Ex Vivo Characterization of Early Secretory Antigenic Target 6–Specific T Cells at Sites of Active Disease in Pleural Tuberculosis

Katalin A. Wilkinson,1,2 Robert J. Wilkinson,2,3 Ansar Pathan,1 Katie Ewer,1 Manyu Prakash,1 Paul Klenerman,1 Nick Maskell,1 Robert Davies,1 Geoffrey Pasvol,2,3 and Ajit Lalvani1

1Nuffield Department of Neurology, University of Oxford, John Radcliffe Hospital, Oxford, 2Wellcome Trust Centre for Research in Clinical Tropical Medicine, Imperial College London, London, and 3Department of Infection and Tropical Medicine, Northwick Park Hospital, Harrow, United Kingdom

Presence of early secretory antigenic target-6 (ESAT-6)–specific, interferon-γ–secreting T cells in blood accurately marks tuberculosis infection. In tuberculous pleural effusions from 10 patients with tuberculosis, these cells were concentrated a mean of 15-fold (standard deviation, ± 6-fold), relative to their level in peripheral blood (P = .014), and displayed rapid effector function. Such cells were absent in 8 control patients with nontuberculous pleural disease. The recruitment of ESAT-6–specific T cells to inflamed tuberculous tissue demonstrates their function in vivo and suggests a novel way to diagnose tuberculous pleuritis.

Eight million new cases of tuberculosis and >2 million deaths due to tuberculosis occur annually [1]. Antigen-specific IFN-γ–secreting CD4 and CD8 T cells are key components of the host response to Mycobacterium tuberculosis [2]. The outcome of infection is partly determined by the interaction of these T cells with M. tuberculosis–infected macrophages at disease sites. However, the mechanisms that govern the trafficking and recruitment of circulating lymphocytes to the site of active disease are not fully understood. Local immune responses can correlate with clinical outcome in tuberculosis [3]. Tuberculous pleuritis is of particular immunological interest, because untreated primary tuberculous pleural effusions can spontaneously resolve, which is consistent with a relatively effective host immune response. The study of T cells from tuberculous pleural fluid provides an opportunity to evaluate a component of the human immune response that is probably involved in mediating containment of M. tuberculosis in vivo.

Among the most interesting antigens of M. tuberculosis are those absent from Mycobacterium bovis bacille Calmette-Guérin vaccine. The most intensively studied of these antigens is early secretory antigenic target-6 (ESAT-6). Circulating ESAT-6–specific T cells in peripheral blood are an accurate marker of M. tuberculosis infection [4, 5]. Murine and human studies have suggested that ESAT-6–specific, IFN-γ–secreting CD4 T cells play a role in protection against M. tuberculosis in vivo [6, 7]. Tuberculosis in the pleural cavity is often locally contained and can resolve without treatment; we thus hypothesized that ESAT-6–specific, Th cell 1–type CD4 T cells might be highly concentrated in pleural fluid. Their presence might also make possible a new approach for rapid diagnosis of tuberculous pleuritis while awaiting results of mycobacterial culture, because only a minority of pleural effusions and biopsy specimens reveal acid-fast bacilli on microscopy [8]. We therefore used an ex vivo enzyme-linked immunospot (ELISPOT) assay to enumerate ESAT-6–specific, IFN-γ–producing T cells directly from specimens from sites of active disease and simultaneously from peripheral blood from 10 patients with tuberculosis and 8 patients with nontuberculous pleural disease.

Patients and methods. Patients were recruited from Northwick Park Hospital in Harrow, London. Ethical approval for the study was obtained from Harrow Research Ethics Committee (file number Harrow LREC 2410). Nine patients had pleural tuberculosis, and 1 patient had tuberculous peritonitis and pleural tuberculosis (6 women and 4 men, all of whom were HIV negative; mean age ± SD, 32 ± 2.8 years). The diagnosis of tuberculosis was confirmed in all cases by culture of M. tuberculosis from pleural fluid, ascites fluid, or pleural biopsy specimens. A heparinized blood sample and a sample from the disease site were obtained from 8 patients at the time of diagnosis; blood samples were not available from 2 patients (patient SD1, who had tuberculous peritonitis, and patient SD2, who had pleural tuberculosis). One patient (patient SD8) had disseminated tuberculosis, which allowed the collection of both pleural and ascitic fluid samples. Eight control patients who had nontuberculous pleural disease (malignant pleural effusion secondary to biopsy–proven mesothelioma or lung cancer) were also included in the study (5 women and 3 men, all of whom
were HIV negative; mean age ± SD, 65 ± 5 years). These patients were from regions of low tuberculosis prevalence, had no past history of tuberculosis, and had no history of contact with patients with tuberculosis.

The ex vivo IFN-γ ELISPOT assay was performed as described elsewhere [9]. PBMCs or pleural fluid cells (3 × 10^6 cells per well) were added to wells in 100 μL of complete medium. Recombinant ESAT-6 (a gift from Adam Whalen at Veterinary Laboratories Agency Weybridge, United Kingdom) and peptides were used at concentrations of 10 μg/mL. The peptides (Research Genetics), labeled p1–p17, were 15 amino acids long, overlapped by 10 residues, and spanned the length of ESAT-6. Twenty micrograms per milliliter PPD (Statens Seruminstitut; batch RT49) and 5 μg/mL PHA as a positive control (ICN Biomedicals) were also added to pairs of ELISPOT wells. The assay was developed after 16 h of incubation. A response was considered positive if the test well contained at least 10 more IFN-γ spot-forming cells (SFCs) than did the negative control well, and this number was at least twice that in negative control wells; the background number of SFCs in negative control wells was always ≤5 SFCs per well. After subtraction of the background value, the number of IFN-γ SFCs specific for each ESAT-6–derived peptide was summed to give the total number of ESAT-6 peptide–specific, IFN-γ SFCs for each individual.

Immunomagnetic depletion of CD4 and CD8 T cells from pleural fluid cells was performed using magnetic beads (Dynabeads; Dynal) as described elsewhere [10]. For CCR7 depletion, sheep anti-rat IgG–coated magnetic beads (M450 Dynabeads) were coated with purified mouse anti-human CCR7 (BD Pharmingen) and were incubated with cells for 30 min on ice. After removing CCR7-positive cells by use of a magnet, the remaining cells were washed, counted, and set up as required. These depletions consistently yielded cells with 97%–99% purity for CD4 and CD8 and with 95%–97% purity for CCR7, as confirmed by analysis with a fluorescent-activated cell sorter.

Results. Although significant numbers of ESAT-6 peptide–specific, IFN-γ–producing T cells were found in PBMCs, patients with tuberculosis invariably had enrichment of such cells in the pleural fluid (n = 8) and ascitic fluid (n = 1) (figure 1). The highest concentration of responding T cells was found in an ascites fluid sample (6167 SFCs per 10^6 ascitic fluid cells); lower concentrations were found in pleural fluid (mean ± SD, 2367 ± 682 SFCs per 10^6 pleural fluid cells; range, 620–4933 SFCs per 10^6 pleural fluid cells; n = 8) and blood (mean ± SD, 218 ± 58 SFCs per 10^6 PBMCs; range, 43–567 SFCs per 10^6 PBMCs; n = 8). By contrast, there were no ESAT-6–peptide–specific, IFN-γ–producing T cells in pleural fluid samples from 7 of 8 nontuberculous patients (mean ± SD, 4 ± 4 SFCs, range, 0–33 SFCs; n = 8). A single control patient had 33 peptide–specific, IFN-γ–producing T cells per 10^6 pleural fluid cells, a value just higher than the cutoff value for a positive result (i.e., 30 SFCs per 10^6 pleural fluid cells). In patients with tuberculosis, the mean concentration (± SD) of antigen-specific, IFN-γ–producing SFCs in the pleural fluid was 15-fold ± 6-fold greater than that of PBMCs (P = .01; n = 8); inclusion of the patient with ascites increased this value to 17-fold ± 6-fold (P = .007; n = 9).

In addition to the higher frequencies of responding T cells, the epitope repertoire was also broader at disease sites (figure 2A and 2B). Several ESAT-6–derived peptides were recognized exclusively in the pleural fluid and ascitic fluid from 6 of 8 patients. Pleural fluid cells recognized a significantly higher number of peptides than did PBMCs (mean ± SD in pleural fluid cells, 8 ± 1.2 peptides [range, 3–13 peptides]; in PBMCs, 4.3 ± 0.6 peptides [range, 2–7 peptides]; P = .02). However, no peptide was exclusively recognized at the disease site in all patients.

In comparing the number of ESAT-6–specific T cells to the number of PPD-specific T cells in the pleural fluid of 6 patients (patients SD4, SD5, SD7, SD12, SD13, and SD14), the ratio of ESAT-6–specific T cells to PPD-specific T cells in pleural fluid
Figure 2. A and B, Comparison of concentrations of early secretory antigenic target 6 (ESAT-6)–specific, IFN-γ–producing spot-forming cells (SFCs) in pleural fluid cells (PFCs) and blood from patient SD4 (A) and in pleural fluid, ascitic fluid, and blood from patient SD8 (B) shows that Mycobacterium tuberculosis–specific T cells have a broader epitope repertoire at the site of disease than in the blood. Both recombinant ESAT-6 antigen (rESAT-6) and ESAT-6–derived peptides (p1–p17) were tested for all patients. For patient SD4, the samples of pleural fluid and blood were obtained simultaneously, whereas for patient SD8, the sample of blood was obtained 8 weeks after samples of ascitic fluid and pleural fluid. C and D, Phenotype of the pleural fluid cells. C, The percentage of rESAT-6–specific IFN-γ SFCs relative to the number of IFN-γ SFCs obtained with undepleted cells (normalized to 100%). In undepleted cells, the numbers of ESAT6–specific, IFN-γ SFCs for the 3 patients were as follows: for patient SD1, 83 SFCs per 10^6 PFCs; for patient SD2, 450 SFCs per 10^6 PFCs; and for patient SD13, 1740 SFCs per 10^6 PFCs. D, The total number of IFN-γ SFCs/10^6 cells positive for ESAT-6–derived peptides (p2, p11, and p12), rESAT-6, PPD, and phytohemagglutinin (PHA) after 6 h and after 16 h of incubation. IFN-γ secretion occurs within 6 h of antigen contact, indicating rapid effector function.

samples was significantly higher than it was in time-matched blood specimens (mean ratio ± SD, 0.96 ± 0.14 vs. 0.54 ± 0.16; P = .031). These data indicate preferential recruitment of ESAT-6–specific, IFN-γ–producing T cells at the disease site. Depletion of cells positive for CD4, CD8, or CCR7 from 3 samples from disease sites (2 pleural fluid sample and 1 ascitic fluid sample) indicated that the majority of IFN-γ–producing T cells at the disease sites are CD4 positive, and a high proportion appear to be CCR7 negative (figure 2C). Furthermore, these cells are able to release IFN-γ within 6 h after antigen contact (figure 2D).

Discussion. Compartmentalization of antigen-specific T cells in pleural fluid has been demonstrated previously, with use of methods that rely on in vitro expansion by antigen stimulation over several days (i.e., with use of proliferation assays and by measurement of cytokines from culture supernatants) [11, 12]. However, the overnight ex vivo ELISPOT assay that we used directly quantitates antigen-specific effector T cells without the need for in vitro expansion and is fast and convenient. Using this ex vivo assay with T cells from disease sites and peripheral blood, we found that ESAT-6–specific, IFN-γ–producing T cells were highly concentrated in tuberculous pleural fluid, and that these cells recognize a broader repertoire of ESAT-6–derived peptides than do T cells from peripheral blood. These T cells were CD4 positive, and a high proportion were CCR7 negative. The cells were able to release IFN-γ within 6 h after antigen contact. Together, these findings show that ESAT-6–specific, Th 1–type effector memory CD4 T cells predominate at sites of active tuberculosis. This preferential recruitment of ESAT-6–specific, IFN-γ–secreting CD4 T cells to sites of replicating bacilli, together with their phenotypic and functional characteristics, suggest that these cells contribute to host defense by containment of M. tuberculosis at the disease site.

It is notable that, although all patients with tuberculosis had ESAT-6–specific, IFN-γ–producing T cells in their pleural fluid, these cells were absent in 7 of 8 control patients with nontuberculous pleural disease, and the concentration of these cells
in the single patient in whom they were detected was very low, just above the cutoff level for a positive result. Thus, in pleural fluid samples, the presence and concentration of ESAT-6–specific T cells, as detected by IFN-γ ELISPOT, appears to be a sensitive and specific marker of active tuberculosis. Increased concentrations of IFN-γ in pleural fluid have shown promise for the diagnosis of tuberculosis that presents with a lymphocytic exudative pleural effusion [13, 14], although these studies did not establish antigenic specificity and used a less-sensitive assay. Because results are available by the morning after sample collection, the ex vivo ELISPOT could help to accelerate the diagnosis of pleural tuberculosis and thereby prevent delays associated with waiting for M. tuberculosis culture results in the majority of cases, in which acid-fast bacilli are not detectable on direct microscopy of pleural specimens [8]. Further validation will require testing of larger numbers of patients with tuberculosis and control patients, including patients with non-tuberculous pleural effusions who have incidental latent tuberculosis infection.

Acknowledgments

We are grateful to the Clinical and Microbiological staff of Northwick Park Hospital, particularly Michael Eddleston, Robert Wall, and Robert Davidson, for assistance in recruitment and diagnosis. We thank Helen Durkan for obtaining follow-up blood samples, and we thank Peter Barnes and Martina Sester for critical review of the manuscript and for helpful comments.

Financial support. K.A.W. was supported by the British Lung Foundation, and R.I.W. was supported by a Wellcome Trust Fellowship in Clinical Science (064261). P.K. and A.L. are Wellcome Trust Senior Fellows in Clinical Science.

Potential conflicts of interest. A.L. is a named inventor on several patents relating to T cell–based diagnosis filed by the University of Oxford. Regulatory approval and commercialization of ELISPOT has been undertaken by a spin-off company of the University of Oxford (T-SPOT TB, Oxford Immunotec Ltd., Abingdon, England) in which A.L. is a shareholder and to which he acts as a nonexecutive scientific advisor. All other authors: no conflicts.

References