Dynamic Antigen-specific T-Cell Responses after Point-Source Exposure to *Mycobacterium tuberculosis*

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Rationale: **The kinetics of *Mycobacterium tuberculosis*–specific Th1-type T-cell responses after *M. tuberculosis* infection are likely to be important in determining clinical outcome.**

Objective: **To investigate the kinetics of T-cell responses, in the context of a point-source school tuberculosis outbreak, in three groups of contacts who differed by preventive treatment status and tuberculin skin test (TST) results: 38 treated TST-positive students, 11 untreated TST-positive staff, and 14 untreated students with negative or borderline TST results.**

Methods: **We used the *ex vivo* IFN-γ enzyme-linked immunospot assay (ELISpot) to track T cells specific for two region of difference 1 (RD1) antigens, early secretory antigenic target 6 and culture filtrate protein 10, for 18 mo after cessation of tuberculosis exposure.**

Main Results: **The treated TST-positive students had an average 68% decline in frequencies of RD1-specific IFN-γ–secreting T cells per year ($p < 0.0001$) and 6 of 38 students had no detectable RD1-specific T cells by 18 mo. No change in frequencies of these cells was observed in the untreated TST-positive staff ($p = 0.38$) and none were ELISpot-negative at 18 mo. Of the 14 untreated students, 7 were persistently ELISpot-positive (all of whom had borderline TST results), and 7 became ELISpot-negative (all but one had negative TST results) during follow-up.**

Conclusions: **The decrease in *M. tuberculosis*–specific T cells and their disappearance in a proportion of treated students likely reflect declining antigenic and bacterial load *in vivo* induced by antibiotic treatment. The observed disappearance of *M. tuberculosis*–specific T cells in the untreated TST-negative contacts suggests that an acute resolving infection may occur in some contacts.**

Keywords: IFN-γ ELISpot; infection; tuberculosis

Th1-type T cells are essential for protection against *Mycobacterium tuberculosis* and for maintenance of long-term immune control and clinical latency. The kinetics and evolution of *M. tuberculosis*–specific Th1-type T cells after *M. tuberculosis* infection are likely to be important in determining the equilibrium between host and pathogen, and subsequent clinical outcome, yet very little is known about the kinetics of antigen-specific T cells in humans after natural infection nor the effect of antibiotic treatment on such responses (1). Studies of human T-cell responses to *M. tuberculosis* have been largely limited to patients with tuberculosis (TB), who had already progressed to active disease at the time of study, and individuals with long-standing latent TB infection (LTBI). T-cell responses have been analyzed longitudinally after *Mycobacterium bovis* bacille Calmette-Guérin (BCG) vaccination (2), but longitudinal tracking of antigen-specific T cells after recent TB exposure has not previously been performed. In contrast, for several viruses, antigen-specific T cells have been tracked after recent infection, and these studies have significantly advanced our understanding of how long-term immune control for chronic viral infections is established (3, 4). We have previously described a large point-source TB outbreak at a school in the United Kingdom where the prolonged infectious period of the source case was known. Detailed conventional and molecular epidemiologic investigations confirmed that the 254 cases of LTBI and 69 secondary cases of active TB resulted from exposure to the single, highly infectious source case and that the background prevalence of TB infection in the community was low (5). This presented a unique opportunity to track *M. tuberculosis*–specific T-cell responses longitudinally in a cohort of contacts recently exposed to the same strain of *M. tuberculosis*.

We quantified antigen-specific Th1-type T cells using the *ex vivo* IFN-γ enzyme-linked immunospot (ELISpot) assay, which measures the frequency of T cells in the peripheral circulation with rapid effector function, as opposed to central memory T cells (6–9). We focused on responses to two secreted antigens encoded by the RD1 gene segment of *M. tuberculosis*: early secreted antigenic target protein (ESAT-6) and culture filtrate protein (CFP-10). These antigens are of special interest because they are strong early targets of Th1-type $CD4^+$ T cells after *M. tuberculosis* infection in animal models; they are implicated as targets of protective immune responses; and they may function as virulence factors (10–16). Because RD1 is absent from *M. bovis* BCG and most environmental mycobacteria, ESAT-6– and CFP-10–specific T cells are highly specific for *M. tuberculosis* and are not cross-reactive with antigens expressed by BCG, which allows tracking of *M. tuberculosis*–specific T cells in BCG-vaccinated populations.

We quantitatively followed *M. tuberculosis*–specific T-cell responses longitudinally for 18 mo in recently infected individuals after point-source exposure. The index case was infectious for 9 mo and follow-up of contacts with detectable RD1-specific IFN-γ–secreting T cells at screening began 3 mo after removal of the index case from the school.

Using the *ex vivo* ELISpot, we identified and tracked the *M. tuberculosis*–specific T-cell response in adult members of staff, adolescent students who were tuberculin skin test (TST)–positive, and a newly defined group of TST-negative ELISpot-positive contacts who did not develop a delayed-type hypersensitivity (DTH) response to purified protein derivative (PPD) and so previously would not have been identified. In accordance with U.K. guidelines, adult TST-positive contacts did not receive chemoprophylaxis, whereas TST-positive students did receive chemoprophylaxis.

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The aims of this study were threefold: first, to assess the evolution of effector T-cell responses in recently exposed contacts; second, to compare effector T-cell responses between treated and untreated TST-positive contacts; and third, to compare the natural dynamics of T-cell responses between TST-positive and TST-negative contacts where neither group was treated.

METHODS

Participants

The epidemiologic features of this outbreak and the contact tracing procedures have been previously described (5). All 24 staff and 145 students found to be positive by the *ex vivo* IFN-γ ELISpot at screening were invited to participate in ELISpot testing and clinical review to be repeated at 6 mo intervals over an 18-mo period (Figure 1A). All TST-positive contacts received chest radiography, which was normal in all contacts except five TST-positive students who showed abnormalities suggestive of active TB. These five students, as well as four students with previous household TB contact, were excluded from the longitudinal analysis (Figure 1B). Our final cohort consisted of 11 TST-positive staff, 38 TST-positive students, and 14 TST-negative students, all with exposure to the same index case over a 9-mo period (Figure 1B). None of these participants had any reported contact with TB cases outside the school.

In accordance with U.K. national guidelines, only TST-positive students received a 3-mo course of rifampin and isoniazid prophylaxis (17). Treatment started when each contact was screened with the TST between March and June 2001. Demographic data collected at screening and clinical data collected every 6 mo at venipuncture were recorded on a standard questionnaire by a study nurse. Any further infectious cases of TB at the school and exposure to any infectious TB cases outside of the school during the follow-up period were also recorded. This study was approved by the Leicestershire Research Ethics Committee.

TST

TST was performed on each participant once by the medical and nursing staff of the outbreak management team using the Heaf test in accordance with national guidelines (17). Cutaneous induration was recorded 1 wk later, as recommended for this application of the TST, and was scored from grade 0 to 4 (18). Grades 0 and 1 were scored as negative; grade 2 (equivalent to a Mantoux response of 5 to 14 mm induration after injection of 10 tuberculin units [TU] of PPD) as negative in BCG-vaccinated contacts and positive in BCG-unvaccinated contacts; and grades 3 and 4 (equivalent to a Mantoux response of at least 15 mm induration) as positive (17, 18). TST results for students at the same school before the school outbreak and from four other schools in the same area at the same time as the outbreak were recorded.

Ex Vivo IFN-γ ELISpot

ELISpot assays were performed as previously described (*see* METHODS in the online supplement) (5, 6, 19–21). Responses to ESAT-6 (17 peptides) that grouped into three pools and CFP-10 (18 peptides)

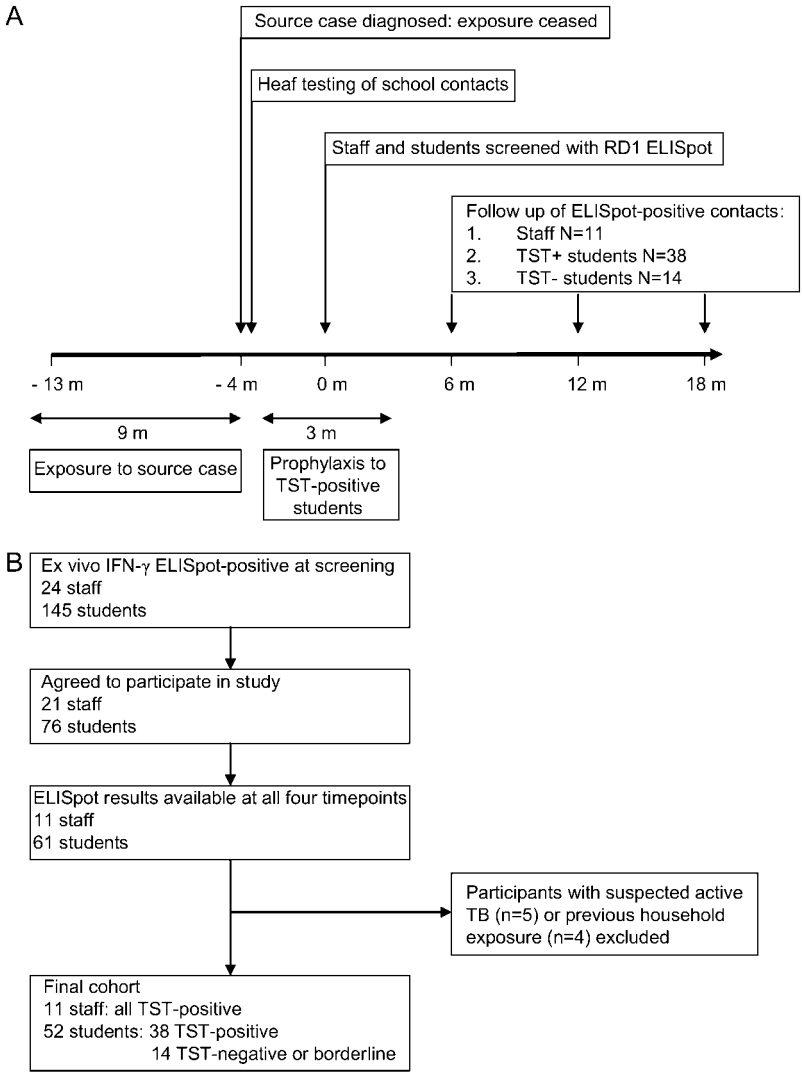

Figure 1. (*A*) Study flow chart of the outbreak and follow-up period. The index case was estimated to be infectious for approximately 9 mo from May 2000 to February 2001 before diagnosis. The index case was then removed from the school and received treatment, ending exposure to contacts at the school. Tuberculin skin testing (TST) began 2 wk later and prophylaxis treatment was given to contacts. Enzyme-linked immunospot assay (ELISpot) testing began in June 2001 and was repeated every 6 mo for 18 mo. (*B*) Study inclusion profile. m = month; TB = tuberculosis.

grouped into three pools were tested. At 6 mo, only those peptide pools to which each participant had responded initially were tested due to logistic constraints, whereas at 12 and 18 mo all six pools were tested. Assays were positive if there was a positive response to at least one of the pools, and positive test wells, with negative control subtracted, were summated to give the total RD1 response. This assay preceded T-SPOT.TB (Oxford Immunotec Ltd, Abingdon, UK) but is very similar to this commercially available test.

Statistical Analysis

Average RD1-specific ESAT-6 and CFP-10 T-cell responses were summarized as geometric mean counts of spot-forming cells (SFCs), expressed together with 95% confidence intervals for each of the participant groups. Rates of change in geometric means were estimated by fitting a linear regression model allowing for repeated measurements on each individual, and the significance of the change was estimated (22). As estimation was undertaken on the log-scale, the exponentiated parameter estimates can be interpreted as symmetrical percentage changes (23), and are standardized to change per year. Trends in the number of ELISpot-positive participants were evaluated using a similar logistic model to produce estimates of odds ratios for the annual change together with tests of statistical significance (22). Differences in the initial T-cell responses between ELISpot-positive and ELISpot-negative contacts at 18 mo within the TST-positive and TST-negative students were compared using *t* tests on log counts and differences in Heaf grades were compared using the χ^2 test for trend (24). Differences in proportions were compared using Fisher's exact test. Breadth of the peptide pool-specific responses was compared within groups using the Mann-Whitney test. All reported p values are two-sided. Analyses were performed using Stata version 9.0 (Stata Corporation, College Station, TX).

RESULTS

Demographic and Clinical Characteristics of Study Participants

The demographics of the 13 staff and 93 students who were *ex vivo* IFN-γ ELISpot-positive at screening and who chose not to take part in the study or were excluded for reasons listed in Figure 1B were similar to those of the 11 staff and 52 students analyzed in this study (*see* RESULTS in the online supplement). For those who did participate, demographic and clinical characteristics for each defined group are shown in Table 1. None of the 63 participants reported previous contact with TB cases outside of the school. Heaf grade results are shown in Tables 1 and 2. The high rates of TB infection at the school resulted from the outbreak and do not reflect the epidemiology of TB in the local community (5). Four other local schools with similar demographic characteristics were screened by TST at the time of the outbreak and the rates of positive skin tests were 1–4%. Moreover, when students at our school were screened 3 yr before the outbreak, only 2.7% were TST-positive. Seventy-three percent of staff were of white ethnicity, whereas the majority of students were of Indian ethnicity. However, similar proportions of staff and students were born in the United Kingdom and were BCG vaccinated. TST-positive students had three times more exposure to the index case than the TST-negative students as quantified from the school timetables (5). Exposure could not be quantified precisely for the staff. No further cases of infectious pulmonary TB occurred in the school during the follow-up period and no study participants had any other known exposure to infectious TB cases outside the school.

Evolution of *M. tuberculosis* Antigen-specific IFN-γ–secreting T Cells in Treated TST-positive Contacts

Thirty-eight students with a positive TST who received chemoprophylaxis showed a significant average decline during 18 mo of follow-up in frequencies of RD1-specific IFN-γ–secreting T cells and T cells specific for ESAT-6 and CFP-10 (Figures 2A and 2D; Table 2). Frequencies of RD1-specific IFN-γ–secreting T cells declined on average by 68% per year (Table 2). Three students had high frequencies of RD1 peptide–specific IFN-γ–secreting T cells at screening, two of which, in addition to a further seven other TST-positive students, showed a transient sizable peak at 6 mo. These high responses were to both ESAT-6 and CFP-10. Frequencies of RD1-specific IFN-γ–secreting T cells became undetectable by 6 mo in two students and undetectable in one student by 12 mo, and remained undetectable in all three students for the rest of the follow-up period. Three other students had no detectable frequencies of RD1-specific T cells by 18-mo follow-up. These six students in total, who were ELISpot-negative at 18 mo, had significantly lower initial RD1-specific IFN-γ–secreting T-cell frequencies compared with the 32 students who were ELISpot-positive at 18 mo (Figure 3; Table 3). This difference was seen in the CFP-10–specific responses but not in responses to ESAT-6. There was no significant difference between the Heaf grades of these two subgroups (Table 3). Four students had no detectable RD1-specific T cells at 6 mo (n = 1) and 12 mo (n = 3) but low frequencies of antigen-specific T cells were detected subsequently (18 mo, n = 4, geometric mean [95% confidence interval (CI)] = 47 [20–109] SFC/10^6 peripheral blood mononuclear cell [PBMC]).

Evolution of *M. tuberculosis* Antigen-specific IFN-γ–secreting T cells in Untreated TST-positive Contacts

The frequency of RD1-specific IFN-γ–secreting T-cell responses within the 11 TST-positive staff showed no significant change during 18 mo of follow-up (Figures 2B and 2D; Table 2). However, there was an increase in CFP-10–specific T-cell responses. Six staff maintained stable frequencies of RD1-specific T cells below 200 per million PBMC throughout follow-up, whereas five staff showed increasing frequencies above 200 per million PBMC. Three staff had no detectable RD1-specific T cells at 6 mo (n = 2) and 12 mo (n = 1) but antigen-specific T cells were detected subsequently (18 mo, n = 3, geometric mean [95% CI] = 159 [2–10,144] SFC/10^6 PBMC).

Evolution of *M. tuberculosis* Antigen-specific IFN-γ–secreting T cells in Untreated TST-negative Contacts

Students with a negative TST who did not receive chemoprophylaxis had a significant decline in RD1-specific T cells during follow-up, which was driven by a significant decline in ESAT-6–specific T cells (Figures 2C and 2D; Table 2). Frequencies of RD1-specific IFN-γ–secreting T cells declined on average by 82% per year. RD1-specific IFN-γ–secreting T cells became undetectable by 6 mo in five students and undetectable by 12 mo in one student and remained undetectable for the rest of the follow-up period. In addition, one student had no detectable frequencies of RD1-specific T cells by 18-mo follow-up. There was a significant difference between the negative Heaf grades of these two subgroups; all the students with a positive ELISpot at 18 mo were Heaf grade 2 at screening, whereas students with a negative ELISpot at 18 mo were Heaf grade 0 (n = 4), Heaf grade 1 (n = 2), and Heaf grade 2 (n = 1) (Table 3). The seven students who were ELISpot-negative at 18 mo did have, on average, lower RD1-specific IFN-γ–secreting T-cell frequencies at the initial time point compared with the seven students who were ELISpot-positive at 18 mo, although this was not significant (Figure 3; Table 3). One student had no detectable frequencies of RD1-specific T cells at 6 and 12 mo, but antigen-specific T cells were detected again at 18 mo (240 SFCs/10^6 PBMC).

Responses to PPD and a Non–*M. tuberculosis* Control Antigen during Follow-up

PPD-specific IFN-γ–secreting T-cell frequencies declined on average by 24% per year in the treated TST-positive students

TABLE 1. DEMOGRAPHIC AND CLINICAL CHARACTERISTICS OF CONTACTS OF THE INDEX CASE

Characteristic	Group		
	TST+ Staff	TST+ Students	TST− Students
n	11	38	14
Age, yr, median (range)	47.0 (31–61)	14.0 (11–15)	13.5 (11–14)
Sex, male, n (%)	2 (18)	22 (58)	9 (64)
Ethnic origin, n (%)			
Indian	3 (27)	29 (76)	13 (93)
White	8 (73)	3 (8)	0
Black African	0	2 (5.0)	1 (7)
Pakistani	0	2 (5.0)	0
Black Caribbean	0	1 (3)	0
Mixed race	0	1 (3)	0
Place of birth, n (%)			
United Kingdom	7 (64)	31 (82)	12 (86)
India	2 (18)	2 (5)	1 (7)
Africa	1 (9)	2 (5.0)	1 (7)
Pakistan	0	2 (5.0)	0
Other	1 (9)	1 (3)	0
BCG vaccinated, n (%)	9 (82)	30 (79)	13 (93)
Exposure to index case, mean h	Not defined	107.5	35.2
Heaf grade, median	3	4	2

Definition of abbreviations: BCG = *Mycobacterium bovis* bacille Calmette-Guérin; TST = tuberculin skin test.

($p = 0.001$; data not shown). However, there was no trend in either of the groups of contacts that were not treated (TST-positive staff, $p = 0.87$; TST-negative students, $p = 0.2$; data not shown).

To determine if 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 frequencies of IFN-γ–secreting T cells to streptokinase-streptodornase (*see* the online supplement) at all follow-up time points. Streptokinase-streptodornase–specific IFN-γ–secreting T-cell frequencies showed no significant trend in either TST-positive or TST-negative students ($p = 0.86$, $p = 0.39$, respectively; data not shown).

Comparison of *M. tuberculosis* Antigen-specific IFN-γ–secreting T Cells among Groups

In the untreated TST-positive staff, there was no significant change in RD1 responses during follow-up, whereas, in contrast, there was a significant decline in responses to RD1 in the TST-positive students who were treated. Comparison of the initial frequencies of RD1-specific IFN-γ–secreting T cells between these two groups showed no significant difference between the staff and TST-positive students (0 mo, geometric mean [95% CI] SFCs/10^6 PBMC = 147 [93–234]; 247 [173–354], respectively; $p = 0.14$). The same comparison between the TST-positive staff and the TST-negative students also showed no significant difference between the initial RD1-specific IFN-γ–secreting T-cell frequencies (0 mo, geometric mean [95% CI] SFCs/10^6 PBMC = 147 [93–234]; 172 [98–305], respectively; $p = 0.66$).

No staff members had responses that became and remained undetectable throughout the 18-mo follow-up. Even though the proportion of TST-positive students who were ELISpot-negative at 18 mo was higher than that of the staff, the difference was not significant (0/11 vs. 6/38, $p = 0.32$), whereas the proportion of TST-negative students who were ELISpot-negative at 18 mo was significantly higher than that of the staff (0/11 vs. 7/14, $p = 0.008$).

TABLE 2. TRENDS OF RD1-, ESAT-6–, AND CFP-10–SPECIFIC IFN-γ–SECRETING T CELLS DURING FOLLOW-UP

	n	Geometric Mean Counts of SFCs (*95% CI*)				Average % Change* per Year (*95% CI*)	Test for Trend
		0 mo	6 mo	12 mo	18 mo		
TST+ students							
RD1	38	247 (173 to 354)	235 (116 to 476)	68 (32 to 147)	56 (26 to 119)	−68 (−78 to –53)	$p < 0.0001$
ESAT-6	38	43 (20 to 93)	47 (19 to 115)	8 (3 to 21)	8 (3 to 19)	−75 (−86 to –55)	$p < 0.0001$
CFP-10	38	57 (25 to 130)	122 (55 to 268)	29 (13 to 66)	20 (9 to 47)	−60 (−75 to –34)	$p < 0.0001$
TST+ staff							
RD1	11	147 (93 to 234)	45 (9 to 220)	99 (25 to 393)	212 (89 to 506)	+45 (−36 to +231)	$p = 0.38$
ESAT-6	11	18 (4 to 96)	6 (1 to 37)	6 (1 to 46)	8 (1 to 54)	−40 (−75 to +40)	$p = 0.23$
CFP-10	11	39 (9 to 171)	19 (4 to 99)	76 (21 to 269)	154 (70 to 341)	+202% (+6 to +757)	$p = 0.04$
TST− students							
RD1	14	172 (98 to 305)	23 (3 to 170)	9 (2 to 50)	14 (2 to 101)	−82 (−93 to –53)	$p = 0.0005$
ESAT-6	14	53 (16 to 181)	9 (2 to 48)	2 (1 to 9)	4 (1 to 18)	−85 (−95 to –57)	$p = 0.0004$
CFP-10	14	19 (4 to 99)	18 (3 to 119)	4 (1 to 22)	10 (2 to 62)	−50 (−82 to +8016)	$p = 0.18$
		Participants ELISpot-positive, n (%)				Odds Ratio for Annual Change (*95% CI*)	Test for Trend
TST+ students	38	38 (100)	35 (92)	32 (84)	32 (84)	0.15 (0.04 to 0.61)	$p = 0.008$
TST+ staff	11	11 (100)	9 (82)	10 (91)	11 (100)	1.33 (0.16 to 11.0)	$p = 0.79$
TST− students	14	14 (100)	8 (57)	7 (50)	7 (50)	0.07 (0.01 to 0.38)	$p = 0.002$

Definition of abbreviations: CI = confidence interval; SFCs = spot-forming cells; TST = tuberculin skin test.
* % change computed using sympercents (23).

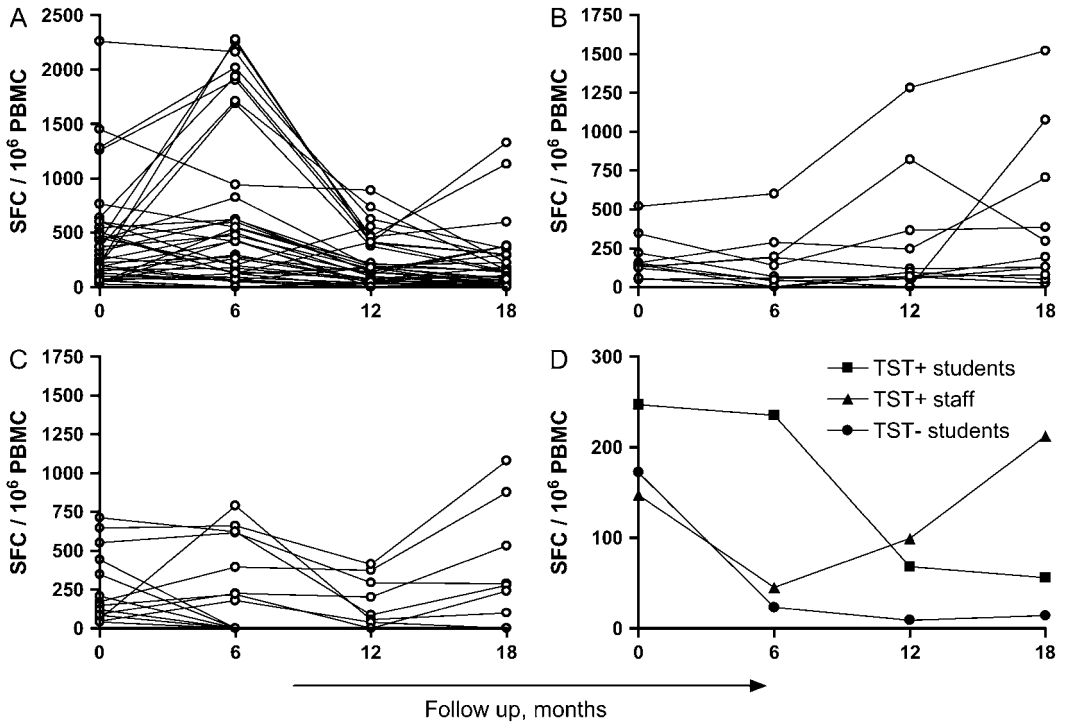

Figure 2. Trends in the frequencies of RD1-specific IFN-γ–secreting T cells differed between the groups of contacts. RD1-specific IFN-γ–secreting T cells were enumerated every 6 mo for 18 mo beginning 3 mo after exposure to the source case had ceased in contacts with detectable RD1 responses at screening. Contacts were defined into three groups: (*A*) TST-positive students, who received chemoprophylaxis; (*B*) TST-positive staff, who did not receive chemoprophylaxis; and (*C*) TST-negative students, who did not receive chemoprophylaxis. The number of contacts with frequencies of RD1-specific IFN-γ–secreting T cells below the detection threshold of the *ex vivo* ELISpot, defined as ELISpot-negative, at 0, 6, 12, and 18 mo, respectively, were 0, 3, 6, and 6 for TST-positive students; 0, 2, 1, and 0 for TST-positive staff; and 0, 6, 7, and 7 for TST-negative students. (*D*) Geometric mean IFN-γ ELISpot responses of treated TST-positive students (*squares*), untreated TST-positive staff (*triangles*), and TST-negative students (*circles*). PBMC = peripheral blood mononuclear cell; SFC = spot-forming cells.

Change in the Breadth of Response to *M. tuberculosis* Peptides during Follow-up

To assess whether the breadth of response to the three ESAT-6 pools and the three CFP-10 pools in contacts who were RD1 ELISpot-positive at all four time points changed over time, we analyzed the median number of pools recognized at each time point within and between the TST-positive treated students, TST-positive untreated staff, and the TST-negative untreated students. No pattern emerged over time within the groups or between the groups. We also compared the breadth of response at screening between contacts that remained RD1 ELISpot-positive at 18 mo with contacts who had turned RD1 ELISpot-negative by 18 mo. There was no significant difference in the median number of RD1 peptide pools recognized at screening between these two subgroups in either the TST-positive contacts or the TST-negative contacts (data not shown).

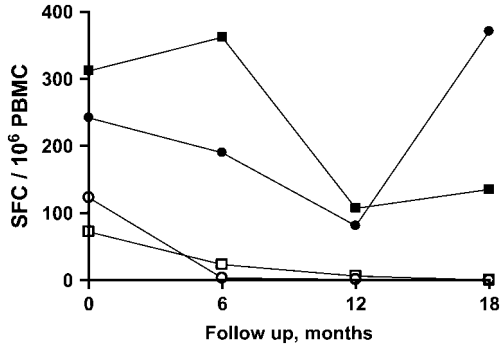

Figure 3. Geometric mean IFN-γ ELISpot responses in TST-positive (*closed squares*) and TST-negative (*closed circles*) students who remained RD1 ELISpot-positive at 18 mo and in the TST-positive (*open squares*) and TST-negative students (*open circles*) who became RD1 ELISpot-negative and remained RD1 ELISpot-negative at 18 mo.

DISCUSSION

It is important to understand the early immune response to *M. tuberculosis* infection, as this period may be crucial in determining clinical outcome. This is particularly true during the first 1 to 2 yr after exposure, when risk of progression to active TB is greatest. We studied the kinetics of *M. tuberculosis*–specific IFN-γ–secreting T cells after point-source exposure during this critical period. It is assumed that the majority of infected contacts acquired infection within the 9 mo when the index case was infectious and at school; the number of contacts infected before the outbreak would have been very low, as the majority were born in the United Kingdom with no previous TB exposure in or outside the school (5).

During treatment for active TB disease the frequency of ESAT-6–specific T cells has been shown to decline in several

TABLE 3. COMPARISON OF TUBERCULIN SKIN TEST AND INITIAL ELISpot RESULTS BETWEEN ELISpot-POSITIVE AND ELISpot-NEGATIVE CONTACTS AT 18 MONTHS

	ELISpot-positive at 18 mo		ELISpot-negative at 18 mo		
SFCs at Screening	n	Geometric Mean SFCs (*95% CI*)	n	Geometric Mean SFCs (*95% CI*)	*t* Test of Log Values
TST+ students					
RD1	32	312 (217 to 449)	6	72 (39 to 132)	p = 0.002
ESAT-6	32	41 (16 to 105)	6	51 (29 to 89)	p = 0.84
CFP-10	32	90 (40 to 202)	6	5 (0 to 74)	p = 0.008
TST+ staff					
RD1	11	147 (93 to 234)	0	—	
ESAT-6	11	18 (4 to 99)	0	—	
CFP-10	11	39 (9 to 171)	0	—	
TST− students					
RD1	7	242 (97 to 606)	7	123 (52 to 289)	p = 0.21
ESAT-6	7	34 (2 to 547)	7	84 (53 to 132)	p = 0.45
CFP-10	7	60 (8 to 467)	7	6 (0 to 108)	p = 0.14
Heaf Grade	n	Grade 0 1 2 3 4	n	Grade 0 1 2 3 4	Test for Trend
TST+ students, n	32	0 0 1* 14 17	6	0 0 1* 2 3	p = 0.53
TST+ staff, n	11	0 0 0 8 3	0	—	
TST− students, n	7	0 0 7† 0 0	7	4 2 1† 0 0	p = 0.004

Definition of abbreviations: CI = confidence interval; SFCs = spot-forming cells; TST = tuberculin skin test.
* *Mycobacterium bovis* bacille Calmette-Guérin unvaccinated contacts.
† *M. bovis* bacille Calmette-Guérin vaccinated contacts.

studies (10, 25–27), and we previously documented an average decline in ESAT-6–specific T cells of 5% per week of treatment (10). However, no studies, to our knowledge, have measured the frequencies of RD1-specific T cells during and after completion of treatment for latent infection. In the TST-positive students who received 3 mo of chemoprophylaxis, there was a significant decline in RD1-specific T-cell responses. Sixteen percent of treated TST-positive students became RD1 ELISpot-negative during follow-up. Antibiotic therapy of active TB greatly reduces bacterial load, which is reflected by the decline in ESAT-6–specific T cells measured by the *ex vivo* IFN-γ ELISpot (7, 10, 26). Although the bacterial burden in latent infection is much lower than in active disease, it seems likely that the decline in RD1-specific T cells observed in the TST-positive students may reflect decreasing numbers of viable bacilli during chemoprophylaxis. This relationship however, would not explain the transient increase in the frequency of RD1-specific T cells secreting IFN-γ at 6 mo of follow-up, after completion of chemoprophylaxis, in nine of the students. A transient increase in the response to recombinant ESAT-6 and CFP-10 antigen has also recently been observed early during preventive treatment for LTBI (28). Although ESAT-6 and CFP-10 are secreted antigens, they are also found stored in the bacterial cell as shown by Pym and colleagues who detected ESAT-6 and CFP-10 in whole-cell lysates and culture supernatants of *M. tuberculosis* (29). Antibiotic-induced killing of the bacilli may release these cytosolic stores into the macrophage *in vivo* resulting in a transient increase in presentation of *M. tuberculosis* antigens, stimulating a larger T-cell response.

In 6 of the 38 students with a positive TST, RD1-specific IFN-γ–secreting T cells became persistently undetectable at various time points using the *ex vivo* ELISpot. It has been suggested that negative *ex vivo* ELISpot results after treatment of active TB indicate successful antibiotic-induced killing of all bacilli and sterile cure. Certainly, the disappearance of RD1-specific effector T cells after treatment of active TB or LTBI is consistent with cessation of antigen stimulation *in vivo*, which in turn would imply absence of viable bacilli. However, it is unclear whether the persistence of positive ELISpot responses after treatment necessarily implies persistence of viable bacilli given that 32 of 38 TST-positive students had RD1-specific ELISpot responses more than a year after completion of preventive treatment. The clinical effectiveness of preventive treatment in reducing the incidence of subsequent active TB implies that the majority of treated students no longer harbor sufficient viable tubercle bacilli to cause disease.

The reason for the persistent RD1-specific T-cell response in the *ex vivo* ELISpot in most treated students is currently unclear. The students who became ELISpot-negative had significantly lower frequencies of RD1-specific IFN-γ–secreting T cells initially compared with TST-positive students who still had detectable frequencies of RD1-specific IFN-γ–secreting T cells at 18 mo. Perhaps, therefore, more individuals may become RD1 ELISpot-negative at a later time point if the declining trend continues. It is also possible that the half-life decay of the effector response may be longer in some individuals than in others and this might explain why many TST-positive students maintained RD1-specific T cells long after treatment. Alternatively, preventive treatment may not have eliminated but instead reduced the number of bacilli to a level where the host is able to maintain long-term control over the residual organisms.

In contrast, in the TST-positive, latently infected staff who did not receive treatment, no significant change in the frequencies of RD1-specific IFN-γ–secreting T cells during follow-up was observed and no TST-positive staff became RD1 ELISpot-negative during follow-up. The persistence of stable levels of circulating effector T cells detected *ex vivo* in the absence of treatment suggests persistent antigen exposure *in vivo*, consistent with ongoing latent infection, as previously observed in the $CD8^+$ T-cell response to ESAT-6 in untreated latently infected contacts (30–32). We know from our previously published work that T cells responding to the RD1 antigens ESAT-6 and CFP-10 are predominantly $CD4^+$ (10, 19, 33), but we were unable to ascertain the phenotype of the T cells in any of the subgroups in this study due to limitations in cell numbers. Although our study is not a randomized controlled trial and TST-positive staff and students were not matched by age and ethnicity, we believe treatment for latent infection in the students accounted for the difference in kinetics of the T-cell response after exposure. In the initial outbreak investigation, no significant associations were

found between the likelihood of being positive by ELISpot and age or ethnic background. However, it may be that these host factors influence the response to treatment of LTBI or long-term control of LTBI through inherited innate immune mechanisms. In addition, because the staff were older than the students, we cannot exclude the possibility that some of them may have been infected through previous unknown exposure and that their T-cell responses reflect remotely acquired, long-term LTBI.

Large-scale studies have shown that 60% of household contacts of sputum smear–positive pulmonary TB cases remain TST-negative and have a lower risk of progression to disease than TST-positive contacts (34). It has been assumed that such individuals do not get infected although they have been exposed to *M. tuberculosis* alongside their TST-positive cohabitants. We identified and followed up TST-negative contacts that had circulating T cells specific for ESAT-6 and CFP-10. The absence of a DTH response to PPD does not rule out infection, whereas the detection of circulating *M. tuberculosis*–specific T cells secreting IFN-γ *ex vivo* strongly suggests *M. tuberculosis* infection has occurred as the T cells must have been primed through intracellular processing and presentation of *M. tuberculosis*–derived peptides in the context of major histocompatibility complex molecules at the surface of the infected macrophage. The TST-negative contacts in our study had, on average, three times less exposure to the index case than the student contacts with a positive TST (5) and may therefore have been infected with a lower dose of *M. tuberculosis* sufficient to induce a detectable T-cell response to *M. tuberculosis* secreted antigens but insufficient to induce a cutaneous DTH response to PPD. We recently identified TST-negative contacts with a positive RD1 ELISpot after brief TB exposure in another setting (20), but this is the first report of clinical and immunologic longitudinal follow-up in such individuals.

A significant decline in RD1-specific T-cell responses was observed in the TST-negative contact group as a whole. If bacterial burden and antigen load have a quantitative relationship with the frequency of *M. tuberculosis*–specific T cells, the declining frequency of *M. tuberculosis*–specific T cells seen in these TST-negative students may reflect a decreasing bacterial burden. This decline is similar to the treated TST-positive students except the TST-negative students did not receive chemoprophylaxis, implying that the bacterial burden in these untreated contacts may have declined spontaneously.

Fifty percent of the TST-negative contacts became RD1 ELISpot-negative. If the loss of circulating *M. tuberculosis*–specific effector T cells in these students reflects cessation of antigen stimulation by viable bacilli *in vivo*, then this raises the possibility that the transient *M. tuberculosis*–specific effector T-cell response in these exposed contacts reflects an acute resolving infection with *M. tuberculosis*. Six of seven of these students had zero or minimal induration in their TSTs; in contrast all seven students who remained ELISpot-positive during follow-up had a Heaf grade 2 (equivalent to a Mantoux response of 5–14 mm after inoculation of 10 TU PPD). This grade 2 Heaf test result, as stipulated by national guidelines, is interpreted as negative in BCG-vaccinated individuals to take into account any PPD-induced response caused by the vaccine. However, we tend to believe the grade 2 Heaf result in the seven BCG-vaccinated contacts who are persistently RD1 ELISpot-positive may represent TB infection. We conclude that the 14 TB-exposed students who were classified as TST-negative probably consisted of two subgroups: students with persistently positive ELISpot responses associated with borderline TST results who probably have "classical" LTBI with persistent dormant bacilli and students with transient *M. tuberculosis*–specific T-cell responses by ELISpot associated with minimal or no TST induration who have an acute resolving infection with *M. tuberculosis*. We believe it unlikely that the transient response we observed could have been induced by tuberculin skin testing. Although PPD contains small amounts of ESAT-6 and CFP-10, we have recently observed that repeated tuberculin testing does not induce false-positive ELISpot results (35). As in all longitudinal studies that follow selected groups, we cannot exclude the possibility that part of the decline in ELISpot responses may have resulted from regression to the mean, through inherent measurement error. However, frequencies of RD1-specific IFN-γ–secreting T cells in the TST-negative, ELISpot-positive group declined on average by 82% per year and the confidence intervals provide 95% confidence that the average decline was between 53 and 93%. This substantial decline exceeds likely regression to the mean effects, making it unlikely that our findings could be explained solely by regression to the mean.

Our inference that disappearance of RD1-specific T cells as measured by IFN-γ ELISpot implies loss of *M. tuberculosis* antigen and bacilli *in vivo* may be flawed. Possible alternative explanations include persistent circulating RD1-specific T cells at frequencies below the detection threshold of the *ex vivo* ELISpot assay (i.e., below 20 per million PBMC); sequestration of these T cells in lung or lymph nodes; or a shifted cytokine secretion profile with loss of IFN-γ secretion. Future experiments will aim to distinguish between these possibilities and to determine whether a pool of *M. tuberculosis*–specific central memory T cells is established after the transient effector response that we have observed. Long-term follow-up of large cohorts of recent contacts who become RD1 ELISpot-negative will help to resolve whether these contacts have cleared infection or whether they carry a risk of progression to active TB; to date, none of the contacts in this study have progressed to active TB.

The phenomenon of transient T-cell responses after infection has also been observed in *M. bovis*–infected cattle where it was associated with clearance of infection. BCG-vaccinated calves were experimentally aerosol-infected with a low dose of *M. bovis* and peripheral antigen-specific IFN-γ–secreting T-cell responses were measured with ELISA and ELISpot. ESAT-6–specific responses initially peaked and then became consistently negative in two of six of the calves. These two calves showed no signs of disease or infection at postmortem in contrast to the calves that maintained persistently detectable ESAT-6–specific responses with TB lung pathology (12). Although a phenotype of acute-resolving infection has not previously been clearly documented with TST, which is a less dynamic test than ELISpot, TST reversions have occasionally been noted in humans in specific settings (36–38).

The significance of the transient disappearance and reappearance of low levels of detectable RD1-specific IFN-γ–secreting T cells in eight contacts (three staff, four TST-positive students, and one TST-negative student) is uncertain. Fluctuating responses around the detection threshold of the *ex vivo* ELISpot assay may precede the sustained disappearance of RD1-specific T cells in some individuals. Alternatively, they may reflect generation of new effector cells on intermittent reexposure *in vivo* to antigens secreted by persisting bacilli, although it is not known whether antigen stimulation of T cells by dormant bacilli *in vivo* is cyclical or continuous. Long-term follow-up of contacts such as these will be required to determine the clinical significance of low fluctuating levels of RD1-specific T cells.

We conclude that there is a dynamic equilibrium between RD1-specific effector T cells as measured by the *ex vivo* IFN-γ ELISpot and the status of dormant tubercle bacilli *in vivo* in latently infected individuals. Preventive chemotherapy, which is believed to kill dormant bacilli, impacts on this equilibrium, resulting in a significant decline in the frequency of RD1-specific

T cells and their disappearance in a proportion of individuals. The quantitative and dynamic readout of the ELISpot suggests that it could be exploited to assess and monitor the impact of novel pharmacologic or immunologic interventions targeted against dormant bacilli. Our second conclusion is that, although TST-negative contacts had lower TB exposure than the TST-positive contacts, a proportion of them mounted transient *M. tuberculosis*–specific effector T-cell responses. Although previously it has been widely assumed that the host response cannot spontaneously clear *M. tuberculosis* infection, our results provide indirect evidence that an acute resolving infection may occur in some contacts. The effector limbs of the host response underlying this phenomenon may have been overlooked but their identification might provide a novel mechanism of action for new TB vaccines.

Conflict of Interest Statement: K.E. is a named inventor on a patent application relating to the use of the RD1-based ELISpot assay. K.A.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.J.D. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. L.A. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. G.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. A.L. is a named inventor on several patents related to T-cell diagnosis filed by the University of Oxford. Regulatory approval of the ELISpot has been undertaken by Oxford Immunotec, in which he is a shareholder and for which he acts as a scientific advisor.

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