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Frequencies of Region of Difference-1 antigen-specific but not PPD-specific IFN-γ-secreting T cells correlate with presence of tuberculosis disease but do not distinguish recent from remote infection

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Conflict of Interest Statement

Professor Lalvani is inventor for several patents underpinning T cell-based diagnosis. The Lalvani ELISpot was commercialised by an Oxford University spin-out company (T-SPOT.*TB*®, Oxford Immunotec Ltd, Abingdon, UK) in which Oxford University and Professor Lalvani have a minority share of equity.

Meetings

This work has been presented aurally at the winter meeting of the Acid Fast Club, U.K. on 12th January 2007: "Frequency of Mycobacterium tuberculosis antigen specific IFN-γ-secreting T cells correlates with presence of pathology in tuberculosis."

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Background

The majority of individuals infected with *M.tuberculosis* achieve lifelong containment of the bacillus. What constitutes this effective host immune response is poorly understood.

Methods

We compared frequencies of IFN-γ-secreting T-cells specific for 5 Region-of-Difference-1-encoded antigens and 1 DosR-encoded antigen in 205 individuals with either active disease (n=167), whose immune responses had failed to contain the bacillus, or with presumed non-recent latent infection (n=38), who had successfully achieved immune control, and a further 149 individuals with recently acquired latent infection.

Results

Amongst subjects with an IFN-γ ELISpot response to one or more RD1-encoded antigens, T-cells from subjects with active disease recognised more peptide pools from those antigens than did subjects with non-recent latent infection (P=0.002). T-cell frequencies to peptide pools were greater in active than non-recent latent infection for summed RD1 peptide pools (P≤0.006), and CFP-10 antigen (P=0.029). Individuals with recently (<6 months) or remotely-acquired (>6 months) latent infection did not differ in numbers of peptide pools recognised, proportions recognising any individual antigen or peptide pool, or antigen-specific T-cell frequencies (P≥0.11). The hierarchy of immunodominance across different antigens was PPD>CFP-10>ESAT-

6>Rv3879c>Rv3878>Rv3873>Acr1, being very similar between active and non-recent latent infection. Responses to the DosR antigen α-crystallin were not associated with latency (P=0.373). In contrast to RD1-specific responses, responses to PPD were not associated with clinical status (P>0.17) but were strongly associated with positive tuberculin skin test results (≥15mm induration, P≤0.01).

Conclusions

Our results suggest RD1-specific IFN-γ-secreting T-cell frequencies correlate with 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.

Introduction

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

IFN-γ is essential for control of infection with MTB, (13, 19) and $CD4^+$ IFN-γ-secreting T cells specific for mycobacterial antigens play a pivotal role(18, 31). Responses to RD1-encoded antigens are of special interest because of the unique features of early secretory antigenic target-6 (ESAT-6) (3, 30) and culture filtrate protein-10 (CFP-10) (5, 7) which appear to be both virulence factors and putative targets of protective immunity,

In primary infection with bovine TB, ESAT-6 specific IFN-γ production, although not proliferative responses, correlated with the severity of disease pathology (39). Likewise, increased ESAT-6 and CFP-10 expression in *M. bovis bacille Calmette-Guerin* (BCG) or *M. microti* were associated with increased pathogenicity in susceptible mice, and correlated with increased RD1-specific T cell responses (11). However RD-1 specific responses induced by vaccination are associated with protective immunity against subsequent challenge with virulent organisms. Thus mice infected with MTB were resistant to re-infection (4), and protection correlated with an accelerated accumulation of IFN-γ-secreting effector T cells responding to Ag85 and ESAT-6 (3). Mice (27)and

guinea pigs (10) vaccinated with BCG::RD1 developed strong $CD4^+$ IFN-γ and proliferative responses to ESAT-6. When challenged these animals had superior protection compared with BCG-vaccinated or unvaccinated mice, with less severe pathology and reduced dissemination of the pathogen (32). The relationship between the magnitude of ESAT-6 responses and disease in humans is unclear and little is known about CFP-10 and almost nothing about the other RD1-encoded antigens. The chaperone protein α-crystallin ("Acr1", "Rv2031c", "HspX", "16-kDa antigen") is up-regulated under oxidative stress (41), and is important for growth in macrophages (14). Encoded by the MTB "dormancy regulon" expressed during natural infection (25), it is up-regulated in latency (35). For these reasons, it has been postulated that Acr1-specific T cell responses may correlate with latency (41) (14).

We recently recruited a cohort of 389 TB suspects, as part of a prospective study of the diagnostic utility of ELISpot responses to RD1 antigens. (15) 205 patients were assigned a definitive diagnosis of active or latent TB, and had not received prior anti-tuberculous chemotherapy. We enumerated IFN-γ-secreting T cells specific for ESAT-6, CFP-10, Rv3879c, Rv3878, Rv3873 (12), PPD and, in a subset, Acr1. We compared responses to these antigens in active TB and LTBI in a blinded, prospective manner to address the following questions: does the frequency of IFN-γ-secreting T cells specific for RD1 antigens, PPD and Acr-1 differ between active TB and LTBI? Does the breadth of the response to RD-1-derived peptides differ between active disease and latent infection? Is the hierarchy of immunodominance similar? And do these responses vary by tuberculin skin test (TST) results?

MATERIALS AND METHODS

Subjects. Adult patients with suspected tuberculosis at two urban hospitals in the U.K., were enrolled prospectively (15). Ethical approval and written informed consent were obtained. 7 patients were known to be HIV positive (15). Demographic and clinical details of the 205 subjects with active TB, the 38 subjects with presumed non-recent LTBI and the 149 subjects with recent asymptomatic infection are shown in Table I.

205 subjects were recruited who responded to at least 1 of 5 RD1 antigens on ELISpot, and in whom a diagnosis of active tuberculosis was either confirmed or excluded unambiguously using clinical or microbiological criteria (Figure 1) (15). Inclusion in this study was predicated on a response to one or more of the 5 RD1 antigens because quantitative comparisons between responses can only be carried out between subgroups with a response. 167 subjects had active tuberculosis, comprising 134 culture-confirmed cases, and 33 cases with highly probable tuberculosis, defined as clinical and radiological features highly suggestive of TB unlikely to be caused by other disease, with a clinical decision to treat, an appropriate response to therapy and supportive histology where available (15). Blood samples were drawn prior to, or within one week of initiating therapy.

38 subjects in whom active TB was excluded and definitive alternative diagnoses identified were deemed to have latent tuberculosis infection on the basis of ELISpot responses to one or more of the five RD1-encoded antigens: of these 31 had a response to ESAT-6 or CFP-10 peptides. No recent (within 6 months) history of contact with TB could be elicited (40), there was no prior history of treatment for tuberculosis, and infection was therefore assumed to be non-recent. These were all patients referred to the

same clinics with suspected tuberculosis, but in whom all microbiological samples were smear- and culture-negative.

To investigate whether comparisons may be affected by recency of infection, immunological data were also compared with data from a prospective community based cohort of 979 children in Istanbul, Turkey who were recent (<6 months) household contacts of adults with sputum smear-positive pulmonary TB (38) (Figure 1). In order to avoid comparing young children whose cellular immune systems may not be mature relative to adults, we restricted our comparison to the subjects in the cohort aged >10 yrs. 149 children aged 10 to 16 years (median 13 yrs, IQR 11-14), in whom active TB was excluded and who responded to either ESAT-6 or CFP-10 on ELISpot, were recruited during 18 months from October, 2002 (Figure 1). Ethical approval was granted by the Institutional Review Board of Marmara University School of Medicine, Ankara, the Turkish Ministry of Health, Ankara, and the WHO Steering Committee on Research Involving Human Subjects, Geneva. Written informed consent was provided by the child's parents or legal guardians.

Ex vivo IFN-γ Enzyme-linked immunospot (ELISpot) assay. 40mL heparinized blood was drawn and $10.25x10^6$ peripheral blood mononuclear cells (PBMCs) plated onto pre-coated interferon-γ ELISpot plates (Mabtech AB, Stockholm, Sweden) at $2{\cdot}5x10^5$ PBMC per well. Duplicate wells contained no antigen (negative control), 5 µg/mL phytohemagglutinin (positive control; ICN Biomedical, Aurora, OH, USA), 100 iu/mL streptokinase-streptodornase (SKSD, Wyeth Farna, SA, Spain), 20 µg/mL PPD (Statens Serum Institut, Copenhagen, Denmark), 10 µg/mL recombinant ESAT-6 and CFP-10 antigens (Lionex GmbH, Germany). Further duplicate wells contained 6 peptide pools incorporating five to seven overlapping 15mer peptides

spanning the length of ESAT-6 (3 pools) or CFP-10 (3 pools), and 45 peptides from selected regions of Rv3873 (2 pools), Rv3878 (2 pools) and Rv3879c (3 pools) (Research Genetics, Huntsville, Ala.) at 10 µg/mL as previously described (16, 26, 34). These 15mer peptides are predominantly recognized by CD4 T cells as previously described,(31, 37) but a minority of these peptides also contain CD8 T cell epitopes (12, 37). After 16 hour incubation at 37ºC in 5% carbon dioxide, plates were developed as previously described (16, 34). Spot forming cells (SFCs) were counted using an automated ELISpot reader (AID-GmbH, Straßberg, Germany) with predefined settings. Predefined thresholds of a mean 5 SFC/well (peptides) or 10 SFC/well (whole antigens) more than, and twice the mean of, the negative control wells were used, as in our 9 previous studies in 2,506 participants (15). Assays were performed and read by operators blind to TST results and personal identifiers, and counter-signed by a second scientist.

Tuberculin skin testing (TST). Tuberculin skin testing was performed and read by experienced TB nurses, blind to ELISpot data, using the Mantoux method with 10 tuberculin units of PPD-S (Evans Vaccines, Liverpool, U.K.), read at 72 hours, or in 58 subjects, by the Heaf method (Bignall Surgical Instruments, Littlehampton, U.K.) and concentrated PPD (100,000 tuberculin units/mL, Evans Vaccines) read at one week (15). We considered induration of 15 mm or greater on the Mantoux test or grade 3 or 4 on the Heaf test (which is considered equivalent to the 15-mm threshold(2)) to be a positive result (15).

Statistical methods. Proportions were compared using χ^2 and Fisher's exact tests where appropriate. Non-parametric continuous variables were compared with Mann Whitney *U* (unpaired data), and Wilcoxon signed-rank (paired data) tests, and associations tested using Spearman's rank correlations. Two-tailed P-values <0.05 were

considered significant. Analyses used GraphPad Prism 4 (GraphPad Software Inc, CA) and SPSS version 13.0 (SPSS Inc, Chicago, IL).

RESULTS

RD1 antigen-specific but not PPD-specific T cell responses are stronger in active than in non-recent latent TB infection. Amongst these 205 subjects, all of whom responded to at least 1 RD1 antigen, those with active disease recognised a greater number of RD1 peptide pools than those with presumed non-recent latent infection: (median number of pools recognised 4 versus 3 respectively; P=0.002) (Figure 2). There was a trend for any given RD1 antigen or peptide pool to be recognised by a greater proportion of subjects with active disease than with latent infection, and these differences were significant for recombinant ESAT-6 (rESAT-6) antigen, ESAT-6 peptide pools and rCFP-10 antigen (Figure 3A,B). In contrast, the proportion of subjects responding to mitogen and the non-TB antigen SKSD did not differ between active and latent infection.

Amongst responding ELISpot wells, significantly higher T cell frequencies were observed in active than in non-recent latent infection for rCFP-10 antigen, and for peptide pools from the RD1 antigens CFP-10, ESAT-6, Rv3878 and Rv3873 (Figure 3C,D). In contrast, T cell frequencies in response to PPD and the non-TB antigen SKSD were not higher in active than latent infection. Interestingly, frequencies of γ-secreting T cells in response to mitogen were lower in active than in latent infection (P=0.01).

Responses to α-crystallin (Acr1) were tested in a subgroup of 77 subjects. Proportions of subjects responding to acr1 did not differ significantly between active and non-recent latent groups (22/61 v 3/16 respectively, Fisher's exact P=0.153), nor did Acr1 specific T cell frequencies (median SFCs in responding wells 90 v 68 respectively Mann Whitney P=0.446).

Hierarchy of immunodominance in active and latent infection. When the strength of responses to specific antigens was compared amongst individuals responding

to those antigens, a hierarchy of immunodominance was observed (Figure 4). The hierarchy of responses to antigens, in decreasing order of T cell frequencies, was PPD, CFP-10, ESAT-6 and Acr1. For responses to peptide pools CFP-10 responses dominated over ESAT-6, followed by Rv3879c, Rv3878 and Rv3873. Differences between successive antigens were highly significant amongst patients with active TB ($P \leq 0.007$ in all cases except Rv3879c versus Rv3878) and the same hierarchy was observed amongst non-recent latently infected subjects, although fewer differences were statistically significant, presumably because of the much lower sample size.

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

T cell responses were greater amongst subgroups positive to tuberculin skin testing. Proportions of individuals with TST $\geq$ 15mm were higher amongst active than amongst non-recent latent cases (79.7% v 35.3%, P<0.0001). Nonetheless, when data were reanalysed including only subjects known to be TST positive (n=132) the trends in the above observations were the same – the mean frequencies and proportions of subjects responding to each antigen were again higher in active than latent infection in virtually every case - although in these smaller groups these trends no longer reached statistical significance (Data not shown).

Next we compared the strength of immune responses amongst patients with active TB and non-recent latently infected subjects combined, stratified by ≥15mm TST responses (n=167). Higher summed T cell frequencies from RD1 responding individuals were observed in the TST-positive subgroup when compared with the TST-negative subgroups for MTB 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, and summed peptide pools from ESAT-6, CFP-10, Rv3873, and all ESAT-6/CFP-10 or all RD1 pools summed ($P<0.05$ in all cases). When these 167 subjects were further stratified into active and latent groups similar trends were observed: summed T cell frequencies were higher in 15mm TST-positive than TST-negative groups for virtually every comparison, although most differences did not reach significance amongst the non-recent latent subgroup which was small (n=34, data not shown).

Amongst cases of active tuberculosis, neither strength nor breadth of immune responses differed according to clinical subtype. When active cases were assigned to 5 subgroups according to site of disease – lymphatic, pulmonary, pulmonary and pleuropulmonary, localised 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 recognised nor summed RD1-specific T cell frequencies (Kruskal-Wallis ANOVA $P=0.165$ and $P=0.082$ respectively). Nor were any significant differences observed when the same immunological variables were compared between patient subgroups stratified by pulmonary *versus* extrapulmonary disease, thoracic *versus* disseminated and localised extrapulmonary disease ($P\geq0.084$ in all cases), or culture-positive *versus* culture-negative cases ($P \geq0.06$).

Neither strength nor breadth of immune responses differed between recent and non-recent latent infection. No significant differences were observed between those with subjects with infection acquired recently (n=149 recent household contacts) or remotely (n=31 adults with presumed non-recent exposure responding to ESAT-6 or CFP-10) in the breadth or strength of the immune response, namely the median number of ESAT-6 and CFP-10 peptide pools recognised (P=0.97), nor in the proportion recognising any individual antigens or peptide pool (P>0.14 in all cases, data not shown) nor in the T cell frequencies amongst wells responding to PPD, ESAT-6 or CFP-10 peptides (P≥0.23) nor for PHA (P=0.11, data not shown). Conversely T cell responses from active cases were broader (median number of ESAT-6 and CFP-10 pools recognised 3 v 2, P=.001) and stronger (summed ESAT-6 and CPF-10 peptide pool cell frequencies, P≤.001) than in those with recently acquired latent infection (Figure 6).

PPD-specific T cell responses are associated with TST result of ≥15mm, but not with clinical status. The response to RD1 antigens is higher in active than non-recent latent infection, and notwithstanding the association of RD1 response with 15mm TST, the relationship of the strength of RD1 response with disease remained even after stratification by TST result in the TST-negative group (shown for summed ESAT-6/CFP-10 peptides in Figure 7A). In contrast, responses to PPD are strongly associated with TST (P≤0.025), but there was no association with clinical status (P>0.17, Figure 7B), nor 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 quantified the proportion of subjects where such a result depended on a weak response near the threshold of the assay. Overall in only 4.9% of subjects (10/205) did the result

depend on a weak response (20-39 SFC/million) in a single pair of duplicate wells, which might therefore have been considered a borderline result. This proportion was lower amongst active than non-recent latent cases (3.0% 5/167 v 13.2% 5/38 respectively, P=0.009). Lastly, subgroup analysis revealed that the main findings of the paper were unaffected by method used for skin testing, taking Heaf grades 3 and 4 as equivalent to 15 mm or more induration on Mantoux testing(2) (data not shown).

DISCUSSION

In this prospective cohort study of tuberculosis suspects with active and latent tuberculosis infection, we found that higher frequencies of RD1-specific IFN-γ-secreting T cells and a wider breadth of 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 (≥15mm) TST responses, as previously described in studies assessing the diagnostic potential of interferon-gamma release assays in parallel with TST. In contrast, PPD-specific responses were strongly associated with ≥15mm TST response, but not with clinical status. T cell responses to the non-TB antigen (SKSD) did not correlate with either clinical status or TST. PHA responses were however lower in active disease, presumably reflecting a non-specific cellular immune-suppression, associated with active TB, which may arguably be mediated by regulatory T cells (8, 21).

Taken together these results would be consistent with the hypothesis that RD1-specific T cell responses are specifically associated with tuberculosis disease and not merely part of a generalized increase in T cell responses to MTB 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 characterise active TB, as opposed to latent infection. This would be consistent with findings in cows (39) where in addition RD-1 specific IFN-γ 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 are unlikely to arise from bias in selection of the patients with active tuberculosis or LTBI.

Despite the differing strength and breadth of responses between latent and active TB, exactly the same hierarchy of immunodominance was observed in both conditions. Whether using whole antigens or peptide pools we found significantly stronger and more frequent responses to CFP-10 than to ESAT-6. We also found that T cell responses to ESAT-6 were significantly stronger than responses to Rv3879c and Rv3878 which in turn were stronger than responses to Rv3873. To our knowledge this is the first definition of the hierarchy of immunodominance for these 5 RD1-encoded gene products. The dominance of CFP-10 over ESAT-6 is surprising since these antigens form a 1:1 heterodimeric complex (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-γ T cell responses in active disease suggest a degree of correlation with pathology, rather than with protective immunity. However, it still possible that strong RD1-specific IFN-γ 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 those individuals who subsequently develop active disease might initially have had weaker early responses with slower kinetics, allowing uncontrolled bacillary replication soon after initial infection. At later stages in disease, by the time of clinical presentation, the RD1-specific response would be progressively driven by increasing antigen and bacterial load and would consequently be much larger. (23) 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, (6, 24) suggesting that factors other than the magnitude, such as the rapidity of the response, may be critical.

Few studies have previously directly compared T cell frequencies in active and latent infection.(20, 22, 31) Each were case-control studies where the subjects with LTBI were recently exposed to TB and were epidemiologically distinct populations from the patients with active TB. An early case-control study by our group suggested higher T cell frequencies in latently infected recent household contacts compared with active culture-positive pulmonary TB patients (31). However, this small study only examined ESAT-6-specific responses and more than a third of the active TB cases had received ≥4 weeks of treatment, which is known to decrease the strength of ESAT-6-specifiic *ex vivo* IFN-γ ELISpot responses. In the present study, the first cohort of latently infected individuals had remote TB exposure, at least six months prior to enrolment and in most cases many years previously, and most had been resident in the UK for at least a year, where annual risk of infection is estimated at less than 0.01% (1). Since recruitment, none 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 the additional cohort with recent (<6 month) TB exposure, indicating that the lower frequency of RD1-specific T cells in latent infection compared with active TB is not restricted to remotely acquired infection. More recently, another case-control study by Janssens *et al* used commercial T-SPOT.*TB* kits to compare total T-SPOT.*TB* responses in 58 HIV-negative cases of culture-confirmed tuberculosis with 127 T-SPOT.*TB*-positive TB contacts with recent (8-12 weeks) TB exposure(1, 22). They observed higher

summed T cell frequencies in culture-confirmed tuberculosis than in contacts, the lowest being amongst the TST negative subgroup, consistent with our observations. However that study did not evaluate any remotely infected contacts with stable LTBI and did not distinguish between ESAT-6 and CFP-10 responses, and neither did it assess responses to other novel RD1 antigens or to PPD or any DosR-encoded antigen. The present study is the first to compare responses to a range of RD1-encoded antigens in active TB and LTBI and to define the hierarchy of immunodominance for RD1 antigens in both active TB and LTBI.

Our study is also the first to compare MTB antigen-specific T cell responses between remotely-acquired and recently-acquired asymptomatic MTB infection. Given that the risk of progression to active TB is much higher in 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-γ 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 finding.

It has been suggested that cellular immune responses to the DosR-encoded antigen, Acr1, might be preferentially associated with LTBI (14), but we found no such association. Previous data from British (41) and African cohorts (14), although not in a Dutch cohort(14), have suggested relatively stronger responses to Acr1 in latency than in active disease. However in our study responses to Acr1 did not differ significantly between the latent and active groups, but rather strongest responses to Acr1 were observed in active TB. Responses to ESAT-6 were stronger than to Acr1 in both latent and active infection.

We defined latently infected persons as those with an IFN-γ ELISpot response to ESAT-6, CFP-10 or one of 3 novel RD1 antigens (15, 26). As the RD1 genomic segment is present predominantly in the mycobacteria from the MTB complex, and absent both from *M. Bovis* BCG, and most environmental mycobacteria, T cell responses to these antigens are considered to be generally highly specific for MTB complex, as validated in recent studies (9, 15, 26). This enabled detection of an additional 7 ESAT-6/CFP-10-negative individuals as latently infected. Conceivably 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 remains impossible to known 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-positives.

It is possible that the high proportion of negative 15mm TST results amongst the RD1 ELISpot-positive individuals with presumed LTBI could be due to waning of the TST after remote exposure to MTB many years ago, as widely documented (17, 36). This is consistent with the demographics of our latently infected cohort, in which the TST-negative subjects tended towards being older (median 54 v 40 years, P=0.14) than those who were TST-positive.

The differential frequencies of RD1 antigen-specific T cells observed suggests that cellular immune responses might be exploited to distinguish active from latent TB infection but the overlap between the frequencies of IFN-γ-secreting T cells in these two phases of infection does not allow for clear discrimination. However, the profile of cytokine secretion of MTB antigen-specific T cells may be more informative. For example, the shift from dominance of IFN-γ only secreting T cells towards co-dominance

of antigen specific IL-2 and IL-2/IFN-γ-secreting $CD4^+$ T cells 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 6 cytokines by real-time RT-PCR may distinguish between active and latent TB infection, although the study sample size was small (42). Given that RD1-specific IFN-γ-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 from latent infection. In summary, whilst PPD-specific Th1-type T cells, a marker of global response to response to MTB, are not associated with clinical status, the Th1 response to the 5 RD-1-encoded antigens we studied is closely related to presence of disease and is characterized by a consistent hierarchy of immunodominance.

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Figure Legends

Table 1

Demographic and clinical characteristics of the study populations

BCG, bacilli Calmette-Guérin; HIV, human immunodeficiency virus.

a) 134 culture confirmed, 33 highly probable: clinical and radiological features highly suggestive of TB unlikely to be caused by other disease and a decision to treat made by a clinician and appropriate response to therapy and histology supportive where available. Histological findings available in 15/33 cases and supportive in all 15 cases, comprising granulomas n=13, epithelioid cells n=6, caseation n=5, necrosis with acute inflammation n=1

b) Tuberculin skin test. Induration of 15 mm or greater on the Mantoux test or grade 3 or 4 on the Heaf test was considered a positive result.

c) Asthma (2), epilepsy (2), ischaemic heart disease (2), viral hepatitis (2), carpal tunnel syndrome (1), chronic obstructive pulmonary disease (1), idiopathic thrombocytopaenic purpura (1), iron deficiency anaemia (1), pernicious anaemia (1), schizophrenia (1)

d) Ischaemic heart disease (4), asthma (3), intravenous drug use (1), multiple sclerosis (1), osteoarthritis (1), Wegener's granulomatosis (1). Only one patient, suffering from Wegener’s granulomatosis, was receiving therapeutic immunosuppression, with 1g BD of mycophenolate mofetil and 10-15 mg of oral prednisolone.

Figure 1.

Study Flow Chart

TB, tuberculosis; RD-1, region of difference-1; ELISpot, enzyme-linked immunospot.

*Failed positive control n=11, high background n=1, peptide contamination n=1, inconclusive assay n=1, defective plate n=1

†Positive to one or more RD-1 pools on ELISpot, no evidence of active TB; with specific risk factors and 15mm TST positive n=12, with specific risk factors and 15mm TST negative n=21, without specific risk factors and 15mm TST negative or unknown n=5

Figure 2.

Number of RD-1 peptide pools recognised. 13 RD-1 peptide pools were tested. *A*, Subjects with active tuberculosis: median number of pools recognised = 4 (n = 167). *B*, Subjects with non-recent latent tuberculosis: median number of pools recognised = 3 (n = 38). Medians of distributions were significantly different (P=.002, Mann Whitney *U* test).

Figure 3.

A and B. Proportion of subjects with active or non-recent latent tuberculosis responding to recombinant antigens *(A)* or peptide pools from 5 RD-1 antigens *(B)*. Bars show proportions with standard errors. Where proportions are significantly different between active and latent disease, P values are given (Pearson's χ^2).

C and D. T cell frequencies in responding wells from subjects with active or non-recent latent tuberculosis responding to recombinant antigens *(C)* or RD-1 peptide pools *(D)*. Data are skewed; bars show medians and inter-quartile ranges. Where proportions are

significantly different between active and latent disease, P values are given (Mann-Whitney U).

ACR1, alpha-crystallin-1; CFP-10, culture filtrate protein-10; ESAT-6, early secretory antigenic target-6; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; PPD, purified protein derivative; r, recombinant; SFC, spot forming colony; SKSD, streptokinase-streptodornase

Figure 4.

Hierarchies of immunodominance. A and B. T cell frequencies responding to recombinant antigens are compared in responding individuals in active *(A)* and non-recent latent disease *(B)*. C and D. T cell frequencies responding to RD-1 peptide pools are compared in responding individuals in active *(C)* and latent disease *(D)*. Graphs show medians and inter-quartile ranges. Where proportions are significantly different between active and non-recent latent disease, P values are given (Wilcoxon rank).

ACR1, alpha-crystallin-1; CFP-10, culture filtrate protein-10; ESAT-6, early secretory antigenic target-6; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; PPD, purified protein derivative; r, recombinant; SFC, spot forming colony; SKSD, streptokinase-streptodornase

Figure 5.

Correlation of hierarchies of immunodominance in active and non-recent latent disease. *(A)* Proportion of subjects responding to each antigen, and *(B)* Median T cell frequencies according to antigen or peptide pool in active and in latent disease.

ACR1, alpha-crystallin-1; CFP-10, culture filtrate protein-10; ESAT-6, early secretory antigenic target-6; PBMC, peripheral blood mononuclear cells; PPD, purified protein derivative; r, recombinant; r_s Spearman's rank correlation; SFC, spot forming colony

Figure 6.

Comparison of responses between those with active disease *(n=167)*, recently acquired latent infection *(n=149)*, and remotely acquired latent infection *(n=31)*. *(A)* Number of ESAT-6 or CFP-10 peptide pools recognised. 6 peptide pools were tested in this analysis. Medians of distributions were significantly different between active and recently acquired infection (P=.001, Mann Whitney *U* test). *(B)* summed T cell frequencies from subjects responding to peptide pools from early secretory antigenic target-6 and culture filtrate protein-10. Graphs show medians and inter-quartile ranges. P values for differences are given (Mann Whitney U).

PBMC, peripheral blood mononuclear cells; SFC, spot forming colony

Figure 7.

Summed T cell frequencies from subjects responding to *(A)* summed peptides from early secretory antigenic target-6 and culture filtrate protein-10 or to *(B)* PPD are shown stratified by tuberculin skin test result in active and non-recent latent infection. Graphs

show medians and inter-quartile ranges. Where differences are statistically significant, P values are given (Mann Whitney U).

PBMC, peripheral blood mononuclear cells; PPD, purified protein derivative; r, recombinant; SFC, spot forming colony; TST, 15mm tuberculin skin test

TABLE 1

DEMOGRAPHIC AND CLINICAL CHARACTERISTICS OF THE STUDY POPULATIONS

Characteristic	Active infection		Recent latent infection		Non-recent latent infection	
Total	167[a]		149		38	
Age (years) (median, interquartile range):	30 (25 to 41)		13 (11 to 14)		51 (31 to 63)	
	%	n	%	n	%	n
Sex:						
Male	59.3	99	52.3	78	71.1	27
Ethnic origin:						
Indian Sub-continent	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:						
BCG vaccinated	58.1	97	79.9	119	39.5	15
BCG vaccination status	11.4	19	20.0	30	31.6	12

unknown						
Tuberculin skin test[b]:						
Positive	63.5	106	76.5	114	31.6	12
Unknown	20.3	34	0	0	10.5	4
Comorbidity:						
None	82.0	137	96.6	144	57.8	22
Previous tuberculosis	9.0	15	0	0	0	0
Diabetes	5.4	9	0	0	21.1	8
HIV infection	4.2	7	0	0	0	0
Sarcoid	1.8	3	0	0	0	0
Alcohol dependency	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[c]	3.4%	5	28.9	11[d]

1

2