

## Enumeration of T Cells Specific for RD1-Encoded Antigens Suggests a High Prevalence of Latent *Mycobacterium tuberculosis* Infection in Healthy Urban Indians

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Knowledge of the prevalence of latent *Mycobacterium tuberculosis* infection is crucial for effective tuberculosis control, but tuberculin skin test surveys have major limitations, including poor specificity because of the broad antigenic cross-reactivity of tuberculin. The *M. tuberculosis* RD1 genomic segment encodes proteins, such as early secretory antigenic target (ESAT)-6, that are absent from *M. bovis* bacille Calmette-Guérin (BCG) and most environmental mycobacteria. We recently identified circulating ESAT-6-specific T cells as an accurate marker of *M. tuberculosis* infection. Here, interferon- $\gamma$ -secreting T cells specific for peptides derived from ESAT-6 and a second RD1 gene product, CFP10, were enumerated in 100 prospectively recruited healthy adults in Bombay (Mumbai), India. Eighty percent responded to  $\geq 1$  antigen, and many donors had high frequencies of T cells that were specific for certain immunodominant peptides. In contrast, of 40 mostly BCG-vaccinated, United Kingdom-resident healthy adults, none responded to either antigen. This study suggests an 80% prevalence of latent *M. tuberculosis* infection in urban India.

One third of the world's population is believed to be latently infected with *Mycobacterium tuberculosis* [1]. Accurate determination of the prevalence of latent infection is essential for an improved understanding of the epidemiology of tuberculosis and for the design and evaluation of tuberculosis control strategies [2]. However, estimates of the prevalence of latent *M. tuberculosis* infection have hitherto been based on the tuberculin skin test (TST), which has multiple major limitations [2–5]. Intradermal inoculation of purified protein derivative (PPD), a crude precipitate of *M. tuberculosis* culture supernatant that contains >200 antigens widely shared among mycobacteria other than *M. tuberculosis*, including *M. bovis* bacille Calmette-Guérin (BCG) and many environmental mycobacteria, elicits a local cutaneous de-

layed-type hypersensitivity response in sensitized individuals. The broad antigenic cross-reactivity of PPD is responsible for the poor specificity of the TST; a positive reaction is consistent with BCG vaccination, exposure to environmental mycobacteria, or *M. tuberculosis* infection [3, 4, 6, 7]. Moreover, repeated inoculation of tuberculin, as is often required in nonresponders at risk of exposure to *M. tuberculosis*, can itself boost a delayed-type hypersensitivity response to PPD; this booster effect is another cause of false-positive TST reactions [2, 5]. The intrinsic sensitivity of the TST for detecting latent *M. tuberculosis* infection is not known, because there is no definitive test against which to compare it; however, for patients with active tuberculosis, it is 75%–90% sensitive, and its sensitivity falls to <50% in patients with disseminated disease [5].

Recent advances in *M. tuberculosis* genomics, using subtractive hybridization [8] and DNA microarrays [9], have identified a genomic segment, RD1, that is present in *M. tuberculosis* complex but absent from all strains of *M. bovis* BCG and almost all environmental mycobacteria. RD1 gene products offer the potential for the development of new diagnostic tests that might differentiate *M. tuberculosis* infection from BCG vaccination and exposure to environmental mycobacteria.

Early secretory antigenic target (ESAT)-6 is a secreted protein encoded by RD1 that has been shown to be highly immunogenic in animal models [10–13] and patients with tuberculosis [14–16]. Using a sensitive ex vivo ELISPOT assay for interferon (IFN)- $\gamma$  [17], we recently identified ESAT-6 peptide-specific IFN- $\gamma$ -secreting CD4 T cells as an accurate marker of *M. tuberculosis* infection in 96% of patients with bacteriolog-

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Informed consent was obtained from all patients and healthy individuals participating in this study. Ethical approval was obtained from the Ethics Committee of the P. D. Hinduja National Hospital and Medical Research Centre, Mahim, Bombay, India, and the Central Oxford Research Ethics Committee.

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ically confirmed active disease ( $n = 47$ ). We also observed that a high proportion (85%) of TST-positive household contacts of patients with sputum smear-positive pulmonary tuberculosis ( $n = 26$ ) had circulating ESAT-6 peptide-specific T cells (authors' unpublished data and [18]). The presence of these antigen-specific T cells in heavily exposed healthy contacts suggests a new approach to the detection of latently infected individuals. Having validated this T cell-based approach for detecting *M. tuberculosis* infection in a nonendemic setting, we were curious to determine the prevalence of latent *M. tuberculosis* infection in an endemic region. To our knowledge, all estimates of the prevalence of latent infection have hitherto been based on TST surveys.

India, with a population of ~1 billion, has ~1.8 million new cases of tuberculosis annually, more than any other country and accounting for one quarter of the global burden [1]. The tuberculosis epidemic is exacerbated by high rates of multidrug-resistant tuberculosis and a rapidly expanding human immunodeficiency virus (HIV) epidemic, both of which are most advanced in major cities [19]. Accurate estimates and surveillance of the prevalence of latent *M. tuberculosis* infection in urban India will, therefore, be important for the development of tuberculosis control strategies over the next several years. Bombay (Mumbai), one of the world's largest and most overcrowded cities, with a population of 15 million (and projected to reach 27 million within 5 years), has a high burden of tuberculosis and is the epicenter of India's HIV epidemic.

The aims of this study were, first, to confirm whether the ESAT-6-based ex vivo ELISPOT assay could identify bacteriologically confirmed tuberculosis cases in India and to compare the frequency of ESAT-6-specific T cells in Indian patients with the frequencies previously enumerated in tuberculosis patients in the United Kingdom (UK). Second, we applied our antigen-specific T cell-based approach to estimate the prevalence of latent *M. tuberculosis* infection in urban India. We therefore studied 100 prospectively recruited healthy adults resident in Bombay; in addition to the ESAT-6-based ex vivo ELISPOT assay, we also used a panel of peptides spanning the length of CFP10, a second RD1 gene product [20], which has recently been reported to be immunogenic in patients with active tuberculosis [21, 22]. Finally, we applied the same approach to a series of prospectively recruited healthy adults resident in a nonendemic country, the UK. For all subjects, IFN- $\gamma$ -secreting PPD-specific T cells, as well as ESAT-6- and CFP10-specific T cells, were enumerated, so that the frequencies of IFN- $\gamma$ -secreting T cells specific for each of these antigen preparations could be compared between healthy residents of tuberculosis endemic and nonendemic regions.

## Subjects and Methods

**Study population.** All subjects were recruited prospectively in Bombay and Oxford. A heparinized blood sample was drawn from each subject after obtaining informed consent.

We recruited 50 patients, 33 male and 17 female, with clinical and radiographic features consistent with tuberculosis and with positive cultures for *M. tuberculosis* from  $\geq 1$  clinical specimen. Of these, 45 patients had pulmonary tuberculosis, 4 had tuberculous lymphadenitis, and one had tuberculous pleuritis. Six patients (5 with pulmonary and one with pleural tuberculosis) were HIV antibody positive. None of the other 44 patients had clinical features suggestive of HIV infection. The mean age of the patients was 33 years (range, 14–69 years). The patients were ethnically diverse, comprising Maharashtrians ( $n = 28$ ), Gujaratis ( $n = 6$ ), Muslims ( $n = 6$ ), South Indians ( $n = 4$ ), Punjabis ( $n = 3$ ), Uttar Pradeshis ( $n = 2$ ), and Sindhis ( $n = 1$ ). Thirty-five patients (70%) had received <1 month's therapy or no treatment at the time of venipuncture for ELISPOT assays; the remaining 15 patients were at later time points in their treatment course (<1 year).

One hundred eight healthy residents of Bombay were prospectively recruited at an executive health checkup clinic; all were corporate executives who had been requested by their employers to undergo medical examinations for their health insurance, and none had self-referred with symptoms. Seven subjects disclosed a history of tuberculosis, and one was resident in Zambia: these 8 subjects were excluded. The remaining 100 subjects, 78 male and 22 female, all had normal chest radiographic findings, and none had any features of HIV infection. The mean age was 47 years (range, 18–70 years). The executives were ethnically diverse, comprising Gujaratis ( $n = 36$ ), Maharashtrians ( $n = 22$ ), South Indians ( $n = 14$ ), Sindhis ( $n = 7$ ), Parsis ( $n = 6$ ), Punjabis ( $n = 6$ ), Marwaris ( $n = 4$ ), Muslims ( $n = 3$ ), and Bengalis ( $n = 2$ ).

Forty healthy adult residents, 21 men and 19 women, were recruited in Oxford, UK. None had lived in a tuberculosis-endemic country for >1 month, and none had a history of tuberculosis or any known tuberculosis contact. All were ethnically whites, and the mean age was 32 years (range, 23–49 years). Thirty-three (82%) had a BCG scar or a history of BCG vaccination.

Five bacteriologically confirmed tuberculosis patients were followed up in the UK, and ESAT-6 peptide-specific IFN- $\gamma$ -secreting T cells were enumerated at different time points during antituberculosis chemotherapy. Three of these patients had pulmonary tuberculosis, one had pleural disease (patient DD1), and one had tuberculous lymphadenitis (patient DD8).

**ESAT-6- and CFP10-derived peptides.** Seventeen peptides spanning the length of the ESAT-6 molecule and 18 peptides spanning the length of the CFP10 molecule were purchased (Research Genetics). Each peptide was 15 amino acids long and overlapped its adjacent peptide by 10 residues. Identity was confirmed by mass spectrometry and purity by high-performance liquid chromatography. Sequence homology searches of the SwissProt and translated GenBank databases of all known protein sequences confirmed that the sequences of these peptides are uniquely restricted to the ESAT-6 and CFP10 proteins of *M. tuberculosis* complex. A response to  $\geq 1$  of these 35 peptides was scored as indicative of *M. tuberculosis* infection.

**Ex vivo ELISPOT assay for single-cell IFN- $\gamma$  release: enumeration of circulating ESAT-6 peptide-specific T cells from peripheral blood.** The ELISPOT assay, based on the principle of a sandwich capture ELISA, detects IFN- $\gamma$  molecules in the immediate vicinity of the T cell from which they are secreted, while they are still at a high concentration. After development, each resulting spot thus

represents the "footprint" of an individual antigen-specific IFN- $\gamma$ -secreting T cell, or spot-forming cell (SFC). The ex vivo ELISPOT assay for IFN- $\gamma$  is sufficiently sensitive to detect antigen-specific T cells directly from peripheral blood, without the need for a prior in vitro stimulation step. Moreover, since the ex vivo ELISPOT assay enumerates antigen-specific T cells with rapid effector function, only short incubation periods are required [17].

Peripheral blood mononuclear cells (PBMC) were separated from 15 mL of blood by standard means and were suspended in RPMI supplemented with 2 mM L-glutamine, 100  $\mu$ g/mL ampicillin and 50  $\mu$ g/mL gentamicin, and 10% heat-inactivated fetal calf serum (complete medium [R10]; Sigma).

As described elsewhere [23], 96-well polyvinylidene difluoride-backed plates (MAIP545; Millipore), precoated with 15  $\mu$ g/mL of anti-IFN- $\gamma$  monoclonal antibody (MAb) 1-D1K (Mabtech), were blocked with R10 for 2 h. A total of  $2.5 \times 10^5$  PBMC ( $3 \times 10^5$  for the UK subjects) was added in 100  $\mu$ L R10/well, and each of the 35 ESAT-6- and CFP10-derived peptides was added individually to single wells, at 10  $\mu$ g/mL. PPD (batch RT49; Statens Serum-institut) was also tested, at 20  $\mu$ g/mL, in duplicate wells. Phytohemagglutinin (ICN Biomedicals) was added, at 5  $\mu$ g/mL, to duplicate positive control wells, and no peptide was added to duplicate negative control wells. Whole recombinant ESAT-6 (a kind gift of A. Whalen and M. Vordermeier, Veterinary Laboratories Agency, Addlestone, UK) was added, at 10  $\mu$ g/mL, for 11 tuberculosis patients and all healthy adults.

After a 14-h incubation at 37°C, 5% CO<sub>2</sub> plates were washed with PBS 0.05% Tween-20 (Sigma). Next, 100  $\mu$ L of 1  $\mu$ g/mL biotinylated anti-IFN- $\gamma$  MAb, 7-B6-1-biotin (Mabtech), was added. After 2 h, plates were washed, and streptavidin-alkaline phosphatase conjugate (Mabtech) was added, to a dilution of 1:1000. After 1 h and further washing, 50  $\mu$ L of chromogenic alkaline phosphatase substrate (Biorad), diluted 1:25 with deionized water, was added. After 20 min, plates were washed and allowed to dry.

Responses were scored as positive if test wells contained  $\geq 5$  IFN- $\gamma$  SFCs more than negative control wells and this number was at least twice that in negative control wells. Although the person performing the assays was not blinded to the patients' tuberculosis status, the readout in SFCs was quantitative, criteria for a positive response were stringent and predefined, and SFCs in negative control wells were always  $< 15$ , so that positive responses were clear-cut. Positive and negative responses were immediately recognizable by direct inspection of the plate, before precise enumeration with a magnifying lens. ELISPOT assay wells in which the number of IFN- $\gamma$  SFCs is  $> 250$  cannot be precisely enumerated, because the spots coalesce with each other. Therefore, 250 SFCs per well (equivalent to 1000 SFCs/ $10^6$  PBMC) was taken as the upper limit for accurate quantitation. The total number of ESAT-6- and CFP10-specific T cells in a given individual was calculated by summing all the IFN- $\gamma$  SFCs specific for each of the different ESAT-6- or CFP10-derived peptides (after subtraction of the background number of SFCs in the negative control wells), respectively, in that individual.

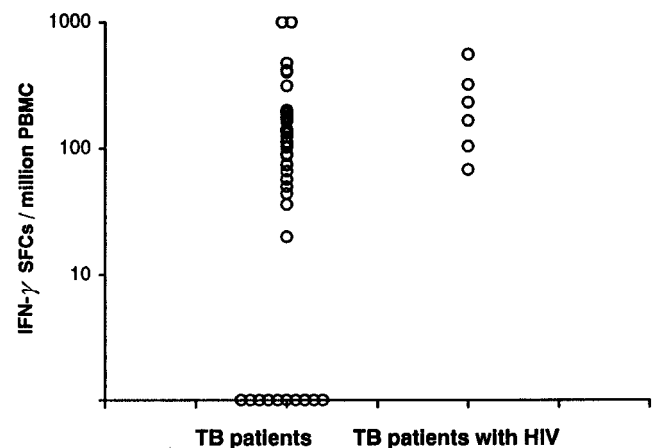
**HIV antibody testing.** HIV antibodies were detected by a rapid test with recombinant protein (Immunocomb II; Organics Ltd.), followed by 2 different microtiter ELISA tests with synthetic peptides from different antigens (Laboratory Systems and Innogenetics).

**Tuberculin skin testing.** One tuberculin unit of PPD (1:10,000;

Span Diagnostics) was injected intradermally in the flexor surface of the forearm, to raise a pale white bleb. Cutaneous induration was measured with a ruler at 72 h, and induration  $> 10$  mm was taken to be positive.

## Results

**High frequencies of circulating ESAT-6 peptide-specific IFN- $\gamma$ -secreting T cells in Indian tuberculosis patients.** A high proportion of Indian tuberculosis patients have circulating ESAT-6 peptide-specific IFN- $\gamma$ -secreting T cells, as determined by ELISPOT assay, and the ESAT-6-based ex vivo ELISPOT also detects tuberculosis patients with HIV infection. Figure 1 shows that 40 of 50 patients with bacteriologically confirmed cases of tuberculosis responded in the ESAT-6-based ex vivo ELISPOT assay. All 6 tuberculosis patients with HIV coinfection responded. The median ESAT-6 peptide-specific T cell count in the 40 patients who responded was 128 per  $10^6$  PBMC (interquartile range [IQR], 73–208/ $10^6$  PBMC). Many patients responded to multiple peptides. Positive responses were clear-cut, compared with negative control wells, and, among a total of 40 positive responses, only 2 tuberculosis patients gave borderline positive responses (20 peptide-specific T cells per  $10^6$  PBMC). Of the 11 patients tested against whole recombinant ESAT-6, including 4 patients with HIV coinfection, all responded to recombinant antigen, as well as to  $\geq 1$  ESAT-6-derived peptides. Six tuberculosis patients (including 3 with HIV infection) underwent TST: all 6 tested negative by Mantoux test but positive in the ESAT-6-based ex vivo ELISPOT assay.



**Figure 1.** Frequencies of early secretory antigenic target (ESAT)-6 peptide-specific and purified protein derivative-specific interferon (IFN)- $\gamma$ -secreting T cells enumerated by ex vivo ELISPOT assay in 50 patients with culture-confirmed tuberculosis (TB) in India. For each individual, the number of peptide-specific T cells for each of the ESAT-6-derived peptides was summated. HIV, human immunodeficiency virus; PBMC, peripheral blood mononuclear cells; SFC, spot-forming cell.

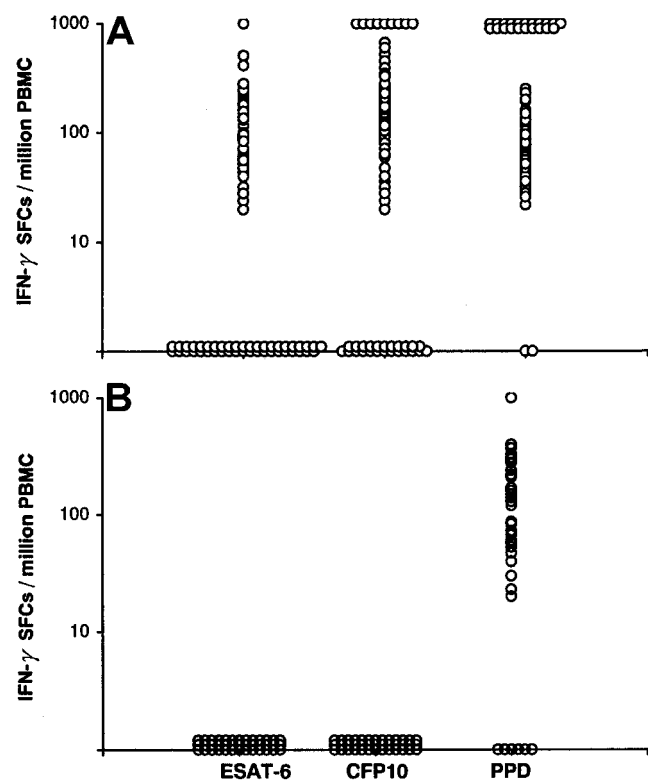
*High frequencies of RD1-encoded antigen-specific IFN- $\gamma$ -secreting T cells in 80% of healthy Indian adults.* One hundred healthy adults with normal chest radiographic findings and no history of tuberculosis were tested by ESAT-6 and CFP10 peptide-based ex vivo ELISPOT assay for IFN- $\gamma$ . Fifty-six had ESAT-6 peptide-specific and 76 had CFP10 peptide-specific T cells detectable in peripheral blood (figure 2A). Overall, 80% responded to either ESAT-6 or CFP10 (table 1). For the responders, the median frequency of ESAT-6 peptide-specific T cells was 86/10<sup>6</sup> PBMC (IQR, 43–156/10<sup>6</sup> PBMC), and the median frequency of CFP10 peptide-specific T cells was 158/10<sup>6</sup> PBMC (IQR, 84–261/10<sup>6</sup> PBMC). These antigen-specific T cell frequencies are as high as (in the case of ESAT-6) or even higher than (in the case of CFP10) the frequency of PPD-specific IFN- $\gamma$ -secreting T cells among the 98 individuals who responded to PPD: median, 86/10<sup>6</sup> PBMC (IQR, 56–222/10<sup>6</sup> PBMC). CFP10 peptide-specific T cell frequencies were especially high, and 9 individuals had >1000 CFP10-specific IFN- $\gamma$ -secreting T cells per 10<sup>6</sup> PBMC. The combined frequency of IFN- $\gamma$ -secreting T cells specific for ESAT-6- and CFP10-derived peptides in the 80 individuals who responded to either of these 2 antigens (median, 208/10<sup>6</sup> PBMC; IQR, 116–357/10<sup>6</sup>) is higher than the frequency of IFN- $\gamma$ -secreting PPD-specific T cells. Thus, in healthy residents of a tuberculosis-endemic area, these 2 antigens alone are a more potent target of IFN- $\gamma$ -secreting T cells ex vivo than is PPD, which contains >200 antigens.

Of the 56 healthy individuals who responded to ESAT-6-derived peptides, most ( $n = 47$ ) also responded to whole recombinant antigen, which suggests that processing and presentation of recombinant antigen occur when it is added exogenously to PBMC in ELISPOT assays. This is consistent with our previous observations [18] and supports the finding that the peptide-specific responses are not artefactual. We have observed that *M. tuberculosis*-infected individuals who respond to ESAT-6-derived peptides, but not whole antigen, in the ex vivo ELISPOT assay have ESAT-6-specific CD8 T cells; whole antigen is not recognized because exogenously added ESAT-6 is not processed through the major histocompatibility complex (MHC) class I antigen-processing pathway, whereas vaccinia virus recombinant for ESAT-6 will present to CD8 T cells [23, 24]. Thus, in the 9 individuals in whom peptide-specific responses were not accompanied by a response to exogenously added antigen, the T cell response to ESAT-6 was probably CD8 mediated.

*Absence of ESAT-6- and CFP10-specific T cells in healthy residents of a nonendemic area.* Forty healthy adult UK residents with no history of tuberculosis were tested prospectively by ESAT-6 and CFP10 peptide-based ex vivo ELISPOT assay for IFN- $\gamma$ . None of the donors reported any tuberculosis contact. Figure 2B shows that no donors responded to any of the CFP10 or ESAT-6 peptides. By contrast, 33 of 40 subjects responded to PPD in the ex vivo ELISPOT assay for IFN- $\gamma$ , with a median frequency of 83 PPD-specific T cells per 10<sup>6</sup>

PBMC (IQR, 53–215/10<sup>6</sup> PBMC), which was very similar to the frequency of PPD-specific T cells seen in the healthy Indian adults. The high prevalence of PPD-specific responses in the unexposed UK residents probably reflects the fact that 33 of the 40 residents were BCG vaccinated.

*Epitope mapping of ESAT-6 and CFP10 in Indian tuberculosis patients and healthy adults.* Many Indian patients and healthy adults responded to multiple peptides, and, as illustrated in figure 3A and 3B, ESAT-6 contains multiple T cell epitopes, which are concentrated at the amino and carboxy termini of the molecule. These highly immunogenic peptides are mostly CD4 T cell epitopes, because T cell lines have been generated in vitro against peptides ESAT-6<sub>1–15</sub>, ESAT-6<sub>6–20</sub>, ESAT-6<sub>16–30</sub>, ESAT-6<sub>21–35</sub>, ESAT-6<sub>31–45</sub>, ESAT-6<sub>46–60</sub>, ESAT-6<sub>51–65</sub>, ESAT-6<sub>56–70</sub>, ESAT-6<sub>66–80</sub>, ESAT-6<sub>71–85</sub>, ESAT-6<sub>76–90</sub>, and ESAT-6<sub>81–95</sub>, and, in each case, peptide-specific responses were abrogated by immunomagnetic depletion of CD4 T cells (data not shown). We have previously identified 8-mer and 9-mer HLA class I-



**Figure 2.** Frequencies of early secretory antigenic target (ESAT)-6 peptide-specific, CFP10 peptide-specific, and purified protein derivative (PPD)-specific interferon (IFN)- $\gamma$ -secreting T cells enumerated by ex vivo ELISPOT assay in 100 healthy adults, with no history of tuberculosis and normal chest radiographic findings, resident in Bombay (Mumbai), India (A), and 40 healthy adults resident in the United Kingdom (B). For each individual, the number of peptide-specific T cells for each of the ESAT-6- or CFP10-derived peptides was summed. PBMC, peripheral blood mononuclear cells; SFC, spot-forming cell.

**Table 1.** Summary of summated responses to ESAT-6- and CFP10-derived peptides and purified protein derivative (PPD) by ex vivo ELISPOT assay for interferon (IFN)- $\gamma$  in healthy adults resident in India and the United Kingdom.

Variable	Bombay (Mumbai), India				Oxford, United Kingdom			
	ESAT-6	CFP10	ESAT-6 or CFP10	PPD	ESAT-6	CFP10	ESAT-6 or CFP10	PPD
No. of adults tested by ex vivo ELISPOT	100	100	100	100	40	40	40	40
No. (%) of adults positive	56 (56)	76 (76)	80 (80)	98 (98)	0 (0)	0 (0)	0 (0)	33
Frequency of antigen-specific IFN- $\gamma$ - secreting T cells <sup>a</sup>	86 (43–156)	158 (84–261)	208 (116–357)	86 (56–222)	0	0	0	83 (53–215)

NOTE. Median frequencies of T cells refer to antigen-specific T cell frequencies in responders only.

<sup>a</sup> Per 10<sup>6</sup> peripheral blood mononuclear cells, median (interquartile range)

restricted CD8 epitopes in peptides 4 and 14 [23, 24], but, because these are strictly restricted by HLA-A6802 and HLA-B52, respectively, only a small proportion of infected people respond. In contrast to these CD8 epitopes, many of the CD4 epitopes were recognized by T cells from a relatively large percentage of the 150 ethnically diverse Indians in this study. For 4 of these peptides (ESAT-6<sub>1–15</sub>, ESAT-6<sub>6–20</sub>, ESAT-6<sub>51–65</sub>, and ESAT-6<sub>71–85</sub>), we have used anti-HLA-DR, -DQ, and -DP MAbs to block presentation to CD4 T cells in ELISPOT assays. In all tuberculosis patients and contacts in whom this was tested ( $n = 14$ ), the responses were MHC class II restricted. The pattern of ESAT-6 epitopes to which the tuberculosis patients responded is similar to that for the healthy subjects (figure 3A and 3B).

Analysis of the T cell response to the 18 overlapping CFP10 peptides in the 100 residents of Bombay has generated the first epitope map of CFP10 (figure 3C). This reveals 2 strikingly immunodominant domains, CFP<sub>51–70</sub> and CFP<sub>71–90</sub>; almost 50% of healthy Indians responded to peptides from these 2 regions, and 15%–20% of Indians responded to CFP<sub>16–30</sub> and CFP<sub>41–60</sub>, identifying these peptides as additional immunogenic regions.

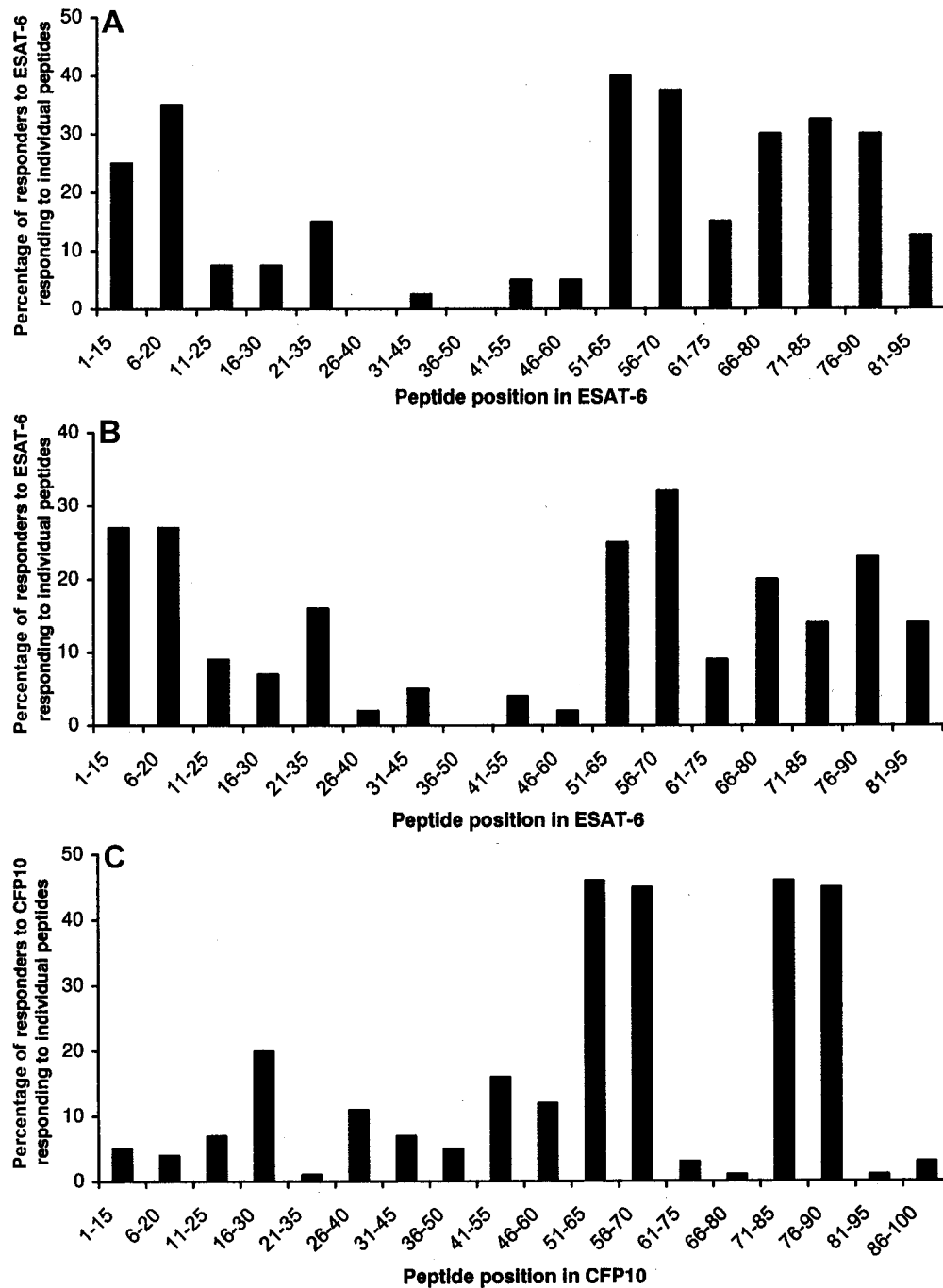
*Progressive decline in frequency of ESAT-6 peptide-specific IFN- $\gamma$ -secreting T cells during antituberculosis treatment.* Figure 4 shows the progressive decline in the frequency of ESAT-6 peptide-specific IFN- $\gamma$ -secreting T cells during antituberculosis treatment in a series of patients with culture-confirmed tuberculosis who were followed up longitudinally in the UK ( $n = 5$ ). All patients had drug-sensitive isolates of *M. tuberculosis* and responded well to therapy.

## Discussion

We recently identified ESAT-6-specific IFN- $\gamma$ -secreting T cells as an accurate marker of *M. tuberculosis* infection in patients with bacteriologically confirmed active disease and in latently infected TST-positive healthy household contacts of patients with sputum smear-positive tuberculosis. 18 CFP10 shares the same species specificity as ESAT-6 and is thus absent from all strains of *M. bovis* BCG and the vast majority of environmental mycobacteria [8, 9, 20]. Here we demonstrate that 80% of our sample of healthy Indian adults harbor circulating IFN- $\gamma$ -secreting T cells specific for  $\geq 1$  of these 2 RD1-encoded antigens, ESAT-6

and CFP10. This suggests that  $\geq 80\%$  of residents of Bombay have been exposed to, or are infected with, *M. tuberculosis*. The striking absence of ESAT-6- and CFP10-specific T cells in all the unexposed UK residents supports this conclusion, and the fact that 33 of these 40 subjects were BCG vaccinated indicates, moreover, that this approach is highly specific and is not confounded by BCG vaccination. Given that all the Bombay residents were asymptomatic and had normal chest radiographic findings, *M. tuberculosis* infection in these individuals would, by definition, be latent rather than active.

Might the high prevalence of ESAT-6- and CFP10-specific T cells in this population result from exposure to a cross-reactive environmental antigen unrelated to *M. tuberculosis* that is present in India but absent in the UK? We consider this unlikely, because sequence homology searches of the SwissProt and translated GenBank databases with each of the ESAT-6- and CFP10-derived peptides indicate that the sequences of all these peptides are uniquely restricted to ESAT-6 and CFP10 of the *M. tuberculosis* complex. However, the *esat6* gene (and therefore, very likely, the gene for CFP10, which is within the same operon as *esat6* [20]) is present in 4 atypical mycobacteria: *M. kansasii*, *M. szulgai*, *M. flavescens*, and *M. marinum* [25]. Thus, it is conceivable that the ESAT-6- and CFP10-specific responses could result from infection with one of these organisms. To account for the striking dichotomy of ESAT-6- and CFP10-specific responses between India and the UK, an environmental mycobacterium would have to cause widespread infection in Bombay but be absent in southern England. However, none of these mycobacteria fit this geographic distribution, nor are they specifically associated with tropical climates. Rather, *M. kansasii* is the most common non-HIV-associated nontuberculous mycobacterium isolated from patients in southern England [26] and is a recognized isolate from tap water in cities in this region [27]. In contrast, *M. kansasii* disease has not been reported from Bombay, although this may reflect underreporting by local laboratories. The absence of RD1-encoded antigen-specific T cells in the 40 UK residents thus makes *M. kansasii* exposure an unlikely cause for the difference in responses between the UK and India. *M. marinum* infection is usually acquired by trauma experienced in a saltwater or freshwater setting or while handling fish [27]; these scenarios would be rare among the corporate executives attending our institution in Bombay to undergo medical checkups for

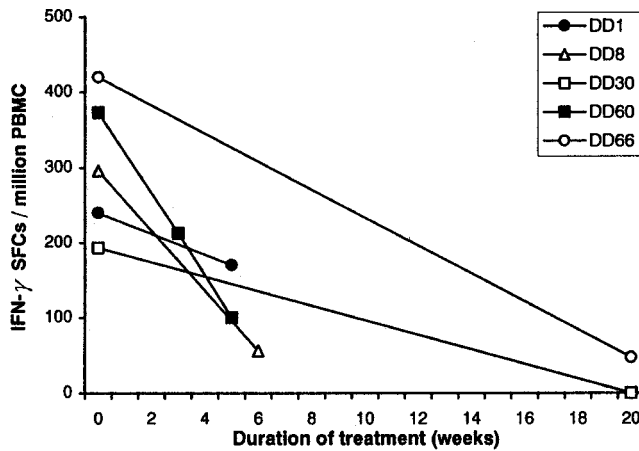


**Figure 3.** Epitope maps of early secretory antigenic target (ESAT)-6 in tuberculosis patients in India (*A*) and in healthy Indian adults resident in Bombay (Mumbai) (*B*) and epitope map of CFP10 in healthy Indian adults resident in Bombay (*C*). For each antigen, the number of individuals responding to a given peptide is expressed as a percentage of the total number of subjects responding to that antigen.

their health insurance. *M. szulgai* and *M. flavescens* are very rare causes of infection in humans. *M. szulgai* has been isolated from patients throughout the world, including the UK, but environmental isolates have not been reported [27]. *M. flavescens* has been found in water supplies in several European countries

[28–31] and thus, as with *M. kansasii*, cannot account for the widespread sensitization to ESAT-6 and CFP10 in Bombay residents and the striking absence of sensitization in the UK donors.

The circulating IFN- $\gamma$ -secreting T cells specific for ESAT-6 and CFP10 were capable of rapid effector function, because



**Figure 4.** Progressive decline in frequency of early secretory antigenic target (ESAT)-6 peptide-specific interferon (IFN)- $\gamma$ -secreting T cells during antituberculosis chemotherapy in a series of 5 patients with culture-confirmed tuberculosis who were followed up longitudinally. For each individual, the number of peptide-specific T cells for each of the ESAT-6-derived peptides, enumerated by the ex vivo ELISPOT assay, was summated at each time point. PBMC, peripheral blood mononuclear cells; SFC, spot-forming cell.

they released IFN- $\gamma$  in the ex vivo ELISPOT assay without requiring prior in vitro stimulation [17]. This suggests that the donors are latently infected with tubercle bacilli that continue to secrete ESAT-6 and CFP10, because high frequencies of circulating effector T cells are probably maintained by recent encounter with antigen in vivo [32]. Because ongoing, active secretion of ESAT-6 and CFP10 requires metabolically active and viable bacilli, the presence of circulating ESAT-6- and CFP10-specific effector T cells in healthy donors suggests that they are latently infected and were not merely exposed at some point in the past. Nonetheless, we cannot rule out the possibility that circulating IFN- $\gamma$ -secreting T cells undergo repeated in vivo stimulation as a result of long-term persistence of ESAT-6 and CFP10 in dendritic cells in the absence of viable bacilli. However, longitudinal follow-up of tuberculosis patients in the UK shows that the frequency of ESAT-6 peptide-specific IFN- $\gamma$ -secreting CD4 T cells declines progressively during the course of antituberculosis chemotherapy (figure 4), which suggests that the frequencies of these T cells are maintained in vivo by ESAT-6 secreted by viable bacilli.

TST surveys in India have generated widely varying results, with the prevalence of TST positivity in adults ranging from 38% to 81% [1, 33-35]. This wide variation reflects, in part, poor standardization of the PPD preparations used, differences in methods, inconsistency in reading skin test results, misclassifications, and the instability of delayed-type hypersensitivity over time [4, 36]. These limitations, together with the intrinsic poor specificity of PPD, render estimates of the prevalence of latent *M. tuberculosis* infection by TST surveys doubtful [36]. Our novel, quantitative approach has generated a more objec-

tive estimate that is not confounded by prior BCG vaccination and is unlikely to be affected by environmental mycobacterial infection. The 80% prevalence in corporate executives is higher than would have been expected on the basis of past TST surveys, especially considering that the latter have all been carried out in rural areas or urban slums. Interestingly, the prevalence of positive results, as determined by RD1-encoded antigen-based ELISPOT assay, was higher in men (64 [82%] of 78) than in women (16 [73%] of 22), which parallels the difference between the sexes seen in TST surveys. This estimate of the prevalence of latent infection is, thus, unexpectedly high for both men and women, particularly if one takes into account the relative affluence and lack of domestic overcrowding in our population sample.

In contrast to the ELISPOT assay for IFN- $\gamma$  that incorporates the 2 RD1-encoded antigens, the use of PPD does not differentiate significantly between the Bombay-resident and UK-resident populations, with 98% and 85% of these populations, respectively, responding to PPD (figure 2A and 2B). The 83% (33 of 40) prevalence of PPD-specific responses in the tuberculosis-unexposed UK residents very likely results from 33 of the 40 residents' being BCG vaccinated. Thus, unlike ESAT-6- and CFP10-specific T cells, PPD-specific IFN- $\gamma$ -secreting T cells do not discriminate between latently infected residents of a tuberculosis-endemic area and unexposed, BCG-vaccinated residents of a nonendemic region; this underscores the practical advantage of using defined antigens with a highly restricted species distribution.

Among the 50 patients with active tuberculosis who were tested with ESAT-6-derived peptides only, 80% had ESAT-6 peptide-specific T cells, as determined by ex vivo ELISPOT. It is not clear why this response rate is lower than that found among a group of patients with active tuberculosis who were recruited in the UK, in whom 45 (96%) of 47 patients responded to  $\geq 1$  ESAT-6-derived peptides, but the following 3 factors are likely to be relevant. First, peptide ESAT-6<sub>1-15</sub> was an immunodominant and permissively recognized epitope in the previous UK study, with 60% of patients responding to this peptide alone, whereas in Bombay, only 25% of patients responded to this peptide (figure 3A). This probably reflects the different HLA backgrounds of the 2 populations. Although 50% of the UK tuberculosis patients were from the Indian subcontinent, none were Maharashtrians, and the remaining 50% were Afro-caribbeans or whites. Of the tuberculosis patients recruited in India, 56% were Maharashtrians, and the remainder were from other Indian ethnic groups. Thus, peptide ESAT-6<sub>1-15</sub> might be restricted by HLA class II alleles that are rare among Maharashtrians. Second, the median frequency of total ESAT-6-specific T cells in the Bombay patients who responded (128 cells/ $10^6$  PBMC; IQR, 72-208/ $10^6$  PBMC) was somewhat lower than that in the London pulmonary tuberculosis patients (177/ $10^6$  PBMC; IQR, 104-392/ $10^6$  PBMC). Bombay patients would, therefore, be more likely to have very low numbers of circulating ESAT-

6-specific T cells, and, if the absolute frequency of ESAT-6-specific T cells in a given patient were below the threshold of our assay ( $20/10^6$  PBMC), such patients would not be identified by the ex vivo ELISPOT assay. Third, the 2 UK patients who did not respond had very advanced pulmonary tuberculosis with cachexia, and, whereas this was rare in the UK patients, many of the Bombay patients presented at an advanced stage of disease. Interestingly, all 6 tuberculosis patients with HIV infection had detectable ESAT-6 peptide-specific T cells, as determined by ex vivo ELISPOT. This preliminary result is the first demonstration of ESAT-6-specific T cells in HIV-infected tuberculosis patients, and it suggests that the clinical and epidemiologic utility of the ex vivo ELISPOT assay for *M. tuberculosis* antigen-specific T cells may be maintained even in HIV-infected individuals.

Finally, the frequency of ESAT-6- and CFP10-specific T cells among healthy urban Indians, as well as the prevalence of positive responses, was also very high (table 1). The median frequency of total CFP10 peptide-specific T cells for the CFP10 responders ( $158$  cells/ $10^6$  PBMC; IQR,  $84$ – $261/10^6$ ) was actually higher than the median frequency of PPD-specific T cells for the PPD responders ( $86/10^6$  PBMC; IQR,  $56$ – $222$ ), whereas the median frequency of total ESAT-6 peptide-specific T cells ( $86/10^6$  PBMC; IQR,  $43$ – $156$ ) was similar to that for PPD. Many of these responses were, moreover, directed against discrete immunodominant regions within the ESAT-6 and CFP10 molecules (figure 3A and 3B). Immune responses in patients with active tuberculosis probably contribute to pathogenesis and tissue destruction, as well as to protection. This intense and highly focused T cell response in residents of a tuberculosis-endemic region who are free of active tuberculosis indicates that these RD1-encoded antigen-specific T cells are not necessarily associated with active disease per se. Rather, the presence of these T cells in latently infected healthy subjects is consistent with a protective role in the long-term control of *M. tuberculosis*.

In conclusion, we have demonstrated that 80 of 100 healthy adults in Bombay have high frequencies of T cells specific for 2 RD1-encoded *M. tuberculosis* antigens, which suggests that  $\geq 80\%$  of this urban population may be latently infected with *M. tuberculosis*. This represents the first estimate of the prevalence of latent *M. tuberculosis* infection, in an endemic region, that uses a T cell-based approach with antigens of tightly defined species specificity.

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