Discovery of dormancy associated antigens of Mycobacterium tuberculosis : novel targets for the development of post-exposure or therapeutic tuberculosis vaccines

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Chapter 2

Human T-cell responses to 25 novel antigens encoded by genes of the dormancy regulon of *Mycobacterium tuberculosis*

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Abstract

The dormancy (DosR) regulon of *Mycobacterium tuberculosis* is expressed in vitro during hypoxia and low-dose nitric oxide stimulation. Tubercle bacilli are thought to encounter these conditions in humans during latent infection. In this study, immune responses were evaluated to the 25 most strongly induced DosR-regulon-encoded proteins, referred to as latency antigens. Proliferation assays were performed using *M. tuberculosis*-specific T-cell lines and peripheral blood mononuclear cells (PBMC) from tuberculosis (TB) patients, tuberculin skin test positive (TST+) individuals and uninfected controls. All 25 latency antigens were able to induce production of interferon-gamma (IFN-γ) by T-cell lines. Eighteen latency antigens were also recognized by PBMC of *M. tuberculosis*-infected individuals, which indicates expression of the DosR-regulon during natural infection. Differential analysis showed that TST+ individuals recognized more latency antigens and with a stronger cumulative IFN-γ response than TB patients, while the opposite profile was found for culture filtrate protein-10. In particular Rv1733c, Rv2029c, Rv2627c and Rv2628 induced strong IFN-γ responses in TST+ individuals, with 61%, 61%, 52% and 35% responders, respectively. In conclusion, several new *M. tuberculosis* antigens were identified within the DosR-regulon. Particularly strong IFN-γ responses to latency antigens were observed in latently infected individuals, suggesting that immune responses against these antigens may contribute to controlling latent *M. tuberculosis* infection.

Introduction

Tuberculosis (TB) is a major threat to global health with an estimate of four persons dying of TB every minute. Surveys with tuberculin skin tests (TST) suggest that one-third of the world's population is latently infected with *Mycobacterium tuberculosis*. This enormous reservoir of latent TB, from which most cases of active disease arise, embodies a major obstacle in achieving worldwide control of TB [1]. In particular when taken into account the currently ongoing AIDS pandemic, as in HIV-1 infected individuals risk of progression from a latent infection to active TB increases significantly, up to 8-10% per year [1,2]. The available vaccine against TB, *M. bovis* bacillus Calmette-Guérin (BCG), only provides significant protection against severe TB disease in young infants and establishment of *M. tuberculosis* infection [3], but affords limited and highly variable protection against pulmonary TB in adults, which is mostly due to reactivation of latent *M. tuberculosis* [4,5].

Both $CD4^+$ and $CD8^+$ T-cells and the cytokines IFN-γ and TNF-α play an important role in the immune response against mycobacteria and in controlling latent infection [6,7]. During latent infection, tubercle bacilli are typically contained within immune-mediated granulomas [8]. However, before the era of chemotherapy, it has been shown that live tubercle bacilli can also be present in macroscopically normal lung tissue [9]. This finding was recently confirmed by in-situ PCR [10], but these tubercle bacilli could not be visualized using Ziehl-Neelsen staining. It has been reported that this is caused by distinct cell-wall alterations of tubercle bacilli during persistence [11].

It is generally thought that persisting tubercle bacilli are subjected to nutrient and oxygen deprivation, microbicidal molecules such as reactive oxygen intermediates and nitrogen intermediates, and immune-mediated killing (reviewed in [12,13]). However, little is known about antigen-specific human T-cell responses against persisting mycobacteria, particularly in the context of latent infection and protection against TB disease. Previous studies of tubercle bacilli cultured under low oxygen report that HspX, also known as 16-kDa alpha-crystallin homologue (Acr) or Rv2031c, is highly expressed during these conditions [14,15]. Cellular immune responses to HspX have been observed in latently infected individuals, while antibodies to this antigen were predominantly found in persons with active TB disease [16]. More recently, HspX was found to be part of the so-called dormancy (DosR) regulon of *M. tuberculosis* comprising 48 genes [15,17-20]. Expression of the DosR regulon is observed as part of the adaptive response of *M. tuberculosis* to hypoxia, including gradual oxygen depletion, which results in distinct stages of non-replicating persistence in the so-called Wayne model [21], and to low-dose nitric oxide stimulation [19]. These in vitro culture models represent proxies of conditions that tubercle bacilli are thought to encounter in vivo during persistence in immune competent hosts [13,22]. This is supported by observations that genes of the DosR regulon are upregulated in IFN-γ activated *M. tuberculosis*-infected murine macrophages and in lungs of chronically infected mice [20,22,23]. In humans, increased levels of mRNA of HspX were found in lungs of patients with chronic active TB [23].

The functions of most DosR regulon encoded proteins are unknown [14,24]. At present it is also not known to which extent DosR regulon encoded proteins are expressed during the different stages of natural *M. tuberculosis* infection in human, nor is it known whether these proteins are targeted as part of the protective immune response to *M. tuberculosis*. To start addressing these questions we have selected 25 of the most strongly expressed proteins of the DosR regulon, further referred to as latency antigens, and evaluated IFN-γ responses and proliferation of human T-cells in TB patients, TST positive (TST+) individuals and *M. tuberculosis*-uninfected controls.

Materials and Methods

Study subjects

Our primary goal was to assess the potential immunogenicity of novel candidate *M. tuberculosis* latency antigens. Therefore, we selected a wide spectrum of *M. tuberculosis* infected individuals, as is described in detail below. In total, three populations were studied: TB patients, TST^+ healthy individuals and *M. tuberculosis* uninfected healthy individuals.

The group of TB patients (n=20) consisted of 11 persons with TB during treatment (mean duration of treatment: 10 weeks, range 2-25) and 9 with cured TB who were treated between 4-63 years before blood sampling (mean interval 29 years). Eleven patients had pulmonary and 9 had extra-pulmonary TB. The mean age was 46 years (range 17-75 years), 14 were male. Eight TB patients were of Dutch origin, 9 of African and 3 of Asian origin. TB patients were either HIV-1 seronegative or had no risk factors for exposure to HIV.

All 23 TST+ healthy individuals were of Dutch origin and were not BCG vaccinated. They had a documented TST result of ≥ 10 mm induration, mostly (n=14) after contact with a case of smear-positive pulmonary TB. The mean age was 37 years (range 21–63), 14 were male. From 12 persons, blood was drawn within 6 months after TST conversion, of whom only 5 were treated with isoniazid. In the remaining 11 TST+ individuals, the mean interval between conversion and blood sampling was 5.4 years (range 2-8). Only 2 of these remote TST converters had received isoniazid. Up to the time of this writing, none of the TST+ individuals had developed active TB after a mean period of 5.5 years (range 2.5-11) since TST conversion. These individuals are regarded as latently infected persons who most likely have acquired natural immunity against development of TB disease.

As a control group, 21 uninfected healthy individuals were studied; none of whom were BCG vaccinated or had had any known exposure to TB. They were either TST negative (n=18; in the others no TST was done) and/or did not respond to *M. tuberculosis*-specific proteins ESAT-6 and CFP-10 in an IFN-γ ELISPOT (known from a previous project [25]). All controls were of Dutch origin with an average age of 30 years (range 22-44), 7 were male.

Blood samples were collected after written informed consent was obtained. PBMC were isolated and stored in liquid nitrogen as previously described [26]. The study

protocol (P207/99) was approved by the institutional review board of the Leiden University Medical Center.

M. tuberculosis antigens and peptides

M. tuberculosis H37Rv was grown for 24 hours in tubes with tightly screwed caps, harvested and lysed as previously described [15]. The culture filtrate of this low oxygen culture was concentrated with a centriprep-concentrator and protein concentration was determined by the BCA test (Pierce, Rockford, Illinois). The lysate from *M. tuberculosis* cultured under standard aerated conditions was provided by the RIVM (Bilthoven, the Netherlands).

DosR regulon encoded genes were selected on basis of their RNA expression level in microarray experiments [19], as most of these 48 genes are hypothetical open reading frames with unknown biological function. The 25 most strongly induced genes were selected and sequences were obtained from http://genolist.pasteur.fr/TubercuList (Table 1). Genes were cloned and proteins were overexpressed in *Escherichia coli* and purified, as previously described [27]. Size and purity were checked by gel electrophoresis and Western blotting with anti-His antibodies. Residual endotoxin levels were determined with a Limulus Amebocyte Lysate assay (Cambrex) and were found to be below 50 IU/mg recombinant protein. Protein batches were tested for non-specific T-cell stimulation in lymphocyte stimulation assays using PBMC of BCG-unvaccinated, TST-negative healthy donors.

Synthetic peptides from culture filtrate protein 10 (CFP-10) were produced as previously described [26].

T-cell lines

Eight long-term T-cell lines were generated with either lysate (n=4) or culture filtrate (n=4) of *M. tuberculosis* grown under low oxygen conditions, using PBMC obtained from two cured TB patients and two TST+ individuals known to respond to HspX. In addition, 4 T-cell lines were made by stimulating PBMC from three TB patients and one TST+ individual with lysate from *M. tuberculosis* cultured under standard aerated conditions. T-cell lines were generated as previously described [26].

Proliferation assays and IFN-γ detection

T-cells ($1.5x10^4$/well) were cultured with autologous or HLA-DR matched irradiated PBMC ($5x10^4$/well) for 3 days and PBMC ($1.5x10^5$/well) were cultured for 6 days in the presence or absence of antigen, as previously described [26]. All latency antigens (Table I) were tested at a final concentration of 0.33 μM (average concentration 9.3 μg/ml, range 3.1-24) in order to allow for direct comparison of immunogenicity between proteins which vary considerably in size (9-74 kDa). Standard *M. tuberculosis* lysate, hypoxic lysate and culture filtrate were used at 1 μg/ml and the peptide-pool of CFP-10 at 1 μg/ml/peptide. The same antigens and identical batches were used throughout all experiments. Due to a shortage of the Rv1733c batch the number of study subjects that could be tested for this antigen was limited to 17 controls, 18 TST+ persons and 16 TB patients. After culture, supernatants were collected for the detection of IFN-γ and proliferation of T-cells was measured by [^{3}H]-thymidine incorporation as described elsewhere [26]. The concentration of IFN-γ was measured

by ELISA (U-CyTech, Utrecht, The Netherlands; detection limit of 20 pg/ml). The mean value of unstimulated cultures was subtracted from the mean value of stimulated cultures.

Statistical analysis

For comparison of the proportion of responders in each study group, the chi-square test was used. For all study subjects who were able to respond to an antigen, levels of IFN-γ production were evaluated non-parametrically using Kruskal-Wallis and Mann-Whitney tests for comparison of study groups. The Wilcoxon-signed-rank test was performed to compare the strength of the response between two antigens for each individual. In controls responses to latency antigens were correlated to the response to *M. tuberculosis* lysate using a Spearman rank correlation. A regression analysis was done to compare the cumulative IFN-γ response to latency antigens between groups, while controlling for the effect of age, general responsiveness of T-cells and time between initial infection and blood sampling.

Results

Human T-cell lines recognize latency antigens

Long-term T-cell lines were generated against either lysate (n=4) or culture filtrate (n=4) of *M. tuberculosis* grown under low oxygen conditions or standard aerated conditions (n=4). All lines responded strongly to the preparations of *M. tuberculosis* grown under hypoxic conditions. Readouts included T-cell proliferation (*data not shown*) and IFN-γ production. Figure 1a shows the individual IFN-γ responses to 25 latency antigens of all generated *M. tuberculosis*-specific T-cell lines. Both T-cell lines generated from cured TB patients as well as from TST+ individuals were able to recognize a large number of latency antigens.

In order to ensure the selection of all proteins with possible immunogenic capacity for further evaluation (see below), we used a relatively low level of IFN-γ as cut-off value (50 pg/ml above background) and calculated the number of T-cell lines that responded to each latency antigen (Figure 1b). This figure shows that all 25 latency antigens were recognized by at least 1 out of 12 T-cell lines tested and 21 antigens by at least 4 T-cell lines. Latency antigen Rv2031c (HspX), and Rv2032 (Acg) were most frequently recognized by 75% of tested T-cell lines (median IFN-γ levels among responding lines: 507 and 129 pg/ml respectively). The observed responses to latency antigens by T-cell lines generated with the standard-aerated *M. tuberculosis* lysate are explained by the finding of HspX in this preparation (Western blot analysis; *data not shown*), indicating the expression of the DosR regulon. Most latency antigens were recognized by T-cell lines raised with hypoxic-lysate as well as with hypoxic culture filtrate, suggesting that some latency antigens might also be found extra-cellularly. This is in line with previous findings showing that Rv0569, Rv2623 and Rv2626c proteins were present in culture filtrate of *M. tuberculosis* grown under hypoxic conditions [15]. In conclusion, using human T-cell lines specific for *M. tuberculosis*, we show that all 25 novel mycobacterial latency antigens are potentially able to elicit cellular immune responses in *M. tuberculosis*-infected individuals.

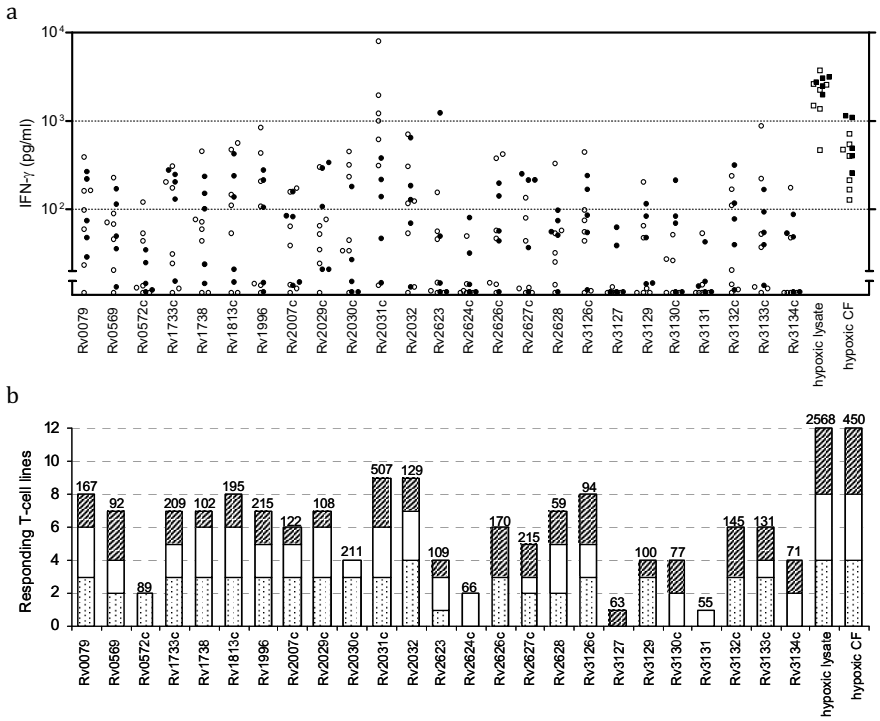

Figure 1. Long-term T-cell lines (n=12) responding to 25 M. tuberculosis latency antigens, hypoxic-M. tuberculosis lysate and culture filtrate (CF)*. Hypoxic lysate and CF were prepared from M. tuberculosis H37Rv which was grown for 24 hours in tubes with tightly screwed caps. 8 lines were generated by stimulating PBMC obtained from TST converters (n=2) or cured TB patients (n=2) with either hypoxic-CF (n=4, cross-hatched bar) or hypoxic-lysate (n=4, white bar). Additionally, PBMC from one TST converter and 3 cured TB patients were stimulated with lysate of M. tuberculosis, which was grown under standard, aerated conditions (n=4, dotted bar).* ***(a)*** *IFN-γ responses of all 12 M. tuberculosis-specific T-cell lines. Dots indicate the IFN-γ response minus the IFN-γ value in the unstimulated cultures. Lines were either generated from PBMC of TST converters (closed circles (•)) or cured TB patients (open circles (o)).* ***(b)*** *Bars indicate the number of lines responding to a latency antigen with an IFN-γ production of at least 50 pg/ml. The number at the top of each bar indicates the median IFN-γ production of these responding lines.*

Recognition of latency antigens by PBMC of *M. tuberculosis* infected individuals

Subsequently, direct peripheral blood ex-vivo stimulation assays were performed using PBMC from 20 TB patients, 23 TST+ individuals and 21 uninfected controls. In order to identify those proteins with a strong capacity to induce IFN-γ production, we calculated for each antigen the proportion of individuals who responded to the antigen with an IFN-γ production level exceeding 100 pg/ml (Table 1). This analysis revealed that 18 of the 25 latency antigens were recognized by at least 10% of either TB patients or TST+ individuals (Table 1). Of these, Rv1733c, Rv2029c and Rv2627c, were even recognized by the majority of TST+ individuals. The remaining 7 antigens tested (Rv0572c, Rv2007c, Rv2623, Rv2624c, Rv3127, Rv3131, Rv3134c) were not or very poorly recognized by PBMC from *M. tuberculosis*-infected individuals. These

Table 1. Immunogenicity of latency antigens[a]

Latency antigen	Gene name	Product	Response (%) [b] HC (n=21)	TB (n=20)	TST+ (n=23)
Rv0079		HP	14	10	26
Rv0569		CHP	-	5	22
Rv0572c		HP	10	5	-
Rv1733c		possible transmembrane protein	41	50	61
Rv1738		CHP	-	11	13
Rv1813c		CHP	14	15	17
Rv1996		CHP	14	11	4
Rv2007c	*fdxA*	probable ferredoxin FDXA	-	-	9
Rv2029c	*pfkB*	probable phosphofructokinase PfkB	29	25	61*
Rv2030c		CHP	14	15	26
Rv2031c	*hspX*	heat shock protein HspX	5	20	-*
Rv2032	*acg*	CHP Acg	14	20	30
Rv2623	*TB31.7*	CHP TB31.7	-	-	-
Rv2624c		CHP	-	-	-
Rv2626c		CHP	14	10	30
Rv2627c		CHP	38	30	52
Rv2628		HP	10	16	35
Rv3126c		HP	19	10	30
Rv3127		CHP	-	-	4
Rv3129		CHP	19	21	35
Rv3130c		CHP	-	-	13
Rv3131		CHP	-	-	4
Rv3132c	*devS*	2-component sensor histidine kinase	24	15	17
Rv3133c	*dosR*	2-component transcriptional regulatory protein	14	32	30
Rv3134c		CHP	-	-	4

[a] Abbreviations: HC, healthy controls; TB, TB patients; TST+, tuberculin skin test positive individuals;
HP, hypothetical protein; CHP, conserved hypothetical protein. Annotations are from http://genolist.pasteur.fr/TubercuList/.

[b] Percentage of individuals with an IFNγ response of ≥100 pg/ml.

-, in none of the study subjects an IFNγ response of ≥100 pg/ml was observed.

*P < 0.05, $\chi 2$ test comparing TB patients with TST+ individuals.

antigens were also the weakest inducers of IFN-γ production among the above tested T-cell lines. Similar results were obtained when proliferation was analysed (*data not shown*).

When comparing the proportion of strong responders among study groups, most latency antigens were recognized by a larger proportion of TST+ individuals as compared to TB patients and controls (Table 1); this trend was only statistically significant for Rv2029c when comparing TST+ persons with TB patients (P=0.02).

HspX was relatively poorly recognized, and more frequently by TB patients than by TST+ individuals (P=0.02) (Table 1). Although no further statistical analysis was possible because of small numbers, it was interesting to note that TST+ individuals who showed at least some response to HspX (IFN-γ of 20-100 pg/ml) all had had recent TST conversion (<6 month).

Recognition of latency antigens by uninfected controls

Somewhat unexpectedly, 16 of the 25 latency antigens were also recognized by T-cells from a minority of *M. tuberculosis*-uninfected controls (Table 1). Controls were BCG-unvaccinated, were TST-negative and did not recognize CFP-10 and ESAT-6 (known from a previous project [28]). Therefore, we hypothesized that observed responses to

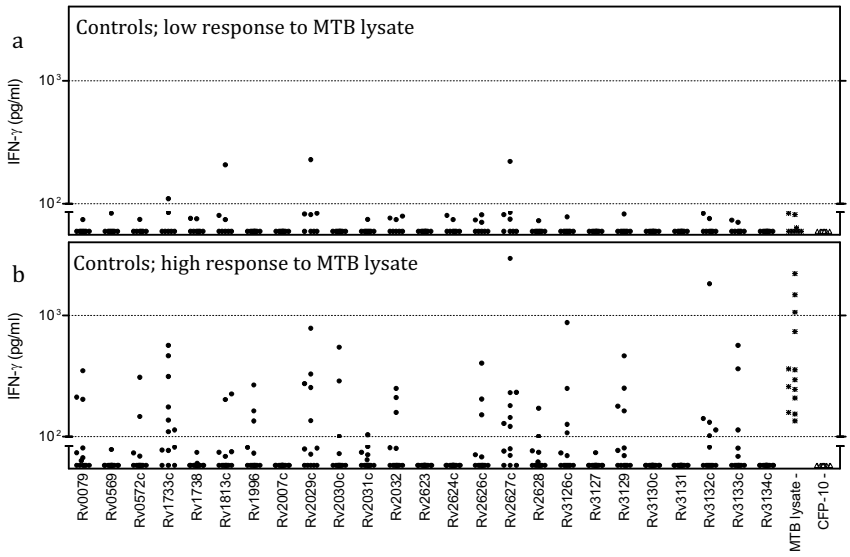

Figure 2. IFNγ responses of M. tuberculosis uninfected healthy controls to 25 latency antigens, M. tuberculosis (MTB) lysate and CFP-10. (a) *Controls with a low IFNγ response (< 100 pg/ml) to standard M. tuberculosis lysate (n = 8)* ***(b)*** *Controls with a strong IFN-γ response (> 100 pg/ml) to standard M. tuberculosis lysate (n = 13). None of the controls responded to the M. tuberculosis specific antigen CFP-10. MTB lysate, M. tuberculosis lysate.*

latency antigens in controls were not caused by infection with *M. tuberculosis* complex species, but more likely by previous exposure to other mycobacteria. Indeed, thirteen 62%) of 21 *M. tuberculosis*-uninfected controls appeared to strongly respond to *M. tuberculosis* lysate (IFN-γ >100 pg/ml; median level 591 pg/ml IFN-γ), which is most likely due to exposure to non-tuberculous mycobacteria. Significant responses to latency antigens were almost exclusively observed in this group of control subjects (Figure 2b), while in the remaining 8 control subjects with low responses to mycobacterial lysate only sporadically low responses to latency antigens were observed (Figure 2a). Responses to latency antigens indeed correlated significantly to the response to *M. tuberculosis* lysate (Spearman R=0.56, P =0.02).

IFN-γ responses to frequently recognized latency antigens and CFP-10

Rv1733c, Rv2029c, Rv2627c and Rv2628 were particularly broadly recognized, predominantly by TST+ individuals. IFN-γ responses to those 4 antigens were analysed in more detail as shown in Figure 3. For comparison of the magnitude of the IFN-γ response between groups, only those individuals were analysed who were able to recognize the antigen with an IFN-γ response above the detection level of the assay. Median IFN-γ production among responders in the group of TST+ individuals in response to Rv1733c, Rv2029c, Rv2627c and Rv2628 was 564, 382, 195 and 160 pg/ml, respectively, while in the group of TB patients lower IFN-γ responses were found, with medians of 160, 137, 194 and 79 pg/ml, respectively (Figure 3). This difference was statistically significant for Rv1733c (P=0.008). In order to compare responses to latency antigens with responses to a *M. tuberculosis*-specific antigen

outside the DosR regulon, PBMC of the same individuals were also stimulated with a peptide pool of CFP-10, an antigen that is secreted by tubercle bacilli during active replication. In contrast to latency antigens, CFP-10 induced significantly stronger IFN-γ responses among TB patients as compared to TST+ individuals, with a median among responders of 242 and 59 pg/ml, respectively (P = 0.042) (Figure 3).

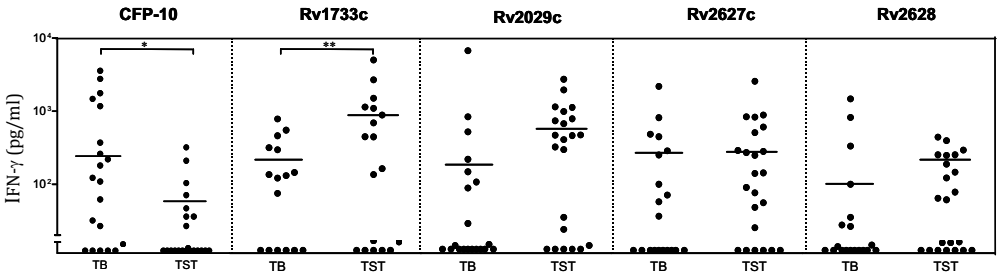

Figure 3. IFN-γ response profiles to the four best recognized M. tuberculosis latency antigens and the M. tuberculosis-specific secreted antigen CFP-10. *IFN-γ production by PBMC from TB patients (TB) and TST positive individuals (TST) in response to Rv1733c, Rv2029c, Rv2627c Rv2628 and CFP-10 (peptide pool) is shown. Horizontal lines indicate the median IFN-γ production among persons who are able to respond to the antigen. Responses were compared using a Mann-Whitney U test; *, P < .05; ** P <.01*

To analyze this difference in antigen recognition pattern between TB patients and latently infected individuals further, we compared the strength of the immune response to the 4 best recognized latency antigens versus CFP-10 within each individual, using the Wilcoxon signed rank test. In TST+ individuals IFN-γ responses to Rv1733c, Rv2029c, Rv2627c and Rv2628 were higher than to CFP-10 (P<0.01, P<0.01, P<0.01 and P=0.07, respectively). The opposite antigen recognition profile was found for TB patients, irrespective of whether they had active or cured disease: there was a stronger IFN-γ response to CFP-10 than to Rv1733c, Rv2029c, Rv2627c and Rv2628 (P=0.19, P=0.01, P=0.04 and P<0.01, respectively). Subsequently, for each individual the IFN-γ response to CFP-10 was subtracted from the response to Rv1733c such that a positive value would indicate that the response to Rv1733c is stronger while a negative value would indicate a stronger response to CFP-10. The same was done for Rv2029c, Rv2627c and Rv2628. This complementary analysis revealed that in TST+ individuals the mean difference in IFN-γ response between CFP-10 and Rv1733c, Rv2029c, Rv2627c and Rv2628 was 451, 308, 184 and 44 pg/ml respectively, while in TB patients negative values were obtained (-373, -365, -458 and -536 pg/ml respectively (P<0.01, P=0.04, P=0.01 and P=0.02).

Cumulative IFN- y response to antigens of the dormancy regulon

As each individual was found to be able to recognize a different set of latency antigens, we calculated the cumulative IFN-γ response to all 25 latency antigens to capture the overall immune response to the tested set of DosR regulon encoded antigens within each person. This analysis showed that a significantly higher cumulative IFN-γ

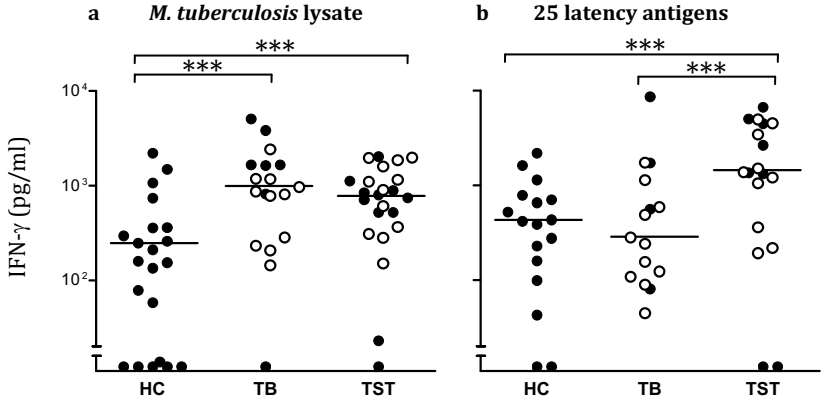

Figure 4. IFN-γ response profiles to M. tuberculosis lysate and the group of 25 latency antigens. *IFN-γ production by PBMC from uninfected healthy controls (HC), TB patients (TB) and TST positive individuals (TST) in response to* ***(a)*** *M. tuberculosis lysate and* ***(b)*** *the cumulative IFN-γ production to 25 latency antigens. TB patients during treatment are indicated with open circles (o) and cured TB patients with closed circles (•). TST+ persons with recent exposure are indicated with open circles (o) and TST+ with a more remote exposure with (•). Horizontal lines indicate the median IFN-γ production among responders, and responses were compared using a Mann-Whitney U test; ***, P< 0.01.*

response to the antigens encoded by the DosR regulon was seen in the group of TST+ individuals compared to TB patients (median IFN-γ of 1453 and 263 pg/ml respectively, *P*=.003) and to controls (median IFN-γ of 434 pg/ml, *P*=.003)(Figure 4). In contrast, for whole *M. tuberculosis*-lysate no significant differences were observed between TB patients and TST+ individuals but their responses were significantly higher when compared to healthy controls (*P*<0.01) (Figure 4). Figure 4 visualizes responses in TB patients both during active disease and following cure, but subgroups were too small for separate statistical analysis. Subgroup analysis was also done for the group of TST converters which was split up into those with recent exposure to *M. tuberculosis* and those who had been exposed more than 2 years prior to the time of blood sampling. Subsequently, a regression analysis was done in which cumulative IFN-γ responses to latency antigens were compared between the two groups, while controlling for the effect of age, general responsiveness of T-cells (by adjusting for the response to *M. tuberculosis*-lysate and PHA) and possible waning of immunity (by adjusting for the time between initial infection with *M. tuberculosis* and the time of blood sampling). This analysis showed that, also after adjusting for all of the above-mentioned variables, a significantly higher cumulative IFN-γ response to the antigens of the DosR regulon was observed in TST+ individuals compared to TB patients.

Finally, response profiles also differed between the two groups of *M. tuberculosis*-infected individuals with regard to the absolute number of latency antigens that were recognized: TST+ persons recognized significantly more latency antigens (mean of 5.4) than TB patients (mean of 2.4) (*P*=0.04).

Discussion

The present study provides the first description of human T-cell responses to a large series of proteins encoded by the recently described dormancy (DosR) regulon of *M. tuberculosis.* All 25 tested latency antigens were able to induce human T-cell responses, using *M. tuberculosis*-specific T-cell lines. Eighteen of these latency antigens were recognized by PBMC from *M. tuberculosis*-infected individuals, indirectly confirm that DosR regulon encoded proteins are expressed during natural *M. tuberculosis* infection in humans. Our results indicate than TST+ individuals tend to recognize more latency antigens, and have a stronger cumulative IFN-γ response when compared to individuals who developed TB. In contrast, IFN-γ responses to CFP-10, a *M. tuberculosis*-specific antigen secreted during active replication, were significantly stronger in TB patients.

Out of the 18 latency antigens that were recognized by PBMC of *M. tuberculosis*-infected individuals only HspX had been previously identified as a *M. tuberculosis* antigen [16]. Of these newly identified antigens, Rv1733c, Rv2029c, Rv2627c and Rv2628 were most frequently recognized, by 61%, 61%, 52% and 35% of TST+ individuals, respectively. These antigens were found to induce relatively strong IFN-γ responses when compared to the well-known immunodominant secreted antigen CFP-10.

Positive responses to latency antigens in TB patients were not entirely unexpected as most patients go through a phase of latent infection before progression to active TB disease occurs; memory immune responses induced during this latent phase may remain detectable thereafter. In addition, during TB disease at least part of the bacilli are expected to encounter hypoxic conditions or to be exposed to nitric oxide which leads to upregulation of the dormancy regulon, as suggested by the finding of variable levels of HspX transcripts in different regions of the lungs of patients with chronic, active TB [22,23]. Furthermore, as recently has been observed for bacilli during aerated stationary phase in vitro, it is possible that in TB patients expression of the DosR is partial or altered, resulting in a more limited response to latency antigens [29].

We hypothesize that immune responses to latency antigens may contribute to the control of persistent *M. tuberculosis* infection and that changes in protein expression profile during latency result in an altered repertoire of *M. tuberculosis* antigens available for T-cell recognition. This could then be reflected in differences in antigen recognition patterns of responding T-cells. The observation that in TST+ individuals IFN-γ responses to the 4 most frequently recognized latency antigens were significantly higher than to CFP-10, while the opposite antigen recognition profile was found for TB patients, is consistent with this hypothesis. Furthermore, latently infected individuals recognized significantly more latency antigens and had a stronger IFN-γ production in response to the group of latency antigens when compared to TB patients. However, our study was not designed to specifically demonstrate a causal relationship between the observed immune responses and protection against TB disease, as this would require the follow-up of untreated latently infected persons and detailed analysis of the kinetics of responses in those who will progress to active TB

versus those who will remain healthy. The observed association between IFN-γ responses to latency antigens and natural protection needs to be further investigated, but we would like to suggest that these antigens may be interesting candidates for evaluation in post-exposure vaccination models.

An unexpected finding of the study was the recognition of latency antigens by some of the uninfected controls. None of the controls were BCG vaccinated, had any identified risk of exposure to TB or responded in vitro to the *M. tuberculosis*-specific antigens ESAT-6 or CFP-10. However, more than half of the controls recognized *M. tuberculosis* lysate, which is most likely explained by exposure to mycobacteria other than *M. tuberculosis.* Responses to latency antigens were nearly exclusively seen in this subgroup of controls, whereas virtually no responses were seen in the mycobacterium non-responsive group, suggesting that responses to latency antigens could result from cross-reactivity to non-tuberculous mycobacteria. It is interesting to note that other mycobacteria, like *M. bovis* and BCG, *M. microti* and *M. smegmatis* have a very similar dormancy regulon [30,31]. Moreover, there may be homologues in other bacteria than mycobacteria causing cross-reactivity. Of note, Rv1733c encodes a possible transmembrane protein, which bears homology with a hypothetical protein of *Streptomyces coelicolor;* and Rv2029c, which is a predicted phosphofructokinase involved in glycolysis, is expected to have homologues in other (myco) bacteria. It is not known whether the observed cross-reactive immune response to latency antigens among *M. tuberculosis* naïve persons contributes to the natural protection that develops in 90% of individuals who are infected with *M. tuberculosis* and remain free of disease; but this is may be an important topic for further studies [5,32].

In our study we tested proteins in equimolar concentrations, to allow for direct comparison of human T-cell responses to different antigens including the previously defined antigen HspX and newly identified latency antigens. The results indicated that several of the latter induced higher responses in a larger proportion of subjects than HspX did. We observed that HspX, at the concentration tested, was recognized by only 20% of TB patients whereas in TST+ persons IFN-γ responses were very infrequent and of a low level. Of note however, several T-cell lines strongly recognized the identical protein batch of HspX. The apparent discrepancy with a previous studie, in which recognition of HspX by T-cells of TST+ individuals was demonstrated [16,28], may be related to differences in readout methodology and to antigen concentrations tested, since relatively low concentrations of antigens were used. Finally, a difference in study populations could be an additional factor, as immune responses to HspX may have waned if the infection with *M. tuberculosis* was remote. The observed responses to HspX were all in recent converters, supporting the latter hypothesis.

In this study we focused on IFN-γ responses by T-cell lines and PBMC. The essential role of IFN-γ in the protective immunity to mycobacteria has been clearly demonstrated by studies in gene-knockout mice and by the increased susceptibility to mycobacteria in individuals with genetic defects in the IFN-γ/interleukin-12/23 pathway [33,34]. Nonetheless, future studies on latency antigens should also address production of other cytokines than IFN-γ [6]. In addition, besides CD4 responses, we are currently studying CD8 T-cells specific for mycobacterial latency antigens, as several studies indicate that CD8 T-cells are important for controlling latent *M. tuberculosis* infection [7,35].

In conclusion, the DosR regulon was found to encode a high number of *M. tuberculosis* antigens with strong T-cell and IFN-γ inducing capacity. A trend was observed towards stronger IFN-γ responses to a larger number of latency antigens in latently infected individuals as compared to individuals who developed TB disease, whereas the opposite profile was found for CFP-10. These findings are consistent with the hypothesis that immune responses to latency antigens may contribute to natural protection against TB disease. However, subsequent prospective studies of large cohorts of latently infected individuals are needed to determine how strongly T-cell responses to these newly identified latency antigens are associated with TB latency and with protection against progression towards active disease. If so, latency antigens may be promising tools for vaccination against TB reactivation.

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