

## Frequencies of Region of Difference 1 Antigen-Specific but Not Purified Protein Derivative-Specific Gamma Interferon-Secreting T Cells Correlate with the Presence of Tuberculosis Disease but Do Not Distinguish Recent from Remote Latent Infections<sup>∇</sup>

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The majority of individuals infected with *Mycobacterium tuberculosis* achieve lifelong immune containment of the bacillus. What constitutes this effective host immune response is poorly understood. We compared the frequencies of gamma interferon (IFN- $\gamma$ )-secreting T cells specific for five region of difference 1 (RD1)-encoded antigens and one DosR-encoded antigen in 205 individuals either with active disease ( $n = 167$ ), whose immune responses had failed to contain the bacillus, or with remotely acquired latent infection ( $n = 38$ ), who had successfully achieved immune control, and a further 149 individuals with recently acquired asymptomatic infection. When subjects with an IFN- $\gamma$  enzyme-linked immunospot (ELISpot) assay response to one or more RD1-encoded antigens were analyzed, T cells from subjects with active disease recognized more pools of peptides from these antigens than T cells from subjects with nonrecent latent infection ( $P = 0.002$ ). The T-cell frequencies for peptide pools were greater for subjects with active infection than for subjects with nonrecent latent infection for summed RD1 peptide pools ( $P \leq 0.006$ ) and culture filtrate protein 10 (CFP-10) antigen ( $P = 0.029$ ). Individuals with recently acquired (<6 months) versus remotely acquired (>6 months) latent infection did not differ in numbers of peptide pools recognized, proportions recognizing any individual antigen or peptide pool, or antigen-specific T-cell frequencies ( $P \geq 0.11$ ). The hierarchy of immunodominance for different antigens was purified protein derivative (PPD) > CFP-10 > early secretory antigenic target 6 > Rv3879c > Rv3878 > Rv3873 > Acr1, and the hierarchies were very similar for active and remotely acquired latent infections. Responses to the DosR antigen  $\alpha$ -crystallin were not associated with latency ( $P = 0.373$ ). In contrast to the RD1-specific responses, the responses to PPD were not associated with clinical status ( $P > 0.17$ ) but were strongly associated with positive tuberculin skin test results ( $\geq 15$ -mm induration;  $P \leq 0.01$ ). Our results suggest that RD1-specific IFN- $\gamma$ -secreting T-cell frequencies correlate with the presence of disease rather than with protective immunity in *M. tuberculosis*-infected individuals and do not distinguish recently acquired asymptomatic infection from remotely acquired latent infection.

The immune response is responsible for both bacillary containment in latent tuberculosis infection (LTBI) and immunopathology in active tuberculosis (TB). Comparing key immune responses for these two states may therefore help us dissect which responses mediate or correlate with disease and protection. It is therefore of particular interest to compare the immune responses in individuals with latent infection, who have successfully achieved immune control, and individuals with

active disease, in whom the immune response has failed to contain the bacterium but immunopathology is present.

Gamma interferon (IFN- $\gamma$ ) is essential for controlling *Mycobacterium tuberculosis* infection (11, 17), and CD4<sup>+</sup> IFN- $\gamma$ -secreting T cells specific for mycobacterial antigens play a pivotal role (16, 31). Responses to region of difference 1 (RD1)-encoded antigens are of special interest because of the unique features of early secretory antigenic target 6 (ESAT-6) (1, 30) and culture filtrate protein 10 (CFP-10) (3, 5), which appear to be both virulence factors and putative targets of protective immunity.

In a primary bovine TB infection, ESAT-6-specific IFN- $\gamma$  production, although not a proliferative response, correlated with the severity of disease pathology (39). Likewise, increased ESAT-6 expression and CFP-10 expression in *Mycobacterium*

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*bovis* bacillus Calmette-Guerin (BCG) or *Mycobacterium microti* infections were associated with increased pathogenicity in susceptible mice and correlated with increased RD1-specific T-cell responses (9). However, RD1-specific responses induced by vaccination are associated with protective immunity against subsequent challenge with virulent organisms. Thus, mice infected with *M. tuberculosis* were resistant to reinfection (2), and protection correlated with accelerated accumulation of IFN- $\gamma$ -secreting effector T cells responding to Ag85 and ESAT-6 (1). Mice (27) and guinea pigs (8) vaccinated with BCG::RD1 developed strong CD4<sup>+</sup> IFN- $\gamma$  and proliferative responses to ESAT-6. When challenged, these animals had superior protection compared with BCG-vaccinated or unvaccinated mice, as well as less severe pathology and reduced dissemination of the pathogen (32).

However, the relationship between the magnitude of ESAT-6 responses and disease in humans is unclear; little is known about CFP-10, and almost nothing is known about the other RD1-encoded antigens. The chaperone protein  $\alpha$ -crystallin ("Acr1," "Rv2031c," "HspX," "16-kDa antigen") is up-regulated under oxidative stress conditions (41) and is important for growth in macrophages (12). Encoded by the *M. tuberculosis* "dormancy regulon" expressed during natural infection (25), this protein is upregulated during conditions of in vitro stress (35). For these reasons, it has been postulated that Acr1-specific T-cell responses may correlate with latency (12, 41).

We recently recruited a cohort of 389 individuals suspected to have TB as part of a prospective study of the diagnostic utility of enzyme-linked immunospot (ELISpot) responses to RD1 antigens. (13). A total of 205 patients were given a definitive diagnosis of active or latent TB and had not received antituberculous chemotherapy. We enumerated IFN- $\gamma$ -secreting T cells specific for ESAT-6, CFP-10, Rv3879c, Rv3878, Rv3873 (10), and purified protein derivative (PPD) and, in a subset, IFN- $\gamma$ -secreting T cells specific for Acr1. We compared responses to these antigens in patients with active TB and patients with LTBI in a blinded, prospective manner to address the following questions. Are the frequencies of IFN- $\gamma$ -secreting T cells specific for RD1 antigens, PPD, and Acr-1 different in patients with active TB and patients with LTBI? Do the breadth of the response to RD1-derived peptides in patients with active disease and the breadth of the response to RD1-derived peptides in patients with latent infection differ? Are the hierarchies of immunodominance similar? Do these responses vary with the tuberculin skin test (TST) results? Do responses differ between recently and remotely acquired latent infections?

(Part of this work was presented at the winter meeting of the Acid Fast Club, United Kingdom, on 12 January 2007.)

#### MATERIALS AND METHODS

**Subjects.** Adult patients suspected to have TB at two urban hospitals in the United Kingdom were enrolled prospectively (13). Ethical approval and written informed consent were obtained. Seven patients were known to be human immunodeficiency virus positive (13). Demographic and clinical details for the 205 subjects with active TB, the 38 subjects with presumed nonrecent LTBI, and the 149 subjects with recent asymptomatic infections are shown in Table 1.

A total of 205 subjects were recruited who responded to at least one of five RD1 antigens in an ELISpot assay and for whom a diagnosis of active TB was either confirmed or excluded unambiguously using clinical or microbiological

TABLE 1. Demographic and clinical characteristics of the study populations

Characteristic	Active infection	Recent latent infection	Nonrecent latent infection
Total no. of subjects	167 <sup>a</sup>	149	38
Age (yr)			
Median	30	13	51
Interquartile range	25–41	11–14	31–63
% (no.) of males	59.3 (99)	52.3 (78)	71.1 (27)
Ethnic origin [% (no.)]			
Indian subcontinent	61.1 (102)	0.0 (0)	47.4 (18)
Black	22.8 (38)	0.0 (0)	31.6 (12)
Caucasian	8.4 (14)	0.0 (0)	18.4 (7)
Turkish	0.0 (0)	100 (149)	0.0 (0)
Other	7.8 (13)	0.0 (0)	2.6 (1)
BCG vaccination status [% (no.)]			
Vaccinated	58.1 (97)	79.9 (119)	39.5 (15)
Unknown	11.4 (19)	20.0 (30)	31.6 (12)
TST [% (no.)] <sup>b</sup>			
Positive	63.5 (106)	76.5 (114)	31.6 (12)
Unknown	20.3 (34)	0 (0)	10.5 (4)
Comorbidity [% (no.)]			
None	82.0 (137)	96.6 (144)	57.8 (22)
Previous TB	9.0 (15)	0 (0)	0 (0)
Diabetes	5.4 (9)	0 (0)	21.1 (8)
Human immunodeficiency virus infection	4.2 (7)	0 (0)	0 (0)
Sarcoid	1.8 (3)	0 (0)	0 (0)
Alcohol dependence	1.2 (2)	0 (0)	2.6 (1)
Chronic renal failure	0.6 (1)	0 (0)	2.6 (1)
Carcinoma	0 (0)	0 (0)	2.6 (1)
Other	9.0 (15) <sup>c</sup>	3.4 (5)	28.9 (11) <sup>d</sup>

<sup>a</sup> The infections of 134 of the subjects were confirmed by culture, and 33 subjects had highly probable active infections (they had clinical and radiological features highly suggestive of TB unlikely to be caused by other disease, and a decision to treat was made by a clinician; there was an appropriate response to therapy and supportive histology where available). Histological findings were available for 15 of the latter 33 cases, and they were supportive in all 15 cases; the subjects had granulomas ( $n = 13$ ), epithelioid cells ( $n = 6$ ), caseation ( $n = 5$ ), and necrosis with acute inflammation ( $n = 1$ ).

<sup>b</sup> Induration of  $\geq 15$  mm in the Mantoux test or grade 3 or 4 in the Heaf test was considered a positive result.

<sup>c</sup> Two subjects had asthma, two subjects had epilepsy, two subjects had ischemic heart disease, two subjects had viral hepatitis, one subject had carpal tunnel syndrome, one subject had chronic obstructive pulmonary disease, one subject had idiopathic thrombocytopenic purpura, one subject had iron deficiency anemia, one subject had pernicious anemia, and one subject had schizophrenia.

<sup>d</sup> Four subjects had ischemic heart disease, three subjects had asthma, one subject used drugs intravenously, one subject had multiple sclerosis, one subject had osteoarthritis, and one subject had Wegener's granulomatosis. Only one patient, who was suffering from Wegener's granulomatosis, was receiving therapeutic immunosuppression therapy consisting of 1 g twice daily of mycophenolate mofetil and 10 to 15 mg of oral prednisolone.

criteria (Fig. 1) (13). Inclusion in this study was predicated on a response to one or more of the five RD1 antigens because quantitative comparisons of responses can be carried out only for subgroups with a response. A total of 167 subjects had active TB; 134 of these subjects had culture-confirmed cases, and 33 subjects had highly probable TB, defined as clinical and radiological features highly suggestive of TB unlikely to be caused by another disease, along with a clinical decision to treat, an appropriate response to therapy, and supportive histology where available (13). Blood samples were drawn prior to or within 1 week of initiation of therapy.

Thirty-eight subjects for whom active TB was excluded and definitive alterna-

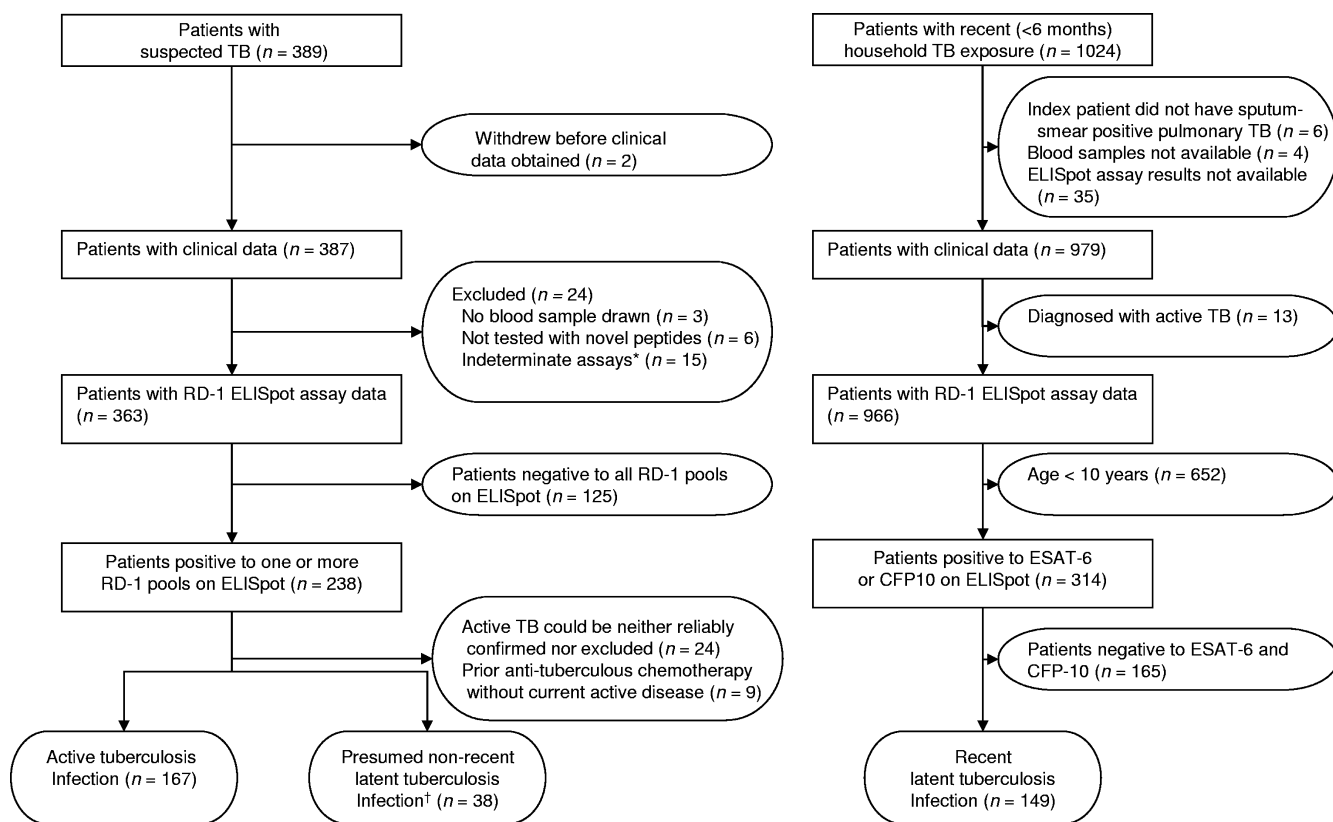


FIG. 1. Study flow chart. For the indeterminate assays (indicated by an asterisk) there were 11 failed positive control assays, 1 assay with a high background level, 1 assay with peptide contamination, 1 inconclusive assay, and 1 defective plate. Of the subjects positive for one or more RD1 pools in the ELISpot assay and with no evidence of active TB (indicated by a dagger), 12 had specific risk factors and a positive 15-mm TST, 21 had specific risk factors and a negative 15-mm TST, and 5 had no specific risk factors and a negative 15-mm TST or unknown TST results.

tive diagnoses were obtained were deemed to have LTBI on the basis of ELISpot responses to one or more of the five RD1-encoded antigens; 31 of these subjects had a response to ESAT-6 or CFP-10 peptides. No recent history (within 6 months) of contact with individuals with TB was elicited (40), there was no prior history of treatment for TB, and infection was therefore assumed to be nonrecent. These subjects were all patients who were referred to the same clinics with suspected TB but for whom all microbiological samples were smear and culture negative.

To investigate whether comparisons might have been affected by how recently infection occurred, immunological data were also compared with data for a prospective community-based cohort of 979 children in Istanbul, Turkey, who had had recent (<6 months) household contact with adults with sputum smear-positive pulmonary TB (38) (Fig. 1). In order to avoid comparing young children whose cellular immune systems may not be mature to adults, we restricted our comparison to the subjects in the cohort who were >10 years old. A total of children who were 10 to 16 years old (median, 13 years; interquartile range, 11 to 14 years), for whom the possibility of active TB was excluded, and who responded to either ESAT-6 or CFP-10 in ELISpot assays were recruited during an 18-month period beginning in October 2002 (Fig. 1). Ethical approval was granted by the Institutional Review Board of Marmara University School of Medicine, Ankara, Turkey, the Turkish Ministry of Health, Ankara, Turkey, and the WHO Steering Committee on Research Involving Human Subjects, Geneva, Switzerland. Written informed consent was provided by each child's parents or legal guardians.

**Ex vivo IFN- $\gamma$  ELISpot assay.** Forty milliliters of heparinized blood was drawn, and  $10.25 \times 10^6$  peripheral blood mononuclear cells were plated onto precoated IFN- $\gamma$  ELISpot plates (Mabtech AB, Stockholm, Sweden) at a concentration of  $2.5 \times 10^5$  peripheral blood mononuclear cells per well. Duplicate wells contained no antigen (negative control), 5  $\mu\text{g}/\text{ml}$  phytohemagglutinin (PHA) (ICN Biomedical, Aurora, OH) (positive control), 100 IU/ml streptokinase-streptodornase (SKSD) (Wyeth Farna, SA, Spain), 20

$\mu\text{g}/\text{ml}$  PPD (Statens Serum Institut, Copenhagen, Denmark), or 10  $\mu\text{g}/\text{ml}$  recombinant ESAT-6 and CFP-10 antigens (Lionex GmbH, Germany). Other duplicate wells contained six peptide pools consisting of five to seven overlapping 15-mer peptides spanning the length of ESAT-6 (three pools) or CFP-10 (three pools) and 45 peptides from selected regions of Rv3873 (two pools), Rv3878 (two pools), and Rv3879c (three pools) (Research Genetics, Huntsville, AL) at a concentration of 10  $\mu\text{g}/\text{ml}$  as previously described (14, 26, 34). The 15-mer peptides are recognized predominantly by CD4 T cells, as previously described (31, 37), but a minority of the peptides also contain CD8 T-cell epitopes (10, 37). After 16 h of incubation at 37°C in 5% carbon dioxide, plates were developed as previously described (14, 34). Spot-forming cells (SFCs) were counted using an automated ELISpot reader (AID-GmbH, Straßberg, Germany) with predefined settings. Predefined thresholds that were means of 5 SFCs/well (peptides) and 10 SFCs/well (whole antigens) more than, and twice the mean for, the negative control wells were used, as in our nine previous studies involving 2,506 participants (13). Assays were performed and data were read by operators blinded to the TST results and personal identifiers, and the results were confirmed by a second scientist.

**TST.** The TST was performed and the results were read by experienced TB nurses blinded to ELISpot data. The method used was the Mantoux method with 10 tuberculin units of PPD-S (Evans Vaccines, Liverpool, United Kingdom), with the results read at 72 h, or (with 58 subjects) the Heaf method (Bignall Surgical Instruments, Littlehampton, United Kingdom) with concentrated PPD (100,000 tuberculin units/ml; Evans Vaccines), with the results read at 1 week (13). We considered induration of  $\geq 15$  mm in the Mantoux test or grade 3 or 4 in the Heaf test (which is considered equivalent to the 15-mm threshold [23]) a positive result (13).

**Statistical methods.** Proportions were compared using  $\chi^2$  and Fisher's exact tests where appropriate. Nonparametric continuous variables were compared by using the Mann-Whitney U (unpaired data) and Wilcoxon signed-rank (paired data) tests, and associations were tested using Spearman's rank correlations.

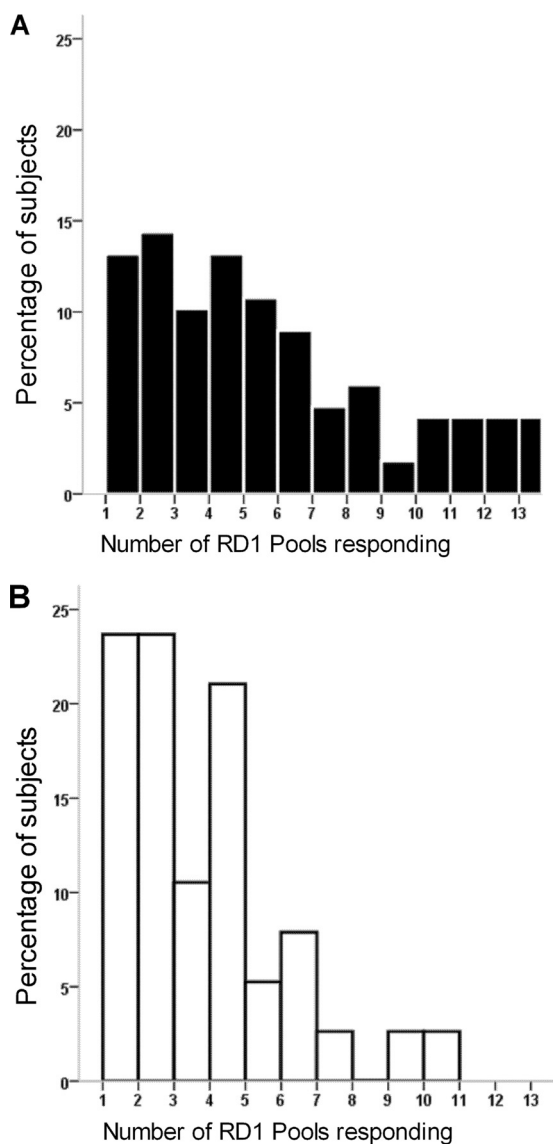


FIG. 2. Number of RD1 peptide pools recognized. Thirteen RD1 peptide pools from five antigens were tested. (A) Subjects with active TB (median number of pools recognized, 4;  $n = 167$ ). (B) Subjects with nonrecent latent TB (median number of pools recognized, 3;  $n = 38$ ). The medians of the distributions were significantly different ( $P = 0.002$ , Mann-Whitney U test).

Two-tailed  $P$  values of  $<0.05$  were considered significant. Analyses were performed with GraphPad Prism 4 (GraphPad Software Inc., CA) and SPSS version 13.0 (SPSS Inc., Chicago, IL).

## RESULTS

**RD1 antigen-specific T-cell responses, but not PPD-specific T-cell responses, are stronger in subjects with active TB than in subjects with nonrecent LTBI.** Of the 205 subjects who responded to at least one RD1 antigen, those with active disease recognized a greater number of RD1 peptide pools than those with a presumed nonrecent latent infection (median numbers of pools recognized, four and three, respectively;  $P = 0.002$ ) (Fig. 2). There was a trend for any given RD1 antigen or

peptide pool to be recognized by a greater proportion of subjects with active disease than of subjects with latent infection, and the differences were significant for recombinant ESAT-6 antigen, ESAT-6 peptide pools, and recombinant CFP-10 antigen (Fig. 3A and B). In contrast, the proportions of subjects responding to mitogen and the non-TB antigen SKSD were not different for subjects with active infection and subjects with latent infection.

In the responding ELISpot wells, significantly higher T-cell frequencies were observed for subjects with active infection than for subjects with nonrecent latent infection for recombinant CFP-10 antigen and for peptide pools of the RD1 antigens CFP-10, ESAT-6, Rv3878, and Rv3873 (Fig. 3C and D). In contrast, the T-cell frequencies in response to PPD and the non-TB antigen SKSD were not higher for subjects with active infection than for subjects with latent infection. Interestingly, the frequencies of IFN- $\gamma$ -secreting T cells in response to mitogen were lower in subjects with active infection than in subjects with latent infection ( $P = 0.01$ ).

Responses to  $\alpha$ -crystallin (Acr1) were tested using a subgroup of 77 subjects. The proportions of subjects responding to Acr1 were not significantly different for the active infection group and the nonrecent latent infection group (22/61 and 3/16, respectively;  $P = 0.153$ , Fisher's exact test), nor were the Acr1-specific T-cell frequencies significantly different (the median numbers of SFCs in responding wells were 90 and 68, respectively;  $P = 0.446$ , Mann-Whitney U test).

**Hierarchy of immunodominance for active and latent infections.** When the strengths of the responses to specific antigens in individuals responding to these antigens were compared, a hierarchy of immunodominance was observed (Fig. 4). The hierarchy of responses to antigens, in decreasing order of T-cell frequency, was as follows: PPD, CFP-10, ESAT-6, Acr1. For the responses to peptide pools, the CFP-10 responses were greater than the ESAT-6 responses, followed by the Rv3879c, Rv3878, and Rv3873 responses. Differences between successive antigens were highly significant for patients with active TB ( $P \leq 0.007$  in all cases except for the comparison of Rv3879c and Rv3878), and the same hierarchy was observed for subjects with nonrecent latent infections, although fewer differences were statistically significant, presumably because of the much smaller sample size.

The hierarchies of immunodominance for active and nonrecent latent infections were compared further and found to be highly correlated with the stages of infection ( $r_s = 0.933$  and  $P < 0.0005$  for the proportion of subjects responding to each specific antigen;  $r_s = 0.717$  and  $P < 0.03$  for cell frequencies) (Fig. 5). In each case PPD dominated the hierarchy, followed by CFP-10, which in all cases was the most immunodominant of the five RD1 antigens tested. The summed peptide responses to antigen pools were greater than the responses to the corresponding whole antigens.

**T-cell responses were greater for subgroups that were TST positive.** The proportions of individuals with TST results of  $\geq 15$  mm were higher for active cases than for nonrecent latent cases (79.7% versus 35.3%;  $P < 0.0001$ ). Nonetheless, when data were reanalyzed so that only data for subjects known to be TST positive ( $n = 132$ ) were included, the trends were the same as those described above; the mean frequencies and proportions of subjects responding to each antigen were higher

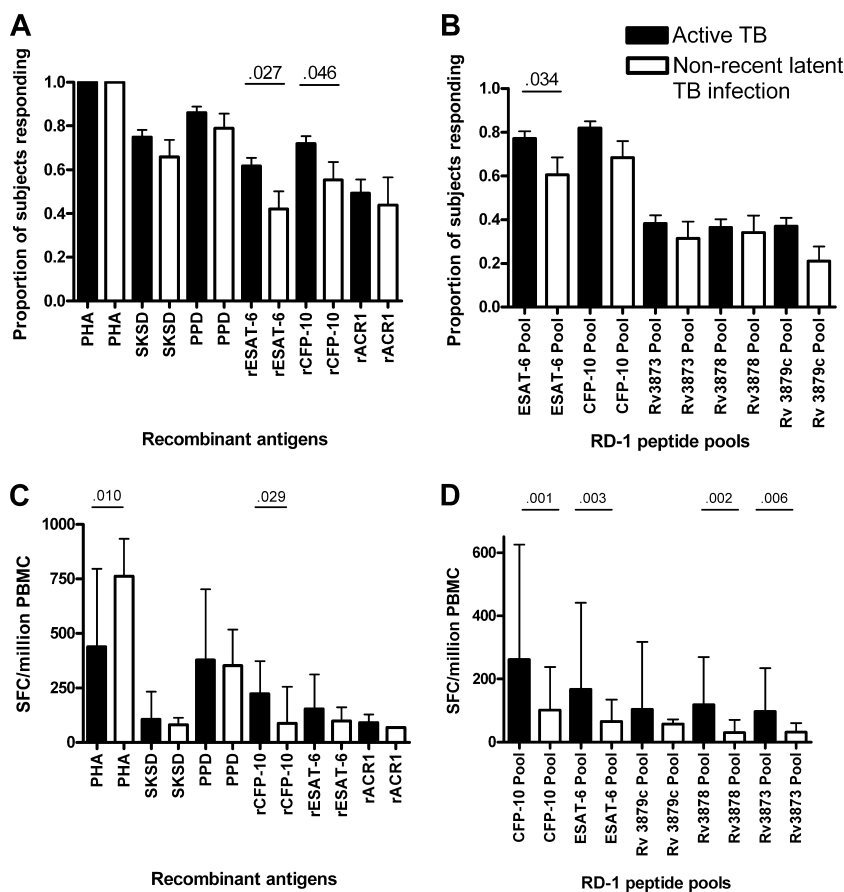


FIG. 3. (A and B) Proportion of subjects with active or nonrecent latent TB responding to recombinant antigens (A) or pools of peptides from five RD1 antigens (B). The bars indicate the proportions, and the error bars indicate standard errors. Where the proportions for active disease and latent disease are significantly different,  $P$  values are indicated (Pearson's  $\chi^2$  test). (C and D) T-cell frequencies in responding wells for subjects with active or nonrecent latent TB responding to recombinant antigens (C) or RD1 peptide pools (D). The data are skewed; the bars indicate the medians, and the error bars indicate the interquartile ranges. Where the proportions for active disease and latent disease are significantly different,  $P$  values are indicated (Mann-Whitney U test). rACR1, recombinant Acr1; rESAT-6, recombinant ESAT-6; rCFP-10, recombinant CFP-10; PBMC, peripheral blood mononuclear cells.

for subjects with active infection than for subjects with latent infection in virtually every case, although with the smaller groups the differences were no longer statistically significant (data not shown).

Next we compared the strengths of the immune responses for patients with active TB and patients with nonrecent latent infections combined, stratified by TST responses of  $\geq 15$  mm ( $n = 167$ ). Higher summed T-cell frequencies for RD1-responding individuals were observed for the TST-positive subgroup than for the TST-negative subgroups for *M. tuberculosis*-specific antigens but not for the control stimuli PHA and SKSD (data not shown). Differences were significant for PPD, ESAT-6 antigen, CFP-10 antigen, summed peptide pools for ESAT-6, CFP-10, and Rv3873, and all ESAT-6–CFP-10 and all RD1 pools summed ( $P < 0.05$  in all cases). When the 167 subjects were further divided into active and latent groups, similar trends were observed; the summed T-cell frequencies were higher for 15-mm TST-positive groups than for TST-negative groups for virtually every comparison, although most differences were not significant for the nonrecent latent subgroup, which was small ( $n = 34$ ) (data not shown).

**For cases of active TB, neither the strength nor the breadth of the immune responses differed according to the clinical subtype.** When active cases were assigned to five subgroups according to the site of disease (lymphatic, pulmonary, pulmonary and pleuropulmonary, localized extrapulmonary, and disseminated), we did not find any association between the site of disease and the median number of ESAT-6 and CFP-10 peptide pools recognized or the summed RD1-specific T-cell frequency ( $P = 0.165$  and  $P = 0.082$ , respectively, Kruskal-Wallis analysis of variance). Nor were any significant differences observed when the same immunological variables were compared for different patient subgroups stratified by pulmonary disease versus extrapulmonary disease, thoracic disease versus disseminated and localized extrapulmonary disease ( $P \geq 0.084$  in all cases), or culture-positive cases versus culture-negative cases ( $P \geq 0.06$ ).

**Neither the strength nor the breadth of immune responses differed when recent infection was compared with nonrecent latent infection.** There were no significant differences between subjects with an infection that was acquired recently (149 individuals with recent household contact responding to ESAT-6

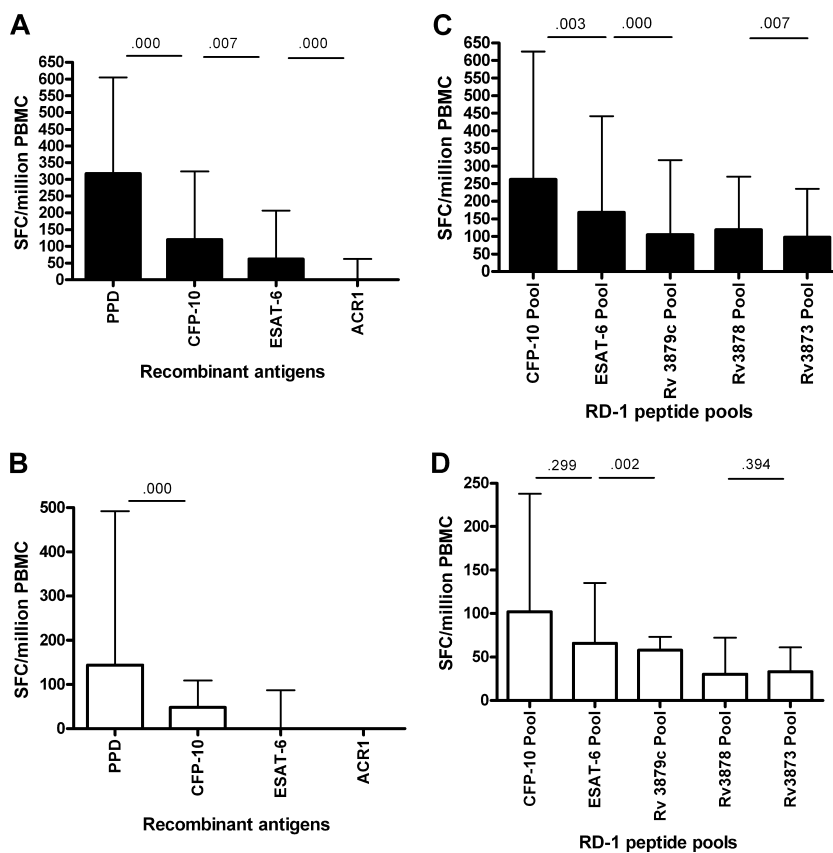


FIG. 4. Hierarchies of immunodominance. (A and B) Comparisons of T-cell frequencies in response to recombinant antigens in responding individuals with active disease (A) and in responding individuals with nonrecent latent disease (B). (C and D) Comparisons of T-cell frequencies in response to RD1 peptide pools in responding individuals with active disease (C) and in responding individuals with latent disease (D). The bars indicate the medians, and the error bars indicate the interquartile ranges. Where the proportions for active disease and nonrecent latent disease are significantly different,  $P$  values are indicated (Wilcoxon signed-rank test). PBMC, peripheral blood mononuclear cells.

or CFP-10) and subjects with infection that was acquired remotely (31 adults with presumed nonrecent exposure responding to ESAT-6 or CFP-10) in the breadth or strength of the immune response (i.e., the median number of ESAT-6 and CFP-10 peptide pools recognized) ( $P = 0.8$ ) (Fig. 6A), in the proportion of subjects recognizing any individual antigen or peptide pool ( $P > 0.14$  in all cases) (data not shown), or in the summed T-cell frequencies for ESAT-6 and CFP-10 peptide pools ( $P = 0.5$ ) (Fig. 6B) or for PHA ( $P = 0.11$ ) (data not shown). Conversely, the T-cell responses for subjects with active infections were broader (median numbers of ESAT-6 and CFP-10 pools recognized, three and two;  $P = 0.001$ ) and stronger (summed ESAT-6 and CPF-10 peptide pool cell frequencies;  $P \leq 0.001$ ) than the T-cell responses for subjects with recently acquired latent infections (Fig. 6).

**PPD-specific T-cell responses are associated with TST results of  $\geq 15$  mm but not with clinical status.** The response to RD1 antigens is greater in subjects with an active infection than in subjects with a nonrecent latent infection, and notwithstanding the association of the RD1 response with a TST result of 15 mm, the relationship between the strength of the RD1 response and disease remained even after stratification by TST results for the TST-negative group (shown for summed ESAT-6 and CFP-10 peptides in Fig. 7A). In contrast, re-

sponses to PPD are strongly associated with TST results ( $P \leq 0.025$ ), but there was no association with clinical status ( $P > 0.17$ ) (Fig. 7B) or with age (Pearson's  $r = -0.54$ ,  $P = 0.49$ ) or ethnicity ( $P > 0.19$  for all comparisons).

**A small proportion of RD1 ELISpot responses are near the threshold of the assay.** As our study cohort was defined by ELISpot response to at least one RD1 antigen, we determined the proportion of subjects for which such a result depended on a weak response near the threshold of the assay. Overall, in only 4.9% of the subjects (10/205) did the result depend on a weak response (20 to 39 SFCs/ $10^6$  peripheral blood mononuclear cells) in a single pair of duplicate wells, which might have been considered a borderline result. This proportion was lower for active cases than for nonrecent latent cases (3.0% [5/167] versus 13.2% [5/38];  $P = 0.009$ ). Lastly, subgroup analysis revealed that the main findings of the study were not affected by the method used for skin testing, when Heaf grades 3 and 4 were considered equivalent to induration of  $\geq 15$  mm in Mantoux tests (23) (data not shown).

## DISCUSSION

In this prospective cohort study of patients suspected to have active TB or LTBI, we found that higher frequencies of RD1-

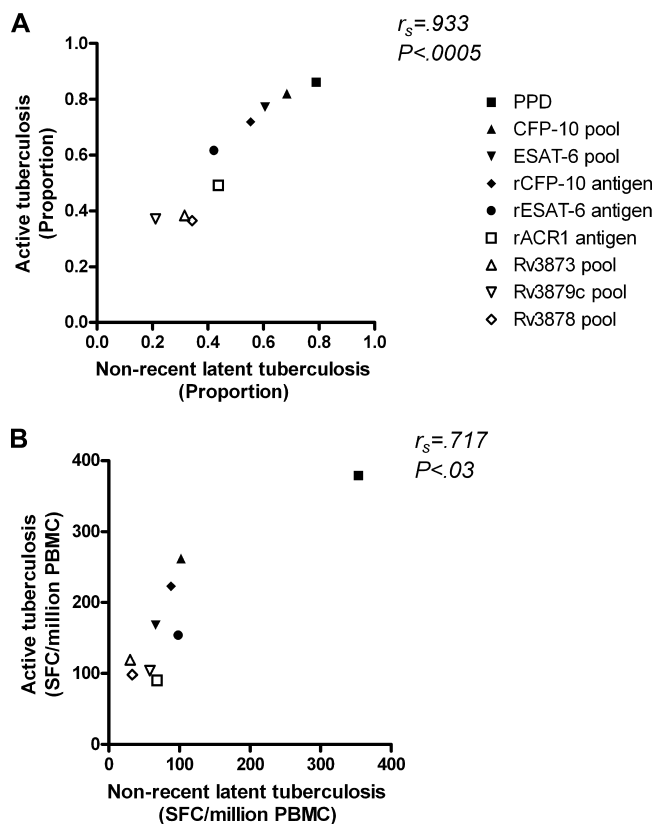


FIG. 5. Correlation of hierarchies of immunodominance for subjects with active disease and subjects with nonrecent latent disease. (A) Proportion of subjects responding to each antigen and (B) median T-cell frequencies according to antigen or peptide pool for subjects with active disease and subjects with latent disease. rCFP-10, recombinant CFP-10; rESAT-6, recombinant ESAT-6; rACR1, recombinant Acr1; PBMC, peripheral blood mononuclear cells;  $r_s$ , Spearman's rank correlation.

specific IFN- $\gamma$ -secreting T cells and a broader T-cell response to RD1 antigen-derived peptide pools correlated with the presence of disease. Stronger RD1-specific T-cell responses were also associated with positive TST responses ( $\geq 15$  mm), as

previously described in studies assessing the diagnostic potential of IFN- $\gamma$  release assays in parallel with TST. In contrast, PPD-specific responses were strongly associated with TST responses of  $\geq 15$  mm but not with clinical status. T-cell responses to a non-TB antigen (SKSD) did not correlate with either clinical status or TST results. The PHA responses were, however, lower in subjects with active disease, presumably reflecting nonspecific cellular immune suppression associated with active TB, which is in part mediated by regulatory T cells (6, 19).

Taken together, these results are consistent with the hypothesis that RD1-specific T-cell responses are specifically associated with TB disease and not merely part of a generalized increase in T-cell responses to *M. tuberculosis* during active TB, since PPD-specific responses were not associated with clinical status. The Th1-type immune response to RD1-encoded antigens in vivo, but not the response to PPD, could thus be preferentially linked to the bacillary replication and inflammation that characterize active TB, as opposed to latent infection. This would be consistent with findings for cows (39) which showed that RD1-specific IFN- $\gamma$  responses correlated with disease pathology scores, although we did not observe correlations with clinical pathology scores in our study. Importantly, because our main cohort was from the same prospectively recruited study population, the immunological differences observed were unlikely to have arisen from bias in selection of the patients with active TB or LTBI.

Despite the differences in strength and breadth between the responses of subjects with latent TB and the responses of subjects with active TB, exactly the same hierarchy of immunodominance was observed for both conditions. Whether we used whole antigens or peptide pools, we observed significantly stronger and more frequent responses to CFP-10 than to ESAT-6. We also found that the T-cell responses to ESAT-6 were significantly stronger than the responses to Rv3879c and Rv3878, which in turn were stronger than the responses to Rv3873. To our knowledge, this is the first definition of the hierarchy of immunodominance for these five RD1-encoded gene products. The dominance of CFP-10 over ESAT-6 is surprising since these antigens form a 1:1 heterodimeric com-

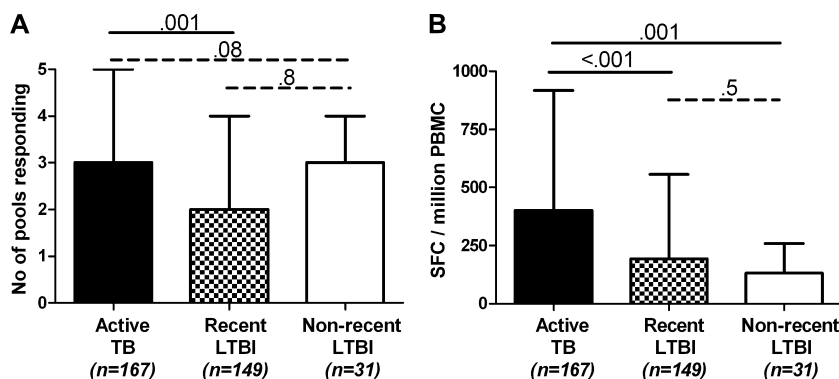


FIG. 6. Comparison of responses of subjects with active disease ( $n = 167$ ), subjects with recently acquired latent infection ( $n = 149$ ), and subjects with remotely acquired latent infection ( $n = 31$ ). (A) Number of ESAT-6 or CFP-10 peptide pools recognized. Six peptide pools were tested in this analysis. The medians of the distributions for subjects with active infection and subjects with recently acquired infection were significantly different ( $P = 0.001$ , Mann-Whitney U test). (B) Summed T-cell frequencies for subjects responding to peptide pools of ESAT-6 and CFP-10. The bars indicate the medians, and the error bars indicate the interquartile ranges.  $P$  values for differences are indicated (Mann-Whitney U test). PBMC, peripheral blood mononuclear cells.

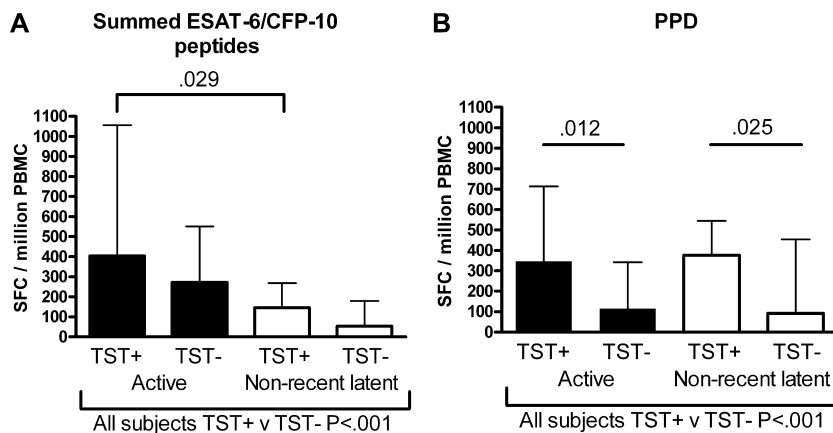


FIG. 7. Summed T-cell frequencies for subjects responding to (A) summed peptides of ESAT-6 and CFP-10 or (B) PPD, stratified by TST results for active and nonrecent latent infections. The bars indicate the medians, and the error bars indicate the interquartile ranges. Where differences are statistically significant,  $P$  values are indicated (Mann-Whitney U test). PBMC, peripheral blood mononuclear cells.

plex (33). Notably, none of the responses to any of the antigens or peptide pools, including Acr1, correlated preferentially with LTBI.

Our observations of stronger RD1-specific IFN- $\gamma$  T-cell responses in subjects with active disease suggest that there is a correlation with pathology rather than with protective immunity. However, it still possible that strong RD1-specific IFN- $\gamma$  T-cell responses that develop rapidly to high levels in the early phases of infection may be protective, resulting in bacillary containment, long-term immune control, and development of latent infection. By contrast, the individuals who subsequently developed active disease might initially have had weaker early responses with slower kinetics, allowing uncontrolled bacillary replication soon after initial infection. At later stages of the disease, by the time of clinical presentation, the RD1-specific response would be progressively driven by increasing antigen and bacterial loads and consequently would be much greater (22). Large longitudinal studies prospectively correlating sequential peripheral blood and pulmonary T-cell responses from the point of initial TB exposure with clinical outcome would be required to distinguish between these possibilities. Moreover, recent evidence suggests that even very small immune responses may be protective (4, 24), suggesting that factors other than the magnitude of the response, such as the rapidity of the response, may be critical.

A few previous studies have directly compared T-cell frequencies in subjects with active infections and subjects with latent infections (18, 21, 31). Each of these studies was a case-control study in which the subjects with LTBI were recently exposed to TB and were epidemiologically distinct from the patients with active TB. An early case-control study by our group suggested that the T-cell frequencies in latently infected subjects with recent household contact were higher than those in patients with active culture-positive pulmonary TB (31). However, this small study examined only ESAT-6-specific responses, and more than one-third of the subjects with active TB cases had received  $\geq 4$  weeks of treatment, which is known to decrease the strength of ESAT-6-specific ex vivo IFN- $\gamma$  ELISpot responses. In the present study, subjects in the first cohort of latently infected individuals had been remotely ex-

posed to TB (at least 6 months prior to enrollment and in most cases many years previously), and most of them had resided in the United Kingdom for at least 1 year, where the annual risk of infection is estimated to be less than 0.01% (20). Since recruitment, none of these subjects have developed active TB, confirming that they are clinically in a state of long-term immune control. Interestingly, we found no differences in the strength or breadth of RD1-specific T-cell responses between this remotely infected cohort with stable LTBI and a cohort recently exposed to TB (<6 months), indicating that the lower frequency of RD1-specific T cells in subjects with latent infection than in subjects with active TB is not restricted to individuals who acquired the infection remotely. More recently, in another case-control study Janssens et al. used commercial T-SPOT.TB kits to compare the total T-SPOT.TB responses of 58 human immunodeficiency virus-negative subjects with culture-confirmed TB with the responses of 127 T-SPOT.TB-positive subjects who were recently (8 to 12 weeks) exposed to TB (21). They observed higher summed T-cell frequencies in subjects with culture-confirmed TB than in subjects with TB contact, and the lowest frequencies were those for the TST-negative subgroup, consistent with our observations. However, Janssens et al. did not evaluate any remotely infected contacts with stable LTBI and did not distinguish between ESAT-6 and CFP-10 responses, nor did they assess responses to other novel RD1 antigens, to PPD, or any DosR-encoded antigen. The present study is the first study to compare responses to a range of RD1-encoded antigens in subjects with active TB and subjects with LTBI and to define the hierarchy of immunodominance for RD1 antigens in both subjects with active TB and subjects with LTBI.

Our study is also the first study to compare the *M. tuberculosis* antigen-specific T-cell responses of subjects with remotely acquired asymptomatic *M. tuberculosis* infection and the antigen-specific T-cell responses of subjects with recently acquired asymptomatic *M. tuberculosis* infection. Given that the risk of progression to active TB is much higher for recently infected persons, it is important to identify immune responses that can distinguish recent infection from remotely acquired stable LTBI. Our data indicate that RD1 antigen-specific IFN- $\gamma$  T-



cell responses cannot distinguish these clinically distinct states. However, given the differences in age and ethnicity between our two latently infected cohorts, further studies are required to corroborate our findings.

It has been suggested that cellular immune responses to the DosR-encoded antigen Acr1 might be preferentially associated with LTBI (12), but we found no such association. Previous data for British (41) and African cohorts (12), but not data for a Dutch cohort (12), have suggested that the responses to Acr1 are stronger in subjects with latent disease than in subjects with active disease. However, in our study the responses to Acr1 in the latent and active disease groups did not differ significantly; rather, the strongest responses to Acr1 were observed in subjects with active TB. The responses to ESAT-6 were stronger than the responses to Acr1 in both subjects with latent infection and subjects with active infection.

We defined latently infected persons as persons with an IFN- $\gamma$  ELISpot response to ESAT-6, CFP-10, or one of three novel RD1 antigens (13, 26). As the RD1 genomic segment is present predominantly in the mycobacteria belonging to the *M. tuberculosis* complex and not present in either *M. bovis* BCG or most environmental mycobacteria, T-cell responses to these antigens are generally considered to be highly specific for the *M. tuberculosis* complex, as validated in recent studies (7, 13, 26). Use of the novel RD1 antigens enabled detection of an additional seven ESAT-6- and CFP-10-negative individuals as latently infected individuals. Conceivably, the responses of latently infected subjects with negative skin tests might represent false-positive ELISpot responses to the RD1 antigens, and in the absence of a "gold standard" test for latent infection, it is still not possible to know definitively who does and who does not have LTBI. Nonetheless, given that very few ELISpot responses in our cohort were close to the assay threshold, it seems unlikely that positive ELISpot results were false-positive results.

It is possible that the high proportion of negative 15-mm TST results for the RD1 ELISpot-positive individuals with presumed LTBI could have been due to waning of the TST response after remote exposure to *M. tuberculosis* many years previously, as widely documented (15, 36). This possibility is consistent with the demographics of our latently infected cohort, in which the TST-negative subjects tended to be older than the subjects who were TST positive (median ages, 54 and 40 years, respectively;  $P = 0.14$ ).

The differential frequencies of RD1 antigen-specific T cells observed suggest that cellular immune responses might be exploited to distinguish active TB infection from LTBI, but the overlap between the frequencies of IFN- $\gamma$ -secreting T cells in these two phases of infection does not allow clear discrimination. However, the profile of cytokine secretion of *M. tuberculosis* antigen-specific T cells may be more informative. For example, the shift from dominance of T cells secreting only IFN- $\gamma$  toward codominance of antigen-specific CD4<sup>+</sup> T cells secreting IL-2 and IL-2 plus IFN- $\gamma$  during treatment of active disease (29) suggests that a dual cytokine signature may correlate better with antigen load than measurement of only one cytokine. Indeed, one recent study suggested that simultaneous measurement of transcription of six cytokines by real-time reverse transcription-PCR may distinguish between active TB infection and LTBI, although the study sample size was small

(42). Given that RD1-specific IFN- $\gamma$ -secreting T cells correlate better with pathology than with protective immunity, measuring cytokine secretion profiles of RD1 antigen-specific T cells may in the future enable accurate discrimination of active infection from latent infection. In summary, while PPD-specific Th1-type T cells, a marker of a global response to *M. tuberculosis*, are not associated with clinical status, the Th1 response to the five RD-1-encoded antigens that we studied is closely related to the presence of disease and is characterized by a consistent hierarchy of immunodominance.

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