Title:

Local immunodiagnosis of pulmonary tuberculosis by enzyme-linked immunospot

Authors:
Claudia Jafari\(^1\), Martin Ernst\(^2\), Alan Strassburg\(^1\), Ulf Greinert\(^1\), Barbara Kalsdorf\(^1\), Detlef Kirsten\(^3\) and Christoph Lange\(^1\)

Affiliation
\(^1\)Division of Clinical Infectious Diseases and \(^2\)Division of Immune Cell Analytics, Research Center Borstel; \(^3\)Hospital Großhansdorf, Center for Pulmonary Medicine and Thoracic Surgery

Address for correspondence:
Christoph Lange MD PhD, Div. of Clinical Infectious Diseases, Research Center Borstel, Parkallee 35, 23845 Borstel, Germany. Email: clange@fz-borstel.de FAX: +49 4537 188 313

Key words: Broncho-alveolar lavage, CFP-10, ELISPOT, ESAT-6, lymphocytes, tuberculosis

Short Title: MTB-specific T cells in pTB

Manuscript word count: 1727

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Abstract:

Lymphocytes are crucial in the immune defense against *Mycobacterium tuberculosis* (MTB) infection. We ascertained whether MTB-specific lymphocytes are selectively compartmentalised in the lungs of patients with minimal active pulmonary tuberculosis (pTB).

We prospectively recruited patients with smear-negative, MTB culture-confirmed pTB. Differential cell counts, immunophenotyping with monoclonal antibodies against cell surface markers CD4, CD8, CD4CD45RA, CD4CD45R0, CD38, HLADR, CD19, CD3, CD57, CD16 and MTB-specific enzyme linked immunospot assays (ELISPOT) with early-secretory-antigenic-target-6 (ESAT-6) and culture-filtrate-protein-10 (CFP-10) of peripheral blood mononuclear cells (PBMCs) and broncho-alveolar lavage mononuclear cells (BALMCs) were performed.

Among 12 patients with culture-confirmed smear-negative pTB, no differences were found in the distribution of total CD4 or CD8 T cells in peripheral blood or BAL. Activated HLA DR+ cells as well as memory CD4CD45R0+ T cells were expanded among cells of the BAL. Compared with a group of control patients with alternative pulmonary pathologies, there was no significant difference in lymphocyte subpopulations. However, ESAT-6 and CFP-10 specific lymphocytes were concentrated with a median of 9.9 and 8.9 times more in BAL compared to peripheral blood in patients with pTB.

MTB-specific T cells are highly selectively compartmentalised at the site of infection in active pTB.

Abstract word count: 195
**Introduction**

T-lymphocytes play an important role in the adaptive immune defence against *Mycobacterium tuberculosis* (MTB) by direct interaction with alveolar macrophages [1, 2]. In healthy adult humans the total number of T-lymphocytes is estimated to be $300 \times 10^9$ [3]. Approximately 2% of T-lymphocytes are found in the peripheral blood whereas nearly 10% reside in the lungs [3]. In active tuberculosis (TB), memory T-cells clonally expand upon antigen encounter and are recruited to the site of the infection [4-10]. These MTB-specific memory T-cells produce IFN-$\gamma$ [11] and other cytokines of a Th1-type immune response [12]. Prompt production of IFN-$\gamma$ following specific antigen presentation is provided predominantly by the population of effector T-cells [11] which typically have a CD4CD45RO+, CD27- and CCR7- phenotype and are end-differentiated for a rapid antigen-specific cytotoxic immune response [11, 13].

Recently, major advances have been made in the immunodiagnosis of MTB-infection by development of T-cell IFN-$\gamma$ release assays (TIGRAs). Enumerating of early secretory antigenic target-6 kDa (ESAT-6) and culture filtrate protein-10 (CFP-10) specific T-lymphocytes from peripheral blood by enzyme-linked immunospot (ELISPOT) or by enzyme-linked immunosorbent assay (ELISA) has been shown to be more sensitive and specific for the immunodiagnosis of active and latent TB infection (LTBI) than the tuberculin skin test (TST) [14-18]. However, as effector T-cells only occur in low frequencies in the peripheral blood, it is not surprising that a clear distinction between active TB, LTBI or past treated TB has not been possible when TIGRAs are performed with cells from the peripheral blood alone [19, 20]. In contrast, counting antigen-specific cells from the site of the infection has been shown to be a promising method to distinguish active TB infection from LTBI [21-24].

To gain a better understanding about the magnitude of recruitment of lymphocytes from the blood into the human lung during active pulmonary TB (pTB) we analyzed
immunophenotypes and numbers of MTB-specific T-lymphocytes in prospectively recruited patients with a suspected diagnosis of pTB and negative acid fast bacilli (AFB) sputum microscopy.

**Methods**

**Subjects**

Patients with 3 negative AFB smear results, pulmonary infiltrates by chest X-ray or thoracic-computed tomography (CT) and suspected pTB were enrolled. Peripheral blood mononuclear cells (PBMCs) were obtained by a venous blood draw. Bronchoalveolar lavage mononuclear cells (BALMCs) were obtained by bronchoscopy [25]. Only patients with culture-confirmed smear-negative active pTB were assigned to the TB group, all patients with presumptive but smear and culture-negative TB were excluded from the study. Patients with negative MTB cultures and alternative diagnosis were assigned to the non-TB group. Healthy controls were not included in the study. The study was approved by the ethics committee of the University of Luebeck Medical School, and all patients gave written informed consent.

**Immunophenotyping**

Lymphocyte subsets were enumerated in PBMC obtained from freshly drawn blood and in BALMCs using directly labeled murine monoclonal antibodies against CD4, CD8, naive CD4CD45RA, memory CD4CD45R0, CD38, HLA DR, CD19, CD3, CD57, and CD16 (DakoCytomation, Hamburg) by two colour flow cytometry (FACSCalibur, Becton-Dickinson, Heidelberg).

**Cell Preparations and Elispot assays**
Cell Preparations ELISPOT assays for human IFN-γ were performed as described previously [22]. Numbers of spot forming cells (sfc) per million lymphocytes were calculated from net numbers of sfc among PBMCs and BALMCs by blood and BAL differential cell counts.

**Statistical analysis**

Statistical tests were performed using non-parametric testing as exploratory analyses without adjustment for multiple testing, with nominal significance defined as p<0.05. To avoid a mathematical error due to division by zero we assigned values of “0” a value of “0.1” when calculating ratios of ESAT-6 and CFP-10 specific cells among lymphocytes from PBMCs and BALMCs.

**Results**

**Patient characteristics**

Forty patients with suspected pTB were prospectively enrolled. Twelve patients had the diagnosis of pTB confirmed by a positive MTB culture from sputum or BAL (eight patients had been included in a previous report [22]). Twenty-five patients with negative MTB cultures were diagnosed with etiologies other than pTB. Nine of them had less than 1 % lymphocytes in the BAL or not enough lavage fluid available for FACS analysis so they also had to be excluded from the study. The diagnoses of the remaining 16 patients from the non-TB control group were sarcoidosis (n = 4), pneumonia (n = 4), former mycobacterial infection without reactivation (n = 4), cryptogenic organizing pneumonia (COP; n = 2), bronchogenic carcinoma (n = 1) and rheumatoid arthritis (n = 1). Seven non-TB patients had positive ELISPOT results on peripheral blood and were diagnosed with LTBI. Patients with pTB were younger (31.1 vs. 50.7 years, p<0.018) and they were more often male (10/12) when compared to patients with alternative diseases (10/16) (p<0.021).
ELISPOT results

The median number of ESAT-6 and CFP-10 specific lymphocytes in the peripheral blood and in the BAL of patients with pulmonary TB was 64 (IQR: 35-167) and 149 (IQR: 85-259)/1000000 lymphocytes and 598 (IQR: 244-2593) and 1653 (IQR: 549-4511)/1000000 lymphocytes respectively. The median number of ESAT-6 and CFP-10 specific lymphocytes in the peripheral blood and in the BAL of patients in the non-TB group was 3 (IQR: 0-33) and 6 (IQR: 0-32)/1000000 lymphocytes and 0 (IQR: 0-150) and 0 (IQR: 0-73)/1000000 lymphocytes respectively (Fig. 1).

Compartmentalisation of ESAT-6 and CFP-10 specific lymphocytes in the lungs of patients with pTB

In patients with pTB, the median ratio of ESAT-6 and CFP-10 specific SFC/1000000 lymphocytes between BAL and peripheral blood lymphocytes was 9.9 (IQR 3-37) and 8.9 (IQR 6-32) respectively. In contrast, the median ratio of ESAT-6 and CFP-10 specific SFC/1000000 lymphocytes between BAL and peripheral blood lymphocytes of control patients was 0 (IQR 0-13) and 0 (IQR 0-7) respectively (Fig. 2).

Immunophenotype

Blood and BAL immunophenotypes are shown in Table 1. There was no significant difference in immunophenotyping of PBMCs or BALMCs between patients with pulmonary TB and controls (data not shown).

Discussion

Recently, a MTB-specific ELISA (QuantiFeron-Gold-in-tube, Cellestis, Carnegie, Australia) and an ELISPot (T-Spot.TB, Oxford Immunotec, Abingdon, UK) have been developed for the diagnosis of tuberculosis [26-28]. However, there has been surprisingly little discussion
on what these assays actually measure. While the ELISA quantifies the IFN-γ production following MTB-specific antigen contact in whole blood, it is possible to enumerate single MTB-specific cells with the ELISPOT assay. Since antigen presenting monocytes and dendritic cells, which are not a source of IFN-γ production, are assayed together with lymphocytes in the ELISPOT assay, results of SFCs in the ELISPOT assay should however be normalized to lymphocyte numbers to achieve more accurate results. Only a minority of the lymphocytes in the human body are found in the peripheral blood [29] and the frequency of MTB-specific T cells in the peripheral blood is very low (approximately 0.002 % – 0.4 % in respect to ESAT-6 and CFP-10 antigen specific T cells) even in active TB [21, 22]. It is not surprising therefore that is has not been possible to discriminate with these assays between active TB and LTBI in routine clinical practice [27]. Following antigen encounter naive cytotoxic T-lymphocyte (CTL) precursors are primed and acquire effector function. They undergo expansion and travel to sites of infection where they mediate pathogen clearance by killing infected cells and secreting effector cytokines. Subsequently most effector CTLs die [30]. Discrimination of active TB from LTBI may therefore be possible by immunodiagnosis when frequencies of antigen-specific cells from the site of the infection and the peripheral blood are compared by MTB-specific ELISPOT [21, 22]. Compartmentalisation of antimycobacterial immune responses at the site of infection during active TB has been previously described [8, 12, 21, 31-38]. In healthy household contacts of patients with tuberculosis [33] and in patients with active pTB, antigen–specific lymphocytes are concentrated in the lungs and on challenge produce helper T-cell type 1 cytokine host responses [34]. However, the magnitude of antigen-specific lymphocyte concentration had not been quantified in pTB so far. In the present study, MTB-specific T-lymphocytes were found to be concentrated by a factor of approximately 1 log among lymphocytes derived by BAL from patients with active smear-negative pTB compared to lymphocytes from the peripheral blood. This concentration of MTB-specific T-cells among lymphocytes in the BAL compared to the peripheral blood is
in the same order of magnitude as the concentration of MTB-specific T-cells among lymphocytes in the pleural effusion compared to the peripheral blood in TB pleurisy [21]. Of note, in agreement with previous findings [6] no other differences in the immunophenotype among PBMCs or BALMCs of patients with active pTB and controls could be found in our study. When immunophenotype of PBMCs and BALMCs were directly compared, there were higher frequencies of CD4CD45R0 memory T cells and HLADR+ activated T-cells and a lower frequency of CD4CD45RA naïve T-cells, CD19 B cells and CD16 NK cells among BALMCs compared to PBMCs, consistent with earlier reports [6, 39, 40]. However, these differences were not restricted to patients with pTB as the same differences between PBMCs and BALMCs were also observed in patients with other pulmonary pathologies. In active pTB antigen-specific T-cells are therefore expanded among other lymphocytes in the lungs and it is possible that most of the lymphocytes found at the site of the infection in pTB are in fact not MTB-specific. Limitations of our study need to be addressed. The influence of age or sex on MTB-specific immune responses cannot be excluded. Only patients with limited disease smear-negative TB were included in the study. The findings of the immunophenotypes may have been different, if patients with more advanced disease or patients with immunosuppressions had been included. It also remains unclear, whether concentration of MTB-specific T-cells at the site of the infection is due to active recruitment of circulating cells or due to local proliferation and expansion of few specific precursors or a combination of both [32].

In conclusion, antigen-specific T-lymphocytes are specifically concentrated at the site of the infection in active tuberculosis and discrimination of active pulmonary tuberculosis from LTBI may be possible by comparing the frequencies of antigen-specific T lymphocytes in the peripheral blood and among lymphocytes in the BAL by ELISPOT. As high numbers of antigen-presenting cells may be present among mononuclear cells from extrasanguinuous
compartments, MTB-specific ELISPOT results should however be normalized to numbers of lymphocytes in the assay.

Future studies should evaluate whether local immunodiagnosis of tuberculosis by ELISPOT is also appropriate for extrapulmonary manifestations of TB like meningitis, peritonitis or pericarditis.


34. Schwander SK, Torres M, Sada E, Carranza C, Ramos E, Tary-Lehmann M, Wallis RS, Sierra J, Rich EA. Enhanced responses to Mycobacterium tuberculosis antigens by


Fig. 1: Numbers of spot-forming cells (sfc) after stimulation with ESAT-6 and CFP-10 antigen in a *Mycobacterium tuberculosis*-specific enzyme-linked immunospot (ELISPOT). Numbers of sfc are shown as per 1000 000 lymphocytes of peripheral blood lymphocytes and broncho-alveolar lavage lymphocytes respectively.

![Graph showing numbers of sfc after stimulation with ESAT-6 and CFP-10 antigen in blood and BAL.](image)

Fig. 2: Ratio of ESAT-6 and CFP-10 specific lymphocytes in BAL and peripheral blood. Black bars represent ESAT-6 specific lymphocytes, white bars represent CFP-10 specific lymphocytes.

![Graph showing ratio of ESAT-6 and CFP-10 specific lymphocytes in blood and BAL.](image)