Clinical & Cellular Immunology

Smits et al., J Clin Cell Immunol 2015, 6:4
http://dx.doi.org/10.4172/2155-9899.1000341

Research Article Open Access

Immunological Signatures Identifying Different Stages of Latent *Mycobacterium tuberculosis* Infection and Discriminating Latent from Active Tuberculosis in Humans

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Received date: April 25, 2015; **Accepted date:** July 10, 2015; **Published date:** July 17, 2015

Abstract

Objectives: One third of the world population is considered latently infected with *Mycobacterium tuberculosis* (LTBI) and sterilizing this reservoir of bacteria that may reactivate is required for tuberculosis (TB) elimination. The group of individuals with LTBI is heterogeneous with some of them being more at risk to develop TB disease than others. Improved diagnosis of subjects with LTBI is needed, allowing to differentiate subjects with LTBI from those with active TB, and to select among LTBI subjects those who are more at risk to develop active TB. We have characterized at the cellular level both the quantitative and qualitative T cell responses to different mycobacterial antigens in selected populations of infected subjects in order to identify new biomarkers that could help to identify *M. tuberculosis*-infected subjects and to stratify them in risk groups for reactivation of the infection.

Methods: lymphoblast frequencies and cytokine production (IFN-γ, TNF-α, IL-2) among $CD4^+$ and $CD8^+$ T cells were analyzed by flow cytometry after *in vitro* stimulation with the latency antigen heparin-binding haemagglutinin (HBHA) or early-secreted antigen Target-6 (ESAT-6) of peripheral blood mononuclear cells from clinically well characterized *M. tuberculosis*-infected humans (28 LTBI, 22 TB disease,12 controls). The LTBI group defined according to the Center for Disease Control guidelines was subdivided into QuantiFERON-TB Gold in-Tube (QFT) positive and negative subgroups.

Results: similar to TB patients, QFT^+ LTBI subjects had higher proportions of HBHA-induced TNF-$\alpha^{single+}$ $CD4^+$ lymphocytes than QFT^- LTBI subjects ($p<0.05$). Compared to LTBI subjects, TB patients had higher frequencies of ESAT-6-induced $CD8^+$ lymphoblasts ($p<0.001$), higher proportions of ESAT-6-induced IFN-γ^+TNF-α^+ $CD4^+$ T lymphocytes ($p<0.05$), and lower proportions of HBHA-induced IFN-γ^+TNF-α^+IL-2^+ ($p<0.05$) $CD4^+$ T lymphocytes.

Conclusions: these data provide new biomarkers to discriminate active TB from LTBI, and more interestingly, help to identify LTBI subjects with increased likelihood to develop TB disease.

Keywords: *Mycobacterium tuberculosis*; Latent tuberculosis stratification; Heparin-binding haemagglutinin; Early-secreted-antigen-6; Multifunctional T cells; $CD4^+$ T lymphocytes; $CD8^+$ T lymphocytes; Lymphoblasts; IFN-γ; TNF-α; IL-2

Introduction

Tuberculosis (TB) remains a major global health problem being the second leading cause of death from infection, after human immunodeficiency virus infection [1]. The World Health Organization (WHO) estimates that 9.0 million people developed TB in 2013 and that 1.5 million died, including 360 000 people who were infected with human immunodeficiency virus (HIV) [1]. In addition to clinical TB, it is estimated that one third of the world population is latently infected with *Mycobacterium tuberculosis*. Most latently TB infected (LTBI) subjects control the infection thanks to appropriate immune responses, and they do not develop any sign of disease. Nevertheless, these LTBI subjects have a lifetime risk of about 10% to reactivate their infection, leading to progression to TB disease, this risk being higher in different clinical conditions associated with immune deficiency [2,3]. Despite being asymptomatic, these subjects constitute an enormous reservoir of *M. tuberculosis* and TB elimination requires focus on sterilizing the pool of LTBI individuals [4,5]. The WHO recommends systematic screening of contacts of TB patients and high-risk groups in

order to achieve the post 2015 tuberculosis strategy aiming to reduce the tuberculosis incidence by 90% by 2035 compared with the 2015 incidence [6]. Improving diagnostic tools of LTBI is therefore urgently needed [7,8], and more research should be done to identify biomarkers able to predict the progression from LTBI to active TB disease.

Identification of LTBI is achieved by immunological tests that re-challenge the individual acquired immune response with mycobacterial antigens, either *in vivo* (tuberculin skin test, TST), or *in vitro* (interferon-gamma release assay, IGRA). Unfortunately these tests do not differentiate subjects with LTBI from those suffering from active TB disease [2] and they poorly predict the risk for the development of TB in subjects with LTBI [7].

However, several recent evidences support the notion that LTBI is not a single defined state but comprises a heterogeneous and dynamic spectrum of interaction patterns between host and pathogen [8]. This heterogeneity may lead to different outcomes, ranging from clearing the infection to pre-reactivation of the disease, probably reflecting various states of bacterial behaviour between dormancy and steady state replication [8-11]. The identification of these different LTBI subgroups is important, as it may help to target preventive therapy only to those subjects that have the highest risk to reactivate their infection [4,5]. We have previously reported that, in contrast to TST and the commercialized IGRA (QuantiFERON-TB Gold In-Tube assay or QFT-GIT and T-SPOT.TB), the IFN-γ response to the mycobacterial latency antigen heparin-binding haemagglutinin (HBHA) provides good discrimination between subjects with LTBI and patients with active TB [12,13]. We have further proposed a risk stratification of subjects with LTBI based on the combination of IFN-γ responses to HBHA and to the Early-Secreted Antigen Target-6 (ESAT-6) [14]. Individuals who secrete high levels of IFN-γ in response to HBHA but not in response to ESAT-6 represent a significant proportion of LTBI subjects in low TB incidence countries [12,14,15]. We suggested that these individuals are able to control their infection, whereas those with low HBHA-specific, but high ESAT-6-specific responses may be at risk of reactivation [14,15].

In this study, we extend the characterization of the immune responses of the subgroups of subjects with LTBI previously defined by combining the IFN-γ responses to HBHA and to ESAT-6, to a more profound quantitative and qualitative analysis at the cellular level of the T cell responses. We analyzed by flow cytometry whether different cytokines (IFN-γ, interleukin-2 (IL-2) and tumor-necrosis-factor-alpha (TNF-α)) were produced alone or in combination by $CD4^+$ or $CD8^+$ T lymphocytes after stimulation with HBHA and ESAT-6. We identified different immune signatures between the two subgroups of subjects with LTBI and also between patients with active TB and subjects with LTBI, providing new evidence of the existence of a spectrum of *M. tuberculosis* infection.

Materials and Methods

Study population

Fresh blood samples were prospectively collected in Belgium, a low-TB incidence country, from 50 subjects recruited among health care workers and medical students who spontaneously reported to be non-infected or LTBI, and from 31 patients referred by the clinicians as possible clinical TB (Supplementary Figure 1A). These individuals were retrospectively classified as non-infected, LTBI or presenting with active TB after careful analysis of the results of the tuberculin skin tests (TST), the *M. tuberculosis* exposure risk factors, the clinical, radiological and microbiological data. Nineteen subjects were rejected and for reasons summarized in supplementary Figure 1A and a summary of the demographic and clinical characteristics of the 62 included subjects is given in Table 1. As recommended in Belgium, LTBI diagnosis was based on TST results, chest X-ray and potential risk factors [16] whereas interferon-γ release assays (IGRA) are not used for the clinical evaluation of subjects and their potential LTBI status. The TST was performed using 2 IU of PPD RT23 (Tuberculin Purified Protein Derivative, Statens Serum Institute, Copenhagen, Denmark) and an induration ≥ 10 mm was considered positive for subjects with a major *M. tuberculosis* exposure risk factor, while a cut-off of 15 mm was used for all others, as recommended by the CDC [17]. The QFT-GIT (Qiagen, Antwerp, Belgium) was however performed for comparison purposes and it scored positive for only 10/28 LTBI subjects defined by the above mentioned criteria (Table 1). Detailed informations on LTBI subjects are reported in supplementary Figure 1B. TB diagnosis was based either on microbiological proof or high clinical suspicion with favorable response to anti-TB treatment, and blood sampling was performed before or within maximum 5 days of treatment. Five TB patients under treatment for more than 5 days were included as a separate cohort. Non-infected controls were defined by a negative TST reaction. The ethics committee ULB-Hôpital Erasme approved the study and each participant signed an informed consent form.

Antigen stimulation

Peripheral blood mononuclear cells (PBMC) were isolated and cultured as previously described [18]. Fresh PBMC were stimulated for five days with HBHA (10 µg/ml; purified as previously described [19], ESAT-6 (Lionex, Braunschweig, Germany; 10 µg/ml) or PPD, (Statens Serum Institute, Copenhagen, Denmark; 4 µg/ml) used as a control of previous mycobacterial exposure. No antigen addition and Staphylococcus enterotoxin B (SEB; Sigma–Aldrich, St. Louis, MO; 0.5 µg/ml) were used as negative and positive controls, respectively. QFT was performed on whole blood according to manufacturer's instructions.

Antigen-induced lymphoblasts

Lymphoblasts induced by antigen stimulation were distinguished from resting lymphocytes based on size and granularity [20]. Gating strategy and a representative example are shown in Supplementary Figure 2 and 3, respectively.

Intracellular cytokine staining and polychromatic flow cytometry

During the last 16 hours of culture, Brefeldin A (3 µg/ml) and Golgistop (1/2000 final dilution; both from BD Biosciences, Mountain View, CA) were added to block cytokine secretion. Labeling of dead cells, fixation and permeabilization were performed as previously described [18]. Cells were labeled and acquired on a FACS CantoII flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (Treestar, Ashland, OR) as shown in supplementary Figure 2.

Subject characteristics	Healthy controls	LTBI individuals	Untreated TB patients	Treated TB patients
N	12	28	17	5
Median age (years) [range]	26 [24-61]	41 [22-58]	32 [20-53]	23 [20-52]
sex				
female n (%)	10 (83,3)	19 (67,9)	7 (41,2)	3 (60)
male n (%)	2 (16,7)	9 (32,1)	10 (58,8)	2 (40)
Ethnic origin				
Caucasian n (%)	8 (66,7)	17 (60,7)	5 (29,4)	2 (40)
North African n (%)	4 (33,3)	4 (14,3)	3 (17,6)	2 (40)
Sub Saharian n (%)	0 (0)	5 (17,9)	7 (41,2)	0 (0)
Hispanic n (%)	0 (0)	2 (7,1)	2 (11,8)	1 (20)
BCG status				
Vaccinated>10 y ago n (%)	0 (0)	18 (64,3)	0 (0)	1 (20)
Vaccinated date unknown n (%)	0 (0)	3 (10,7)	2 (11,8)	0 (0)
Unvaccinated n (%)	12 (100)	4 (14,3)	7 (41,2)	3 (60)
Unknown n (%)	0 (0)	3 (10,7)	8 (47,1)	1 (20)
TST[a] [range]				
Negative n (%)	12 (100)	0 (0)	1 (5,9)	0 (0)
Positive n (%)	0 (0)	28 (100)	5 (29,4)	2 (40)
Unknown n (%)	0 (0)	0 (0)	11 (64,7)	3 (60)
Detailed status				
Recent LTBI n (%)[b]	-	5 (17,9)	-	-
Remote LTBI n (%)[c]	-	15 (53,6)	-	-
Unknown n (%)	-	8 (28,6)	-	-
Pulmonary TB n (%)	-	-	10 (58,8)	2 (40)
Disseminated TB n (%)	-	-	7 (41,2)	3 (60)
Treatment (days) [range]	-			90 [15-180]
Diagnosis				
Culture positive n (%)	-	-	13 (76,5)	3 (60)
Culture negative n (%)	-	-	4 (23,5)	2 (40)
QFT[d]				
Negative n (%)	-	18 (64,3)	-	-
Positive n (%)	-	10 (35,7)	-	-

[a]TST: tuberculin skin test; [b]Recent *M. tuberculosis* infections indicate infections<2years; [c]Remote *M. tuberculosis* infections indicate infections ≥ 2years; [d]QFT: QuantiFERON-TB Gold in-tube assay

Table 1: Demographic and clinical data of subject subgroups

Analysis of cytokine profile

Among responders, cytokine profiles were compared using SPICE software, representing relative frequencies of cells producing all 7 combinations of the three examined cytokines [20]. Responders were defined based on total frequency of cytokine-producing cells (IFN-γ and/or TNF-α and/or IL-2). Two criteria were used: a response ≥ 2 times the background (unstimulated sample), and a background-corrected response above an antigen-specific threshold, defined by the 75th percentile of the non-infected controls (0.35%, 0.2%, 1% for $CD4^+$ T cells, and 0.15%, 0.15%, 0.2% for $CD8^+$ T cells, for HBHA, ESAT-6 and PPD, respectively).

Statistical analysis

Data were analyzed using GraphPad Prism version 5.00 for Windows (San Diego, CA) or IBM SPSS statistics version 21 (Chicago, IL). Kruskal-Wallis-test, followed by two-by-two comparisons (Dunn's post-test) were applied for continuous variables and Fisher's exact-test for categorical variables. Cytokine profiles were compared with SPICE (Student's t-test) [21]. A $p<0.05$ was considered significant.

Results

Antigen-induced lymphoblasts

We first compared the frequencies of lymphoblasts among $CD4^+$ and $CD8^+$ T cells, between non-infected controls, LTBI subjects and TB patients, as readout of antigen-specific activation. The frequency of HBHA-induced lymphoblasts among $CD4^+$ T cells was significantly higher for LTBI subjects and TB patients than for non-infected controls ($p \leq 0.01$), but no significant differences were noted between LTBI subjects and TB patients (Figure 1A). Similar results were obtained for the HBHA-induced $CD8^+$ lymphoblasts, but the frequencies were lower (Figure 1B). In contrast, the frequencies of ESAT-6-induced $CD4^+$ lymphoblasts were higher in the active TB group compared to the LTBI group ($p \leq 0.01$) with however positive results in both groups (Figure 1C). Separate analysis of the QFT^+ and QFT^- LTBI subjects indicated that only the QFT^+ subgroup had ESAT-6-induced $CD4^+$ lymphoblasts, with frequencies that were similar to those of the TB patients. ESAT-6-induced $CD8^+$ lymphoblasts were found almost exclusively in TB patients ($p \leq 0.001$) (Figure 1D), suggesting that this may be a biomarker of TB disease. ROC curve analysis for the frequencies of $CD8^+$ lymphoblasts in TB patients compared to controls and LTBI subjects indicated a sensitivity of 94% of this biomarker for TB disease with a specificity of 90% (area under the curve 0.93 – Fisher's exact $p<0.0001$).

Antigen-induced cytokine-producing cells

We next compared the frequencies of cytokine-producing cells (IFN-γ, TNF-α, IL-2) among the $CD4^+$ and $CD8^+$ T lymphocytes after stimulation with HBHA or ESAT-6. *In vitro* stimulation of the PBMC with HBHA induced higher frequencies of IFN-γ-containing $CD4^+$ T lymphocytes in infected compared to non-infected subjects (Figure 2A). The frequencies were slightly lower for TB patients than for LTBI subjects, in agreement with previous results obtained with HBHA-IGRA [12,13]. Higher frequencies of HBHA-induced TNF-α-containing-$CD4^+$ lymphocytes were also noted in infected compared to non-infected subjects ($p<0.001$ and $p<0.01$ for LTBI and TB, respectively), whereas no differences between the groups were observed for the HBHA-induced IL-2-containing $CD4^+$ lymphocytes (Figure 2A).

The frequencies of ESAT-6-induced IFN-γ-producing cells were also higher in infected compared to control subjects, with a significant increase in TB patients compared to LTBI and to control subjects ($p<0.05$ for LTBI and controls, Figure 2B), confirming previous IGRA results [12]. Only few LTBI subjects produced IFN-γ in response to ESAT-6 in this cohort, and therefore there was no significant difference between LTBI subjects and non-infected controls. TB patients were also characterized by higher frequencies of ESAT-6-induced TNF-α-containing $CD4^+$ T lymphocytes compared to LTBI and non-infected subjects ($p<0.01$ for LTBI and controls, Figure 2B).

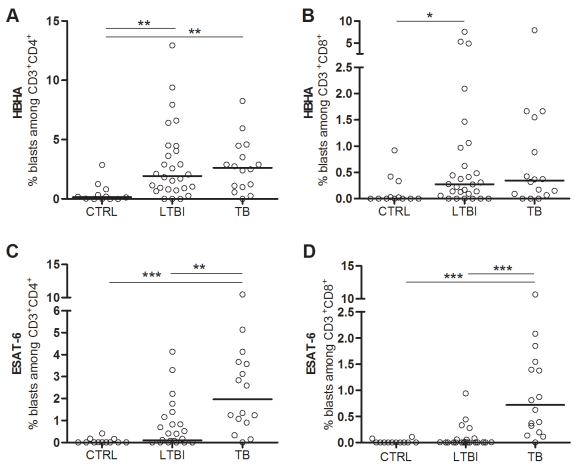

Figure 1: Comparison of the percentages of blast cells among PBMC induced by mycobacterial antigens in healthy controls (CTRL, n=12), LTBI subjects (LTBI, n=28) and TB patients (TB, n=17). Panel A and B represent frequencies of $CD3^+CD4^+$ (A) and $CD3^+CD8^+$ (B) expanded T-lymphoblasts generated after long-term stimulation of PBMC with HBHA. Panel C and D represent frequencies of ESAT-6 expanded $CD3^+CD4^+$ (C) and $CD3^+CD8^+$ (D) T lymphoblasts. Frequencies of blasts obtained in unstimulated cultures were subtracted. Horizontal lines represent median frequencies. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

Antigen-induced cytokine production by $CD8^+$ T cells was low, both in response to HBHA and to ESAT-6, and no significant differences between groups were observed (Figures 2C and 2D). As an internal control, the frequencies of PPD-induced cytokine producing cells were also analysed. PPD induced higher frequencies of IFN-γ-, TNF-α- or IL-2-producing $CD4^+$ T lymphocytes for infected compared to non-infected subjects, but TB cases could not be discriminated from LTBI subjects (Supplementary Figure 4A), confirming results previously reported for the analysis of PPD-induced IFN-γ secretion [12]. The frequencies of PPD-induced cytokine-producing $CD8^+$ lymphocytes were very low, but the frequencies of IL-2-producing $CD8^+$ cells appeared to be higher for TB patients than LTBI subjects and controls ($p<0.01$) (Supplementary Figure 4B).

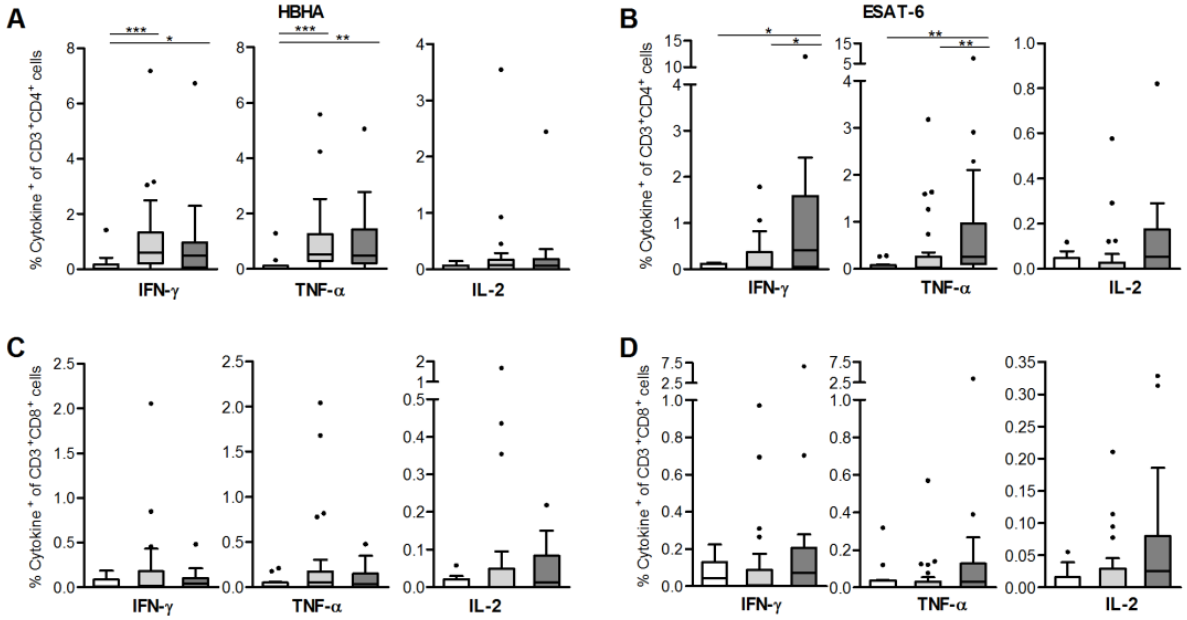

Figure 2: Cytokine expression after long-term stimulation with *M. tuberculosis* antigens. Frequencies of IFN-γ-, TNF-α- or IL-2-expressing cells among $CD3^+CD4^+$ (A, B) or $CD3^+CD8^+$ (C, D) T cells in response to HBHA (A-C) or ESAT-6 (B, D) are represented. Uninfected controls (n=12), LTBI subjects (n=28) and TB patients (n=17) are represented by white, light grey and dark grey boxplots, respectively. Boxplots represent interquartile range (IQR), with whiskers (75^{th} percentile + 1.5 IQR and 25^{th} percentile – 1.5 IQR) and outliers (dots). Individual data were background subtracted before being summarized in the figures. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

Quality of antigen-specific T cell responses

We further analyzed the quality of the antigen-specific T cell responses by determining the frequencies of multi-functional $CD4^+$ and $CD8^+$ T cells producing all possible combinations of IFN-γ, TNF-α and IL-2. Cytokine profiles were expressed as the relative frequencies of each cytokine combination among cytokine-producing cells for which the total cytokine response was above the threshold level defined in the Material and Methods section. HBHA-specific cytokine profiles of LTBI subjects were heterogeneous and overall comparable to those of TB cases, with three dominant T cell subtypes both within $CD4^+$ and $CD8^+$ T lymphocytes, double positive IFN-γ^+TNF-α^+ cells, IFN-$\gamma^{single+}$, and TNF-$\alpha^{single+}$ cells (Figures 3A and 3B). The only significant difference between TB patients and LTBI subjects was a low but significantly increased frequency of triple positive IL-2^+IFN-γ^+TNF-α^+ $CD4^+$ T lymphocytes in LTBI subjects (p=0.05; Figure 3A). The numbers of ESAT-6-responding LTBI subjects were low, due to the cohort characteristics of the LTBI subjects (see Material and Methods section). Those who responded to ESAT-6 had lower frequencies of double positive IFN-γ^+TNF-α^+ $CD4^+$ lymphocytes than the TB patients (p=0.012; Figure 3C). The numbers of responders with ESAT-6-induced $CD8^+$ T cell responses were low, with no significant difference between the two groups (Figure 3D). In response to PPD, the relative frequencies of TNF-$\alpha^{single+}$ $CD4^+$ cells were increased in TB patients compared to LTBI subjects (p=0.048, Supplementary Figure 5A), as were the frequencies of double positive IFN-γ^+TNF-α^+ $CD8^+$ cells ($p \leq 0.001$, Supplementary Figure 5B). In contrast, the frequencies of triple positive IL-2^+IFN-γ^+TNF-α^+ cells and of IFN-$\gamma^{single+}$ $CD4^+$ T lymphocytes were higher for LTBI subjects than for TB patients (p=0.001 and p=0.039 respectively, Supplementary Figure 5A).

LTBI subgroups

As shown above, the LTBI cohort was heterogeneous, since only roughly one third of them responded to ESAT-6 and QFT. We therefore compared the quality of the HBHA-induced responses between the QFT^+ LTBI and QFT^- LTBI subjects. The two LTBI subgroups showed important differences. Similar to the patients with active TB, the HBHA-induced $CD4^+$ T cell cytokine profile of QFT^+ LTBI subjects was characterized by a high frequency of TNF-$\alpha^{single+}$ $CD4^+$ T lymphocytes compared to the QFT^- LTBI subjects (p=0.006, Figure 4A). In contrast, patients with active TB and QFT^+ LTBI subjects had lower frequencies of IFN-$\gamma^{single+}$ $CD4^+$ T cells compared to QFT^- LTBI subjects, the difference being statistically significant only between the two groups of LTBI subjects (p=0.018, Figure 4A). This resulted in significantly higher ratios of IFN-$\gamma^{single+}$ over TNF-$\alpha^{single+}$ cells for QFT^- compared to QFT^+ LTBI subjects and TB patients (p=0.0104, Figure 4B). As shown for a limited number of patients, this ratio was restored upon TB treatment (Figure 4B).

The frequency of HBHA-induced triple positive IL-2^+IFN-γ^+TNF-α^+ cells was highest in the QFT^- LTBI subjects, followed by the QFT^+ LTBI subjects, and it was lowest in the TB patients (Figure 4A). The analysis of the heterogeneity of the $CD8^+$ T cell responses was limited by the restricted number of responders (8 QFT^- and 3 QFT^+ LTBI subjects, 4 TB patients), but revealed the same trend of increased IFN-$\gamma^{single+}$ and decreased TNF-$\alpha^{single+}$ frequencies for QFT^- LTBI subjects, although this did not reach statistical significance (data not shown).

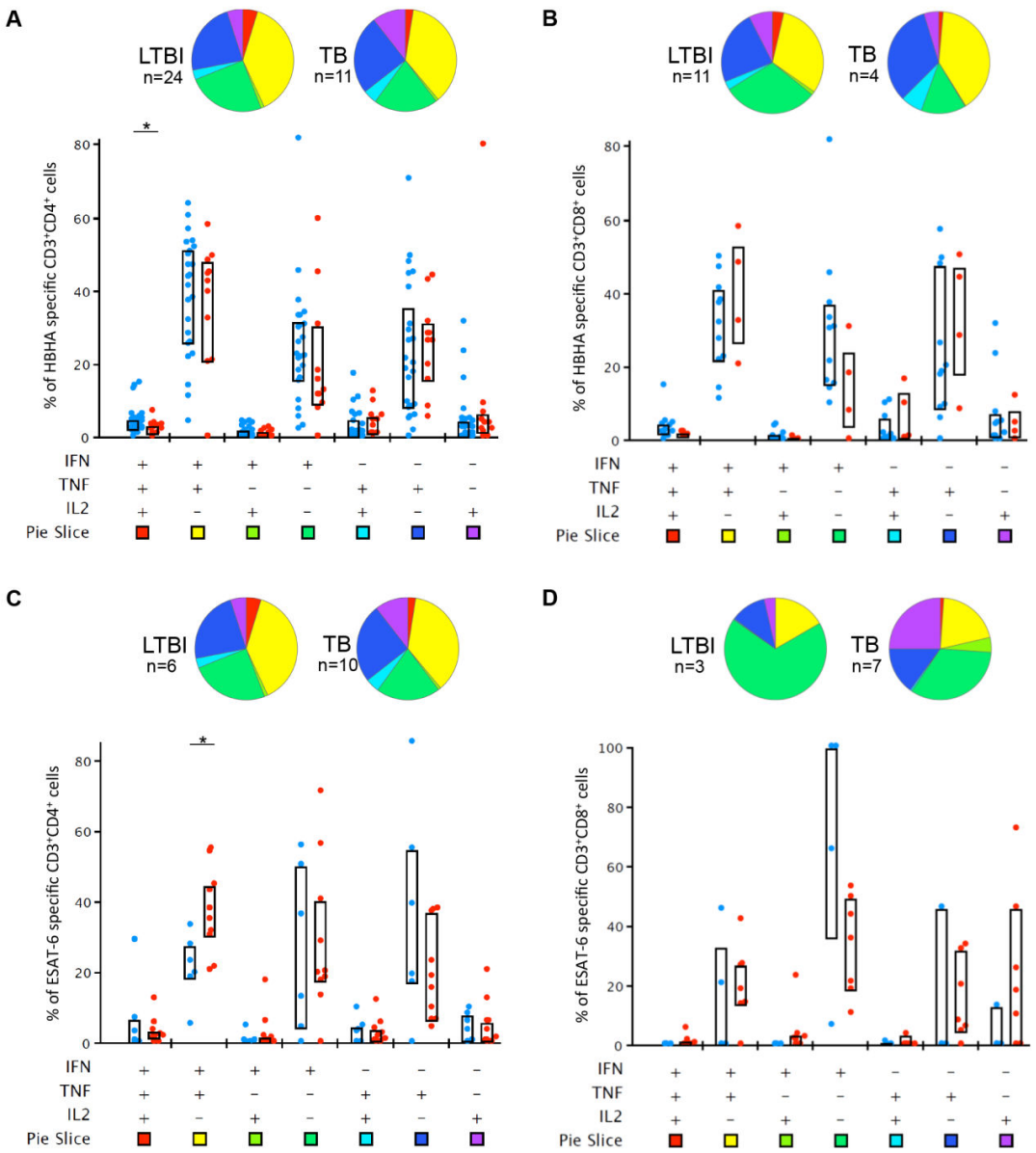

Figure 3: Qualitative analyses of *M. tuberculosis*-specific $CD3^+CD4^+$ and $CD3^+CD8^+$T cell responses by polychromatic flow cytometry. Cytokine expression profiles of PBMC stimulated with HBHA (A, B) or ESAT-6 (C, D) were determined by the combined analysis of IFN-γ, TNF-α and IL-2. For all seven possible combinations of these cytokines shown on the x-axis, the frequencies among *M. tuberculosis*-specific $CD3^+CD4^+$ (A, C) and $CD3^+CD8^+$ (B, D) T cells are shown on the y-axis for LTBI subjects (blue) and TB patients (red). Subject subgroups were compared using the SPICE software: *$p \leq 0.05$. Pie charts summarize the data, and each slice corresponds to the mean of the proportion of the *M. tuberculosis*-specific $CD3^+CD4^+$ or $CD3^+CD8^+$ T cells positive for each combination of expressed cytokines using the color-code shown below the x-axis. Results are shown for responders as defined in the methods section.

Discussion

The heterogeneity of *M. tuberculosis* infection states beyond the two extremes of active and latent TB, is now widely accepted [8-12,22]. LTBI subjects are usually defined by their detectable adaptive immune responses to mycobacterial antigens in the absence of any clinical manifestation and/or radiological abnormalities. The reference test for their identification was initially the TST that is still the gold-standard in Belgium. More recently, the commercial IGRAs QFT and T-SPOT.TB (Immunotec, UK) assay are also used in several countries to detect subjects with LTBI [2]. However, as also observed in this study,

several groups have reported that these tests do not detect all subjects with LTBI [12,14,23,24].

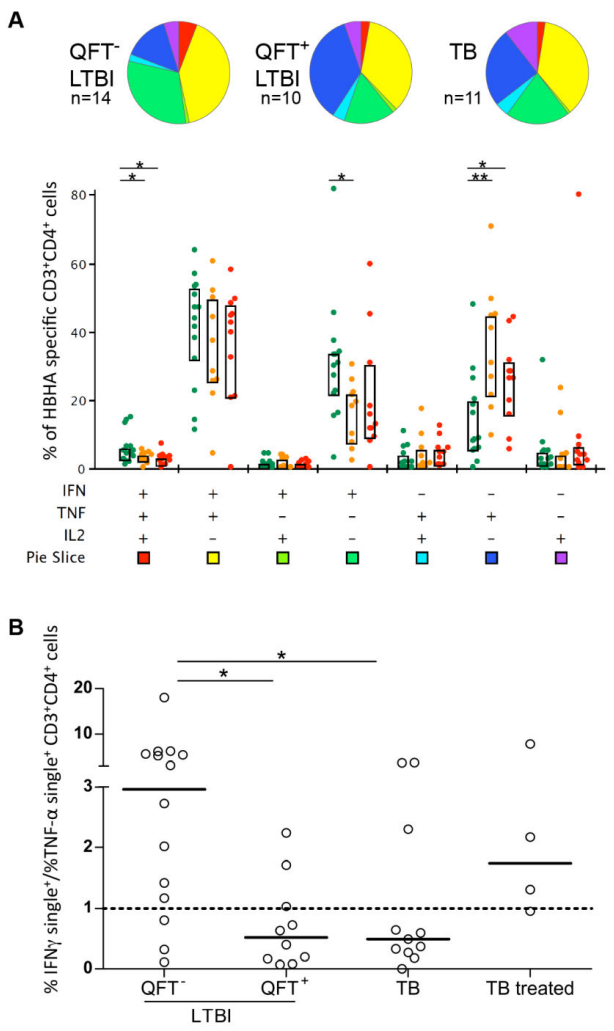

Figure 4: Qualitative analyses of HBHA-specific $CD3^+CD4^+$ T cell responses by polychromatic flow cytometry in LTBI subgroups and TB patients. (A) Cytokine expression profiles of PBMC stimulated with HBHA were determined by the combined analysis of IFN-γ, TNF-α and IL-2. For all seven possible combinations of these cytokines shown on the x-axis, the frequencies among HBHA-specific $CD3^+CD4^+$ T cells are shown on the y axis for QFT^- LTBI subjects (green, n=14), QFT^+ LTBI subjects (orange, n=10), and TB patients (red, n=11). Subject subgroups were compared using the SPICE Software: *p≤0.05; **p≤0.01. Pie charts summarize the data, and each slice corresponds to the mean of the proportion of the HBHA-specific $CD3^+CD4^+$ T cells positive for each combination of expressed cytokines using the color-code shown below the x-axis. (B) Ratio of the frequency of IFN-$\gamma^{single+}$- over the frequency of TNF-$\alpha^{single+}$ $CD4^+$ T lymphocytes after stimulation with HBHA. Results are shown for responders as defined in the methods section. QFT, QuantiFERON Gold in-tube.

These *M. tuberculosis*-infected individuals comprise some with subclinical infection, others who maintain the bacteria in a dormant stage but remain susceptible to develop TB disease, and still others in whom the anti-TB immune response may be strictly localized or of insufficient magnitude to be systemically detected [10]. On the other hand, individuals who may have cleared the infection but still have detectable adaptive memory immune responses are identified by immune response-based diagnosis, clearly illustrating the limitation of currently available diagnostic tools [22].

Biomarkers able to differentiate different stages within latency are urgently needed in order to prioritize those LTBI subjects with the highest risk to reactivate the infection as the primary target population for preventive therapy [1]. Such biomarkers are still elusive today [7,22,25]. Based on a longitudinal follow-up of the IFN-γ responses to HBHA and ESAT-6, we previously proposed a classification of LTBI subjects in three sub-groups: (i) those who are positive for HBHA but lose this response over time, most likely due to clearance of the bacteria, (ii) those with a stable IFN-γ response to HBHA over time, often without response to ESAT-6, and (iii) those who respond to both antigens with alternating dominance of intensity over time [14]. We proposed that this latter group presents the highest risk for reactivation, as illustrated by case reports, especially when the HBHA response declines [14].

In the current study we show that the quality of the T lymphocyte immune response to *M. tuberculosis* antigens is also heterogeneous among LTBI subjects. The subgroup of LTBI subjects with a positive QFT test presents a similar profile of T lymphocyte response to the latency antigen HBHA than patients with TB disease, characterized by a high frequency of TNF-$\alpha^{single+}$ $CD4^+$ T lymphocytes. In contrast, the QFT^- LTBI subgroup was characterized by a dominance of IFN-$\gamma^{single+}$ $CD4^+$ T lymphocytes. Considering the important role of IFN-γ in the defense against *M. tuberculosis* [2,10] and the reported association of TNF-$\alpha^{single+}$ cells with active TB in response to other antigens [26,27], these observations strengthen our previously proposed risk stratification based on the absence or presence of IFN-γ responses in the QFT test or to ESAT-6, compared to HBHA [14]. According to the current model in which TB is represented as a dynamic spectrum of infection states [8-11,22], combinations of different biomarkers may be assigned to different states of infection, thus positioning the QFT^+ LTBI subgroup, especially with low HBHA responses, as an intermediate between QFT^- LTBI subjects less likely to progress on the one side and TB patients on the other. The heterogeneous quality of the immune response to different antigens is likely due to different levels of bacterial replication among LTBI subjects associated with changes in *M. tuberculosis* gene expression profiles (22). This is probably a result of the physiological ability of the bacilli to modify their gene expression in response to different micro-environmental conditions at different anatomical sites within the infected host. Dormancy of the bacteria is associated with the expression of latency antigens, such as HBHA. The effector memory phenotype of the HBHA-induced IFN-γ-producing $CD4^+$ T cells reported previously strongly suggests that this immune response reflects the persistence of *M. tuberculosis* antigens [13]. We rule out that the HBHA-induced immune responses described here in the QFT^- LTBI subjects may have resulted from a previous BCG vaccination that was reported in most LTBI subjects included in this study. Several previous studies have indicated that HBHA-IGRA results are not influenced by BCG vaccination in infancy in TST^+ LTBI adult subjects and can therefore be used to detect LTBI subjects regardless of their BCG vaccination status [12,13]. In this study we therefore identified LTBI by TST, using very strict criteria for

their classification as LTBI, with a minimum induration size of 15 mm, knowing that more than 10 years after a BCG vaccination, this size is very unlikely the result of a vaccine-induced response only. Exceptionally, an induration of more than 10 mm was used for subjects with major documented *M. tuberculosis* exposure.

Biomarkers that clearly differentiate TB disease from latent TB are also urgently needed to help identifying subtle forms of active TB, and extensive research is performed to achieve this goal both at the cellular level and by transcriptomic approaches [10]. Using flow cytometry we also identified here potential biomarkers to differentiate TB disease from LTBI. Compared to LTBI subjects, TB patients were characterized by elevated T lymphocyte responses to ESAT-6, namely a high proportion of $CD8^+$ lymphoblasts and a high proportion of double positive IFN-γ^+TNF-α^+ $CD4^+$ lymphocytes. In contrast, the proportion of HBHA-specific triple positive IFN-γ^+TNF-α^+IL-2^+ $CD4^+$ lymphocytes was lower in TB patients compared to LTBI individuals. The proportion of PPD-specific triple positive IFN-γ^+TNF-α^+IL-2^+ $CD4^+$ lymphocytes was also higher in LTBI compared to TB patients, whereas TB patients had higher proportions of TNF-$\alpha^{single+}$ $CD4^+$ lymphocytes, but the clinical value of PPD-induced responses is limited as they are not specific for *M. tuberculosis* infection. If confirmed by larger studies, the ESAT-6-induced $CD8^+$ T lymphoblasts provide a promising easy-to-perform immunological test for the identification of patients with active TB. It will also be interesting to investigate whether this is applicable to young children and for HIV-infected subjects, two patient populations for which TB diagnosis remains particularly difficult [28,29]. Concerning the qualitative differences in immune responses observed between TB disease and LTBI, conflicting findings were previously reported, particularly on the role of IFN-γ^+TNF-α^+IL-2^+ triple positive T cells, which has been proposed as a hallmark of TB disease [30-32], as well as of LTBI [33-35]. In addition, recent vaccine trials have revealed that these multifunctional triple positive cells not necessarily offer protection in humans [36,37]. Although a number of differences in the experimental setup can be identified between these studies, none of them can directly explain these conflicting results. In line with what has been described by Marin et al. [27], we found the same dominant T cell subpopulations when stimulation time was reduced to overnight incubation as compared to five days (data not shown), indicating that incubation time by itself does not explain the heterogeneity. However, the nature of the antigen, such as an antigen associated to the replicative or the latent status of the bacteria, and peptide versus protein antigens, certainly influences which T cell subtypes are most dominant among antigen-specific cells [22,38], while the choice of the study population examined (low versus high TB burden countries) most certainly also influences the observed immune responses.

In conclusion, this study provides further evidence that the T lymphocyte response to ESAT-6 compared to HBHA may help to identify patients with TB disease and to differentiate them from LTBI subjects. In addition it shows that the quality of the immune response to the latency antigen HBHA may be helpful to distinguish LTBI subjects with relatively low or high risk to reactivate their infection. Longitudinal follow-up of different LTBI subjects is ongoing to confirm the clinical utility of these biomarkers.

Acknowledgements

This work was supported by the European Community within the 7th framework program (FP7) NEWTBVAC (grant HEALTH-2009-2.3.2-2), by INNOV IRIS from the Region de Bruxelles-Capitale, and by the Fonds de la Recherche Scientifique Médicale (FRSM, grant n°T.0147.13). V.C. was supported by a fellowship from the Fonds National de la Recherche Scientifique (FNRS), C.W-T was supported by a fellowship from the Fond Erasme and F.M. was partially supported by a grant from the FNRS. We thank all the volunteers who participated in this study and the clinicians involved in the recruitment of patients, particularly Annie Drowart, Kinda Schepers, Sabine Allard, Sara Debulpaep, Inge Muyle, Charlotte Martin and Agnès Libois. We thank Gaëlle Pottier and Sébastien Vanderseypen for technical assistance.

Conflict of Interest Disclosure

The authors declare no commercial or financial conflict of interest.

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This article was originally published in a special issue, entitled: **"T-cell Immunology"**, Edited by Mark S. Kindy, The Medical University of South Carolina, USA