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Human Leukocyte Antigens A*3001 and A*3002 Show Distinct Peptide-Binding Patterns of the *Mycobacterium tuberculosis* Protein TB10.4: Consequences for Immune Recognition▽†

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High-tuberculosis (TB)-burden countries are located in sub-Saharan Africa. We examined the frequency of human leukocyte antigen (HLA) alleles, followed by recombinant expression of the most frequent HLA-A alleles, i.e., HLA-A*3001 and HLA-A*3002, to study differences in mycobacterial peptide presentation and $CD8^+$ T-cell recognition. We screened a peptide library (9-mer peptides with an 8-amino-acid overlap) for binding, affinity, and off-rate of the *Mycobacterium tuberculosis*-associated antigen TB10.4 and identified only three TB10.4 peptides with considerable binding to HLA-A*3001. In contrast, 22 peptides bound to HLA-A*3002. This reflects a marked difference in the binding preference between the two alleles, with A*3002 tolerating a more promiscuous peptide-binding pattern and A*3001 accommodating only a very selective peptide repertoire. Subsequent analysis of the affinity and off-rate of the binding peptides revealed a strong affinity (8 nM to 7 μM) and moderate off-rate (20 min to 3 h) for both alleles. Construction of HLA-A*3001 and HLA-A*3002 tetramers containing selected binding peptides from TB10.4, including a peptide which was shared among both alleles, QIMYNYPAM ($TB10.4_{3–11}$), allowed us to enumerate epitope-specific T cells in HLA-A*3001- and HLA-A*3002-typed patients with active TB. HLA-A*3001 and HLA-A*3002 major histocompatibility complex-peptide complexes were recognized in individuals with active TB, irrespective of their homozygous HLA-A*3001 or HLA-A*3002 genetic background. The antigen-specific T cells exhibited the $CD45RA^+$ $CCR7^+$ precursor phenotype and the interleukin-7 receptor (CD127), which were different from the phenotype and receptor exhibited by the parental $CD8^+$ T-cell population.

Tuberculosis (TB) is a major worldwide problem, with approximately 10 million individuals being newly infected and 2 million deaths occurring every year. The highest TB incidences are found in sub-Saharan Africa and Southeast Asia (10, 24). This calls for a better understanding of the immune response directed against *Mycobacterium tuberculosis* in blood samples obtained from individuals of African descent. This may aid with the development of new and better diagnostics as well as the identification of biomarkers, which will help to monitor novel TB vaccine candidates, since currently, only large and costly phase II and III clinical trials can define the effectiveness of vaccines in African countries with a high TB burden. Dissection of the biology in major histocompatibility complex (MHC)-peptide presentation will contribute to a better profiling of successful immune responses, i.e., the responses in individuals who have been exposed yet who appear to be protected from TB, and less successful responses in individuals with latent or active TB (24).

Many parts of the immune system play an important roles in *M. tuberculosis*-directed responses (16). The adaptive anti-*M. tuberculosis* immune response is dependent on presentation of disease-associated immunogenic peptides via the MHC molecules to T-cell receptors (TCRs) from $CD4^+$ and $CD8^+$ T cells (52). T-cell recognition of a peptide derived from a protein in the presence of a costimulatory signal leads to T-cell activation and proliferation. Identification of novel antigenic peptides recognized by $CD8^+$ T cells and presented via the trimeric, heavy-chain β_2-microglobulin peptide-MHC class I complex (28) will therefore be of great value to better understand the cytotoxic T-lymphocyte (CTL)-mediated anti-*M. tuberculosis* immune response.

MHC class I is the most polymorphic molecule in the human body, with over 2,000 different alleles identified in humans to date (39). The majority of the polymorphism lies within exons 2 and 3, coding for the peptide-binding pocket of the MHC class I molecule. Most of these alleles are associated with different peptide-binding preferences, summarized as a peptide-binding motif (14). The allele distribution and frequency differ considerably between ethnic populations, and therefore, our efforts to increase our knowledge of the disease-related epitopes associated with population-relevant, different MHC class I molecules and to understand the cellular immune response in individuals of different ethnic backgrounds need to include the most frequent alleles in individuals of African descent. The human leukocyte antigen (HLA)-A*30 family is

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one of the most frequent allele families in individuals from an African background. It consists of 31 alleles, of which the most common are HLA-A*3001 and HLA-A*3002 (9, 34). Individual MHC class I alleles may have an impact on disease susceptibility, and differences may guide vaccination efforts, as has been described for the antigen-specific (melanoma antigen recognized by T cells 1 [MART-1]) immunization of patients with melanoma: peptide vaccination elicits cellular immune responses in HLA-A*0202, -A*0204, -A*0205, and -A*0207 but not in individuals with HLA-A*0209 (15).

Although only 4 amino acids situated in the peptide-binding pocket differentiate between HLA-A*3001 and HLA-A*3002, preferences in peptide selection have been observed (26, 27). MHC class I-binding peptides have been identified for HLA-A*3001 (such as peptides that originated from vaccinia virus) and for HLA-A*3002 (e.g., peptides originating from *Plasmodium falciparum* and the severe acute respiratory syndrome-associated coronavirus) (12, 50). Regarding TB, four peptides presented by HLA-A*3001 have been identified: RPKPD YSAM and RVRQAWDTL from a hypothetical protein, RA WGRRLMI from antigen 85B (Ag85B), and RGRIGRTYL from glucose dehydratase (50) Thus far, no *M. tuberculosis* epitopes have been identified for HLA-A*3002, in part due to the lack of detailed knowledge concerning the peptide-binding motifs and the inability to perform affinity and off-rate analyses.

We chose *M. tuberculosis* protein TB10.4 (RV0288) as a paradigm, since it is a component of several novel candidate vaccines against TB (11, 37). TB10.4 represents an early secreted virulence factor and is present in virulent *M. tuberculosis* strains and in the *M. bovis* bacillus Calmette-Guérin (BCG) vaccine strains (48).

Here, we used recombinant MHC molecules to screen a peptide library from TB10.4 for binding affinity and off-rate using the African MHC class I alleles HLA-A*3001 and HLA-A*3002. Novel binding peptides were characterized by affinity and off-rate studies, and subsequent tetramer constructs were used to visualize ex vivo antigen-specific $CD8^+$ T cells in patients with acute pulmonary TB.

MATERIALS AND METHODS

Cloning. Bacterial expression vectors (pET24d+ and pHN1) containing the nucleotide sequences for the soluble part of the heavy chain of the MHC class I allele HLA-A*3002 and the light-chain β_2-microglobulin were obtained from Beckman Coulter, San Diego, CA. The gene for HLA-A*3001 was obtained by altering the HLA-A*3002 sequence using a site-directed mutagenesis kit (Stratagene, La Jolla, CA). The mutations c282g, a299t, a301g, and c526t were made, which created HLA-A*3001.

Recombinant proteins. Recombinant MHC class I molecules were produced as described before (1, 17). Briefly, the heavy and light chains were produced as inclusion bodies in *Escherichia coli* BL21(DE3)pLys (Invitrogen, Carlsbad, CA) and solubilized in an 8 M urea buffer, pH 6.5 (Sigma-Aldrich Sweden AB, Stockholm, Sweden). MHC class I heavy and light chains were folded with an allele-specific peptide (JPT Peptide Technologies GmbH, Berlin, Germany) for 3 days in 100 mM Tris–400 mM arginine–5 mM EDTA buffer, pH 8.0. The peptides KTKDIVNGL (F-actin-capping protein beta) for A*3001 and KIQNF RVYY (human immunodeficiency virus [HIV] integrase) for A*3002 were used (26, 40). Folded MHC class I-peptide monomers were concentrated using a stirred cell device (Millipore, Billerica, MA) and were biotinylated using the enzyme BirA (Avidity, Aurora, IL). The biotinylated monomers were concentrated and affinity purified using an avidin column (Thermo Fisher Scientific, Rockford, IL).

Epitope screening assay (i) Coating of plates. Ninety-six-well plates (Greiner-Bio One GmbH, Frickenhausen, Germany) were first coated with biotinylated bovine serum albumin (BSA), followed by avidin (from Beckman Coulter). After the plates were blocked with 1% BSA, they were coated with 0.5 μg/ml biotinylated monomer.

(ii) Binding assay. Nonamer peptides overlapping by 8 amino acids (aa) covering the whole TB10.4 sequence (total, 88 peptides) were synthesized by JPT Peptide Technologies GmbH. Peptide-binding, affinity, and off-rate experiments were performed in duplicate in the MHC monomer-coated plates as reported before (5, 51). Monomer-coated plates were stripped of the placeholder peptide, leaving the heavy chain free to associate with a candidate peptide after addition of β_2-microglobulin. Peptide binding was detected as fluorescence, after 18 h of incubation at 21°C with a conformational HLA-A30-specific antibody (Ab; IgM, clone 0273HA; One Lambda Inc., Canoga Park, CA) and a fluorescence-labeled secondary antibody (goat anti-mouse IgM-Alexa Fluor 488, clone A21042; Invitrogen). The binding of each candidate peptide was compared with the binding of an appropriate control peptide (KTKDIVNGL for A*3001 and KIQNF RVYY for A*3002). A more detailed analysis of the binding characteristics of each individual peptide was performed using affinity and off-rate assays.

(iii) Off-rate assay. The stability of the trimeric heavy-chain β_2-microglobulin–peptide complex was analyzed by incubating the refolded MHC molecules at 37°C for eight different times (0 h, 0.5 h, 1 h, 1.5 h, 2 h, 4 h, 6 h, and 8 h). The off-rate is expressed as a half-life ($t_{1/2}$) value, which is defined as the time point when 50% of the initial peptide concentration has dissociated from the HLA-peptide molecule complexes.

(iv) Affinity assay. The affinity is measured by incubating each MHC class I allele with different concentrations (10^{-4} to 10^{-9} M) of each individual peptide overnight and then calculating the peptide quantity needed to achieve 50% binding saturation (the 50% effective dose [ED_{50}] value).

(v) Calculations. The values of peptide binding, affinity, and off-rate were calculated using iTopia system software. Sigmoidal dose-response curves were generated using Prism (version 4.0) software (GraphPad).

Cellular analysis. A total of 359 healthy individuals from Cape Town, South Africa, volunteered to participate in the study to determine MHC frequency. Peripheral blood mononuclear cells (PBMCs) from individuals were two-digit MHC class I typed at the University Blood Bank, University of Mainz. From these, 19 HLA-A*30-positive samples were selected and subsequently four-digit typed at the Blood Bank, Karolinska University Hospital, Huddinge, Sweden.

PBMCs from patients diagnosed with active pulmonary tuberculosis were obtained from the University of Pretoria in Pretoria, South Africa, and institutional review board approval and informed consent were documented (reference no. 45/2008, Faculty of Health Sciences, University of Pretoria, South Africa, and from the ethical committee in Stockholm, Sweden, with reference number 2008/1208-31/3). The PBMCs were HLA typed using sequence-specific primer (SSP) typing kits (One Lambda Inc.). Four specimens positive for HLA-A*3001 and four positive for HLA-A*3002 were identified in 25 MHC class I-typed PBMC samples.

Positive MHC-binding peptides were used to construct recombinant MHC class I molecules. These molecules were subsequently used to prepare tetramers, as previously described (1). A tetramer (KTKDIVNGL) loaded with a self-epitope derived from F-actin-capping protein beta and an epitope (KIQNFR VYY) from the HIV type 1 (HIV-1) integrase were also constructed as control reagents. Most individuals were found to react to HLA-A*30–KTKDIVNGL–MHC–peptide complexes (up to 1% in $CD3^+$ $CD8^+$ T cells; data not shown). This reagent was therefore used as a positive-control tetramer; the HIV integrase epitope served as the negative tetramer. MHC class I monomers containing the selected epitopes were incubated together with fluorescence-labeled streptavidin at a ratio of 4:1 at room temperature for 30 min. The following six *M. tuberculosis* tetramers were constructed and labeled with streptavidin-phycoerythrin (PE) and streptavidin-allophycocyanin (APC): $A^*3001_{QIMYNYPAM}$-PE, $A^*3001_{LVRAYHAMS}$-PE, $A^*3002_{QIMYNYPAM}$-PE, $A^*3002_{QIMYNYPAM}$-APC, $A^*3002_{IMYNYPAML}$-PE, and $A^*3002_{AMEDLVRAY}$-PE.

Cellular analyses were performed using a fluorescence-activated cell sorter (Gallios flow cytometer; Beckman Coulter). Tetramer-positive events were identified in the $CD3^+$ $CD8^+$ $CD4^-$ lymphocyte population using the following Abs: CD3 peridinin chlorophyll protein (clone SP-34-2; Becton Dickinson, Franklin Lakes, NJ), CD8a Alexa Fluor 750 (clone T8), and CD4 Pacific Blue (clone SFC112T4D11) (Beckman Coulter). Cells in the $CD3^+$ $CD8^-$ $CD4^+$ compartment were excluded from enumeration of $CD3^+$ $CD8^+$, tetramer-positive events. Subsequent analyses of the nature of the tetramer-positive events were performed using the following Abs: CD127 Alexa Fluor 488 (clone HCD12; Nordic Biosite, Täby, Sweden), CD107a APC-Alexa Fluor 700 (clone H4A3), CD45RA phycoerythrin (PE)-Texas Red (ECD) (clone 2H4; Beckman Coulter), and CCR7 PE-cyanin 7 (PC7) (clone 3D12; Becton Dickinson). Statistical significance between different T-cell populations was evaluated as a matrix using Excel 2007 software (Microsoft, Redmond, WA) and the two-sided Student *t* test.

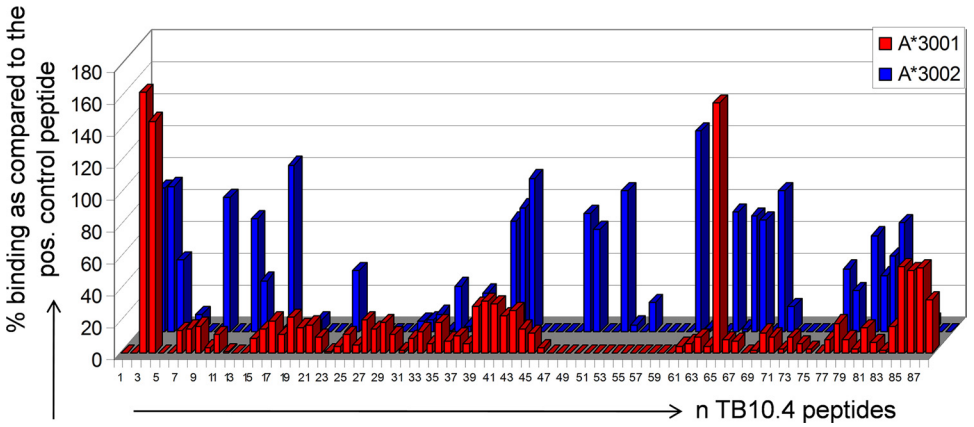

FIG. 1. MHC class I-binding peptides identified by binding to recombinant MHC class I molecules. The *x* axis indicates peptides 1 to 88 (the amino acid sequences of the positive binders can be found in Table 1), and the *z* axis indicates the percent binding for the respective peptides compared with that of an allele-specific positive-control peptide (KTKDIVNGL, A*3001; KIQNFRVYY, A*3002). Red, binding to HLA-A*3001; blue, binding to HLA-A*3002.

RESULTS

HLA prevalence. We analyzed the MHC class I allele frequency in PBMCs from the 359 healthy individuals from Cape Town, South Africa. The most common HLA-A serotypes were A*02 (30.1%), A*30 (28.1%), A*24 (18.7%), and A*68 (18.7%); the most common HLA-B serotypes were B*15 (27.6%), B*58 (20.6%), B*07 (18.1%), and B*44 (14.8%); and the most common HLA-Cw serotypes were Cw*07 (37.9%), Cw*04 (28.1%), Cw*06 (27.3%), and Cw*02 (17.3%) (see Table S1 in the supplemental material). For A*30-positive specimens, we subsequently performed four-digit typing to decipher the nature of the actual allele. The following percentages were identified in the HLA-A*30 cohort: A*3001, 53%; A*3002, 21%; A*3003, 0%; and A*3004, 26%. HLA-A*30 was also found to be the most frequent MHC class I allele in individuals with pulmonary TB (our unpublished data).

Identification of MHC-binding peptides from TB10.4. The 88 overlapping peptides from TB10.4 were tested for binding to two HLA molecules: HLA-A*3001 and HLA-A*3002. Peptides with binding 30% greater than that of an allele-specific positive-control peptide were considered candidate peptides. The peptide-binding pattern was very different between the two alleles, since only 3/88 (3.4%) peptides were identified to be binders to HLA-A*3001 and 22/88 (25%) were identified to be binders to HLA-A*3002 (Fig. 1 and 2 and Table 1): HLA-A*3001 exhibits a much more restricted peptide-binding pattern than HLA-A*3002.

The three HLA-A*3001-binding peptides QIMYNYPAM ($TB10.4_{3–11}$), IMYNYPAML ($TB10.4_{4–12}$), and LVRAY HAMS ($TB10.4_{65–73}$) exhibited high MHC-binding values, ranging from 147% to 163% of the value for the positive-control peptide. All peptides binding to HLA-A*3001 were also identified to bind to HLA-A*3002. We segregated the HLA-A*3002-binding peptides in two different groups: high binders (binding >70% of the level of binding for the positive control) and intermediate binders (binding 30% to 70% of the level of binding for the positive control). In the first group, we identified 16 peptides, with AMEDLVRAY ($TB10.4_{61–69}$) and DMAGYAGTL ($TB10.4_{17–25}$) showing even better binding than the positive control (126% and 105% of that for the positive control, respectively) (Table 1). In the second group, we identified six additional peptides. Positively binding peptides for this allele were found throughout the whole amino acid sequence of TB10.4.

Affinity and off-rate studies. The affinity of a peptide is defined as the binding strength between the nominal peptide

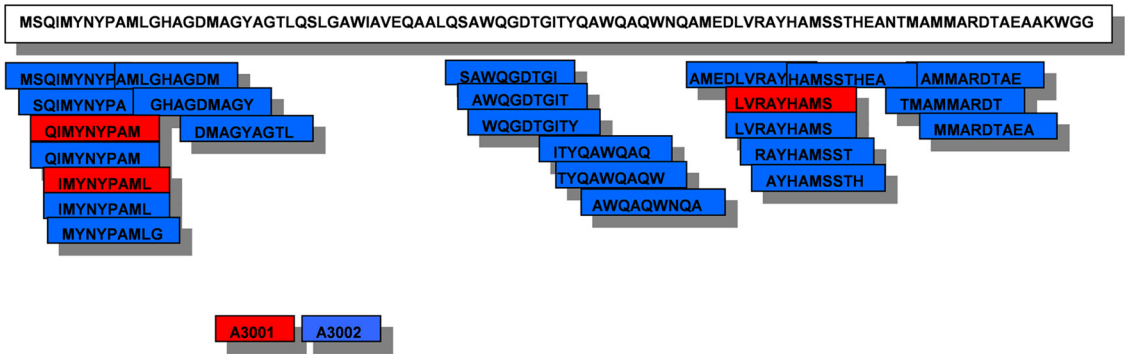

FIG. 2. Alignment of MHC class I-binding peptides within the amino acid sequence of TB10.4. Red, peptides identified for HLA-A*3001; dark blue, peptides identified for HLA-A*3002.

TABLE 1. MHC binding peptides in TB10.4

Peptide no.	Sequence	Peptide name	Relative binding (%) of the following HLA class I allele[a]:	
			A*3001	A*3002
1	MSQIMYNYP	$TB10.4_{1–9}$	0	**75**
2	SQIMYNYPA	$TB10.4_{2–10}$	0	*46*
3	QIMYNYPAM	$TB10.4_{3–11}$	**163**	**90**
4	IMYNYPAML	$TB10.4_{4–12}$	**146**	**91**
5	MYNYPAMLG	$TB10.4_{5–13}$	0	*45*
10	AMLGHAGDM	$TB10.4_{10–18}$	4	**84**
13	GHAGDMAGY	$TB10.4_{13–21}$	0	**71**
17	DMAGYAGTL	$TB10.4_{17–25}$	20	**105**
41	SAWQGDTGI	$TB10.4_{41–49}$	29	**70**
42	AWQGDTGIT	$TB10.4_{42–50}$	23	**78**
43	WQGDTGITY	$TB10.4_{43–51}$	26	**96**
49	ITYQAWQAQ	$TB10.4_{49–57}$	0	**74**
50	TYQAWQAQW	$TB10.4_{50–58}$	0	*64*
53	AWQAQWNQA	$TB10.4_{53–61}$	0	**88**
61	AMEDLVRAY	$TB10.4_{61–69}$	4	**126**
65	LVRAYHAMS	$TB10.4_{65–73}$	**157**	**75**
67	RAYHAMSST	$TB10.4_{67–75}$	7	**73**
68	AYHAMSSTH	$TB10.4_{68–76}$	0	**70**
70	HAMSSTHEA	$TB10.4_{70–78}$	13	**88**
80	TMAMMARDT	$TB10.4_{80–88}$	3	*60*
82	AMMARDTAE	$TB10.4_{82–90}$	7	*48*
83	MMARDTAEA	$TB10.4_{83–91}$	2	*68*

[a] Peptides with a relative binding of >30% of that for the positive controls are marked as follows: boldface numbers indicate relative binding of >70% of that for the positive controls, and italic numbers indicate binding of between 30 and 69% of that for the positive controls.

and the MHC molecule. For HLA-A*3001, the affinities ranged from 2 μM to 6 μM, and for HLA-A*3002 they ranged from 8 nM to 7 μM. Generally, affinities were higher for peptides binding to HLA-A*3002 than for peptides binding to HLA-A*3001 (Fig. 3 and Table 1). This was also found to be true concerning three peptides that bound to both HLA-A*3001 and HLA-A*3002 but which exhibited a higher affinity toward HLA-A*3002 (4 μM, 5 μM, and 2 μM for HLA-A*3001 versus 40 nM, 8 nM, and 2 μM for HLA-A*3002, respectively) (Table 2).

The dissociation rate was moderately fast and varied from 30 min to 1.8 h for the HLA-A*3001 allele. HLA-A*3002 had a slightly wider range of off-rates, with peptide SQIMYNYPA ($TB10.4_{2–10}$) dissociating most rapidly with a $t_{1/2}$ of only 20 min and with peptide IMYNYPAML ($TB10.4_{4–12}$) having a $t_{1/2}$ value for binding of more than 3 h. No general difference concerning the dissociation rates between the two alleles could be identified (Fig. 4 and Table 2).

Cellular analysis of selected TB10.4 epitopes. We constructed tetrameric MHC class I-peptide complexes to evaluate whether the TB10.4 candidate peptides serve as target epitopes for $CD8^+$ T cells from patients with pulmonary TB. Five different TB10.4 tetramers presenting four different MHC class I peptides were constructed: two for HLA-A*3001 (QIM YNYPAM [$TB10.4_{3–11}$] and LVRAYHAMS [$TB10.4_{65–73}$]) and three for HLA-A*3002 (QIMYNYPAM [$TB10.4_{3–11}$], IM YNYPAML [$TB10.4_{4–12}$], and AMEDLVRAY [$TB10.4_{61–69}$]). The peptides were chosen on the basis of their high affinity values and slow dissociation rates, important parameters for tetramer stability. Since the peptide QIMYNYPAM ($TB10.4_{3–11}$) bound relatively stably to HLA-A*3001 and HLA-A*3002, we constructed two different tetramers presenting the identical peptide. Tetramers were used to stain for epitope-specific $CD8^+$ T cells in the HLA-A*30-typed patients.

We were able to identify antigen-specific $CD8^+$ T cells binding to the entire panel of TB10.4 epitopes loaded onto the novel tetramer molecules presented in the current report. The epitope LVRAYHAMS (presented by A*3001) showed the strongest $CD8^+$ T-cell recognition, followed by the epitope QIMYNYPAM (presented by A*3002), which had antigen-specific T-cell frequencies of up to 0.89% of all $CD8^+$ T cells (Table 3). We could not identify any general differences in the frequency of MHC-peptide-specific T cells in PBMCs from patients who were typed to be HLA-A*3001 concerning the recognition of the $A*3001_{QIMYNYPAM}$ and $A*3001_{LVRAYHAMS}$ MHC-peptide complexes and peptides presented by the A*3002 allele. The same was true regarding PBMCs from HLA-A*3002-positive patients, who recognized the A*3001 and the A*3002 tetramers at equal levels (Table 3).

Next, we addressed the question of whether the peptide QIMYNYPAM ($TB10.4_{3–11}$) presented by HLA-A*3002 or by HLA-A*3001 was recognized by the same or different T cells (from an HLA-A*3002-positive or HLA-A*3001-positive patient) One way to address this question is the *ex vivo* sorting of MHC-tetramer antigen-specific T cells, followed by TCR CDR3 analysis (21), yet the frequency of tetramer-reactive T cells was too low to allow such an approach to be used. We used an alternate way to test whether T cells recognizing HLA-A*3002- or HLA-A*3001-peptide tetramer complexes exhibit the same immune phenotype using markers for CD45RA and CCR7. The majority (70%) of the total $CD8^+$ T-cell population in PBMCs obtained from patients with pulmonary TB exhibited a peripheral memory effector phenotype ($CD45RA^-$ $CCR7^-$). In contrast, *M. tuberculosis* antigen-specific T cells, defined by tetramer staining, showed a different phenotype with a higher percentage of precursor cells ($CD45RA^+$ $CCR7^+$) (28 to 43%), central memory cells ($CD45RA^-$ $CCR7^+$) (13 to 25%), and terminally differentiated cells ($CD45RA^+$ $CCR7^-$) (8 to 31%) (Fig. 5A). We did not observe significant differences concerning the phenotype of TB10.4 QIMYNYPAML epitope-specific T cells and the MHC class I-restricting molecule, i.e., HLA-A*3001 and HLA-A*3002 (Fig. 5B).

CD45RA/CCR7 marker analysis was followed by examination of the interleukin-7 (IL-7) receptor (CD127) and CD107a expression in total $CD8^+$ T cells compared to that in tetramer-positive T cells (Fig. 5C). Expression of the IL-7 receptor appears to be crucial for $CD8^+$ memory T-cell generation (23), and CD107a expression reflects T-cell degranulation (35). The majority of $CD8^+$ T cells stained negative for the IL-7-receptor yet were positive for the degranulation marker CD107a (up to 61%). This was different in tetramer-reactive T cells, which showed a strong simultaneous expression of the IL-7 receptor and CD107a (51 to 67%). CD07a and CD127 expression analysis did not show differences in $A*3001_{QIMYNYPAM}$ ($TB10.4_{3–11}$)- or $A*3002_{QIMYNYPAM}$ ($TB10.4_{3–11}$)-specific $CD8^+$ T-cell interaction (Fig. 5D).

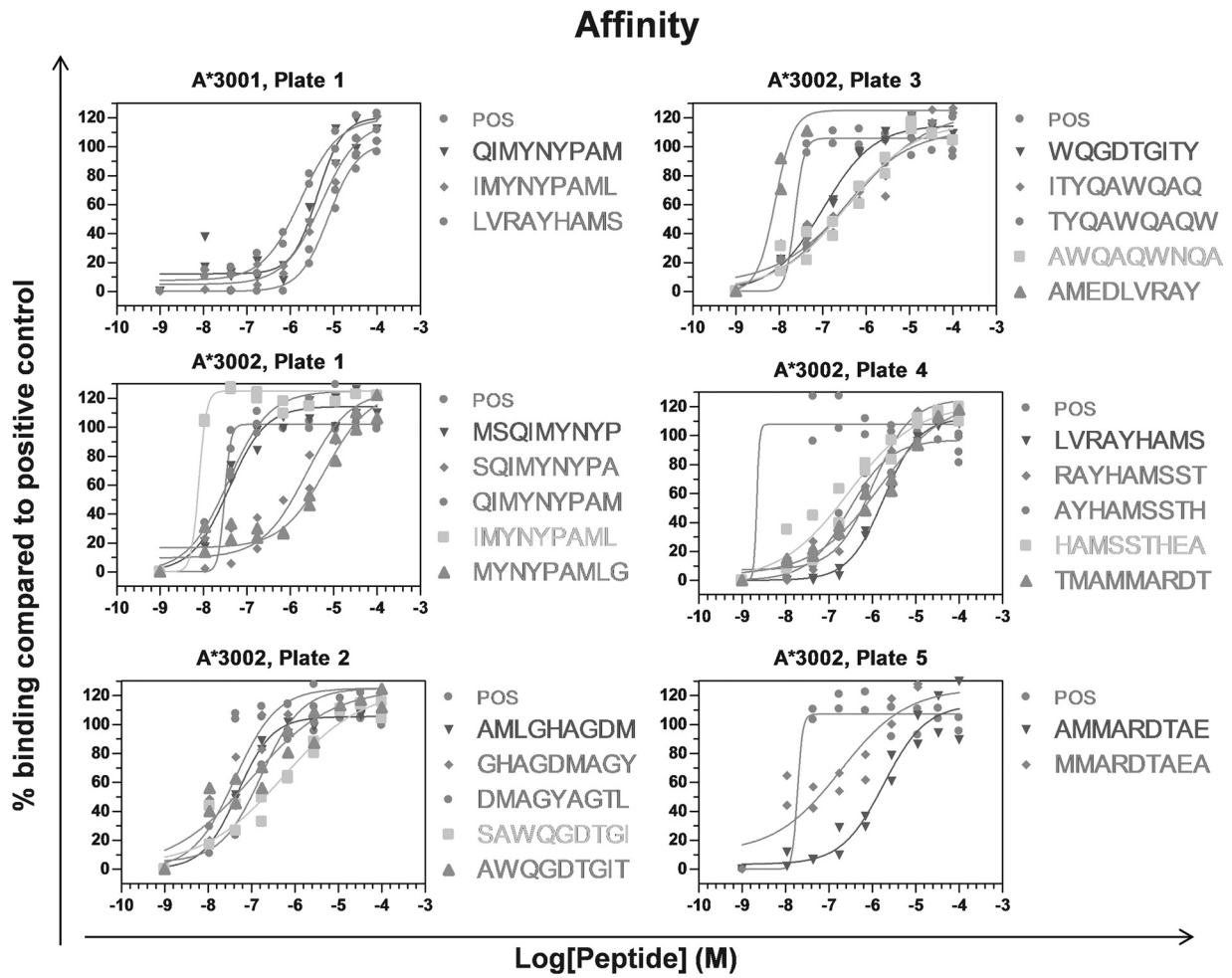

FIG. 3. Affinity data for the peptides positive for binding to HLA-A*3001 and HLA-A*3002. The affinity graph shows individual peptide binding for different peptide concentrations (in M) compared with that for a positive control. The ED_{50} value can be calculated from these curves using sigmoidal curve fitting using GraphPad Prism software.

DISCUSSION

Tuberculosis, particularly in conjunction with HIV infection, imposes a global health threat and is certainly a prime target for vaccine development. We addressed the need to develop tools which enable molecular and cellular studies of the MHC class I-peptide interaction and T-cell recognition in PBMCs from individuals of African descent and produced tetramer tools based on the HLA-A*3001 and HLA-A*3002 alleles, which are common in Africa (34). We identified a high prevalence of these two alleles and subsequently proceeded to produce recombinant proteins, which allowed us to identify MHC class I-binding peptides from an *M. tuberculosis* target protein, which has been reported to induce T-cell responses upon BCG vaccination, upon natural infection, or in response to TB10.4 vaccination (4, 8, 13, 33, 47).

The difference between HLA-A*3001 and HLA-A*3002 is constituted by four amino acids; Q70H, V76E, D77N, and W152R (39). Three of these amino acids (those at positions 70, 77, and 152) are situated in the α helices, which form the sides of the peptide-binding cleft. They may therefore directly affect peptide binding, affinity, and off-rate, since the amino acid substitutions lead to changes in size, electrical charge, and polarity. These amino acids are situated in the B, E, and F pockets, respectively (41), and have previously been reported to be important for peptide interactions (7) by forming contact with amino acid numbers 6, 7, and 9 of the binding peptide (22). The last differentiating amino acid (that at position 76) is situated adjacent to the peptide-binding cleft and has a protruding side chain, which could potentially affect T-cell recognition.

The biochemical features of HLA-A*3001 concerning peptide presentation are still unsolved. Several conflicting reports exist regarding the peptide-binding specificity. One report suggests the peptide-binding preference reflected in the A*3001 peptide-binding motif to be X(Y/F)XXXXXXL (26), but Lamberth et al. reported another motif for HLA-A*3001, i.e., XX XXXXX(K/V/R) (27). Both studies indicated that amino acid 9 is the central anchor point concerning peptide binding. A different study suggested that amino acid residue 3 may be important as an anchor residue for peptide-MHC interaction (45). Our study cannot confirm any of these motifs, since we identified only three positively binding peptides for this allele,

TABLE 2. Affinity and off-rate data for selected TB10.4 peptides

Peptide no.	Sequence	Peptide name	HLA class I allele[a]			
			A*3001		A*3002	
			Affinity	Off-rate	Affinity	Off-rate
1	MSQIMYNYP	$TB10.4_{1–9}$			4.0E−08	2.3
2	SQIMYNYPA	$TB10.4_{2–10}$			2.0E−06	0.3
3	QIMYNYPAM	$TB10.4_{3–11}$	**4.0E−06**	**0.8**	**4.0E−08**	**1.1**
4	IMYNYPAML	$TB10.4_{4–12}$	5.0E−06	0.5	**8.0E−09**	**3.1**
5	MYNYPAMLG	$TB10.4_{5–13}$			7.0E−06	0.6
10	AMLGHAGDM	$TB10.4_{10–18}$			5.0E−08	1.2
13	GHAGDMAGY	$TB10.4_{13–21}$			4.0E−08	1.3
17	DMAGYAGTL	$TB10.4_{17–25}$			2.0E−07	2.2
41	SAWQGDTGI	$TB10.4_{41–49}$			5.0E−07	1.1
42	AWQGDTGIT	$TB10.4_{42–50}$			1.0E−07	1.1
43	WQGDTGITY	$TB10.4_{43–51}$			1.0E−07	1.7
49	ITYQAWQAQ	$TB10.4_{49–57}$			5.0E−07	0.6
50	TYQAWQAQW	$TB10.4_{50–58}$			3.0E−07	0.3
53	AWQAQWNQA	$TB10.4_{53–61}$			4.0E−07	0.3
61	AMEDLVRAY	$TB10.4_{61–69}$			**8.0E−09**	**2.1**
65	LVRAYHAMS	$TB10.4_{65–73}$	**2.0E−06**	**1.8**	2.0E−06	1
67	RAYHAMSST	$TB10.4_{67–75}$			1.0E−06	1.2
68	AYHAMSSTH	$TB10.4_{68–76}$			3.0E−07	0.4
70	HAMSSTHEA	$TB10.4_{70–78}$			3.0E−07	0.6
80	TMAMMARDT	$TB10.4_{80–88}$			2.0E−06	0.9
82	AMMARDTAE	$TB10.4_{82–90}$			2.0E−06	0.6
83	MMARDTAEA	$TB10.4_{83–91}$			2.0E−07	0.6

[a] Affinity is reported as an ED_{50} value (M), and off-rate is reported as a $t_{1/2}$ value (h), as described in Materials and Methods. Tetramers were constructed for the epitopes marked in boldface numbers.

and all peptides exhibited different amino acids at the C-terminal position, as well as at positions 2 and 3. The data in this report indicate, rather, that A*3001 may, as suggested by Sidney et al. (45), be able to use different arrangements of primary and secondary anchor residues.

The peptide-binding motif X[**YFLV**]XXXXXX[**Y**] has been proposed for HLA-A*3002 (26), indicating a preference for tyrosine residues at the C-terminal position and a preference for hydrophobic or aromatic amino acids at position 2. A similar motif which describes the C-terminal position, with aromatic or hydrophobic amino acids being the most important part for peptide binding, has been proposed by Sidney et al. (46). Our results agreed in part with these findings; i.e., 20 of the 22 HLA-A*3002-binding peptides evaluated in this study exhibited aromatic, hydrophobic, or small uncharged amino acids in the C-terminal position. We identified 10 different amino acids at position 2 of the HLA-A*3002-binding peptides. The amino acids at positions 1 and 3 also showed great variations. This indicates that HLA-A*3002 appears to utilize multiple secondary anchor residues in the C- and N-terminal regions and may therefore show a promiscuous binding motif. This may explain the large number of positive HLA-A*30-binding peptides identified in this study. It also underlines that the use of *in silico* predictive programs using common binding motifs for the alleles HLA-A*3001 and HLA-A*3002 may underestimate the number of candidate binding peptides. The actual measurement of the MHC class I-peptide interaction appears to be necessary to objectively identify target peptides from a protein.

We found that the major difference between the two HLA-A*30 alleles is the very restricted peptide-binding profile of HLA-A*3001 compared to the profile of HLA-A*3002. HLA-A*3002 has previously been described to be an allele with a broad binding pattern (38). In contrast, HLA-A*3001 has been proposed to behave similarly to the HLA-A*03 alleles (27) with more restricted peptide-binding patterns, yet no direct comparison has been performed, to our knowledge. It is likely that the amino acid substitutions, which differ between HLA-A*3001 and HLA-A*3002, lead to profound differences in the structure of the peptide-binding cleft, e.g., the introduction of the big and bulky tryptophan at position 152 in HLA-A*3001, a position contributing to the shape of the E pocket. This might affect the ability to accommodate big amino acids at the C-terminal end. In fact, all the identified HLA-A*3001-binding peptides (this study) exhibit relatively small amino acids at positions 8 and 9. The D77N and W152R substitutions lead to a change of electric charge from a more negative charge in HLA-A*3001 to a more positive charge in HLA-A*3002. This may be the reason why one of the A*3001 peptide-binding motifs showed a preference for positively charged amino acids at the C terminus (although we could not denitrify peptides with such characteristics). The confirmation of these theories has to await the crystal structures of these proteins, which are not available at this time. In summary, the substantial differences between HLA-A*3001 and HLA-A*3002 underline the value of accurate four-digit typing of the HLA-A*30 family in the context of susceptibility to or protection from disease (31). For instance, HLA-A*30 has been identified as one of the risk factors for the development of cytomegalovirus (CMV) disease after allogeneic hematopoietic stem cell transplantation (25). It is not clear if this association is due instead to the limited capacity of HLA-A*3001 to present CMV peptides to $CD8^+$ T cells.

Many of the TB10.4 peptides that were identified as good

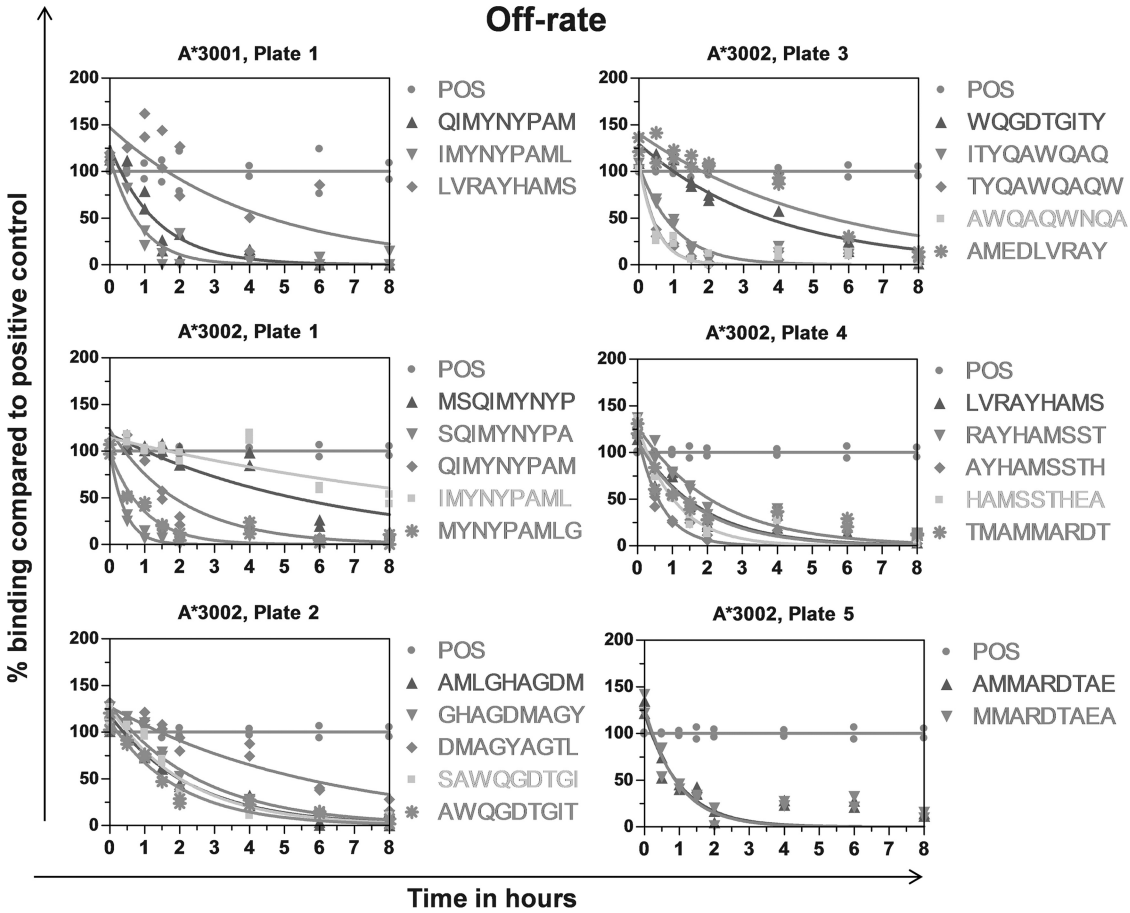

FIG. 4. Off-rate data for the peptides positive for binding to HLA-A*3001 and HLA-A*3002. The off-rate graph shows individual peptide off-rates (in hours) compared with that for an allele-specific positive control. The $t_{1/2}$ value was calculated using sigmoidal curve fitting. Curves were fitted using GraphPad Prism software.

binders to the HLA-A*30 alleles are promiscuous peptides, and they have previously been described to be binders to one or several other MHC class I alleles, e.g., MSQIMYNYP ($TB10.4_{1–9}$) (HLA-A*0101 and HLA-B*1501) (4), WQGDT GITY ($TB10.4_{43–51}$) (HLA-B*1501) (50), and MMARD TAEA ($TB10.4_{83–91}$) (A-A*0201, B*0702, and B*1501) (4). The promiscuity is indeed remarkable for peptides QIMYN YPAM ($TB10.4_{3–11}$) and IMYNYPAML ($TB10.4_{4–12}$). These are altogether presented by no less than seven different MHC class I molecules, including the HLA-A*30 alleles. Only seven of the positive MHC class I-binding peptides for HLA-A*3001 and HLA-A*3002 bound exclusively to one or both of these

TABLE 3. Prevalence of epitope-specific T cells identified by tetramer staining[a]

Patient	Allele	% tetramer-positive events in $CD3^+$ $CD8^+$ cells				
		A*3001		A*3002		
		QIMYNYPAM	LVRAYHAMS	QIMYNYPAM	IMYNYPAML	AMEDLVRAY
ABFA7239A	A*3001	0.13	0.05	0.17	0.1	0.23
ABFA7246A	A*3001	0.11	0.1	0.14	0.07	0.06
ABFA7242A	A*3001	0.31	0.11	0.41	0.09	0.12
BM06	A*3001	0.06	0.89	0.1	0.03	0.27
ABFB1286A	A*3002	0.08	0.1	0.11	0.1	0.07
ABFA7768A	A*3002	0.17	ND[b]	0.19	0.22	0.14
ABFA1266A	A*3002	0.01	0.11	0.38	0.19	0.19
YEN0015	A*3002	0.14	0.84	0.47	0.1	0.49

[a] PBMCs from individuals with TB were incubated with MHC-matched MHC class I-TB10.4 tetramers and stained for T-cell markers. Results are reported as percent tetramer-positive events in the $CD3^+$ $CD8^+$ T-cell population; negative gating was performed to exclude $CD4^+$ T cells. $A*3001_{KTKDIVNGL}$ (self-F-actin-capping protein beta) was the positive control tetramer, for which the rate of tetramer-positive events was 0.01. The $A*3002_{KIQNFRVYY}$ (HIV integrase) epitope was the negative tetramer for which the rate of tetramer-positive events was 1.2.

[b] ND, not determined.

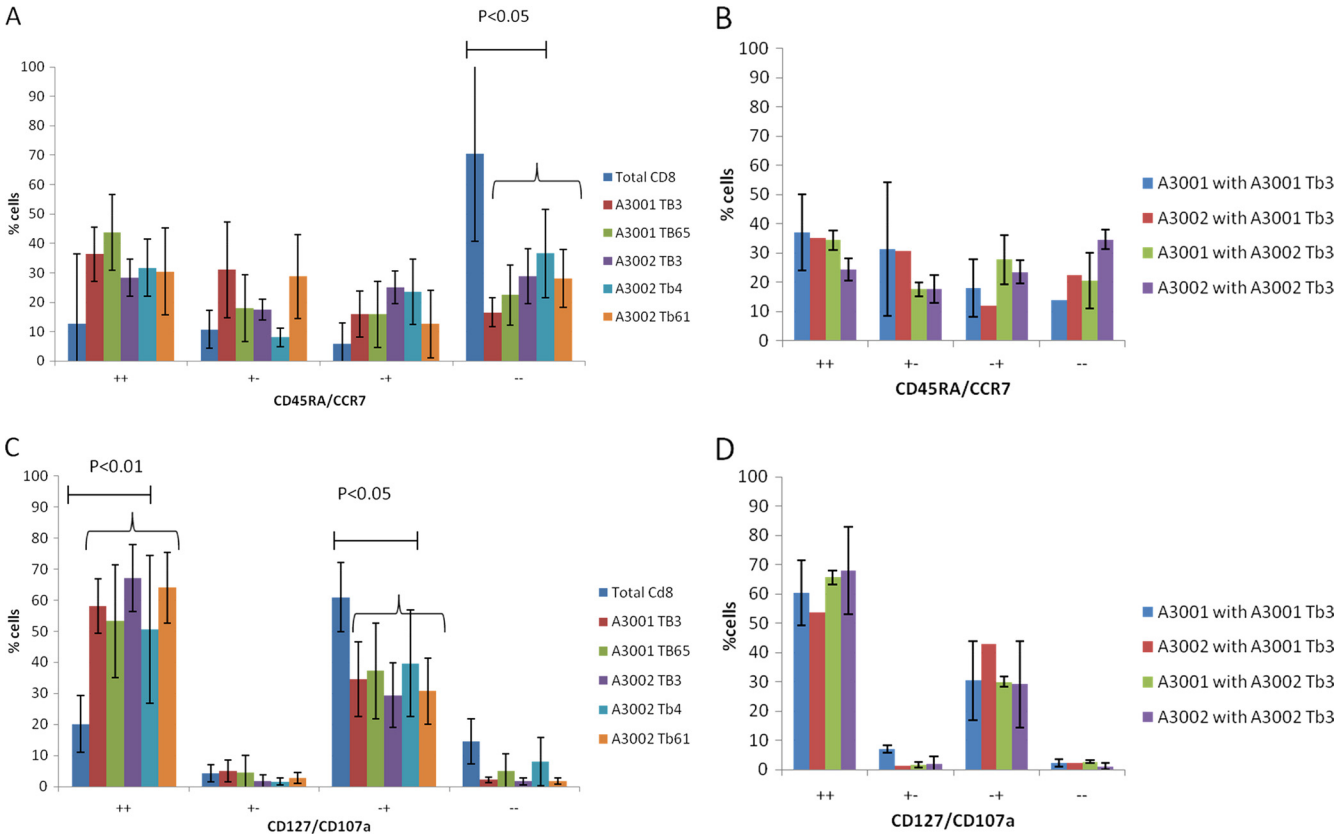

FIG. 5. (A and B) Phenotypic analysis of tetramer-positive T cells recorded in the $CD3^+$ $CD8^+$ compartment showing the proportion (percent) of precursor $CD45RA^+$ and $CCR7^+$ cells (++), terminally differentiated $CD45RA^+$ and $CCR7^-$ cells (+−), central memory $CD45RA^-$ and $CCR7^+$ cells (−+), and effector memory $CD45RA^-$ and $CCR7^-$ (−−) cells. (A) Percent T cells residing in the different maturation/differentiation compartments as the average for PBMCs obtained from eight patients, as follows: in all $CD3^+$ $CD8^+$ cells (blue), in $A^*3001_{QIMYNYPAM}$-PE-positive cells (red), in $A^*3001_{LVRAYHAMS}$-PE-positive cells (green), in $A^*3002_{QIMYNYPAM}$-PE tetramer-positive cells (purple), in $A^*3002_{IMYNYPAML}$-PE-positive cells (light blue), and in $A^*3002_{AMEDLVRAY}$-PE tetramer-positive cells (orange). (B) Phenotypic analysis in cross-recognition of $CD8^+$ T cells binding to either $A^*3001_{QIMYNYPAM}$ or $A^*3002_{QIMYNYPAM}$ molecules in PBMCs from patients with an A*3001 (blue and green) or an A*3002 (red and purple) background. (C and D) Proportion (percent) of $CD127^+$ and $CD107a^+$ (++), $CD127^+$ and $CD107a^-$ (+−), $CD127^-$ and $CD107a^+$ (−+), and $CD127^-$ and $CD107a^-$ (−−) cells in all $CD3^+$ $CD8^+$ cells (blue), in $A^*3001_{QIMYNYPAM}$-PE-positive cells (red), in $A^*3001_{LVRAYHAMS}$-PE-positive cells (green), in $A^*3002_{QIMYNYPAM}$-PE tetramer-positive cells (purple), in $A^*3002_{IMYNYPAML}$-PE-positive cells (light blue), and in $A^*3002_{AMEDLVRAY}$-PE tetramer-positive cells (orange) (C). (D) Phenotypic analysis based on CD107a and CD127 expression on T cells which recognize $A^*3001_{QIMYNYPAM}$ and $A^*3002_{QIMYNYPAM}$ tetramer complexes from PBMCs obtained from patients with an A*3001 (blue and green) or an A*3002 (red and purple) background. The statistics were calculated using Student's two-sided *t* test.

alleles. We identified epitope-specific T cells for the epitope IMYNYPAML ($TB10.4_{4–12}$), which is presented by four different MHC molecules, including that encoded by the African allele, HLA-A*3002 (4). It is important to dissect if such a multiallelic focus on a certain target peptide is beneficial or rather is detrimental in the context of immune protection in infectious diseases for individuals with a certain MHC class I genotype, which would allow different MHC molecules to present the same peptide simultaneously to CD8 T cells.

The time frame which describes the binding of a certain peptide species to selected MHC class I molecules is biologically important, since it reflects the time frame in which the epitope would be available for T-cell priming (and for target recognition in the effector phase of the immune response). This can be studied using the concepts of affinity and dissociation time. Previous studies using recombinant MHC molecules have shown that for most MHC alleles the affinity (ED_{50}) lies between 100 μM and 10 nM (4, 5). This is true for the peptides analyzed for HLA-A*3001, yet we identified here some peptides with an even higher affinity (ED_{50}, ~8 nM). Previous reports suggested a correlation between high affinity and a dominant T-cell response (44). However, the distinct yet low frequency of tetramer-reactive T cells does not suggest a dominant recognition in the context of acute pulmonary TB. The frequency of these target-specific T cells is similar to that of antigen-specific T cells in cancer; a low frequency of peptide-reactive T cells in the peripheral circulation does not preclude clinical efficacy (3). Not mutually exclusively, HLA-A*3001- or HLA-A*3002-restricted T cells may show different frequencies after initiation of anti-*M. tuberculosis* therapy; immune reconstitution, e.g., TCR zeta-chain recovery (42); and T-cell redistribution. Tetramer-guided identification of *M. tuberculosis*-directed T-cell responses may be critical in acute pulmonary TB, often associated with T-cell anergy (36) and dysfunctional cytokine production (36, 51). It may also be helpful to trace *M. tuberculosis*-reactive T cells in PBMCs from

patients with HIV-*M. tuberculosis* coinfection, since less than 200 $CD4^+$ T cells/μl blood are associated with false-negative results in interferon (IFN)-based immunological diagnostic test systems (49).

The aim of the current study was not to map the detailed T-cell response in individuals with different clinical presentations of TB but to determine if HLA-A*3001/HLA-A*3002-binding peptides were recognized by $CD8^+$ T cells from patients with TB and if HLA-A*3001-presented peptides were recognized from T cells obtained from an HLA-A*3002-positive individual. The entire panel of MHC class I-TB10.4 peptide complexes was recognized by antigen-specific T cells in PBMCs from patients with pulmonary TB, irrespective of the HLA-A*3001 or HLA-A*3002 background. This observation differed from that from a previous study, in which no cross-recognition was observed using HIV peptides and $CD8^+$ T cells from HLA-A*3002- and HLA-A*3001-positive subjects defined by gamma IFN enzyme-linked immunospot assay (18). This may reflect either the nature of the HIV peptides (which may not bind to the picky HLA-A*3001 allele) or, not mutually exclusively, the lack of IFN production in HLA-A*3001/HLA-A*3002-restricted T cells. We did not detect general differences in T-cell recognition by applying an A*3001 tetramer with an A*3001-binding peptide using PBMCs from an A*3002-positive patient. This was also true using the same peptide, QIMYNYPAM ($TB10.4_{3-11}$), which bound either to HLA-A*3001 or to HLA-A*3002.

The phenotype defined by CD45RA, CCR7, CD107a, and CD127 expression of the tetramer-positive $CD8^+$ T cell appears to be similar for T cells recognizing TB10.4 T-cell epitopes, with the majority of the CD8 T cells exhibiting a precursor compartment profile, i.e., $CD45RA^+$ and $CCR7^+$ expression. This finding is in line with previous findings regarding marker analysis of tetramer-specific T cells recognizing another *M. tuberculosis* antigen, i.e., Ag85B (20), yet it differs from the findings of other studies which showed that the majority of antigen-specific T cells against *M. tuberculosis* may reside in the memory ($CD45RA^-$) compartment (51) or the terminally differentiated effector population ($CD45RA^+$ $CCR7^-$) compartment (8). A possible explanation for the high number of antigen-specific precursor $CD8^+$ T cells in our study may be the increase in lymphopoiesis in patients with TB. Not mutually exclusively, it could also be due to the recycling of terminally differentiated cells to precursor $CD8^+$ T cells; i.e., the cells revert to a quiescent state indistinguishable from precursor cells, on the basis of CD45RA and CCR7 expression, but they are not truly naïve (2, 6). They may therefore also show different telomere lengths or exhibit a different T-cell receptor excision cycle content.

The phenotypes of the TB10.4 epitope-reactive T cells specific for the epitope QIMYNYPAM presented by either HLA-A*3001 or HLA-A*3002 appear to be similar and to not be influenced by the restricting molecule (Fig. 5B). This is also true for expression of the IL-7 receptor and the cytotoxicity marker CD107a (Fig. 5D). There is a difference of only a single amino acid between HLA-A*3001 and HLA-A*3002 (position 76) protruding out from the peptide-binding pocket. It may therefore be possible that the same T-cell population recognizes the identical peptide bound to either HLA-A*3001 or HLA-A*3002. However, we would require further studies to examine in greater detail the T-cell frequency of HLA-A*3001-restricted T cells reacting to a broader array of HLA-A*3002-presented peptides using tetramer molecules, since certain peptide species may not have been presented to the T-cell repertoire in an HLA-A*3001-homozygous individual. Some TCRs may recognize their target peptides irrespective of the MHC presenting allele, as described for HLA-A3 and HLA-A11 (30). Not mutually exclusively, the different peptide-binding grooves of HLA-A*3002 and HLA-A*3001 might influence the conformation of the binding peptide, thereby altering the surface exposed to the TCR (32), which influences T-cell recognition. A different exposure of the peptide backbone to responder T cells may affect T-cell recognition and subsequent T-cell effector functions. Flexibility in the TCR itself may even contribute to MHC class I-peptide recognition patterns: some TCR Vβ families may even allow effective recognition of point mutations in target peptides and contribute to immune protection (19, 29, 43).

In summary, we used recombinant MHC class I molecules to identify 3 novel target peptides for HLA-A*3001 and 22 candidate peptides for HLA-A*3002 in the *M. tuberculosis* TB10.4 protein. We described marked differences concerning binding characteristics, with a very restricted binding pattern in HLA-A*3001 compared with that in HLA-A*3002, and constructed MHC class I-peptide tetramer complexes to visualize antigen-specific T cells in peripheral blood from patients with pulmonary TB. The availability of these reagents will aid further work on intervention strategies by molecular design for pathogens, i.e., *M. tuberculosis* or HIV, prevalent in the African population.

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M.M. is a cofounder of Thymed, covering immune diagnostics.

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