serum (Gemini Bio-products, Sacramento, CA) containing 10% dimethyl sulfoxide, and cryo-preserved 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 utilized according the manufacturer's instructions (Sequence-Specific Oligonucleotides (SSO) typing; One Lambda, Canoga Park, CA). Where needed, PCR based methods were used to provide high resolution sub-typing. (Sequence-Specific Primer (SSP) typing; One Lambda, Canoga Park, CA).

Ex vivo IFNγ ELISPOT assay

PBMC were incubated at a density of 2×10^5 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 (Immobilon-P; Millipore, Bedford, MA) coated with 10 μg/ml anti-IFNγ 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 IFNγ mAb (clone R4-6A2; Mabtech) for 2 h. The spots were developed using Vectastain ABC peroxidase (Vector Laboratories, Burlingame, CA) and 3-amino-9-ethylcarbazole (Sigma-Aldrich, St.Lois, MO) and counted by computer-assisted image analysis (KS-ELISPOT reader, Zeiss, Munich, Germany). The level of statistical significance was determined with a Student's 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 10^6 were ≥20, the stimulation index ≥2, and $p<0.05$.

Magnetic bead separation

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

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

HLA Restriction

To determine the HLA locus restriction of identified epitopes, antibody inhibition assays were performed. $CD4^+$ T cells were purified by positive selection together with effluent cells (at a ratio 2:1) were incubated with 10 μg/ml of antibodies (Strategic Biosolutions, Windham, ME) against HLA-DR (LB3.1), DP (B7/21) or DQ (SVPL3) 30 min prior to peptide addition. IFNγ cytokine production against positive peptides was then measured in an ELISPOT assay as described above. The pan MHC class I antibody (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 manufacturer's instructions. Purified cells (~10×10^6) were incubated in 0.5 ml PBS containing 0.5% BSA and 2 mM EDTA pH 8.0 (MACS buffer) with a 1:50 dilution of class II tetramer for 2 h at room temperature. Cells were then stained for cell surface antigens using anti-CD4-FITC, anti-CCR7-APCEFluor780, anti-CD45RA-EFluor450 and anti-CD8, -CD14, -CD19-PerCPCy5.5 (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 of anti-PE microbeads (Miltenyi Biotech) 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). The column was washed three times with 3 ml of MACS buffer, and after removal from the magnetic field, cells were collected with 5 ml of MACS buffer. Before acquiring of samples 7AAD (BD Biosciences) were added to exclude dead cells. Samples were acquired on an LSR II flow cytometer (BD Immunocytometry Systems) and analyzed using FlowJo software.

Results

Ex vivo T cell responses to common TB antigens 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) antigens. For this purpose we measured *ex vivo* production of IFNγ by PBMCs from 18 LTBI donors, utilizing ELISPOT assays and 15-mer peptides overlapping by 10 amino acids spanning the entire length of the proteins. The peptides were arranged in 11 antigen specific pools (2 per antigen, except Rv2031c which had 3) of approximately 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 since this antigen is included in the QuantiFERON-TB Gold In-Tube test. When the data was 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 three or four 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 2 out of 4 independent experiments, the antigen/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 antigens.

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

While the data shown above clearly establishes an immunodominance hierarchy within the set of antigens analyzed, it was unclear whether this dominance might be reflective of a large(r) number of different epitopes being recognized in the more dominant antigens or, alternatively, whether dominance at the antigen 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 identified. The patterns of reactivity derived on the basis of these experiments are shown in Fig. 2*A*, 3*A*, 4*A* and *B*. 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 antigens 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 antigens, 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 antigen of the four recognized antigens had the fewest antigenic regions; however the two immunodominant antigens (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 antigens.

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 antigens might contain fewer or no HLA class II binding peptides as compared to the more dominant CFP10, ESAT-6 and Rv2031c antigens, 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 Supplementary Table 1. Next, the LTBI donors analyzed were HLA typed by a high-resolution Luminex-based method (Supplementary Table 2), 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 6 or more donor HLA class II types (Supplementary 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.

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 No. 1000492). 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 antigen contained a minimum of 9 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) antigens 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 antigens simply containing no or fewer HLA class II binding peptides as compared to the more dominant antigens, 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. 15mers overlapping by 14 amino acids) was analyzed. In general, the same minimal epitope core sequence was recognized by the different donors responding to a given antigenic region (Fig. 2*B*, *C*, 3*B*, *C*, and 4*C*, *D*), defined by the pattern of reactivity to the peptides overlapping by 14 amino acids. 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-$6_{14–28}$ is compared to 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-$6_{23–37}$ is compared to 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-$6_{22–29}$ (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 Fig. 2*D*, *E*, 3*D*, *E*, and 4*E*, *F*.

Taken together, these results define the minimal epitope core sequences contained within ESAT-6, CFP10, Rv2031c, and Rv1038c. Most importantly the data presented indicate that the immunodominant antigenic regions corresponded to the same core sequence, similarly recognized by different donors, rather than to multiple overlapping but distinct regions.

CD4 and HLA Class II restriction of selected epitopes

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. 5*A*, a total loss of peptide reactivity was observed in all of the $CD4^+$ T cell-depleted PBMC fractions, while $CD8^+$ 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 utilized. For this purpose, the capacity of antibodies specific for HLA-DR, DP or DQ to inhibit the epitope-specific response was tested (Representative data; Fig. 5*B*). 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 No. 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 (<100nM) or intermediate (<1000nM) 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-$6_{3–17}$, $CFP10_{40–54}$, $CFP10_{72–86}$, $Rv2031c_{106–120}$, and $Rv1038c_{28–42}$), 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-$6_{70–84}$, $Rv2031c_{111–125}$). These data suggest that promiscuous recognition in the context of multiple HLA class II molecules maybe a mechanism significantly contributing to epitope immunodominance.

Conversely, in the case of 3 other epitopes (ESAT-$6_{18–32}$, ESAT-$6_{73–87}$, 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, 4 out of 5 donors expressing DRB5*01:01 responded to $CFP10_{52–66}$, and 3 out of 5 donors expressing DQB1*03:01 responded to ESAT-$6_{72–87}$, as listed in the “no. donors responding” column (Table II), thus further strengthening the proposed restrictions.

On average each of these three single locus restricted peptides bound 6.3 (±1.2) alleles. By contrast, the promiscuously restricted peptides bound 12.3 (±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 while they are selectively restricted by one HLA class II molecule, they are frequently and strongly recognized in the context of that particular HLA molecule.

Phenotypic characterization of selected epitopes

In order 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 $CFP10_{52–66,}$ the experiments above indicated DRB5*01:01 as the restriction element. In the case of the promiscuous epitope $CFP10_{40–54}$, DRB1*01:01 was indicated as one of the likely restricting elements, since 3 of 4 donors that responded express DRB1*01:01 and $CFP10_{40–54}$ 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 utilized (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 3 donors at frequencies 0.1 to 0.5% (Mean of 0.33 SD±0.23) above background for DRB1*01:01 $CFP10_{40–54}$ and in 6 donors at frequencies 0.1 to 10% (Mean of 2.54 SD±3.97) above background for DRB5*01:01 $CFP10_{52–66}$. Only a small number of background tetramer-positive 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 $CFP10_{52–66}$ (Fig. 6*A*),

which confirmed that tetramer specificity was derived from the epitope and HLA molecule combination.

The memory subset phenotype was addressed using antibodies to CD45RA and CCR7 (37–39). As shown in Fig. 6*B* and *C*, DRB1*01:01 $CFP10_{40–54}$ epitope-specific tetramer-positive $CD4^+$ 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 naïve ($CCR7^+CD45RA^+$) or effector T cells ($CCR7^+CD45RA^+$). Similarly for the DRB5*01:01-$CFP10_{52–66}$ response, epitope-specific tetramer-positive $CD4^+$ 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 naïve ($CCR7^+CD45RA^+$) or effector T cells ($CCR7^-CD45RA^+$) (Fig. 6*C*).

Discussion

While 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*, despite the fact that 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 antigens equally recognized with similar prevalence (response frequency) and magnitude of responses, or does significant variation exist from one antigen to the next? 2) If significant variability exists, is it possible to identify additional antigens that might enhance the antigenic composition of the IGRAs? 3) What are the molecular features of antigen recognition at the population level, i.e. what determines which antigens 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 tetrameric 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 utilized TB antigens 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. While direct *ex vivo* responses to ESAT-6 and/or CFP10 have been investigated previously (9, 11, 12, 40, 41), most studies utilized 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 antigen 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 utilized short-term T cell cultures (13, 14), which could explain the discrepancy between this study and earlier work, and highlights the necessity of revisiting

antigen reactivity with approaches more closely reflective of the techniques used in the assays, i.e. *ex vivo* detection of IFNγ.

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

This study provides, to the best of our knowledge, the first actual molecular HLA binding affinity data for these commonly utilized TB antigens 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 which might play a role in antigen recognition are the function of the protein, expression levels, stage of expression, antigen presentation etc., factors which 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 antigens. 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 antigens 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 antigens and epitopes to make room for highly prevalent epitopes derived from additional antigens. A substantial number of LTBI patients are negative for the IGRA antigens. This is consistent with the fact that IGRA is by necessity a cocktail of different antigens. Our results further emphasize that an approach based on inclusion of only one of the IGRA antigens is not feasible and rather suggest that inclusion of additional epitopes and antigens 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 utilized 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 antigens are a minor component of the *ex vivo* IFNγ response measured by IGRAs, and is consistent with CD8 restricted epitopes for ESAT-6 and CFP10 having been defined using T cell lines (5, 11, 49). Antigen-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 predominantly effector memory cells, $CD45RA^-CCR7^-$ (37–39), which is consistent with previous studies in HIV-positive 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), as there is no accurate test for antigen 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 antigens 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 previously described (54). It would perhaps be unlikely to detect central memory cells in PBMC samples, as 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 reactivation. In conclusion, these data demonstrate how the epitopes identified following the approaches described herein can be used to develop tetrameric reagents and suggest that definition and production of cocktails 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 utilized TB antigens shows that different antigens are recognized with drastically different prevalence (response frequency) and magnitude of responses. The recognition of Rv2031c also suggests that it is possible to identify additional antigens that might enhance the antigenic composition of IGRAs. Furthermore, a limited number of epitopes are recognized in the context of multiple donors (prevalent) or even across different HLAs (promiscuous), suggesting that eliminating poorly or not recognized antigens and epitopes to make room for highly prevalent epitopes derived from additional antigens 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this article

TB	tuberculosis
ESAT-6	early secreted antigenic target 6kDa
CFP10	culture filtrate protein 10kDa
IGRA	interferon gamma release assay
LTBI	latent tuberculosis infection
SFC	spot forming cell
T_{EM}	T effector memory

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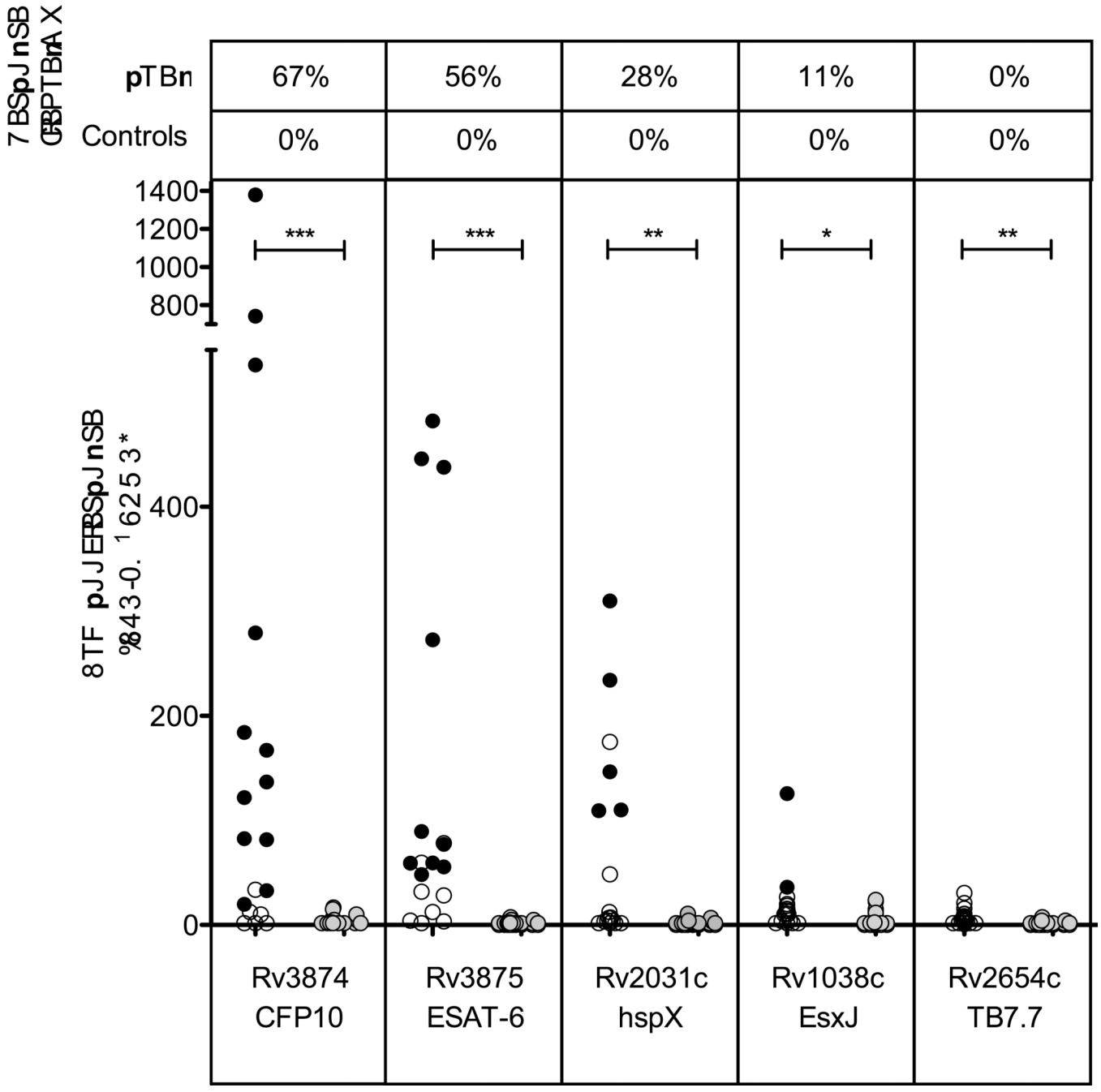
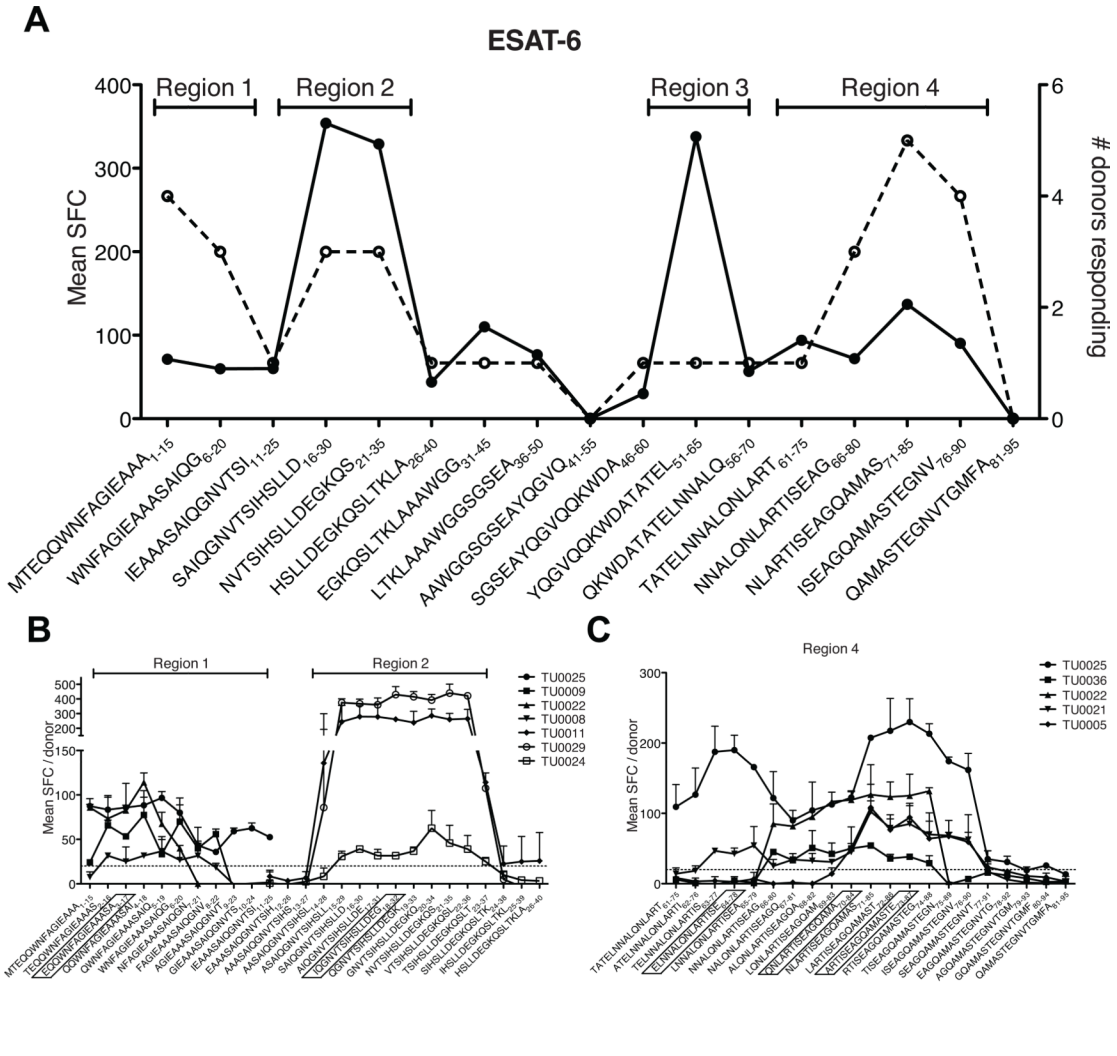


FIGURE 1. Differential *ex vivo* responses to commonly utilized TB antigens

PBMC from either LTBI donors (black (positive responses) and white (negative responses) dots) or control donors (grey dots) were incubated with 1μg/ml pools of peptides from indicated proteins, after which the number of IFNγ-producing cells were enumerated in an ELISPOT assay. Top panel shows response frequency in LTBI and control donors to Rv3874 (CFP10), Rv3875 (ESAT-6), Rv2031c (hspX), Rv1038c (EsxJ) and Rv2654c (TB7.7). Graph shows sums of pool responses (SFC/10^6 PBMC) per antigen for LTBI vs. control donors for indicated antigens. Pool responses were averaged from at least three independent experiments per donor and then summed to calculate response per antigen. For

each individual experiment pools were tested in triplicate. *** $p<0.0001$, ** $p<0.01$, * $p<0.05$ (One-tailed Mann-Whitney test).



D

Region 1 and 2		
Donor	**Optimal epitope**	**Sequence**
TU0008	ESAT-6 $_{3-17}$	EQQ**WNFAGIEAAAS**A
TU0009	ESAT-6 $_{3-17}$	EQQ**WNFAGIEAAAS**A
TU0022	ESAT-6 $_{3-17}$	EQQ**WNFAGIEAAAS**A
TU0025	ESAT-6 $_{3-17}$	EQQ**WNFAGIEAAAS**A
TU0011	ESAT-6 $_{18-32}$	IQGN**VTSIHSLL**DEG
TU0024	ESAT-6 $_{18-32}$	IQGN**VTSIHSLL**DEG
TU0029	ESAT-6 $_{18-32}$	IQGN**VTSIHSLL**DEG

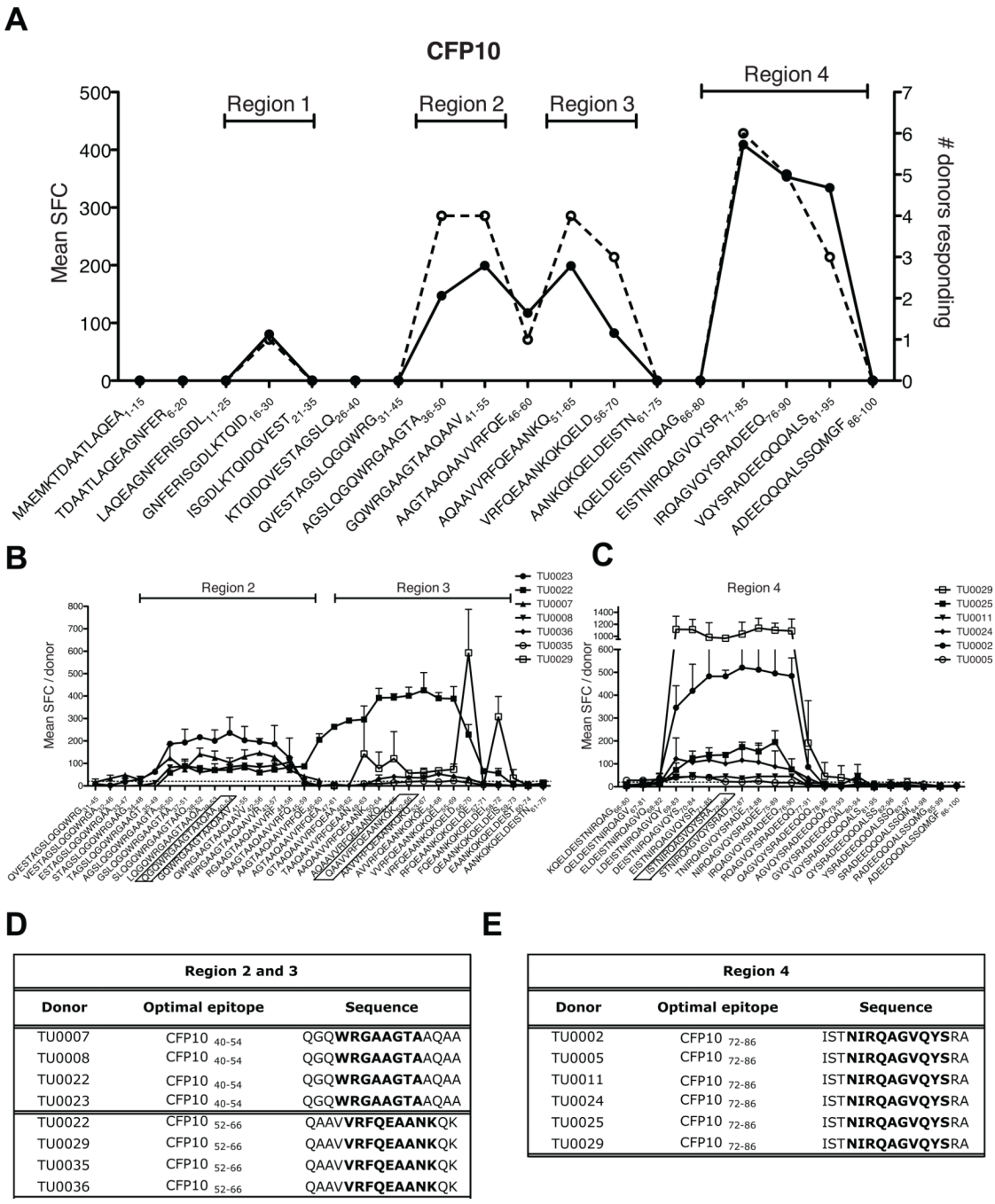
E

Region 4		
Donor	**Optimal epitope**	**Sequence**
TU0021	ESAT-6 $_{64-78}$	E**LNNALQNLARTIS**E
TU0025	ESAT-6 $_{64-78}$	E**LNNALQNLARTIS**E
TU0022	ESAT-6 $_{70-84}$	QNLA**RTISEAG**QAMA
TU0036	ESAT-6 $_{70-84}$	QNLA**RTISEAG**QAMA
TU0005	ESAT-6 $_{73-87}$	ART**ISEAGQAMAS**TE
TU0021	ESAT-6 $_{73-87}$	ART**ISEAGQAMAS**TE
TU0025	ESAT-6 $_{73-87}$	ART**ISEAGQAMAS**TE

FIGURE 2. Determination and characterization of antigenic regions from ESAT-6

PBMC from LTBI donors were incubated with 10μg/ml peptides from ESAT-6, and the number of IFNγ-producing cells was enumerated in an ELISPOT assay. ESAT-6 has four antigenic regions, indicated by capped lines. The number of donors tested for each region were n=7 for region 1 and 2, n=5 for region 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-mers 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-mers 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* and *C*). Selected epitopes identified from Region 1, Region 2 and Region 4, showing donors responding, position in protein and sequence. Minimal epitope core sequence is highlighted in bold (*D* and *E*).



D

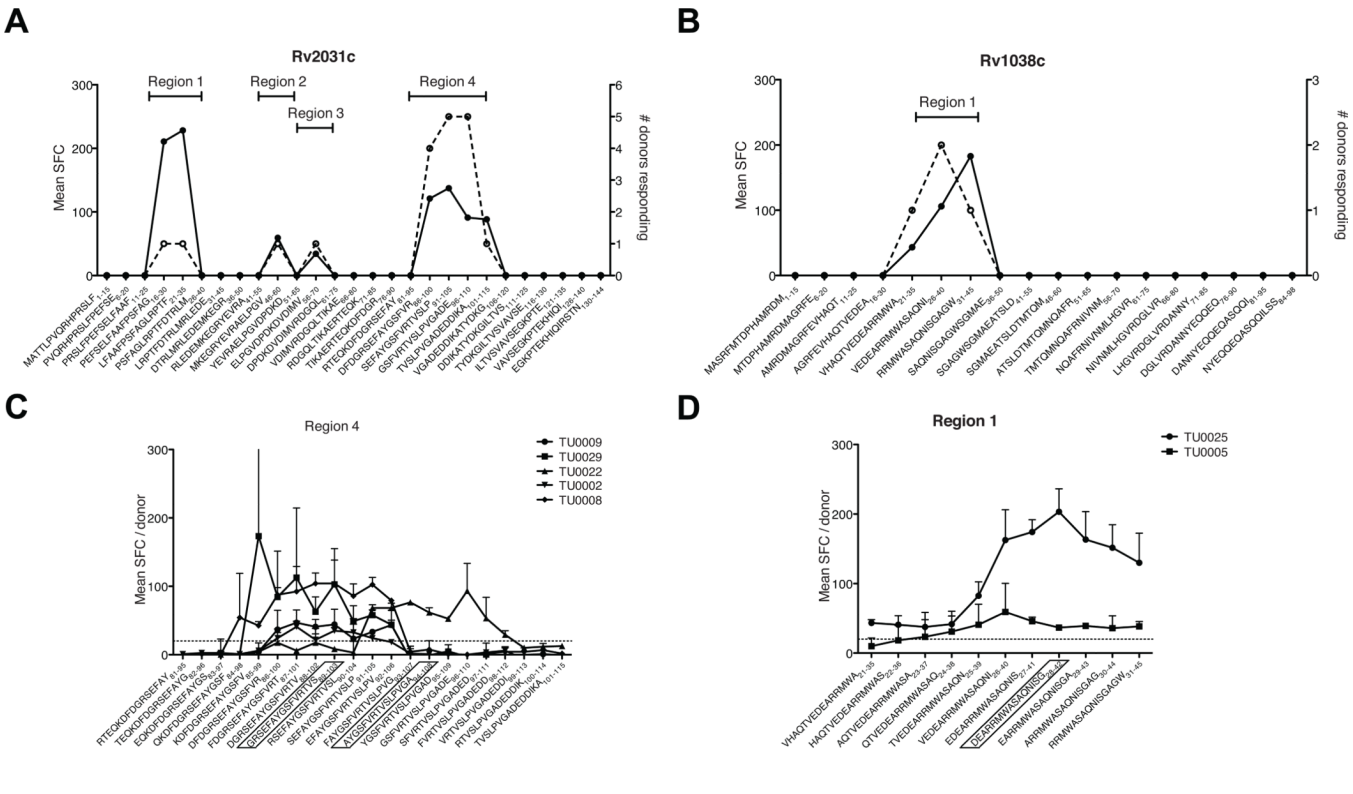
Region 2 and 3		
Donor	**Optimal epitope**	**Sequence**
TU0007	CFP10 $_{40-54}$	QGQ**WRGAAGTA**AQAA
TU0008	CFP10 $_{40-54}$	QGQ**WRGAAGTA**AQAA
TU0022	CFP10 $_{40-54}$	QGQ**WRGAAGTA**AQAA
TU0023	CFP10 $_{40-54}$	QGQ**WRGAAGTA**AQAA
TU0022	CFP10 $_{52-66}$	QAAV**VRFQEAANK**QK
TU0029	CFP10 $_{52-66}$	QAAV**VRFQEAANK**QK
TU0035	CFP10 $_{52-66}$	QAAV**VRFQEAANK**QK
TU0036	CFP10 $_{52-66}$	QAAV**VRFQEAANK**QK

E

Region 4		
Donor	**Optimal epitope**	**Sequence**
TU0002	CFP10 $_{72-86}$	IST**NIRQAGVQYS**RA
TU0005	CFP10 $_{72-86}$	IST**NIRQAGVQYS**RA
TU0011	CFP10 $_{72-86}$	IST**NIRQAGVQYS**RA
TU0024	CFP10 $_{72-86}$	IST**NIRQAGVQYS**RA
TU0025	CFP10 $_{72-86}$	IST**NIRQAGVQYS**RA
TU0029	CFP10 $_{72-86}$	IST**NIRQAGVQYS**RA

FIGURE 3. Determination and characterization of antigenic regions from CFP10

For description see legend for Fig. 2. CFP10 has four antigenic regions, indicated by capped lines (n=5 for region 1 and 2, n=9 for region 3 and 4) (*A*). Minimal epitope core sequence within each antigenic region for CFP10 (*B* and *C*). Selected epitopes identified from Region 2, Region 3 and Region 4, showing donors responding, position in protein and sequence. Minimal epitope core sequence is highlighted in bold (*D* and *E*).



E

Region 4		
Donor	**Optimal epitope**	**Sequence**
TU0002	Rv2031c $_{106-120}$	GRS**EFAYGSFVR**TVS
TU0008	Rv2031c $_{106-120}$	GRS**EFAYGSFVR**TVS
TU0009	Rv2031c $_{106-120}$	GRS**EFAYGSFVR**TVS
TU0029	Rv2031c $_{106-120}$	GRS**EFAYGSFVR**TVS
TU0022	Rv2031c $_{111-125}$	AYG**SFVRTVSLP**VGA

F

Region 1		
Donor	**Optimal epitope**	**Sequence**
TU0005	Rv1038c $_{28-42}$	DEA**RRMWASAQNI**SG
TU0025	Rv1038c $_{28-42}$	DEA**RRMWASAQNI**SG

FIGURE 4. Determination of minimal epitopes from Rv2031c and Rv1038c

For description see legend for Fig. 2. Rv2031c has four antigenic regions (n=1 for region 1, n=3 for region 2 and 3 and n=5 for region 4) and Rv1038c has one antigenic region (n=2), indicated by capped line (*A* and *B*). Minimal epitope core sequence within each antigenic region for Rv2031c and Rv1038c (*C* and *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* and *F*).

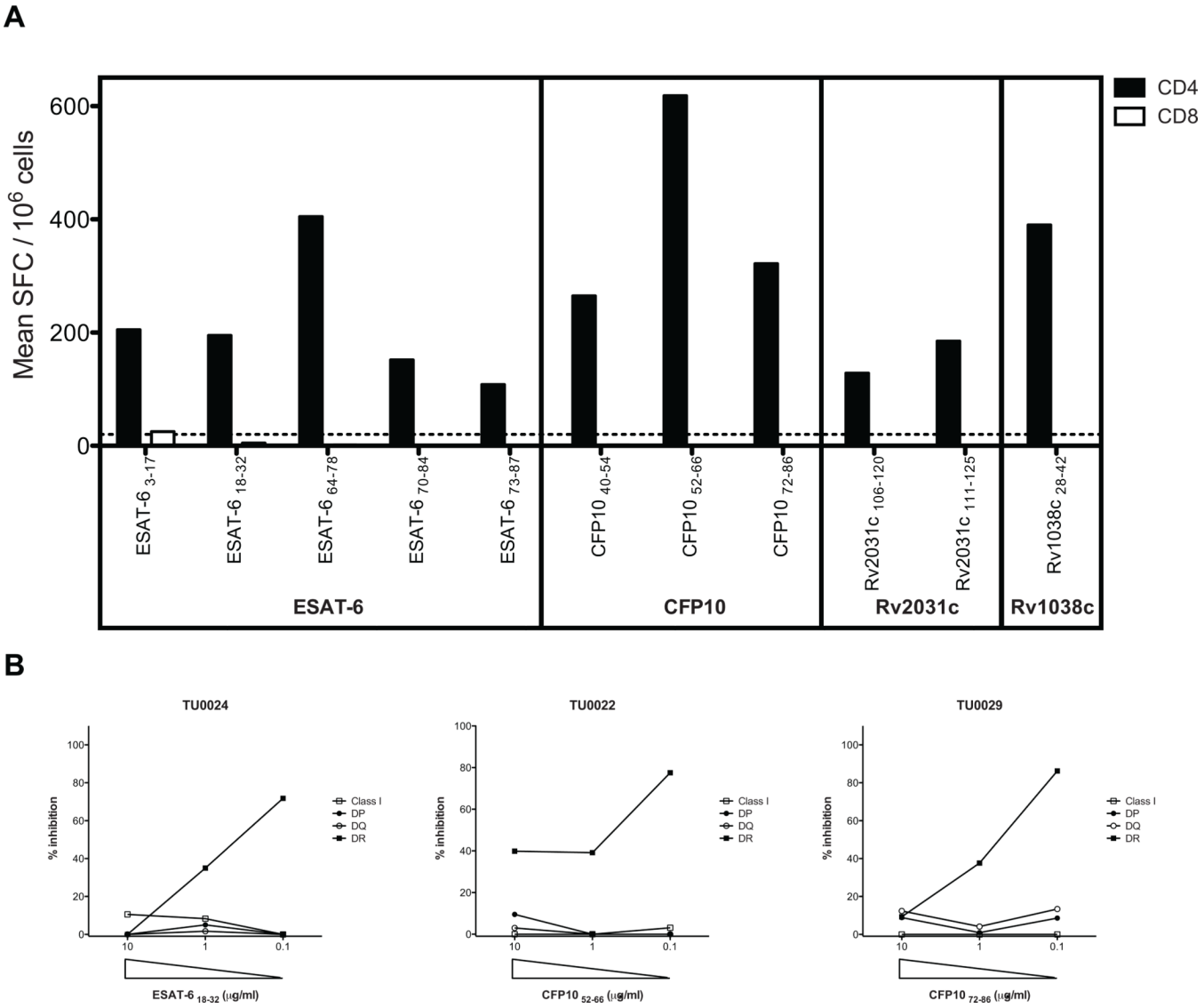


FIGURE 5. Characterization of epitopes

CD4 T cells are the dominant cell type responding to tested epitopes. PBMCs were depleted of $CD8^+$ (black columns) or $CD4^+$ (empty columns) T cells and were incubated with 10μg/ml of indicated peptides and the number of IFNγ-producing cells was enumerated in an ELISPOT assay. Shown are the net SFC/10^6 cells for representative donors for at least two independent experiments. Each individual peptide was tested in triplicate. The dotted line indicates the 20 net SFC/10^6 cells threshold used to define positivity (*A*). Determination of restricting locus. Purified $CD4^+$ T cells and remaining PBMC cells (2:1 ratio) were incubated with antibodies against HLA-DR, DP, DQ or pan MHC Class I and stimulated with peptide at indicated concentrations. The number of IFNγ producing cells was determined by ELISPOT. Shown are percentage inhibitions by antibodies at different peptide concentrations for representative donors (*B*).

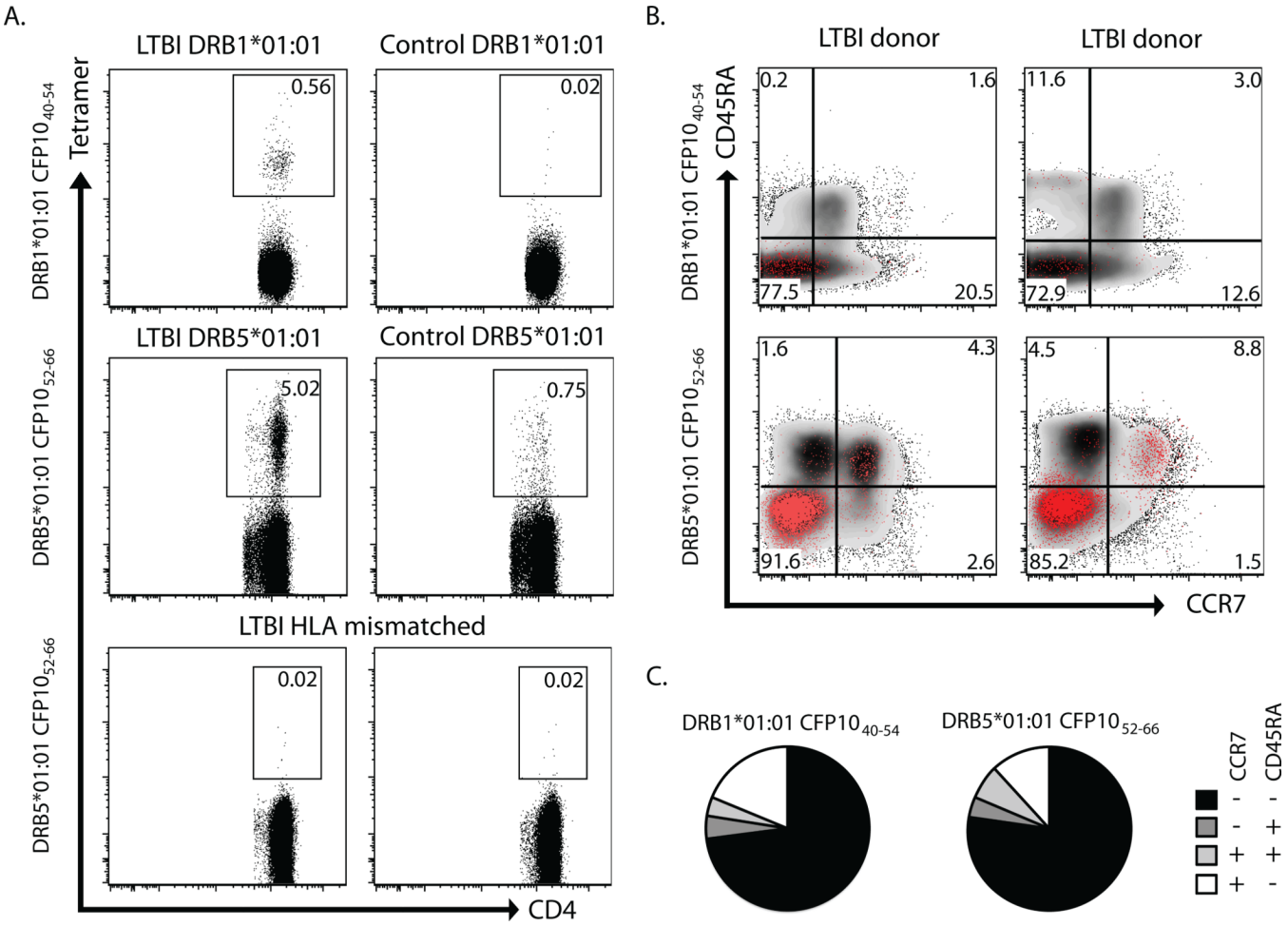


FIGURE 6. HLA restriction and memory phenotype of CFP10-specific CD4$^+$ T cells using MHC Class II tetramers

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-positive cells were isolated following magnetic bead enrichment. Plots are gated on CD4$^+$ T cells and the numbers indicate the percentages tetramer-positive cells isolated from each donor's CD4$^+$ population. DRB1*01:01 CFP10$_{40–54}$ n=3 LTBI donors and DRB5*01:01 CFP10$_{52–66}$ n=6 LTBI donors (*A*). Memory phenotype of tetramer-positive 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-positive 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 CFP10$_{40–54}$ (n=3) and DRB5*01:01 CFP10$_{52–66}$ (n=6). Effector memory T cells are CCR7$^-$ and CD45RA$^-$, central memory T cells are CCR7$^+$ and CD45RA$^-$, naïve T cells are CCR7$^+$ and CD45RA$^+$ and effector T cells are CCR7$^-$ and CD45RA$^+$ (*C*).

Table I

Summary of number HLA molecules bound by 15-mers overlapping by 10 amino acids covering ESAT-6, CFP10, Rv2031c, Rv2654c and Rv1038c

Antigen	Total no. peptides	No. peptides binding ≥20% of alleles
CFP10	18	9
ESAT-6	17	9
Rv2031c	27	14
Rv1038c	18	9
Rv2654c	15	11

Table II

Summary of epitope characteristics

Donor HLA type and HLA binding pattern										
Epitope		**Donor**	**SFC[1]**	**Restricting HLA locus**	**DRB1/3/4/5, DQB1, DPB1**				**Inferred restricting HLA allele**	**No. donors expressing allele resp.**
PROMISCUOUS	ESAT-6 $_{3–17}$	TU25	85	**DQ, DR**	DRB1*08:04	DQB1*02:01	DQB1*03:19			
		TU9	53	**DR**	DRB1*01:01	DRB1*08:02				
		TU22	82	**DQ**	DQB1*06:02					
		TU8	25	undet.	DRB1*01:01	DRB1*08:02	DQB1*04:02			
	CFP10 $_{40–54}$	TU23	236	**DR**	DRB1*01:01	DRB1*11:01	DRB3*02:02			
		TU7	103	**DP, DQ, DR**	DRB1*01:01	DRB1*08:02	DQB1*04:02			
		TU22	70	**DP**	DPB1*124:01					
		TU8	85	**DR**	DRB1*01:01	DRB1*08:02				
	CFP10 $_{72–86}$	TU24	101	**DR**	DRB1*04:11	DRB1*04:03	DRB4*01:03	DRB4*01:03		
		TU5	23	undet.	DRB1*11:02	DRB1*13:04	DRB3*03:02	DRB3*03:02		
					DQB1*03:01	DQB1*03:19	DPB1*02:01	DPB1*17:01		
		TU2	483	**DP, DQ, DR**	DRB1*04:02	DRB1*08:02	DRB4*01:03			
		TU25	139	**DQ**	DQB1*03:19					
		TU11	45	**DP, DQ, DR**	DRB1*04:10	DRB1*15:01	DRB4*01:03	DRB5*01:01		
					DQB1*06:02	DPB1*03:01				
		TU29	968	**DR**	DRB1*04:11	DRB1*15:02	DRB4*01:03	DRB5*01:02		
	Rv2031c $_{106–120}$	TU2	35	**DP, DR**	DRB1*04:02	DRB1*08:02	DPB1*04:01			
		TU9	44	**DP**	DPB1*04:01					
		TU8	104	**DP, DQ, DR**	DRB1*01:01	DRB1*08:02	DPB1*04:01			
		TU29	103	**DP, DR**	DRB1*04:11	DRB1*15:02	DRB5*01:02	DPB1*02:01		
	Rv1038c $_{28–42}$	TU5	37	**DP, DQ**	DQB1*03:01	DQB1*03:19	DPB1*02:01	DPB1*17:01		
		TU25	203	**DP**	DPB1*01:01	DPB1*17:01				
SINGLE HLA	ESAT-6 $_{70–84}$	TU36	50	**DP**	DPB1*03:01	DPB1*18:01				
		TU22	119	**DP**	DRB1*07:01	DRB4*01:03	DPB1*124:01			
	Rv2031c $_{111–125}$	TU22	62	**DR**	DRB1*07:01	DRB1*15:03	DRB5*01:01			

Donor HLA type and HLA binding pattern										
Epitope		**Donor**	**SFC[1]**	**Restricting HLA locus**	**DRB1/3/4/5, DQB1, DPB1**				**Inferred restricting HLA allele**	**No. donors expressing allele resp.**
	ESAT-6 $_{18–32}$	TU24	31	**DR**	DRB1*04:11	DRB1*04:03	DRB4*01:03	DRB4*01:03	DRB4*01:03	3 of 8
		TU11	261	**DR**	DRB1*04:10	DRB1*15:01	DRB4*01:03		DRB4*01:03	
		TU29	430	**DR**	DRB1*04:11	DRB1*15:02	DRB4*01:03		DRB4*01:03	
	ESAT-6 $_{73–87}$	TU21	85	**DQ**	DQB1*03:19				DQB1*03:19	3 of 5
		TU5	94	**DQ**	DQB1*03:19	DQB1*03:01			DQB1*03:01[2], DQB1*03:19	
		TU25	230	**DQ**	DQB1*03:19				DQB1*03:19	
	CFP10 $_{52–66}$	TU36	37	**DR**	DRB1*15:01	DRB5*01:01			DRB5*01:01	4 of 5
		TU35	18	undet.	DRB1*09:01	DRB1*15:01	DRB4*01:01	DRB5*01:01	DRB5*01:01	
					DPB1*01:01	DPB1*14:01				
		TU22	401	**DR**	DRB1*15:03	DRB4*01:03	DRB5*01:01		DRB5*01:01	
		TU29	56	**DR**	DRB1*04:11	DRB1*15:02	DRB4*01:03	DRB5*01:02	DRB5*01:02[2]	
UNDET.	ESAT-6 $_{64–78}$	TU25	190	undet.	DRB1*08:04	DQB1*03:19	DPB1*01:01	DPB1*17:01		
		TU21	43	undet.	DRB1*08:04	DRB1*12:01	DRB3*02:07	DQB1*03:19		
					DPB1*18:01					

Binding

STRONG	(<100nM)
INTERMEDIATE	(100-999nM)
NOT AVAILABLE	-

[1] Mean SFC/10^6 PBMC, average of 2 independent experiments

[2] Variant alleles that have an identical peptide binding region to an allele in the assay panel and the inferred restricting allele