Identification of T-Cell Antigens Specific for Latent Mycobacterium Tuberculosis Infection

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Abstract

**Background:** T-cell responses against dormancy-, resuscitation-, and reactivation-associated antigens of Mycobacterium tuberculosis are candidate biomarkers of latent infection in humans.

**Methodology/Principal Findings:** We established an assay based on two rounds of in vitro restimulation and intracellular cytokine analysis that detects T-cell responses to antigens expressed during latent M. tuberculosis infection. Comparison between active pulmonary tuberculosis (TB) patients and healthy latently M. tuberculosis-infected donors (LTBI) revealed significantly higher T-cell responses against 7 of 35 tested M. tuberculosis latency-associated antigens in LTBI. Notably, T cells specific for Rv3407 were exclusively detected in LTBI but not in TB patients. The T-cell IFNγ response against Rv3407 in individual donors was the most influential factor in discrimination analysis that classified TB patients and LTBI with 83% accuracy using cross-validation. Rv3407 peptide pool stimulations revealed distinct candidate epitopes in four LTBI.

**Conclusions:** Our findings further support the hypothesis that the latency-associated antigens can be exploited as biomarkers for LTBI.

Citation: Schuck SD, Mueller H, Kunitz F, Neher A, Hoffmann H, et al. (2009) Identification of T-Cell Antigens Specific for Latent Mycobacterium Tuberculosis Infection. PLoS ONE 4(5): e5590. doi:10.1371/journal.pone.0005590

Editor: Derya Unutmaz, New York University School of Medicine, United States of America

Received February 25, 2009; Accepted April 20, 2009; Published May 18, 2009

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Funding: Part of this work received financial support from the Bill and Melinda Gates Foundation, Grand Challenges to S.H.E. Kaufmann, T.H.M. Ottenhoff, K.L.M.C. Franken, and M. Jacobsen. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

In the vast majority of individuals, specific cellular immunity against M. tuberculosis is capable of controlling infection leading to latent M. tuberculosis infection (LTBI) [1]. LTBI is thought to be associated with a dormancy/non-replicating state of low metabolic activity of the pathogen. Dormancy-related (DosR) antigens as well as proteins expressed during reactivation and resuscitation of dormant bacilli are candidate biomarkers for LTBI and disease reactivation [2,3,4]. A limited number of studies tested latency-associated antigens in immunologic assays. These showed that DosR antigens induced T-cell cytokine expression in humans [5] and mice [6]. Resuscitation-promoting factors (rpf) induced immune responses in mice [7] but have not been tested in humans. Recent studies based on M. tuberculosis knock-out strains revealed that Rv3407, a protein which is not expressed in M. bovis BCG at detectable abundance [8], is under the control of two rpf [9]. Neither Rv3407 nor reactivation-associated antigens (i.e. Rv0104, Rv1115 [10]) have been tested for immunogenicity so far. For reasons of comprehensibility we use the term latency-associated antigens for M. tuberculosis proteins involved in dormancy, resuscitation, and reactivation of M. tuberculosis.

We tested the immunogenicity of 35 latency-associated antigens using different assays based on intracellular cytokine staining for IFNγ and IFNγ-ELISA. IFNγ is a crucial mediator of protection against tuberculosis which strongly depends on T helper type-1 immunity. IFNγ activates infected macrophages at the site of bacterial residence - an essential mechanism for the killing of mycobacteria [11]. IFNγ release in response to immunodominant antigens (i.e. ESAT6, CFP10) is used in standard tests for M. tuberculosis infection (IFNγ release assays, IGRA) [12]. IGRA as well as standard intracellular cytokine staining methods are based on short-term incubation between 6 and 24 h [13]. Principally short-term assays detect recent M. tuberculosis infection while prolonged in vitro stimulation increases sensitivity for LTBI [14,15,16]. Yet, these assays remain insufficient as robust correlates of protection against M. tuberculosis [17]. Therefore, biomarkers which reliably predict protective immunity are urgently needed [18]. Antigens predominantly expressed by dormant M. tuberculosis during LTBI are promising candidate immune markers of protection [5]. We therefore decided to develop an assay based on two rounds of in vitro restimulation, to determine IFNγ production in response to M. tuberculosis latency-associated antigens in LTBI, TB patients, and tuberculin skin test
(TST)-negative donors. Differentially expressed proteins were then tested for their capacity to discriminate between LTBI and TB patients. Finally, overlapping peptide pools were applied to identify immunogenic epitopes of the most promising candidate, Rv3407.

Results

Two-rounds of in vitro restimulation detect T-cell responses against latency-associated antigens

We assessed 29 antigens associated with *M. tuberculosis* dormancy, resuscitation, and reactivation (in short latency, Table 1) for their potential to elicit recall responses after 16 h short-term in vitro stimulation of PBMC from LTBI. We detected IFNγ expression in CD4+CD45RO+ memory T cells from LTBI after stimulation with PPD from *M. tuberculosis* and, to a minor degree, with *M. tuberculosis*-immunodominant proteins ESAT6_CFP-10, TB10.4, and Ag85a (figure 1A). Notably, none of the latency-associated antigens induced detectable cytokine expression after 16 h in LTBI (figure 1A) and no T-cell responses against *M. tuberculosis*-specific antigens were detected in 10 tuberculin skin test (TST)-negative donors (data not shown).

Increased T cell-derived IFNγ responses after prolonged in vitro incubation have been described [5,14,16]. Since our own previous experiments indicated that a second stimulation before measurement with the same antigen is optimal for intracellular cytokine detection (data not shown), we combined 7 days of stimulation with restimulation 16 h prior to analysis. Frequencies of IFNγ-expressing T cells in PBMC from LTBI were markedly increased after stimulation with immunodominant proteins or PPD for 7 days (figure 1B, upper graph). In contrast to the short-term single stimulation assay, latency-associated antigens induced IFNγ expression in memory T cells from the majority of LTBI in the long-term restimulation assay (figure 1B, upper graph). Two of these latency-associated antigens, namely Rv0569 and Rv3407, induced IFNγ expression comparable to the ESAT6_CFP-10 fusion protein, the immunodominant antigen which induced the highest frequencies of cytokine-expressing cells in LTBI. In contrast, none of the antigens induced detectable IFNγ expression in T cells from TST-negative donors in this assay (figure 1B, lower graph). Hence, the “7-day two rounds of restimulation” assay detects specific T-cell responses in LTBI which are missed by the 16h short-term assay.

Comparisons for the individual donor T-cell responses after 7 days and two rounds of restimulation revealed comparable results against three selected immunodominant antigens (i.e. ESAT6_CFP-10, TB10.4, Ag85a) and three latency-associated antigens (i.e. Rv0569, Rv1734, Rv2003) (data not shown). Considering significantly higher proportions of T cells specific for immunodominant antigens in the short-term assay, a simply ‘boosted’ T-cell response by prolonged stimulation would not explain equal proportions since this would have led to higher proportions of T cells specific for immunodominant antigens in the 7-day assay.

Analyses of IFNγ in supernatants by ELISA at day 7 following two rounds of restimulation revealed a similar tendency for PPD, immunodominant, and latency-associated *M. tuberculosis* proteins in LTBI (figure 2A). In contrast to intracellular staining, a considerable amount of IFNγ was also detected in supernatants of PBMC from some TST-negative donors after stimulation with latency-associated antigens (figure 2B). Therefore intracellular measurement of IFNγ after two rounds of in vitro restimulation revealed higher specificity as compared to the IFNγ ELISA.

Significantly stronger T-cell responses in LTBI as compared to TB patients against latency-associated antigens

The expression of the vast majority of antigens used in this study has been associated with latent stages of *M. tuberculosis* infection. Consequently, we addressed the question whether these antigens are differentially recognized by T cells from patients with active pulmonary TB and LTBI. Eleven latency-associated antigens (i.e. Rv0569, Rv1734, Rv1733c, Rv2003, Rv2005c, Rv2006, Rv0140, Rv1009, Rv1884c, Rv2450c, and Rv3407), which induced strong responses in LTBI, were selected for these experiments. Seven of these latency-associated antigens induced significantly higher T-cell responses in LTBI as compared to TB patients (P<0.001 for

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**Table 1. List of proteins candidates.**

| Name            | Category                                      |
|-----------------|-----------------------------------------------|
| ESAT6_CFP-10    | Immunodominant *M. tuberculosis* proteins     |
| TB10.4          |                                               |
| Rv03019c        |                                               |
| Ag85A           |                                               |
| Ag85B           |                                               |
| Hsp65           |                                               |
| Rv0081          | DosR* regulon-encoded *M. tuberculosis* proteins [2] |
| Rv0569          |                                               |
| Rv0573c         |                                               |
| Rv1733c         |                                               |
| Rv1734          |                                               |
| Rv1735c         |                                               |
| Rv1736c         |                                               |
| Rv1996          |                                               |
| Rv1997_C term   |                                               |
| Rv1997_N term   |                                               |
| Rv1998          |                                               |
| Rv2003          |                                               |
| Rv2005c         |                                               |
| Rv2006          |                                               |
| Rv2023          |                                               |
| Rv2023          |                                               |
| Rv2624c         |                                               |
| Rv2625c         |                                               |
| Rv2628          |                                               |
| Rv3126c         |                                               |
| Rv3129          |                                               |
| Rv3133c         |                                               |
| Rv0140          | *M. tuberculosis* reactivation-associated proteins [10] |
| Rv1115          |                                               |
| Rv0867c         | *M. tuberculosis* resuscitation promoting factors [30] |
| Rv1009          |                                               |
| Rv1884c         |                                               |
| Rv2450c         |                                               |
| Rv3407          | *M. tuberculosis* resuscitation-associated protein [19] |

*M. tuberculosis* dormancy-related antigens.
doi:10.1371/journal.pone.0005590.t001
Rx1733c, Rx2003, Rx2005c, Rx0140, and Rx3407; \( P < 0.01 \) for Rx1009; \( P < 0.05 \) for Rx2450c (figure 3A).

We set a threshold of 0.2% IFN-\( \gamma \)^+CD4^+CD45RO^+ T cells (i.e. 20-fold above the assumed flow cytometric detection threshold of 0.01%) to define positive T-cell responses against latency-associated antigens in LTBI and TB patients for the most promising candidates (\( P < 0.001 \)) (Table 2). Latency-associated antigens induced T-cell responses in the range of 45.5 to 72.7% of LTBI patients. A specific T-cell response against Rx2003 was detected in the vast majority of LTBI (16 of 22) but also induced

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**Figure 1.** IFN-\( \gamma \)-expressing CD4^+CD45RO^+ T cells after 16 h and 7 days restimulation with immunodominant and latency-associated antigens from LTBI and TST-negative controls. Intracellular cytokine expression after 16 h restimulation in PBMC from LTBI (A), and 7 days – including two rounds of in vitro restimulation – in PBMC from LTBI (B, upper graph) and TST-negative donors (B, lower graph) are shown. Scatter plots indicate mean and standard deviation. Percentages of IFN-\( \gamma \)-expressing CD4^+CD45RO^+ memory T cells are indicated on the y-axis for SEB, PPD from *M. tuberculosis*, and tested antigens (x-axes). Background values of non-stimulated controls were subtracted for each individual donor. The most promising candidate Rx3407 is underlined. PPD: purified protein derivative of *M. tuberculosis*; SEB: Staphylococcus enterotoxin B. doi:10.1371/journal.pone.0005590.g001
positive responses in 25% (5 of 20) of the TB patients. Notably, the resuscitation-associated antigen Rv3407 [19] induced positive T-cell responses in 12 LTBI (55%) but in none of the TB patients. These results prompted us to determine whether T-cell responses against \textit{M. tuberculosis} antigens are sufficient for classification of TB patients and LTBI.

T-cell responses against latency-associated antigens discriminate between LTBI and TB patients

We included the 11 immunogenic latency-associated antigens together with ESAT6, CFP-10, and PPD in a discrimination approach. Random forest analyses together with leave-1-out cross-validations across all possible combinations were applied to determine the prediction accuracy and relative importance of each factor for classification. These analyses revealed a cross-validated prediction accuracy of 83% between TB patients and LTBI (figure 3B). The relative feature importance for discrimination showed that Rv3407 was by far the most influential factor for classification (figure 3C). Consequently we designed overlapping peptide pools of Rv3407 to identify immunogenic epitopes.

Identification of immunogenic peptides of Rv3407

The matrix design of the overlapping peptide pools leads to six ‘vertical’ and five ‘horizontal’ pools. Each of the 29 peptides of Rv3407 was present in two pools (Table 3). In 6 out of 10 LTBI tested – those with the highest T-cell response against Rv3407 – a minimum of 0.2% IFN$\gamma$-expressing CD4$^+$ T cells was detected after stimulation with at least two of the peptide pools (figure 4). In four LTBI (LTBI-A to LTBI-D) a major immunogenic epitope could be identified (figure 4A-D) while in two LTBI (LTBI-E, LTBI-F) with the weakest response against the Rv3407 protein no prominent epitope-specific T-cell responses were observed (figure 4E-F). Notably peptide 6 (LRQHASRYLARVEAG) identified in LTBI-A induced IFN$\gamma$ expression in about 1% of CD8$^+$ memory T cells, as well (data not shown). Therefore distinct peptide epitopes in different LTBI were recognized by antigen-specific T cells and ongoing studies will determine whether this is due to differential HLA-phenotypes.

We consider the detection of specific T-cell immunity against latency-associated antigens and the identification of immunogenic epitopes an initial step towards definition of reliable biomarkers for protective immunity against TB.
T-cell immunity in LTBI is crucial for protection against resuscitation and reactivation of *M. tuberculosis*. We established two rounds of *in vitro* restimulation assay that induced specific T-cell immunity against latency-associated antigens which were not detected in a short-term T-cell assay. These intracellular IFN-γ responses were significantly increased in LTBI as compared to TB patients. This assay was highly specific since IFN-γ-expressing memory T cells were only detected in PBMC from LTBI and not from TST-negative donors.

We identified a subgroup of latency-associated antigens that induced significantly stronger T-cell responses in LTBI as compared to TB patients. This is in accordance with a previous study that showed increased responses against the latency-associated antigens.

**Table 2. Positive T-cell responses against latency-associated antigens.**

| Antigen     | LTBI (%) | TB patients (%) |
|-------------|----------|-----------------|
| Rv2003      | 16 (72.7) | 5 (25)          |
| Rv1733c     | 15 (68.2) | 2 (10)          |
| Rv3407      | 12 (54.5) | 0 (0)           |
| Rv2005c     | 12 (54.5) | 3 (15)          |
| Rv0140      | 10 (45.5) | 2 (10)          |

1 Number of donors with more than 0.2% IFN-γ-producing T cells.

doi:10.1371/journal.pone.0005590.t002

**Discussion**

T-cell immunity in LTBI is crucial for protection against resuscitation and reactivation of *M. tuberculosis*. We established two rounds of *in vitro* restimulation assay that induced specific T-cell immunity against latency-associated antigens which were not detected in a short-term T-cell assay. These intracellular IFN-γ responses were significantly increased in LTBI as compared to TB patients. This assay was highly specific since IFN-γ-expressing memory T cells were only detected in PBMC from LTBI and not from TST-negative donors.

We identified a subgroup of latency-associated antigens that induced significantly stronger T-cell responses in LTBI as compared to TB patients. This is in accordance with a previous study that showed increased responses against the latency-
assessed antigen Rv1733c in LTBI as compared to TB patients [5]. The remaining candidates (i.e. DosR regulon-encoded proteins Rv2003 and Rv2005c, the reactivation-associated protein Rv0140, the resuscitation promoting factors Rv2450c and Rv3407, as well as Rv3407) have not been tested so far. Rv3407 improved the vaccine efficacy of BCG suggesting that subunit vaccination can be considered for TB patients although it is likely that most TB patients undergo a phase of latent infection prior to active disease. As an initial attempt to address this point we determined T-cell responses twice (prior to and under drug treatment) for a subgroup of TB patients (data not shown) and detected increased frequencies for some of the latency proteins in patients under drug treatment. Therefore the lack of latency-associated T cells at least for some antigens seems to be restricted to the acute TB phase. This could be explained i) by the recruitment of these T cells to the lung or ii) increased T-cell regulation during the acute TB phase.

In contrast to intracellular IFNγ analyses, some IFNγ was also detected in supernatants of PBMC from a subgroup of TST-negative donors after stimulation with latency-associated antigens as described previously [5]. The second round of restimulation did not cause this higher background in TST-negative donors, since supernatants collected at day 6 from a subgroup of uninfected donors showed similar IFNγ abundance as compared to the day 7 time point after restimulation (data not shown). Using flow cytometry, we excluded the possibility that other immune cell populations (e.g. NK cells, NKT cells, CD8+ T cells) were the source of IFNγ (data not shown). A possible explanation would be exhaustion or induced cell death (e.g. by apoptosis) after restimulation 16 h prior to analysis [25]. Alternatively, these donors could be false negative in the T-Spot TB® although we have no evidence to support this assumption, or these responses reflect cross-reactivity towards antigens expressed by commensal bacteria [26].

In an initial attempt to characterize the T-cell responses against latency-associated antigens we compared the T-cell responses against immunodominant and latency-associated antigens from individual donors. While immunodominant antigens ESAT6_CFP-10, TB10.4, Ag85a induced significantly stronger T-cell responses in the 16-h short-term assay as compared to latency-associated antigens (i.e. Rv0569, Rv1734, Rv2003), comparable proportions of IFNγ-producing T cells were detected in the 7-day two rounds of restimulation assay. We conclude that a simply ‘boosted’ T-cell response by prolonged stimulation and two rounds of restimulation did not cause this effect as this would lead to higher proportions of T cells specific for immunodominant antigens. Distinct maturation stage of T-cell populations specific to higher proportions of T cells specific for latency-associated antigens we compared the T-cell responses to two rounds of restimulation did not cause this effect as this would lead to higher proportions of T cells specific for immunodominant antigens. Distinct maturation stage of T-cell populations specific for latency-associated antigens may account for these findings.

In an initial attempt to characterize the T-cell responses against latency-associated antigens we compared the T-cell responses against immunodominant and latency-associated antigens from individual donors. While immunodominant antigens ESAT6_CFP-10, TB10.4, Ag85a induced significantly stronger T-cell responses in the 16-h short-term assay as compared to latency-associated antigens (i.e. Rv0569, Rv1734, Rv2003), comparable proportions of IFNγ-producing T cells were detected in the 7-day two rounds of restimulation assay. We conclude that a simply ‘boosted’ T-cell response by prolonged stimulation and two rounds of restimulation did not cause this effect as this would lead to higher proportions of T cells specific for immunodominant antigens. Distinct maturation stage of T-cell populations specific for latency-associated antigens may account for these findings.

This may lead to differences in the activation threshold prerequisite for in vitro cytokine expression. Previous studies demonstrated higher sensitivity of a 6-day whole blood assay for
T-cell responses in LTBI suggesting that prolonged in vitro restimulation preferentially induced central memory T cells [14,16]. This supports the notion that T cells specific for latency-associated antigen have a distinct maturation/activation status. Detailed investigation of both T-cell populations will be performed in the future, including global gene expression profiling.

**Materials and Methods**

**Ethics Statement**

The clinical investigations have been conducted according to the principles expressed in the Declaration of Helsinki. All donors gave written informed consent and the local ethics committee of the University Hospital Berlin at Charité approved this study (205-18.1; 205-18.2; 205-18.3).

**Human subjects**

Peripheral blood (40 ml) was obtained from 22 LTBI and 20 patients with active pulmonary TB. LTBI and TB patients were recruited at HELIOS clinic Emil-von-Behring and the Charité, both in Berlin; and at the Asklepios Clinic, Munich-Gauting. Diagnosis of LTBI was based on positive TST (>10 mm) and positive T-Spot TB™. TB diagnosis was based on patient history, chest X-ray, TST, and mycobacterial culture. Ten *M. tuberculosis* non-infected (TST-negative) donors were recruited among volunteers of the Max Planck Institute for Infection Biology, Berlin. All
donors were Caucasians. There was no gender bias and only minor age differences between both study groups (Table 4).

Recombinant M. tuberculosis proteins and synthetic peptide pools

Recombinant *M. tuberculosis* proteins (Table 1) were expressed in *Escherichia coli* and purified as described previously [5,27]. Synthetic 15-mer peptide pools representing Rv3407 were generated with an overlap of 12 amino acids using combinatorial chemistry (Jerini). The pools were designed as matrix pools as described before [28]. The peptides are listed in Table 3.

Cell culture assays

Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation (Biocoll, Biochrom) following manufactur-

| Table 4. Characteristics of LTBI, TB patients and non-infected donors. |
|-----------------|-----------------|-----------------|
|                 | LTBI¹           | TB patients²    | Non-infected donors³ |
| Total number    | 22             | 20             | 10             |
| Female          | 11             | 10             | 5              |
| Male            | 11             | 10             | 5              |
| Age range years (median) | 28–64 (47) | 26–63 (41) | 24–55 (36) |

¹healthy latency *M. tuberculosis*-infected donors.
²patients with active pulmonary TB.
³healthy tuberculin skin test (TST)-negative donors.

doi:10.1371/journal.pone.0005590.t004

Figure 5. Gating procedures of flow cytometry analyses to determine protein candidate specific T cell proportions. Representative analyses from a patient with Tb (A) and an LTBI (B) are shown. Open red circles and dot plot connected by red arrows indicate the sequence of analysis steps. First, lymphocytes were gated using size (forward scatter; FSC) and granularity (side scatter, SSC). These cells were then analyzed for CD4 expression. CD4⁺ T cells were analyzed for IFNγ CD45RO expression for each stimulation (without stimulus, w/o; protein 3; protein 11; SEB). Proportions of CD45ROhigh IFNγ expressing CD4⁺ T cells (upper right quadrants) were determined. The background of non-stimulated T cells (w/o) was subtracted for analyses. doi:10.1371/journal.pone.0005590.g005
er’s instructions and 2×10^5 cells were cultured in 200 μL medium A (RPMI-1640 (GIBCO, Invitrogen) with 10% human serum (Sigma-Aldrich), 100 μg/ml penicillin, 100 μg/ml streptomycin, 1 mM L-glutamine and 10 mM HEPES (all PAA laboratories)) using 96-well round-bottom plates (NUNC). Each well was surrounded by wells filled with sterile water (200 μl) to avoid drying effects. In the short-term assay we stimulated PBMC for 16 h with different recombinant M. tuberculosis proteins (5 μg/ml, see Table 1), purified protein derivative (PPD) of M. tuberculosis (5 μg/ml) (Statens Serum Institute), Staphylococcus enterotoxin B (SEB) (1 μg/ml) (Sigma-Aldrich), and synthetic peptide pools (5 μg/ml peptide) at 37°C and 5% CO2. After 4 h of incubation brefeldin A (10 μg/ml) (Sigma-Aldrich) was added.

In the long-term assay the same stimuli were applied at the beginning except for SEB that was added on day 6 to a well of non-stimulated cultured cells. For each of the other stimuli the same antigen was added on day 6 to a well of non-stimulated cultured cells. For each of the other stimuli the same antigen was added on day 6 to a well of non-stimulated cultured cells. For each of the other stimuli the same antigen was added on day 6 to a well of non-stimulated cultured cells.

Afterwards, for both assays, cells were fixed and permeabilized using cytotox/fixperm™ (BD Biosciences) following manufacturer’s instructions and stained with the following fluorochrome-labeled monoclonal antibodies: α-CD3 (Pacific Blue) (BioLegend), α-CD4 (APC-Cy7), α-CD8 (PerCP), α-CD45RO (PO7-Cy7), α-IFNγ (APC) (all BD Biosciences). After staining for 45 min at 4°C, cells were washed twice in cytperm/wash, and once in PBS containing 10% FCS. Measurements and analyses were performed using a LSRII flow cytometer and FACS-Diva software (both BD Biosciences). Examples of the procedures of analysis are shown in Figure 5A for a TB patient and Figure 5B for an LTBI. About 18000 CD4+ CD45RO+ T cells were collected for each sample. For background determination values, of non-stimulated controls were subtracted from the different stimuli of each individual donor.

Cytokine analyses in culture supernatants by ELISA
PBMC (5×10^6) were added to 96-well round-bottom plates (NUNC) in 200 μl medium. Cells were stimulated for 7 days as described in the previous section but without adding brefeldin A. We harvested 110 μl of cell culture supernatant at day 7. The IFNγ ELISA (BD Biosciences) was performed according to manufacturer’s guidelines. Plates were analyzed by measuring extinction at 450 nm using an ELISA plate reader (Molecular Devices).

Donor classification and significance analyses
The discriminatory power for classifying TB patients and LTBI was investigated using random forest analysis [29] based on the proportion of IFNγ-expressing CD4+, CD45RO+ T cells against 13 selected stimuli (i.e. PPD, ESAT6, CFP10, RV3659, RV1732c, RV1734, RV2003, RV2005c, RV2006, RV0140, RV1809c, RV2450c, and RV3407). We determined the relative feature importance for discrimination using a leave-1-out cross validation for all possible combinations of genes and assessed the proportion of correctly classified patients in the left-out group.

For significance analyses the Mann-Whitney U-test was used. Significance of two-sided p-values is indicated as follows: * P<0.05, ** P<0.01, and *** P<0.001.

Acknowledgments
We thank PD Dr. T. Bauer, Head of the Clinic of Pneumonology, HELIOS Klinikum Emil von Behring, Berlin, for supporting this study and M.L. Grossman for carefully reading the manuscript. This work is part of the Ph.D. thesis of S.D. Schuck.

Author Contributions
Conceived and designed the experiments: SMD JF. Performed the experiments: SMD HM. Analyzed the data: SDR MJ. Contributed reagents/materials/analysis tools: FK AN HH KF DR THMO SHEK. Wrote the paper: SBS THMO SHEK MJ.

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