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Dynamic Relationship between IFN-γ and IL-2 Profile of *Mycobacterium tuberculosis*-Specific T Cells and Antigen Load[1]

Kerry A. Millington,*† John A. Innes,‡ Sarah Hackforth,‡ Timothy S. C. Hinks,† Jonathan J. Deeks,§ Davinder P. S. Dosanjh,† Valerie Guyot-Revol,† Rubamalaar Gunatheesan,† Paul Klenerman,¶ and Ajit Lalvani[2]*†

Distinct IFN-γ and IL-2 profiles of Ag-specific CD4$^+$ T cells have recently been associated with different clinical disease states and Ag loads in viral infections. We assessed the kinetics and functional profile of *Mycobacterium tuberculosis* Ag-specific T cells secreting IFN-γ and IL-2 in 23 patients with untreated active tuberculosis when bacterial and Ag loads are high and after curative treatment, when Ag load is reduced. The frequencies of *M. tuberculosis* Ag-specific IFN-γ-secreting T cells declined during 28 mo of follow-up with an average percentage decline of 5.8% per year ($p = 0.005$), while the frequencies of Ag-specific IL-2-secreting T cells increased during treatment ($p = 0.02$). These contrasting dynamics for the two cytokines led to a progressive convergence of the frequencies of IFN-γ- and IL-2-secreting cells over 28 mo. Simultaneous measurement of IFN-γ and IL-2 secretion at the single-cell level revealed a codominance of IFN-γ-only secreting and IFN-γ/IL-2 dual secreting CD4$^+$ T cells in active disease that shifted to dominance of IFN-γ/IL-2-secreting CD4$^+$ T cells and newly detectable IL-2-only secreting CD4$^+$ T cells during and after treatment. These distinct T cell functional signatures before and after treatment suggest a novel immunological marker of mycobacterial load and clinical status in tuberculosis that now requires validation in larger prospective studies. ***The Journal of Immunology,*** **2007, 178: 5217–5226.**

Antigen-specific memory T cell responses are phenotypically and functionally heterogeneous (reviewed in Refs. 1–5). Based on their phenotypic expression of CCR7, CD62L, and CD45RA and their ability to produce cytokines and proliferate, they have been subdivided into effector (T_{EM})[3] and central (T_{CM}) memory T cells. IFN-γ is predominantly produced by T_{EM} while IL-2 (IL-2) is predominantly produced by T_{CM} (6).

The IFN-γ and IL-2 cytokine profile of CD4$^+$ T cells has been studied in a number of viral infections with different viral loads and persistence. High viral load in acute CMV and HIV-1 infection and in chronic progressive HIV-1 infection was associated with a dominance of CD4$^+$ T cells secreting IFN-γ-only (7–10). Ag persistence with low Ag load in chronic hepatitis C virus, HSV, EBV, CMV, and HIV-2 infections and in HIV-1 long-term nonprogressors was associated with CD4$^+$ T cells secreting IFN-γ-only, IFN-γ/IL-2, and IL-2-only (7, 8, 11, 12). Ag clearance of past influenza infection was also associated with CD4$^+$ T cells secreting IFN-γ only, IFN-γ/IL-2, and IL-2 only but with a dominance of CD4$^+$ T cells secreting IFN-γ/IL-2 (13). In contrast, in another model of Ag clearance, past tetanus toxoid vaccination, CD4$^+$ T cells specific for tetanus toxoid predominantly secreted IL-2 only (8). When HIV-1 viral load was manipulated with the administration of antiretroviral therapy or therapy interruptions, a shift between these cytokine patterns was observed (8). These studies suggest that there is an association between distinct CD4$^+$ T cell IFN-γ and IL-2 cytokine secretion patterns and viral load (14), with different Ag loads characterized by distinct functional signatures of CD4$^+$ T cells.

The relationship between T cell functional signatures and Ag load has not hitherto been investigated in bacterial infections. We decided to assess the relationship between the IFN-γ and IL-2 cytokine secretion profile of Ag-specific T cells and bacterial load within a single disease entity and chose tuberculosis as a model. Unlike the case in several chronic viral infections where viral load can be readily quantified, there is no direct quantitative measure of mycobacterial load. However, bacterial and Ag load are high in active disease and decline substantially after successful treatment when the individual has recovered. We hypothesized that the balance between the frequencies of IFN-γ-secreting T cells and IL-2-secreting T cells would change after treatment of active tuberculosis and that patients with untreated active tuberculosis would have distinct T cell IFN-γ and IL-2 functional profiles from successfully treated patients. Although *Mycobacterium tuberculosis*-specific IFN-γ-secreting T cells have previously been enumerated during treatment of active tuberculosis (15–18), they have not been

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[3] Abbreviations used in this paper: T_{EM}, effector memory T cell; BCG, bacille Calmette-Guérin; CFP-10, culture filtrate protein; CI, confidence interval; ESAT-6, early secreted antigenic target protein; PPD, purified protein derivative; SFC, spot-forming cell; SKSD, streptokinase streptodornase; T_{CM}, central memory T cell; TST, tuberculin skin test.

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tracked beyond the completion of therapy and the frequencies of IL-2-secreting T cells have not been reported.

Using ELISPOT assays for IFN-γ and IL-2, we tracked the frequencies of T cells specific for two *M. tuberculosis*-specific Ags, early secreted antigenic target protein 6 (ESAT-6) and culture filtrate protein 10 (CFP-10), encoded by the region of difference 1, an *M. tuberculosis* genomic segment that is absent from all strains of *Mycobacterium bovis* bacille Calmette-Guérin (BCG) vaccine (19). We studied 23 active tuberculosis cases during 28 mo of follow-up. To identify and quantify functional T cell subsets, we simultaneously measured ESAT-6- and CFP-10-specific IFN-γ and IL-2 secretion at the single-cell level using an ultrasensitive cytokine secretion assay in patients with active tuberculosis before, during, and after their antituberculosis treatment.

Materials and Methods

Participants

Twenty-three consenting adult patients with active tuberculosis were prospectively recruited at Birmingham Heartlands Hospital (Birmingham, U.K.) and longitudinally followed up for 28 mo. After obtaining written informed consent, the first heparinized blood sample was drawn before or within the first week of antituberculosis treatment. The demographic data at screening and the clinical data at screening and at all follow-up time points (3, 6, 12, 18, and 28 mo) were recorded on a standard questionnaire by the study research nurse. The 12-mo follow-up blood sample was drawn 11–16 mo following treatment initiation; the 18-mo follow-up blood sample was drawn 17–21 mo following treatment initiation, and the 28-mo sample was drawn 25–32 mo after treatment initiation. None of the patients we followed up reported contact with other tuberculosis cases after their diagnosis. Four additional patients with active tuberculosis were recruited at Birmingham Heartlands Hospital and Northwick Park Hospital (London, U.K.) to study cross-sectionally. Ethical approval was granted by the East Birmingham Local Research Ethics Committee (Birmingham, U.K.), the Harrow Research Ethics Committee (Harrow, U.K.), and the Central Oxford Research Ethics Committee (Oxford, U.K.).

Of the patients tested for HIV infection, all were HIV Ab negative. Of the remaining patients, none had clinical or laboratory features specifically suggestive of HIV infection. A tuberculin skin test (TST) was performed and read by an experienced tuberculosis nurse or doctor using the Mantoux method with 0.1 ml (10 tuberculin units) of purified protein derivative (PPD) (Evans Vaccines). Cutaneous induration was measured after 72 h with a ruler. A response was scored as positive if the diameter of induration was 15 mm or greater irrespective of BCG vaccination status. For logistical reasons, in some cases the Heaf method was used instead with the standard multiple puncture, six-needle, disposable head Heaf gun (Bignall Surgical Instruments) and concentrated PPD (100,000 tuberculin units per ml; Evans Vaccines). Induration was measured 1 wk later as recommended for this application of the TST. A response was scored from grade 0 to grade 4 and grades 3 or 4 were recorded as positive irrespective of BCG vaccination status in accordance with United Kingdom national guidelines.

Control participants with no known history of *M. tuberculosis* exposure were recruited from departmental immunology research laboratories.

Table I. *Demographic and clinical characteristics of patients with active tuberculosis followed longitudinally (n = 23)*

Characteristics	Numerical Data
Demographic characteristics	
Median age in years (range)	35 (17–83)
Female gender (%)	9 (39)
Ethnic origin[a]	
Indian subcontinent	18
Black African	3
Indonesian	1
White	1
Clinical characteristics	
Basis of diagnosis[b]	
M. tuberculosis culture-positive	12
Histological appearance of granulomas	8
Clinical/radiological findings highly suggestive of tuberculosis	3
Site of disease	
Lymphatic	8
Pulmonary	7
Pleural	3
Disseminated[c]	2
Genitourinary	1
Joint	1
Meningeal	1
Tuberculin skin test	
Positive[d]	22
ND	1

[a] All participants were born in their country of ethnic origin except for one Pakistani who was born in the United Kingdom.
[b] Diagnosis is equivalent to American Thoracic Society class 3 (23).
[c] Meningeal and pleural, $n = 1$; pulmonary and colonic, $n = 1$.
[d] Mantoux ≥15 mm, $n = 10$; Heaf grade ≥3, $n = 12$.

Purification of lymphocytes from peripheral blood

Thirty milliliters of blood was available at all of the time points except in active untreated tuberculosis where only 10 ml were available in half of the patients. PBMCs were isolated from heparinized whole blood by Ficoll-Paque PLUS density gradient centrifugation (Amersham Biosciences), washed twice in RPMI 1640 (Sigma-Aldrich), and suspended in R10 (RPMI 1640 (Sigma-Aldrich) supplemented with 1 mM sodium pyruvate (Invitrogen Life Technologies), 2 mM L-glutamine, and 10% heat-inactivated FCS (Sigma-Aldrich)). PBMCs were cryopreserved in 90% FCS (Sigma-Aldrich).

Ex vivo IFN-γ ELISPOT

The in-house ex vivo IFN-γ ELISPOT assays were performed as previously described (20). Briefly, precoated IFN-γ ELISPOT plates (Mabtech) were seeded with 2.5×10^5 PBMCs per well; duplicate wells contained no peptide (negative control), 5 μg/ml PHA (positive control; MP Biomedicals), 20 μg/ml tuberculin PPD (States Serum Institut), 100 U/ml streptokinase, 26 U/ml streptokinase streptodornase (SKSD) (Wyeth Farma), or one of six pools containing 15-mer peptides overlapping by 10 aa (10 μg/ml final concentration of each peptide; Louisiana State University Health Science Center Core Laboratories, New Orleans, LA) spanning the length of ESAT-6 (17 peptides) or CFP-10 (18 peptides) in three pools for each Ag. After 18 h of incubation at 37°C in 5% carbon dioxide, plates were developed with an alkaline phosphatase-conjugated IFN-γ detection Ab (Mabtech) followed by the chromogenic substrate BCIP/NBT Plus (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium; Moss). Spot-forming cells (SFCs) were counted using an automated ELISPOT reader (Autoimmun Diagnostika). Settings for the intensity and size of a counted spot were predefined and the same settings were used throughout. Responses were scored as positive if the test wells contained a mean of at least five SFCs more than the mean of the negative control wells and was at least twice the mean of the negative control wells. This threshold is similar to that used in the commercially available assay T-SPOT. *TB*, which is based on our ELISPOT assay system and uses a threshold of six SFCs more than the negative control when the negative control has no more than five SFCs. Positive ESAT-6 and CFP-10 peptide pool responses were summated for each participant to give the ESAT-6/CFP-10 response.

Ex vivo IL-2 ELISPOT

The ex vivo IL-2 ELISPOT was performed in the same manner as the ex vivo IFN-γ ELISPOT with the following differences. The membrane of a MAIPS4510 plate (Millipore) was prewet with 50 μl of 70% ethanol per well for 2 min at room temperature. Wells were washed five times with 200 ml/well sterile water and incubated overnight at 4°C with 10 μg/ml coating IL-2 Ab (Mabtech). Wells were washed five times with 200 ml/well sterile PBS (Sigma-Aldrich) and blocked for 30 min at room temperature with R10. After incubation with stimulus for 18 h (see above), plates were developed with 1 μg/ml biotin-conjugated IL-2 detection Ab (Mabtech) for 2 h at room temperature and then streptavidin-ALP (Mabtech) diluted 1/1000 in PBS for 1 h at room temperature followed by the chromogenic substrate BCIP/NBT Plus (Moss). SFCs were counted and interpreted as described above. Settings for the intensity and size of a counted spot were predefined and the same settings were used throughout.

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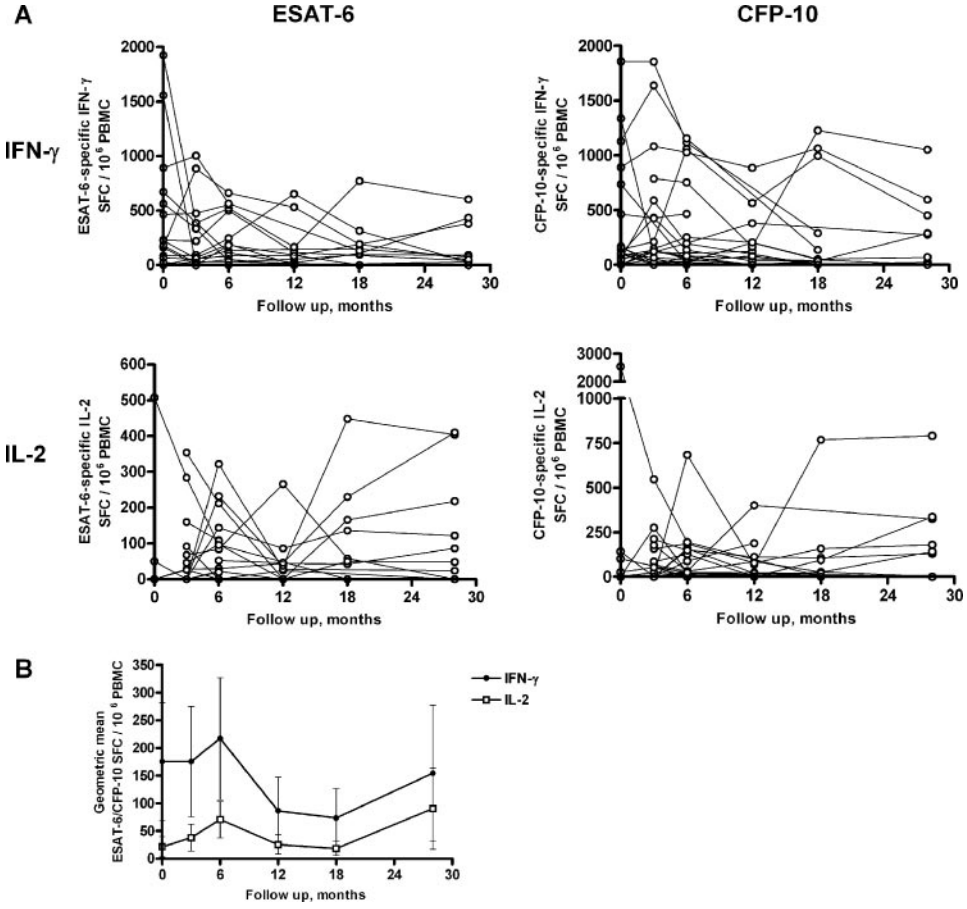

FIGURE 1. Frequencies of ESAT-6- and CFP-10-specific IFN-γ- and IL-2-secreting T cells in 23 patients with active tuberculosis followed up for 28 mo during and after successful treatment. *A*, Frequencies of ESAT-6- and CFP-10-specific IFN-γ- and IL-2-secreting T cells were enumerated by ex vivo ELISPOT at 0, 3, 6, 12, 18, and 28 mo after diagnosis of active tuberculosis disease. *B*, Geometric means of ESAT-6/CFP-10-specific IFN-γ and IL-2 SFCs.

Ex vivo detection and magnetic bead enrichment of IFN-γ and IL-2 single and dual secreting Ag-specific cells

PBMCs (5×10^6) in a 48-well plate were rested at 37°C in 5% carbon dioxide overnight. Media alone, 1 μg/ml staphylococcal enterotoxin B (positive control; Sigma-Aldrich), or 10 μg/ml peptide pool (ESAT-6 or CFP-10) were added to the cells for 5 h. Cells were transferred into a 15-ml Falcon tube and washed with MACS buffer (PBS, 0.5% BSA, and 2 mM EDTA (Sigma-Aldrich)). After centrifugation at 1400 rpm for 10 min at 4°C, the cell pellet was resuspended in 80 μl of cold RH5 (RPMI 1640 supplemented with 5% heat-inactivated human AB serum (Blood Transfusion Service; Bristol, U.K.)). Ten microliters of IFN-γ catch reagent and 10 μl of IL-2 catch reagent (Miltenyi Biotec) were added per test condition and incubated on ice for 5 min. Five milliliters of warm RH5 was added and cells were slowly rotated for 45 min at 37°C in 5% carbon dioxide. An equal volume of cold MACS buffer was added and incubated on ice for 10 min. Cells were washed with MACS buffer and after centrifugation the cell pellet was resuspended in 80 μl of cold MACS buffer and stained with 10 μl of allophycocyanin-conjugated IFN-γ-specific detection Ab, 10 μl of PE-conjugated IL-2-specific detection Ab (Miltenyi Biotec), 5 μl of FITC-conjugated anti-$CD4^+$ Ab (eBioscience), 4 μl of PerCP-conjugated anti-CD14, and 4 μl of PerCP-conjugated anti-CD19 (BD Pharmingen) for 15 min on ice.

Table II. *Trends of ESAT-6, CFP-10, ESAT-6/CFP-10, and SJSD-specific IFN-γ-secreting T cells during follow-up*

	Geometric Mean Counts of SFCs (95% CI)[a]			Tested for Trend	Geometric Mean Counts of SFCs (95% CI)[a]			Tested for Trend
	0 mo	3 mo	6 mo	0–6 mo	12 mo	18 mo	28 mo	0–28 mo
ESAT-6								
IFN-γ	24 (6–98) **23**	11 (3–44) **22**	21 (6–81) **22**	$p = 0.77$	8 (2–40) **16**	8 (2–40) **17**	27 (5–145) **12**	$p = 0.04$
IL-2	2 (0–9) **10**	5 (2–20) **19**	11 (3–37) **21**	$p = 0.06$	4 (1–14) **16**	5 (1–20) **17**	12 (2–81) **12**	$p = 0.83$
CFP-10								
IFN-γ	60 (18–208) **23**	70 (21–227) **22**	76 (26–220) **22**	$p = 0.75$	1 (1–1) **16**	18 (4–85) **17**	18 (2–149) **12**	$p = 0.002$
IL-2	5 (1–53) **10**	11 (3–43) **19**	30 (12–75) **21**	$p = 0.06$	6 (2–27) **16**	5 (1–20) **17**	11 (1–92) **12**	$p = 0.41$
ESAT-6/CFP-10								
IFN-γ	111 (32–384) **23**	119 (38–370) **22**	161 (60–430) **22**	$p = 0.57$	44 (9–206) **16**	36 (7–178) **17**	57 (8–399) **12**	$p = 0.005$
IL-2	5 (1–58) **10**	22 (6–86) **19**	55 (23–134) **21**	$p = 0.02$	14 (3–60) **16**	9 (2–44) **17**	31 (4–237) **12**	$p = 0.72$
SKSD								
IFN-γ	19 (8–44) **21**	22 (12–41) **22**	25 (12–51) **22**	$p = 0.26$	28 (10–81) **16**	23 (10–51) **17**	19 (7–54) **12**	$p = 0.73$
IL-2	89 (0–8, 250, 569) **2**	24 (12–46) **17**	28 (13–61) **18**	$p = 0.85$	34 (14–85) **14**	35 (14–89) **13**	49 (25–95) **12**	$p = 0.29$

[a] Boldfaced type indicates the number of patients (*n*).

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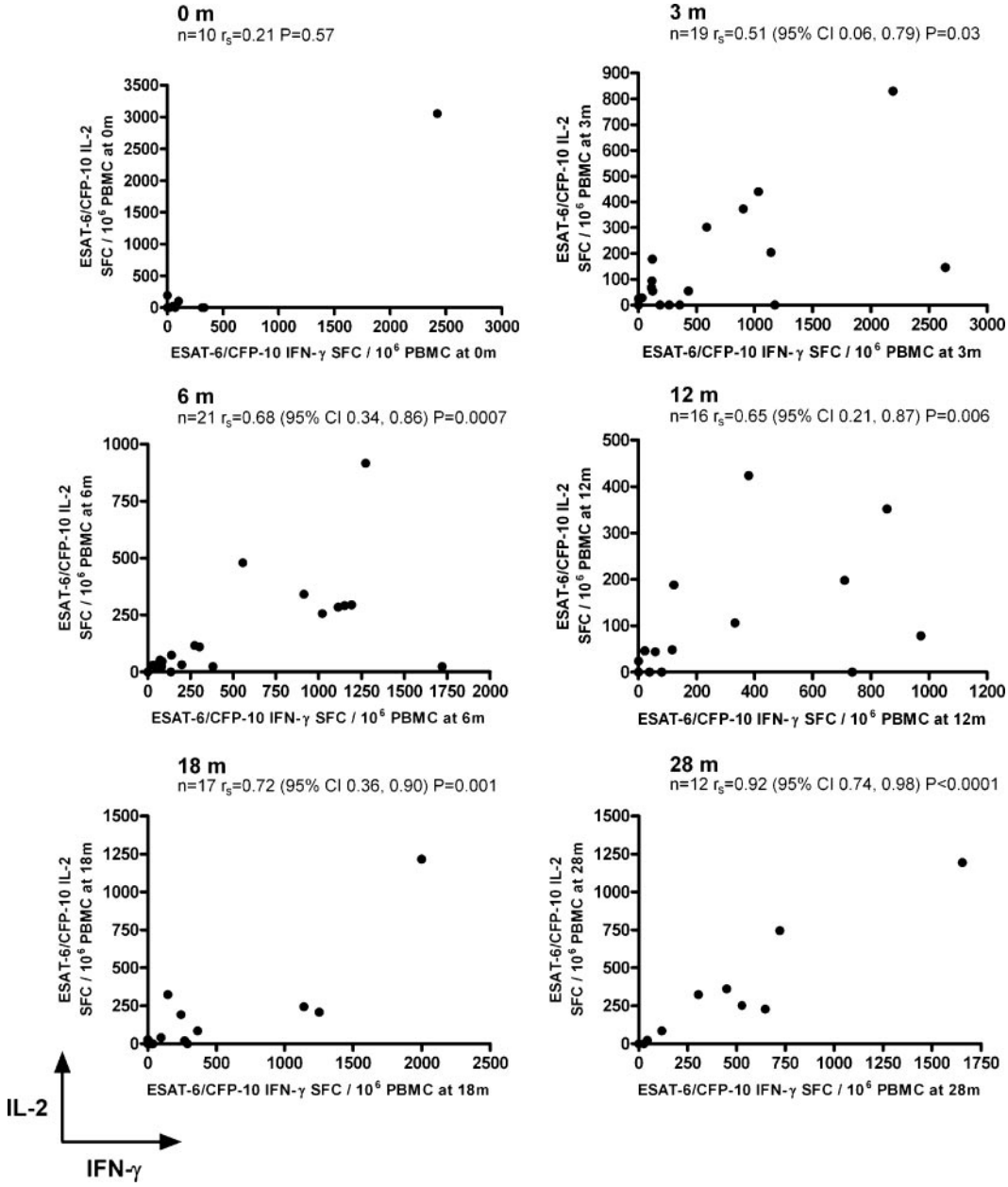

FIGURE 2. Correlation between ESAT-6/CFP-10-specific IFN-γ- and IL-2-secreting T cells measured separately with the ex vivo IFN-γ and IL-2 ELISPOT assays at 0, 3, 6, 12, 18, and 28 mo of follow-up. There is an increasing positive association between the frequency of T cells secreting IFN-γ and the frequency of T cells secreting IL-2 at corresponding time points during the follow-up.

Cells were washed with MACS buffer and cell pellets were resuspended in 80 μl of cold MACS buffer. Superparamagnetic microbeads (20 μl) conjugated to monoclonal mouse anti-PE and anti-allophycocyanin Abs (Miltenyi Biotec) were added and incubated for 15 min at 4°C to 8°C. Cells were washed in cold MACS buffer and 10% of the cell sample was removed for pre-enrichment flow cytometric analysis. The remaining cells were positively selected by passing through two MS^+ columns (Miltenyi Biotec). Cells bound to microbeads are uniformly retained in the column once a certain threshold of bound microbeads has been reached. Dead cells were excluded by staining with ViaProbe 7-aminoactinomycin D (BD Pharmingen) just before acquisition. Flow cytometric acquisition was performed on a FACSCalibur device and analysis was performed using FlowJo version 6.1 software (Tree Star). The frequency of $CD4^+$ T cells secreting IFN-γ and/or IL-2 was calculated by dividing the number of postenrichment $CD4^+$ T cells secreting IFN-γ and/or IL-2 by the number of $CD4^+$ T cells in the pre-enrichment sample multiplied by 9 (to account for the fact that 90% of the cells were used for the enrichment).

Statistical analysis

Average ESAT-6/CFP-10-, ESAT-6-, CFP-10-, and SKSD-specific T cell responses were summarized as geometric mean counts of SFCs expressed together with 95% confidence intervals (CI). Rates of change in geometric means over the 28 mo of follow-up were estimated by fitting a linear regression model allowing for repeated measurements on each individual and the significance of change was estimated (21). Because the estimation was undertaken on the log scale, the exponentiated parameter estimates can be interpreted as symmetrical percentage changes (22) and are standardized to change per year. Comparisons of T cell responses between contacts followed for 28 mo and contacts followed up for <28 mo and between ELISPOT-positive and ELISPOT-negative contacts at 28 mo were compared using *t* tests on log counts. Spearman's rank correlation coefficient was used to test the association between frequencies of IFN-γ- and IL-2-secreting ESAT-6/CFP-10-specific T cells at corresponding time points. Differences in proportions were compared using the χ^2 test. Comparisons of IFN-γ, IFN-γ/IL-2, and IL-2 secretion between active tuberculosis and

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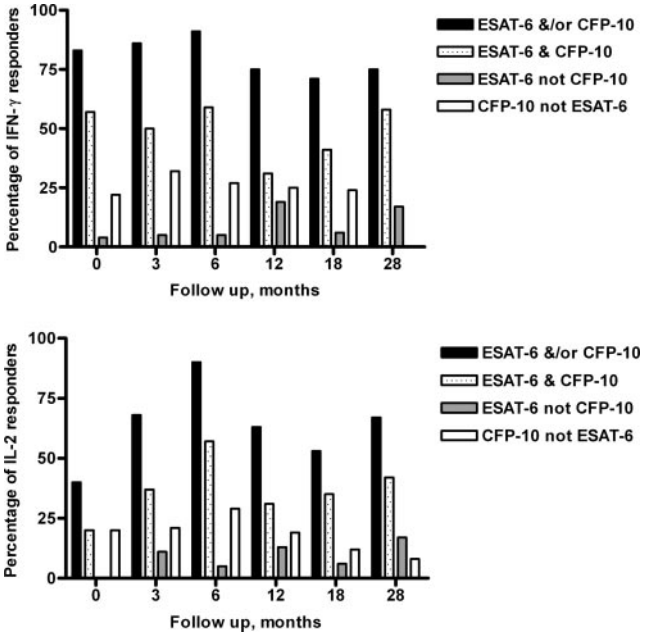

FIGURE 3. Proportion of patients with IFN-γ- or IL-2-secreting T cells as defined by a positive ex vivo ELISPOT response in active tuberculosis and during follow-up. The percentages of patients that responded to ESAT-6 and/or CFP-10, ESAT-6 and CFP-10, ESAT-6 alone, or CFP-10 alone are shown.

during and after treatment were tested using the Mann-Whitney *U* test. $p < 0.05$ was considered significant. Analyses were performed using Stata version 9.2.

Results

Demographic and clinical characteristics of patients with active tuberculosis and control participants

Demographic and clinical characteristics of the 23 patients with active tuberculosis followed up are summarized in Table I. All participants had clinical and radiological findings consistent with active tuberculosis (American Thoracic Society class 3 (23)). Diagnosis was confirmed by bacteriological isolation of *M. tuberculosis* in 12 participants and a further eight cases had histological appearances of granulomas and clinical and radiological findings strongly suggestive of tuberculosis. Three participants were classified as having highly probable tuberculosis on the basis of clinical and radiological features highly suggestive of tuberculosis that were unlikely to be caused by another disease, and a decision was made by the attending physician to initiate antituberculosis chemotherapy, which resulted in an appropriate response to therapy. The site of disease in the 23 participants represented a broad clinical spectrum (Table I). All participants were treated in accordance with British Thoracic Society guidelines and received therapy for 6 mo or, in the case of two patients with tuberculosis meningitis and one patient with joint tuberculosis, therapy for 12 mo (24). Treatment was successful in all participants as evidenced by no clinical or radiographic evidence of current disease, the completion of antituberculosis chemotherapy, and sterile mycobacterial cultures. Nine participants were followed up for six time points, five participants for five time points, six participants for four time points, and three participants for three time points of the study. IFN-γ and IL-2 ELISPOT assays and clinical assessments were performed at each of these time points. However, it was only possible to do IL-2 ELISPOT assays in ~50% of patients at the first study time point when active tuberculosis was diagnosed because of limited blood sample volume (see *Materials and Methods*). One participant died during the study but cause of death was attributed to cancer and not tuberculosis; otherwise missed follow-up time points were for logistical reasons only.

The four active tuberculosis patients studied cross-sectionally at the pretreatment time point only had a median age of 29 (range 21–62) and all were male. Three patients were of African and one was of Indian ethnicity and birth. All four had smear and culture-confirmed pulmonary tuberculosis. Three were TST positive (20-, 22-, and 24-mm induration) and two were BCG-vaccinated.

The 13 control participants were healthy laboratory personnel from regions with a low prevalence of tuberculosis (United Kingdom, France, Canada, Israel, and New Zealand) and no known exposure to *M. tuberculosis*. Eleven were Caucasian and two were of Indian ethnicity. The median age was 29 years (range 20–47), eight were female, and 11 were BCG vaccinated. All tested negative by the ESAT-6/CFP-10-specific IFN-γ ELISPOT assay, indicating the absence of *M. tuberculosis* infection (20, 25, 26).

Frequencies of ESAT-6 and CFP-10-specific IFN-γ- and IL-2-secreting T cells during follow-up

Frequencies of ESAT-6- and CFP-10-specific IFN-γ- and IL-2-secreting T cells for each participant are shown in Fig. 1*A*. The frequencies of ESAT-6/CFP-10-specific IFN-γ-secreting T cells declined during 28 mo of follow-up with an average percentage decline per year of 5.8% (95% CI, −9.6 to −1.7%, $p = 0.005$; Table II). The frequencies of IFN-γ-secreting T cells specific for CFP-10 declined more during 28 mo of follow-up than the frequencies of IFN-γ-secreting T cells specific for ESAT-6 (an average decline of 8.2% (95% CI, −13.0% to −3.1%) $p = 0.02$ compared with 4.1% (95% CI, −7.8 to −0.2%, $p = 0.04$; Table II). Four patients were ex vivo ESAT-6/CFP-10-specific, IFN-γ ELISPOT-negative when diagnosed with active tuberculosis. Excluding these four patients, the frequencies of ESAT-6/CFP-10 peptide-specific IFN-γ-secreting T cells declined during 28 mo of follow-up with an average percentage decline per year of 8.1% (95% CI, −11.6% to −4.4%, $p < 0.0001$, $n = 19$). Cases followed up for 28 mo ($n = 12$) and cases not followed up for 28 mo ($n = 11$) showed no significant difference in the initial frequencies of ESAT-6/CFP-10-specific IFN-γ-secreting T cells (0 mo, geometric means (95% CI) SFC/10^6 PBMC 142 (21, 956) vs 84 (12, 575), respectively; $p = 0.67$).

Frequencies of ESAT-6 and CFP-10-specific IL-2-secreting T cells were generally lower than IFN-γ-secreting T cells (Fig. 1, *A* and *B*) and showed a significant increase during treatment over 6 mo ($p = 0.02$; Table II).

The pattern in the geometric mean frequencies of T cells secreting IFN-γ and IL-2 mirrored each other and became progressively closer to each other from 12 mo onwards (Fig. 1*B*). In addition, there was a statistically significant positive correlation between the frequencies of IFN-γ-secreting and IL-2-secreting T cells after 3 mo of treatment that became progressively stronger over time (Fig. 2).

To determine whether the changes in the *M. tuberculosis*-specific T cell response during follow-up were reflective of *M. tuberculosis*-specific immunity and not of generalized fluctuations in T cell recall response, we quantified the frequencies of IFN-γ-secreting and IL-2-secreting T cells specific for the nontuberculosis Ag SKSD during follow-up. There was no significant trend in frequencies of SKSD-specific IFN-γ-secreting or IL-2-secreting T cells during follow-up (Table II).

ESAT-6 and CFP-10-specific IFN-γ-secreting T cell responses have previously been demonstrated to be *M. tuberculosis* specific because they are absent in unexposed controls (15, 16, 25–27). In

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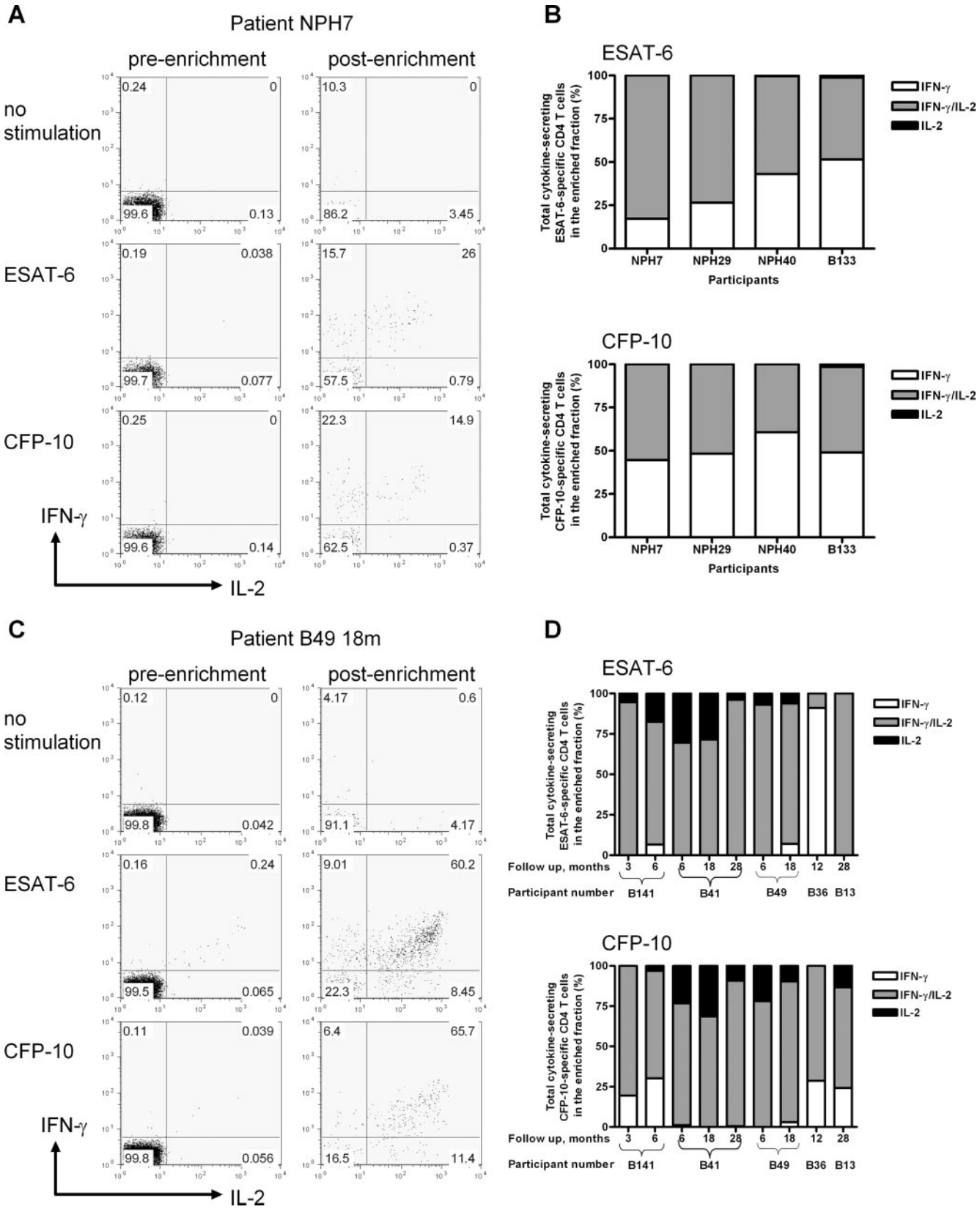

FIGURE 4. The IFN-γ and IL-2 cytokine profile of $CD4^+$ T cells in active tuberculosis and during and after antituberculosis therapy. Representative dot plots from an active tuberculosis patient (*A*) and a different patient 18 mo after initiation of treatment (*C*). The percentages plotted indicate the relative proportions of $CD4^+$ cells producing IFN-γ and/or IL-2 in the enriched fraction with background levels of nonspecific cytokine production subtracted in active tuberculosis (*B*) and during follow-up (*D*). ESAT-6- and CFP-10-specific IFN-γ- and IFN-γ/IL-2-secreting $CD4^+$ T cells codominated in active tuberculosis when bacterial load was high, whereas IFN-γ/IL-2-secreting T cells dominated during and after treatment when the viable bacterial load was reduced or cleared with the loss of IFN-γ-only secreting $CD4^+$ T cells and the appearance of IL-2-secreting $CD4^+$ T cells. These data were reproducibly observed in four active tuberculosis cases pretreatment and in five patients at nine follow-up time points in total during and after their treatment. The four active tuberculosis cases and three follow-up patients had pulmonary disease and the remaining follow-up patients had lymphatic disease.

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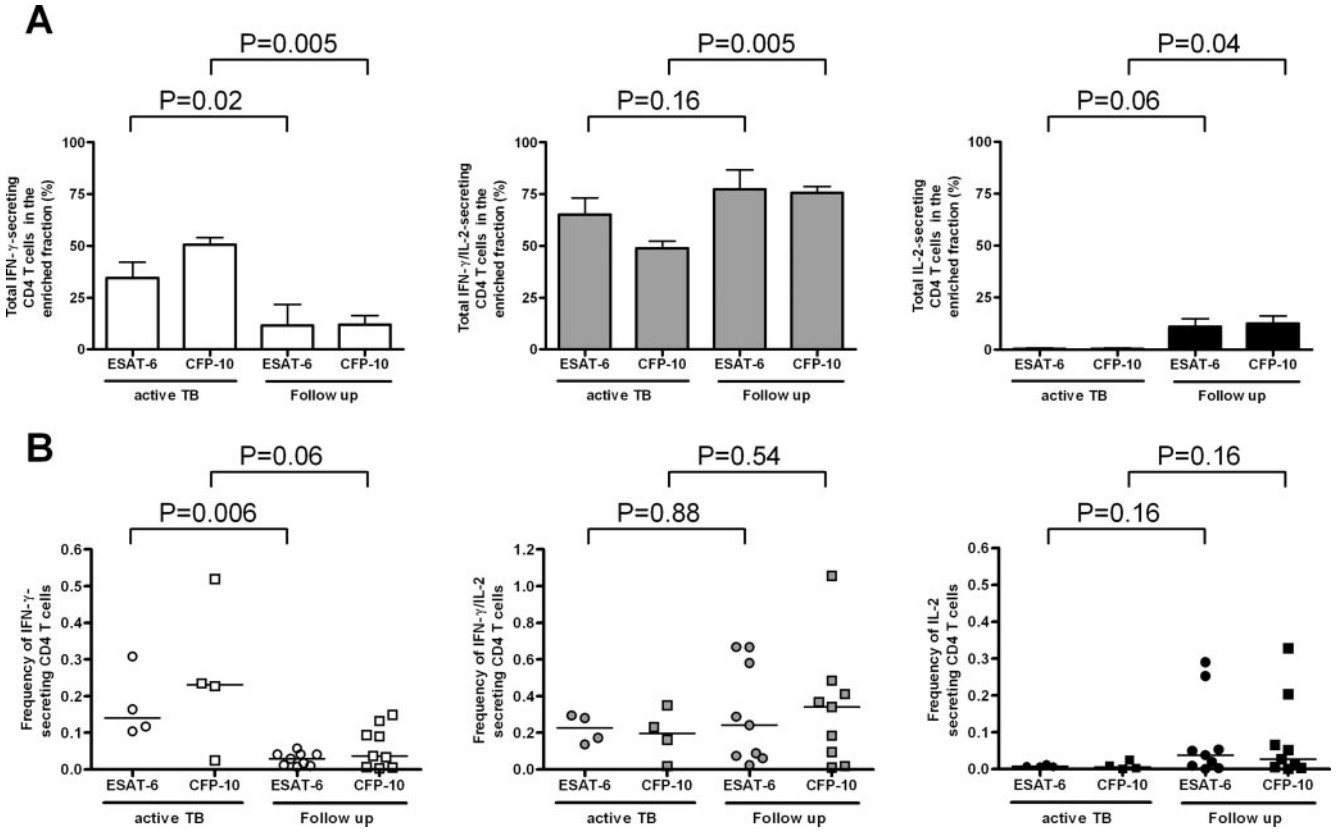

FIGURE 5. The proportion and frequency of ESAT-6- and CFP-10-specific $CD4^+$ T cell IFN-γ and/or IL-2 responses in active tuberculosis compared with follow-up time points (during and after treatment). Shown are the proportions of ESAT-6- and CFP-10-specific $CD4^+$ T cells secreting IFN-γ and/or IL-2 in the enriched fraction (*A*) and the frequencies of these cells as determined after magnetic enrichment of IFN-γ and IL-2-secreting cells (*B*), where the horizontal lines indicate the median, positive response. The *p* values indicate whether there is a statistical difference between active tuberculosis and follow-up.

this study we observed that ESAT-6- and CFP-10-specific IFN-γ-secreting and IL-2-secreting T cell responses are *M. tuberculosis* specific, as none of the 13 healthy controls responded to these peptides in either the ex vivo IFN-γ ELISPOT or in the IL-2 ELISPOT (all responses were <20 SFCs per 10^6 PBMCs; data not shown).

Proportion of patients with IFN-γ and IL-2 responses to ESAT-6 vs CFP-10 in active tuberculosis and during follow-up

Four patients (pulmonary, $n = 2$; pleural, $n = 1$; and lymphatic, $n = 1$) were ESAT-6/CFP-10 IFN-γ ELISPOT-negative when diagnosed with active tuberculosis but became positive in each case 3 mo into treatment (Fig. 3). Four patients turned ESAT-6/CFP-10 IFN-γ ELISPOT-negative (at 3 mo, $n = 2$; at 6 mo, $n = 1$; and at 18 mo, $n = 1$), i.e., the frequency of ESAT-6- and CFP-10-specific T cells decreased to levels below 20 SFCs per 10^6 PBMCs, which we defined as the threshold of detection of Ag-specific T cells by the ex vivo ELISPOT, and they remained ELISPOT-negative for at least two consecutive time points and during the rest of their follow-up. We also used the cytokine secretion assay with enrichment of IFN-γ- and IL-2-secreting cells in three of the four participants at time points when the ex vivo IFN-γ ELISPOT had turned negative. No $CD4^+$ T cells specific for ESAT-6 or CFP-10 secreting either of these cytokines could be detected (data not shown). Two participants became ESAT-6/CFP-10 peptide IFN-γ ELISPOT-negative at 3 and 12 mo, respectively, but became IFN-γ ELISPOT-positive again 3 to 6 mo later. One participant turned ESAT-6/CFP-10 peptide IFN-γ ELISPOT-negative at 18 mo but did not attend the 28-mo time point.

Most patients were ex vivo ESAT-6/CFP-10 IFN-γ ELISPOT-positive when diagnosed with active tuberculosis and during their follow-up (Fig. 3). Only 40% of patients with active tuberculosis were ex vivo ESAT-6/CFP-10 IL-2 ELISPOT-positive at diagnosis; however, the proportion of ex vivo ESAT-6/CFP-10 IL-2 ELISPOT-positive patients significantly increased to 90% after 6 mo of treatment ($p = 0.01$). In general, more patients responded to CFP-10 alone in the IFN-γ and IL-2 ELISPOT assays than to ESAT-6 alone during the first 6 mo after treatment initiation; thereafter, the proportions of patients responding either to CFP-10 or ESAT-6 alone were similar (Fig. 3).

Sensitive detection of $CD4^+$ T cells secreting IFN-γ, IL-2 or both cytokines at the single-cell level

Because the lower limit of detection of conventional flow cytometric techniques is ~0.02 to 0.05% (28, 29), the detection of IFN-γ and IL-2 at the single-cell level using these techniques would be difficult in several of our patients in whom the frequencies of Ag-specific IFN-γ- or IL-2-secreting T cells were <0.05%. However, the cytokine secretion assay with magnetic bead enrichment of cytokine-secreting cells has demonstrated reliable detection of the ultra-low frequencies of cytokine secreting T cells (13, 30, 31). Because this technique specifically enriches for cytokine-secreting cells of interest, it reduces background staining. We know from our previously published work that T cells responding to ESAT-6 and CFP-10 are predominantly $CD4^+$ (16, 25, 32); thus, although $CD4^-$ T cells also contribute to the *M. tuberculosis*-specific IFN-γ response, we gated on live $CD4^+$ lymphocytes.

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To define an appropriate lower limit of detection for this assay in the tuberculosis model, we assessed the background level of the staining of $CD4^+$ T cells secreting IFN-γ and IL-2 following magnetic bead enrichment. We recruited seven healthy unexposed controls and stimulated their PBMCs with ESAT-6 and CFP-10 peptides. The median frequency of ESAT-6-specific $CD4^+$ T cells secreting IFN-γ was 0% (range 0–0.009), 0.0005% for those secreting IFN-γ/IL-2 (range 0–0.002), and 0% for those secreting IL-2 (range 0–0.001). The median frequency of CFP-10-specific $CD4^+$ T cells secreting IFN-γ was 0% (range 0–0.007), 0% for those secreting IFN-γ/IL-2 (range 0–0.002), and 0.001% for those secreting IL-2 (range 0–0.005). Based on the distribution of these data compared with the distribution of frequencies detected in the four active tuberculosis cases, we defined the lower limit of detection of ESAT-6 and CFP-10-specific $CD4^+$ T cells using magnetic bead enrichment as 0.007% of $CD4^+$ T cells.

PBMC from four untreated patients with active tuberculosis were stimulated for 5 h with either ESAT-6 peptides or CFP-10 peptides. After cell surface capture of secreted IFN-γ and/or IL-2, cells were magnetically enriched for IFN-γ and/or IL-2-positive cells. Representative dot plots are shown in Fig. 4*A*. The relative proportions of $CD4^+$ T cells producing IFN-γ and/or IL-2 in the postenrichment fraction with background levels of nonspecific cytokine production subtracted in all four active tuberculosis cases are shown in Fig. 4*B*. $CD4^+$ T cells secreting IFN-γ-only and IFN-γ/IL-2 codominated the response to both ESAT-6 and CFP-10. Nine follow-up samples from five patients 3 mo into treatment and thereafter are shown in Fig. 4, *C* and *D*. $CD4^+$ T cells secreting IFN-γ/IL-2 dominated the response to both ESAT-6 and CFP-10 with a substantial decrease in IFN-γ-only secreting $CD4^+$ T cells and the appearance of IL-2-only secreting $CD4^+$ T cells.

Quantitative comparison of functional T cell subsets before and after the initiation of treatment

We compared the relative proportions and frequencies of ESAT-6 and CFP-10-specific IFN-γ-, IFN-γ/IL-2-, and IL-2-secreting $CD4^+$ T cells between samples from active tuberculosis patients before the initiation of treatment and samples from the patients after the initiation of treatment (i.e., after at least 3 mo of treatment). The relative proportions of ESAT-6- and CFP-10-specific $CD4^+$ T cells secreting IFN-γ only were significantly higher in untreated active tuberculosis compared with follow-up during and after treatment ($p = 0.02$ and $p = 0.005$, respectively; Fig. 5*A*). In contrast, the relative proportion of ESAT-6- and CFP-10-specific $CD4^+$ T cells secreting IL-2 only were higher during and after treatment compared with pretreatment ($p = 0.06$ and $p = 0.04$, respectively). The relative proportion of $CD4^+$ T cells secreting both cytokines was significantly higher during follow-up compared with untreated active tuberculosis for CFP-10-specific T cells ($p = 0.005$) and nonsignificantly different for ESAT-6-specific T cells ($p = 0.16$).

Similarly, the absolute frequencies of ESAT-6- and CFP-10-specific IFN-γ-only secreting $CD4^+$ T cells were higher in untreated active tuberculosis compared with those during follow-up ($p = 0.006$ and $p = 0.06$, respectively; Fig. 5*B*), whereas the frequencies of ESAT-6- and CFP-10-specific IL-2-only secreting $CD4^+$ T cells were higher during follow-up compared with pretreatment, although the difference did not reach statistical significance ($p = 0.16$ for each Ag). The frequencies of $CD4^+$ T cells secreting both IFN-γ and IL-2 did not change significantly between active tuberculosis and during follow-up (Fig. 5*B*). Some of the frequencies detected could also have been detected with conventional flow cytometric techniques; however, within every individual tested the frequencies of at least one of the functional subsets were below the detection limit of conventional flow cytometric techniques.

Discussion

The aim of this study was to assess the relationship between the frequency and functional signature of Ag-specific T cells secreting IFN-γ and IL-2 and Ag load in tuberculosis. Our statistical analysis revealed a significant decline in the frequency of ESAT-6/CFP-10-specific T cells secreting IFN-γ over 28 mo and a significant increase in the number of ESAT-6/CFP-10-specific T cells secreting IL-2 during 6 mo of treatment. These contrasting dynamics for the two cytokines led to a progressive convergence of the frequencies of IFN-γ- and IL-2-secreting cells over 28 mo during and after treatment. From simultaneous analysis of IFN-γ and IL-2 secretion at the single-cell level in a subset of our cohort using the ultra-sensitive cytokine secretion assay, we found this was due to a change in the functional signature of *M. tuberculosis*-specific T cells over time. There was a shift in the IFN-γ and IL-2 cytokine profile from a codominance of IFN-γ-only and IFN-γ/IL-2-secreting T cells in active tuberculosis to a dominance of IFN-γ/IL-2-secreting T cells and the appearance of IL-2-only secreting T cells during and after treatment. Thus, IFN-γ measured with the ex vivo ELISPOT is secreted by two functional subsets of $CD4^+$ T cells with different relative proportions in untreated active tuberculosis compared with those during and after treatment.

The decline in ESAT-6- and CFP-10-specific IFN-γ T cell responses ex vivo is consistent with the decline observed during treatment of active tuberculosis in other studies (15–18, 33). However, in our study, which is the longest longitudinal study of Ag-specific T cells in tuberculosis to date, the decline in ESAT-6- and CFP-10-specific IFN-γ-secreting T cells was only statistically significant over 28 mo, whereas in previous studies the decline in ESAT-6- or CFP-10-specific IFN-γ-secreting T cells was significant over 6 or 12 mo using different statistical analyses (Table II) (16, 18, 33). An increase in IFN-γ secretion from cultured PBMCs during antituberculosis therapy has been observed in other studies (34–38) where IFN-γ was measured after several days in vitro culture. These results reflect IFN-γ derived from in vitro Ag-stimulated proliferating memory T cells. The increased IFN-γ production after treatment with these assays likely reflect the fact that Ag-specific lymphoproliferation is inhibited by the nonspecific immunosuppression associated with active, untreated tuberculosis (16). This readout is different from that of the direct ex vivo enumeration of IFN-γ-secreting T cells (18 h) as performed in this study (28, 39–41).

Harari et al. (8) showed that functional T cell heterogeneity is associated with changes in HIV Ag load, and we have now demonstrated that functional T cell heterogeneity is also associated with changes in *M. tuberculosis* Ag load. In active disease IFN-γ is secreted from two functional subsets of IFN-γ-only and IFN-γ/IL-2 dual secreting T cells, whereas after treatment IFN-γ is predominantly secreted from one subset of IFN-γ/IL-2 dual secreting T cells. These observations would not have been possible from the IFN-γ and IL-2 ELISPOT assays alone. It is possible that IFN-γ/IL-2 functional profiles might correlate with specific clinical parameters such as disease severity or the anatomical site of the disease. However, the small number of patients within each clinical subgroup precluded statistically meaningful comparisons, which would require a larger study population. Although our data are from cases of active tuberculosis not paired with follow-up samples, the demographic and clinical characteristics of the untreated patients and the patients tested during and after treatment were similar.

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The IFN-γ and IL-2 profile after treatment is similar to a model of Ag clearance, past influenza infection (13). The model suggests that after antituberculosis treatment viable bacilli and Ag may be cleared. This is consistent with the clinical observation that <10% of tuberculosis patients relapse within the first year after completing antituberculosis treatment (42), and none of the patients we studied relapsed during 28 mo of follow-up. Interestingly in a different model of Ag clearance where infection with live organisms is not involved, past tetanus toxoid vaccination, IL-2-only secreting T cells persisted years after vaccination with minimal levels of IFN-γ/IL-2 dual secreting T cells. Although detailed phenotyping of *M. tuberculosis*-specific IFN-γ- and IL-2-secreting T cells was beyond the scope of this study, previous studies have identified a relationship between the function and phenotype of memory $CD4^+$ T cells and proposed that the IL-2-only secreting cells are typical of T_{CM} that persist after Ag clearance while the IFN-γ/IL-2- and IFN-γ-only secreting T cells are typical of T_{EM} (6, 8, 43).

IFN-γ is an important mediator of macrophage activation and resistance to *M. tuberculosis* infection and is therefore crucial in the effector response to this intracellular pathogen (44–46). The paradoxical functions of IL-2 could explain why IL-2 is secreted from cells also secreting IFN-γ in both the early and the later stages of infection and why IL-2 is secreted when the Ag load has declined or been cleared by treatment (47). Dual IFN-γ/IL-2-secreting cells can support their own expansion because IL-2 is a potent T cell growth factor. The presence of these cells in active tuberculosis when the Ag load is high may therefore suggest their involvement in the initiation phase of the immune response through the expansion of effector cells that may further differentiate into IFN-γ-only secreting cells. The relative increase in the proportion of $CD4^+$ T cells secreting both cytokines during and after treatment may reflect the maintenance of a stable effector response. The secretion of IL-2 when the Ag load is reduced or cleared may reflect its function in the termination of T cell responses. This proposed signaling function augments the growth and survival of regulatory T cells that control inflammatory responses (48). Regulatory T cells express high levels of CD25, the IL-2 receptor α-chain, and have recently been described in active tuberculosis where they suppressed IFN-γ-secreting ESAT-6- and CFP-10-specific T cells ex vivo (49).

As a quantitative measure of T cell function, the IFN-γ ELISPOT assay for diagnosis of *M. tuberculosis* infection (15, 16, 20, 25, 26, 50) holds promise as a tool for tracking Ag load and monitoring disease activity (16, 17, 40). But T cell function defined solely by the quantification of IFN-γ secretion may prove to be an insufficient biomarker of Ag load and clinical disease status, and other measures of T cell function will probably be required in addition (14). Through the simultaneous measurement of IFN-γ and IL-2 secretion, we noted a shift in the cytokine profile of *M. tuberculosis*-specific T cells associated with the treatment-induced change from high to low Ag load. Our data thus suggest that IFN-γ and IL-2 functional signatures are associated with Ag load and clinical disease status.

We propose that this dynamic functional signature could be used as an immunological marker of mycobacterial load. The existing clinical, radiological, and microbiological parameters used to monitor the response to treatment in active tuberculosis have several limitations. The IFN-γ and IL-2 functional signature could be used to evaluate new therapies for active tuberculosis in clinical trials where new biomarkers are urgently needed. In latent tuberculosis infection for which there are no clinical, radiological, or microbiological parameters for assessing the response to therapy, the IFN-γ and IL-2 functional signature could be used to monitor the impact of conventional and novel preventive treatments as well as new vaccines. The IFN-γ and IL-2 functional signature could also be used to monitor individuals infected with *M. tuberculosis* at a high risk of progression to active tuberculosis, e.g., patients with HIV coinfection or on anti-TNF therapy, to guide the early initiation of treatment as Ag load increases before clinical reactivation. The question of whether such changes in the *M. tuberculosis* Ag-specific T cell functional signature predict specific clinical outcomes is the subject of our ongoing prospective studies.

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Disclosures

Ajit Lalvani is named inventor for several patents underpinning T cell-based diagnosis filed by the University of Oxford since 1996. Regulatory approval and commercialization of the Lavani ELISPOT test (T-SPOT.*TB*) has been undertaken by a University of Oxford spin-out company (Oxford Immunotec), in which Ajit Lalvani has a share of equity and to which he acts as scientific advisor in a nonexecutive capacity. The University of Oxford has a share of equity in Oxford Immunotec.

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