Recruitment of *Mycobacterium tuberculosis* specific CD4+ T cells to the site of infection for diagnosis of active tuberculosis

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Abstract. Nemeth J, Winkler H-M, Zwick RH, Rumetshofer R, Schenk P, Burghuber OC, Graninger W, Ramharter M, Winkler S (Medical University of Vienna, Vienna, Austria, Universitätsspital Basel, Basel, Switzerland, Otto-Wagner Hospital; Medical University of Vienna, Vienna; Austria). Recruitment of *Mycobacterium tuberculosis* specific CD4+ T cells to the site of infection for diagnosis of active tuberculosis. *J Intern Med* 2008; doi: 10.1111/j.1365-2796.2008.02012.x

Context. Accurate and early diagnosis of active tuberculosis (TB) is problematic as current diagnostic methods show low sensitivity (acid-fast bacilli smears), are time-consuming (culture of biological samples) or show variable results [*Mycobacterium tuberculosis* (MTB)-specific PCR].

Objectives. In the course of infection, MTB-specific T cells clonally expand at the site of infection and may thus be used as diagnostic marker for active disease.

Design. In this cohort study, the frequency of MTB-specific, interferon (IFN)-γ expressing CD4+ T cells obtained from peripheral blood and the site of disease in 25 patients with suspected TB was assessed ($n = 11$, bronchoalveolar lavage; $n = 7$, pleural fluid; $n = 1$, ascites; $n = 1$, joint fluid; $n = 5$, cerebrospinal fluid).

Results. Amongst 15 patients who showed proven active TB infection, a striking increase of MTB-specific T cells was detected at the site of infection compared with peripheral blood (median increase: 28.5-fold, range: 7.25–531 fold; median of IFN-γ-producing CD4+ T cells from blood: 0.02%, range: 0–0.52%; median of IFN-γ-producing CD4+ T cells from the site of infection: 1.81%, range: 0.29–6.55%, $P < 0.001$).

Main outcome measure. Recruitment of MTB-specific T cells to the site of infection yielded a sensitivity of 100% and specificity of 90%, irrespective of the compartment affected.

Conclusions. The accumulation of MTB-specific T cells at the site of infection may prove as useful diagnostic marker for an accurate and rapid diagnosis of active TB.

Keywords: ESAT-6, IFN-γ, T cell, tuberculosis.

Introduction

Tuberculosis (TB) remains one of the leading causes of death affecting more than 8 million persons annually.

Although TB it is typically a disease of the lungs, which serves both as port of entry and as the major site of disease manifestation, *Mycobacterium tuberculosis* (MTB) has the ability to disseminate to various

extrapulmonary sites [1]. To date, diagnosis of pulmonary TB relies on either time-consuming (culture) or insensitive (microscopy) methods often leading to deferred initiation of treatment. Tuberculous pleuritis and tuberculous meningitis share that direct detection of MTB by acid fast staining is hampered by slow numbers of bacteria leading to unacceptably low sensitivity for direct bacillary detection and PCR [2, 3]. The clinical course of MTB infection is characterized to a large extent by the interaction of T cells with MTB-harbouring macrophages. Most importantly, MTB-specific T cells are recruited and clonally expand at the site of infection [4, 5].

Recently introduced enzyme-linked immunospot and ELISA have been developed to rapidly detect MTB-specific T cells using the MTB-specific antigen early secretory antigenic target (ESAT)-6. ESAT-6-specific T cells in peripheral blood are shown to be an accurate marker of infection. However, distinction between active and latent TB is not possible by the assessment of ESAT-6 specific T cells in peripheral blood [6, 7]. In this report, we tested the hypotheses that ESAT-6 specific T cells are accumulated at the site of infection and may serve as rapid and accurate diagnostic marker for active TB.

Patients and methods

Patients

Patients presenting with signs and symptoms suggestive for TB were eligible for this study. Patient recruitment was confined to the wards of the Medical University of Vienna (Department for Infectious Diseases and Respiratory Medicine) and the Department of Respiratory and Critical Care Medicine at the Otto-Wagner Hospital in Vienna, Austria. Biological samples were obtained by broncho-alveolar lavage (broncho-alveolar fluid, $n = 11$) or puncture (pleural effusion, $n = 7$; ascites, $n = 1$; arthritis, $n = 1$). Cerebrospinal fluid was assessed in case of suspected tuberculous meningitis ($n = 5$). Investigators performing laboratory procedures were blinded with regards to other diagnostic procedures for TB.

Active TB was defined by the presence of either of the following criteria: detection of acid-fast bacilli in Ziehl–Neelsen staining of sputum or aspirates, chest X-rays or computed tomography unambiguous signs for TB, positive mycobacterial PCR, necrotizing granulomatous inflammation without other causes, clinical response to tuberculostatic drugs and clinical history suggestive of active TB, including at least three of the following symptoms: night sweat, cough lasting for more than 3 weeks, unintended weight loss, malaise, fever, lassitude and known exposure to open TB. Written informed consent was obtained from all participating individuals. Ethical clearance was given by the Ethics Committee of the Medical University of Vienna and the Viennese KAV.

Methods

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by Ficoll-diatrizoate centrifugation. Cells obtained from the site of disease were filtered through a 100 μm membrane (Becton Dickinson, Mountain View, CA, USA), spinned down and washed twice in phosphate-buffered saline. The following assay procedures were equal for both PBMC and cells from the site of disease: cells were cultured in ultra culture medium (Bio Whittaker, Walkersville, MD, USA) supplemented with L-glutamine (2 mmol L^{-1}; Sigma, St. Louis, MI, USA), gentamicin (170 mg L^{-1}; Sigma) and 2-mercaptoethanol (3.5 μL L^{-1}; Merck, Darmstadt, Germany) and plated out in 24-well cell culture plates at 2×10^6 per well. Cells were stimulated with ESAT-6 (Statens Serum Institute, Copenhagen, Denmark) (final concentration, 5 μg mL^{-1}). To amplify T-cell receptor signalling and to facilitate the initial phase of T-cell activation, the co-stimulatory mAb CD28 (R&D Systems, Minneapolis, MN, USA) was added at a final concentration of 5 μg mL^{-1}. Cells cultured without antigen served as negative controls.

Cells were stimulated for 18 h at 37 °C in 5% CO_2, brefeldin A (Sigma, final concentration, 10 μg mL^{-1}) was added after 6 h to block protein secretion. Cells were then harvested on ice, fixed with 2% formalde-

hyde and resuspended in Hank's balanced salt solution (Sigma), supplemented with bovine serum albumin (0.3%) and sodium acid (0.1%).

Four colour staining was performed with mouse or rat anti-human-mAb and the respective isotype controls labelled with fluorescein-isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC) and peridinin chlorophyll (PerCP). Cell walls were permeabilized with saponin (0.1%) (Sigma), resuspended with 50 μL of saponin-buffered antibody dilutions and incubated for 25 min and protected from light exposure. The following mAb were used: anti-IFN-γ [clone: B27] (FITC-labelled), anti-tumour necrosis factor (TNF)-α [clone: mAb11] (PE-labelled) (both obtained from Pharmingen, San Diego, CA, USA); anti-CD4 [clone: RPA-T4] (APC-labelled), anti-CD8 [clone: SK1] (PerCP-labelled) (both purchased from Becton Dickinson). Appropriate antibody dilutions were detected by titration and turned out to be 1 : 50 for anti-CD4, 1 : 20 for anti-CD8, 1 : 100 for anti-TNF-α and 1 : 1000 for interferon (IFN-γ).

Cells were gated by their forward-side scatter characteristics and additionally defined as $CD4^+$ and $CD8^+$ cells. Cells fulfilling both criteria (lymphocyte and $CD4^+$ or $CD8^+$) were further analysed for their cytokine production pattern. At least 10^5 cells were measured. Data were analysed with Cell Quest software (Becton Dickinson) and results were expressed as the percentage of cytokine-producing cells in the $CD4^+$ and $CD8^+$ population. To assure specificity, spontaneous cytokine production of the controls was subtracted from cytokine production of the stimulated cells. Statistical analysis was performed using a commercially available software package (SPSS 13.0 for Windows; SPSS Inc., Chicago, IL, USA). Wilcoxon–Mann–Whitney *U*-test was applied for group differences.

Results

In all, 25 patients (7 female) were included in this study (median age 47, 18–84). Fifteen patients (Table 1) were classified as having active TB accord-

Table 1 Patient characteristics

Patient	Age	Sex	Country of origin	Final diagnosis	Confirmation of TB	Origin of T cells	Enrichment
1	27	w	Pakistan	TB	Yes (culture)	BAL	[2.44]
2	32	m	Pakistan	TB	No (granulomatous pneumonia)	BAL	28.5 [1.71]
3	18	w	Unknown	TB	Yes (culture)	BAL	28 [0.56]
4	34	m	Peru	TB	Yes (culture)	BAL	29.4 [4.71]
5	21	m	India	TB	No	BAL	[1.81]
6	47	m	Austria	TB of the joint	No (granulomatous inflammation)	Joint fluid	[0.64]
7	26	m	Turkey	peritoneal TB	Yes (culture)	Ascites	[6.55]
8	84	m	Austria	TB	No (lymphocytic exsudate)	Pleural fluid	103.25 [53.69]
9	18	w	Pakistan	Tuberculous meningitis	Yes (culture)	CSF	[3.42]
10	21	m	Turkey	Tuberculous meningitis	Yes (culture)	CSF	5 [0.15]
11	48	m	Austria	TB	Yes (culture)	Pleural fluid	138.5 [2.77]
12	52	m	Austria	TB	Yes (culture)	Pleural fluid	13.5 [1.22]
13	56	m	Turkey	TB	Yes (culture)	BAL	[0.64]
14	18	m	India	TB	No (pleuritis)	Pleural fluid	531 [5.31]
15	52	m	India	TB	No (lymphocytic exsudate)	Pleural fluid	7.25 [0.29]

IFN, interferon; MTB, *Mycobacterium tuberculosis*; TB, tuberculosis; BAL, broncho-alveolar lavage; CSF, cerebrospinal fluid; w, women; m, men.
Response at the site of infection without peripheral reactivity was interpreted as positive. Percentage of $CD4^+$ T cells producing MTB-specific IFN-γ at the site of infection reported in brackets.

ing to routine diagnostic methods (acid-fast bacilli in sputum: $n = 6$; culture positive: $n = 9$; PCR-positive: $n = 6$). TB patients were suffering from other diseases (pneumonia, $n = 2$; lung cancer, $n = 2$; rheumatoid arthritis, $n = 1$; fever of unknown origin, $n = 1$; meningitis because of *Listeria monocytogenes*, $n = 1$; neuroborreliosis, $n = 1$; unspecified inflammation of the central nervous system, $n = 1$; chronic obstructive pulmonary disease, $n = 1$).

Interferon-γ producing CD4$^+$ T cells were detectable at the site of infection (median: 1.81%, range: 0.29–6.55%) in all patients diagnosed with active TB (Fig. 1). Nine participants suffering from TB (60%) showed reactivity of CD4$^+$ T cells after ESAT-6 stimulation in peripheral blood (median: 0.02%, range: 0.02–0.52%). Amongst patients, a striking increase of ESAT-6-specific T cells was detectable at the site of infection when compared with peripheral

blood (median of increase: 28.5-fold, range: 7.25–531 fold; $P < 0.001$ compared to peripheral blood).

The flow cytometry assay was interpreted as positive when ≥0.02% of CD4$^+$ T cells were expressing IFN-γ following ESAT-6 stimulation. To assure specificity, spontaneous cytokine production in unstimulated control wells was subtracted from cytokine production after stimulation with ESAT-6. Enrichment at the site of infection was interpreted as positive, if the frequency of ESAT-6-specific IFN-γ-expressing T cells at the site of infection was at least two times higher than in peripheral blood. Likewise, any response at the site of disease without peripheral reactivity was interpreted as positive.

In the control group, three individuals (33.3%) showed significant IFN-γ responses in CD4$^+$ T cells from peripheral blood after ESAT-6 stimulation
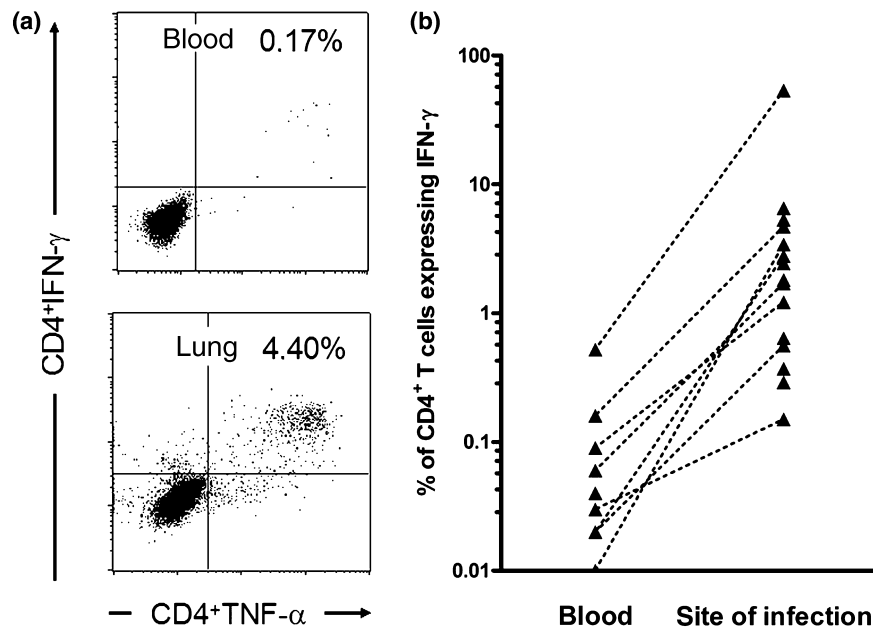

Fig. 1 Enrichment of *Mycobacterium tuberculosis* (MTB)-specific T cells at the site of infection. (a) Representative two parameter dot plots displaying the frequency of MTB-specific CD4$^+$ T cells from peripheral circulation (blood) and from site of infection (lung). Cells were stimulated with early secretory antigenic target and MAb CD28. Numbers indicate the percentage of interferon (IFN)-γ producing CD4$^+$ T cells. (b) Enrichment of MTB-specific, IFN-γ producing CD4$^+$ T cells at the site of infection ($n = 15$) compared with peripheral blood during active tuberculosis. The response of individual pairs is linked on the graph by jittered lines. MTB-specific T cells are increased at the site of disease (median of increase: 28.5-fold, range: 7.25–531 fold). TNF-α, tumour necrosis factor.

(0.02%, 0.05% and 0.09%, respectively). However, two of them did not display any enrichment of MTB-specific T cells at the site of infection. The third one with an 11-fold enrichment of T cells at the site of infection was assigned to the control group because of negative results in routine diagnostic tests. Staining of $CD4^+$ T cells with TNF-α in addition to IFN-γ was not associated with a higher diagnostic accuracy, as was staining with CD8 (data not shown). Sensitivity and specificity of our in-house assay was 100% and 90%, respectively.

Discussion

To date, diagnosis of active TB is difficult and time consuming. Recently implemented novel immunological *in vitro* assays have failed to discern between latent and active disease [6–8]. In patients with pulmonary affection, broncho-alveolar cells but not PBMCs showed enhanced reactivity towards mycobacterial antigens [4, 5]. Thus, it is suggestive that whilst only a small number of MTB-specific T cells are found in peripheral circulation, highly activated, antigen-specific effector T cells accumulate at disease site and rapidly produce Th-1-type cytokines [4, 5, 9–12].

In this report, we assessed an in-house cell culture assay followed by flow cytometry to enumerate the percentage of $CD4^+$ T cells obtained from the site of infection producing IFN-γ after ESAT-6 stimulation. Our data indicate that the preferential recruitment of $CD4^+$ T cells may allow distinguishing between patients with active TB and those with nontuberculous diseases. MTB-specific IFN-γ response in peripheral blood was low amongst patients (60%) with active TB, well corresponding to recent findings [8]. In contrast, patients suffering from active TB showed a striking enrichment of MTB-specific T cells at the site of infection. Interestingly, 3 out of 10 patients with other diseases than TB showed IFN-γ responses after ESAT-6 stimulation. Two of them had no accumulation of MTB-specific T cells at the site of infection detectable. This finding underlines the hypothesis that the hallmark of active disease is not the presence of MTB-specific T cells in peripheral blood but rather the accumulation of these cells at the compartment affected. Thus, the reactivity of T cells to MTB-specific antigens such as ESAT-6 in peripheral blood without enrichment at the site of disease is indicative of latent infection but not active TB.

Currently, diagnostic standards for active TB are far from perfect. This may also be seen as limitation in our study as the lack of a reliable gold standard further complicates thorough assessment of novel diagnostic approaches. Further drawbacks of our method include the need for invasive procedures to obtain biological samples from the site of infection. This may prove as practical limitation for many resource poor settings where TB is of high public health importance. However, the fact that our novel diagnostic approach provides definite results within 24 h may – *per se* – be seen as a major potential advantage, for example, in case of tuberculous meningitis, where early treatment is mandatory. We conclude that in line with recent findings [9–12] for routine purposes, enumeration of MTB-specific, IFN-γ secreting $CD4^+$ T cells from the site of infection can provide a new tool to tremendously accelerate diagnosis of active TB.

Conflict of interest statement

Acknowledgements

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