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Cellular Immune responses during latent tuberculosis : immunodiagnosis and correlates of protection

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Chapter 8

Recognition of stage-specific mycobacterial antigens differentiates between acute or latent infection with *M. tuberculosis*

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ABSTRACT

M. tuberculosis is estimated to infect 80-100 million people annually, the vast majority of whom do not develop clinical tuberculosis, but instead maintain the infection in a latent state. These individuals generally become positive to a tuberculin skin test and may develop clinical tuberculosis at a later date, particularly if their immune system is compromised. Latently infected individuals are interesting for two reasons: first, they comprise an important reservoir of *M. tuberculosis*, which needs to be considered for tuberculosis control. Second, if detected prior to recrudescence of the disease, they represent a human population that is making a protective immune response to *M. tuberculosis*, which is very important for defining correlates of protective immunity. In this study, we show that while responsiveness to the ESAT-6 antigen is a good marker for *M. tuberculosis* infection, a strong response to the 14 kDa Rv2031c-encoded antigen (HspX or α -crystallin) is largely restricted to the latently infected individuals, offering the possibility of differential immunodiagnosis of, or therapeutic vaccination against, TB.

INTRODUCTION

Tuberculosis (TB) is easily transmitted by inhalation of infectious *M. tuberculosis* bacilli from the air and is one of the leading causes of death in adults between the ages of 15-45 (1). The global incidence rate of TB is increasing by approximately 0.4% per year (1) driven by factors such as the HIV/AIDS epidemic, poverty, and increasing population density. Despite the existence of effective treatment regimens, control of TB is complicated by the chronic nature of the disease. Only 5-10% of recently-exposed individuals develop clinically active TB in the first 2 years after exposure and this, together with the often transient nature of exposure, makes accurate tracing of recently exposed and potentially infected individuals extremely difficult.

A higher percentage of exposed individuals become infected, but are able to limit the growth of the mycobacteria and become latently infected. The exact incidence of latent infection remains unclear, but it is estimated that up to 2 billion people globally may harbor latent TB infections (1-3). Latency can only be understood as a dynamic process – an active host immune response is essential to keep the disease in the latent stage, as demonstrated by the high rate of TB recrudescence in individuals with HIV infection (4) or the reactivation of the latent infection in individuals given anti-TNF- α therapy (5). The decline of the immune system due to old age or malnutrition also increases the risk of latent disease becoming clinically significant.

Thus, latently-infected individuals provide a highly relevant population to study what human protective immune responses against *M. tuberculosis* look like. However, identifying these people has been problematic in the past. The classical definition of latent infection (conversion to positivity in the PPD-based skin test after exposure to *M. tuberculosis*, without clinical illness) is of limited reliability, especially in TB-endemic countries, where widespread BCG vaccination or high levels of exposure to environmental mycobacteria often lead to skin test positivity. We have therefore used immune recognition of the *M. tuberculosis* virulence factor ESAT-6 (Early Secreted Antigen Target, 6 kDa) encoded by *esat6* Rv3875, to define *M. tuberculosis* infection (6;7). ESAT-6 is essential for the bacteria to survive and spread *in vivo*, (8;9) is expressed early in infection and recognized by most TB patients (10). Although immune responses to ESAT-6 by themselves cannot separate acute or latent TB (7;11), recognition by healthy individuals without recent *M. tuberculosis* exposure is currently the best operational definition of latent *M. tuberculosis* infection (3;6;7).

Using this approach we have recently shown that latently-infected healthy individuals produce elevated levels of mRNA for Th1 cytokines, such as IFN- γ , and in particular, the antagonistic splice variant of IL-4, IL-4 δ 2 (6;7). This implicates IL-4 in the clinical form of illness, a hypothesis supported by recent publications showing elevated IL-4 production in individuals with clinical illness or progressing towards clinical illness ((12-14) and

Demissie *et al.*, submitted). However, *M. tuberculosis* is not a passive participant in this process. The bacteria respond to the host immune response by controlling their own gene expression and may interfere with the host's immune response as well (15-18).

Typically, in latent infection, bacteria are present only in low numbers. The physical microenvironment where the bacteria survive has not been characterized in detail, but is thought to include restricted access to nutrients and to oxygen and low pH, together with elevated levels of hydrolytic enzymes, and reactive nitrogen and oxygen species released by the host's immune response. The abundance of regulatory proteins in the *M. tuberculosis* genome (19), may explain the ability of the pathogen to adapt to this hostile environment (15;20) by upregulating so-called latency genes. One of the most prominent of these is Rv2031c (also known as α -crystallin, HspX, or the 16 kDa antigen) whose importance is demonstrated by the reduced ability of bacteria deficient for this gene to grow in macrophages (21). Rv2031c is clearly expressed during infection in humans, as it is recognized by sera from a majority of TB patients (22). Moreover, production of Rv2031c appears to increase as the bacteria go into the metabolically resting stage (23) and decrease as they revert to exponential growth (24). It therefore serves as the prototypic "latency-associated antigen"

In this report, we compared immune responses to Rv2031c with responses to ESAT-6 in TB patients, contacts with well-defined recent exposure to TB and healthy individuals with and without evidence of prior infection with *M. tuberculosis*. The data, collected at multiple sites in TB-endemic and non-endemic regions suggest that the ratio of immune responses to ESAT-6 and Rv2031c may be characteristic of different phases of *M. tuberculosis* infection. These results offer hope that specific diagnostic assays can be developed to distinguish between progressive and latent TB, even in individuals who are currently asymptomatic and suggest that vaccination aimed at boosting immunity to latency-phase antigens may be feasible.

MATERIAL AND METHODS

Study sites and subjects

The study sites included Hossana and Butajira in the Southern Region of Ethiopia, Fajara, in the Gambia, and Leiden in the Netherlands. The African cohorts analyzed are from an ongoing, multi-centre longitudinal study being carried out in Africa (VACSEL/VACSIS). These cohorts have been previously described in detail (6;7;11). Briefly, healthy household contacts (HHC) of TB patients (TB) were recruited from TB clinics. In both cases, blood was drawn on the first visit, before treatment of index cases commenced. Community controls (CC) were randomly selected from the same neighborhoods and prior TB disease or contact with TB excluded by questionnaire. Pulmonary TB was confirmed

by the presence of acid-fast bacilli in at least two of three consecutive sputum samples, or one positive sputum sample and a positive culture. Active TB was excluded in all healthy participants (HHC and CC) on entry to the study by radiological and clinical examination, sputum microscopy and culture as previously described (11).

In the Netherlands, TB patients with pulmonary- and extra-pulmonary tuberculosis were recruited at the clinic and blood samples were taken from 9 patients during active tuberculosis and from 10 patients 1 - 8 years after successful treatment. Exposed, non-BCG vaccinated individuals with a tuberculin skin test (TST) of >10 mm induration were recruited based on TB control program records as non-endemic latently-infected individuals. Twenty of these had not received any prophylactic antibiotic, whereas three subjects had received INH treatment 1-3 years post-conversion. At the time of writing, all TST positive persons have remained free of clinical TB (12 years mean follow-up). Dutch community controls were randomly selected and were TST negative, unvaccinated individuals with no known history of exposure to TB.

Blood samples were obtained from all donors at entry to the study. All participants in Africa were screened for HIV by repeated ELISA and samples from HIV-positive individuals were excluded from the study. Pre- and post-test counseling was offered to all these participants. Only adults (18 years or older) who had given written consent were included in the study and this work was performed under a study protocol approved by the institutional and national ethical review boards.

Sample preparation

Venous blood (15-30 ml) was drawn into a Venoject tube containing heparin (Terumo, Leuven, Belgium) using a butterfly needle and gently mixed by inversion. PBMC were enriched as previously described (11;25) by centrifugation over Ficoll-Hypaque (Pharmacia Biotech) and washing in complete medium (RPMI-1640 supplemented with 5% heat inactivated pooled human AB serum, 1% L-glutamine and 1% penicillin/streptomycin) before use for *in vitro* assays. Samples were then frozen in liquid nitrogen. Frozen cell samples were thawed, washed multiple times in complete medium before use. In all cases, viable cells were counted and diluted to 2×10^6 viable lymphocytes/ml in complete medium and 100 μ l per well used for both ELISA and ELISpot.

Antigen Preparations

All recombinant antigens were produced using the same protocol previously described (26). Briefly, the full-length genes were PCR-amplified from cloned *M. tuberculosis* genomic DNA and subcloned. His-tagged proteins were expressed in *Escherichia coli* XL-1 Blue and purified essentially as described previously (27). Purified recombinant antigen was solubilised and stored at -80°C until use.

IFN- γ Enzyme-Linked Immunosorbent Assay (ELISA)

In vitro restimulation of PBMC with either PPD (20 $\mu\text{g}/\text{ml}$) or recombinant antigen (2 $\mu\text{g}/\text{ml}$) was carried out using the same protocol as previously described (11). The supernatants were harvested at day 5 after stimulation and stored at -80°C until assayed. The levels of IFN- γ were assayed from duplicate culture supernatants using Capture mAb 1-D1K and signaling mAb 7-B6-1, in accordance with the manufacturer's instructions (Mabtech, AB., Sweden) and concentrations of cytokine were calculated using the standard curve generated from recombinant human IFN- γ or IL-4 (Life Technologies, Paisley, UK). Results are expressed in pg/ml and the cut-off for positivity was set at three standard deviations above the mean of unstimulated wells (110 pg/ml). The difference between duplicate wells was consistently less than 10% of the mean.

Enzyme-Linked Immunospot Assay (ELISpot)

The assay was performed as previously described (6). For this study, we tested ESAT-6 or Rv2031c (10 $\mu\text{g}/\text{ml}$) (Statens Serum Institut, Copenhagen, Denmark), PHA (5 $\mu\text{g}/\text{ml}$) as positive control or media as negative control. Cells were recovered from liquid nitrogen and serially titrated, in duplicate wells of anti-human IFN- γ precoated ELISpot plates (MAIPS45; Millipore). ELISpot plates were counted using an AID plate reader (Autoimmun Diagnostika, Strasburg, Germany). The mean number of spot-forming cells (SFC) per well for each antigen was calculated, the mean SFC of the negative control subtracted and transformed to SFC per 10^6 cells. A positive response to antigen was taken as twice the background with more than 10 spot forming units (SFU).

Statistics

Comparisons between groups were assessed by the Kruskal-Wallis and Dunnett's multiple comparisons tests. The Mann-Whitney test was used for analyses within groups. In all instances, a P value < 0.05 was considered significant.

RESULTS

Assessment of IFN- γ responses induced by early and late stage antigens from *M. tuberculosis*

The Ethiopian study cohort was divided into three groups based on their clinical status: index cases (designated TB, $n = 44$) healthy household contacts (designated HHC, $n = 82$) and community controls (designated CC, $n = 20$). Cells from the peripheral blood of each group were stimulated *in vitro* with either ESAT-6 (a so-called early stage antigen) or RV2031c (the prototypic late stage antigen) and the IFN- γ responses assessed by ELISA. As can be clearly seen in Figure 1, all three groups contained a wide range of ESAT-6

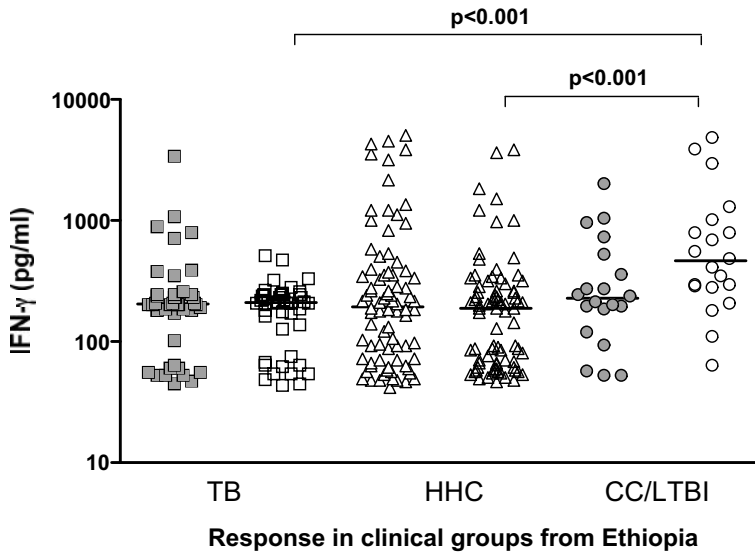


Figure 1

In vitro IFN- γ responses to restimulation with ESAT-6 (Filled points) or Rv2031c (Unfilled points) of PBMC from TB (n =44), HHC (n = 82) and CC (n =20) clinical groups from Ethiopia, as assessed by ELISA. Results are individual responses, expressed in pg/ml. The medians of the groups are shown, and levels of cytokine which were significantly different between groups are indicated.

responsive individuals, but although responses to Rv2031c were also present in all three groups, the median response was significantly higher in the CC group. A similar pattern was observed for the Gambian cohort (TB, n=12, HHC, n = 32, CC, n = 20), but using the ELISpot assay instead of ELISA. While these assays are not exactly comparable in terms of magnitude, the general trends observed are comparable (28). ESAT-6 responses were not significantly different, but the number of cells responding to Rv2031c in the CC group was significantly elevated compared to the other two clinical groups (Figure 2).

However, responses to the group as a whole tell us little about individual responses. We therefore visualized the results by plotting the IFN- γ response to the two antigens against each other for every antigen-responsive individual. When the responses were compared for each individual, a pattern was discernible, with immune responses to ESAT-6 tending to dominate responses to Rv2031c in subjects from the TB group, while individuals in the CC group were more biased towards Rv2031c, even if responsive to ESAT-6. This yielded a line of regression for each study group, and as shown in Figure 3A, in the Ethiopian cohort, there was a gradation from the acute TB group to the CC group, with the HHC group in between. This is made plain by comparing the slopes of the lines, as shown in

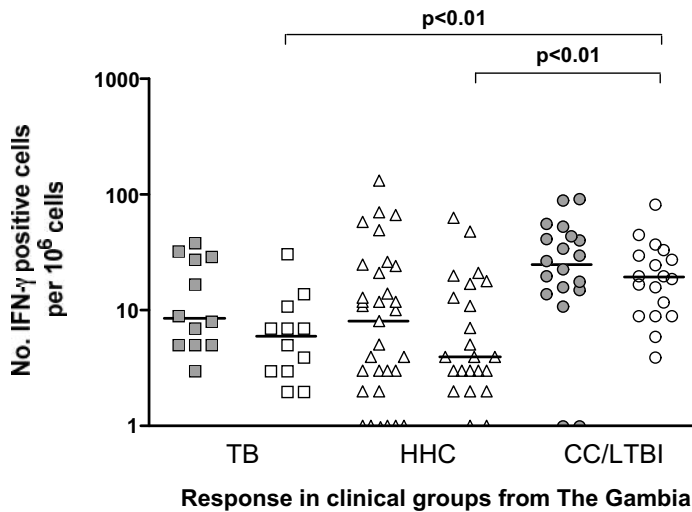


Figure 2

In vitro IFN- γ responses to restimulation with ESAT-6 (Filled points) or Rv2031c (Filled points) of PBMC from TB (n = 12), HHC (n = 32) and CC (n = 20) clinical groups from The Gambia, as assessed by ELISpot. Results are individual responses, expressed as number of spots per 10^6 PBMC. The medians of the groups are shown, and groups in which numbers of IFN- γ producing cells were significantly different are indicated.

Figure 3B. The same type of analysis for the Gambian cohort, using the ELISpot data, yielded the same conclusion (Figure 3C).

Elevated Rv2031c responses are associated with latent infection

The data presented above suggest that there are substantial numbers of ESAT-6 responsive individuals in each group. For TB patients and household contacts this is hardly surprising and the incidence of positivity in the latter group from The Gambia matches that of a recent, larger study, supporting the representative nature of the data shown here. However, the number of ESAT-6 positive subjects among the community control groups can only be plausibly explained by the assumption that in a TB-endemic community, there will be cases of unsuspected latent TB infection even among those healthy individuals without identified exposure to TB cases. This finding is consistent with a large body of earlier studies (3;7;29;30) and this group was therefore designated CC/LTBI (to designate Latent TB Infection). To evaluate this further, we divided the CC group from the Ethiopian cohort into ESAT-6 high responders (3 or more standard deviations above the mean of unstimulated wells) who were presumptively latently infected (CC-Hi) or low responders (CC-Lo) and re-analyzed the data from the IFN- γ ELISA. As shown in Figure 4A, the response to Rv2031c was with the response to ESAT-6 in all groups (consistent with the hypothesis that the response is due to *M. tuberculosis* infection). However, the

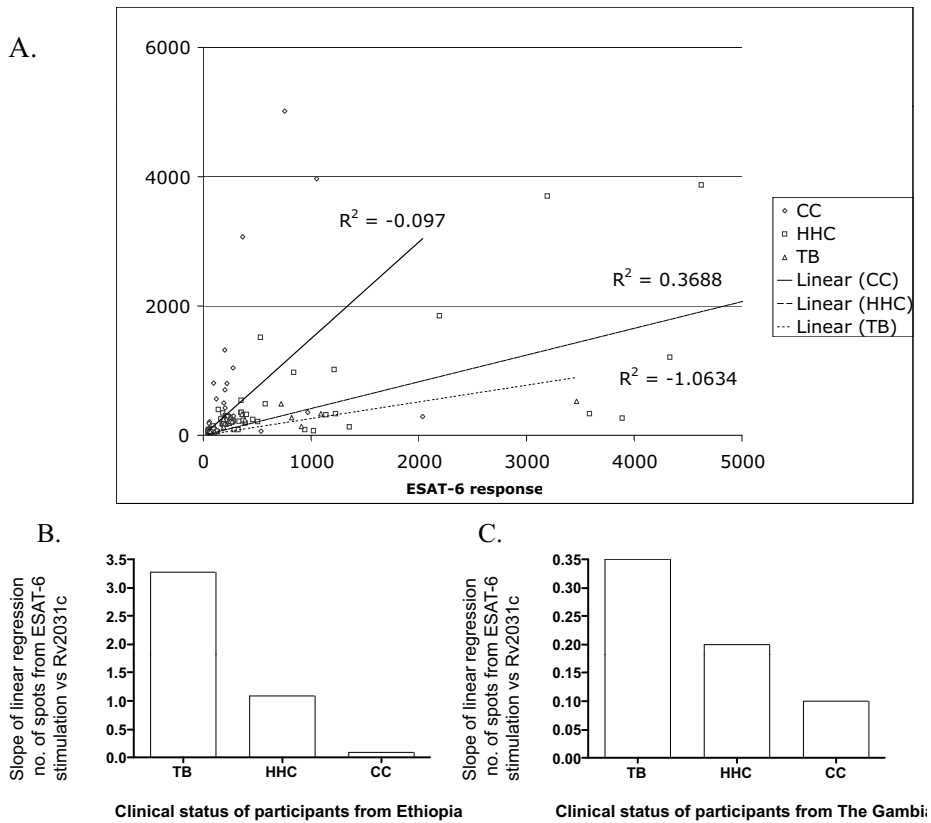
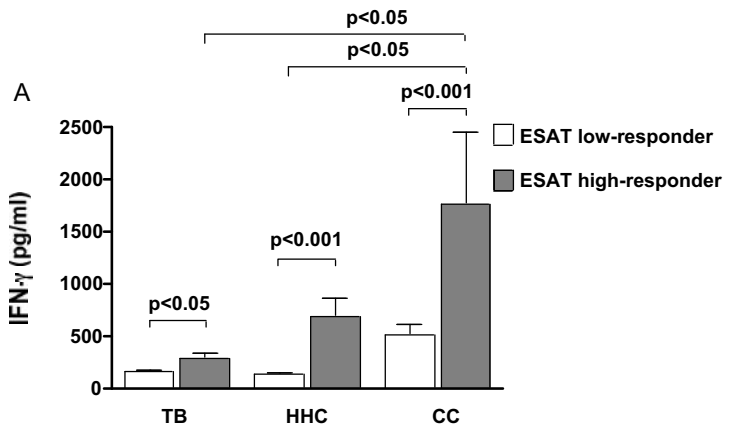


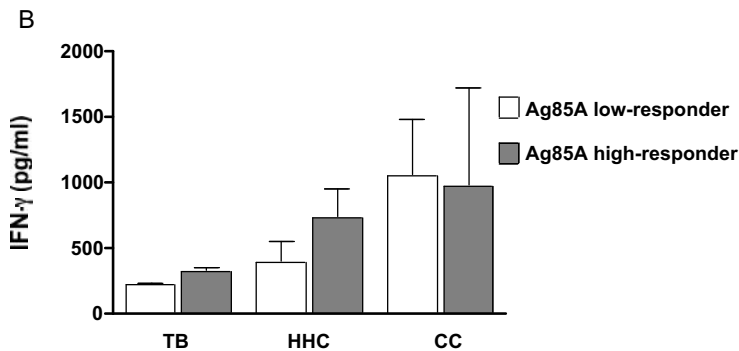
Figure 3

Regression analysis of the levels of the IFN- γ produced in response to ESAT-6 or Rv2031c in clinical groups from Ethiopia (A). The slopes of the line of regression for this analysis (B) and an identical analysis performed on ELISpot data from The Gambia (c) show the bias of individual responses to one or the other antigen.

magnitude of the response was greatest in the ESAT-6 responsive (and therefore presumably latently-infected healthy individuals). Analysis of individual responses demonstrated that in the CC group, the strongest Rv2031c responders are also the strongest responders to ESAT-6, as indicated by the relatively low ratio of ESAT-6 to Rv2031c responses shown in Figure 3. To ensure that this correlation was not simply coincidental, we segregated the CC group using the same criteria, but based on the response to the shared mycobacterial antigen Ag85A, instead of ESAT-6. As can be seen in Figure 4B, there were no significant differences between the clinical groups, when separated on the basis of reactivity towards Ag85A. These data indicate that the responses against Rv2031c seen in the strongly ESAT-6-responsive individuals from the CC group are consistently associated with responses to ESAT-6, but are particularly elevated in those subjects thought to have latent infection with *M. tuberculosis*. An analysis of the Gambian cohort using the



RV2031c response in ESAT-6-responsive and non-responsive individuals from Ethiopia

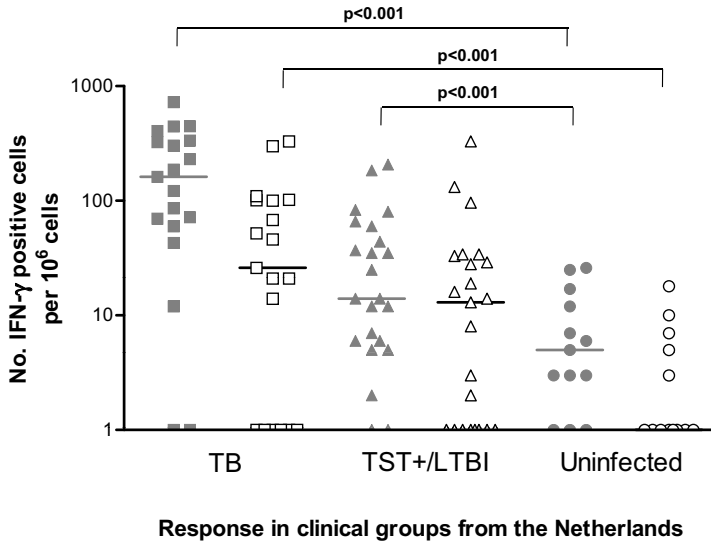


RV2031c response in Ag85A-responsive and non-responsive individuals from Ethiopia

Figure 4

In vitro IFN- γ responses to restimulation with Rv2031c of PBMC from the community control groups from Ethiopia after segregation into ESAT-6 high (CC-Hi) and low responders (CC-Lo) (A) or Ag85A high (CC-Hi) and low (CC-Lo) responders (B), as assessed by ELISA. Results are means and standard deviations, expressed in pg/ml. Responses that were significantly different between groups are indicated.

same analysis led to the same conclusion, with the median response to Rv2031c among the high ESAT-6 responder CC group (45 spots/ 10^6 PBMC) being significantly higher than in the low responder CC group (17 spots/ 10^6 PBMC, $p < 0.005$) and significantly higher than the ESAT-6 high responders from the TB or HHC groups ($p < 0.01$, data not

**Figure 5**

In vitro IFN- γ responses to restimulation with ESAT-6 (Filled points) or Rv2031c (Unfilled points) of PBMC from TB patients ($n = 19$), healthy TST+ contacts ($n = 23$) and uninfected controls ($n = 13$) from the Netherlands, as assessed by ELISpot. Results are individual responses, expressed as number of spots per 10^6 PBMC. The levels of IFN- γ producing cells that were significantly different between groups are indicated.

shown). Thus, this analysis shows that in both African cohorts the strongest Rv2031c responses are found in latently-infected healthy individuals (ESAT-6 responders in the CC/LTBI group). Even in those TB patients who strongly respond to ESAT-6, the response to Rv2031c was relatively low compared to the latently infected individuals in the CC group. This indicates that the lower Rv2031c responses observed in the TB patients can not be entirely attributed to the generalized suppression of immune responses which can be observed during active disease. Since the CC group from both African cohorts was selected as much as possible to avoid recent TB exposure, the responses to ESAT-6 in this group is most easily explained by latent infection arising from a prior, unidentified encounter with an infectious TB case.

Immune responses to ESAT-6 and Rv2031c in clinical cohorts from a non TB-endemic setting

All of the data presented above are from TB-endemic countries, where although it is unlikely that the ESAT-6 responses in the CC group are due to a recent exposure, the possibility cannot be ruled out. Therefore we carried out a similar analysis in a non-TB endemic setting. We recruited 19 tuberculosis patients, 23 latently-infected (TST positive)

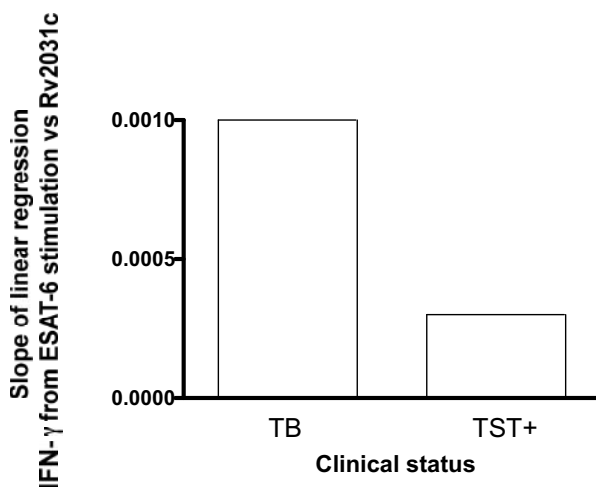


Figure 6

Regression analysis of the levels of the IFN- γ produced in response to ESAT-6 or Rv2031c in clinical groups from The Netherlands, plotted against one another. The slopes of the line of regression for this analysis show the bias of individual responses to one or the other antigen.

individuals and 13 healthy, non-BCG vaccinated, TST negative controls (Uninfected). As before, we investigated T cell recognition of Rv2031c and ESAT6 in the different groups by IFN- γ ELISpot assay. Unlike the CC groups from Ethiopia and the Gambia, the unexposed group from the Netherlands would not be expected to contain a latently-infected subset. Not unexpectedly therefore (31), the Dutch uninfected group had no significant response to either ESAT-6, or to Rv2031c, suggesting that (at least in a developed world setting) exposure to environmental mycobacteria was not sufficient to generate any cross-reactivity against *M. tuberculosis*-derived Rv2031c (Figure 5). However, the responses of the acute and latent TB groups were also noticeably different to the parallel groups derived from the TB-endemic countries. The TB patient group had significantly higher responses to ESAT-6 than the uninfected group and the TST+ (latently-infected) group. In contrast, the Rv2031c responses in the latently-infected, TST+ group were not significantly different from the acutely infected TB group (though both groups were significantly more responsive to both ESAT-6 and Rv2031c than the uninfected group).

To further analyze the pattern of antigen recognition in TB patients versus latently infected individuals, the number of T cells specific for the early stage antigen ESAT-6 were plotted against the number of T cells specific to the late stage antigen RV2031c, as was done for the cohorts from TB-endemic regions, above. The lines of regression had quite different slopes between the two groups (Figure 6) with the same bias as seen in the

cohorts from TB endemic countries. These results confirm that individuals with acute infection and demonstrate a relatively strong T cell response towards ESAT-6 compared to Rv2031c, while this is not seen in TST-converters, who are assumed to be latently-infected.

DISCUSSION

The course of a *M. tuberculosis* infection is very complex, with only 5-10% of infections thought to progress to active TB in a short time. In most of the infected individuals, some bacteria are believed to survive and enter a quiescent phase resulting in latent infection, which can reactivate later in life. So an infection with *M. tuberculosis* can give rise to disease anywhere from months to decades after infection. The estimated one third of the world's population that harbors latent TB therefore represents an immense infection reservoir and future source of disease transmission (1). Characteristically, *M. tuberculosis* initially enters the body by infecting alveolar macrophages. The acute phase of infection is characterized by rapid bacterial growth and the development of an immune response dominated by recognition of bacterial antigens actively secreted in the first growth phase, such as ESAT-6 (32). The development of an cell-mediated, inflammatory response is clearly involved in arresting bacterial growth and subsequently restricting the infection to the latent stage, as is shown by the significant increased risk of reactivation attendant on HIV infection (4) or inhibition of TNF- α (33).

Though an obligatory aerobic organism, *M. tuberculosis* is able to adapt to and survive in the hypoxic and hostile environment of host macrophages. In this regard, it has been shown that *M. tuberculosis* undergoes a dramatic change in gene transcription characteristic of non-replicating persistence (15;20). When grown *in vitro* in oxygen-depleted cultures as well as in cultures exposed to nitric oxide, *M. tuberculosis* up-regulates overlapping, characteristic sets of genes (16;23;34) among which is the gene encoding the small 16kDa heat shock protein, Rv2031c. Expression of this protein is increased by a factor of between approximately 4 and 7 during the stationary growth phase (23) and appears to be crucial for the survival of the organism (21). Recently, North and colleagues used real time PCR in a mouse model of TB infection to demonstrate that when the immune response reaches the point where it inhibits the growth of the bacteria, the transcription of a number of genes is down-regulated, while that of Rv2031c is up-regulated (35).

Accordingly, in the present study we have compared recognition of the late-expressed antigen Rv2031c with an antigen (ESAT-6) expressed early during bacterial multiplication and also (at a reduced level) later in infection. Immune recognition of ESAT-6 is known

to be highly specific for exposure to members of the tuberculosis complex (3;36-39) so it serves as a marker for prior *M. tuberculosis* infection. The data presented here demonstrate that Rv2031c, a protein which is known to be induced during stress-restricted growth of *M. tuberculosis in vitro*, was most strongly recognized by T-cells from individuals with evidence of previous *M. tuberculosis* infection, but who had neither symptoms nor any evidence of recent exposure to infectious TB cases – a profile most consistent with Latent TB. This strongly suggests that Rv2031c is indeed expressed by *M. tuberculosis* in humans during latent infection. Moreover, in persons with acute TB or recent TB infection, the Th1 immune response, as measured by IFN- γ , while including an Rv2031c-responsive component, is biased towards ESAT-6, while in persons who have become latently infected after exposure to *M. tuberculosis*, the immune response is predominantly targeted towards Rv2031c. This led to observation of a consistent gradient in the ESAT-6/Rv2031c response ratio in the study groups, with a high ratio in TB patients, a low ratio in CC from endemic regions (due almost entirely to latently-infected individuals) and an intermediate ratio in the HHC groups (recently-exposed, healthy individuals). Moreover, this bias was detectable in study groups of different ethnicity drawn from three geographically-distinct sites, with very different incidence rates of TB. The differences in the magnitude of the responses that were seen may reflect the environment in which the cohorts live, or it may reflect the nature of the patient's infection. In general, TB patients in Ethiopia and the Gambia are self-referred to the clinic and very often present with an advanced state of the disease, compared to TB patients in Europe. This is associated with a generalized suppression of immune responses (11;25;30) which may explain why the African TB patients had relatively lower responses to both ESAT-6 and Rv2031c compared to their Dutch counterparts, while the immune responses in the latently infected groups (who were all healthy) were comparable for both sites.

In this analysis, we have focused on cell-mediated immune responses to Rv2031c, whereas most previous work has looked at humoral responses. The most recent study of this kind, which tested serological responses to a panel of antigens, including Rv2031c reached exactly the same conclusion as we have done (40). This strongly suggests that this is a general phenomenon related to *M. tuberculosis* infection. Other studies are also compatible with this hypothesis – while antibody to Rv2031c increased in TB patients during treatment, it was initially low, suggesting that humoral responses to this antigen are also minimal during active disease (41), while chronically exposed, but healthy individuals, who may have been latently-infected had high titres of antibody against this antigen. (42). Interestingly, the strong association of Rv2031c with ESAT-6 reactivity and therefore with *M. tuberculosis* infection, implies that exposure to other mycobacteria, whether in the environment, or from BCG vaccination does not induce strong reactivity to Rv2031c, even though genes encoding analogues are present in other mycobacteria. At

first glance, this appears to contradict earlier work (43), but since the BCG vaccines in that study were also exposed to *M. tuberculosis*, it is impossible to determine whether the immune responses are due to BCG vaccination or latent TB infection. Our work would suggest the latter – and this is in agreement with the conclusions of that study, where the authors write that in the case of *M. tuberculosis* “...prolonged containment may be partially mediated by IFN- γ -producing CD4+ T cells responding to the 16 kDa antigen that is expressed by non-replicating bacilli” (43).

There are several implications of these findings. The first is that the differences in antigen recognition described here and the differences in cytokine expression patterns previously described (6;7) are both consistent with the identification of ESAT-6 responsive, healthy individuals without recent TB contact as latently-infected. In addition, these data indicate that a strong IFN- γ response to Rv2031c correlates with a certain level of protection against TB disease, consistent with earlier suggestions (43;44). If validated, this opens the possibility that generating (or boosting) immune responses against antigens expressed during the latent phase of infection might reduce the reactivation of latent infection. While this is speculative, therapeutic vaccines based on this concept are already being developed by a number of groups. Finally, these data also provide proof of concept for an immunodiagnostic test that could potentially allow clinicians to identify a *M. tuberculosis* infection by ESAT-6 responsiveness and estimate the risk that it is progressive or latent by determining the ratio of the response to ESAT-6 and Rv2031c. Such an immunodiagnostic test would make it more feasible to identify and treat active TB in a very early stage, which could finally reduce the transmission of *M. tuberculosis*. This is of particular importance in TB-endemic countries, where the current priority is on treating active TB and where the huge reservoir of latent infection is likely to greatly reduce the specificity of immunodiagnosis based on RD antigens (3;7;29;30). However, longitudinal studies will be needed to confirm whether ESAT-6/Rv2031c response ratios are indeed predictive for progression to active TB disease. Further, it is likely that multiple early and late stage antigens will be required in order to obtain sufficient sensitivity for such an immunodiagnostic test. In this regard, it is encouraging that immune responses to CFP10, another early antigen from *M. tuberculosis*, (3;45) show a very similar pattern to those presented here for ESAT-6, albeit CFP10 was only tested with a subset of the samples tested for ESAT-6 (data not shown) and that other antigens may mimic the pattern seen here with Rv2031c(40). Proteomic and transcriptomic analyses have identified more than 200 genes whose transcription is altered by transition to non-replicating hypoxic growth conditions, so it will take some time – and much work - to assess the diagnostic potential of these antigens, alone or in combination.

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