influence epidemiological and clinical outcomes of the disease (Caws et al., 2008; Parwati et al., 2010; Valway et al., 1998). Strains with diverse epidemiological and/or genotypic backgrounds were found to induce different infection outcomes and cytokine profiles in several *in vitro* (Manca et al., 2004; Mathema et al., 2012; Theus et al., 2005) and *in vivo* (Dormans et al., 2004; López et al., 2003; Mathema et al., 2012; Tsenova et al., 2005) infection models, clearly indicating that the genetic intra-species variability is linked to phenotypic and functional variability. However, the experimental parameters that accurately reflect the epidemiological success of different *M. tuberculosis* strains remain undefined. Herein we aim to investigate whether the differences in virulence, regarded as epidemiological success, of two closely related Haarlem MDR *M. tuberculosis* strains, namely strains M and 410, could be associated with their ability to grow and elicit cytokine production in two *in vitro* models of human macrophage infection.

2. Materials and methods

2.1. M. tuberculosis strains and epidemiological background

Three *M. tuberculosis* strains were used: MDR strains M and 410 from the collection kept at the Reference Laboratory for Mycobacteria at the INEI-ANLIS "Carlos G. Malbrán" in Buenos Aires, and the laboratory strain H37Rv, which was included as reference. Isolate 6548 representative of strain M had been obtained in 1998 from a male HIV positive patient hospitalized at the epicenter of the outbreak. Isolate 410 was obtained earlier in the MDR-TB epidemic from a female patient who was HIV negative and had no other known co-morbidity. She remained infectious and was hospitalized in several health centers in the outbreak area between diagnosis and death. All four isolates from this patient available to genotyping (1992, 1995, 1998, 1999) had the same unique IS*6110* RFLP pattern, which was not detected again by the national MDR-TB surveillance system (Yokobori et al., 2012). Strain H37Rv was kindly provided by de Kantor (former head, TB laboratory, INPPAZ PAHO/WHO). Strains were grown in Middlebrook 7H9 broth (Becton Dickinson, USA) with ADC enrichment supplemented with 100 U/ml penicillin with agitation for 15–21 days. Clumps were disaggregated mechanically using glass beads, and after 2 h of settling, the supernatant was harvested, aliquoted and stored at −80 °C until use. To minimize virulence loss, we only used stock cultures which had undergone three or less recultures after primary isolation. Bacterial concentration of each strain was determined by serial dilution and plating for each strain. Recultures used in this study were re-submitted to standard IS*6110* DNA fingerprinting (van Embden et al., 1993) and spoligotyping (Kamerbeek et al., 1997) (Supplementary Fig. 1). Additional information related to the selected strains is resumed in Supplementary Table I.

2.2. Cell culture and differentiation

Human monocyte-derived macrophages (MDM) were obtained from buffy coats of six healthy volunteers who gave written informed consent (Servicio de Hemoterapia, Hospital Fernández, Buenos Aires). All donors were seronegative for HIV, hepatitis B, syphilis and Chagas disease; all received BCG vaccination in childhood but PPD skin test status was unknown. The Ethics Committee of the Academia Nacional de Medicina approved all experimental procedures. Peripheral blood mononuclear cells were purified in a Ficoll-Hypaque gradient, plated at 5×10^6 per well in 24-well plates (GBO, Germany) and allowed to adhere for 2 h at 37 °C. After removal of nonadherent cells by washing with warm saline solution (SS), monocytes were allowed to differentiate in a 5% CO_2 humidified atmosphere at 37 °C for 5 days in RPMI 1640 medium (HyClone, Thermo Scientific, USA) supplemented with 10% fetal calf serum (Natocor, Argentina), hereafter mentioned as complete medium. Around 5×10^5 MDM per well were obtained.

The human myelomonocytic leukemia cell line was used as secondary model of *in vitro* infection. Unlike the more widely used THP-1 line, these monoblasts remain in a pliant state of maturation and, upon standard induction, they promptly differentiate into a macrophage-like, cytokine producing, phenotype (dU937) (Harris and Ralph, 1985). U937 were grown in complete medium supplemented with 100 U/ml penicillin. Cultures were started at a density of 10^5 cells/ml every 3–4 days. For differentiation, cells were harvested at exponential growth phase, washed and suspended in complete medium containing 100nM phorbol 12-myristate 13-acetate (PMA, Sigma, USA). 2×10^5 cells were seeded into culture plates and allowed to differentiate for 3 days before use. Cell viability of MDM and dU937 cells was determined by 0.2% trypan blue exclusion, which routinely exceeded 95%.

2.3. Macrophage infection

Adherent MDM or dU937 cells were infected with *M. tuberculosis* strains H37Rv, M or 410 on day 0. Bacterial stocks were spun down at low speed to remove clumps before preparing the infecting suspensions in complete medium. Strains were added into cell culture plates containing adherent macrophages at a multiplicity of infection (MOI) of 5 viable bacilli per cell in triplicate. After incubation at 37 °C for 3 h, infected cells were washed three times with warm RPMI medium to eliminate free bacteria and were cultured in complete medium.

2.4. Intracellular bacilli replication

Intracellular replication of each *M. tuberculosis* strain was measured by counting colony forming units (CFU) at days 0, 2 and 5 after MDM infection. To this end, ice-cold SS was vigorously pipetted into the wells and the plates were incubated for 10 min at 4 °C to further facilitate detachment of adherent cells. Then, cells from each well were transferred into microtubes, pelleted and resuspended in SS. An aliquot of this suspension was used to determine the cell-associated CFU by lysing MDM with 0.05% Triton X-100 in PBS and plating serial dilutions on 7H10/OADC agar plates. Plates were incubated at 37 °C 5% CO_2 for 3–5 weeks until *M. tuberculosis* colonies became visible and countable. At the same time points MDM were placed onto glass slides, fixed by heat and acid-fast bacilli (AFB) were stained by Ziehl–Neelsen method. The slides were examined at 1000× magnification in an optical microscope. The percentage of infection was calculated as the number of MDM containing at least one AFB among 100 cells per slide. Simultaneously, the number of bacilli inside each infected MDM was counted, and the median bacillary load per MDM was calculated based on the frequency distribution of number of bacilli per infected cell. Samples were assessed in duplicate. dU937 cells were similarly processed to determine CFU counts at days 0, 3 and 7 post-infection.

2.5. Cytokine detection

Culture supernatants of infected and uninfected control MDM and dU937 cells were harvested at different time points (after 4 h of culture for TNF-α and 24 h for IL-10 and IL12p70) and were frozen at −70 °C until assayed with commercial ELISA kits (Ready-SET-Go!, eBioscience, USA) for human TNF-α, IL-10 and IL-12p70 according to the manufacturer's instructions. Sensitivities of the assays were 4.7 pg/ml for IL-10 and 7.8 pg/ml for TNF-α and IL-12p70.

2.6. Flow cytometry

The following fluorochrome conjugated anti-human monoclonal antibodies and their corresponding isotypes were used: fluorescein isothiocyanate-anti-Mannose Receptor (MR, BD), phycoerythrin-anti-CD11b and PerCP/Cy5.5- anti-CD14 (Biolegend, USA). Harvested cells were incubated with the antibodies for 20 min at 4 °C, washed, fixed with 0.5% paraformaldehyde and suspended in Isoflow™ (BD). Fluorescence-labeled cells were measured in a FACScan cytometer (BD) and data were analyzed with FCS Express software (De Novo Software, USA). 20,000 specific events were acquired according to forward and side scatter properties.

2.7. Statistical analysis

Nonparametric Friedman test was performed to compare MDM responses to the different strains within each experimental group, followed by Wilcoxon paired test to compare two groups. Results from dU937 cells were analyzed by ANOVA, followed by paired *t* test. Comparisons among cell types were performed by Mann Whitney test. A value of $P < 0.05$ was assumed as significant.

3. Results

3.1. Strain M grows less than strain 410 in MDM

First, we studied the intracellular growth of the selected strains using human MDM as host cells. No significant differences in MDM-associated CFUs were observed on day 0 (Fig. 1A). Growth lag was observed with both MDR strains until day 2, but by day 5 intracellular growth was observed for all three *M. tuberculosis* strains (day 0 vs. day 5: $P < 0,05$). Strain M showed the lowest median CFU counts at days 2 and 5 but this difference was not statistically significant when compared with strain H37Rv. At day 5 post-infection, strain 410 showed significantly higher CFU counts compared with M. The lower replication rate of the outbreak strain M had also been observed in axenic culture (Supplementary Table I).

Having observed that the growth of strains M and 410 were different, we determined the percentage of infected MDM and the bacillary load for each strain. The lower replication of strain M in MDM was also reflected in the percentage of infected cells and intracellular bacillary loads. In contrast with CFU counts, at day 0 both the percentages of MDM containing AFB and the bacillary loads were significantly lower for strains M and 410 than for H37Rv (Fig. 2A and B), probably owing to differences in bacterial viability or staining pattern. While strain M showed the lowest percentage of infection and number of bacilli per cell throughout the experiment, strain 410 equaled the percentage of infection and surpassed the intracellular bacterial burden of H37Rv at day 2 post-infection.

Given the high degree of variability in the growth of the strains among donors (Supplementary Fig. 2) we proceeded to confirm MDM results in a model with higher reproducibility such as the cell line dU937. CFU were recovered at 0, 3 and 7 days post-infection. Surprisingly, the differences in growth rate between M and 410 were abolished, and both MDR strains grew significantly slower than H37Rv (Fig. 1B).

3.2. Strain M elicits less IL-10 and early TNF-α release than strain 410 in MDM

Next, we evaluated the ability of the selected strains to induce the production of cytokines in MDM. Infection with strain M elicited lower amounts of TNF-α at 4 h post infection than strain 410 and similar to those induced by H37Rv (Fig. 3A). After this time point, TNF-α levels remained high and no significant differences among strains were observed (data not shown). IL-10 was induced after infection with all three strains (Fig. 3B), and again, strain M induced lower amounts than strain 410 and similar to those

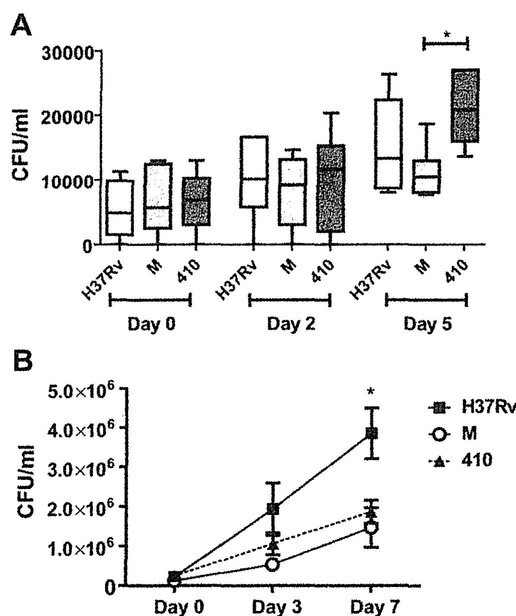

Fig. 1. (A) Intracellular growth of strains H37Rv, M and 410 on days 0, 2 and 5 post-infection in human MDM infected at a MOI of 5. Data are represented as the median and 25th–75th percentiles (box & whiskers plot) of CFU/ml of lysate. ($^*P < 0.05$; paired Wilcoxon test). $n = 6$. (B) Intracellular growth of strains H37Rv, M and 410 on days 0, 3 and 7 post-infection in dU937 cells. Data are represented as mean ± SEM of CFU/ml of lysate. Statistical significance: H37Rv vs. M/410: $^*P < 0.05$; paired *t* test. $n = 6$.

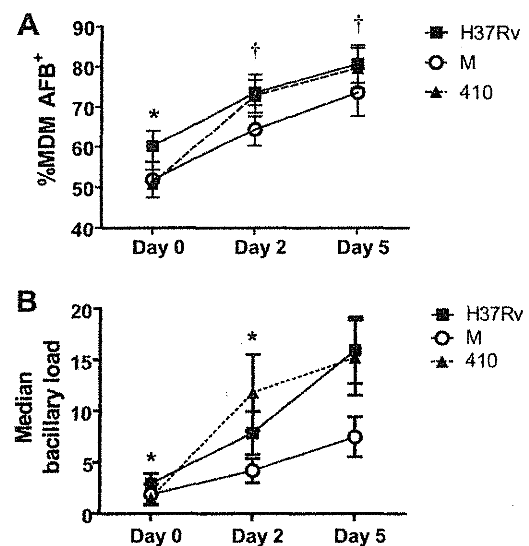

Fig. 2. (A) Percentage of MDM infected with strains H37Rv, M or 410 on day 0, 2 and 5 post-infection was determined by Ziehl–Neelsen staining. Statistical significances: H37Rv vs. M/410: $^*P < 0.05$, H37Rv/410 vs. M: $^{\dagger}P < 0.05$; paired Wilcoxon test. $n = 6$. (B) Intracellular bacterial burden of MDM infected with strains H37Rv, M and 410 on days 0, 2 and 5 post-infection. Data are represented as the mean ± SEM of the median bacillary load from each donor assessed by Ziehl–Neelsen staining. Statistical significance: H37Rv vs. M/410: $^*P < 0.05$; M vs. 410: $^{\dagger}P < 0.05$; H37Rv vs. M: $^{\S}P < 0.05$; paired Wilcoxon test. $n = 6$.

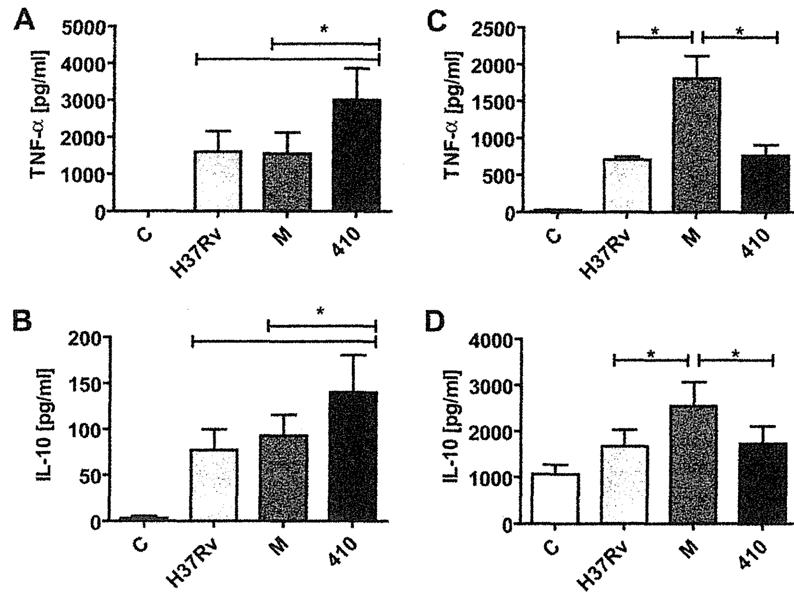

Fig. 3. Cytokine induction by strains H37Rv, M and 410 on MDM (A, B) and dU937 (C, D). TNF-α release at 4 h (A, C) and IL-10 at 24 h post infection (B, D) are shown. Data are represented as pg/ml for each strain assessed by ELISA. Statistical significance: H37Rv/410 vs. M in MDM: $^*P < 0.05$; paired Wilcoxon test. $n = 6$. In dU937 cells: $^*P < 0.05$; paired t test. $n = 3$.

induced by strain H37Rv. No spontaneous release of TNF-α or IL-10 to culture supernatants was detected in the uninfected controls.

In dU937 cells, strain M was the strongest inducer of both TNF-α and IL-10 (Fig. 3C and D), and a high spontaneous release of IL-10 was observed (Fig. 3D).

None of the strains induced detectable levels of IL-12p70 at any time point either in MDM or in dU937 cells (data not shown).

3.3. MDM and dU937differ in critical receptors for M. tuberculosis uptake

Given the discrepancy in growth rates observed between MDM and dU937, we speculated that the differences could be due to critical surface receptors for *M. tuberculosis* uptake. Thus, the expression of CD11b, CD14 and MR was determined. A higher CD11b and CD14 expression was observed in MDM than in dU973 and, while MR was strongly expressed in MDM it was undetectable in dU937 (Supplementary Fig. 3).

4. Discussion

It has been proposed that the most accurate measure of virulence of a given *M. tuberculosis* strain is the extent to which it is able to cause active disease cases in a given period of time, rather than enhanced mortality and disease severity (Brites and Gagneux, 2011). However, experimental parameters such as mortality in animal models are usually assumed to reflect epidemiological success (Dormans et al., 2004; Manca et al., 1999). In line with this, the epidemiological virulence of different strains and lineages is usually expected to correlate with bacterial growth in experimental *in vitro* and *in vivo* infection models. Indeed, highly transmitted strains were shown to grow more rapidly than sporadic strains in human macrophages (Theus et al., 2004; Theus et al., 2006). In particular, the remarkably successful Beijing family strains (Parwati et al., 2010) have shown enhanced ability to grow *in vitro* as well as in animal infection (López et al., 2003; Li et al., 2002; Manca et al., 1999; Theus et al., 2007; Tsenova et al., 2005).

Paradoxically, from an evolutionary point of view, enhanced host mortality might represent a dead end for *M. tuberculosis* (Brites and Gagneux, 2011). In fact, our results challenge the notion that the epidemiological success of a given strain always correlates with a high *in vitro* intracellular growth rate, as we found the opposite in the MDM model presented herein. Results of a recent study by Mathema et al. (2012) endorse our finding. These authors showed that, within a selection of closely related strains of the Euro-American lineage from New York–New Jersey, the strains with slower replication, weaker pro-inflammatory response in primary human monocytes, and milder disease in guinea pigs were the most prevalent in the area. Several other reports failed to prove a definite correlation between enhanced growth rate and epidemiological virulence (Alonso et al., 2010; Hoal-van Helden et al., 2001; Marquina-Castillo et al., 2009; Zhang et al., 1999).

Herein, we also show that, in the MDM model, the successful strain M is a poor inducer of TNF-α and IL-10 compared to 410, critical cytokines for the immune response against *M. tuberculosis* (Flynn and Chan, 2005). In a previous work, we demonstrated that these differences were not related to the antigenic load but were abolished upon heat inactivation, showing that production of these cytokines is dependent on heat labile, probably actively secreted antigens (Yokobori et al., 2012). While a depressed induction of TNF-α has been reported for the extensively studied HN878 Beijing strain (Krishnan et al., 2011; Manca et al., 2004) and other prevalent strains (Mathema et al., 2012; Theus et al., 2005), contrasting results regarding IL-10 induction and its role in *M. tuberculosis* virulence have been reported. Some studies found that clustered strains induce high levels of IL-10 and low levels of TNF-α at early time points favoring an anti-inflammatory profile (Theus et al., 2005). Other studies showed that successful strains induce lower levels of both cytokines (Krishnan et al., 2011). Indeed, our results obtained with MDM are in line with these latter findings. Thus, it is not possible to find a distinctive innate cytokine induction pattern of epidemiologically successful strains as a whole (Alonso et al., 2010; Chacón-Salinas et al., 2005; Manca et al., 2004; Theus et al., 2005; Zhang et al., 1999), and intra-lineage variability could account for this failure (Krishnan et al., 2011; Wang et al., 2010). Herein we also showed that, both strain M and 410 were fully able

to inhibit the macrophage production of IL-12, a trait which is considered a virulence mechanism (Dao et al., 2008; Henao et al., 2007; Nau et al., 2002).

Another important outcome of the current work is that the performance of clinical isolates can differ critically between experimental models. The discrepancies between results in MDM and dU937 cells are probably due to the differential expression of critical surface receptors related to *M. tuberculosis* recognition and uptake (Schäfer et al., 2009). The enhanced replication rate of strain 410 was reestablished when plated on 7H10 agar after dU937 cell lysis, giving rise to countable colonies earlier than strain M (data not shown), also suggesting that the intracellular milieu was determinant. It is known that MR induces a strong inhibitory signal (Kang et al., 2005; Rajaram et al., 2010) and it would be interesting to determine if our strains differ in the usage of this and other receptors.

Although macrophage-like cell lines are widely used in models of infection, in our case dU937 cells fail to show the growth differences between the epidemiologically successful strain and the sporadic. Other limitations of the dU937 model are the high spontaneous release of IL-10 and the null expression of MR. In contrast, macrophages obtained from primary cells did reveal intriguing differences between both *M. tuberculosis* strains, making MDM the choice model despite high variability observed among donors. Differentiated THP-1 cells might constitute an alternative model as these cells can recognize mycobacteria through MR (Diaz-Silvestre et al., 2005).

The higher intracellular growth rate and the cytokine profile induced by 410 in MDM recalls the behavior of strain CDC1551 (Manca et al., 2004; Manca et al., 1999). This strain induced an unusually high rate of skin test conversion (Valway et al., 1998). In line with this, rather than being unable to cause disease in new hosts, strain 410 might have an enhanced ability to go into latency. Evidence supporting this hypothesis is currently lacking, since PPD skin test status among contacts is unknown, and we consider that this is an interesting issue for future research.

We have previously shown that, compared to strain 410, strain M is a poor inducer of CD8 T lymphocyte mediated cytotoxicity (Geffner et al., 2009) and macrophage cell death (Yokobori et al., 2012). Therefore, we speculate that the epidemiologic success of the non-Beijing strain M actually relies on its modest replication rate and cytokine induction, keeping a state of quiescence and remaining rather unnoticed by the host at early stages of infection, as it has recently been described for other successful genotypes (Mathema et al., 2012).

In summary, our study highlights the importance of defining an accurate selection criterion when comparing performance of clinical isolates in experimental models of infection. In addition to lineage specific characteristics, substantial intra-lineage variations have also been reported (Alonso et al., 2010; Homolka et al., 2010; Portevin et al., 2011; Wang et al., 2010). In most publications, strains were selected based on epidemiological features or family/lineage markers but not on both. During the course of *M. tuberculosis* diversification, different strategies might have been developed, and micro-variations in critical functions might further determine the epidemiological fate of a certain genotype (Reed et al., 2004). In fact, in addition to the IS*6110* RFLP pattern, strains M and 410 differ in other genotypic markers like one VNTR-MIRU 15 locus, presence of strain specific mutations and rifampin resistance-conferring mutation in *rpoB* gene (Supplementary Table I). This latter difference shows that the strains diverged from their ancestor before becoming MDR. Further studies are needed to determine the exact relationship between these differences, the results obtained in the current study and the epidemiological divergence of these strains. Even though our study is limited by the number of clinical isolates tested, it underscores the importance of studying *M. tuberculosis* subpopulations taking into account the different levels of variation, from lineage and family to micro-variations in a certain community, which were previously underestimated.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2013.01.007.

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Original Article

Multicolor Flow Cytometric Analyses of CD4^{+} T Cell Responses to *Mycobacterium tuberculosis*-Related Latent Antigens

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SUMMARY: Although IFN-γ release assays (IGRAs) provide increased specificity over tuberculin skin tests, the early and sensitive detection of reactivation of latently infected *Mycobacterium tuberculosis* is required to control tuberculosis (TB). Recently, a multicolor flow cytometry has been developed to study CD4^{+} T cell cytokine responses (IFN-γ/IL-2/TNF-α) to purified protein derivatives (PPD) and *M. tuberculosis*-specific antigens (ESAT-6/CFP-10) and provided useful information regarding anti-TB immunity. However, the diagnostic relevancy remains uncertain. Here, we analyzed three additional CD4^{+} T cell cytokine responses (IL-10/IL-13/IL-17) to latent mycobacterial antigens (α-crystallin, methylated heparin-binding hemagglutinin [HBHA], and mycobacterial DNA-binding protein 1 [MDP-1]) as well as PPD and ESAT-6/CFP-10 in 12 IGRA^{+} TB cases and 8 healthy controls. No significant difference in IFN-γ response was observed between TB cases and controls, which was likely due to the high variation among the individuals. However, we found a significant increase over healthy controls in (i) the IL-2 response to HBHA in recovery stage TB cases, (ii) the number of *M. tuberculosis*-specific polyfunctional CD4^{+} T cells in on-treatment and recovery stage cases, and (iii) the IL-17 response to HBHA and MDP-1 in on-treatment and recovery stage cases. These results suggest that a combination of these T cell cytokine parameters could aid in accurate diagnosis of latent TB infection.

INTRODUCTION

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* infection. *M. tuberculosis* can establish a long-term persistent infection without causing any symptoms; this condition is referred to as latent TB infection (LTBI). It is estimated that one to two billion people worldwide are living with LTBI (1). A minority of people with LTBI develop clinical disease (active TB) when the host TB defense mechanism is altered by poor health conditions such as malnutrition, aging, and immunodeficiency caused by human immunodeficiency virus (HIV) infection (2). The lifetime risk of clinical disease development in LTBI individuals is estimated to be 5–10% (3,4); this group of individuals may benefit from prophylactic treatment. However, because the vast majority of people with LTBI will not develop active TB, it is not practical to treat all LTBI individuals. Therefore, development of a screening method to identify individuals who may benefit from preventative treatment is required.

Clinical IFN-γ release assays (IGRAs) that detect the IFN-γ response to *M. tuberculosis*-specific antigens such as ESAT-6 and CFP-10 have been developed and are widely utilized for the diagnosis of TB because they are more specific than conventional tuberculin skin tests (5). The sensitivity of IGRAs for the detection of active TB is approximately 85%, and the specificities for the detection of active TB and LTBI are greater than 85% and 98–100%, respectively (6); however, the predictive value of IGRAs for the development of active TB from LTBI is only 2.7% (7).

Although ESAT-6 and CFP-10 are the predominant secretory proteins released during the active stage of *M. tuberculosis*, several mycobacterial antigens are known to be associated with the latent stage of *M. tuberculosis* infection. The α-crystallin (Acr) protein is a member of the small heat shock protein family that has chaperone activity in vitro (8). Acr is required for the growth of *M.*

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tuberculosis in cultured macrophages, and its expression increases under hypoxic conditions (9). Heparin-binding hemagglutinin (HBHA) is a protein that functions as an adhesin for epithelial cells (10). Because the transition from the alveolar space to deeper organ sites is a crucial step in the pathogenesis of LTBI, HBHA may serve as a target antigen for the diagnosis of LTBI, as reported previously (11). Mycobacterial DNA-binding protein 1 (MDP-1) belongs to a group of orthologous DNA-binding proteins that constitute 8–10% of total protein in mycobacteria such as *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) and *M. tuberculosis* (12,13). MDP-1 is upregulated in the stationary phase of *M. tuberculocis* infection and induces protective immunity against *M. tuberculosis* infection in mice (12,14). Therefore, in combination with *M. tuberculosis*-specific ESAT-6 and CFP-10, the latency-associated antigens may aid in monitoring the immune response to *M. tuberculosis* in LTBI.

$CD4^+$ T cells produce IFN-γ and play a critical role in controlling persistent *M. tuberculosis* infection. HIV-infected patients with a decreased baseline $CD4^+$ T cell count have a higher incidence of TB (15), and macaques coinfected with *M. tuberculosis* and simian immunodeficiency virus that display a decreased $CD4^+$ T cell count show earlier reactivation of latent *M. tuberculosis* infection (16). There are many subsets of $CD4^+$ T cells, such as T-helper 1 (Th1), Th2, Th17, and regulatory T cells (Tregs), and they produce a unique set of cytokines (17). All $CD4^+$ T subsets cooperate or interfere with each other to control infection, and the dominant subset may differ between active and latent *M. tuberculosis* infection cases. Multi-parameter flow cytometry enables analysis of these cytokine-producing antigen-specific T cells ex vivo. Using this technology, it was proposed that polyfunctional T cells that produce multiple cytokines are associated with protective immunity (18), and T-cell polyfunctionality has been analyzed in individuals with HIV and *M. tuberculosis* double infection (19,20). These studies suggested that the number of *M. tuberculosis*-specific T cells producing a combination of IFN-γ, IL-2, and/or TNF-α may be correlated with the level of *M. tuberculosis* protection; however, evidence that these polyfunctional T cells are directly associated with protection is still lacking. Furthermore, no previously published study has evaluated polyfunctional T cells, including subsets of $CD4^+$ T cells, with a wide range of *M. tuberculosis* antigens such as latent phase proteins.

In this study, we postulated that the profiles of multiple T cell cytokines produced in response to latency-associated antigens vary at distinct clinical stages of *M. tuberculosis* infection and provide diagnostic information that supplements the ESAT-6 and CFP-10 profiles detected by conventional IGRAs. Multicolor flow cytometric analyses of the five $CD4^+$ T cell cytokine responses, such as IFN-γ, IL-2, IL-10, IL-13, and IL-17, to several *M. tuberculosis*-specific antigens, including latency-associated antigens, in active TB (on-treatment), non-active TB (recovery stage and contact cases), and healthy control cases are presented.

MATERIALS AND METHODS

Study subjects: Twelve *M. tuberculosis*-infected cases, including seven cases of TB diagnosed by sputum smear or PCR analysis and five cases with a history of close contact with active TB, were recruited from Tokyo National Hospital (Tokyo, Japan). The characteristics of the *M. tuberculosis*-infected cases, all of which were QuantiFERON TB (QFT) test positive, are shown in Table 1. At the time of evaluation, two of the seven TB cases were receiving on-going treatment with anti-TB drug therapy and five were in the recovery stage after completion of anti-TB drug therapy. One contact case was receiving prophylactic anti-TB treatment.

Table 1. Characteristics of *M. tuberculosis*-infected cases

Case no.	Sex	Age	Anti-TB therapy (duration after treatment)	QFT test	Others
1	male	25	ongoing (6 month)	positive	
2	male	58	none (contact)	positive	
3	male	79	none (contact)	positive	
4	male	53	prophylaxis (contact)	positive	
5	female	82	ongoing (<1 month)	positive	
6	male	31	previous (>6 years)	positive	
7	male	76	previous (>2 years)	positive	
8	male	79	previous (>1 year)	positive	
9	female	36	none (contact)	positive	
10	male	77	previous (>4 years)	positive	HIV+
11	female	46	none (contact)	positive	
12	male	95	previous (>1 year)	positive	

Eight healthy controls with no history of TB infection or exposure, including two males and six females with a mean age of 32.1 years, were recruited from Nagasaki University (Nagasaki, Japan). A blood sample from one of the laboratory staff, who had a previous history of being highly reactive to many mycobacterial antigens without displaying symptoms, was used as a positive control. All the samples included in the present study were obtained with informed consent and with ethical approval from the Institute of Tropical Medicine Nagasaki University Joint Ethics Committee as well as from the research and ethical committees of the National Institute of Infectious Diseases (Tokyo, Japan).

Reagents: Staphylococcal enterotoxin B (SEB) and purified protein derivatives (PPD) of *M. tuberculosis* were purchased from Sigma-Aldrich (St. Louis, Mo., USA) and Japan BCG Laboratory (Tokyo, Japan), respectively.

ESAT-6, CFP-10, Acr, methylated HBHA (mHABA), and methylated MDP-1 (mMDP-1) were recombinant protein products from *Escherichia coli.* The vectors expressing ESAT-6, CFP-10, HBHA, and MDP-1 were produced by a PCR-based approach using a bacterial chromosome. Each PCR product containing the relevant coding region was designed to allow the expression of a C-terminal, 6× histidine-tagged variant of the recombinant protein following ligation into the pET-21b vector (Toyobo, Osaka, Japan). Recombinant *M. tuberculosis* proteins were purified using Ni-NTA columns (1 ml bed volume) (QIAGEN, Germantown, Md., USA), according to the manufacturer's instructions. The endotoxins were excised from recombinant *M. tuberculosis* proteins using EndoTrap columns (Profos AG, Regenburg, Germany), according to the manufacturer's instructions. Residual endotoxin levels

were determined using a Limulus amebocyte lysate test (Lonza, Walkersville, Md., USA) and were found to be below 0.5 EU/10 μg protein. Chemical methylation of the lysine residues in recombinant mHBHA and mMDP-1 was performed as described previously (21).

The following fluorescently labeled monoclonal antibodies (mAbs) were used in this study: anti-CD3-APC-Cy7 (HIT3a), anti-IFN-γ-PE-Cy7 (4S.B3), anti-IL-10-PE (JES3-9D7), anti-IL-17-Alexa Fluor 700 (BL168), anti-TNF-α-PerCP-Cy5.5 (MAb11) (Biolegend, San Diego, Calif., USA), anti-CD4-Pacific Blue (OKT4), anti-IL-2-APC (MQ1-17H12), and anti-IL-13- FITC (PVM13-1) (eBioscience, San Diego, Calif., USA). Where necessary, the relevant isotype control mAb was used. Cell viability was assessed using the LIVE/DEAD kit (Invitrogen, Carlsbad, Calif., USA). Brefeldin-A (BFA) was purchased from Sigma-Aldrich.

In vitro culture: Peripheral blood mononuclear cells (PBMCs) were isolated from 10 ml of heparinized blood by density gradient centrifugation using Lymphoprep solution (AXIS-Shield, Oslo, Norway). After two washes with PBS, cells were resuspended in R10 medium, consisting of RPMI 1640 (Wako Junyaku Co., Tokyo, Japan) supplemented with 10% FBS, penicillin/streptomycin (Invitrogen), and 2 mM L-glutamine. A total of 0.5 to 1 × 10^6 PBMCs in 200 μl of R10 medium were plated into each well of a 96-well round bottom culture plate. Cultures were incubated with no antigen (medium only) or medium containing SEB (16.7 μg/ml), PPD (25 μg/ml), ESAT-6 and CFP-10 (0.9 μg/ml each), Acr (1.4 μg/ml), mHBHA (1.8 μg/ml), or mMDP-1 (7.5 μg/ml). All antigen stimulations were performed in the presence of BFA (1 μg/ml). The cells were incubated overnight (14–16 h) at 37°C in a 5% CO_2 incubator.

Flow cytometry: Following overnight incubation, the culture plate was centrifuged and the supernatant was removed. This was followed by the addition of 20 μl of a previously titrated surface marker cocktail (CD3 and CD4) and LIVE/DEAD reagent. The plate was vortexed and incubated for 30 min at 4°C. After washing with PBS, the cells were permeabilized, fixed with FACS permeabilizing solution (BD Bioscience, San Jose, Calif., USA), and incubated for 20 min at 4°C. The cells were washed three times with Perm/wash solution (BD Bioscience), and 20 μl of a previously titrated anti-cytokine mAbs cocktail containing IFN-γ, IL-2, IL-10, IL-13, and IL-17 was then added. After 30-min incubation at 4°C, the cells were washed and acquired using a FACS Canto II flow cytometer (BD Bioscience). FACS data were reanalyzed using FlowJo software, version 8.8.7 (TreeStar, San Carlos, Calif., USA).

Statistical analysis: Group medians and distributions were analyzed using Wilcoxon matched-pairs signed-rank tests and Mann-Whitney U tests. All analyses were performed using GraphPad Prism software, version 5 (San Diego, Calif., USA). A P value $<0.05\%$ was considered significant.

RESULTS

Assessment of IFN-γ-producing *M. tuberculosis*-specific T cells in fresh and frozen PBMCs: T cells stimulated with recombinant *M. tuberculosis*-related antigens were analyzed by flow cytometry. Because the majority of the Japanese population is immunized with BCG in early childhood, many individuals produce PPD-reactive memory T cells. Interestingly, one asymptomatic volunteer was found to be highly reactive to both PPD and the *M. tuberculosis*-specific antigens, ESAT-6 and CFP-10. This volunteer was a 60-year-old laboratory technician who had worked in a microbiology laboratory for almost 40 years and was therefore at a high risk of *M. tuberculosis* exposure; however, this individual had no history of TB symptoms or signs of respiratory diseases. PBMCs from this individual were used to determine the concentration of *M. tuberculosis*-related antigens required to maximize the T cell response without causing cell toxicity. Because fresh PBMC samples were not always available, levels of several cytokines from both fresh and frozen PBMC samples stimulated with a mixture of ESAT-6 and CFP-10 were compared. Both fresh and frozen PBMCs were stimulated with an ESAT-6/CFP-10 mixture (1.5 μg each) overnight. T cell surface staining with CD3, CD4, and CD8, followed by intracellular staining, was then performed using a multicolor flow cytometer and mAbs targeting IL-2, TNF-α, MIP-1β, and IL-17. The frequencies of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells in fresh PBMCs (Fig. 1A) were approximately 2% and 3.8%, respectively. These frequencies were reduced by more than 50% in the frozen samples (Fig. 1B). The frequency of MIP-1β-producing $CD8^+$ T cells was decreased from 2.79 + 0.81% (3.6% total) in fresh samples (Fig. 1A) to 1.48 + 0.35% (1.83% total) in frozen samples (Fig. 1B). However, the frequencies of IL-2, TNF-α, and MIP-1β-producing $CD4^+$ T cells were similar in fresh and frozen PBMCs. These results strongly suggest that IFN-γ-producing T cells are susceptible to degradation by freeze-thaw procedures. Therefore, subsequent studies were performed using PBMCs obtained from fresh blood samples.

IFN-γ and IL-2 responses of $CD4^+$ T cells to *M. tuberculosis*-related antigens: The IFN-γ and IL-2 responses of $CD4^+$ T cells to various *M. tuberculosis*-related antigens in the 12 *M. tuberculosis*-infected cases and eight healthy controls were investigated by flow cytometry. Double-positive (IFN-γ and IL-2) $CD4^+$ T cells were detected following PPD and ESAT-6/CFP-10 stimulation; the frequency of these double-positive cells was lower for ESAT-6/CFP-10 stimulation than that for PPD stimulation (Fig. 2A). $CD4^+$ T cell responses to other *M. tuberculosis*-related antigens, namely Acr, mHBHA, and mMDP-1, were also investigated (Fig. 2A). In *M. tuberculosis*-infected cases, the IFN-γ response of $CD4^+$ T cells to all *M. tuberculosis*-related antigens tested was significantly higher than that of control cells exposed to medium alone (Fig. 2B). In contrast, the IFN-γ responses to *M. tuberculosis*-related antigens in healthy controls were not statistically significant (Fig. 2B). The latter finding remained the same even if analysis was confined to individuals with a high $CD4^+$ T cell response to PPD. These results indicate that the *M. tuberculosis*-related antigens used in this study can stimulate *M. tuberculosis*-reactive or mycobacteria-reactive $CD4^+$ T cells. Interestingly, IL-2-producing $CD4^+$ T cells were detectable in the absence of antigen in three *M. tuberculosis*-infected cases; however, none were detected in healthy controls (Fig.

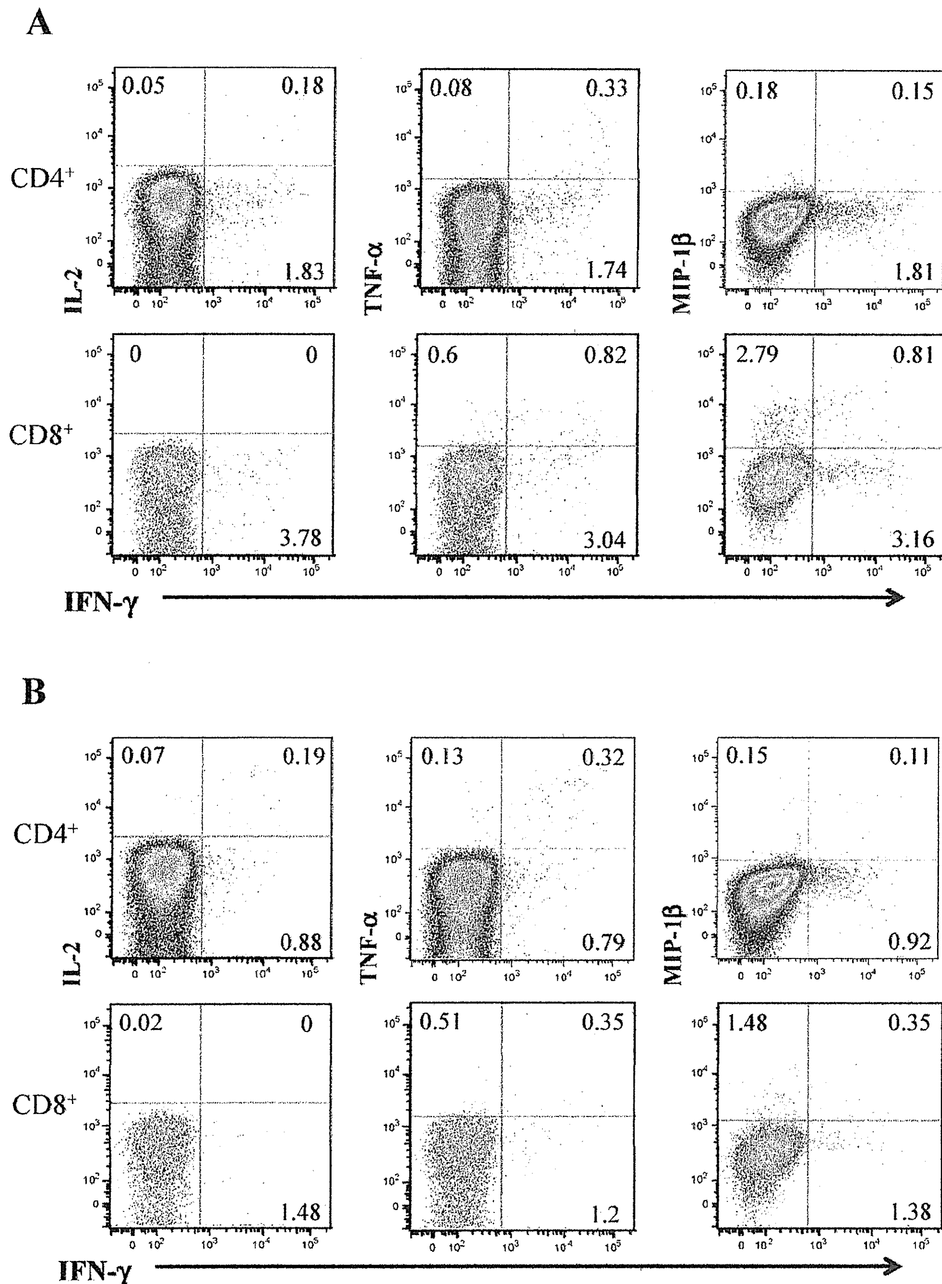

Fig. 1. Flow cytometric analyses of fresh and frozen PBMCs following ESAT-6/CFP-10 stimulation. (A) Fresh PBMCs. (B) Frozen PBMCs. PBMCs were prepared from one asymptomatic high responder. Upper plots display $CD3^+CD4^+$ T cells, and lower plots display $CD3^+CD8^+$ T cells. The plots display the percentage of cells producing IFN-γ and either IL-2, TNF-α, or MIP1-β. Values in the lower right quadrant represent the percentage of IFN-γ-producing cells; values in the upper left quadrant represent the percentage of IL-2, TNF-α, or MIP1-β-producing cells; and values in the upper right quadrant represent the percentage of cells producing both IFN-γ and the relevant cytokine.

2C). Despite this non-specific response, the IL-2 response to PPD in *M. tuberculosis*-infected cases was significantly higher than that of media-exposed control cells ($P = 0.0425$). In healthy controls, a slight IL-2 response to PPD was detected; however, the response was not significant (Fig. 2C). Further analysis of the IL-2 response to mHBHA in *M. tuberculosis*-infected cases grouped by clinical status revealed a significant IL-2 response in recovery stage TB cases compared with that in control cases ($P = 0.0225$, Fig. 2D) and contact cases ($P = 0.0119$, Fig. 2D). Interestingly, two distinct groups were identified, one of which had a high IL-2 response and the other had a low IL-2 response in on-treatment and recovery stage TB cases. This finding suggests that the IL-2-producing $CD4^+$ T cells that are stimulated by mHBHA may indicate ongoing *M. tuberculosis* replication.

Use of polyfunctional $CD4^+$ T cells producing both IFN-γ and IL-2 as an indicator of TB: The polyfunctionality and corresponding high mean fluorescence in-

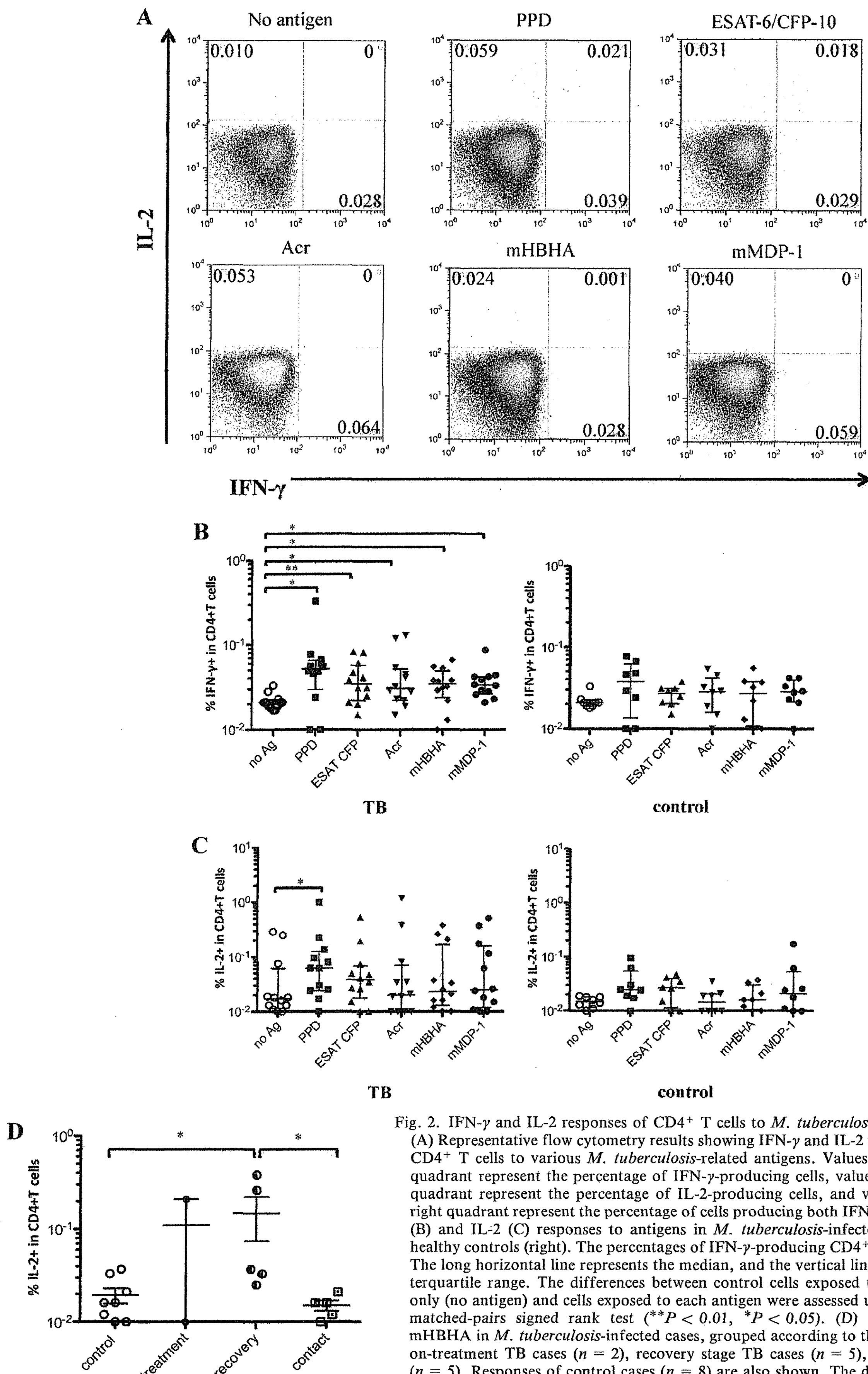

Fig. 2. IFN-γ and IL-2 responses of $CD4^+$ T cells to *M. tuberculosis*-related antigens. (A) Representative flow cytometry results showing IFN-γ and IL-2 responses of $CD3^+$ $CD4^+$ T cells to various *M. tuberculosis*-related antigens. Values in the lower right quadrant represent the percentage of IFN-γ-producing cells, values in the upper left quadrant represent the percentage of IL-2-producing cells, and values in the upper right quadrant represent the percentage of cells producing both IFN-γ and IL-2. IFN-γ (B) and IL-2 (C) responses to antigens in *M. tuberculosis*-infected cases (left) and healthy controls (right). The percentages of IFN-γ-producing $CD4^+$ T cells are shown. The long horizontal line represents the median, and the vertical line represents the interquartile range. The differences between control cells exposed to culture medium only (no antigen) and cells exposed to each antigen were assessed using the Wilcoxon matched-pairs signed rank test (**$P < 0.01$, *$P < 0.05$). (D) IL-2 responses to mHBHA in *M. tuberculosis*-infected cases, grouped according to the disease status as on-treatment TB cases ($n = 2$), recovery stage TB cases ($n = 5$), and contact cases ($n = 5$). Responses of control cases ($n = 8$) are also shown. The differences between each set of samples were assessed using the Mann-Whitney test (*$P < 0.05$).

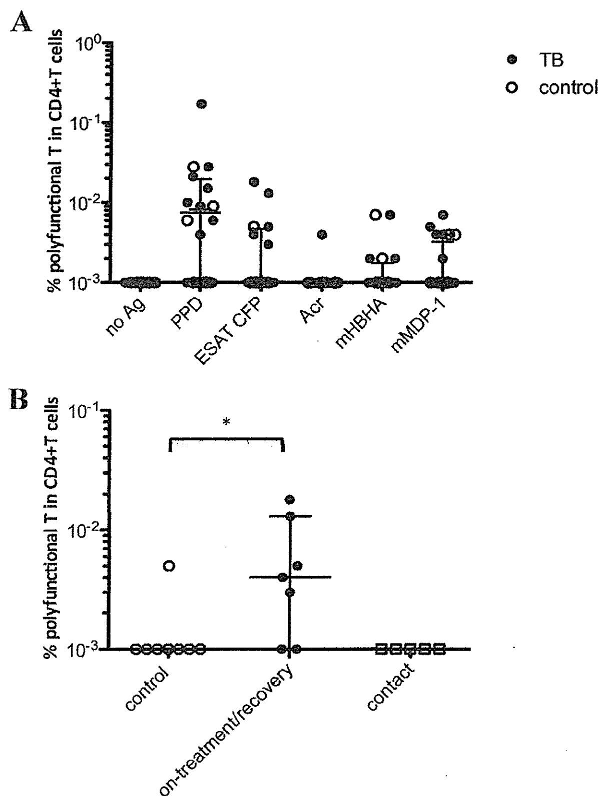

Fig. 3. Responses of polyfunctional CD4$^+$ T cells to *M. tuberculosis*-related antigens. (A) Percentages of polyfunctional (secreting both IFN-γ and IL-2) CD4$^+$ T cells following stimulation by various *M. tuberculosis*-related antigens. Filled circles represent the *M. tuberculosis*-infected cases, and open circles represent the healthy controls. (B) Percentages of polyfunctional CD4$^+$ T cells following stimulation by ESAT-6/CFP-10; *M. tuberculosis*-infected cases are grouped according to the disease status as either TB (on-treatment/recovery; $n = 7$) or contact cases ($n = 5$). Responses of control cases ($n = 8$) are also shown. The differences between control cells exposed to culture medium only and grouped *M. tuberculosis* cases were assessed using the Mann-Whitney test (*$P < 0.05$). (A, B) The long horizontal line represents the median, and the vertical line represents the interquartile range.

tensity of T cells that produce multiple cytokines are highly correlated with protection in various infections and vaccination (26). In this study, polyfunctional CD4$^+$ T cells producing both IFN-γ and IL-2 were detected following all *M. tuberculosis*-related antigen stimulations. No polyfunctional CD4$^+$ T cells were detectable without antigen stimulation. Although the overall frequency of polyfunctional CD4$^+$ T cells following PPD stimulation was higher in the *M. tuberculosis*-infected cases than that in the healthy controls, three of the eight healthy controls showed a high frequency of polyfunctional CD4$^+$ T cells (Fig. 3A). The difference between the frequency of polyfunctional CD4$^+$ T cells in *M. tuberculosis*-infected cases and healthy controls was not significant for the other *M. tuberculosis*-related antigen stimulations (Fig. 3A); however, when the seven on-treatment and recovery stage *M. tuberculosis*-infected cases were analyzed separately from the five *M. tuberculosis* contact cases, the frequency of polyfunctional CD4$^+$ T cells following ESAT-6/CFP-10 stimulation was significantly higher in the on-treatment and recovery stage groups than that in the control group (Fig. 3B; $P = 0.0365$). These results suggest that the polyfunctional CD4$^+$ T cell response to this set of *M. tuberculosis*-specific antigens and not the IFN-γ response alone (Fig. 2B) has a diagnostic value for the detection of on-treatment and recovery stage TB cases.

IL-17A responses to some *M. tuberculosis*-related antigens are associated with active or latent *M. tuberculosis* infection: Compared with control cells exposed to culture medium, the percentage of CD4$^+$ T cells producing IL-17A was slightly but significantly increased by PPD and mHBHA stimulation ($P = 0.0117$ and 0.0233, respectively) (Fig. 4A). This increase was observed only in *M. tuberculosis*-infected cases and not in healthy controls (data not shown). Furthermore, when the on-treatment and recovery stage cases were analyzed separately from the contact cases, the IL-17A responses induced by mHBHA (Fig. 4B) and mMDP-1 (Fig. 4C) were significantly higher ($P = 0.0127$ and 0.0237, respectively) than those in healthy controls in the on-treatment and recovery stage groups only. Overall, these data suggest that an increased frequency of IL-17A-producing CD4$^+$ T cells is a supportive indicator of *M. tuberculosis* infection.

The frequency of IL-10-producing CD4$^+$ T cells is reduced following TB therapy: IL-13-producing CD4$^+$ T cells were not detected in the subjects included in this study. In *M. tuberculosis*-infected cases, the IL-10 responses to ESAT-6/CFP-10, mHBHA, and mMDP-1 were significantly higher than the response to culture medium alone ($P = 0.0413$, 0.0231, and 0.0144, respectively) (Fig. 5A). Notably, the frequency of Acr-stimulated IL-10-producing CD4$^+$ T cells in the recovery stage TB cases was significantly lower than that in the contact cases ($P = 0.0362$) (Fig. 5B). This trend needs to be confirmed with a larger cohort of TB cases.

DISCUSSION

In this study, we performed cross-sectional analysis of cytokine profiles in CD4$^+$ T cells from different clinical stages of TB infection, including on-treatment (active TB), recovery stage, and contact cases (both as non-active TB), following stimulation by PPD, ESAT-6/CFP-10, and other latency-associated *M. tuberculosis* antigens. We demonstrated here that the IFN-γ response alone was unable to distinguish between TB cases and healthy controls, even after exposure to a new set of latency-related *M. tuberculosis* antigens. However, when TB cases were grouped by distinct clinical stages of TB infection, significant differences between the recovery stage TB group and control group and between the recovery stage TB group and contact group were observed in the IL-2 response to mHBHA (Fig. 2D), and significant differences were observed between the recovery stage TB group and control group in the IL-17 response to mHBHA and mMDP-1 (Fig. 4B and 4C). Similarly, the number of *M. tuberculosis*-specific polyfunctional T cells producing both IFN-γ and IL-2 was also significantly different from that of the control group in ESAT-6/CFP-10 stimulation, which is consistent with previous findings (22). Thus, although further studies are necessary, our results indicate that cytokine

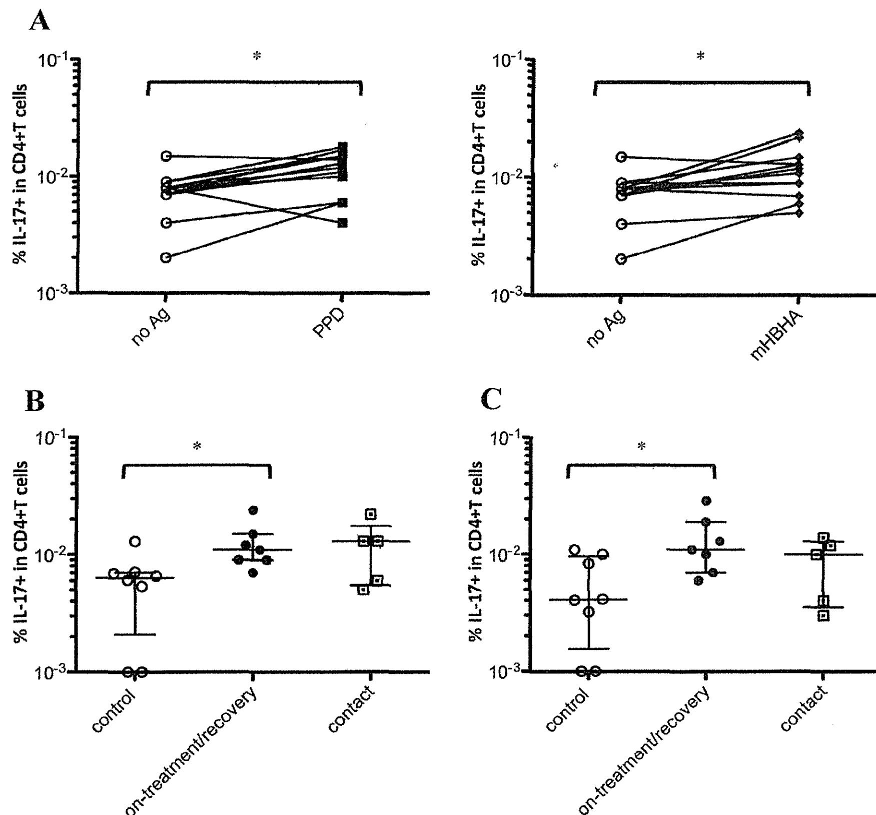

Fig. 4. IL-17 responses of CD4+ T cells to *M. tuberculosis*-related antigens. (A) IL-17 responses to culture medium alone (no antigen), PPD, and mHBHA in *M. tuberculosis*-infected cases. The differences between control cells and each antigen were assessed using the Wilcoxon matched-pairs signed rank test (*$P < 0.05$). (B, C) IL-17 responses to mHBHA (B) and mMDP-1 (C) in TB cases grouped according to the disease status as TB (on-treatment/recovery; $n = 7$) or contact cases ($n = 5$). Responses of control cases ($n = 8$) are also shown. The differences between control cells and each *M. tuberculosis* group were assessed using the Mann-Whitney test (*$P < 0.05$). The long horizontal line represents the median and the vertical line represents the interquartile range.

profiles of various *M. tuberculosis*-related antigen-specific T cells can be useful parameters to assess TB status.

It is known that the clinical treatment of *M. tuberculosis* shifts the single IFN-γ-producing CD4+ T cell response to both a polyfunctional IFN-γ/IL-2 response and a single IL-2 response (23). Therefore, while the dominant IFN-γ response is detectable during the active TB stage, dominant IL-2 responses are more likely to be detected at the non-active (contact and recovery) stage of the disease. Unfortunately, we were unable to address this issue in the present study because only two on-treatment cases were included; therefore, further studies with a larger sample size are warranted. Notably, the IL-2-producing CD4+ T cell response was elevated in some patients prior to *M. tuberculosis* antigen stimulation. Because IL-2 is known to be crucial for the maintenance of Treg cells (24) and proliferation of Th cells (25), we speculated that these IL-2-producing CD4+ T cells are on duty in vivo to regulate inflammatory responses caused by *M. tuberculosis* infection.

Polyfunctional *M. tuberculosis*-specific T cells have a memory function with proliferative capacity (19,26) as well as the ability to produce high quantities of cytokines (27). These functional abilities are necessary to control the propagation of foreign pathogens such as *M. tuberculosis*. Therefore, polyfunctional T cells are expected to be induced in *M. tuberculosis* cases with active bacterial replication, and some studies have reported that polyfunctional T cells are present at higher frequencies in active TB cases than those in healthy controls or latent TB cases (28,29). In the present study, significant levels of ESAT-6/CFP-10-specific polyfunctional CD4+ T cells producing both IFN-γ and IL-2 were detected in TB cases only. An investigation of the TNF-α responses was not performed in this study owing to the color detection limitation of the flow cytometer employed. Recently, a large cohort investigation of ESAT-6- and CFP-10-induced polyfunctional *M. tuberculosis*-specific T cells (producing IFN-γ, IL-2, and TNF-α) in TB patients showed that the TNF-α-producing *M. tuberculosis*-specific CD4+ T cell response is dominant and accurately reflects the active TB state (30). However, because frozen PBMC samples were used in the investigation, IFN-γ-producing CD4+ T cells may have been exclusively lost, as observed in the present study (Fig. 1). Therefore, further studies are warranted to confirm this finding.

CD4+ T cells that secrete IL-17 are considered as Th17 cells (17), which trigger early inflammatory via neutrophil recruitment (31). Because of the methylation at lysine residues, mycobacterial HBHA proteins are resistant to proteolytic degradation by proteases present in bronchoalveolar lavage fluids, and their abundance

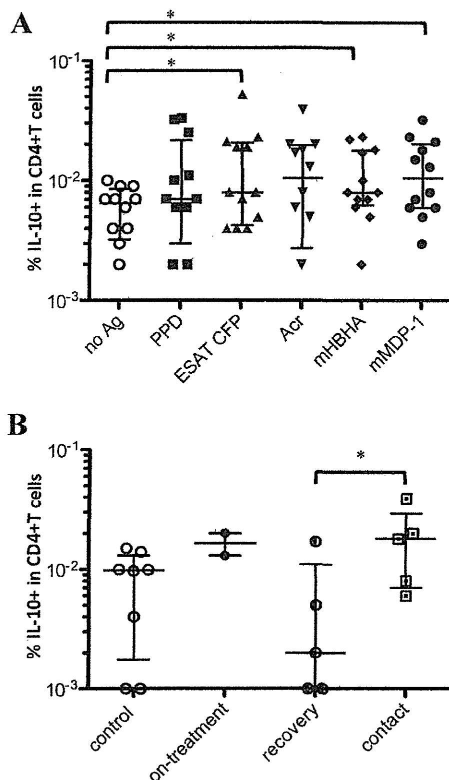

Fig. 5. IL-10 responses of CD4+ T cells to *M. tuberculosis*-related antigens. (A) The percentages of IL-10 secreting CD4+ T cells are shown. The differences between control cells and each antigen were assessed using the Wilcoxon matched-pairs signed rank test (*$P < 0.05$). (B) IL-10 responses to Acr in *M. tuberculosis*-infected cases grouped according to the disease status as on-treatment TB cases ($n = 2$), recovery stage TB cases ($n = 5$), and contact cases ($n = 5$). Responses of control cases ($n = 8$) are also shown. The differences between each set of samples were assessed using the Mann-Whitney test (*$P < 0.05$). (A, B) The long horizontal line represents the median and the vertical line represents the interquartile range.

in the cell membrane is believed to aid the attachment of *M. tuberculosis* to alveolar epithelial cells (32). Therefore, we postulated that HBHA proteins are one of the first antigens that the host immune system encounters and that the IL-17 response to HBHA proteins can be detected in a very early phase of *M. tuberculosis* infection or exposure. In the present study, we detected a small but significant number of IL-17A-producing CD4+ T cells in response to HBHA in TB cases. This is compatible with a previous finding that HBHA-specific memory CD4+ T cells are present in vivo (33). To clarify the role of this IL-17 immune response in the course of *M. tuberculosis* infection, it is worth investigating the IL-17 response to HBHA in a cohort of early *M. tuberculosis* exposure population.

The C-terminal domain of HBHA recognizes the lysine-rich domain to be the same as heparin-sulfate receptors, and MDP-1 has a heparin-binding site that resembles HBHA (34). We hypothesized that mMDP-1 could also induce an early host immune response in a similar manner to mHBHA. As expected, significant responses to mMDP-1 were detected in the on-treatment and recovery stage *M. tuberculosis* cases. Therefore, the Th17 response to HBHA and MDP-1 may be a candidate predictive marker of early *M. tuberculosis* infection.

IL-10 functions as an anti-inflammatory cytokine and has been suggested to contribute to the onset of infectious disease by inhibiting other inflammatory cytokines. IL-10 is also known to directly affect macrophages by inhibiting expression of MHC class II and co-stimulatory molecules (35). A high number of Treg cells, which are a known source of IL-10, are present in human TB granulomas (36). In this study, the IL-10 response was low in recovery stage TB cases and was even lower than that in contact TB cases (Fig. 5B). This result may be due to the migration of IL-10-producing T cells from the blood to local inflammatory sites. Alternatively, *M. tuberculosis*-specific IL-10-producing T cells may have a transient role during early infection and self-antigen-specific IL-10-producing T cells may contribute to halting inflammation (37). The candidate of self-antigen is a dump of *M. tuberculosis* granuloma. Thus, it is considered that the IL-10 response to *M. tuberculosis* antigen simply follows *M. tuberculosis* inflammation and that the IL-10 response may serve as a marker for resolving the disease.

The IL-2 response to mHBHA antigen in recovery stage TB cases was significantly higher than that in healthy controls and contact cases. This finding implies that the IL-2 response to mHBHA is a useful marker of LTBI. Recent analysis of the ratio of HBHA-induced and recombinant ESAT-6-induced IFN-γ responses suggested that latent TB patients can be categorized into three different risk groups (38). Here, we found that the ratio of mHBHA-induced and ESAT-6/CFP-10-induced IFN-γ T cell responses tended to decrease after longer post-treatment durations. Because HBHA molecules are enriched in the *M. tuberculosis* membrane, it is likely that the T cell responses to HBHA accurately reflect the TB burden. On the other hand, ESAT-6 is known to be important for suppressing host immunity by inhibiting macrophage function (39) and may be required when *M. tuberculosis* is present in granulomas. Therefore, the ratio of HBHA-induced to ESAT-6/CFP-10-induced T cell responses may vary at distinct clinical stages of TB.

In conclusion, this study employed various *M. tuberculosis*-related antigens, including a novel methylated MDP-1 antigen, to show that the cytokine profiles of CD4+ T cells differ at each clinical stage of TB. The results indicate that the detection of *M. tuberculosis*-specific polyfunctional T cells reflects the onset of TB. A more accurate prediction of disease onset may be achieved by combining the detection of several cytokines such as IL-10 and IL-17, which are produced in response to various *M. tuberculosis*-related antigens. A combination of this approach with the detection of latent *M. tuberculosis*-related antigens may allow the development of an improved diagnostic test that can more accurately identify *M. tuberculosis*-infected individuals at a higher risk of developing TB and eventually form the basis of a public health measure for controlling *M. tuberculosis* infection. We believe that further analysis of a wide spectrum of T cell cytokine responses to latent *M. tuberculosis*-related antigens will help in the assessment of the disease status of TB patients and the initiation of an early therapeutic inter-

vention.

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Conflict of interest None to declare.

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