Original Article

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

Yoshiro Yamashita[1], Yoshihiko Hoshino[2], Mayuko Oka[4],
Sokichi Matsumoto[5], Haruyuki Ariga[6], Hideaki Nagai[6],
Masahiko Makino[2], Koya Ariyoshi[1], and Yasuko Tsunetsugu-Yokota[3]*

*[1]Department of Clinical Medicine, Institute of Tropical Medicine,
Nagasaki University, Nagasaki 852-8523;
[2]Department of Mycobacteriology, Leprosy Research Center,
National Institute of Infectious Diseases, Tokyo 189-0002;
[3]Department of Immunology, National Institute of Infectious Diseases, Tokyo 162-8640;
[4]Division of Applied Life Science, Graduate School of Life and Environmental Sciences,
Kyoto Prefectural University, Kyoto 606-8522;
[5]Department of Bacteriology, Osaka City University Graduate School of Medicine,
Osaka 545-8585; and
[6]Center for Respiratory Medicine, National Hospital Organization Tokyo National Hospital,
Tokyo 204-8585, Japan*

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.*

*Corresponding author: Mailing address: Department of Immunology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku, Tokyo 162-8640, Japan. Tel: +81-3-4582-2716, Fax: +81-3-5285-1156, E-mail: yyokota@nih.go.jp

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

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	

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.

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 $\times$ 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.
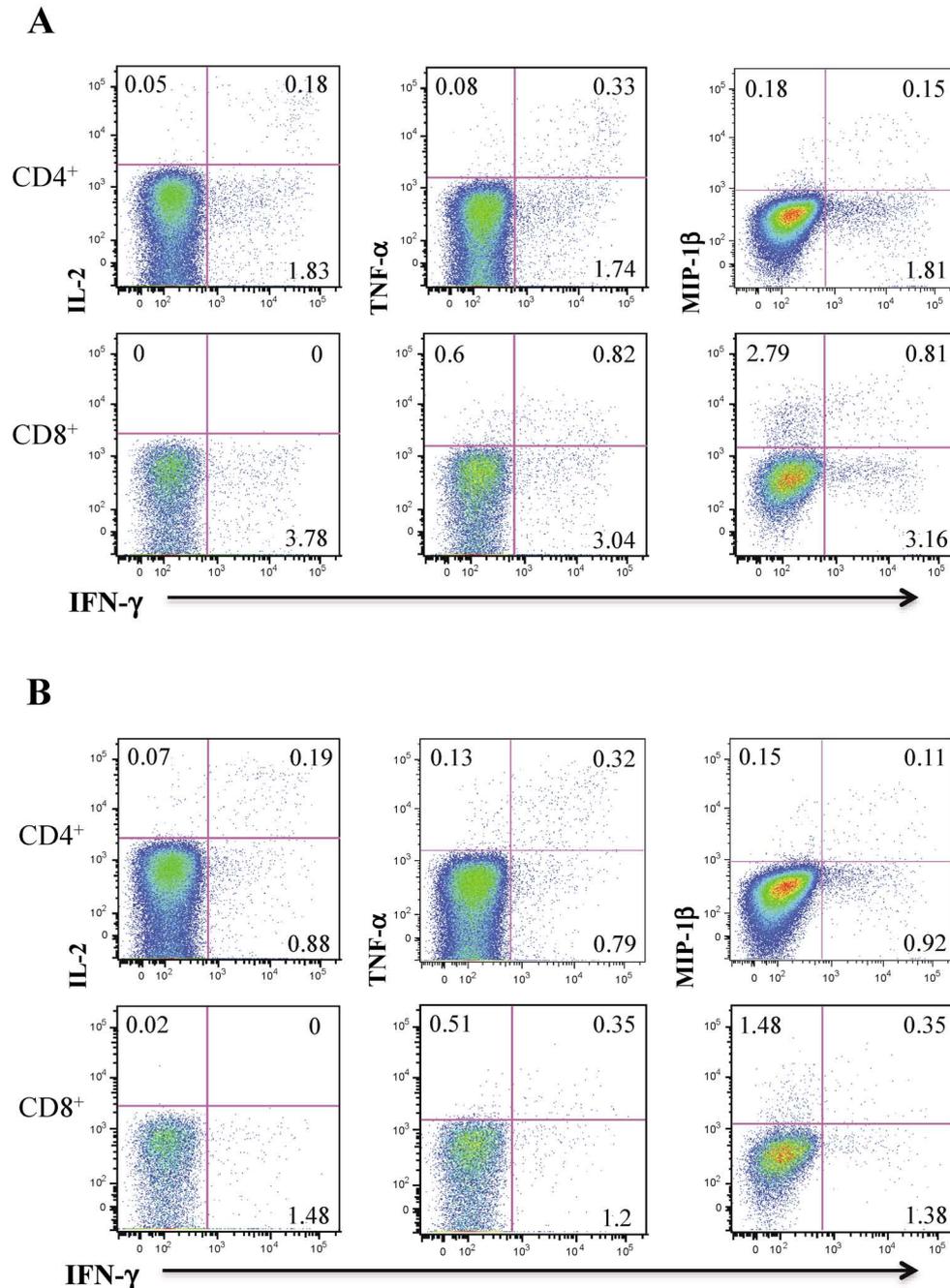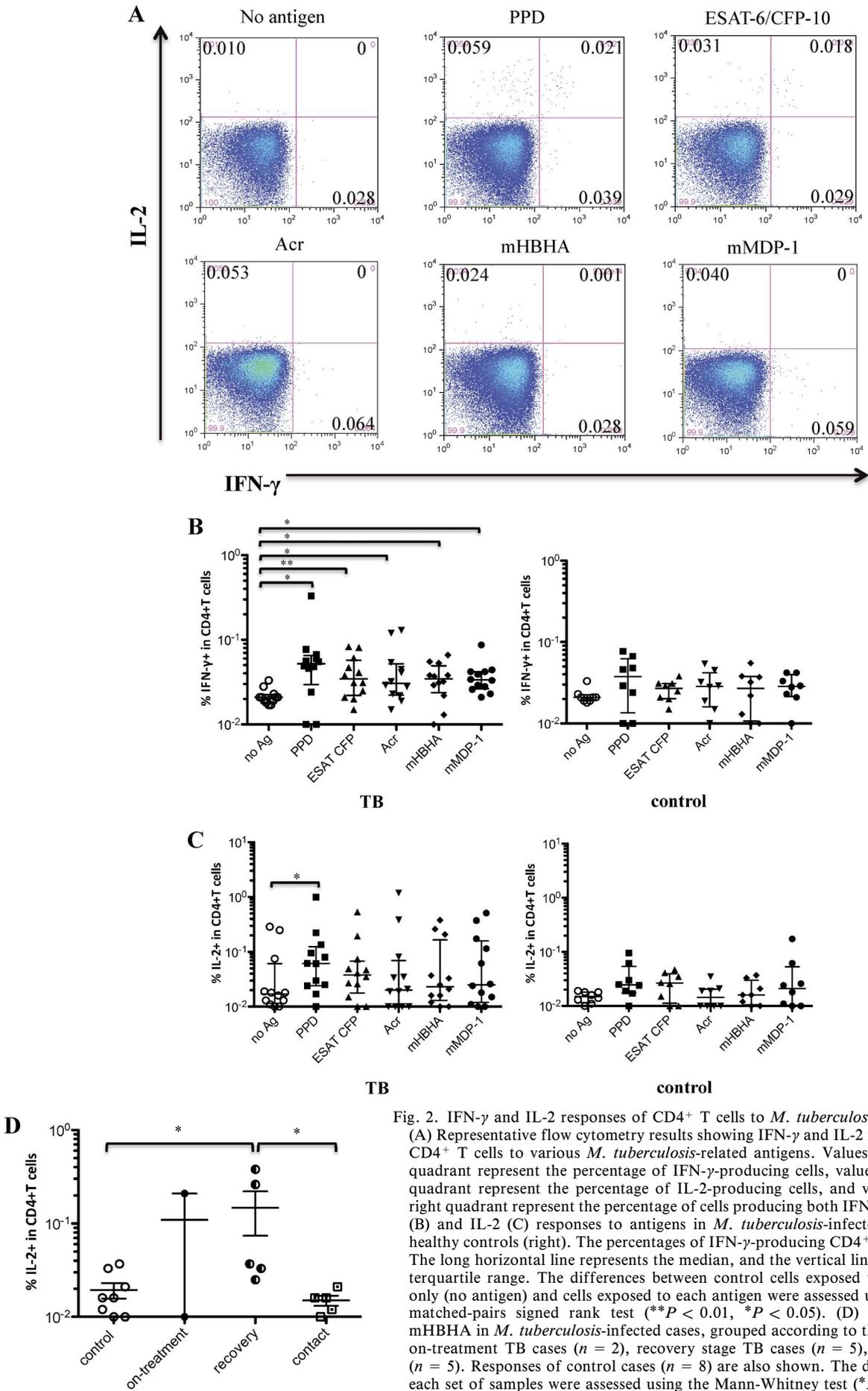
Fig. 1. Flow cytometric analyses of fresh and frozen PBMCs following ESAT-6/CFP-10 stimulation. (A) Fresh PBMCs. (B) Frozen PBMCs. PBMCs were prepared from one asymptomatic high responder. Upper plots display CD3+CD4+ T cells, and lower plots display CD3+CD8+ T cells. The plots display the percentage 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 (*$P < 0.05$).
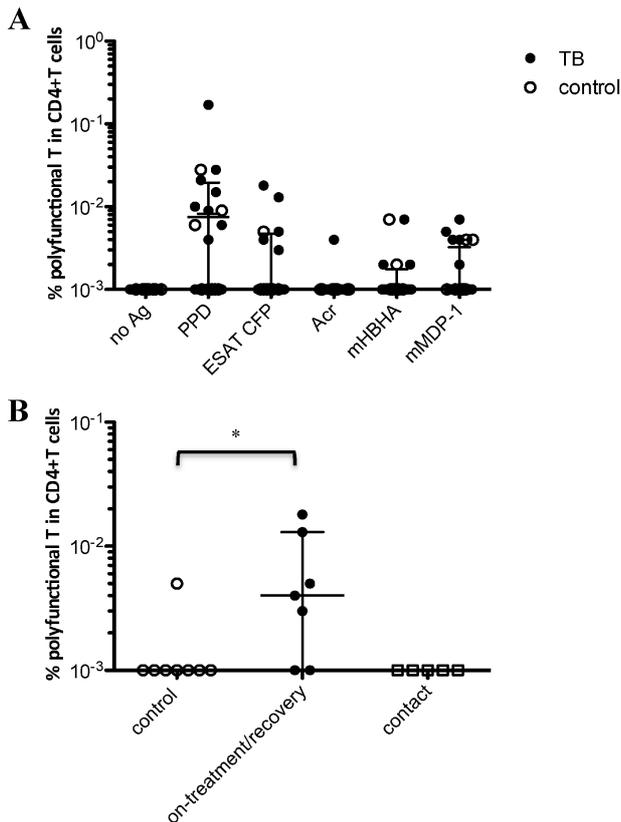
Fig. 3. Responses of polyfunctional CD4+ T cells to *M. tuber-culosis*-related antigens. (A) Percentages of polyfunctional (secreting both IFN-γ and IL-2) CD4+ T cells following stimulation by various *M. tuberculosis*-related antigens. Filled circles represent the *M. tuberculosis*-infected cases, and 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. tuber-culosis*-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
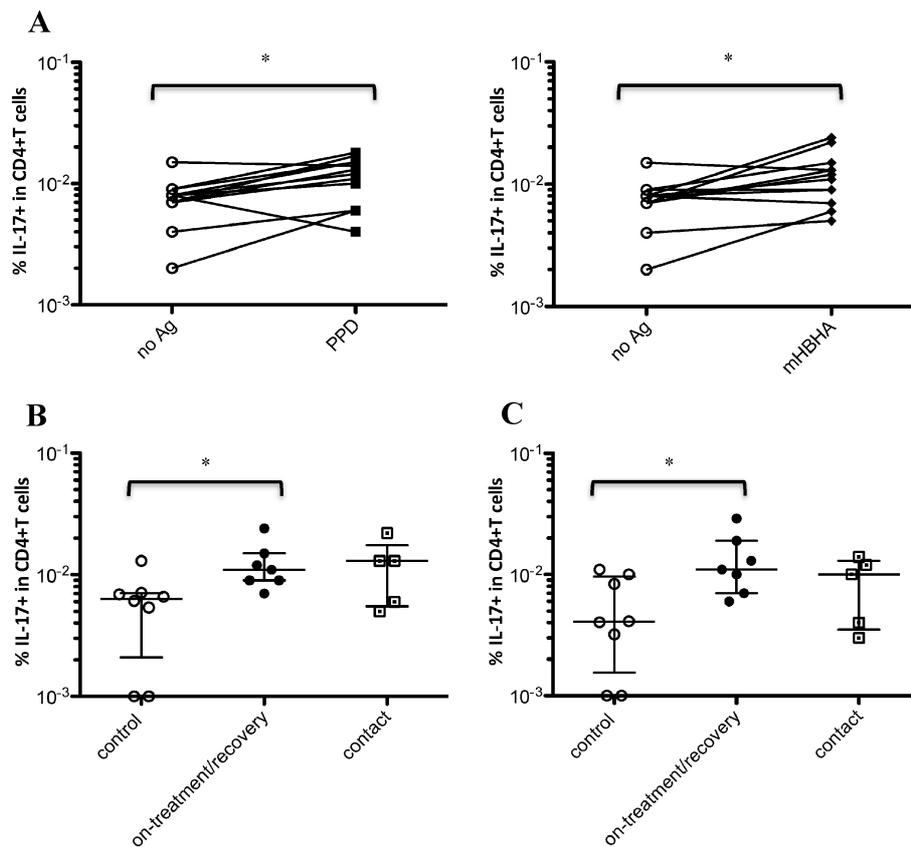
A

B

C

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
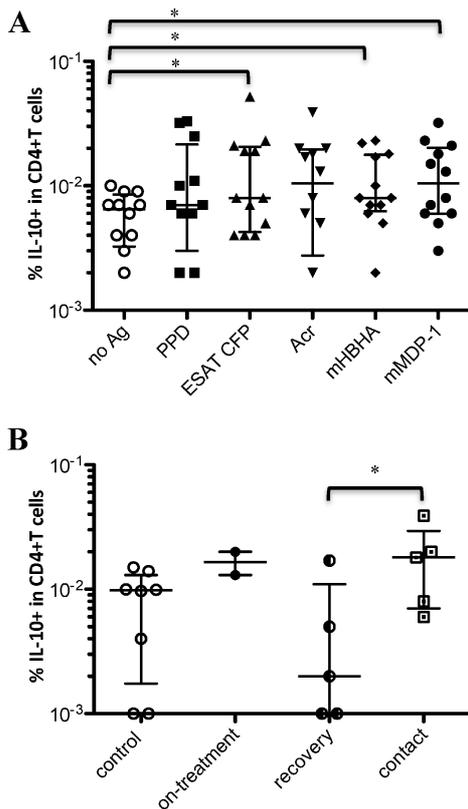
Fig. 5. IL-10 responses of CD4⁺ T cells to *M. tuberculosis*-related antigens. (A) The percentages of IL-10 secreting CD4⁺ T cells are shown. The differences between control cells and each antigen were assessed using the Wilcoxon matched-pairs signed rank test (*$P < 0.05$). (B) IL-10 responses to Acr 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 candi-

date 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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