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ORIGINAL ARTICLE

Impact of MHC class I alleles on the M. tuberculosis antigen-specific CD8$^+$ T-cell response in patients with pulmonary tuberculosis

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*Challenged by scattered understanding of protective immunity to Mycobacterium tuberculosis (MTB), we have mapped peptide epitopes to human leukocyte antigen (HLA)-A*0101, A*0201, A*1101, A*2402, B*0702, B*0801 and B*1501 of the secreted mycobacterial antigen Ag85B, a vaccine candidate that may be associated with immune protection. Affinity (ED_{50}) and half-life ($t_{1/2}$, off-rate) analysis for individual peptide species on HLA-A and HLA-B molecules revealed binding ranges between 10^{-3} and 10^{-7} M. After selection of the best matches, major histocompatibility complex class I/peptide tetramer complexes were constructed to measure the CD8$^+$ T-cell responses directly ex vivo in peripheral blood mononuclear cells (PBMC) derived from 57 patients with acute pulmonary tuberculosis. Three patterns of (allele-) specific CD8$^+$ recognition were identified: (a). Focus on one dominant epitope with additional recognition of several subdominant T-cell epitopes (HLA-A*0301, A*2402, B*0801 and B*1501); (b). Co-dominant recognition of two distinct groups of peptides presented by HLA-B*0702; and (c). Diverse and broad recognition of peptides presented by HLA-A*0201. Peptides that bound with slow off-rates to class I alleles, that is HLA-A*0201, were associated with low frequency of CD8$^+$ T cells in PBMCs from patients with tuberculosis. HLA-B alleles showed fast off-rates in peptide binding and restricted high numbers (up to 6%) of antigen-specific CD8$^+$ T cells in patients with pulmonary tuberculosis.*
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Keywords: *M.tuberculosis; MHC class I; CD8$^+$ T-cells; cellular immunity*

Introduction

To date, neither anti-mycobacterial drugs nor BCG vaccines have provided solutions to the global tuberculosis (TB) problem. Developing and applying better vaccines that protect against lung TB is the only logistically plausible solution, but these efforts are hampered by our incomplete concepts of protective immunity and cellular immune responses induced by vaccination. Although the immune correlates of protection from infection and TB disease remain elusive, experimental evidence supports the hypothesis that induction of Th1 responses and enhancement of a robust CD8 T-cell memory to M*ycobacterium tuberculosis* (MTB) antigens are goals for vaccine developers.[1,2] To understand this part of immunity better and to link the biochemistry of individual major histocompatibility complex (MHC) class I-peptide complexes with the *ex vivo* frequency of MHC class I-restricted and peptide-specific CD8$^+$ T-cells, we decided to study affinity and off-rates for peptides provided from the MTB Ag85B for the most frequent MHC class I alleles in the Caucasian population and to develop MHC-I tetramer reagents for flow cytometry that allow the enumeration of antigen-specific CD8 T cells in specimens from patients with active pulmonary tuberculosis without *ex vivo* manipulation of immune cells.

The selection of Ag85B as target of investigation is justified by its importance for mycobacterial species and its immunogenicity. Ag85B is part of a mycosyltransferase complex and essential for intracellular survival and cell synthesis. Proteins of the highly homologous mycosyltransferase antigen 85 complex (Ag85A, B, C) are secreted from replicating mycobacterial species,[3,4] they are involved in the final step of the mycobacterial cell wall synthesis and in cord factor (trehalose-dimycolate) generation in *M. tuberculosis*.[5] Ag85 members are present in all Mycobacterial species, genetic disruption experiments showed that Ag85A is critical for survival within macrophages.[6] Components of the Ag85 complex play not only a role in mycobacterial pathogenicity, they also present targets for humoral and cellular immune responses. Ag85A has been shown to induce T-cell proliferation and IFN-γ production in healthy (PPD skin-reactive) individuals infected with *M. tuberculosis*

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or *M. leprae* and in patients with clinical active tuberculosis.[7,8] Ag85 complex proteins rank among the top MTB vaccine candidates,[9] they are elevated and readily detectable in serum from patients with active tuberculosis,[10] as well as in cerebrospinal fluid from patients with MTB meningitis.[11] Antigen-specific CD8+ T cells were suggested to control MTB infection and MHC class I-restricted CD8+ T-cells recognizing MTB-associated antigens have been reported.[12–14]

The precise MHC class I-peptide-binding profile and the broader pattern of Ag85 specific cellular immune responses in patients with active tuberculosis has not yet been defined. Only a few human Ag85B-specific CD8+ T-cell epitopes have been identified up to now, novel tools to predict proteasomal cleavage and peptide binding to single alleles may aid to predict novel epitopes,[15] yet objective affinity and off-rate analysis for these *M. tuberculosis* epitopes are lacking and the impact of MHC class I alleles in shaping the T-cell response directed against Ag85B has not been addressed. Therefore, we began a systematic approach by testing peptide epitopes from Ag85B, which bind across the most frequent Caucasian MHC class I molecules. CD8+ T-cell responses directed against Ag85B may serve as a paradigm for the binding characteristics and diversity of T-cell responses in acute bacterial infections, and the dynamic of changes may provide clues about encrypted rules of protective immunity to intracellular pathogens restricted by different MHC class I alleles.

Results

Ag85B epitope identification

A selection of 49 candidate peptides derived from *M. tuberculosis* Ag85B were tested for binding to HLA-A molecules, including HLA-A*0101, A*0201, A*0301, A*1101 and A*2402 (Figure 1 and Supplementary Table S2 in the supplementary (S) data set), and 15 peptides were tested for binding to HLA-B*0702, B*0801 and B*1501 (Figure 1). When an arbitrary cutoff of 30% binding was applied for peptide binding to MHC class I molecules, 1/49 peptides showed binding to HLA-A*0101, 11/49 to HLA-A*0201, 1/49 to HLA-A*0301, 1/49 to HLA-A*1101 and 10/49 to HLA-A*2402. Five out of 15 test peptides bound to HLA-B*0702, 2/15 to HLA-B*0801 and 5/15 to HLA-B*1501. Individual peptide sequences and the location within Ag85B, as well as the corresponding SYFPEITHI score are provided within the supplementary data set (Supplementary Table S2a/b and Figure S5).

Several groups of peptides were selected for more detailed MHC-binding studies, including affinity (ED_{50}) and off-rate ($t_{1/2}$) analysis: (i) peptides that showed more than 30% binding to individual MHC class I molecules, (ii) peptides that were likely to be generated by proteasomal cleavage even if these showed less than 30% binding in the initial score and (iii) peptides that showed binding to more than a single MHC allele derivative (Figure 1 and Table 1). The latter situation could be of particular interest if the cellular immune response would target a peptide species presented by MHC molecules encoded by different alleles. Twenty-one peptides were selected for further binding analysis to

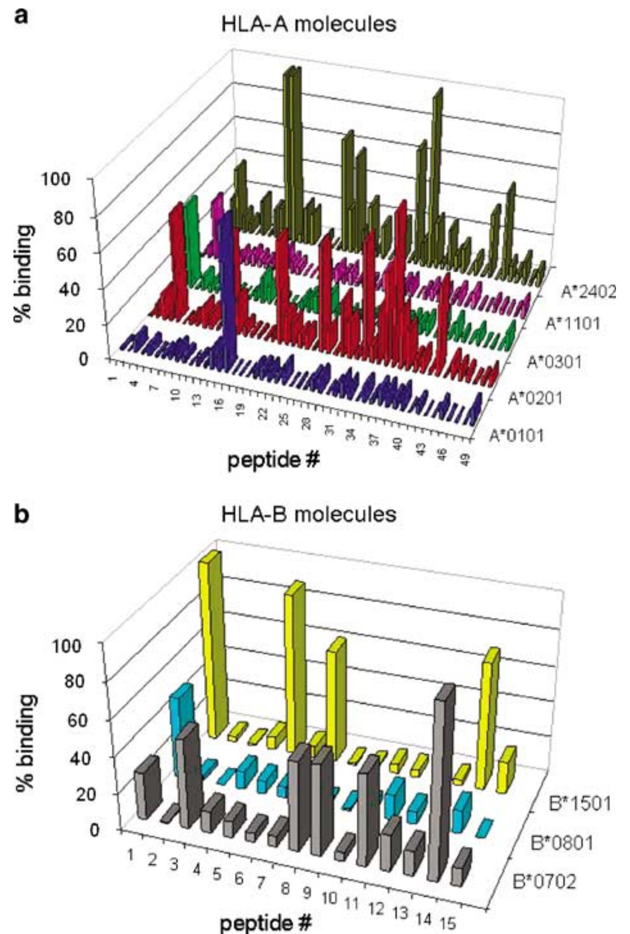


Figure 1 Peptide binding to HLA-A (**a**) and HLA–B (**b**) molecules. The individual peptide sequences are listed in the Supplementary material (Supplementary Table S2). Alleles are listed on the *y* axis, the percentage of binding of test peptides as compared to the control peptide is listed on the *z* axis. Only 100% binding values as compared to control peptide are presented for a few individual peptide species that showed stronger binding. Complete data sets including raw numbers are listed in Supplementary Table S2 (supplemental data).

HLA-A, and 10 peptides were selected for binding to HLA-B allele derivatives (Table 1).

The majority (9/12) of HLA-A*0201 binding peptides showed a half-life ($t_{1/2}$) in the range of 4.6–9.4 h; 3/12 peptides exhibited a $t_{1/2}$ from 12.2 up to 17.2 h (Table 1). The HLA-A*0201 complex is exceptional in this context, since other HLA-A or B molecules exhibit significantly lower MHC/peptide off-rates in the range of 0.5–3.9 h (Table 1 and Figure 2; data for each individual peptide species are presented in Supplementary Figure S6).

Affinity (ED_{50}) analysis revealed that Ag85B peptides bind over 4 logs from 10^{-3} to 10^{-7} M to MHC class I molecule species (Table 1). Eight out of 11 HLA-A*0201 binding peptides (one peptide did not yield an ideal fitting curve, 12 peptides were tested in total) exhibited an ED_{50} value in the range of 10^{-6} M. The majority of peptides which bind to HLA-A molecules showed affinities in the range of 10^{-6} M; one of two peptides that bound to HLA-A*0301 exhibited an ED_{50} value of 10^{-5} M; a similar binding value was obtained for both candidate peptide species that bind to HLA*1101. One of 10 peptides that bound to HLA-A*2402 showed weak

Table 1 Compilation of HLA-A and HLA-B peptide binding results

HLA-allele			*A*0101*		*A*0201*		*A*0301*		*A*1101*		*A*2402*	
No.	*Position*	*Sequence*	ED_{50}	$t_{1/2}$	ED_{50}	$t_{1/2}$	ED_{50}	$t_{1/2}$	ED_{50}	$t_{1/2}$	ED_{50}	$t_{1/2}$
HLA-A alleles												
1	35–43	**AVYLLDGLR**					**1.E-05**	**12.8**	8.E-05	3.5	NA	1.1
2	37–45	**YLLDGLRAQ**			2.E-06	8.3						
3	60–68	**WYYQSGLSI**									1.E-07	1.8
4	61–69	YYQSGLSIV									2.E-07	1.9
5	64–72	SGLSIVMPV			2.E-06	6.6						
6	75–83	**QSSFYSDWY**	5.E-06	2.2								
7	100–108	**FLTSELPQW**			5.E-06	6.3					**2.E-06**	**1.9**
8	108–116	WLSANRAVK									5.E-06	2.2
9	114–122	AVKPTGSAA									3.E-06	3.2
10	126–134	**SMAGSSAMI**			3.E-06	9.4						
11	132–140	AMILAAYHP			NA	15.2						
12	137–145	AYHPQQFIY									2.E-05	1.9
13	143–151	**FIYAGSLSA**			7.E-07	6.7						
14	144–152	**IYAGSLSAL**			4.E-04	4.6					**2.E-07**	**1.8**
15	145–153	YAGSLSALL			8.E-07	12.2						
16	151–159	ALLDPSQGM			2.E-06	9.1						
17	158–166	**GMGPSLIGL**			1.E-06	6.6						
18	160–168	SLIGLAMGD			7.E-06	17.2						
19	167–175	**AMGDAGGYK**					**3.E-06**	**0.3**	**3.E-05**	**0.5**		
20	199–207	**KLVANNTRL**			**6.E-06**	**9.6**					6.E-03	2.8
21	232–240	FVRSSNLKF									4.E-06	1.8

HLA-allele			*B*0701*		*B*0801*		*B*1501*	
No.	*Position*	*Sequence*	ED_{50}	$t_{1/2}$	ED_{50}	$t_{1/2}$	ED_{50}	$t_{1/2}$
HLA-B alleles								
1	18–26	MGRDIKVQF			8.E-06	1.5	2.E-06	1.7
2	21–29	**DIKVQFQSG**			2.E-07	1.6		
3	70–78	**MPVGGQSSF**	5.E-05	1.5				
4	74–83	**GQSSFYSDWY**					4.E-06	1.9
5	92–100	CQTYKWETF					1.E-05	2.6
6	144–152	**IYAGSLSAL**	2.E-05	1.6				
7	160–168	**GPSLIGLAM**	2.E-05	1.7				
8	197–205	**IPKLVANNT**	3.E-05	1.6				
9	230–238	**ENFVRSSNL**			2.E-04	1.6		
10	232–240	**FVRSSNLKF**	2.E-06	1.1			**8.E-06**	**3.9**

Abbreviation: MHC, major histocompatibility complex.
ED_{50} affinity: Selected epitope candidate peptides were incubated at concentrations ranging from 10^{-4} to 10^{-9} M to determine the relative binding affinities for the MHC class I molecule. Affinity is expressed as the quantity of peptide needed to achieve 50% binding saturation; the 'ED_{50}' value, in mol/l (M). $t_{1/2}$ *off-rate***:** The dissociation of bound peptide over time was evaluated. The off-rate is expressed as the $t_{1/2}$ value, the time in hours after which the binding saturation level declined to 50%. Peptides used for monomerization/tetramerization are depicted in bold letters (red). The peptides that bound to different MHC molecules (more than one) are marked in bold numbers (in blue).
Not applicable (NA) designates situations when MHC/peptide interactions measured in duplicates and after three repeats did not yield a plausible exponential decay curve, using the GraphPad Prism statistical software.

binding (6×10^{-3} M), but other peptide species bound to HLA-A*2402 in the range of 10^{-5}–10^{-7} M. In contrast to the candidate peptides binding to HLA-A molecules, the Ag85B peptides exhibited a lower and more limited binding range to HLA-B molecules: four of five peptides evaluated for HLA-B*0702 bound in the range of 10^{-5} M, a single peptide bound with a higher affinity (ED_{50} is 2×10^{-6} M). The three candidate peptides with binding to HLA-B*0801 exhibited an ED_{50} range from 10^{-4} to 10^{-7} M, and two of two peptides for HLA-B*1501 bound with an ED_{50} of 10^{-5} and 10^{-6} M, respectively. Of particular interest were peptides that did bind (although with different ED_{50} and $t_{1/2}$ values) to different MHC class I alleles. Two peptide species were found to bind across different HLA-A molecules, that is AVLLDGLR (aa 35–43) and AMGDAGGYK (aa 167–175) with binding to A*0301 and A*1101. A similar situation was found for the peptide MGRDIKVQF (aa 18–26), with binding to HLA-B*0801 and to HLA-B*1501. For instance, the peptide AVYLLDGLR (aa 35–43) displayed a binding range of 1×10^{-5} M to HLA-A*0301 and 8×10^{-5} M for HLA-A*1101, with a longer half-life (ED_{50}) to A*0301 (12.8 h) as compared to HLA-A*1101 (3.5 h). The peptide AMGGDAGGYK (aa 167–175) had a higher affinity to HLA-A*0301 (ED 3×10^{-6} M) and showed one log difference in binding to HLA-A*1101 (3×10^{-5} M) with a similarly short half-life of 0.3 and 0.5 h, respectively. Other peptides, such as FVRSSNLKF (aa 232–240), bound to three different HLA species, that is to HLA-A*2402 and to two HLA-B molecules (HLA-B*0801 and

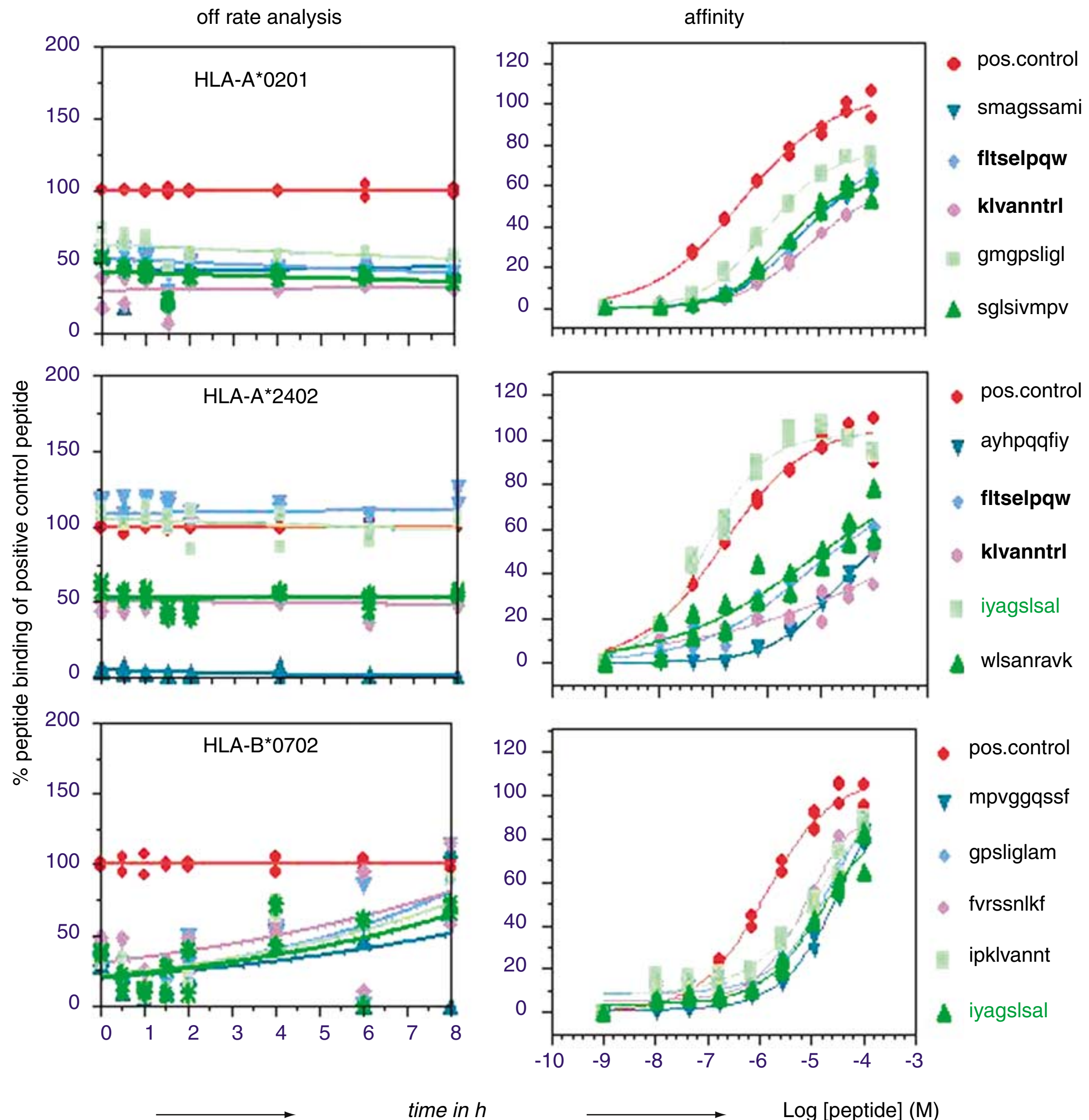


Figure 2 Binding and off-rate analysis of candidate peptides to HLA-A*0201; -A*2402; -B*0702 molecules. Curves are fitted by Graphpad Prism software. Affinity analyses are depicted on the right panel. The ED_{50} value represents the concentration of the test peptide, which yields 50% of the positive control peptide binding. Off-rate analysis are depicted on the left panel. Peptide half-life ($t_{1/2}$) represents time (hours) elapsed for 50% loss of fluorescence intensity as compared to time point 0 at saturation. The peptides FLTSELPQW and KLVANNTRL (illustrated in bold) bound both to HLA-A*0201 and -A*2402, as described earlier. The peptide IYAGSLSAL bound to HLA-A*2402 as well as to -B*0702 (in green). Complete data sets of individual ED_{50} and $t_{1/2}$ values are listed in Supplementary Table S2. Binding and off-rate analysis raw data for each individual test peptide are provided in the supplemental data set, Supplementary Figure S6.

B*1501) with a similar affinity; FVRSSNLKF showed an ED_{50} value of 4×10^{-6} M (to A*2402), 2×10^{-6} M (to B*0702) and 8×10^{-6} M (to B*1501); the relatively short half-life of these MHC class I/peptide complexes was 1.8, 1.1 and 3.9 h, respectively. A different peptide (IYAGSLSAL, aa 144–152) bound to HLA-A*0201, A*2402 and to HLA-B*0702. The peptide IYAGSLSAL (aa 144–152) displayed a comparatively low affinity to B*0702 (2×10^{-5} M, $t_{1/2}$ is 1.6 h), a higher affinity to HLA-A*2402 (2×10^{-7} M) with a similar half-life of 1.8 h, yet a low affinity to HLA-A*0201 (4×10^{-4} M, $t_{1/2}$ is 4.6 h) (see Table 1). Two peptide species were successfully complexed to tetramers which bind to different HLA molecules: AMGDAGGYK (aa 167–175) was successfully folded with HLA-A*0301 and A*1101 and the above listed peptide IYAGSLSAL (aa 144–152), binding to B*0702 and to A*2402. Some patients tested positive for both HLA-A*0301 and A*1101, or A*2402 and B*0702 (see details in Supplementary Table S2a/b). This allowed enumerating CD8+ T cells, which recognize identical peptides presented by different alleles (see Supplementary Table S3).

Other peptides analyzed showed subtle differences in the amino-acid composition and binding to different MHC class I epitopes which cover the identical area in Ag86B, for example the HLA-A*0201 epitope **KLVANNT**RL (aa 199–297; ED_{50}; 6×10^{-6} M; $t_{1/2}$; 9.6 h) and the B*0702 epitope IP**KLVANNT** (aa 197–205; ED_{50}; 3×10^{-5} M; $t_{1/2}$; 1.6 h). A similar situation was found for the epitope **FVRSSNLKF** (aa 232–240) which bound to HLA-A*2402, B*0701, B*1501 (see above) and the peptide EN**FVRSSNL** (aa 230–238) presented by HLA-B*0801

with a low ED50 of 2×10^{-4} M and a half-time of 1.6 h, or the peptide G**QSSFYSDWY** (aa 74–83), presented by HLA-B*1501 with an ED50 value of 4×10^{-6} M and a half-life of 1.9 h (only the decamer bound to HLA-B*1501, not the nonamer QQSSFYSDWY); the nonameric version **QSSFYSDWY** (aa 75–83) was presented by HLA-A*0101 with an ED50 value of 5×10^{-6} M and a half-life of 2.2 h. The localization of the individual candidate T-cell epitopes within Ag85B is compiled in Figure 3c and in Supplementary Figure S8 in the supplementary data. Note that the three essential amino-acids,[5,16] required for the enzymatic mycolyl transferase activity (S at position 126, E at position 229 and H at position 262) are not contained in the epitopes that bound to the eight human MHC class I alleles, except for serine at position 126, which constitutes the HLA-A*0201 binding peptide SMAGASSAMI (aa 126–134).

Presence of tetramer-reactive T-cells in patients with active tuberculosis

Peripheral blood mononuclear cells (PBMC) were collected from 57 (MHC class I-typed) patients with acute pulmonary tuberculosis and analyzed for tetramer binding using 21 different tetramers (see Table 1). HLA-A*0101 and A*1101 were covered with a single tetramer species, HLA-A*0301 and B*1501 with two tetramers, B*2402 and B*0801 with three, A*2402 with four and A*0201 with five tetramers, respectively. The data depicted in Figure 3a–c are expressed as percent of tetramer-positive events in CD3+, CD8+ (CD4−, CD13−, CD19−) T cells. The MHC class I molecules, tetramer-positive cells (in %) and data obtained by using negative control tetramers (using 'placeholder' peptides) for standardization are provided in the supplementary data set (Supplementary Table S3, supplemental data). Figure 3a shows a representative data set for three epitopes covering HLA-B*0801 restricted responses, Figure 3b shows the presence of tetramer-positive CD3+CD8+ T cells for each individual patient ($n = 57$), matched for the MHC class I haplotype, that is the entire array of tetramers has been applied to each MHC class I molecule, depending on the genetic background of each individual patient. Figure 3c shows the presence of tetramer-positive events stratified for each MHC class I molecule. In general, and as validated by the negative control tetramer-binding data, tetramer binding levels for HLA-A*0201 are relatively low. The predominant MHC class I response directed against Ag85B in patients with acute pulmonary tuberculosis is apparently HLA-B restricted, that is the highest frequency of tetramer-reactive T cells has been identified in HLA-B*0702 or to HLA-B*0801 restricted CD8+ T-cell responses (Figure 3b and c). With the exception of HLA-A*0101 and HLA-A*1101, for which we were able to generate and test a only single tetramer, three CD8 T-cell binding patterns were identified: (a) dominance of a single Ag85B epitope–HLA complex recognized by CD8+ T cells in combination with other 'subdominant' T-cell epitopes at lower frequency; (b) co-dominance of two T-cell epitopes among a restricted group of peptides; and (c) diverse and broad recognition of Ag85B peptides from some species without any dominant CD8+ T-cell recognition. The detailed analysis for each MHC class I/peptide complex is depicted in Supplementary Figure S7.

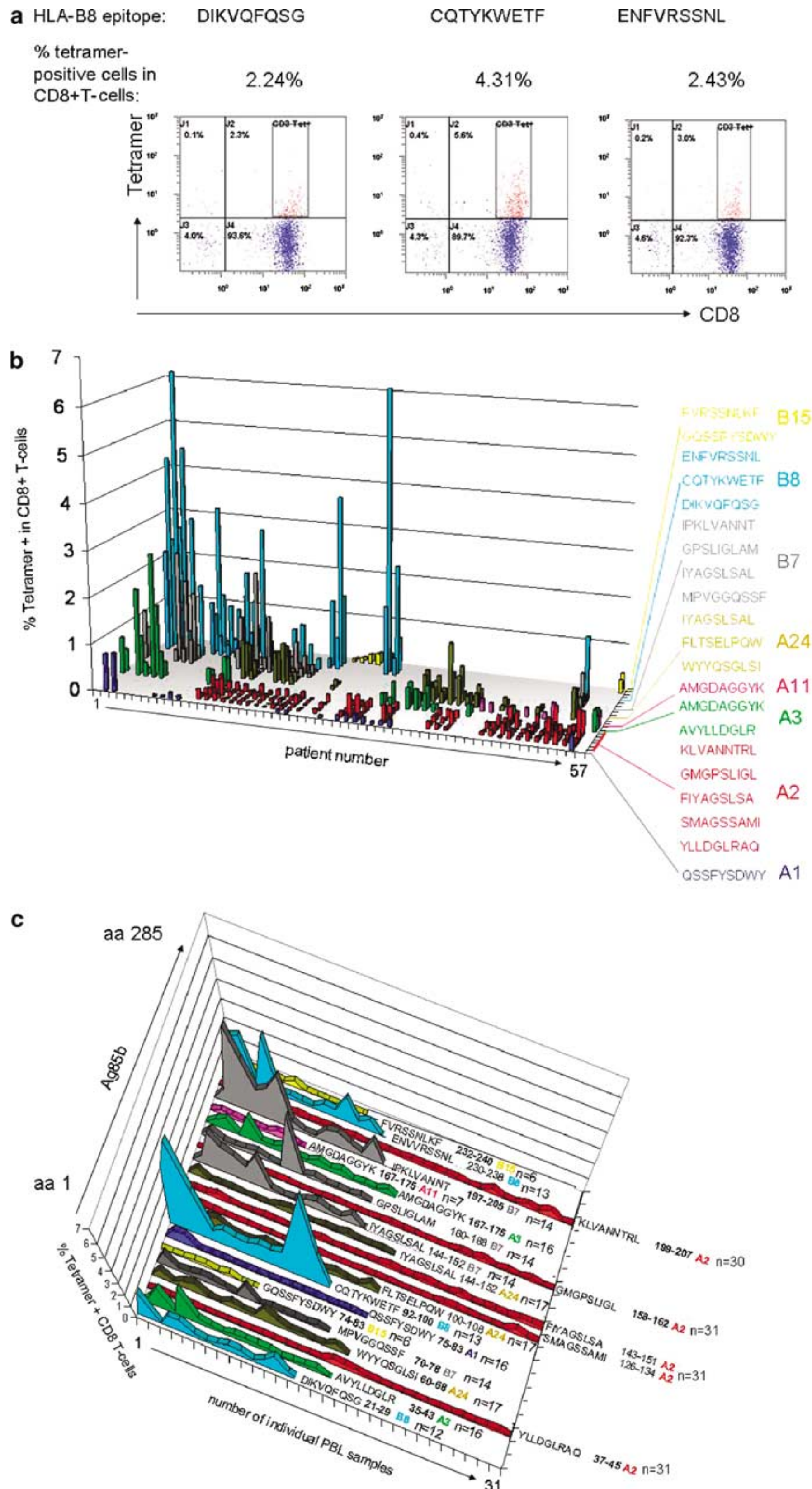
HLA-A*0301, A24*02, B*0801 and B*1501 are represented in category A with a predominant T-cell response. For instance, the CD8+ T-cell reactivity directed against the epitope AVYLLDGLR (aa 35–43) was slightly higher in HLA-A*0301 restricted T-cell responses, as assessed by frequency of tetramer-reactive CD8+ T-cells, yet it was associated with a similar CD8+ T-cell response directed against AMGDAGGYK (aa 167–175). HLA-A*2402 presented peptides showed also a hierarchy for CD8+ T-cell binding, the epitope WYYQSGLSI (aa 60–68) showed the highest, the epitope FLTSELPQW (aa 100–108) an intermediate and the peptide IYAGSLSAL (aa 144–152) the lowest frequency of tetramer-positive CD8+ T-cells, that is the peptide WYYQSGLSI was indicative for an HLA-A*2402 restricted and Ag85B-specific T-cell response (see Figure 4a–c and Supplementary Figure S7). A similar situation appeared to be true for CD8+ T-cells recognizing Ag85B peptides in the context of HLA-B*0801 and HLA-B*1501: CD8+ reactivity to the epitope CQTYKWETF (aa 92–100), presented by HLA-B*0801 (more than 6% tetramer-positive T-cells in individual patients) were indicative for the HLA-B*0801 restricted response to Ag85B, that is the peptides ENFVRSSNL (aa 230–238) and DIKVQFQSGS (aa 21–29) were less frequently recognized. A similar pattern was identified in the HLA-B*1501-restricted T-cell response: the CD8+ T-cell response directed against the peptide FVRSSNLKF (aa 232–240) was indicative for responsiveness to GQSSFYSDWY (aa 74–83).

Two alleles differ in this pattern: HLA-B*0702 showed the pattern of category B and HLA-A*0201 that of the category **C** in CD8+ T-cell recognition. Only two out of four HLA-B*0702 presented T-cell epitopes showed a co-ordinated T-cell frequency, that is the CD8+ T-cell response showed the highest frequency in response to the peptide IPKLVANNT (aa 197–205), in some patients more than 4% of CD8+ T-cells, followed by a similar dynamic of the T-cell response directed against IYAGSLSAL (aa 144–152). Binding of CD8+ T cell cells to IPKLVANNT was not associated with a similar pattern of reactivity to other HLA-B*0702 presented epitopes, that is GPSLIGLAM (aa 160–168) or MPVGGQSSF (aa 70–78), (see Supplementary Table 3 and Supplementary Figure S7). A more diverse pattern emerged in HLA-A*0201 presented T-cell epitopes ('category C recognition'). The T-cell response did not exhibit a focused pattern in regard to the frequency of tetramer-reactive CD8+ T cells. For instance, individual patients showed comparably higher CD8+ T-cell frequencies directed against YLLDGLRAQ (aa 37–45) and not against KLVANNTRL (aa 199–207). Yet, other patients exhibited up to 0.4% FIYAGSLSA (aa 143–151)-reactive T cells not associated with similar CD8+ frequencies directed against the other T-cell epitopes presented by HLA-A*0201. Thus, HLA-A*0201 showed a predominant recognition of single peptide species, that is YLLDGLRAQ (aa 37–45) and KLVANNTRL (aa 199–207) and did not exhibit a co-ordinated pattern for recognition of other HLA-A*0201 binding peptides as compared to the other HLA-A or -B alleles.

Ag85B-reactive T cells reside in the same CD8+ T-cell subset independent of MHC restriction and peptide specificity

Tetramer-reactive T cells were evaluated for T-cell differentiation and homing markers to assess the antigen-85B specific T-cells for their subset phenotype. Of interest was to determine if CD8 T cells that bound to different peptides presented by the same HLA molecule reside within the same T-cell subsets, and if presentation

by a different HLA allele-encoded molecules would affect this distribution. In this experiment, PBMCs from three additional patients (A, B, C) with active tuberculosis were evaluated, and PBMCs from a healthy individual (HLA-B3$^+$, B7$^+$) with a positive PPD-skin test (with no clinical evidence of disease) were used for comparison (see Figure 4a and b). In the healthy donor, the majority of HLA-A*0301 and HLA-B*0702-restricted



T cells, independent of the peptide species, resided in the CD45RA$^+$, CCR7$^+$ CD8$^+$ precursor subset, followed by tetramer-reactive T cells in the CD45RA$^+$, CCR7$^-$ T-cell subset. In contrast, the majority of tetramer-reactive T cells in PBMCs from patients with active tuberculosis resided either in the CD45RA$^-$, CCR7$^-$ (peripheral memory) T-cell subset (patients A and B), or in the CD45RA$^-$, CCR7$^+$ (central memory) T-cell subsets in patient C (Figure 4a). No difference could be observed, either in regard to the MHC restriction or to the specific T-cell epitope. Functional analysis (*in vitro* IL-2 and IFN-γ production) revealed that tetramer-binding T cells in PBMCs obtained from patients with active tuberculosis are little or not responsive to stimulation with the nominal peptide epitope, yet they are partially responsive to PMA/ionomycin stimulation, which leads to IFN-γ, but not to IL-2, production, as determined by intracellular cytokine staining (Figure 4b).

Discussion

By using a systematic approach to epitope discovery in the eight most frequent Caucasian HLA-A and B molecules, and subsequent generation and application of tetramers, we have been able to measure Ag85B-specific CD8$^+$ T-cell response in 57 patients with active tuberculosis across the different MHC class I molecules. Previously, only a few MHC class I-mediated responses have been identified for the Ag85B of *M. tuberculosis*, including three HLA-B35 presented epitopes directed against the closely related antigen Ag85C.[17] At least two HLA-A*0201 restricted CD8$^+$ T-cell epitopes, including FIYAGSLSA (aa 143–151) and KLVANNTRL (aa 199–207) were described in HLA-A2 transgenic mice and human PBMCs after *in vitro* stimulation and expansion.[12] Direct *ex vivo* identification of Ag85-specific T-cell responses without *in vitro* re-stimulation, however, had not yet been reported.

CD4$^+$ T-cells obtained by from healthy, PPD-skin test positive donors have been reported to focus preferentially on the region 92–108 in Ag85B,[18] which provides the epitope FLTSELPQW (aa 100–108) recognized by HLA-A*2402-restricted T cells.

Although high values in binding algorithms, for example SYFPEITHI, are generally associated with good MHC/peptide binding, there are exceptions (see Table 2a/b and Supplementary Figure S5). For instance, the peptide DPTQQIPKL (aa 192–200) showed a high SYFPEITHI score of 21 for HLA-B*0702, but it did not show significant binding (5% of the control peptide) to the recombinant MHC class I protein. In contrast, the peptide YYQSGLSIV (aa 61–69) exhibits a SYFPEITHI score of 10 and showed 128% binding to HLA-A24. As well, cell-based MHC stabilization assays, such as those using T2 for HLA-A2, were found to be only variably useful in terms of HLA-A2 epitope prediction, perhaps partially due to unaccounted cellular processing of candidate peptides.[19–22]

For clinical studies, most attempts to directly enumerate antigen-specific T-cells in specimens are hampered by the limited availability of appropriate peptide epitopes and the limited availability of MHC class I tetramer reagents. Nevertheless, in HLA-A*0201 and B*0702 of CMV-positive carriers, the frequency of CMV-specific CD8$^+$ T-cells is reproducibly higher for the HLA-B*0702 restricted CMVpp65 epitope, as compared to the HLA-A*0201 restricted CD8$^+$ T-cell response. This also implies that the frequency of antigen-specific CD8$^+$T-cells may be grossly underestimated if only HLA-A*0201 restricted T-cell responses were to be analyzed,[23,24] at least in situations when a patient is both HLA-A*0201 and B*0702 positive. The dominant Ag85B-specific immune response restricted by HLA-B alleles applied only for HLA-B*0702 and HLA-B*0801, but not for HLA-B*1501. Thus, although one could speculate that the overall shorter half-life of peptides in HLA-B molecules would result in a differentially better priming and expansion during the 'first wave' of CD8$^+$ T-cell responses directed against Ag85B in patients with active tuberculosis, this is not reflected in the CD8$^+$ T-cell responses against the tested peptides presented by HLA-B*1501. The overall pattern of Ag85B candidate peptide binding to HLA-B molecules, with a short half-life and a comparable lower affinity as compared to HLA-A molecules, reflects the nature of the HLA-B and not the individual composition of the peptides provided from an MTB-associated antigen: detailed binding analysis of candidate peptides derived from the tumor associated antigen survivin, which is over-expressed in a high number of human cancers, showed a similar pattern of differential binding to human HLA-A and -B molecules,[22] other MTB-associated antigens (e.g. TB10.4) exhibit a similar profile (our unpublished data).

The current data suggest that longer peptide half-lives, such as those observed for Ag85B peptides binding to HLA-A*0201, may be associated with a low frequency of antigen-specific CD8$^+$ T cells. Longitudinal analyses of TB patients (after effective anti-mycobacterial therapy) will show how the pattern of immune reactivity with a preferential expansion of CD8$^+$ T cells restricted by certain HLA molecules will change. The preservation of antigen-specific T-cell responses may be associated with exposure to the nominal antigen.[25] This will be of particular interest in the context of the infection with MTB: Ag85B production is associated with live, replicat-

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Figure 3 (**a**) Representative data set for relatively high responses to HLA-B*0801 in CD8$^+$ T cells of patient; 2. T cells were negatively gated on CD4, CD13 and CD19 and positively gated on CD3 and CD8$^+$ T-cells, and tetramer binding was performed. The raw data for each individual patient including the MHC class I typing is listed in Supplementary Table S3 of the supplemental data set. (**b**) Overview of the T-cell recognition pattern in CD8$^+$ T cells of 57 individuals with pulmonary tuberculosis. The HLA-A and -B alleles of each patient are listed in the supplemental data set Supplementary Table S3. Patient ID numbers are on the *x* axis, the % tetramer-positive cells within CD8$^+$ T cells is plotted on the *z* axis, individual peptide species (each HLA molecule is presented in a different color) are listed on the *y*-axis. (**c**) Frequency of tetramer-reactive T cells as stratified for the location of the epitope within the Ag85B protein. The individual peptide species are listed on the *y* axis, each HLA molecule is depicted with a different color (corresponding to **b**). The number of individuals tested is indicated on the *x* axis, the percentage of tetramer-reactive T cells in CD8$^+$ T cells is listed on the *z* axis. Epitopes derived from HLA-B*0801 and HLA-B*0702 exhibited the highest Ag85B-reactive T-cell frequencies. A detailed examination of the hierarchy of MHC/peptide complexes recognized by CD8$^+$ T cells is presented in the supplementary data set in Supplementary Figure S7.

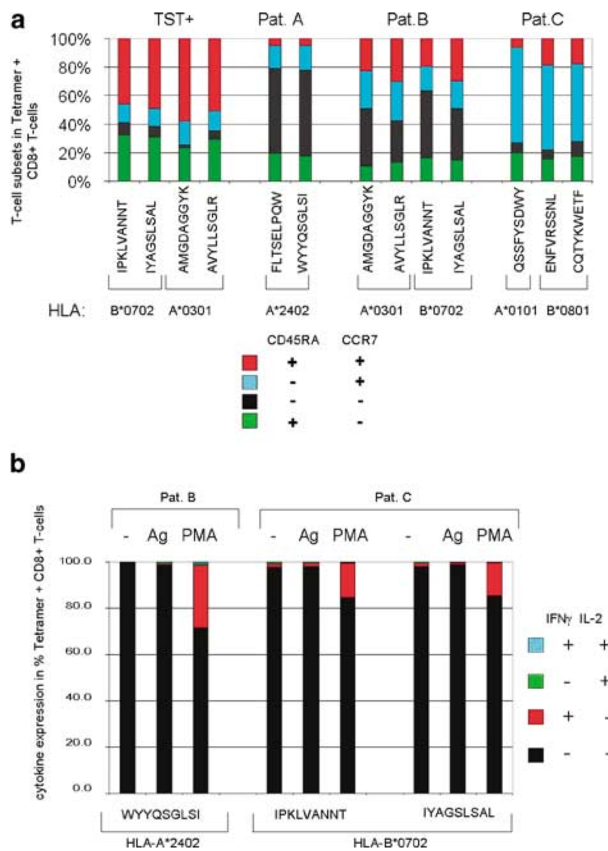

Figure 4 T-cell subset analysis of antigen-specific CD8$^+$ T cells and cytokine expression pattern of tetramer labeled cells. (**a**) PBMCs from healthy, tuberculin skin test (TST)-reactive blood donors were tested for binding to the entire set of MHC/peptide complexes, according to the MHC class I haplotype of the individual in conjunction with CD45RA and CCR7. Results from one representative example are shown on the left (TST +) as percentage of the tetramer labeled cells. In comparison, three patients with active tuberculosis were examined similarly for tetramer-reactive T cells expressing the T-cell homing and differentiation markers CD45RA and CCR7 (designated as Pat. A–C). The majority of tetramer-reactive T cells in healthy individuals reside in the CD45RA$^+$CCR7$^+$ CD8$^+$ precursor T cells, irrespective of the restricting HLA allele or the individual T-cell epitope. A similar consistency in percentage of T-cell subsets to different HLA restricted epitopes was seen in patients with tuberculosis, independent of the individual restricting molecule or the target epitope. In contrast to healthy individuals, the majority of Ag85B-reactive T cells, however, resided in either the central memory (CD45RA$^-$CCR7$^+$) or in the peripheral memory (CD45RA$^-$ CCR7$^-$) T-cell subset. (**b**) Cytokine production in tetramer-reactive T cells in PBMCs from patients with active pulmonary tuberculosis was tested by exposing cells to either the nominal target peptide or by stimulation with PMA/ionomycin, and by subsequent staining with tetramers. Only PMA stimulation, but not peptides, production. Representative data shown from patients B and C resulted in IFN with identical pattern of cytokine responses, independent of the restricting HLA molecule or peptide epitope. Data were expressed as % of cytokine-positive in CD8$^+$ tetramer-reactive T cells.

ing bacteria. This situation will be different upon effective treatment.[3] The pattern of the T-cell response may also be associated with the nature of the antigen presenting cell. Ag85 complex is also available in serum in acute MTB infection, bound to fibronectin and immunoglobulin G.[10] This may impact on antigen uptake, processing and presentation. Arrest of viable *M. tuberculosis* conferred by adaptive immunity in the mouse lung is associated with decreased mRNA levels for the Ag85 complex. Future longitudinal studies with the newly developed tetramer reagents will help to address these basic immunological questions and the impact of the different players orchestrating the cellular immune response, that is (i) nature of the antigen, (ii) antigen processing and presentation, (iii) presence of the antigen and (iv) restricting MHC molecules and the TCR repertoire capable of responding to MHC class I epitopes. The latter point is particularly interesting if the same epitope can be presented by different MHC class I alleles (see Table 1 and Supplementary Table 2).

Based on the CD8$^+$ T-cell recognition pattern of a single antigen, that is Ag85B and eight human MHC class I molecules, the 'immunome' focus in active tuberculosis is diverse and co-dominant for HLA-A*0201 and HLA-B*0702 in respect to Ag85B epitopes in the patient cohort described in the current report. The presence of antigen-specific CD8$^+$ T-cells in either the central and peripheral memory T-cell subsets may rather reflect the cytokine milieu associated with the inflammatory nature of acute tuberculosis infection in contrast to the differential presence of either CMV, EBV or HIV-reactive T cells in the CD8$^+$ T-cell populations that exhibit alternative homing and differentiation characteristics in patients with chronic viral infections.[26] This notion is also supported by the lack of IL-2 and INF-γ production in tetramer-reactive T cells upon peptide stimulation and is consistent with the notion of MTB-antigen-specific anergy in patients with either acute or latent MTB infection.[27,28] Of note, high amount of circulating Ag85 proteins have also been incriminated to participate in antigen-specific anergy in patients with MTB infection due to the interaction of Ag85 complex with T –cell-associated fibronectins.[10,29]

At this time, we are not able to link the presence of dominant HLA-B*0702 or HLA-B*0801-restricted immune responses with better response to therapy, or different patterns of disease severity. All patients studied suffered from pulmonary TB and presented with acid-fast bacilli in the sputum detected by direct microscopy, which indicates at least 10^4 bacilli/ml sputum. Data from HIV-infected individuals suggest that HLA-B*5801, B*0801 and HLA-B*0702 may be associated with more favorable clinical performance, lower viral load and broader recognition of gag epitopes as compared to other MHC alleles.[30] Future prospective studies with individuals with active TB and their respective contacts who are either (i) able to fight off the infection despite exposure, (ii) able to contain the infection (development of latent TB) or (iii) not able to control the infection and present with clinical disease will show the impact of the HLA-restricting elements and MTB Ag85-presented T-cell epitopes. Conceptually, several variables will play a role: the number and strength of MHC class I-restricted T-cell responses (e.g. the dominant response for HLA-B*0702 and HLA-B*0801), the breadth of the antigen-specific immune response defined by the number of epitopes presented by an individual allele[30] and the possibility that individual peptide epitopes can be presented by different MHC class I alleles, such as those described in the current report.

The implications of this study are the following: First, the CD8$^+$ T-cell response would have been underestimated if only a single MHC allele, for example

HLA-A*0201, would have been selected for screening of Ag85B restricted T-cell responses (see Supplementary Figure S8). Second, the CD8$^+$ T-cell response directed against Ag85B can be classified into different categories, on the basis of the restricting element. Third, a prolonged presence of antigen, such as for Ag85B, high affinity peptide binding to individual class I alleles (e.g. HLA-A*0201) and comparably slow MHC class I peptide off-rates appear to be associated with low frequency of antigen-specific CD8$^+$ T-cell responses in the peripheral circulation of patients with clinical TB.

Materials and methods

Cellular analysis

Heparinized blood was drawn at the time of clinical diagnosis from patients with sputum-positive *M. tuberculosis* cultures, PBMCs were separated and stored in liquid nitrogen. All patients showed presence of acid-fast bacilli in sputum samples by direct microscopy and presented with cough and intermittent fever. Informed consent and ethical approval were documented (on file with the reference no. 837.327.99 -2272- 15 November, 1999, University Mainz, Germany). MHC class I and class II genomic typing was performed at the Blood Bank, University of Mainz. Tetramer reagents were prepared from the Immunomics Corporation (Beckman Coulter, San Diego, USA). Tetramer reagents were labeled with PE, except HLA-A2 tetramers that were labeled with APC. Flow cytometric analysis was performed including negative gating on CD4$^+$, CD13$^+$, CD19$^+$ cells to exclude false-positive events. Positive events were identified in the CD3$^+$, CD8$^+$, (CD4-, CD13- and CD19-negative) population. PBMCs were first analyzed for absolute cell numbers and for dead/alive discrimination by flow cytometry. Tetramer binding was analyzed after 30 min incubation at RT, followed by a 10 min incubation step using anti-CD3 (clone CHT1), anti-CD8 (clone T8) (positive gating) and anti-CD4 (clone 13B8.2), anti-CD13 (clone SJ1D1) and anti-CD19 (clone J4.119) for negative gating. Cells were analyzed using an FC500 flow cytometer from Beckman Coulter, and a PARTEC CyflowML. Data are expressed as percent of tetramer-positive events in CD3$^+$, CD8$^+$ (CD4$^-$, CD13$^-$, CD19$^-$) T cells. T-cell differentiation and maturation was evaluated using co-staining of tetramer-positive cells with anti-CD45RA, clone 2H4 and anti-CCR7, clone 150503. To test for functional reactivity, PBMCs were stimulated for 1 h at 37°C with the nominal candidate peptide, followed by a 5 h incubation step in the presence of the stimulating peptide and Golgi blockage with Brefeldin A. Tetramer binding was performed, cells were fixed, permeabilized and stained for intracellular IFN-γ (clone 45.15), or IL-2 (clone MQ1-17H12). Stimulation with PMA/ionomycin was carried out as a positive control. All antibodies were obtained from Beckman Coulter, Krefeld, Germany; except the anti-CCR7 mAb, obtained from BD Pharmingen (Heidelberg, Germany).

MHC class I-binding algorithm

Possible epitopes within the amino-acid sequence of Ag85B for CD8 T cell binding were selected based on the SYFPEITHI program, which is suitable to predict peptides for HLA-A*0101, A*0201, A*0301, A*2402, B*0702 and B*0801 presentation. This algorithm does not cover HLA-A*1101 or B*1501. Peptide candidates binding to either A*1101 or B*1501 were selected based on three-dimensional structure information as well as on MHC-binding peptides, which have been described in the past; for HLA-A*1101: V or I in position 2, M or L in position 3, L or I in position 7 and K or R in position 9; for HLA-B*1501: Q or L in position 2, I or V in position 5, F or Y in position 9. An additional indicator to elect candidate peptides was applied with the program PAProC, which represents a prediction tool for proteasomal cleavage by human proteasomes based on experimental cleavage data.[15] Even if an individual peptide candidate did not yield a high binding score on SYFPEITHI, it was still included in the experimental screening process if it was likely to be produced by the constitutive or the immune proteasome. Test peptides were selected from the *M. tuberculosis* Ag85B (Rv1886c) and custom synthesized (JPT, Berlin, Germany). For binding to HLA-A, 49 peptides were selected, and 15 peptides were selected for binding analysis to HLA-B. Only nonamers were included; decamer peptides were only tested for HLA-B*1501.

MHC peptide-binding assay

Peptide-binding experiments were performed in duplicates in *iTOPIA* 96-well plates coated with individual recombinant MHC class I molecules (HLA-A*0101, A*0201, A*0301, A*1101, A*2402, B*0702, B*0801 and B*1501; Beckman Coulter, San Diego, USA). The screening involved an initial peptide-binding assay that measures the ability of individual peptides species to bind to HLA molecules under standardized, optimal conditions. In this assay, the place holder peptides were stripped from the MHC class I monomer complexes coated on plastic, which leaves the MHC class I heavy chain available as a binding partner for candidate peptides in the presence of human β-2 microglobulin. Peptide binding was studied at a concentration range from 1×10^{-5} to 1×10^{-6} M at 21°C using specific monoclonal antibodies, which recognize only the trimeric, properly folded MHC class I/peptide/β-2 microglobulin complex conformation. Each candidate peptide was tested against an appropriate control peptide, specific for each MHC class I molecule, and results are reported in % binding as compared to the control peptide.

Off-rate and affinity assay

Test peptides identified as 'binders' were further characterized for affinity and dissociation. In general, peptides which showed values equal or greater than 30% of the control peptide were considered. However, some peptides with lower binding values were included as elaborated previously.

Off-rate assay: As this assay evaluated the dissociation of previously bound peptide at defined time points, the off-rate is expressed as the $t_{1/2}$ value and indicates how long a peptide is able to stay in the MHC class I-binding cleft. Candidate peptides were bound to the relevant MHC class I monomer, the MHC class I/peptide/β-2 microglobulin complexes were incubated at 37°C and tested at the indicated time points for specific peptide content. Results are reported in hours required for 50% reduction in peptide loading of the MHC class I molecules.

Affinity assay: Candidate peptides were incubated at concentrations ranging from 10^{-4} to 10^{-9} M to determine their relative binding affinities for each individual MHC class I molecule. Binding affinity is expressed as the peptide quantity needed to achieve 50% binding saturation (the ED_{50} value) of an individual MHC class I molecule species in the iTOPIA plates.

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Supplementary Information accompanies the paper on Genes and Immunity website (http://www.nature.com/gene)