Highly Focused T Cell Responses in Latent Human Pulmonary *Mycobacterium tuberculosis* Infection

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Highly Focused T Cell Responses in Latent Human Pulmonary Mycobacterium tuberculosis Infection

Glenn Tully,* Cornelius Kortsik, Hanni Höhn, Ingeborg Zehbe,* W. E. Hitzler, Claudia Neukirch,* Kirsten Freitag,* Klaus Kayser,‡ and Markus J. Maeurer1*

The elucidation of the molecular and immunological mechanisms mediating maintenance of latency in human tuberculosis aids to develop more effective vaccines and to define biologically meaningful markers for immune protection. We analyzed granuloma-associated lymphocytes (GALs) from human lung biopsies of five patients with latent Mycobacterium tuberculosis (MTB) infection. MTB CD4+ and CD8+ T cell response was highly focused in the lung, distinct from PBL, as assessed by TCR-CDR3 spectratyping coupled with a quantitative analysis of TCR VB frequencies. GALs produced IFN-γ in response to autologous macrophages infected with MTB and to defined MTB-derived HLA-A2-presented peptides Ag85a242–250, Ag85b199–207, early secreted antigenic target 6 (ESAT-6)28–36, 19-kDa Ag88–97, or the HLA-DR-presented ESAT-61–20 epitope. Immune recognition of naturally processed and presented MTB epitopes or the peptide ESAT-61–20 could be linked to specific TCR VB families, and in two patients to unique T cell clones that constituted 19 and 27%, respectively, of the CD4+ and 17% of the CD8+ GAL population. In situ examination of MTB-reactive GALs by tetramer in situ staining and confocal laser-scanning microscopy consolidates the presence of MHC class I-restricted CD8+ T cells in MTB granuloma lesions and supports the notion that clonally expanded T cells are crucial in immune surveillance against MTB. The Journal of Immunology, 2005, 174: 2174–2184.

Disease containment in tuberculosis (TB) involves a complex network of different T cell subsets and their ability to mount and maintain effective immune responses. Our knowledge of all engaged immune events is yet to be complete. The essential role of MHC class II-restricted CD4+ (1) and MHC class I-restricted CD8+ T cells (2, 3) is well established (recently reviewed in Refs. 4 and 5). One of their major weapons is the production of cytokines, e.g., IFN-γ (6) and TNF-α, which can activate macrophages and induce production of bacteriocidal molecules (7). Nonclassically restricted CD8+ lymphocytes, CD4+ CD8−, and CD1-restricted T cells, γδ+ T cells, and NK cells have also been reported to recognize Mycobacterium tuberculosis (MTB)-associated Ags and to be involved in disease control (8–11).

This knowledge is derived largely from studies in mice. Most studies on active or latent human TB to date are limited to the analysis of PBL and do not address the immunological situation at the primary site of MTB infection, the lung. Our objective was to determine which factors are responsible for containing MTB and sustaining latency in the human lung. In five patients with latent MTB infection, we cultivated granuloma-associated lymphocytes (GALs) from lung biopsies and analyzed these cells in regard to their molecular composition, phenotype, frequency, Ag specificity, and function. Furthermore, we took a direct look at the in vivo immunological situation in the lung tissue by confocal laser-scanning microscopy (CLSM), using tetramer technology.

Materials and Methods

Patients

Five patients were recruited from the University Hospital Heidelberg who underwent explorative surgery to rule out a malignant lesion of the lung. The surgical specimens (~10 mm × 8 mm) were analyzed by H&E staining and revealed no evidence of cancer, but granuloma formation associated with a strong T cell infiltrate. No major areas of calcification or caseous alterations were identified. Sputum (obtained after surgery) as well as aliquots from the surgical specimens were cultured according to National Center for Clinical Laboratory Standards standards for 6 wk in a liquid (BACTEC MGT 960; BD Biosciences) and using a solid (Lowenstein-Jensen; BD Biosciences) medium. No viable mycobacteria could be identified. Aliquots from the surgical specimens tested positive by PCR for MTB (Cobas Amplicor M. tuberculosis test; Roche). Thus, patients were diagnosed for latent MTB infection based on: 1) typical pulmonary lesions by x-ray; 2) PCR-based MTB detection; and 3) typical granuloma formation defined by H&E staining. This pattern (in the absence of cultivable bacteria) is consistent with “latent MTB infection,” as reviewed recently (12), which is in accordance with in situ PCR analysis of human lung tissue obtained from individuals (at autopsy) with no clinical evidence of MTB (13). An aliquot of each surgical specimen was cut into small (1 mm × 1 mm) pieces, as described earlier for specimens from patients with colorectal cancer (14). Briefly, tissue sections were placed into 48-well plates supplemented with RPMI 1640, 10% FCS, and antibiotics plus 50 ng of human rIL-7/70mL, kindly provided by A. Minty (Sanofi).

T cells from lung granulomatous lung tissue, which grew out within 48 h, designated as GALs, were subjected to further analysis. For some patients, only a limited number of T lymphocytes could be obtained that did not allow a comprehensive CD4+ or CD8+ T cell analysis in each case. Of note, T cells were not restimulated with peptides or autologous macrophages. All patients tested positive for purified protein derivative reactivity, according to National Center for Clinical Laboratory Standards guidelines. Blood was drawn after informed consent of the individuals enrolled in the study, which has been approved by the local ethics committee (on file with reference 837.327.99 (2272) from November 15, 1999).

Flow cytometry and tetramer analysis

Heparinized blood from patients was drawn and PBMCs were obtained by separation over a Ficoll gradient and stored in liquid nitrogen at 1–5 × 107

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2 Abbreviations used in this paper: TB, tuberculosis; CLSM, confocal laser-scanning microscopy; ESAT, early secreted antigenic target; GAL, granuloma-associated lymphocyte; MTB, Mycobacterium tuberculosis.
cells/vial in 90% FCS and 10% DMSO. Tetramer reagents loaded with the HLA-A2-binding MTB 19-kDa peptide VLDTNPGVEP, the MTB peptide MTB Ag85B KLVANNTLR, the early secreted antigenic target 6 (ESAT-6) peptide LLDEGKQSL, or the HLA-DR4 tetramer complex loaded with the ESAT-6 peptide FAGIEEAASAIQGNV were prepared and obtained from Immunomics (Beckman Coulter). HLA-A2 tetramer complexes loaded with the CMVpp65 epitope NVLPMVATV or the melanoma-associated gp100 peptide YLEPGPPTV (Beckman Coulter) served as control tetramers and were thawed and washed, and tetramer analysis or TCR VB frequency was performed, as described earlier in detail (15).

**Tetramer in situ staining**

Tetramer in situ staining was performed using the identical granuloma lesions subjected to TCR-CDR3 analysis. We only had sufficient amounts of tissue from patients 3 and 4 available to perform T cell in situ detection. A total of 10–20 frozen sections at 10 μm thickness from lung tissue was obtained and incubated at 4°C (final concentration 1 μg/ml diluted in 2% normal goat serum in PBS) overnight with PE-labeled HLA-A2 tetramers loaded with the MTB 19-kDa or the Ag85B epitope, as listed above. The following steps were all performed at room temperature, and primary and secondary antibodies were diluted in Ab-diluent buffer (DakoCytomation). Sections were washed three times in PBS and fixated in 2% formaldehyde solution buffered in PBS for 5 min, followed by three washing steps using PBS. Mouse anti-PE (Sigma-Aldrich) was incubated for 1:100 for 30 min, followed by one washing step with PBS. Cy3-labeling of donkey anti-mouse (Dianova) was used at a dilution of 1/800 for 1 h. Double staining with mouse anti-human CD8α (DakoCytomation; clone C8/144B) was performed at a dilution of 1/50 for 1 h. Before this incubation, a biotinylated step with the animal research kit (DakoCytomation) was applied, according to the manufacturer’s instructions, to avoid cross-reactivation. FITC-labeled streptavidin (DakoCytomation) was applied at 1:50 to detect CD8+ T cells. Tetramer-positive cells appear in red and CD8+ T cells in green. Specimens were photographed with ColorViewXS (Olympus) attached to a Zeiss Axioscope microscope (Zeiss).

**Immunomagnetic cell-sorting and functional assays**

CD4+ or CD8+ T cells were separated from 3 to 5 × 10⁵ PBMCs or GALs using anti-CD4- or CD8-coated immunomagnetic beads (Miltenyi Biotec). TCR VB-sorted T cells were obtained using VB-specific mAbs (Beckman Coulter) directly labeled with PE, followed by anti-PE-directed immunomagnetic bead sorting. T cells were cultured for 48 h in 96-well plates containing 50% AIM-V medium, 50% DMEM (high glucose) obtained from Invitrogen Life Technologies, supplemented with 10% FCS and 50% AIM-V medium, 50% DMEM (high glucose) obtained from Invitrogen Life Technologies, supplemented with 10% FCS and 50 ng/ml human rIL-7. The candidate target ESAT-6-derived peptides AIOGNVTSI (aa 17–25), LLDEGKQSL (aa 28–36), and ATAELNNA (aa 62–70).

**TCR-CDR3 spectratyping**

The TCR-CDR3 composition of either CD4+ or CD8-positive sorted T cells (using immunomagnetic beads) or tetramer-sorted T cells, using either HLA-A2/Ag85B or HLA-DR/ESAT-6 complexes, was conducted, as described (15). These T cells, not restimulated in vitro, were obtained after a 48-h culture period in the presence of IL-7. Of note, IL-7 has been reported not to skew the TCR repertoire (22). Briefly, RNA was extracted from 2 × 10⁵ cells and reverse transcribed into cDNA, amplified by individual TCR VB-specific primer pairs, and a runoff reaction using a fluorophore-labeled TCR-CB-specific primer (PCR conditions: 94°C, 1 min/60°C, 1 min/72°C, 1 min, 40 cycles) was performed. Labeled amplicons were analyzed by DNA fragment analysis using appropriate size standards and a 310 se- qence analyzer and Genescan software (Applied Biosystems). Serial dilutions (10^1, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9) of either CD4+ or CD8+ sorted T cell populations showed that the use of at least 1 × 10⁶ T cells in this assay system results in highly reproducible TCR patterns pertaining to intra- and interassay variations, and does not lead to false positive monoclonal TCR-CDR3 patterns. To identify monoclonal/oligoclonal TCR transcripts, amplicons were subcloned into the TA sequencing vector (Inviro- gen Life Technologies). Plasmid DNA was sequenced by fluorescent dideoxynucleotide chain termination. The identity of the TCR VB sequence was confirmed by either direct sequencing of the PCR amplicon or all subcloned PCR transcripts yielded the identical TCR sequence. If the TCR VA/VB family is oligo- or polyclonal, a Gauss distribution of the CDR3 length each peak represents in-frame transcripts with a given CDR3 length. The area under the curve represents the frequency of a distinct CDR3 length in an individual TCR VA/VB family to condense the information from a single sample analysis, the individual TCR VA/VB families were grouped into a single figure with VB1-VB24 along with the CDR3 length expressed as the number of amino acids. This TCR-CDR3 landscape provides the structural anatomy as defined by the TCR-CDR3 length for each TCR family in a T cell subpopulation. The area under the curve of each CDR3 peak is expressed as the percentage of the entire CDR3 area (100%). For sake of visual clarity, differences in different colors, as in different scale intervals. The CDR3 pattern obtained from CD8+ or CD4+ T cells obtained from granulomatous lesions (GALs) can be compared with the CD4+ or CD8+ sorted PBL obtained at the same time, i.e., during surgery. This TCR perturbation within each CDR3 length is calculated by the areas between the CDR3 distribution in each sample and the control distribution. Positive or negative perturbations may occur in each TCR VA/VA VB CDR3 peak, and are depicted as differences as compared with the control sample. Each perturbation yielding a 10% difference is depicted in a different color. Note that a flat TCR landscape in this analysis implies that no perturbation exists, i.e., the TCR VA/VA VB landscape would yield the identical picture as compared with the control sample. Comparative analysis of TCR-CDR3 length measurements in CD8+ CDR3 (unsorted) GAL was conducted using corresponding freshly harvested lung tissue obtained from two patients.

**CDR3 analysis and TCR VB staining: quantitative TCR analysis**

TCR spectratyping yields only the qualitative, but not the quantitative assessment of a T cell population. A panel of 24 individual mAbs directed against the TCR VB chain (Beckman Coulter) was grouped to three individual anti-VB mAbs either labeled with FITC, PE, or double-labeled with FITC/PE, which can either be gated on energy-coupled dye-CD4, or phycoerythrin-cyanin-5-CD8+ T cells. Thus, the frequency of 24 individual TCR VA/VB families either in the CD4+ or CD8+ T cell populations can be analyzed in eight different tubes, which yields the percentage (%) of a VB family in CD3+ CD8+ T cells. This factor can now be used to correct the CDR3-VB landscape analysis (23). Exclusively, a mAb panel directed against TCR VB chains, but not TCR VA chains, is available.

**Results**

GALs were cultivated from lung biopsies of five patients that presented with a lung lesion of unknown etiology. Pathological diagnosis (typical granuloma formation) and PCR-assisted amplification of MTB revealed latent TB infection in five of five patients (12, 13). No viable mycobacteria could be isolated from sputum, bronchoalveolat, or the biopsy itself. Thus, we had the possibility to analyze PCR-positive, culture-negative human lung granuloma tissue for T cell reactivity to MTB-associated Ags. The aim of this study was to ascertain the aspects involved in sustaining MTB latency in the human lung by analyzing: 1) the
quality of the T cell responses, as determined by TCR-CDR3 spectratyping; 2) the quantity of the T cells in each TCR VB family, as determined in flow cytometry analysis; and 3) the function of the GALs, as defined by cytokine production.

CD4+ GALs and CD8+ GALs are oligoclonal, composed of highly expanded VB families, and constitute an idiosyncratic population as compared with PBL.

After magnetic bead sorting, CD4+ and CD8+ GAL populations were analyzed separately for the objective structural composition of their TCR repertoire, defined by TCR-CDR3 spectratyping complemented by a quantitative assessment of the TCR repertoire measured by flow cytometry. In Fig. 1, the TCR landscapes have been plotted showing 24 VB families on the x-axis, the CDR3 lengths within the family as amino acid counts on the z-axis, and the percentage of CDR3 lengths within the lymphocyte population as a combination of the flow cytometrical enumeration and the area under the curve, as determined by CDR3 analysis on the y-axis.

An augmentation of a single CDR3 length within a VB family is considered to be due to an expansion of T lymphocytes that are possibly involved in a specific immune response. Each patient showed an individual, particular TCR VB profile for both the predominantly oligoclonal CD4+ (Fig. 1, middle panel) and the CD8+ GAL population (Fig. 1, left panel). In some patients, T cells belonging to a distinct oligoclonal or monoclonal VB family constituted a major fraction of the lymphocyte population. For example, in patient 1, VB14-positive CD4+ lymphocytes and in patient 5, VB7-positive T cells accounted for 27 and 19% of all CD4+ GALs, respectively. TCR-CDR3 spectratyping suggested that these VB families were composed of a single T cell clone. We sequenced the individual TCRs (Table I) and proceeded to analyzing their function in latent TB.

Major T cell expansions could also be found in CD8+ GALs: monoclonal VB3-positive T cells in patient 1 (see Table I for TCR sequence) and oligoclonal VB17-positive lymphocytes in patient 3 constituted up to 17 and 9% in CD8+ GAL, respectively. No common expansion of a TCR VB family could be identified in comparing the individual patients or HLA type that might have indicated a preferential VB usage in human MTB containment.

As the panel of 24 VB-specific Abs commercially available covers over 80%, but not the complete VB repertoire (23), we were as yet not able to identify all T cells (in a quantitative fashion) that may be involved in controlling MTB. For example, in patient 1, 20% of CD4+ and 55% of CD8+ GAL could not be quantitatively assessed for VB expression in flow cytometry. These T cells, for which VB frequencies could not be measured, comprised only few

![FIGURE 1](http://www.jimmunol.org/)

Preferential oligo- or monoclonal expansion of distinct TCR VB families in MTB-reactive CD4+ GAL. Left panel, CD8+ GAL: TCR landscapes combine the qualitative information on the TCR repertoire as gained by CDR3 spectratyping and the flow cytometrical enumeration of VB families; 24 VB families have been plotted on the x-axis, the CDR3 lengths within the family are depicted as amino acid counts on the z-axis, and the percentage of CDR3 lengths within the lymphocyte population as a combination of the flow cytometrical enumeration and the area under the curve is shown on the y-axis. Middle panel, TCR VB composition in CD4+ GAL. Right panel, MTB reactivity of GAL is shown as IFN-γ production in response to autologous MTB-infected macrophages after 48 h of coincubation. Functional analysis of CD4+ GAL for patient 5 could not be performed in this setting due to limited amount of autologous macrophages. Controls included macrophages alone or CD4+ GAL plus macrophages. MHC class II-restricted responses could be blocked with the respective mAbs directed against DR, DP, or DQ.
additional VB families by molecular assessment, and thus may also have been clonally expanded (data not shown).

To determine whether the TCR repertoire of the lung is a mere mirror image of the peripheral blood or whether the lung acquires its distinct repertoire, we analyzed both compartments (GAL vs PBL) of patients (except PBL for patient 1 due to limited material) and present the data obtained for patient 2 (for both CD4 and CD8 T cells) and for patient 5 (for total/unsorted PBL and GAL) as a proof of principle in Fig. 2. By subtracting the GAL TCR landscape from the TCR landscape obtained for PBL, we visualized the percent differences, as shown in Fig. 2, a (molecular TCR-CDR3 analysis) and b (molecular TCR-CDR3 analysis corrected by analysis of TCR VB frequencies). The perturbed landscapes indicate that each compartment was indeed composed of a dissimilar TCR repertoire, and that individual TCR families are preferentially expanded within granuloma tissue. Had the repertoires been identical, a flat landscape would have resulted. Unsorted (48-h, IL-7-expanded) GAL showed a similar TCR composition as compared with the corresponding freshly isolated granuloma tissue section (data not shown).

**CD4**^+^ GALs show a Th1 cytokine secretion pattern in response to MTB epitopes presented by autologous macrophages

Before addressing the function of those GALs characterized by an expanded VB family, we determined, in a more general approach, the function of the heterogenous CD4 and CD8 T cells and for patient 5 (for total/unsorted PBL and GAL) assessed the percent differences, as shown in Fig. 2, a (molecular TCR-CDR3 analysis) and b (molecular TCR-CDR3 analysis corrected by analysis of TCR VB frequencies). The perturbed landscapes indicate that each compartment was indeed composed of a dissimilar TCR repertoire, and that individual TCR families are preferentially expanded within granuloma tissue. Had the repertoires been identical, a flat landscape would have resulted. Unsorted (48-h, IL-7-expanded) GAL showed a similar TCR composition as compared with the corresponding freshly isolated granuloma tissue section (data not shown).

**CD4**^+^ GALs and CD8^+^ GALs characterized by an expanded VB family are the major IFN-γ producers

To investigate whether an expansion of a VB family within the CD4^+^ and the CD8^+^ GAL population is indeed indicative of a major functional role in disease combat, we sorted the GALs according to their expanded VB family and analyzed them in cytokine release assays. As elaborated above, TCR-CDR3 spectratyping coupled with flow cytometry allowed us to recognize VB9 and

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**Table I. Monoclonal TCR sequence of highly expanded MTB-reactive GAL in lung tissue**

<table>
<thead>
<tr>
<th>Patient/GAL</th>
<th>End of V Region (Amino Acid Sequence)</th>
<th>CDR3 Region (Nucleotide and Amino Acid sequence)</th>
<th>Joining Region (Amino Acid sequence)</th>
<th>J Family</th>
<th>Reactivity Against</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. 1</td>
<td>CD4” VB14”</td>
<td>PSPQGTSYFCC gcgcagctttggaaggggggggtggtgt</td>
<td>EQFEGPGTTLTVE</td>
<td>BJ 2-1</td>
<td>Naturally processed and presented MTB epitope on autologous macrophages</td>
</tr>
<tr>
<td>No. 1</td>
<td>CD8” VB3”</td>
<td>ASTNQRTSYMLGC gcgcagctttaatggggggggggggtg</td>
<td>NQPPFHGKG</td>
<td>BJ 1-5</td>
<td></td>
</tr>
<tr>
<td>No. 5</td>
<td>CD4” VB7”</td>
<td>SAVLYLC gcgcagcagcaacagtttggggggggtggtgt</td>
<td>ETQYGPGTTL</td>
<td>BJ 2-5</td>
<td>ESAT-61-20</td>
</tr>
</tbody>
</table>

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**FIGURE 2.** The TCR repertoire of lung-derived GALs is distinct from PBL, indicating sequestration of particular T cells to the lung. The molecular composition of the TCR repertoire of lung-residing GALs was compared with that in PBL by subtracting the appropriate TCR landscapes obtained for each compartment by: A, CDR3 spectratyping, or B, CDR3 spectratyping adjusted by the frequencies of VB expression measured in flow cytometry. Data were obtained for five of five patients and are exemplified for patients 2 and 5. The perturbed landscapes indicate the differences in the TCR repertoires. Note that data are obtained in CD4^+^, or CD8^+^ sorted GAL or PBL. Analysis of unsorted GAL vs the corresponding lung tissue revealed no major difference. n.a. = Not applicable (quantitative TCR VB analysis determined by flow cytometry cannot be performed in tissue sections).
14 in patient 1, VB8 and VB9 in patient 2, and VB7 in patient 5 as the major represented VB families in the CD4⁺ GAL population, as well as VB3 in patient 1 in CD8⁺ GALs. We sorted and exposed T cells, expressing the relevant VB families, to MTB-infected autologous macrophages for 48 h, as described above. The production of IFN-γ by these T cells resulted in much higher concentrations (ranging from 1300 to 9955 pg/ml for CD4⁺ GALs, and 1185 pg/ml for CD8⁺ GALs; Fig. 3) than were produced by the whole population of GALs at comparative cell counts. As expected, mAbs directed against MHC class I inhibited cytokine production of VB3⁺ CD8⁺ GALs. In CD4⁺, VB-sorted GALs, IFN-γ production was blocked either by DR, DP, or DQ mAbs, suggesting that the sorted T cells were restricted to a single MHC class II allele.

CD4⁺ GALs, belonging to a certain expanded TCR VB family, recognize ESAT-6₁⁻₂₀

To determine Ag specificity of the sorted CD4⁺ GALs, we analyzed the ability of the individual TCR VB-sorted T cells to recognize MTB-derived DR-binding peptides ESAT-6₁⁻₂₀ and ESAT-6₂⁻₉₅. VB-sorted GALs recognized ESAT-6₁⁻₂₀ and produced IFN-γ in patients 1, 2, and 5, whereas ESAT-6₂⁻₉₅ induced an IFN-γ production above background levels only in VB7⁺ GALs of patient 5 (Fig. 4a).

An increase of peptide concentration evoked a stronger cytokine response, as shown in Fig. 4b, but at the highest peptide concentration tested results varied, ranging from a further increase in patient 2 to suppression of IFN-γ in patient 5. When analyzing the corresponding TCR VB-depleted GAL population in the identical experimental setting, we observed that these GALs did not recognize the ESAT-6₁⁻₂₀ peptide, as no IFN-γ production was measurable above background levels (VB9-negative GALs in patient 1, 4 pg/ml; VB9-negative GAL in patient 2, below detection levels; VB7-negative GALs in patient 5, 45 pg/ml; data not shown in Fig. 4a). Recognition of ESAT-6₁⁻₂₀ was, therefore, restricted to a single VB family in these three patients or even a single VB7⁺ T cell clone in patient 5.

MTB-derived HLA-A2-binding peptides evoke a Tc1 cytokine response in CD8⁺ GALs

Recognition of peptides provided by MTB-associated Ags ESAT-6, 19-kDa Ag, or Ag85a/b was investigated in the CD8⁺ GAL population of HLA-A2⁺ patients (Table II; Fig. 5). MTB-derived HLA-A2-binding peptides Ag85a₁₈⁻₅₆, Ag85a₂₄₂⁻₂₅₀, Ag85b₁₄₃⁻₁₅₂, Ag85b₁₉₉⁻₂₀₇, and 19-kDa Ag₈₈₈⁻₉₇, respectively, were loaded on T2 cells, CD8⁺ GALs were added, and the in vitro cytokine production was measured after 48 h of incubation. A melanoma-derived HLA-A2-binding gp100-derived peptide was used as a negative control.

Although 19-kDa Ag₈₈₈⁻₉₇ evoked moderate cytokine production, peptides Ag85a₂₄₂⁻₂₅₀ and Ag85b₁₉₉⁻₂₀₇ were recognized, as determined by IFN-γ and GM-CSF production. The other peptides had not elicited a much higher cytokine production than the control peptide gp100 in any patient. IL-4 was not detectable in any case (data not shown). In addition, GALs derived from patient 3 were analyzed for reactivity to three A2-binding target peptides provided from ESAT-6. These GALs recognized ESAT-6₂₈⁻₃₆ in a MHC class I-restricted manner and produced substantial amounts of IFN-γ (Fig. 4c).

FIGURE 3. The CD4⁺ and CD8⁺ GALs expressing expanded TCR VB families are MTB reactive, producing increased amounts of IFN-γ as compared with unsorted GALs. In patients 1, 2, and 5, those CD4⁺ or CD8⁺ GALs previously identified as expressing an expanded VB family were sorted according to their VB expression by immunomagnetic bead sorting and exposed to autologous MTB-infected macrophages for 48 h. IFN-γ production (picograms per milliliter) was measured in supernatants by ELISA. HLA restriction was confirmed by addition of mAbs directed against either MHC class I, DR, DP, or DQ.
of IFN-γ and GM-CSF (Fig. 6). ESAT-61–20 and ESAT-662–70 elicited minor cytokine production. The presence of peptide-reactive GALs was substantiated by HLA-A2 tetramer staining in GAL: CD8+ GAL from patient 3 stained positive for the ESAT-6 peptide LLDEGKQSL (2.4%), and 1.1 and 1.6% for the 19-kDa and the Ag85b tetramer, respectively (data not shown).

MTB-reactive GALs in situ defined by tetramer analysis

We have been able to obtain sufficient serial sections from the granuloma lesions from patients 3 and 4, which allowed us to visualize Ag-specific CD8+ GALs in situ by staining with HLA-A2 tetramers for Ag85b 199–207 and 19-kDa Ag 88–97. TCR-bound tetramers appeared red in CLSM, while mAbs directed against CD8 were FITC labeled and appeared green (Fig. 7). However, tetramer in situ staining allows visualization of the spatial arrangement of the MHC class I/peptide-specific T cells in situ, but does not address the molecular composition of the Ag-specific T cell population. To assess the TCR usage in MHC/peptide-specific T cells, we sorted HLA-2/Ag85b-binding CD8+ GAL from patient 3 and performed TCR-CDR3 analysis (Fig. 8). The comparison of TCR usage in tetramer-sorted and CD8+ GAL shows the presence of a few clonotypes directed against a defined T cell epitope. T cells have been harvested from a granuloma lesion (H&E, parallel section) with marked lymphocytic infiltration (Fig. 31). A few T cell clonotypes constitute also the HLA-DR4-restricted, ESAT-6-specific CD4+ T cell population isolated from a different patient with latent human pulmonary TB (Fig. 8). Tetramer in situ staining (Fig. 7b) suggests that Ag85b-reactive T cells are focused to several loci in the granuloma tissue. As previously observed in murine lymphoid tissue (24), tetramer staining visualized TCRs that seemed clustered at one (or few) pole(s) of the T cell, giving an impression of a bright red dot or dots on the circular cell, while CD8 seemed more equally distributed, giving the cell a homogenous, annular appearance. Upon double expression, the red and green resulted in an orange-yellow coloring of the cells. Staining with gp100-MHC tetramers, as a negative control, led to mere background staining. The tetramer-positive cells were focused to several different loci within the granuloma section. The H&E staining of a parallel tissue section is shown for patient 3.

Discussion

The immunological situation in latent TB has been difficult to understand and research. We lack an apt animal model for this state of disease. Moreover, the results obtained from the analysis of PBL in humans may not necessarily reflect the immune response at the site of infection itself. Several studies report a prominence of Th2-type cytokines, e.g., IL-10 (25–27), in contrast, T cells from other compartments in patients with active TB, such as

Table II. Reference number, age, sex, HLA haplotype, and pathological diagnosis of studied patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex/Age</th>
<th>MHC Class I Haplotype</th>
<th>MHC Class II Haplotype</th>
<th>Pathological Diagnosis</th>
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<td>1</td>
<td>M/65</td>
<td>A<em>26, A</em>29, B<em>45, B</em>52, Cw<em>06, Cw</em>12</td>
<td>DRB1<em>15, DRB1</em>12, DRB3*, DRB5*, DQB1<em>06, DQB1</em>03</td>
<td>Latent MTB infection</td>
</tr>
<tr>
<td>2</td>
<td>M/63</td>
<td>A<em>03, A</em>11, B<em>52, B</em>53, Cw<em>04, Cw</em>12</td>
<td>DRB1<em>01, DRB1</em>11, DRB3*, DQB1<em>05, DQB1</em>03</td>
<td>Latent MTB infection</td>
</tr>
<tr>
<td>3</td>
<td>M/37</td>
<td>A<em>02, A</em>68, B<em>07, B</em>13, Cw<em>01, Cw</em>16</td>
<td>DRB1<em>04, DRB1</em>15, DRB4*, DRB5*, DQB1<em>02, DQB1</em>06</td>
<td>Latent MTB infection</td>
</tr>
<tr>
<td>4</td>
<td>F/41</td>
<td>A<em>02, B</em>18, B<em>40, Cw</em>02, Cw*07</td>
<td>DRB1<em>11, DRB1</em>16, DRB3*, DRB5*, DQB1<em>03, DQB1</em>05</td>
<td>Latent MTB infection</td>
</tr>
<tr>
<td>5</td>
<td>F/50</td>
<td>A<em>02, B</em>44, Cw<em>05, Cw</em>16</td>
<td>DRB1<em>04, DRB1</em>07, DRB4*, DQB1*03</td>
<td>Latent MTB infection</td>
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pleural effusions (28, 29) or T cells isolated by bronchoalveolar lavage (30, 31), showed a marked IFN-γ and/or proliferative response that was lower or even absent in PBL of the same analyzed patients. This phenomenon may be due to sequestration of distinctive MTB-reactive T cells to the site of infection, possibly on account of particular chemokine receptors, such as CCR2 or CCR5 (32). Hence, a closer look at the immunological events in the lung tissue itself is essential for a better comprehension of protective immunity to MTB.

The fine specificity of TCR Ag recognition resides in the CDR3 of the TCR VA and VB chain (33). The clonal expansion of Ag-specific T cells in response to Ag is reflected as a single or a few overrepresented CDR3 peak(s) within a VB family in CDR3 spectratyping (34, 35). Ultimately, this clonal expansion of T cells can translate into an augmentation of the relevant VB family within the VB repertoire, as described for both viral (36) and bacterial (37) infections. We showed that GALs isolated from human lung tissue were characterized by oligoclonal or monoclonal expansions of individual VB families. These TCR VB expansions in GAL could not be observed in corresponding PBL, suggesting that the MTB-reactive T cells had indeed been sequestered to the lung. A comparable compartmentalization has been reported in a study on active human TB, in which the TCR VB repertoire of lymphocytes from pleural fluid differed from that found in PBL (38). The fact that the TCR repertoire analysis in freshly obtained granuloma tissue exhibited a similar pattern as compared with (unsorted) 48-h expanded GAL (see Fig. 2) and that IL-7, used for T cell culture, does apparently not skew the TCR repertoire (22) suggests that the functional TCR composition data indeed reflect the in situ situation.

CD4+ GALs readily recognized MTB-infected autologous macrophages and produced IFN-γ, a key cytokine well established in

FIGURE 5. CD8+ GALs preferentially recognize Ag85a242–250, Ag85b199–207, and 19-kDa Ag19–88 in latent TB. A2-binding MTB-derived peptides were analyzed for their recognition by CD8+ GALs in latent TB. T2 cells were loaded with 1 μg of the relevant peptide and exposed to CD8+ GALs. Supernatants were collected after 48 h and analyzed in respect to IFN-γ, GM-CSF, and IL-4 production (data for IL-4 production not shown).

FIGURE 6. Focused immune responses in latent TB. The HLA-A2-binding ESAT-628–36 peptide is a key target of immune recognition by CD8+ GALs. Three ESAT-6-derived HLA-A2-binding peptides of the ESAT-6 Ag were individually loaded on T2 cells for analyzing the recognition by CD8+ lung-derived GALs in latent TB. ESAT-628–36 evoked a strong cell-mediated immune response as assessed by IFN-γ production, while peptides ESAT-617–25 and ESAT-662–70 were only weakly recognized by lung-derived T cells. IFN-γ and GM-CSF (and IL-4; data not shown) were measured in supernatants after 48 h of incubation. IFN production could successfully be blocked with the relevant anti-MHC class I mAb, but not with the MHC class II (DR-binding) mAb L243.
contributing to immunity against MTB (6, 39). In contrast, CD8\(^{+}\) GAL failed to produce IFN in response to autologous MTB-infected targets (data not shown), despite the fact that CD8\(^{+}\)/H11001\(\text{TCR VB3-sorted T cells recognize the same MTB-infected APCs (Fig. 4). Two mutually not exclusive mechanisms may account for this phenomenon. First, the E:T ratio in the (clonal; see Table I) TCR VB3-sorted T cells may have been superior as compared with the unsorted CD8\(^{+}\)/H11001 population in which the VB3\(^{+}\)/H11001 cells constituted up to 17% (Fig. 1). Second, in vitro infection of autologous macrophages with MTB may rather facilitate presentation of MHC class II-presented epitopes (see Fig. 1).

The frequencies of expanded, MTB-specific T cell clones were unexpectedly high in the lung tissue (reaching up to 27% of CD4\(^{+}\)/H11001 GAL in an individual patient) as compared with our previously published findings for CD8\(^{+}\)MTB Tet-19-kDa Ag\(^{+}\)reactive T cells in PBL of patients with active TB (~2% of CD8\(^{+}\) PBL) (40, 41) or, in respect to tissue-residing lymphocytes, as seen for Melan-A/ MART1\(_{27–35}\)-specific tumor-infiltrating T cells (5.68% of tumor-infiltrating lymphocytes) (42). The frequency differences seem even more pronounced, considering that tetramer analysis or limiting dilution assays used in these studies may reflect frequencies for Ag-specific T cells of diverse T cell clones, while we had observed single T cell clone expansions in GALs. Masopust et al. (43), in contrast, have detected frequencies similar to those in GALs, when analyzing organ-specific tetramer-reactive T cell responses in acute murine listeriosis. These frequencies declined after successful resolution of the disease. In latent MTB infection, T cell frequencies may remain elevated, as the infection is essentially not cleared due to the persistence of bacilli inside macrophages for many years.

**FIGURE 7.** In situ tetramer staining shows a dispersed localization of tetramer-reactive GALs. a, H&E staining for a parallel section from the tissue specimen obtained from patient 3 shows granuloma formation (magnification \(\times200\)). b, A total of 10–20 frozen sections at 10 \(\mu\)m thickness from lung tissue was stained with HLA-A2 tetramers for Ag85b\(_{199–207}\) and 19-kDa Ag\(_{88–97}\) or gp100 as a negative control, costained with CD8 mAbs, and visualized by CLSM. Tetramer-binding GALs appear dispersed within the granuloma; while a homogeneous distribution of CD8 was apparent in GALs, tetramer-stained TCRs seemed localized at one or a few poles of the cell. Data presented for patients 3 and 4. Background staining for the irrelevant control (gp100) tetramer (magnification \(\times400\)). Note that tetramer-guided analysis of HLA-A2-restricted/Ag85b-reactive T cells has been conducted in GAL from patient 3 (see Fig. 8).

**FIGURE 8.** Focused T cell response to defined MHC/peptide complexes. a, Tetramer-guided sorting of HLA-A2-restricted/Ag85b-specific T cells (left) in CD8\(^{+}\) GAL from patient 3 (see Fig. 7). Sufficient CD4\(^{+}\)/H11001 GAL could successfully be harvested from a different (HLA-DR4\(^{+}\)) patient with latent MTB infection, not listed in Table II, and sorted using an HLA-DR4/ESAT-6 tetramer complex (right). b, TCR repertoire analysis in either CD8\(^{+}\), HLA-A2/Ag85b-specific (left) or CD4\(^{+}\), HLA-DR4/ESAT-6-specific T cells isolated from granuloma tissue. Data are presented as percentage of overrepresentation of TCR VB families in tetramer-sorted T cells as compared with CD8\(^{+}\), or CD4\(^{+}\) GAL, respectively. c, Individual VB families could be quantitatively assessed using a panel of TCR VB-specific mAbs in CD4\(^{+}\) GAL (15). TCR VB4 and VB7 are monoclonal in HLA-DR4/ESAT-6-reactive CD4\(^{+}\) GAL.
For three patients diagnosed with latent TB infection, we were able to define the Ag specificity of the major expanded VB family (VB9 in patients 1 and 2, and VB7 in patient 5). These GALs produced increased amounts of IFN-γ in response to naturally processed and presented MTB epitopes as compared with the whole CD4+ GAL population. In patient 5, for example, a single VB7-positive T cell clone (Table I) made up 19% of lung-residing lymphocytes that were dedicated to the recognition of an ESAT-6 epitope. Of note, the same TCR VB7 clone has been identified by TCR-CDR3 typing in the corresponding freshly harvested lung lesion, suggesting that the VB7 family has been clonally expanded in situ (data not shown). The ESAT-6 has been implicated to be sensitive and specific for the detection of active (44–47) or latent (48, 49) MTB infection in humans due to its absence of expression in bacillus Calmette-Guerin and most environmental mycobacterial species (50, 51). Moreover, these expanded lymphocytes were the sole CD4+ T cells that recognized ESAT-61–20 in the whole GAL population. This indicates a highly focused immune response to select MTB Ags in the lung. These data suggest that successful MTB containment, at least in some patients, may be highly focused and restricted to a few TCR clonotypes. This is a situation that was found to be true for successful containment of HIV (52), or more recently, for clinically effective T cell responses in patients with cancer (53).

We were not able to link all expanded TCR VB families to the recognition of naturally processed and presented MTB epitopes in this study. Different repertoires of MTB epitopes may be generated, associated with environmental conditions that direct the MTB transcription machinery of genes necessary for intracellular survival (for review, see Ref. 12). Most likely, overnight infection of autologous macrophages with MTB, used as APCs in the current study, may not reflect Ag processing in granuloma lesions. In addition, we have only been able to address HLA-A2- and HLA-DR4-restricted presentation of defined T cell epitopes; other MHC class I or II alleles may be more effective in shaping a focused, MTB-specific T cell response. Further studies, implementing a broader set of Ag peptides presented by different MHC alleles, are needed to ultimately address the existence of potential immunodominant T cell responses. The immunological control of other recurring or chronic infections of the lung, such as CMV or other crucial MTB-associated target epitopes associated with nonreplicating persistent bacilli (54–56), may likely require the presence of adequately expanded T cells as well.

These data carry implications for vaccine design as well as for the definition of novel surrogate markers associated with immune protection. Given the observations that each organ may be equipped with a different immune response to the same pathogen or more recently, for clinically effective T cell responses in patients with cancer (53).

Substantial evidence exists for the critical role of CD8+ lymphocytes in TB (2, 62, 63). We have also observed clonal expansions of MTB-specific CB8+ GALs in lung tissue, as for example, the TCR VB3-positive T cell clone that constituted 17% of the CD8+ GAL population in patient 1. Our goal was then to determine the major antigenic targets of CD8+ GALs by screening a panel of HLA-A2-presented peptides derived from the secreted Ags 85a, 85b, ESAT-6, and the 19-kDa lipoprotein that had been reported as MTB-associated targets (19–21). We observed a preferential recognition of Ag85a642–650 and Ag85b190–209 as well as ESAT-6236–35, and a moderate reaction to 19-kDa Ag86–97, while Ag85a48–56 and Ag85b143–152 elicited little response, if any at all. Whether this observation is a general trend for lung-derived CD8+ T cells or a distinct feature for the individual patient may need to be further studied in a larger cohort. The major importance of these data is, however, that Ag85a- and Ag85b-specific as well as ESAT-6- and 19-kDa Ag-specific CD8+ T cells are indeed present in the lung (as defined by IFN-γ production upon antigenic stimulation) and not only in peripheral blood, and they are presumably involved in the maintenance of latency. In attempting to visualize these cells ex vivo, we performed in situ tetramer staining, followed by CLSM, and, thereby, achieved an unprecedented exposition of both Ag85b- and 19-kDa Ag-specific CD8+ T cells in human lung tissue without the bias of in vitro culture of T cells. TCR analysis in HLA-A2/Ag85b- or HLA-DR4/ESAT-6-sorted GAL from single patients also suggests a restricted TCR usage (Fig. 8).

In summary, we demonstrate that in human latent TB CD4+ and CD8+, MTB-reactive lymphocytes are present, highly expanded, and functional in the lung tissue. CDR3 spectratyping coupled with flow cytometrical TCR VB analysis is a powerful tool in their identification, and has enabled us to understand that a focused lymphocyte population, distinct from PBL, does govern latency. Key targets are peptides of MTB-secreted Ags such as ESAT-6 and MTB Ag85a/85b. In the future, integration of these Ags into a vaccine may prove crucial for establishing protective immunity to MTB.

References


