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Review

Mycobacterium tuberculosis-specific and MHC class I-restricted CD8+ T-cells exhibit a stem cell precursor-like phenotype in patients with active pulmonary tuberculosis

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SUMMARY

The nature and longevity of the T-cell response directed against Mycobacterium tuberculosis (MTB) are important for effective pathogen containment. We analyzed ex vivo the nature of MTB antigen-specific T-cell responses directed against the MTB secreted antigens Rv0288, Rv1886c, Rv3875, the antigens Rv2958c, Rv2957, and Rv0447c (intracellular, non-secreted enzymes) in blood from Korean patients with active tuberculosis (TB). MTB-specific T-cell function was defined by intracellular cytokine production (interleukin (IL)-2, interferon gamma, tumour necrosis factor alpha, and IL-17) and by multimer-guided (HLA-A*02:01 and HLA-A*24:02) analysis of epitope-specific CD8+ T-cells, along with phenotypic markers (CD45RA and CCR7), CD107a, a marker for degranulation, and CD127 co-staining for T-cell differentiation and homing. Cytokine production analysis underestimated the frequencies of MTB antigen-specific T-cells defined by major histocompatibility complex (MHC) class I–peptide multimer analysis. We showed that MTB antigen-specific CD8+ T-cells exhibit a distinct marker profile associated with the nature of the MTB antigens, i.e., Rv0288, Rv1886c, and Rv3875-reactive T-cells clustered in the precursor T-cell compartment, whereas Rv2958c, Rv2957, and Rv0447c-reactive T-cells were associated with the terminally differentiated T-cell phenotype, in the patient cohort. Rv0288, Rv1886c, and Rv3875-specific CD8+ T-cells were significantly enriched for CD107a+ T-cells in HLA-A*02:01 ($p < 0.0001$) and HLA-A*24:02 ($p = 0.0018$) positive individuals, as compared to Rv2958c, Rv2957, and Rv0447c antigens. CD127 (IL-7 receptor)-expressing T-cells were enriched in HLA-A*02:01-positive individuals for the Rv0288, Rv1886c, and Rv3875 specificities ($p = 0.03$). A high proportion of antigen-specific T-cells showed a precursor-like phenotype (CD45RA+CCR7+) and expressed the stem cell-associated markers CD95 and c-kit. These data show that MTB-specific T-cells can express stem cell-like features; this is associated with the nature of the MTB antigen and the genetic background of the individual.

1. Introduction

Mycobacterium tuberculosis (MTB), the aetiological agent of tuberculosis (TB) affects 8.8 million people and causes an estimated 1.5 million deaths globally per annum.[1] Several factors such as poor BCG vaccine efficacy, challenges in making an accurate diagnosis of TB using available diagnostics, widespread emergence of drug resistance, poor infection control measures, and co-infection with HIV, continue to fuel the TB epidemic.[2–5] Critical to determining protective immune responses is the study of multiple MTB exposures, recurrent MTB re-infections, as well as simultaneous infections with multiple MTB strains.[6] The immunological consequences of these scenarios and their impact on memory immune responses have not yet been examined. A better understanding of protective or ineffective immune responses in TB may aid the design of biologically relevant biomarkers to visualize clinically relevant memory immune responses.

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Different T-cell subsets, including CD4+ and CD8+ T-cells, play a crucial role in MTB containment,[7,8] i.e., by cytokine production or direct cytotoxicity. Th1 cytokines, interferon gamma (IFN-γ) and tumour necrosis factor alpha (TNF-α), are important for optimal MTB control.[8,9] Polyfunctionality of T-cells, i.e., the ability to simultaneously produce interleukin 2 (IL-2), IFN-γ, and TNF-α, has previously been linked to immune protection and/or post-vaccination memory responses in different infectious diseases including HIV.[10] However, in TB, the data available to date are pointing in different directions.[11–14]

T-cells recognize, and are activated by, small parts (peptide stretches) of protein-derived antigens presented via major histocompatibility complex (MHC) glycoproteins (in humans called human leukocyte antigen (HLA)) on antigen-presenting cells (APCs). Many different immunogenic MTB proteins have been identified by their ability to induce antigen-specific T-cell responses, usually defined by cytokine production. Such MTB proteins include the early expressed (secreted) antigens Ag85B (Rv1886c), TB10.4 (Rv0288), and ESAT-6 (Rv3875).[15–19] More recently, we have identified epitopes and T-cell responses (in both CD4+ and CD8+ T-cells) provided from (non-secreted) MTB intracellular enzymes (cyclopropane fatty acid synthase (CFA synthase; Rv0447c) and two glycosyltransferases (Rv2957 and Rv2958)[19–21]), which are preferentially expressed in slow-growing bacteria, including in *Mycobacterium* species. Rv0447c is a key enzyme involved in MTB lipid metabolism, producing factors that make the cell wall impermeable.[22] The two glycosyltransferases belong to the intermediary metabolism; they are involved in the formation of phenolic glycolipids and glycosylated *p*-hydroxy-benzoic acid methyl esters, constituents of the mycobacterial cell envelope and MTB virulence factors.[23] Rv2958c is also believed to be involved in the ability of MTB to survive inside macrophages.[24] These (non-secreted) proteins may show a different pattern of immunogenicity compared to the early expressed (secreted) MTB antigens, yet this has not been formally shown.

Antigen-specific T-cells recognizing MTB antigens have been identified by different immune readouts,[1] for example by using cytokine production, i.e., IFN-γ or TNF-α (by intracellular cytokine staining (ICS), ELISPOT, or ELISA in cell culture supernatant after in vitro stimulation with defined molecular targets). Alternatively, epitope-specific T-cells can directly be identified ex vivo, without the need for functional readouts, using soluble MHC class I–peptide complexes (i.e., MHC multimers). One advantage of this approach is the possibility to assess T-cell frequencies without any in vitro manipulation,[25] in combination with cell-surface markers to determine the phenotype and effector functions of the pathogen-specific T-cells. Based on the cell-surface markers CD45RA and CCR7, mature T-cells can be divided into four different phenotypic compartments. Precursor T-cells express both CD45RA and CCR7; they replenish the T-cell pool, yet produce only limited amounts of cytokines. Central memory T-cells (CD45RA−CCR7+) represent lymph node homing antigen-experienced cells that lack immediate effector functions (except IL-2 production). The third and fourth compartments represent effector memory T-cells (which have down-regulated both cell-surface markers) and terminally differentiated effector cells (CD45RA+CCR7−), which home to anatomical sites of disease.[26,27] The latter populations represent T-cell populations producing IFN-γ and TNF-α with cytotoxic potential.[28]

In the current study, we compared the frequency of MTB antigen-specific CD8+ T-cells in peripheral blood mononuclear cells (PBMCs) detected by ICS to the frequency of antigen-specific T-cells directed to defined MTB epitopes using MHC class I multimers, in order to evaluate whether antigen-specific T-cells are possibly being underestimated in active pulmonary TB. We characterized homing, differentiation, and effector functionality

(CD107a, degranulation), IL-7 receptor (CD127)-mediating survival signals, as well as c-kit (CD117) and CD95, 'stem-ness', in T-cell populations in association with the MHC class I genetic background and the nature of the MTB antigens (i.e., intracellular (non-secreted) enzyme antigens vs. MTB secreted antigens). The results show in which T-cell compartment MTB-specific T-cells reside; a crucial information since the lack of effector T-cells (CD45RA+ CCR7−) has been associated with an increased risk of developing active TB.[8]

2. Materials and methods

2.1. Patient data

Twelve newly diagnosed patients with active pulmonary TB (acid-fast and culture-positive) at St. Mary's Hospital, Seoul, South Korea were enrolled in the study. Eleven patients were male and one was female (patient 12), and they ranged in age from 23 to 73 years (**Supplementary Material**, Table S1). The samples were obtained after diagnosis and after drug susceptibility testing (DST), which were performed in accordance with international guidelines. Institutional review board (IRB) consent was obtained from the Catholic Medical Centre, Seoul, South Korea (Ref. XC09FZZZ0046K; the independent ethics review committee of the Catholic University Seoul, South Korea) and from the ethics committee in Stockholm (Ref. 2011/863-31/2; Stockholm City Ethical Council South Committee). The ethics committees reviewed the study plan, as well as the patient informed consent forms, which are on file with the institutions as stipulated. The patients provided written consent, these papers are on file at the hospital.

Blood samples were drawn from the patients and PBMCs were isolated. The PBMCs were HLA-typed in South Korea for the alleles HLA-A*02 and A*24. Three individuals tested positive for HLA-A*02, three individuals positive for HLA-A*24, and six individuals double-positive for both alleles were included in order to gauge MHC class I multimer complexes. We chose to use material from patients with newly diagnosed active pulmonary TB, since the clinical definition of the spectrum of latent TB is challenging.

2.2. Selection of epitopes for multimer construction

Epitopes from different well-characterized MTB proteins (Rv3875, Rv0288, and Rv1886c) for multimer construction were selected based on (1) previously reported detection of antigen-specific T-cells (A2-Rv3875$_{AMASTEGNV}$,[29] A2-Rv3875 $_{LLDEGKQSL}$,[30] A2-Rv0288$_{IMYNYPAML}$,[31] A24-Rv0288$_{IMYNYPAML}$,[31] A2-Rv1886c$_{KLVANNTRL}$,[17] A2-Rv1886c$_{FIYAGSLSA}$,[17] and A24-Rv1886c$_{IYAGSLSAL}$[17]), and (2) MHC peptide binding data (A24-Rv3875$_{AYQGVQQKW}$ (our own non-published data) and A24-Rv3875$_{ELNNALQNL}$[32]). Epitopes from proteins Rv2957, Rv2958c, and Rv0447c were selected based on high SYFPEITHI scores,[33] which translate into a high likelihood of successful soluble MHC class I antigen–peptide production (A2-Rv2957$_{SIIIPTLNV}$ (score = 26), A24-Rv2957$_{PYNLRYRVL}$ (score = 21), A2-Rv2958c$_{ALADLPVTV}$ (score = 30), A24-Rv2958c$_{KYIAADRKI}$ (score = 25), A2-Rv0447c$_{VLAGSVDEL}$ (score = 31), and A24-Rv0447c$_{KYIFPGGLL}$ (score = 25)).

2.3. Cellular analysis with multimers

Fifteen different MHC class I–peptide multimers were either purchased or constructed in our laboratory: A2-Rv3875$_{AMASTEGNV}$, A2-Rv3875$_{LLDEGKQSL}$, A2-Rv1886c$_{KLVANNTRL}$, A24-Rv1886c$_{IYAGSLSAL}$ (Beckman Coulter, San Diego, CA, USA), A24-Rv3875$_{AYQGVQQKW}$, A24-Rv3875$_{ELNNALQNL}$, A2-Rv2957$_{SIIIPTLNV}$, A24-Rv2957$_{PYNLRYRVL}$, A2-Rv2958c$_{ALADLPVTV}$, A24-Rv2958c$_{KYIAADRKI}$, A2-Rv0447c$_{VLAGSVDEL}$, A24-Rv0447c$_{KYIFPGGLL}$ (Immudex, Copenhagen, Denmark), A2-Rv0288$_{IMYNYPAML}$, A24-Rv0288$_{IMYNYPAML}$, and A2-Rv1886c $_{FIYAGSLSA}$

(constructed by us, as described previously[25]); these were fluorescently labelled with streptavidin–phycoerythrin (PE), streptavidin–allophycocyanin (APC), and fluorescein isothiocyanate (FITC). Cryopreserved PBMCs were stained with the multimers at 37 °C for 30 min. Cellular analyses were performed using a FACS Gallios Flow Cytometer (Beckman Coulter). Multimer-positive events were recorded in the CD3+CD8+CD4− compartment using the following antibodies: anti-CD3-PE/Texas red (ECD) (Clone UCHT1) (Beckman-Coulter), anti-CD8a-APC/Cy7 (Clone SK1) (Becton Dickinson, Franklin Lakes, NJ, USA), and anti-CD4-Pacific orange (Clone S3.5) (Invitrogen, Carlsbad, CA, USA). Cells in the CD3+CD8−CD4+ compartment were excluded from enumeration of CD3+CD8+ multimer+ events. Only multimer responses were reported that were at least three times higher than the negative control and for which we could detect more than an absolute 50 events.

2.4. Analysis of T-cell phenotype

Phenotypic analysis of multimer-positive cells was performed using the following antibodies: anti-CD45RA-PerCP/Cy5.5 (Clone HI100) (Biolegend, San Diego, CA, USA) and anti-CCR7- PE/Cy7 (Clone 3D12) (Becton Dickinson). Degranulation marker analysis was performed using anti-CD107a-Pacific blue (PB) (Clone H4A3) (Biolegend); for IL-7Rα expression, unconjugated anti-CD127 (Clone R34.34) (Beckman Coulter) was labelled using the Zenon-Alexa-700 labelling kit (Invitrogen). Analyses of 'stem cell markers' were performed using anti-CD95-FITC (Clone LT95) (Thermo Scientific/Pierce Biotechnology, Rockford, IL, USA), anti-CD95-Alexa-700 (Clone LT95) (Exbio, Prague, Czech Republic), and anti-CD117-Brilliant violet 421 (Clone 104D2) (Biolegend). Analysis of senescence markers was performed using anti-IL-18R-FITC (Clone H44) (Thermo Fisher Scientific, Waltham, MA, USA), anti-PD-1-PE (Clone EH12.1), anti-CTLA-4-PE/Cy5 (Clone BNI3) (Becton Dickinson), anti-TIM-3-PerCP/eFluor710 (eBioscience, San Diego, CA, USA), and polyclonal anti-LAG-3-APC (R&D Systems, Minneapolis, MN, USA).

2.5. Intracellular cytokine staining (ICS)

Cryopreserved cells were incubated for 6 h together with 10 µg/ml secretion inhibitor (brefeldin A) and either only medium, 25 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1 µg/ml ionomycin (SigmaAldrich, St Louis, MO, USA), or 1 µg/ml peptide mixes of overlapping peptides covering the TB proteins Rv3875 and Rv0288 (JPT Peptide Technologies GmbH, Berlin, Germany, and AERAS Foundation, Rockville, IL, USA). Intracellular cytokine production was subsequently detected after fixation and permeabilization (Beckman Coulter) by flow cytometry analysis in the CD3+CD8+ compartment, using the following antibodies: anti-CD3-PE/Texas red (ECD) (Clone UCHT1) (Beckman Coulter), anti-CD8a-PC/Cy7 (Clone SK1), anti-CD4-PerCP/Cy5.5 (Clone L200), anti-IFN-γ-PE/Cy7 (Clone B27), anti-IL-2-PE (Clone MQ1-17H12), anti-TNF-α-APC (Clone MAb11) (Becton Dickinson), and anti-IL-17-FITC (Clone BL168) (Biolegend).

2.6. Statistics

Statistical significance between different T-cell populations was evaluated using Prism 4.0 (GraphPad Software, La Jolla, CA, USA) using the Mann–Whitney non-parametric test.

3. Results

3.1. Broad recognition of Mycobacterium tuberculosis-reactive and MHC class I-specific T-cells in patients with active TB

We constructed multimeric MHC class I–peptide complexes from six different MTB proteins. We divided the antigens into two groups based on their MTB expression pattern: (1) early secreted antigens: ESAT-6 (Rv3875), TB10.4 (Rv0288), and Ag85B (Rv1886c), and (2) intracellular, non-secreted expressed antigens associated with slow-growing bacteria: glycosyltransferase 1 (Rv2958c), glycosyltransferase 2 (Rv2957), and CFA synthase (Rv0447c). The multimers were constructed using two of the most frequent MHC class I alleles in the Korean population, i.e., HLA-A*02:01 and A*24:02. Flow-based analysis of PBMCs from all patients showed the detection of antigen-specific CD8+ T-cells with frequencies ranging from 0.1% to 6.5% (Table 1). Examples of flow cytometry staining and the gating strategy are provided in the **Supplementary Material** (Figures S1 and S2). The highest frequency of antigen-specific T-cells was found to be associated with the MTB antigen Rv0288 (median 0.3% (0.1–0.9%)), followed by Rv3875 (median 0.3% (0.1–6.5%)) and Rv2958c (median 0.2% (0–1.1%)). The most frequently detected individual epitopes within these antigens were A24-Rv3875$_{ELNNALQNL}$ (median 2.9% (1.5–6.5%)), followed by A24-Rv2957$_{PYNLRYRVL}$ (median 1.3% (0.9–1.8%))

Table 1
Frequencies of MHC class I restricted *M. tuberculosis* CD8+ T-cells in PBMCs from patients with active TB.

	Multimer[a]	Epitope	Patient											
			1 A*02/A*24	2 A*24	3 A*02	4 A*02	5 A*24	6 A*02	7 A*02/A*24	8 A*02/A*24	9 A*24	10 A*02/A*24	11 A*02/A*24	12 A*02/A*24
A2	Rv0288	IMYNYPAML	0.1		0.1	0.3		0.2	0.2	0.6		0.1	0	0.1
	Rv1886c	KLVANNTRL	4.1		0.9	1.1		0.4	0.8	0.7		1.1	1.0	0.2
	Rv1886c	FIYAGSLSA	0.1		0.1	0.2		0.1	0.1	0.1		0.2	0.3	0
	Rv3875	AMASTEGNV	0.7		0.4	0.7		0.6	0.1	0.5		0.5	0.3	0.3
	Rv3875	LLDEGKQSL	0.5		0.2	0.2		0.1	0.1	0.1		0.2	0.1	0.1
	Rv2958c	ALADLPVTV	0.1		0.1	0		0	0	0.1		0.2	0	0.1
	Rv2957	SIIIPTLNV	0.1		0.1	0		0.1	0	0.1		0	0	0.1
	Rv0447c	VLAGSVDEL	0.2		0.1	0.1		0.1	0.1	0.1		0.2	0.2	0.2
A24	Rv0288	IMYNYPAML	0.3	0.4			0.3		0.5	0.9	0.7	0.4	0.4	0.9
	Rv1886c	IYAGSLSAL	0.3	0.2			0.1		0.1	0.1	0	0.1	0.1	0.1
	Rv3875	AYQGVQQKW	0.1	0.1			0.1		0.1	0.3	0.2	0.2	0.2	0.2
	Rv3875	ELNNALQNL	6.5	3.2			2.9		2.5	1.5	1.8	2.9	3.1	1.8
	Rv2958c	KYIAADRKI	0.3	1.1			0.3		0.4	0.4	0.4	0.4	0.8	0.6
	Rv2957	PYNLRYRVL	1.5	1.8			1.2		0.9	1.2	1.3	1.7	1.0	1.4
	Rv0447c	KYIFPGGLL	0.1	0.4			0.1		0.2	0.1	0.2	0.2	0.2	0.1

[a] Frequency of antigen-specific T-cells identified by multimer staining. Peripheral blood mononuclear cells (PBMCs) from individuals with tuberculosis (TB) were incubated with major histocompatibility complex (MHC)-matched MHC class I multimers and stained for T-cell markers. Results are reported as the percentage multimer-positive events in the CD3+CD8+ T-cell population; negative gating was performed to exclude CD4+ T-cells. For cells marked in grey, subsequent phenotypic analysis of the cells was performed. Frequencies of CD8+ T-cells directed against the (negative) control multimers have been deducted.

and A2-Rv1886c$_{KLVANNTRL}$ (median 0.9% (0.2–4.1%)) (**Supplementary Material** Figure S3). We compared the frequencies of antigen-specific T-cells detected in the two groups of MTB antigens. The results showed a higher frequency of CD8+ T-cells detecting epitopes originating from antigens that belonged to the first group A early secreted MTB antigens (i.e., Rv0288, Rv1886c, and Rv3875) as compared to the MTB group B antigens (i.e., Rv2958c, Rv2957, and Rv0447c; intracellular, non-secreted enzymes). Differences in T-cell frequencies were significant for the allele HLA-A*02:01 ($p < 0.0001$); however an opposite trend could be seen for the A*24:02 allele (Figure 1A). The higher frequency of T-cells recognizing the group A antigens was driven by a few immunodominant epitopes derived from the MTB antigen Ag85B (e.g., A2-Rv1886c$_{KLVANNTRL}$).

We studied whether the nature of the restricting MHC allele has any association with the detection of antigen-specific CD8+ T-cells (for the antigens used for analysis in the current study). Therefore, we constructed two different multimers (HLA-A*02:01 and A*24:02) presenting the identical Rv0288 epitope (IMYNYPAML) and compared the frequencies of antigen-specific CD8+ T-cells. We could detect a significantly higher frequency of antigen-specific

cells recognizing the epitope bound to HLA-A*24:02 as compared to the same epitope presented by HLA-A*02:01 in PBMCs from patients with the corresponding MHC class I background ($p = 0.0019$) (Figure 1B). The same observation was found to be true for the comparison of the total frequency of antigen-specific T-cells restricted by HLA-A*24:02 as compared to the epitopes restricted by HLA-A*02:01 ($p < 0.0001$) (Figure 1C).

3.2. Cytokine production in Mycobacterium tuberculosis-reactive T-cells

Next, we studied the effector functions of T-cells from patients with active pulmonary TB in order to test the functionality of MTB-specific CD8+ T-cells. As a first step, we studied the ability of the cells to produce cytokines (examples of flow cytometry staining are provided in the **Supplementary Material** Figure S4). We could detect the production of the Th1 cytokines IL-2 (median 21%), IFN-γ (median 9.3%), and TNF-α (median 19%) as a response to maximal stimulation (PMA/ionomycin) in the CD8+ T-cell population. The maximal production of interleukin 17 (IL-17) was low in CD8+ (median 0.0%) (Figure 2A). We also identified cytokine-producing MTB-reactive CD8+ T-cells (IL-2, IFN-γ, and TNF-α in the range of 0.11–0.19%) responding to the MTB antigens Rv0288 and Rv3875. TNF-α was found to be the most frequently produced cytokine in CD8+ T-cells, followed by IL-2 and IFN-γ. The production of IL-17 was low in response to the MTB antigens, as well as to PMA/ionomycin (Figure 2B). Due to the general low frequency of cytokine-producing cells, polyfunctionality of the CD8+ T-cells could not be assessed in this study.
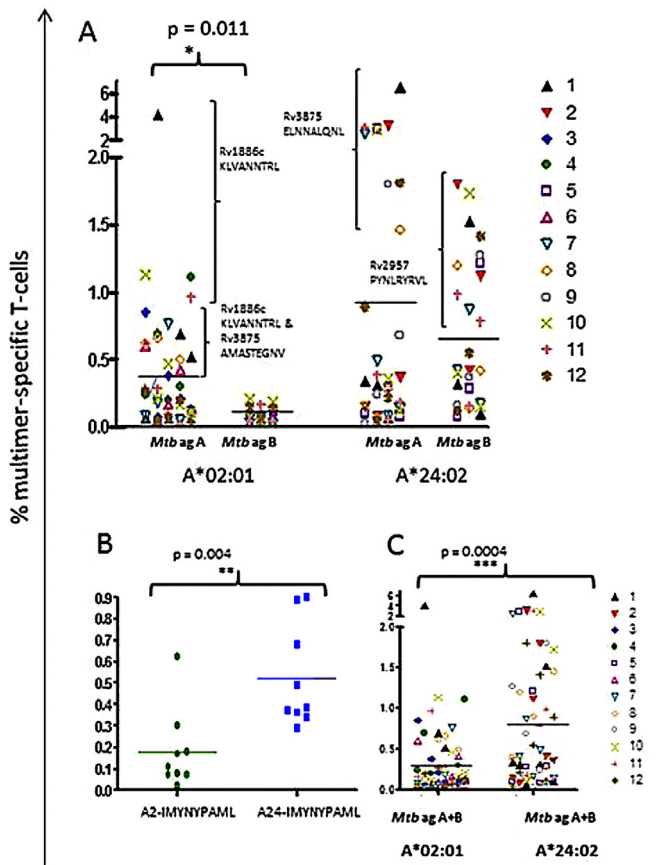
Figure 1. Frequency of multimer-positive CD8+ T-cells. (A) Comparison of the antigen-specific CD8+ T-cells recognizing the MTB antigens of group A (Rv0288, Rv1886c, and Rv3875) (secreted antigens) restricted by A*02:01 ($n = 45$) and A*24:02 ($n = 27$), and the novel MTB antigens of group B (Rv2958, Rv2957, and Rv0447) (intracellular, non-secreted enzymes) restricted by A*02:01 ($n = 32$) and A*24:02 ($n = 27$). (B) Individual detection of antigen-specific T-cells specific for the 'super-epitope' IMYNYPAML divided per restricting allele A*02:01 ($n = 9$) (green) and A*24:02 ($n = 9$) (blue). (C) Individual detection of total (MTB groups A and B) antigen-specific T-cells restricted by either A*02:01 ($n = 72$) or A*24:02 ($n = 59$). Each dot represents the staining in a single patient, and the different patients are shown with different shapes and colours. Significant differences were detected by comparing the median values using the Mann–Whitney non-parametric test.
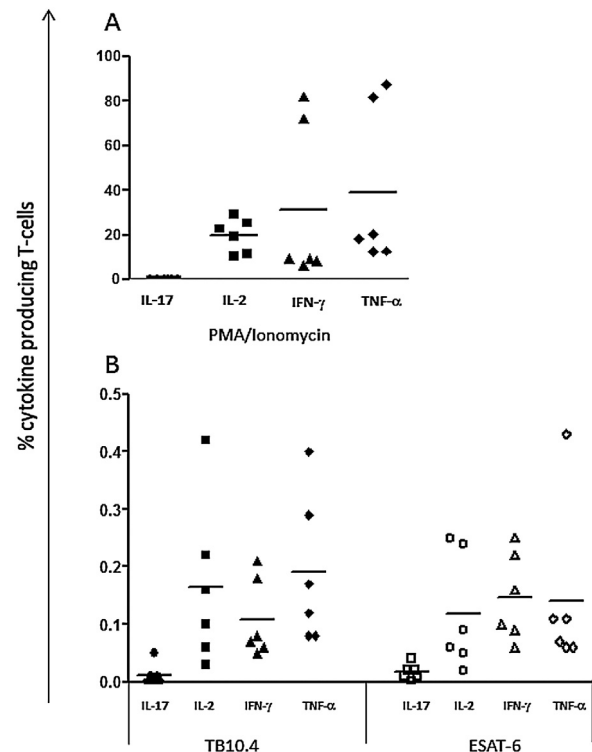
Figure 2. Intracellular cytokine staining (ICS) ($n = 6$). (A) Percentage of CD8+ T-cells producing the cytokines IL-17 (circles), IL-2 (squares), IFN-γ (triangles), and TNF-α (diamonds) in response to maximum stimuli (PMA/ionomycin). (B) Percentage of CD8+ T-cells producing cytokines in response to peptide covering the TB protein Rv0288 (filled symbols) or Rv3875 (open symbols); each dot represents the mean value of two replicates. Frequencies from stimulation with only medium (negative controls) have been subtracted. The black line shows the median value for each cytokine.
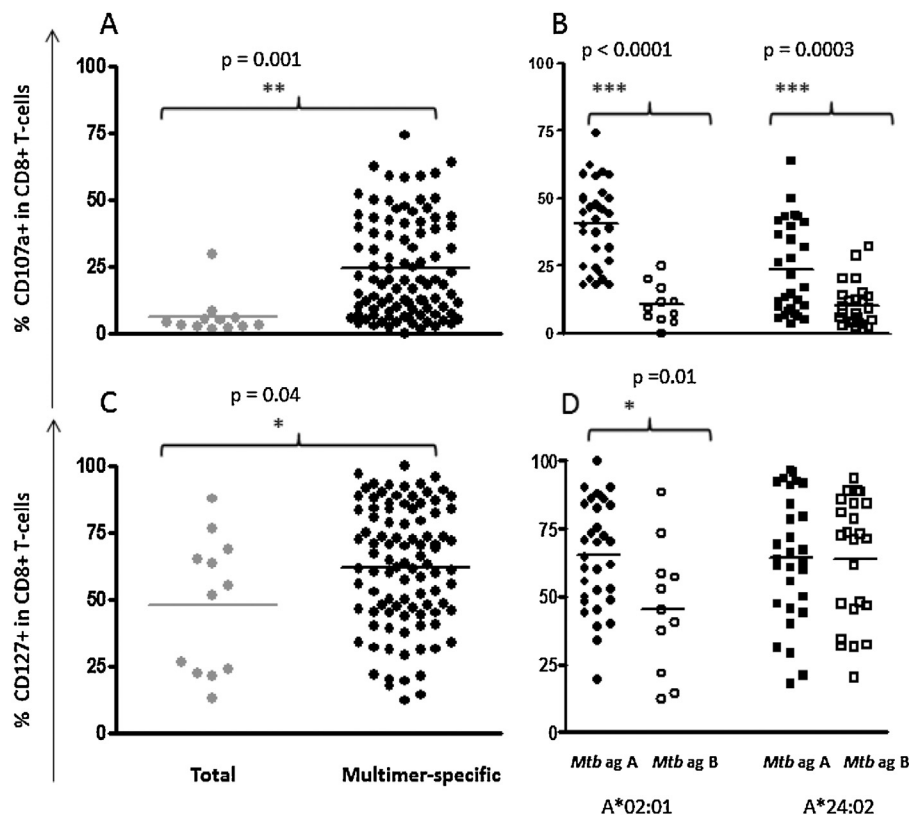
3.3. CD107a and CD127 expression in CD8+ T-cells differentiates between the nature of the Mycobacterium tuberculosis target antigen and MHC class I restriction

Since the frequency of T-cell-producing cytokines in response to MTB antigens was very low, we continued to investigate other survival markers and effector functions in MTB-specific T-cells. The IL-7 receptor α-chain (CD127) is an essential cell-surface receptor in the generation of CD8+ memory cells, while CD107a is a marker of degranulation on CD8+ T-cells. The frequency of CD107a-positive T-cells can be used to gauge ex vivo antigen-specific T-cells with degranulation properties. Examples of flow cytometry staining and the gating strategy are provided in the **Supplementary Material** (Figure S2). We could detect significantly higher frequencies of CD107a+ multimer-positive (i.e., MTB epitope reactive) CD8+ T-cells ($p < 0.0001$) as compared to total CD8+ T-cells (Figure 3A). Divided into the two groups of MTB antigens listed above, we could detect a significantly higher frequency of CD107a+ ($p = 0.0018$) CD8+ T-cells responding to the group A (secreted antigens Rv0288, Rv1886c, and Rv3875) epitopes as compared to the group B antigens (enzymes, non-secreted Rv2958c, Rv2957, and Rv0447c). However, we also found higher frequencies of CD107a-positive T-cells in Rv2958c-, Rv2957-, and Rv0447c-specific T-cells as compared to CD107a expression on total CD8+ T-cells (Figure 3B). There was a similar trend for the marker CD127: a higher frequency of CD127+ MTB antigen-specific CD8+ T-cells could be found as compared to the total CD8+ T-cell population (Figure 3C). We identified a higher frequency of CD127-positive T-cells specific for the group A antigens (early secreted proteins) in HLA-A*02:01-restricted T-cells ($p = 0.03$), while for the A*24:02-restricted T-cells no differences were detected concerning CD127 expression and reactivity to early (secreted) antigens and the intracellular (non-secreted) antigens (Figure 3D).

3.4. Comparison of multimers and ICS: cytokine production analysis underestimates Mycobacterium tuberculosis-reactive T-cells

Analysis of MTB-specific T-cells showed that we can (1) detect antigen-specific T-cells directed towards several different MTB antigens, (2) detect low cytokine production in response to MTB antigens, and (3) detect indirect effector functions indicated by CD107a expression (recent degranulation). We compared the CD8+ T-cells, directed to a single epitope in an MTB protein identified by multimer staining, with T-cells producing cytokines in response to overlapping peptides covering the identical (full length) protein. We were able to detect higher frequencies of antigen-specific T-cells by the use of multimers as compared to intracellular cytokine production ($p < 0.0001$) (Figure 4A). For Rv0288, we compared antigen-specific T-cells recognizing the epitope IMYNYPAML, both restricted by HLA-A*02:01 and A*24:02, to the production of IL-17, IL-2, IFN-γ, and TNF-α. In both cases, we could detect higher frequencies of CD8+ T-cells directed to a single epitope as compared to CD8+ T-cells (from the identical blood sample) producing cytokines in response to epitopes spanning the entire target protein (Figure 4B). The frequency of antigen-specific T-cells by multimer staining for the epitopes from Rv3875 was higher compared to the frequency of CD8+ T-cells producing cytokines. For a single epitope (A*24-Rv3875-6$_{ELNNALQNL}$) we were in fact able to detect more than 10 times higher frequencies of antigen-specific T-cells compared to T-cells producing cytokines (Figure 4C).

Figure 3. Increased frequencies of cells expressing the degranulation marker CD107a (A) and the IL-7 receptor CD127 (C) in antigen-specific CD8+ T-cells ($n = 95$) (black) versus total CD8+ T-cells ($n = 12$) (grey). Expression of CD107a (B) and CD127 (D) by CD8+ T-cells recognizing the MTB antigens of group A (Rv0288, Rv1886c, and Rv3875) (A*02:01 $n = 31$ and A*24:02 $n = 29$) (closed symbols) and the MTB antigens of group B (Rv2958, Rv2957 and Rv0447) (A*02:01 $n = 11$ and A*24:02 $n = 24$) (open symbols) restricted by A*02:01 (circles) and A*24:02 (squares). Each dot represents an individual multimer in one individual TB patient. The black line shows the median value for each group. The Mann–Whitney non-parametric test was performed and significant values were calculated based on the following p-values: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
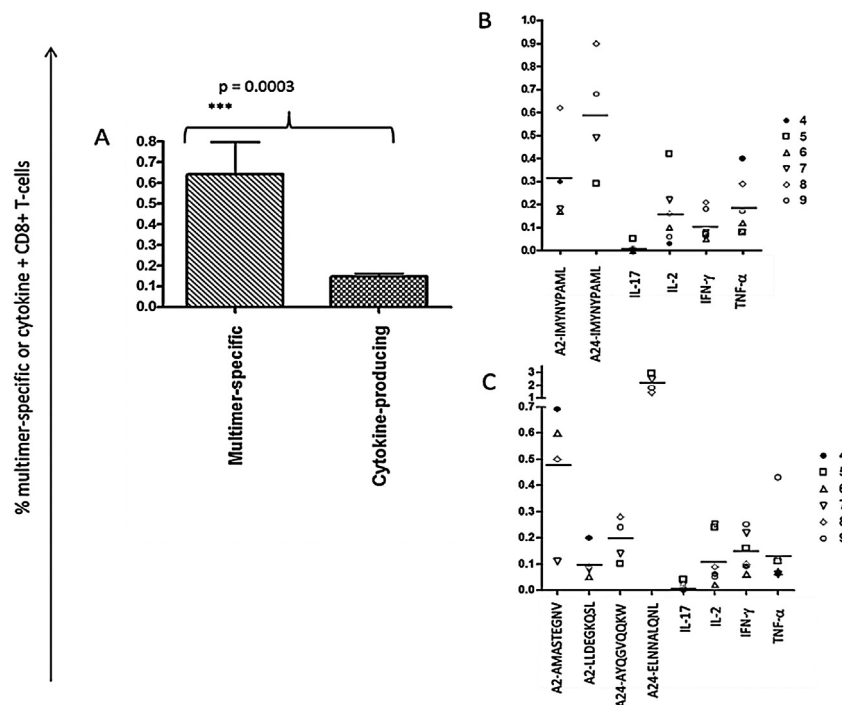
Figure 4. Comparison of the frequency of cytokine-producing CD8+ T-cells and antigen-specific T-cells. (A) Comparison of the total frequency of CD8+ antigen-specific T-cells ($n = 24$) (striped) and CD8+ T-cells producing cytokines (IL-2, IFN-γ, and TNF-α) ($n = 36$) (chequered). The Mann–Whitney non-parametric test was performed and significant values were calculated based on the following *p*-values: $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (B) Comparison of CD8+ T-cells recognizing the IMYNYPAML epitope from the TB protein TB10.4 restricted by A*02:01 ($n = 4$), and A*24:02 ($n = 4$) and CD8+ T-cells producing IL-17 ($n = 6$), IL-2 ($n = 6$), IFN-γ ($n = 6$), and TNF-α ($n = 6$) in response to stimulation with peptides covering the whole Rv0288 sequence. (C) Comparison of CD8+ T-cells recognizing multimers A2-Rv3875$_{AMASTEGNV}$ ($n = 4$), A2-Rv3875$_{LLDEGKQSL}$ ($n = 4$), A24-Rv3875$_{AYQGVQQKW}$ ($n = 4$), A24-Rv3875$_{ELNNALQNL}$ ($n = 4$), and CD8+ T-cells producing IL-17 ($n = 6$), IL-2 ($n = 6$), IFN-γ ($n = 6$), and TNF-α ($n = 6$) in response to stimulation with peptides covering the whole Rv3875 sequence. Each dot represents the staining in a single patient and the different patients are shown with different shapes.

3.5. Phenotypic analysis of antigen-specific T-cells shows that a majority of the Mycobacterium tuberculosis-reactive T-cells reside in the precursor T-cell compartment

We could detect a median of approximately 15% (5–81%) of cells with a precursor phenotype, approximately 30% (9–74%) of T-cells cells with a terminal differentiated phenotype, 4.6% (0.4–10%) with central memory phenotype, and 26% (6–65%) with an effector memory phenotype (the gating strategy and representative staining from raw data are provided in the **Supplementary Material** Figure S2). However, the patient-to-patient differences were high, most likely due to the large age differences between the patients (age range 23–73 years). Within the antigen-specific T-cell compartments, we could detect significantly higher percentages of MTB-specific T-cells with a precursor phenotype ($p = 0.02$) and a trend towards a higher percentage of central memory cells and lower percentages of terminally differentiated cells and effector memory cells (Figure 5A). We could also detect an increase in the percentage of precursor T-cells recognizing the group A (secreted) antigens as compared to the group B (non-secreted) antigens. This situation was mirrored in the opposite recognition pattern concerning terminally differentiated CD8+CD45RA+CCR7– T-cells. The differences were significant for the A*02:01-restricted T-cells ($p = 0.02$ respective 0.04), yet it was also possible to detect a similar trend for the A*24:02-restricted cells (Figure 5B and 5C). For the other two phenotypic subsets (central memory and effector memory), no significant differences could be detected (**Supplementary Material** Figure S5).

3.6. Stem cell marker c-kit and CD95 expression in Mycobacterium tuberculosis-specific T-cells with a precursor-like phenotype

We could identify a high frequency of antigen-specific T-cells with a precursor-like phenotype; these T-cells were examined in greater detail, i.e., using the cell-surface markers CD95 (FAS receptor) and c-kit (the gating strategy and representative staining are provided in the **Supplementary Material** Figure S2). We were able to detect a significant number of CD95-positive T-cells in MTB antigen-specific T-cells compared to CD95 expression in the total CD8+ T-cell compartment expressing a precursor phenotype (CD45RA+CCR7+) ($p = 0.03$) (Figure 6A). This was due to T-cells recognizing antigens provided from the early (secreted) MTB proteins Rv0288, Rv1886c, and Rv3875 (group A) as compared to (non-secreted) antigens associated with cellular metabolism ($p = 0.02$) (Figure 6B). For the c-kit marker, we could detect an upregulation of c-kit on MTB antigen-specific T-cells recognizing individual MHC class I–peptide antigens, yet we could not identify statistically robust differences. A trend towards higher expression of c-kit+ on T-cells in response to group A compared to group B antigens could be detected as well (Figure 6C and 6D). The c-kit-positive cells were further analyzed for expression of senescence markers (PD-1, CTLA-4, TIM-3, and LAG-3), as well as the expression of the IL-18R in PBMCs from five TB patients. We could not detect a stronger expression of these senescence markers on CD45RA+CCR7+c-kit+ T-cells. The same PBMCs were also tested for their ability to produce cytokines (IL-2, TNF-α, IFN-γ) in response to stimulation with either PMA/ionomycin or peptides derived from the MTB protein Rv0288. The results show that CD8+ T-cells produced cytokines in response to maximal stimulation, yet this was not found to be true for the CD45RA+CCR7+c-kit+ T-cells, which hardly produced any cytokines at all (representative data are provided in the **Supplementary Material** Figure S6).

4. Discussion

Antigen-specific CD8+ T-cells have recently been a focus of attention in human TB, especially in the context of anti-TNF-α
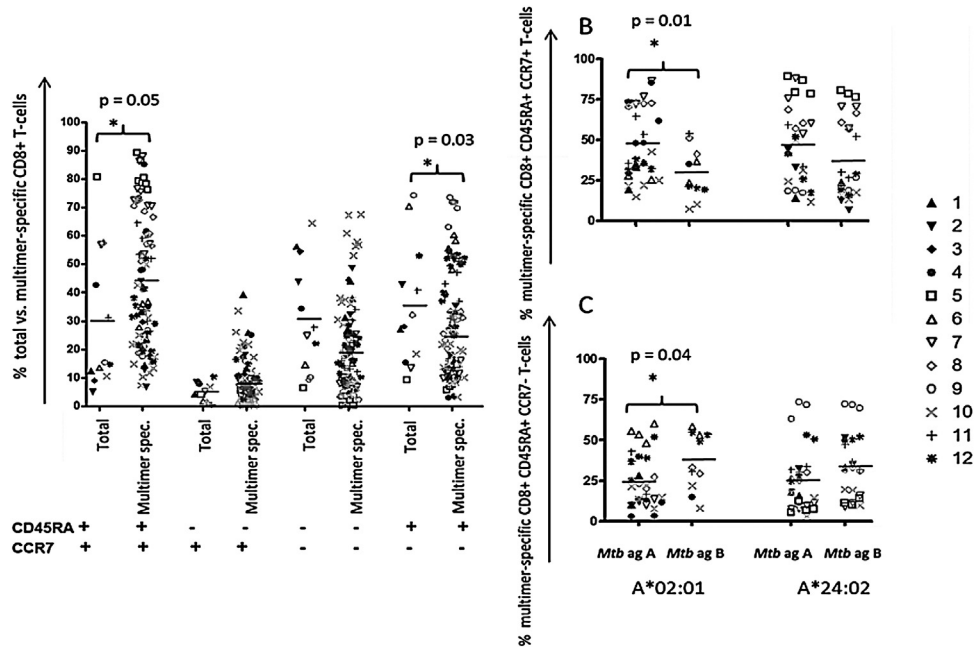
Figure 5. Frequencies of total CD8+ T-cells in different T-cell compartments. (A) Total CD8+ T-cells ($n = 12$) vs. antigen-specific CD8+ T-cells ($n = 95$) belonging to the different phenotypic compartments: precursor (CD45RA+CCR7+), central memory (CD45RA−CCR7+), effector memory cells (CD45RA−CCR7−), and terminally differentiated cells (CD45RA+CCR7−). Comparison of the frequencies of the antigen-specific CD8+ T-cells recognizing the MTB antigens of group A (Rv0288, Rv1886c, and Rv3875) and the MTB antigens of group B (Rv2958, Rv2957, and Rv0447) restricted by A*02:01 ($n = 31$ vs. $n = 11$) and A*24:02 ($n = 29$ vs. $n = 24$) belonging to the (B) precursor (CD45RA+CCR7+) and (C) terminally differentiated (CD45RA+CCR7−) compartments. Each dot represents the staining in a single patient and the different patients are shown with different shapes. The Mann–Whitney non-parametric test was performed and significant values were calculated based on the following p-values: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
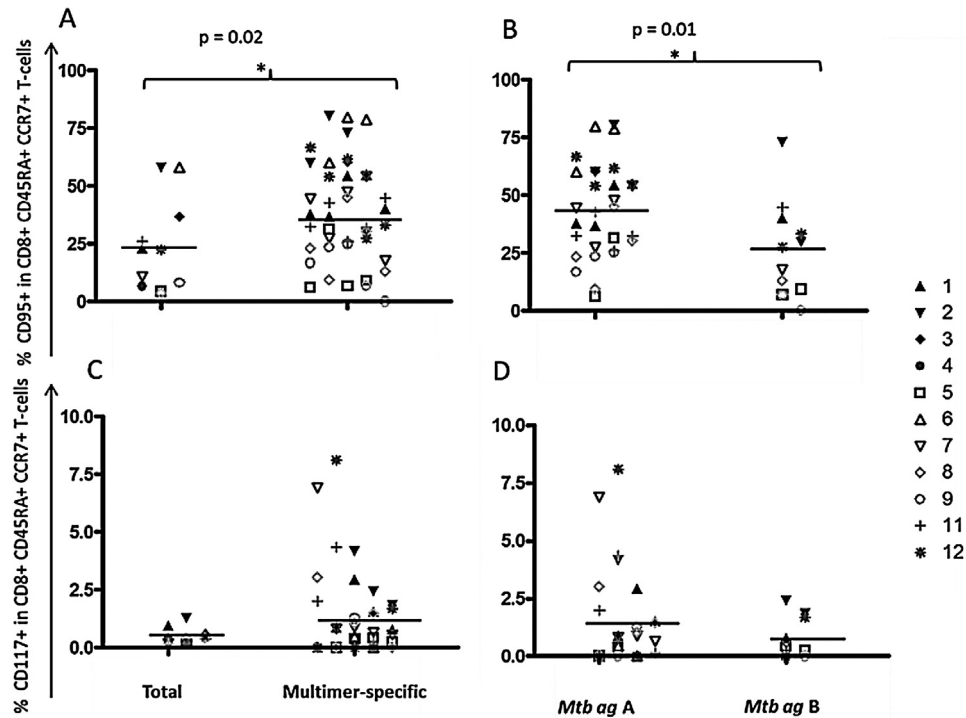
Figure 6. Expression of the 'stem cell markers' CD95 and c-kit. (A) Expression of CD95 on antigen-specific ($n = 42$) vs. total CD8+CD45RA+CCR7+ T-cells ($n = 11$). (B) Comparison of the frequencies of the antigen-specific CD8+CD45RA+CCR7+ T-cells recognizing the MTB antigens of group A (Rv0288, Rv1886c, and Rv3875) ($n = 30$) and the MTB antigens of group B (Rv2958, Rv2957, and Rv0447) ($n = 12$). (C) Expression of c-kit on the naïve (CD45RA+CCR7+) total CD8+ T-cells ($n = 11$) and antigen-specific CD8+ T-cells ($n = 42$). (D) c-kit on antigen-specific T-cells recognizing the MTB group A proteins (Rv0288, Rv1886c, and Rv3875) ($n = 30$) and proteins Rv2958, Rv2957, and Rv0447 ($n = 12$) belonging to the CD8+CD45RA+CCR7+ compartment. Each dot represents the staining in a single patient and the different patients are shown with different shapes. The Mann–Whitney non-parametric test was performed and significant values were calculated based on the following p-values: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

treatment. This treatment leads not only to TNF-α reduction, but also to depletion of terminally differentiated (CD45RA+ CCR7−) CD8+ T-cells,[8] which links T-cell phenotype to a clinically relevant endpoint: higher risk of developing active TB.

This study did not address the evaluation of the diagnostic potential of the MHC class I–peptide multimers, since we did not include PBMCs from patients with latent TB, or from uninfected and/or from non-MTB exposed individuals. Instead, we evaluated ex vivo detection, characterization, and functional analysis (cytokine production and degranulation) of antigen-specific CD8+ T-cells directed against a broad panel of 15 different MHC class I–peptide complexes[17,31,34,35] presenting epitopes from six different MTB proteins presented by the most common Asian MHC class I alleles (HLA-A*02:01 and A*24:02)[36] in patients with active pulmonary TB.

Although some MHC class I presented MTB epitopes (A2-Rv1886c$_{KLVANNTRL}$, A24-Rv3875$_{ELNNALQNL}$, and A24-Rv2957$_{PYNLRYRVL}$) were found to be associated with a more prominent CD8+ T-cell response, we could not identify a dominant CD8+ T-cell response directed towards a single MTB epitope as described for viral pathogens (e.g., Epstein–Barr virus, cytomegalovirus, or HIV) (reviewed in Yewdell and Bennink[37]). The MTB response, at least restricted by HLA-A*02:01 and HLA-A*24:02, was characterized by a low frequency of MTB antigen-specific T-cells in blood from 12/12 individuals (with active pulmonary TB). However, since the lung is the actual site of infection, the immunological profile and recognition pattern might differ between lung lymphocytes and the PBMCs used in this study, as reported previously.[30] Immuno-dominant CD8+ T-cell responses have been described for other infectious diseases where CD8+ T-cells are important, e.g., in HIV-directed immune responses restricted by HLA-B27 and HLA-B57.[38] The preferential use of these MHC class I restricting elements is associated with T-cell polyfunctionality, increased avidity of the cognate TCR (T-cell receptor) –MHC–peptide recognition, and enhanced T-cell clonal turnover.[39] Most of the HLA-A*02:01 MTB epitopes have been analyzed concerning their biochemical interaction with MHC class I molecules,[17,31] and have not shown aberrant low MHC class affinity or off-rates as compared to viral epitopes. We hypothesize that the low frequency of MTB-reactive T-cells, restricted by HLA-A*02:01 and A*24:02, is rather reflecting the available T-cell receptor repertoire in patients with active TB and not the intrinsic biochemical characteristics of MHC class I MTB peptide complexes. In addition, we cannot rule out T-cell cross-recognition of CD8+ T-cells directed against the frequently recognized epitope A24-Rv3875$_{ELNNALQNL}$ from CD8+ T-cells recognizing 'self-epitopes' or epitopes derived from other pathogens (e.g., the A24-Rv3875$_{ELNNALQNL}$ exhibits high (89%) identity with the same amino acid stretch present in Streptococcus agalactiae and Helicobacter winghamensis; the latter pathogen is associated with gastroenteritis[40]). Since some of the presented epitopes express single nucleotide polymorphism between different clinical isolates,[41] we cannot rule out that the infected individuals' MTB isolates affected the antigen-specific T-cell recognition. However, we do not have the detailed DNA sequences of the individual MTB isolates available.

Th1 cytokines, i.e., IL-2, IFN-γ, and TNF-α, play a central role in cellular immunity and the control of active TB. However, the frequencies and impact of single cytokine-producing T-cells, as well as polyfunctional CD4+ and CD8+ cells, tend to vary between different studies. Latently infected individuals, exposed household contacts, and active TB cases seem to have different cytokine profiles: T-cells from patients with active TB appear to produce higher amounts of cytokines including TNF-α and IFN-γ in CD4+ and CD8+ T-cell compartments. IL-2, on the other hand, seems to be more frequently produced in blood from household contacts[14,42,43] in response to MTB antigens. In the current study, we

were able to detect a low, yet clearly distinguishable, cytokine production in response to stimulation with the MTB antigens Rv0288 and Rv3875. The frequencies were lower than, or approximately in the same range as, those identified in response to different MTB proteins in other studies (purified protein derivative (PPD),[13] PPD/Rv3874/Rv3875,[44] Rv1886c/Rv3875/Rv2031c[14]).

On the other hand, we were able to identify higher frequencies of antigen-specific T-cells by use of MHC class I multimers in the same blood samples, compared to T-cells producing cytokines. Either these T-cells are non-functional or they exert a different immune effector function. Based on the up-regulation of the degranulation marker CD107a, these functions might include cytotoxicity. The discrepancy between cytokine-producing cells (IFN-γ) and cytolytic cells has previously been reported in the context of HIV, where CD8+ T-cells tend to exhibit either one of these functions, but rarely both together.[45] These results imply a potential underestimation of antigen-specific cells if T-cell tests are based solely on the detection of Th1 cytokines, with a risk of overlooking T-cells expressing a different cytokine profile or cytotoxic functions.

Much of the current work related to the identification of antigen-specific immune responses in different settings (active-latent MTB, TB in children, HIV co-infected individuals, and TB vaccine settings) has to a high degree focused on a limited number of very well-studied MTB proteins (e.g., Rv0288, Rv1886c, and Rv3875), while MTB codes for more than 4000 different proteins. In this study, we compared differences between these well-studied antigens and three recently identified immunogenic proteins, which are rather expressed in slow-growing bacterial species and are not secreted. The novel epitopes identified in these proteins might not be exclusively present in mycobacteria, but may also occur in other slow-growing bacteria and amoebas, e.g., Streptomyces sp and Dictyostelium sp; cross-recognition of epitopes might therefore occur, in particular since the CFA synthase from these species shows a high homology to the MTB-derived CFA synthase (56% respective 37%) and at least one of the Rv0447c-derived epitopes (Rv0447c$_{KYIFPGGLL}$) perfectly matches an epitope derived from Streptomyces sp and Dictyostelium sp. More work may be needed to elucidate how multiple exposures, co-infection with other pathogens, or even harmless commensals shape the immune reactivity pattern in patients exposed to MTB, in particular since CD8+ T-cells directed against these (non-secreted) MTB targets reside in the terminally differentiated T-cell subset. The fact that differences are more profound for epitopes restricted by the allele HLA-A*02:01 than A*24:02 could be explained either by intrinsic peptide-specific properties or intra allele-specific differences. In general, the fact that the frequencies of antigen-specific T-cells were higher, restricted by the allele HLA-A*24:02, may reflect the success of this allele in antigen presentation and subsequently the high frequency of this allele in the Korean population.[36]

Recent studies detecting MTB-reactive T-cells in blood from children and active and/or latently infected adult individuals, have shown the phenotype of antigen-specific CD8+ T-cells in patients with TB seems to be highly variable.[12,17,29,42,43,46,47] However, somewhat surprisingly, many of these studies reported high frequencies of antigen-experienced T-cells with a precursor-like phenotype.[12,43,46,47] In line with these findings, we could also detect that the majority of the MTB-specific cells belonged to the precursor compartment (CD45RA+CCR7+). Several mechanisms may apply, e.g., increased lymphopoiesis, as well as activated cells reverting back to a 'naïve'-like phenotype without actually being precursor cells.[26,43] Recent studies have also identified antigen-experienced human T-cell populations to viral (influenza and cytomegalovirus) and tumour antigens, bearing many characteristics of precursor T-cells including CD45RA+CCR7+CD127+. At the

same time, these cells express memory and stem cell-like markers such as CD95+ and/or c-kit and have the ability to differentiate into diverse subsets of memory and effector cells.[48,49] CD95 (Fas receptor) is a receptor with both pro- and anti-apoptotic characteristics, depending on the cellular context, and it is also involved in the modulation of T-cell activation.[50] c-kit, on the other hand, is expressed mainly on different types of hematopoietic progenitor cells and plays a role in cell survival, proliferation, and differentiation.[51] The increased frequencies of antigen-specific naïve cells (CD45RA+CCR7+) expressing the CD95 and c-kit cell-surface described in the current report, suggest that these precursor-like cells belong to a stem cell memory compartment. The higher than average expression of the IL-7 receptor molecule CD127 (described here) would also be in the line with these observations. Other investigators[52] argued that the c-kit-positive stem cell memory T-cells identified by Turtle et al.[49] represent, in fact, a subset of effector-type cells, since they produce IFN-γ, express the senescence marker KLRG1, exhibit shorter telomere length, and tested negative for the phenotypic markers CD45RA and CCR7. A considerable frequency of the c-kit-positive T-cells in the current study, however, expressed both of the phenotypic markers (CD45RA/CCR7), while they did not express other senescence markers, nor did they produce cytokines in response to maximal stimulation. Therefore, we suggest that these T-cells belong to a different T-cell compartment as compared to the c-kit+ T-cells described by Turtle and co-workers. Identification of these multipotent precursor-like memory cells in TB could be highly relevant, particularly in the development of novel or improved TB vaccines; the clinical relevance of CD45RA+CCR7+c-kit MTB-specific T-cells will need to be further examined in longitudinal studies in patients responding to MTB treatment and by evaluating household contacts of index persons (who have likely been exposed to MTB, yet are protected).

The higher frequencies of antigen-specific CD8+ T-cells directed against the secreted antigens (at least in HLA-A*02:01-mediated responses) could reflect a dominant response to actively multiplying MTB. In addition, we could identify differences concerning the phenotype between the two groups of antigens (early expressed secreted antigens vs. non-secreted enzymes), in contrast to data reported by Lindestam Arlehamn et al. in regard to MTB-specific CD4+ T-cell responses in latently infected individuals.[53] The dominant immune recognition, as well as the T-cell phenotype (i.e., lower frequency of terminally differentiated effector cells) could indicate that the T-cell response directed against secreted MTB antigens is less functional and that the (secreted) antigens may act as decoy antigens to subvert cellular immune responses, as previously suggested by Baena and Porcelli.[54]

To summarize, we detected ex vivo MHC class I-restricted and MTB antigen-specific T-cells. The frequencies of these cells were associated with the nature of the antigen (secreted versus non-secreted), with the MHC restricting element, and with T-cell effector and homing functions including T-cells with stem cell-like features. Ex vivo identification of multimer-reactive T-cells showed that functional T-cell assays underestimate the frequency of MTB-reactive T-cells (at least in patients with active, not yet treated, pulmonary TB). Manipulation of MTB-restricted T-cells towards a precursor phenotype may hold the key to future targeted therapies aimed at expanding long-term immune effector T-cells. The data also call for a more detailed examination of potentially MTB cross-reactive T-cells induced by other pathogens or mycobacteria other than tuberculosis (MOTT).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijid.2014.12.017.

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