Eur Respir J 2008; 31: 261–265
DOI: 10.1183/09031936.00096707

Local immunodiagnosis of pulmonary tuberculosis by enzyme-linked immunospot

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ABSTRACT: **Lymphocytes are crucial in the immune defence against *Mycobacterium tuberculosis* (MTB) infection. The aim of the present study was to ascertain whether or not MTB-specific lymphocytes are selectively compartmentalised in the lungs of patients with minimal active pulmonary tuberculosis (PTB).**

Patients with smear-negative MTB-culture-confirmed PTB were prospectively recruited. Differential cell counts, immunophenotyping with monoclonal antibodies directed against the cell surface markers CD4, CD8, CD4CD45RA, CD4CD45R0, CD38, human leukocyte antigen DR, CD19, CD3, CD57 and CD16 and MTB-specific enzyme-linked immunospot assays of peripheral blood mononuclear cells and bronchoalveolar lavage (BAL) mononuclear cells with 6-kDa early secretory antigenic target and culture filtrate protein 10 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 fluid (BALF). Activated human leukocyte antigen-DR-positive cells, as well as memory CD4CD45R0-positive T-cells, were expanded among cells of the BALF. Compared with a group of control patients with alternative pulmonary pathologies, there was no significant difference in lymphocyte subpopulations. However, 6-kDa early secretory antigenic target- and culture filtrate protein 10-specific lymphocytes were more concentrated, with a median BALF:peripheral blood ratio of 9.9 and 8.9, respectively, in patients with PTB.

***Mycobacterium tuberculosis*-specific T-cells are highly selectively compartmentalised at the site of infection in active pulmonary tuberculosis.**

KEYWORDS: **Bronchoalveolar lavage, culture filtrate protein 10, enzyme-linked immunospot, lymphocytes, 6-kDa early secretory antigenic target, tuberculosis**

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Received:
July 30 2007
Accepted after revision:
October 17 2007

STATEMENT OF INTEREST
None declared.

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 3.0×10^{11} [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 interferon (IFN)-γ [11] and other cytokines of the type-1 T-helper cell-type immune response [12]. Prompt production of IFN-γ following specific antigen presentation is predominantly provided by the population of effector T-cells [11], which typically have a CD4CD45R0-positive, CD27-negative and CC chemokine receptor 7-negative phenotype and are end-differentiated for a rapid, antigen-specific cytotoxic immune response [11, 13].

Since the late 1990s, major advances have been made in the immunodiagnosis of MTB infection with the development of T-cell IFN-γ release assays. Enumeration of 6-kDa early secretory antigenic target (ESAT)-6 and culture filtrate protein (CFP)-10-specific T-lymphocytes from peripheral blood by enzyme-linked immunospot (ELISPOT) assay or 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, since effector T-cells only occur at low frequency in peripheral blood, it is not surprising that a clear distinction between active TB, LTBI and previously treated TB has not been possible when T-cell IFN-γ release assays have been performed with cells from the peripheral blood alone [19, 20]. In contrast, counting antigen-specific cells from

European Respiratory Journal
Print ISSN 0903-1936
Online ISSN 1399-3003

the site of the infection has been shown to be a promising method for distinguishing active TB infection from LTBI [21–24].

In order to gain a better understanding about the magnitude of recruitment of lymphocytes from the blood into the human lung during active pulmonary TB (PTB), immunophenotypes and numbers of MTB-specific T-lymphocytes were analysed in prospectively recruited patients with a suspected diagnosis of PTB and negative acid-fast bacillus sputum microscopy results.

METHODS

Subjects

Patients with three negative acid-fast bacillus smear results, pulmonary infiltrates on chest radiography or thoracic computed tomography and suspected PTB were enrolled. Peripheral blood mononuclear cells (PBMCs) were obtained by 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 an 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 Lübeck Medical School (Lübeck, Germany), and all patients gave written informed consent.

Immunophenotyping

Lymphocyte subsets were enumerated among PBMCs obtained from freshly drawn blood and BALMCs using directly labelled murine monoclonal antibodies directed against CD4, CD8, naive CD4CD45RA, memory CD4CD45R0, CD38, human leukocyte antigen DR, CD19, CD3, CD57 and CD16 (DakoCytomation, Hamburg, Germany) by two-colour flow cytometry (FACSCalibur; Becton-Dickinson, Heidelberg, Germany).

Cell preparations and ELISPOT assays

Cell preparations for ELISPOT assays of human IFN-γ were performed as described previously [22]. The numbers of spot-forming cells (SFCs) per million lymphocytes were calculated from net numbers of SFCs among PBMCs and BALMCs by means of blood and BAL fluid (BALF) differential cell counts.

Statistical analysis

Statistical tests were performed using nonparametric testing for exploratory analyses, without adjustment for multiple testing, with nominal significance defined as having a p-value of <0.05. In order to avoid mathematical errors due to division by zero, a value of 0 was assigned a value of 0.1 when calculating ratios of ESAT-6- and CFP-10-specific cells among lymphocytes among PBMCs and BALMCs.

RESULTS

Patient characteristics

Forty patients with suspected PTB were prospectively enrolled. Twelve had the diagnosis of PTB confirmed by positive MTB culture from sputum or BALF (eight of these patients had been included in a previous report [22]). A group of 25 patients with negative MTB culture results were diagnosed with aetiologies other than PTB; nine of which had <1% lymphocytes in their BALF or not enough BALF available for the fluorescence-activated cell sorter analysis and so 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 organising pneumonia (n=2), bronchogenic carcinoma (n=1) and rheumatoid arthritis (n=1). Seven non-TB patients gave positive ELISPOT results with peripheral blood, and were diagnosed with LTBI. Patients with PTB were younger (31.1 *versus* 50.7 yrs; $p<0.018$) and more often male (10 out of 12) than patients with alternative diseases (10 out of 16; $p<0.021$).

ELISPOT assay results

The median number of ESAT-6- and CFP-10-specific lymphocytes in patients with PTB was 64 (interquartile range (IQR) 35–167) and 149 (85–259) per million lymphocytes,

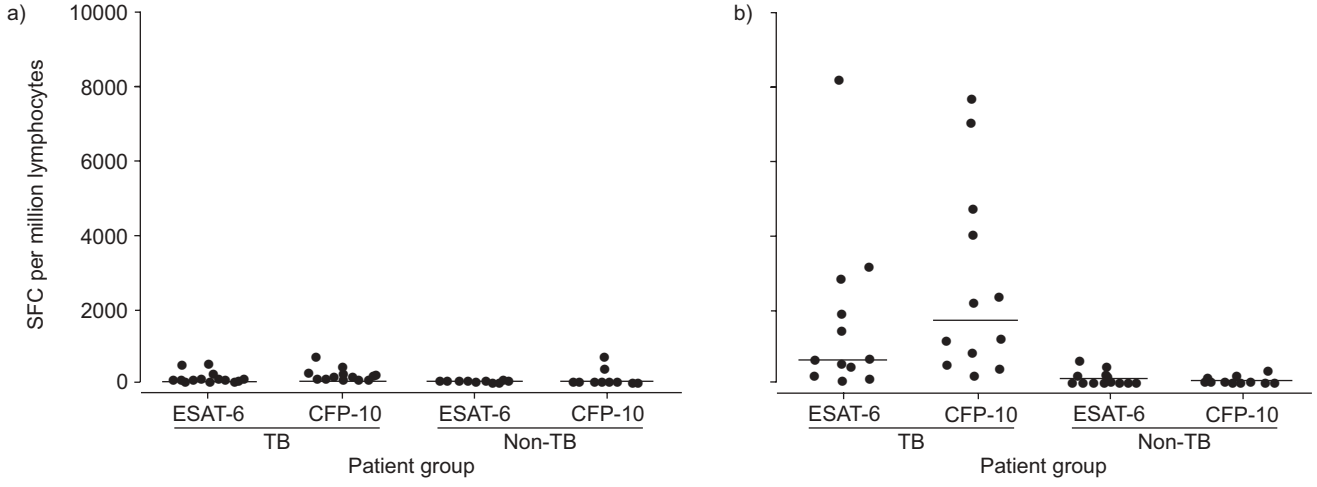

FIGURE 1. Concentration of interferon-γ-releasing cells after stimulation with 6-kDa early secretory antigenic target (ESAT)-6 and culture filtrate protein (CFP)-10 measured using a *Mycobacterium tuberculosis*-specific enzyme-linked immunospot assay in a) peripheral blood lymphocytes and b) bronchoalveolar lavage lymphocytes. Horizontal bars represent medians. TB: tuberculosis; SFC: spot-forming cell.

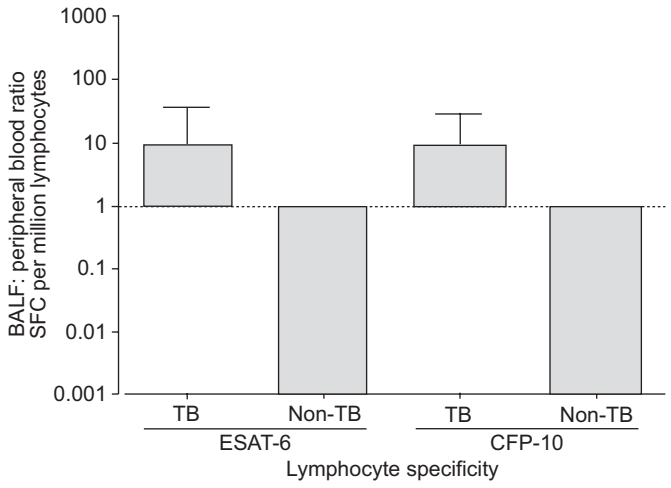

FIGURE 2. Ratio of 6-kDa early secretory antigenic target (ESAT)-6- and culture filtrate protein (CFP)-10-specific lymphocytes in bronchoalveolar lavage fluid (BALF) and peripheral blood (-----: 1:1 ratio). Data are presented as median and interquartile range. The BALF: peripheral blood ratio is close to 0 for both antigens in non-tuberculosis (TB) patients. SFC: spot-forming cell.

respectively, in the peripheral blood, and 598 (244–2,593) and 1,653 (549–4,511) per million lymphocytes, respectively, in BALF. The median number of ESAT-6- and CFP-10-specific lymphocytes in patients in the non-TB group was 3 (0–33) and 6 (0–32) per million lymphocytes, respectively, in the peripheral blood, and 0 (0–150) and 0 (0–73) per million lymphocytes, respectively, in BALF (fig. 1).

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

In patients with PTB, the median (IQR) BALF:peripheral blood ratio of ESAT-6- and CFP-10-specific SFCs per million lymphocytes was 9.9 (3–37) and 8.9 (6–32), respectively. In contrast, the median BALF:peripheral blood ratio of ESAT-6- and CFP-10-specific SFCs per million lymphocytes in control patients was 0 (0–13) and 0 (0–7), respectively (fig. 2).

Immunophenotype

Blood and BALF immunophenotypes are shown in table 1. There was no significant difference in the immunophenotyping results of PBMCs or BALMCs between patients with PTB and controls (data not shown).

DISCUSSION

Since the late 1990s, an MTB-specific ELISA (QuantiFERON-TB-Gold In-Tube; Cellestis, Carnegie, Australia) and ELISPOT assay (T-Spot.*TB*; Oxford Immunotec, Abingdon, UK) have been developed for the diagnosis of TB [26–28]. However, there has been surprisingly little discussion as to what these assays really measure. Although the ELISA quantifies IFN-γ production following MTB-specific antigen contact in whole blood, it is possible to enumerate single MTB-specific cells using 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, ELISPOT assay results should be normalised to lymphocyte numbers in order to achieve more accurate results. Only a minority of the lymphocytes in the human body are found in peripheral blood [29], and the frequency with which MTB-specific T-cells occur in peripheral blood is very low (~0.002–0.4% with respect to ESAT-6- and CFP-10-specific T-cells) even in active TB [21, 22]. It is not surprising, therefore, that it has not been possible to discriminate between active TB and LTBI in routine clinical practice using these assays [27].

Following antigen encounter, naïve cytotoxic T-lymphocyte 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

TABLE 1 Peripheral blood mononuclear cell (PBMC) and bronchoalveolar lavage mononuclear cell (BALMC) immunophenotypes in patients with culture-positive tuberculosis (TB) and other pulmonary diseases

Lymphocyte surface marker	TB group			Non-TB group		
	PBMCs	BALMCs	p-value	PBMCs	BALMCs	p-value
Subjects n	12	12		16	16	
CD4	38 (26–49)	52 (20–59)	NS	32 (21–42)	37 (20–58)	NS
CD8	39 (36–51)	35 (31–67)	NS	40 (29–48)	34 (22–54)	NS
CD4/CD45RA	17 (10–25)	1 (1–3)	0.012	12 (5–25)	2 (2–4)	0.001
CD4/CD45R0	17 (13–26)	50 (18–57)	0.013	15 (11–22)	35 (18–57)	0.011
CD38	55 (43–69)	35 (26–60)	0.012	50 (40–66)	34 (24–44)	0.041
HLA-DR	33 (24–52)	48 (41–80)	0.017	33 (24–41)	59 (37–70)	0.031
CD19	11 (8–17)	2 (1–5)	0.005	11 (7–24)	4 (3–6)	0.027
CD3	75 (62–78)	76 (75–84)	NS	69 (47–74)	75 (59–85)	NS
CD57	34 (17–53)	22 (19–32)	NS	33 (20–50)	32 (23–44)	NS
CD16	12 (11–20)	5 (1–8)	0.007	18 (12–31)	7 (5–14)	0.008
CD4:CD8 ratio	0.75 (0.6–1.7)	1.66 (0.3–1.8)	NS	0.72 (0.5–1.2)	1.18 (0.4–2.7)	0.049

Data are presented as median (interquartile range) percentage of cells bearing marker, unless otherwise stated. HLA: human leukocyte antigen; NS: nonsignificant.

secreting effector cytokines. Subsequently, most effector cytotoxic T-lymphocytes die [30]. It may, therefore, be possible to discriminate active TB from LTBI using immunodiagnosis, comparing the frequencies of antigen-specific cells from the site of infection and peripheral blood using MTB-specific ELISPOT analysis [21, 22]. Compartmentalisation of antimycobacterial immune responses at the site of infection during active TB has previously been described [8, 12, 21, 31–38]. In healthy household contacts of patients with TB [33], and in patients with active PTB, antigen-specific lymphocytes are concentrated in the lungs, and, on challenge, produce type-1 helper T-cell cytokine host responses [34]. However, to date, the magnitude of antigen-specific lymphocyte concentration has not been quantified in PTB.

In the present study, MTB-specific T-lymphocytes were found to be concentrated by a factor of approximately log 1 among lymphocytes derived by BAL from patients with active smear-negative PTB compared with those from the peripheral blood. This concentration of MTB-specific T-cells among lymphocytes in BALF compared with peripheral blood is of the same order of magnitude as the concentration of MTB-specific T-cells among lymphocytes in pleural effusion compared with peripheral blood in TB pleurisy [21]. It is of note that, in agreement with previous findings [6], no other differences in immunophenotype among PBMCs or BALMCs from patients with active PTB and controls could be found in the present study.

When the immunophenotype of PBMCs and BALMCs were directly compared, there were higher frequencies of CD4CD45R0 memory T-cells and human leukocyte antigen DR-positive activated T-cells and a lower frequency of CD4CD45RA-naive T-cells, CD19 B-cells and CD16 natural killer cells among BALMCs than among PBMCs, consistent with earlier reports [6, 39, 40]. However, these differences were not restricted to patients with PTB; 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 infection in PTB are, indeed, not MTB-specific. The limitations of the present study need to be addressed. An influence of age or sex on MTB-specific immune responses cannot be excluded. Only patients with limited smear-negative TB were included in the present study. The findings regarding immunophenotypes may have been different if patients with more advanced disease or with immunosuppression had been included. It also remains unclear as to whether the concentration of MTB-specific T-cells at the site of infection is due to active recruitment of circulating cells or local proliferation and expansion of a few specific precursors or a combination of both [32].

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

Future studies should evaluate whether local immunodiagnosis of tuberculosis by enzyme-linked immunospot assay is also appropriate for extrapulmonary manifestations of tuberculosis, such as meningitis, peritonitis or pericarditis.

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