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Characterization of CD4/CD8+ αβ and Vγ2Vδ2+ T cells in HIV-negative individuals with different *Mycobacterium tuberculosis* infection statuses

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ABSTRACT

Background: The immune responses of T cell subsets among patients with different *Mycobacterium tuberculosis* (*M.tb*) infection statuses [i.e., active tuberculosis (ATB), latent tuberculosis infection (LTBI) and non-infection (healthy control, HC)] have not been fully elucidated in HIV-negative individuals. Specifically, data are limiting in high tuberculosis epidemic regions in China. To investigate the distributions and functions of T cell subsets (i.e., CD3+, CD4+, CD8+ αβ and Vγ2Vδ2+ T cells) in HIV-negative subjects with different *M.tb* infection statuses, we conducted a case-control study that enrolled 125 participants, including ATB patients ($n = 46$), LTBI subjects ($n = 34$), and HC ($n = 45$).

Results: An IFN-γ release assay (IGRA) was employed to screen LTBI subjects. Whole blood cell surface staining and flow cytometry were used to detect phenotypic distributions of T cells in the peripheral blood mononuclear cells (PBMCs) and tuberculous pleural fluid mononuclear cells (PFMCs). PPD and the phosphorylated antigen HMBPP were employed as stimulators for the detection of *M.tb* antigen-specific T cell functions via intracellular cytokine staining (ICS). The absolute numbers of T cell subsets, including CD3+ CD4+, CD3+ CD8+ αβ and Vγ2Vδ2+ T cells, were significantly reduced in active tuberculosis compared with latent tuberculosis or the healthy controls. Importantly, PPD-specific CD3+ CD4+ and CD3+ CD8+ αβ T cells and HMBPP-specific Vγ2Vδ2+ T cells in ATB patients were also significantly reduced compared to the LTBI/HC subjects ($P < 0.05$). In contrast, the proportion of CD4+ T cells in PFMCs was higher compared to PBMCs, while CD8+ and Vγ2Vδ2+ T cells in PFMCs were lower compared to PBMCs (all $P < 0.05$). PPD-specific CD4+ T cells predominated among CD3+ T cells in PFMCs.

Conclusions: Cellular immune responses are impaired in ATB patients. Antigen-specific CD4+ T cell may migrate from the periphery to the lesion site, where they exert anti-tuberculosis functions.

1. Introduction

Approximately one-third of the world's population (or two billion people) is estimated to be infected with *Mycobacterium tuberculosis* (*M.tb*). About 5–10% of infected people develop TB disease during their lifetime, mostly within 5 years after a new infection [1]. The risk that a new or latent infection will progress to disease is increased by a compromised immune system.

M.tb as an intracellular pathogen that has a complex relationship with the host. When the natural immune response cannot control the growth of *M.tb*. There is continuous exposure to *M.tb* antigens. Then, the organism starts to induce acquired immune responses, with a predominance of cell-mediated immune responses [2,3]. CD4+ T cells play an important role primarily through the secretion of interferon-gamma (IFN-γ), interleukin-2 (IL-2), tumor necrosis factor-α (TNF-α) and other cytokines that are involved in immune control [4–6]. CD8+ T cells and γδ T cells play protective roles through the secretion of IFN-γ, perforin and granzyme [5–13]. Our previous studies in HIV/AIDS patients showed that cytokine secretion by *M.tb* antigen-specific CD8+ and Vγ2Vδ2+ T cells was greatly enhanced in subjects with latent tuberculosis infection compared with subjects with active

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tuberculosis. This enhanced response was likely to help the HIV-1-infected host effectively inhibit pathogen replication into a latent state [14].

However, the immune responses of T cell subsets among subjects with different *M.tb* infection statuses, such as active, latent and no *M.tb* infection, have not been fully elucidated in HIV-negative individuals. Specifically, data are limiting in the high tuberculosis epidemic regions of China. Therefore, we conducted a case-controlled study to investigate the distributions and functions of T cell subsets, including CD3+, CD4+, CD8+ αβ and Vγ2Vδ2+ T cells, in HIV-negative subjects with different *M.tb* infection statuses. We further analyzed the immune responses at the lesion sites of *M.tb* infection (pleural effusion of patients with tuberculous pleurisy).

2. Materials and methods

2.1. Study population

One hundred and twenty-five individuals were recruited in this study, including active tuberculosis (ATB) patients ($n = 46$), latent tuberculosis infection (LTBI) subjects ($n = 34$), and healthy controls (HC) ($n = 45$). The ATB patients were recruited from January 1, 2011, to October 31, 2011, from Wuxi No. 5 People's Hospital. LTBI subjects were recruited from the close contacts of ATB patients, and the healthy controls were recruited from volunteers at Fudan University.

This study was approved with written consent by the Ethics Committee of Huashan Hospital, Fudan University, with approval number of 2011-247. Written informed consent was obtained from all of the participants.

2.2. Criteria for ATB, LTBI and HC inclusion

We employed an IFN-γ release assay (IGRA) for tuberculosis to distinguish BCG vaccination from *M.tb* infection. The individuals were divided into three groups based on the IGRA assay. ATB patients included patients with active pulmonary tuberculosis ($n = 27$) and tuberculous pleurisy ($n = 19$). All patients with pulmonary tuberculosis were sputum acid-fast bacillus (AFB) smear- or culture-positive, and treatment naïve or anti-tuberculosis treated with a duration of less than 1 week. Confirmed tuberculous pleurisy was diagnosed with *M.tb* culture-positive in the pleural fluid and/or pleural biopsy. Thirty-four individuals were diagnosed with LTBI based on a positive IGRA and no evidence of active tuberculosis (e.g., clinical manifestations of pulmonary and extrathoracic tuberculosis and abnormal chest radiographs). Forty-five individuals were healthy controls who had negative IGRA results and no evidence of active tuberculosis. All enrolled participants were HIV-negative, had not been diagnosed with cancer, diabetes, autoimmune diseases or other chronic infections (i.e., chronic HBV/HCV infection), and had not received immune modulator treatments.

2.3. Immunofluorescence staining and flow cytometric analysis

Blood samples collected freshly from all groups of participants were handled, and analyzed by phenotyping and intracellular cytokine staining (ICS) at the biocontainment laboratory [12]. Peripheral blood mononuclear cells (PBMCs) were isolated from heparin-anticoagulated blood by density gradient sedimentation using Lympholyte-H (Cedarlane Laboratories Ltd, Ontario, Canada). For cell-surface staining, 100 μL of anticoagulated blood was treated with red blood cell (RBC) lysis buffer and washed twice with 5% fetal bovine serum (FBS)-phosphate-buffered saline (PBS) prior to staining [14]. PBMCs were stained with up to four Abs (conjugated to FITC, PE, allophycocyanin, pacific blue, and PE-Cy7) for at least 10 min at room temperature. After staining, the cells were fixed with 2% formaldehyde-PBS prior to analysis on a BD FACS Aria flow cytometer (BD Bioscience, San Diego, CA, USA). Lymphocytes were gated based on forward-scatter and side-scatter properties; at least 20,000 gated events were analyzed using the FCS EXPRESS 3 Software (De Novo Software, Glendale, CA, USA). Absolute cell numbers were calculated based on flow cytometry data and complete blood counts. The following mouse anti-human mAbs were used: Vγ2 (7A5) and Vδ2 (15D) (Thermo Scientific, Rockford, MD, USA); CD3 (SP34, SP34-2), CD8 (RPA-T8), CD28 (CD28.2), CD49d (9F10) and IFN-γ (4S.B3) (BD Pharmingen, San Diego, CA, USA); and CD4 (OKT4) (BD Bioscience, San Diego, CA, USA). The secondary Ab (PE-conjugated goat anti-mouse IgG; Beckman Coulter, Marseille, France) was used for indirect staining.

2.4. Intracellular cytokine staining

ICS was performed using the standard protocol as recently described [13–15]. For the ICS assay, 10^6 PBMCs plus the costimulatory mAbs CD28 (1 μg/mL) and CD49d (1 μg/mL) were incubated with purified protein derivative (PPD) (25 μg/mL), phosphoantigen (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) (40 ng/mL) or media alone in a 200 μL final volume for 1 h at 37 °C in a 5% CO_2 atmosphere, followed by an additional 5 h of incubation in the presence of brefeldin A (GolgiPlug; BD Bioscience). After staining the cell surface markers CD3/CD4/CD8/Vγ2 for at least 15 min at room temperature, the cells were permeabilized for 45 min (Cytofix/cytoperm; BD Biosciences) at 4 °C and stained another 45 min for IFN-γ at room temperature prior to resuspending in 2% formaldehyde-PBS. HMBPP belongs to phosphoantigen and only TCRs bearing both the Vγ2 and Vδ2 elements can recognize HMBPP. The phosphoantigen compound HMBPP used in the study was 98% pure and specifically stimulated the activation/expansion of Vγ2Vδ2 T cells but not other cell subpopulations [12].

2.5. IFN-γ release assay (IGRA)

The IGRA used in this study was the QuantiFERON-TB Gold In-Tube (QFT-GIT) test. The QFT-GIT was performed according to the manufacturer's instructions (QuantiFERON-TB Gold In-Tube, Cellestis Ltd., Carnegie, Australia). Briefly, a 3 mL venous blood sample was collected from each participant on the day of pleural effusion collection and aliquoted into three tubes (TB-specific antigen, mitogen and nil tubes, respectively). The samples were incubated at 37 °C in a humidified 5% CO_2 incubator for 24 h. On the second day, the tubes were centrifuged at 3000 rcf for 10 min, and the plasma was collected and stored at 4 °C until the IFN-γ assay was performed using an enzyme-linked immunosorbent assay (ELISA). The optical density (OD) of each test was read using a 450 nm filter with a 620 nm reference filter using the ELISA plate reader.

The results were interpreted as positive, negative or indeterminate using the QFT-GIT analysis software developed by the company (QFT-GIT, Cellestis Ltd., Carnegie, Australia). If IFN-γ secretion in response to TB antigen was $\geqslant$0.35 IU/mL after subtracting the nil control, the sample was considered positive for QFT-GIT. If the value was <0.35 IU/mL, it was considered negative. If the negativity was associated with a poor PHA response (i.e., IFN-γ secretion in response to mitogen was <0.5 IU/mL), it was considered an indeterminate or invalid result for QFT-GIT. Subjects with IFN-γ secretion >8.0 IU/mL in the nil control samples were also considered indeterminate for QFT-GIT.