Response to Rv2628 latency antigen associates with cured tuberculosis and remote infection

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ABSTRACT

Interferon-gamma-release assays based on RD1 antigens have improved diagnosis of latent tuberculosis infection (LTBI). However, these tests cannot discriminate between recently-acquired infection (higher risk of progression to active tuberculosis) and remote LTBI. The objective of this study was to evaluate the interferon-gamma-T-cell responses to *M. tuberculosis*-DosR regulon-encoded antigens (latency antigens) compared with QuantiFERON TB-Gold In-tube (QFT-IT) in subjects at different stages of tuberculosis. We studied 16 individuals with remote LTBI and 23 with recent infection. We also analyzed 15 controls unexposed to *M. tuberculosis*, 50 with active tuberculosis and 45 patients with cured-tuberculosis. Results indicate that subjects with remote LTBI had significantly higher interferon-gamma whole blood responses to *M. tuberculosis*-latency antigen Rv2628 than individuals with recent infection, active tuberculosis and controls (p<0.003), whereas no significant differences between these groups were found for other latency antigens tested (Rv2626, Rv2627, Rv2031c, Rv2032). The proportion of responders to Rv2628 was 5-fold higher among QFT-IT-positive-individuals with remote infection than among those with recently-acquired infection. These data suggest that responses to *M. tuberculosis*-latency antigen Rv2628 may associate to immune-mediated protection against tuberculosis. In contact tracing investigations, these preliminary data may differentiate recent (positive-QFT-IT results without responses to Rv2628) from remote infection (positive to both tests).

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INTRODUCTION

According to surveys with tuberculin skin tests (TST), the World Health Organization (WHO) estimated that approximately one-third of the world's population is latently infected with *Mycobacterium tuberculosis.* This enormous reservoir of latent tuberculosis infection (LTBI), from which most cases of active tuberculosis (TB) arise, embodies a major obstacle in achieving worldwide control over TB [1].

The introduction of T-cell-based interferon (IFN)-γ release assays (IGRAs), using antigens belonging to *M. tuberculosis* region of difference (RD)1 [including early secreted antigenic target (ESAT)-6 and culture filtrate protein 10 (CFP)-10], may represent a significant step towards improved LTBI diagnosis [2-4]. There is evidence that commercial IGRAs are highly sensitive in detecting LTBI [2] and have a high specificity that is unaffected by Bacillus Calmette Guerin (BCG) vaccination. Although IGRAs were designed as assays for LTBI, these tests do not discriminate between active disease and LTBI [5, 6]. Moreover IGRAs do not distinguish between a recently acquired infection and remote LTBI [7]. This information may have important clinical implications, since recently acquired infection carries a significantly increased risk of progression to active disease compared to remote LTBI [8].

During LTBI, tubercle bacilli are contained within immune-mediated granulomas [9]. It is thought that persisting tubercle bacilli are subjected to nutrient and oxygen deprivation [10, 11]. As part of the adaptive response of *M. tuberculosis* to hypoxia, expression of the DosR regulon is observed. The functions of most DosR regulon encoded-proteins, further referred to here as latency antigens, are unknown [12, 13]. However, recently it has been shown that certain latency antigens are more frequently recognized in TST-positive individuals without active TB than in diseased patients, while the opposite profile was found for CFP-10 and ESAT-6 [13-17]. In particular, it has been shown that Rv2627c, Rv2628 and Rv3407 induced strong long-term IFN−γ responses in TST-positive individuals. These studies, in which an experimental setting involving long-term (6-7 days) stimulation of frozen/fresh PBMC or whole blood was used, suggest a contribution of immune responses against the latency antigens to controlling LTBI.

Lately, both short-term and long-term whole blood IGRAs [17-19] have been shown to be accurate in detecting immune responses to *M. tuberculosis* [2] and thus useful for diagnostic purposes. Therefore, the objective of this proof of principle study was to evaluate the whole blood IFN−γ response to the recently-described antigens of latency (Rv2626c, Rv2627c, Rv2628, Rv2031c, Rv2032) in day-1 (short response) and day-7 (long-term response) assays in the different stages of TB. Long-term response was assessed to be in line with the results reported in the literature. We studied subjects with remote and recent infection, individuals with cured TB, patients with active TB and controls not exposed to *M. tuberculosis*. All enrolled individuals were also tested by a commercial IGRA [QuantiFERON-TB Gold In tube (QFT-IT) and by purified protein derivative (PPD) as control antigen.

MATERIAL AND METHODS

Study population

The following individuals were enrolled: 1) "controls", individuals with no risk of *M. tuberculosis* infection who tested TST-negative and QFT-IT-negative; 2) "recent infection", individuals who reported household or equivalent close contact (work) [20] with smear-positive pulmonary TB patients in the previous 3 months, tested TST-positive and QFT-positive, negative chest x-Ray for pulmonary lesions and no prior preventive therapy; 3) "remote LTBI", individuals who tested TST-positive and QFT-IT-positive and reported household or equivalent contact with patients with smear-positive pulmonary TB at least 3 years before enrolment and who did not receive preventive therapy; 4) "cured TB", individuals with culture-positive pulmonary TB who completed a 6 month course of treatment and were culture-negative upon treatment completion. The patients were evaluated at completion of therapy; 5) "active TB"; individuals diagnosed with TB (either with a positive culture for *M. tuberculosis* from sputa or with a positive *M. tuberculosis*-specific RNA amplification (MTD Test, Gen-probe, San Diego, USA) from biopsy specimens and/or biological fluids) who started specific treatment

less than 8 days before enrolment. Remote and recent infection subjects were comparable in terms of exposure (they all were close contacts). Individuals who tested positive to human immunodeficiency virus antibody test (3 subjects) or were on immunosuppressive drugs (2 subjects), were not included in the study. Upon enrolment, demographic and epidemiological information was collected through a structured questionnaire. The study was approved by our ethical committee and all enrolled individuals provided written informed consent.

M. tuberculosis antigens

DosR regulon encoded genes were selected on the basis of their RNA expression level in microarray experiments [13]. Genes were cloned and proteins were overexpressed in Escherichia coli and purified, as previously described [21].

TST

TST was administered by the Mantoux procedure using 5 IU of purified protein derivative (Chiron, Siena, Italy). Results were read after 72 hrs. Indurations of at least 5 mm or ≥10mm were scored positive for close contacts or for the other conditions respectively [22].

Whole blood enzyme linked immunosorbent assays with antigens of M. tuberculosis expressed during latency

1-day (short) response. 0.5 ml per well of heparinized whole blood was seeded in a 48-well plate (Corning Costar, Corning Incorporated, New York, NY, USA) and treated with phytohaemagglutinin (PHA) at 5 µg/ml (Sigma, St Louis, MO, USA) and the following proteins: Rv2628 and Rv2031c at 1 µg/ml, PPD (batch RT 47, Staten Serum Institut, Copenhagen, Denmark), Rv2626, Rv2627 and Rv2032 at 5 µg/ml. The concentrations

provided were chosen after titration of the different reagents (data not shown). Samples were then incubated

for 24 hrs. At day 1, plasma was harvested and stored at -20°C until tested.

7-day (long-term) response. We used the previously described methodology [17, 18]. Briefly, at the

day of collection, an aliquot of heparinized blood was diluted 5-fold using RPMI 1640 supplemented with

penicillin, streptomycin and 2mM L−glutamine (the last four products are from Euroclone Ltd, United

Kingdom) and plated into 48-well plates (Corning Costar) and stimulated as above described. The day-7

diluted plasma was harvested following incubation at 37°C and stored at -20°C until tested.

IFN−γ determination. IFN−γ from day-1 and day-7 plasma was evaluated by a commercial ELISA (CMI,

Cellestis Limited, Carnegie, Victoria, Australia) and data are presented as IU/ml after subtraction of the

appropriate control. For values above the highest standard, we repeated the assay with diluted plasma

samples.

Commercially available IGRA

QuantiFERON TB-Gold In tube (QFT-IT) (Cellestis Limited) was performed and its results were scored as

indicated by the manufacturer (cut-off value for a positive test was 0.35 IU/ml).

Statistical analysis

The main outcome of the study was the evaluation of IFN−γ production in response to antigenic stimulation,

expressed as continuous (IU/ml) or dichotomous (positive/negative) measures. For continuous measures,

mean±standard deviation (SD) of IFN−γ production was calculated, t-test was used for pair-wise comparisons

and ANOVA was used to compare means among the different groups. The results were confirmed when

evaluated by the Mann Withney test for pair-wise comparisons and by the Kruskall Wallis test for multiple

comparisons after calculation of the median and interquartile range (data not shown). For pair-wise

comparisons a post-hoc analysis with Bonferroni correction was used and differences were considered significant at p values ≤0.05.

For dichotomous measures, chi square was used. For pair-wise comparisons we used the Bonferroni correction and differences were considered significant at p values ≤0.05. The cut-off value for definition of positivity of the whole blood assay based on Rv2628 was defined by receiver-operator characteristic analysis. SPSS v 14 for Windows (SPSS Italia Srl, Bologna, Italy) and Prism 4 software (Graphpad Software 4.0, San Diego, CA, USA), were used in the analysis.

RESULTS

Characteristics of the population

We studied 15 controls, 16 individuals with remote LTBI, 50 patients with active TB, 45 subjects with cured TB and 23 with recent infection. Age, gender, BCG vaccination, origin and TST are reported among the different groups (Table 1). The majority were male, BCG-vaccinated, almost 40% were from Western Europe and the pulmonary localization was the most represented in those with active TB.

In vitro short-term IFN–γ response to the M. tuberculosis latency antigens Rv2626, Rv2627, Rv3031c, Rv3032 and Rv2628: quantitative analysis.

We evaluated the IFN-γ-specific responses to Rv2628, Rv2626c, Rv2627c, Rv2031c, Rv2032 in the different groups. All the subjects responded to the mitogen: controls [mean: 15.7 IU/ml; SD 7.6], recent infection (mean: 15.1 IU/ml; SD 8.4), remote LTBI (mean: 15.7 IU/ml; SD 8.4), active TB (mean: 10.0 IU/ml; SD 8.6) and cured TB (mean: 15.3 IU/ml; SD 10.9). Significant difference was found for overall comparison among the different groups (p=0.026) while no significant difference was found for pair-wise comparisons.

Analyzing the IFN-γ response to Rv2628, a significant difference was found for overall comparison among the different groups (p<0.001). For pair-wise comparisons, the response to Rv2628 (figure 1) was significantly higher in those with remote LTBI (mean: 5.3 IU/ml; SD 5.9) compared to recent infection (mean:

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0.3 IU/ml; SD 0.5) (p=0.001) and compared to active TB (mean: 1.1 IU/ml; SD 3.4) (p=0.002), whereas no significant difference was observed in comparison to cured TB (mean: 3.1 IU/ml; SD 5.1) (p=0.547). Also close to significance was the difference between cured TB and recent infection (p=0.056). Moreover, the difference between cured TB and recent infection (p=0.056) was close to significance. Finally, significant difference was found between controls (mean: 0.1 IU/ml; SD 0.1) and subjects with remote LTBI (p=0.003).

Regarding the other latency antigens tested, Rv2626c, Rv2627c, Rv2031c, Rv2032, no significant IFN-γ differences were found neither considering all the groups simultaneously, nor performing pair-wise comparisons (Figure 2A-D).

As an internal control, we evaluated IFN-γ response in the QFT-IT, whose antigens are secreted-antigens and associated with the replicative status of the mycobacteria, and to PPD, a non-specific mixture of mycobacterial antigens. Quantitative responses measured by QFT-IT are shown in Figure 2E. Among individuals with LTBI, who were selected on the basis of a QFT-IT-positive response, no significant difference was observed by comparing recent infection (mean: 9.2 IU/ml; SD 10.0) to remote LTBI (mean: 10.3 IU/ml; SD 8.4). The proportion of individuals with a positive response to QFT-IT was similar between active TB [36/50 (72.0%)] and cured TB [31/45 (68.9%)] and no significant difference was observed when the responses in these two groups were compared in quantitative terms (mean: 5.5 IU/ml; SD 9.8 and 4.0 IU/ml; SD 6.0, respectively).

Response to PPD was analyzed in a large portion of the subjects enrolled (14/15 of the controls; 8/23 of those with recent infection; 10/16 of the remote LTBI; 34/50 of the active TB; 34/45 of the cured TB). When quantitative data were analyzed, a significant difference in the response to PPD was found considering all the groups simultaneously (p=0.004). For pair-wise comparisons, a significant difference was found between controls (mean: 5.5 IU/ml; SD 7.4) and remote LTBI (mean: 17.7 IU/ml; SD 6.2) (p=0.004) and between controls and cured TB (mean: 13.0 IU/ml; SD 9.6) (p=0.041) (Figure 2F). No significant differences were found

between controls and recent infection (mean: 13.8 IU/ml; SD 6.3) or between controls and active TB (mean: 10.3 IU/ml; SD 7.1) or between active and cured TB or remote LTBI and recent infection.

All together, these data suggest that IFN-γ responses to Rv2628 are most frequently found in those able to control *M. tuberculosis* replication, either naturally (remote LTBI) or after chemotherapy (cured TB), compared to those with active *M. tuberculosis* replication, either controlled, but acute (recent infection) or uncontrolled (active disease). These responses are different than those found by all other evaluated antigens including the remaining *M.tuberculosis* latency antigens (Rv2626c, Rv2627c, Rv2031c, Rv2032), QFT-IT-antigens (ESAT-6, CFP-10, TB7.7) as well as PPD, since these responses did not significantly differ among the above-mentioned groups.

In vitro short-term assay IFN–γ responses to M. tuberculosis latency antigen Rv2628: qualitative analysis

Based on the significant difference found in the quantitative analysis, we performed a receiver-operator characteristic (ROC) analysis for the response to Rv2628 in order to evaluate its potential use in discriminating the different stages of TB. In this analysis, we used the remote LTBI and the active TB as comparator groups because they represented the best examples of *M. tuberculosis*-containment or -active replication, respectively. Significant results for area under the curve (AUC) analysis were obtained (AUC, 0.85; 95% confidence interval (CI), 0.75–0.96, p<0.001). For scoring purposes we chose a cut-off to maximize the sum of sensitivity and specificity. We found that a cut-off of 0.5 predicted LTBI with 76.0% sensitivity (95% CI, 61.8%–86.9%) and 87.5% specificity (95% CI, 61.6%–98.4%).

Finally, based on the cut-off value found for the responses to Rv2628, we scored the results as negative and positive. Significant difference was found among the different groups (p<0.001). As shown in table 2, the highest proportion of positive results was found among remote LTBI (14/16, 87.5%) who were positive to QFT-IT by definition, followed by cured TB (24/45; 53.3%) who resulted 68.9% positive to QFT-IT (table 3). Lower proportions of positive response to Rv2628 were found in individuals with active TB (12/50,

24.0%) and in those with recent infection (4/23; 17.3%); proportion of positive response to QFT-IT in these two groups were 72.0% and 100% (by definition), respectively. None of the controls responded to Rv2628 and resulted negative to QFT-IT by definition.

A pair-wise comparison of positivity rate for Rv2628 in the five groups of individuals enrolled is also shown in table 2. Proportions of positive responses among remote LTBI were significantly higher than those recorded among active TB, recent infection and controls, while the difference with cured LTBI was not statistically significant. A significant difference was also found between cured TB compared to recent infection or controls, whereas the difference between cured and active TB (p=0.056) was close to significance.

In vitro long-term IFN–γ responses to the M. tuberculosis latency antigens

Long-term IFN–γ response was assessed to be in line with the results reported in the literature, although performed with different experimental settings. [17-20]. Long-term IFN–γ response to Rv2628 was significantly different among the groups analyzed simultaneously (p<0.001). Moreover this response was significantly higher in those with remote LTBI (mean: 12.1 IU/ml; SD 13.0) compared to those with recent infection (mean: 2.9 IU/ml; SD 5.3) (p=0.002), controls (mean: 0.55 IU/ml; SD 1.6) (p<0.001) and active TB (mean: 1.7 IU/ml; SD 3.2) (p<0.001). Similar to the findings reported for the short-term responses, a significant difference was found between the response found in active TB compared to cured TB (p=0.019) and close to the significance levels was the difference found between cured TB and controls (p<0.058). Results similar to those described for the short responses were found when analyzing the qualitative data (data not shown). Regarding the other latency antigens (Rv2626c, Rv2627c, Rv2031c, Rv2032), no significant difference in terms of IFN-γ release was found for all the comparisons performed (data not shown). Regarding PPD, a significant difference was found for overall comparison among the different groups (p=0.001). For pair-wise comparisons, significant differences were found only between controls (mean: 8.6 IU/ml; SD 7.4) and remote LTBI (mean: 21.1 IU/ml; SD 4.6) (p=0.001) or controls and cured TB (mean: 6.8 IU/ml; SD 9.0) (p=0.007).

DISCUSSION

In the present proof of principle study we analyzed IFN$-\gamma$-T-cell responses of individuals at different stages of *M. tuberculosis* infection/disease and uninfected controls to a series of proteins expressed during *M. tuberculosis* dormancy (Rv2626c, Rv2627c, Rv2628, Rv2031c, Rv2032). We also evaluated the response to *M. tuberculosis* early phase secreted antigens (ESAT6, CFP10 and TB7.7) as assessed by QFT-IT and to a mixture of mycobacterial antigens as present in PPD.

Among all antigens evaluated, Rv2628 induced a significantly higher IFN$-\gamma$ response, and increased proportions of positive responses to this antigen were found in remote LTBI and cured TB compared to recent infection and active TB. Among QFT-IT-positive individuals with remote LTBI, the proportion of responders to Rv2628 was almost 5-fold higher compared to QFT-IT-positive recently-*M. tuberculosis*-infected individuals (87.5% vs 17.3% respectively). When analyzed in quantitative terms, similar results were obtained by using long- or short-term assay. However, the whole blood, day-1 format appears more appealing for potential use as a diagnostic tool. Together, these results suggest that the response to Rv2628 can be used to characterize LTBI as a remote vs. recently acquired infection. This can be helpful in the clinical management of recently acquired infection, since persons with this condition may have the greatest benefit from preventive therapy [8].

The *M. tuberculosis* latency antigens used in the present study are encoded by the *M. tuberculosis dosR* regulon, which has been shown to play a critical role in preparing *M. tuberculosis* for the metabolic shift-down associated with bacterial dormancy [23, 24]. At present, the exact role of the *dosR* regulon products in human *M. tuberculosis* infection is not completely understood. Rv2031c, the archetypal *dosR* regulon product, is a small heat-shock protein, and cellular immune responses to Rv2031c have been observed in individuals with LTBI [25]. More recently, Leyten *et al.* studied human T cell responses to a set of the 25 *dosR* encoded proteins, using long-term cultured thawed PBMC [14]. The results provided first support for the immunogenicity

of *dosR* regulon proteins in human PBMC stimulation assays of TST+ individuals. Subsequently, the latency antigens Rv1733c, Rv2029c, Rv2627c and Rv2628 were identified as the most frequently recognized antigens in latently infected individuals [14, 16]. Similarly, IFN-γ response to Rv3407 has recently been shown as a response specific for LTBI [15]. Our results confirm that cellular immune response to these latency antigens can be found *in vitro*, and show that this response can also be efficiently detected by means of a whole- blood 1-day assay. We also found that the response to Rv2628 antigen was more clearly associated with a latent infection, as previously shown using other experimental approaches by Leyten [14] and Black [15] and confirmed by Roupie [26] in mice. However, from all the studies reported, it is presently unclear why the response to Rv2628 may provide a better discrimination between the different stages of TB infection. Among the studies performed on human samples [14-17], Schuck [15] did not show any immunogenic activity of Rv2628. It may likely due to the selection of LTBI subjects that have not been clearly defined as recently or remotely infected.

Detectable responses to *M. tuberculosis* latency antigens, although limited, were also found in active TB patients. This is probably due to the fact that these antigens are expressed in response to immune and physiological stress experienced by the bacteria, which eventually result in latency, and immune responses to the antigens induced during this phase may remain detectable [23, 26, 27].

As previously shown [14], we also found low levels of recognition of latency antigens Rv2626c and Rv2627c by some of the uninfected controls not correlated with BCG-vaccination (data not shown). This result may most likely be explained by exposure to mycobacteria other than *M. tuberculosis* that have similar dormancy regulon [28, 29 and Lin et al, submitted]. The Dos regulon is also present in BCG, but in the present study, vaccination does not seem to be associated with induction of responses to Rv2628, either in controls or in the subjects with recent infection, as previously reported [30].

Our study has a number of limitations. Individuals with recent infection were the contacts of sputum smear-positive TB patients in the last 3 months. By definition, they were all TST-positive. However a TST conversion was documented in only 8 of them, so we cannot rule out that misclassification may have occurred in some cases. Although the association of the results of immune response with a status of latency which we describe is highly likely, the number of individuals enrolled in this proof of principle study is limited. This indicates the need to confirm the data in larger independent studies. Moreover the groups enrolled were not fully matched for region of origin. To evaluate if the results could have been influenced by differences in ethnic distribution of different groups, we repeated the analysis by including only those who originated from Western European countries, which represented the largest population group. In this group we found the same associations observed in the overall population (data not shown). This observation suggests that our results are most likely not biased by ethnicity or country of origin. These results were obtained evaluating the response to Rv2628 at a single time point; however an overtime evaluation of those with active TB and recent infection is crucial to understand the meaning of this study in a clinical context and ongoing studies are in process. Finally, several comparisons were performed to assess the possible association of each antigen with the stage of TB, and this may have increased the chance of "false positive" associations. However, the differences in responses to Rv2628 between those able to control *M. tuberculosis* replication, either naturally (remote LTBI) or after chemotherapy (cured TB), and those with active *M. tuberculosis* replication, either controlled, but acute (recent infection) or uncontrolled (active disease) were still highly significant after applying the correction for multiple comparisons.

In conclusion, the whole blood short-term IFN-γ response to Rv2628 is frequently detected in remote LTBI, but it is rarely found in recently acquired infection. These results may have important clinical practical implication for risk stratification when deciding to initiate preventive therapy for LTBI.

ACKNOWLEDGEMENTS

REFERENCES

1. WHO. Global tuberculosis control: surveillance, planning, financing. In: *WHO r Geneva* 2008;1-294.
2. Pai M, Zwerling A, Menzies D. Systematic review: T-cell-based assays for the diagnosis of latent tuberculosis infection: an update. *Ann Intern Med* 2008; 149: 177-184.
3. Lalvani A. Diagnosing tuberculosis infection in the 21st century: new tools to tackle an old enemy. *Chest* 2007;131: 1898-1906.
4. Ferrara G, Losi M, Meacci M, Meccugni B, Piro R, Roversi P, Bergamini BM, D'Amico R, Marchigiano P, Rumpianesi F, Fabbri LM, Richiedi L. Routine hospital use of a new commercial whole blood interferon-gamma assay for the diagnosis of tuberculosis infection. *Am J Respir Crit Care Med* 2005;172: 631-635.
5. Goletti D, Stefania C, Butera O, Amicosante M, Ernst M, Sauzullo I, Vullo V, Cirillo D, Borroni E, Markova R, Drenska R, Dominguez J, Latorre I, Angeletti C, Navarra A, Petrosillo N, Lauria FN, Ippolito G, Migliori GB, Lange C, Girardi E. Accuracy of immunodiagnostic tests for active tuberculosis using single and combined results: a multicenter TBNET-Study. *PLoS ONE* 2008; 3: e3417.
6. Goletti D, Carrara S, Vincenti D, Saltini C, Rizzi EB, Schininà V, Ippolito G, Amicosante M, Girardi E. Accuracy of an immune diagnostic assay based on RD1 selected epitopes for active tuberculosis in a clinical setting: a pilot study. *Clin Microbiol Infect* 2006;12: 544-550.
7. Goletti D, Parracino MP, Butera O, Bizzoni F, Casetti R, Dainotto D, Anzidei G, Nisii C, Ippolito G, Poccia F, Girardi E. Isoniazid prophylaxis differently modulates T-cell responses to RD1-epitopes in contacts recently exposed to Mycobacterium tuberculosis: a pilot study. *Respir Res* 2007; 27: 8:5.
8. Marais BJ, Gie RP, Schaaf HS, Beyers N, Donald PR, Starke JR. Childhood pulmonary tuberculosis: old wisdom and new challenges. *Am J Respir Crit Care Med* 2006;173: 1078-90.
9. Ulrichs T, Kosmiadi GA, Trusov V , Jorg S, Pradl L, Titukhina M, Mishenko V, Gushina N, Kaufmann SH. Human tuberculous granulomas induce peripheral lymphoid follicle-like structures to orchestrate local host defence in the lung. *J Pathol* 2004; 204: 217-228.
10. Wayne LG, Hayes LG. Nitrate reduction as a marker for hypoxic shiftdown of Mycobacterium tuberculosis. *Tuber Lung Dis* 1998;79: 127-132.
11. Tufariello JM, Chan J, Flynn JL. Latent tuberculosis: mechanisms of host and bacillus that contribute to persistent infection. *Lancet Infect Dis* 2003;3: 578-590.
12. Yuan Y, Crane DD, Barry CE 3rd. Stationary phase-associated protein expression in Mycobacterium tuberculosis: function of the mycobacterial alpha-crystallin homolog. *J Bacteriol* 1996;178: 4484-4492.
13. Park HD, Guinn KM, Harrell MI, Liao R, Voskuil MI, Tompa M, Schoolnik GK, Sherman DR. Rv3133c/dosR is a transcription factor that mediates the hypoxic response of Mycobacterium tuberculosis. *Mol Microbiol* 2003;48: 833-843.
14. Leyten EM, Lin MY, Franken KL, Friggen AH, Prins C, van Meijgaarden KE, Voskuil MI, Weldingh K, Andersen P, Schoolnik GK, Arend SM, Ottenhoff TH, Klein MR. Human T-cell responses to 25 novel antigens encoded by genes of the dormancy regulon of Mycobacterium tuberculosis. *Microbes Infect* 2006;8: 2052-2060.
15. Schuck SD, Mueller H, Kunitz F, Neher A, Hoffmann H, Franken KL, Repsilber D, Ottenhoff TH, Kaufmann SH, Jacobsen M. Identification of T-cell antigens specific for latent mycobacterium tuberculosis infection. *PLoS ONE* 2009;4: e5590.
16. Black GF, Thiel BA, Ota M, Parida SK, Adegbola R, Boom WH, Dockrell HM, Franken KL, Friggen AH, Hill PC, Klein MR, Lalor MK, Mayanja H, Schoolnik G,Stanley K, Weldingh K, Kaufmann SH, Walzl G, Ottenhoff TH; the GCGH Biomarkers for TB Consortium. Immunogenicity of novel DosR regulon-encoded candidate antigens of Mycobacterium tuberculosis in three high-burden populations in Africa. *Clin Vaccine Immunol* 2009; 16: 1203-1212.
17. Weir RE, Gorak-Stolinska P, Floyd S, Lalor MK, Stenson S, Branson K, Blitz R,Ben-Smith A, Fine PE, Dockrell HM. Persistence of the immune response induced by BCG vaccination. *BMC Infect Dis* 2008; 25: 8:9.

18.   Lawn SD, Bangani N, Vogt M, Bekker LG, Badri M, Ntobongwana M, Dockrell HM, Wilkinson RJ, Wood R. Utility of interferon-gamma ELISPOT assay responses in highly tuberculosis-exposed patients with advanced HIV infection in South Africa. *BMC Infect Dis* 2007: 28; 7:99.

19. Geluk A, Lin MY, van Meijgaarden KE, Leyten EM, Franken KL, Ottenhoff TH, Klein MR. T-cell recognition of the HspX protein of Mycobacterium tuberculosis correlates with latent M. tuberculosis infection but not with M.bovis BCG vaccination. *Infect Immun* 2007; 75: 2914-2921.

20. National Collaborating Centre for Chronic Conditions. Tuberculosis: clinical diagnosis and management of tuberculosis, and measures for its prevention and control. London (UK): Royal College of Physicians; 2006

21. Franken KL, Hiemstra HS, van Meijgaarden KE, Subronto Y, den Hartigh J, Ottenhoff TH, Drijfhout JW. Purification of his-tagged proteins by immobilized chelate affinity chromatography: the benefits from the use of organic solvent. *Protein Expr Purif* 2000;18: 95-9.

22. American Thoracic Society. Diagnostic standards and classification of tuberculosis in adults and children. *Am J Respir Crit Care Med* 2000;161: 1376–95.

23. Voskuil MI, Schnappinger D, Visconti KC, Harrell MI, Dolganov GM, Sherman DR, Schoolnik GK. Inhibition of respiration by nitric oxide induces a Mycobacterium tuberculosis dormancy program. *J Exp Med* 2003;198: 705-713.

24. Schnappinger D, Ehrt S, Voskuil MI, Liu Y, Mangan JA, Monahan IM, Dolganov G, Efron B, Butcher PD, Nathan C, Schoolnik GK. Transcriptional Adaptation of Mycobacterium tuberculosis within Macrophages: Insights into the Phagosomal Environment. *J Exp Med* 2003;198: 693-704.

25. Demissie A, Leyten EM, Abebe M, Wassie L, Aseffa A, Abate G, Fletcher H, Owiafe P, Hill PC, Brookes R, Rook G, Zumla A, Arend SM, Klein M, Ottenhoff TH, Andersen P, Doherty TM, The VACSEL Study Group. Recognition of stage-specific mycobacterial antigens differentiates between acute and latent infections with latent infections with Mycobacterium tuberculosis. *Clin Vaccine Immunol* 2006;13: 179-186.

26. Roupie V, Romano M, Zhang L, Korf H, Lin MY, Franken KL, Ottenhoff TH, Klein MR, Huygen K. Immunogenicity of eight dormancy regulon-encoded proteins of Mycobacterium tuberculosis in DNA-vaccinated and tuberculosis-infected mice. *Infect Immun* 2007;75: 941-9.

27. Shi L, Jung YJ, Tyagi S, Gennaro ML, North RJ. Expression of Th1-mediated immunity in mouse lungs induces a Mycobacterium tuberculosis transcription pattern characteristic of nonreplicating persistence. *Proc Natl Acad Sci U S A* 2003;100: 241-246.

28. Boon C, Li R, Qi R, Dick T. Proteins of Mycobacterium bovis BCG induced in the Wayne dormancy model. *J Bacteriol* 2001;183: 2672-2676.

29. Mayuri, Bagchi G, Das TK, Tyagi JS. Molecular analysis of the dormancy response in Mycobacterium smegmatis: expression analysis of genes encoding the DevR-DevS two-component system, Rv3134c and chaperone alpha-crystallin homologues. *FEMS Microbiol Lett* 2002; 211:231-237.

30. Lin MY, Ottenhoff TH. Host-pathogen interactions in latent Mycobacterium tuberculosis infection: identification of new targets for tuberculosis intervention. *Endocr Metab Immune Disord Drug Targets* 2008; 8: 15-29.

FIGURE LEGEND

Figure 1. IFN$-\gamma$ response to *M. tuberculosis* Rv2628 by short (1-day) stimulation of whole blood in controls, remote LTBI, recent infection, patients with TB disease and successfully treated TB patients (cured TB) is shown. Remote LTBI and recent infection subjects were selected to be positive to QFT-IT. Horizontal lines indicate the mean IFN$-\gamma$ production in response to the antigen. Responses were compared using ANOVA with Bonferroni correction for pair-wise comparisons; significant p values are reported ($p<0.05$).

FIGURE 1

Rv 2628

Figure 2A-F. IFN$-\gamma$ response to *M. tuberculosis* Rv2626c, Rv2627c, Rv2031c, Rv2032, QFT-IT and PPD by short (1-day) stimulation of whole blood in controls, remote LTBI, recent infection, patients with TB disease and successfully treated TB patients (cured TB) is shown. Remote LTBI and recent infection subjects were selected to be positive to QFT-IT. Horizontal lines indicate the mean IFN$-\gamma$ production in response to the antigens. Responses were compared using ANOVA with Bonferroni correction for pair-wise comparisons; significant p values are reported (p<0.05).

FIGURE 2

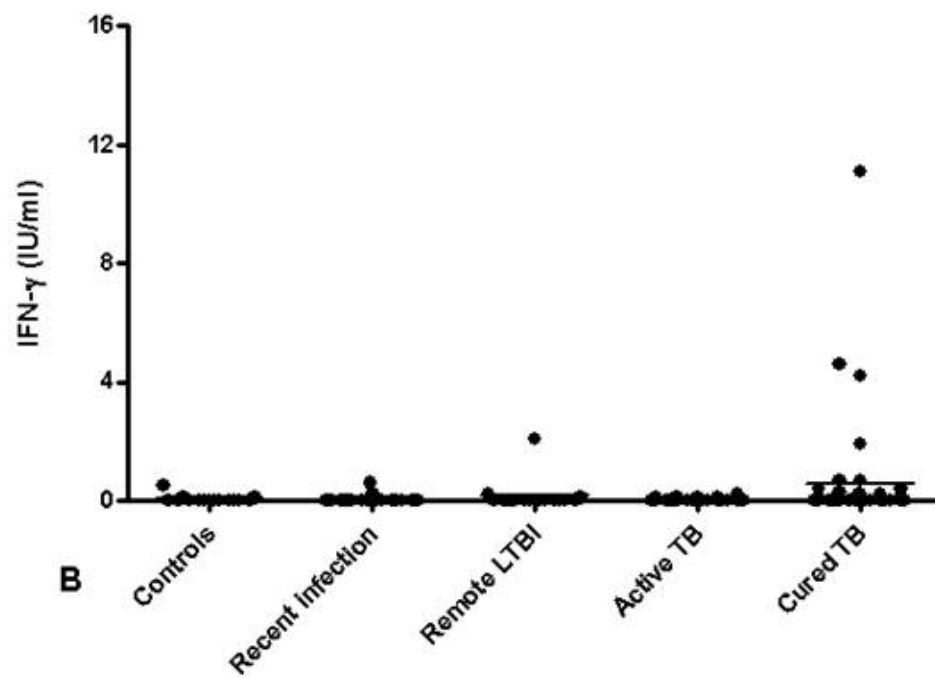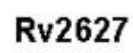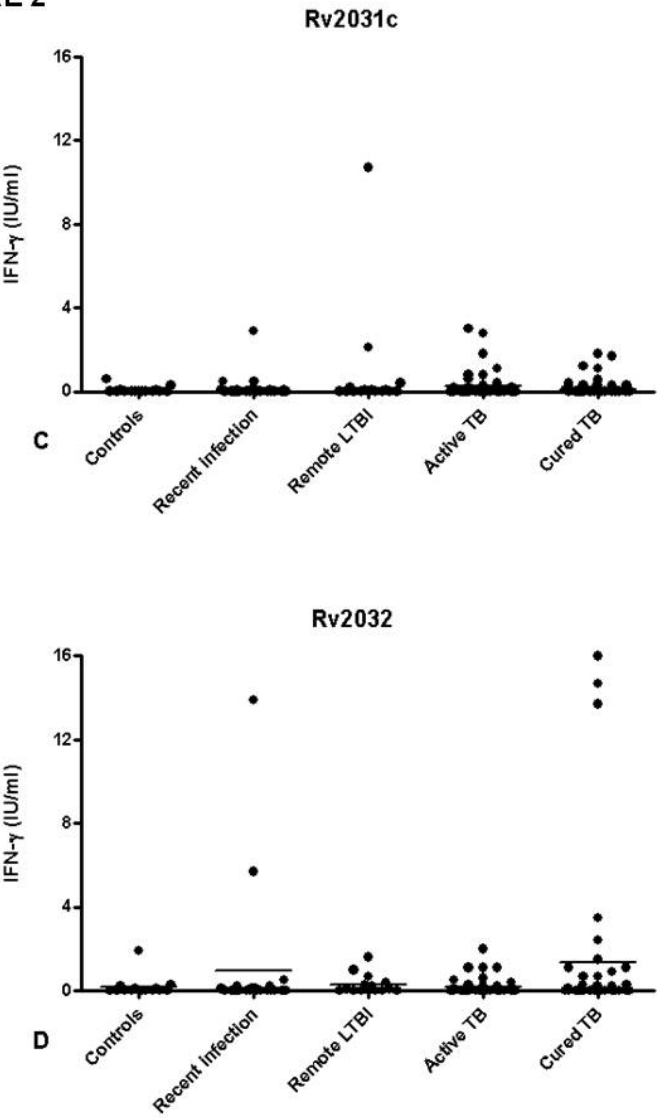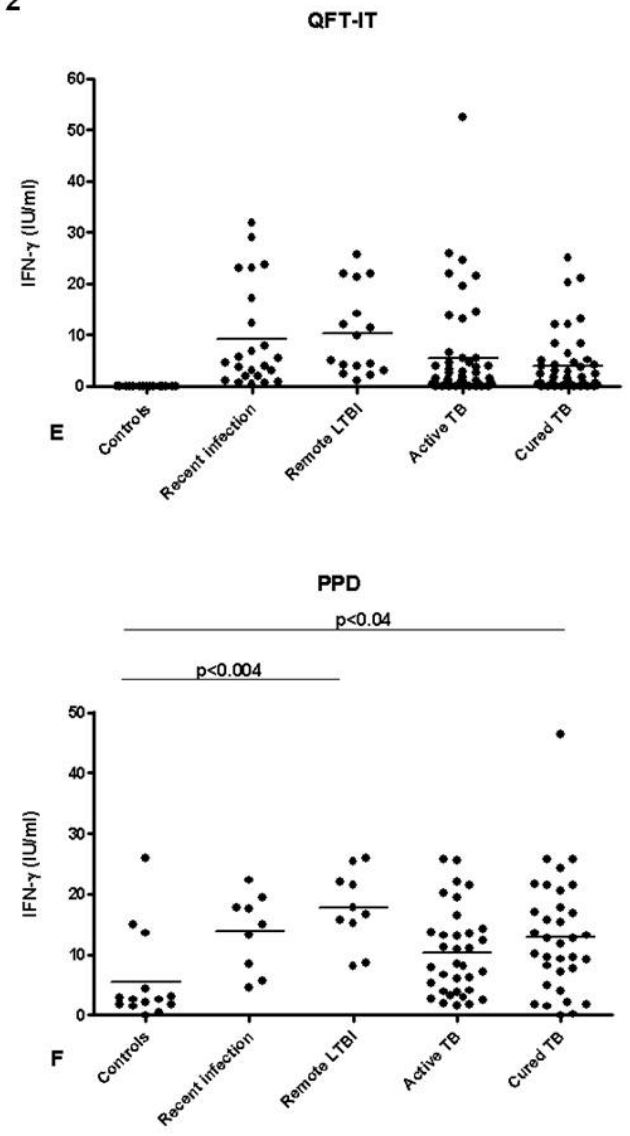

Rv2626

Rv2627

FIGURE 2

Rv2031c

Rv2032

FIGURE 2

QFT-IT

E

PPD

F

Table 1. Demographic and clinical characteristics of the subjects enrolled in the study.

	Total	Controls	Remote LTBI	Recent contacts	Active TB	Cured TB
	N. 149 (%)	N. 15 (%)	N. 16 (%)	N. 23 (%)	N. 50 (%)	N. 45 (%)
Age y mean (±SD)	38.1±1.2	34.2±3.1	50.5±3.7	38.3±3.4	34.8±1.8	38.5±2.3
Female Gender	69 (46.3)	10 (66.7)	4 (25.0)	12 (52.2)	21 (42.2)	22 (48.9)
BCG vaccinated	95 (63.8)	6 (40.0)	3 (18.3)	14 (60.9)	41 (82.0)	31 (68.9)
Origin						
Western Europe	59 (39.6)	12 (80.0)	13 (81.3)	9 (39.1)	9 (18.0)	16 (35.6)
Eastern Europe	56 (37.6)	2 (13.3)	2 (12.5)	9 (39.1)	24 (48.0)	19 (42.2)
Asia	18 (12.1)	-	1 (6.3)	3 (13.0)	10 (20.0)	4 (8.9)
Africa	10 (6.7)	-	-	2 (8.7)	4 (8.0)	4 (8.9)

Latin America	6 (4.0)	1 (6.7)	-	-	3 (6.0)	2 (4.4)
Confirmed TB: localization						
Pulmonary	41 (27.5)	-	-	-	41 (82.0)	-
Extra-pulmonary	5 (3.3)	-	-	-	5 (10.0)	-
Pulmonary and extra-pulmonary	4 (2.6)	-	-	-	4 (8.0)	-
TST mean±SD	ND	1±2.6	28.6±8.9	17.1±11.1	ND	ND

Footnotes: TB: tuberculosis; LTBI: latent tuberculosis infection; TST: tuberculin skin test; ND: not done; SD: standard deviation.

Table 2. IFN–γ response to Rv2628 after 1 day of *in vitro* stimulation in controls and patients at different stage of *M.tuberculosis* infection/disease and p-values for pair-wise comparison of proportion of response among groups.

Group	positive over total (%)	Comparator group			
		Cured TB	Active TB	Recent infection	Controls
		p-value*			
Remote LTBI	14/16 (87.5%)	p=0.178	p<0.001	p<0.001	p<0.001
Cured TB	24/45 (53.3%)	-	p=0.056	p=0.048	p=0.001
Active TB	12/50 (24.0%)	-	-	p=1.000	p=0.537
Recent LTBI	4/23 (17.3%)	-	-	-	p=1.000
Controls	0/15 (0%)	-	-	-	-

Footnotes: TB: tuberculosis; LTBI: latent tuberculosis infection. *The hypothesis of equal proportion was tested by Fisher exact test with Bonferroni correction. *P value was significant if ≤0.05.

Table 3. Accuracy of the response to QuantiFERON TB-Gold In-Tube and RV2628 for the diagnosis of the different status of tuberculosis.

QuantiFERON TB-Gold In-Tube vs IFN-γ response to Rv2628

	QuantiFERON TB Gold In Tube	IFN-γ response to Rv2628	p-value*
Active TB	36/50 (72.0)	12/50 (24.0)	P<0.0001
Past TB	31/45 (68.9)	24/45 (53.3)	P=0.1

Footnotes: TB: tuberculosis; IFN: Interferon. *The hypothesis of equal proportion was tested by Fisher exact test.