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Biological functions of *Mycobacterium tuberculosis*-specific CD4$^+$T cells were impaired by tuberculosis pleural fluid

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ABSTRACT

The local milieu at the site of *Mycobacterium tuberculosis* infection that modulates T-cell functions is the main battleground for the host to build counter-*M. tuberculosis* immune responses. CD4$^+$T cells are enriched predominantly in tuberculosis pleurisy and their roles are of considerable importance, but their nature and functional profiles linked with local condition remain elusive. Here we evaluated the functions of *M. tuberculosis*-specific CD4$^+$T cells from the major three profiles: cytokines production, cell activation and division. Results showed that pleural fluid (PF) from tuberculosis patients in a dose dependent manner inhibited the production of IFN-γ, IL-2 and TNF-α by *M. tuberculosis*-specific peptides or BCG activated CD4$^+$T cells from pleural fluid mononuclear cells (PFMCs). Surface staining for activation molecules indicated that PF could also blunt cell activation process. CFSE labeling showed that antigen-specific CD4$^+$T cell division ceased following co-incubation with PF. Pre- or post-treatment with PF could disturb subsequent cell activities. The strong inhibitory effect mediated by PF on CD4$^+$T cells was functional predominance. Moreover, application of inhibitors of IDO, adenosine, neutralizing Abs to IL-10 and TGF-β could partially reverse IFN-γ production. Our current research provided novel information that the functions of antigen-specific CD4$^+$T cells coincubated with PF were apparently impaired, which were distinct from cells that cultured in fresh culture medium. We concluded that CD4$^+$T cell mediated antigen-specific cellular immune response that occurred locally might be impaired by PF.

1. Introduction

Tuberculous pleurisy is one of the most common forms of extrapulmonary tuberculosis [1]. It is caused by severe delayed-type hypersensitivity in response to *Mycobacterium tuberculosis* infection showing clear signs of leakage into the pleural space [1,2]. The process involves the accumulation of fluid enriched in proteins and the migration of immune cells that are generally lymphocytic, with a predominance of T lymphocytes, particularly CD4$^+$T cells [2,3]. Characterization of the specific cellular immune response in the exudates may provide insight into the functional profiles of host immunity and relevant pathophysiological mechanisms.

Several studies reported that tuberculous pleural fluid is a Th1-dominant environment [2,4]. The type 1 cytokines IFN-γ and TNF-α predominate at the site of disease in patients with pleurisy [5,6]. Other studies have focused on the functions of *M. tuberculosis*-specific CD4$^+$T cells at the site of local infection. In vitro functional studies demonstrated that CD4$^+$T cells are typically of the Th1 type when stimulated with *M. tuberculosis* specific antigens, in that they are potent IFN-γ producer [7,8]. IFN-γ plays a central part in protect against tuberculosis [9–11]. Humans with genetic mutations of the IL-12-dependent IFN-γ production pathway display susceptibility to poorly pathogenic mycobacteria [12,13]. IFN-γ-producing antigen-specific CD4$^+$T cells have a significant role in establishing anti-*M. tuberculosis* specific cellular immune response and hence considered crucial for protection against tuberculosis.

Despite the concomitant heightened levels of Th1-type mediators and the robust Th1 immune response mediated by antigen-specific CD4$^+$T cells, host immune responses still cannot cause satisfactory control, let alone eradication, of tubercle bacilli. *M. tuberculosis* actively impairs the protective immune response as an immunologic escape strategy during infection [14–17]. Evidence suggested that mycobacteria could impair antigen presentation to reduce T-cell stimulation [17–19]. The functions of macrophages, such as phagosome maturation, as well as the generation of ROI and RNI, could also be inhibited by *M. tuberculosis* [20,21]. Although most evidence favors a dominant role for the Thl cytokines at the site of infection, other data suggest that a broad spectrum of

Abbreviations: PF, pleural fluid; PFMC, pleural fluid mononuclear cells; BAL, bronchoalveolar lavage; 1-MT, 1-methyl-tryptophan.

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cytokines may contribute to anti-*M. tuberculosis* immune defenses. Bronchoalveolar lavage (BAL) fluid from tuberculosis patients contained several immunosuppressive factors, resulting in decreased Th1 cytokines production, as well as inhibiting T-cell functional development [22]. IL-10 and TGF-β are two classically potential deactivators of the immune response in tuberculosis [14,22,23]. Moreover, the increasing levels of IDO observed in infectious and allergic diseases might inhibit Th1-type immune response and induce T-cell tolerance [24]. Clinical and experimental studies have demonstrated that the level of adenosine correlated with the magnitude of pulmonary inflammation [25]. Adenosine has been implicated in the pathogenesis of chronic lung diseases such as asthma [26] and chronic obstructive pulmonary disease [27].

However, most of the research focused on either local condition or in vitro analysis of the functions of cells at the site of infection, which seemed limited to evaluate host immune responses when separated cells from their local condition. The microenvironment is thought to be a critical determinant of the phenotype and activity of local cells. Whether the microenvironment is beneficial or harmful to local immune response correlates with the presentation and outcome in tuberculosis pleurisy. Our preliminary studies have demonstrated that PF could lead to the dysfunction of T cells isolated from normal donors and inhibition of the differentiation process and functions of Th1 cells [28]. But detailed functional analysis of antigen-specific $CD4^+$T cells linked with their local milieu has not been reported and required to be further analyzed. Only a better understanding of the interaction between immune cells and local condition will lead to novel and more effective immunotherapy.

In this study, we analyzed functions of antigen-specific $CD4^+$T cells isolated ex vivo directly from pleurisy of untreated tuberculosis patients and found that dysfunctional cellular immunity mediated by $CD4^+$T cells was directly correlated with the local milieu. Furthermore, application of 1-methyl-tryptophan (1-MT), caffeine, anti-IL-10 and anti-TGF-β neutralizing Abs into PF could partially rescue T-cell functions.

2. Materials and methods

2.1. Patients

A total of 31 patients with newly diagnosed tuberculosis pleurisy at the Chest Hospital of Guangzhou were enrolled in the study. The diagnosis was based on positive cultures for *M. tuberculosis* either in cultures of pleural fluid or pleural biopsy tissue, clinical and radiological features and a good response to anti-tuberculosis treatment. The participants comprised 20 men and 11 women, with a mean age of 40.7 years (range, 18–92 years). Patients with positive results of HIV, hepatitis B virus (HBV), hepatitis C virus (HCV), or with a history of autoimmune diseases were excluded from the study. All pleurisy samples were obtained during diagnostic thoracocentesis before the initiation of chemotherapy and taken after permission from the patients. Pleurisy samples were subjected to routine biochemical analysis, including tests for total protein (range, 32–70.1 g/L), glucose (range, 2.3–15.1 mmol/L), lactate dehydrogenase (range, 127–1262 U/L) and ADA (range, 5.9–60.3 U/L). The leukocyte counts in tuberculosis pleurisy ranged from 0.5×10^9 to 14.9×10^9 cells/L and the purity of lymphocytes ranged from 39% to 100%. All individuals involved in this study obtained adequate informed consent. The study was approved by the Medical School Review Board at Sun Yat-sen University, China.

2.2. mAbs and reagents

The following antibodies were used for cell surface and intracellular staining: allophycocyanin (APC)-labeled IFN-γ, phycoerythrin (PE)-labeled anti-CD3, anti-CD69-PE, anti-IL-2-PE, peridinin chlorophyll protein (PerCP)-labeled anti-CD4, fluorescein isothiocyanate (FITC)-labeled anti-CD25, PE-cy7-conjugated TNF-α and isotype-matched control antibodies were purchased from BD Biosciences Pharmingen (San Jose, CA, USA). Purified anti-CD28mAb was also purchased from BD Biosciences Pharmingen. BCG was purchased from Shanghai Institute of Biological Products (Shanghai, China). To fully reflect the functional profile of *M. tuberculosis*-specific $CD4^+$T cells, six highly immunogenic and largely HLA-DR-restricted peptides in China populations, four derived from ESAT-6 and two from the CFP-10 protein were selected. The synthetic peptides of 20 amino acids (aa) in length were obtained from Shenzhen Hanyu manufacture (Shenzhen, China). The sequences of the peptides are as follows: p1, ESAT-6 1–20 (MTEQQWNFAGIEAAASAIQG); p2, ESAT-6 31–50 (EGKQSLTKLAAAWGGSGSEA); p3, ESAT-6 61–80 (TATELNNALQNLARTISEAG); p4, ESAT-6 71–90 (NLARTISEAGQAMASTEGNV); p5, CFP-10 51–70 (AQAAVVRFQEAANKQKQELD); p6: CFP-10 71–90 (EISTNIRQAGVQYSRADEEQ).

2.3. Cell isolation and preparation of PF

Pleurisy samples from tuberculosis patients were centrifuged at 2000 rpm for 10 min at room temperature. Cell-free PF was separated and stored at −80 °C for further use. Cell pellets were suspended and then isolated by Ficoll-Hypaque (Tianjin HaoYang Biological Manufacture, Tianjin, China) density gradient centrifugation at 2000 rpm for 20 min. Pleural fluid mononuclear cells (PFMCs) were collected and washed twice in Hank's balanced salt solution. The cells were suspended at a concentration of 2×10^6/mL in complete RPMI 1640 medium (Gibco, Grand Island, NY, USA) containing 10% heat-inactivated fetal calf serum (Sijiqing, China), 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol. All were purchased from Gibco.

2.4. Enzyme-linked immunosorbent assay (ELISA)

The cell culture supernatants were harvested and assayed for the production of IFN-γ, IL-2 and TNF-α by ELISA according to the manufacturer's protocol (BD Pharmingen, San Diego, CA). The detection limits of the IL-2, TNF-α and IFN-γ assay kits was 7.8 pg/mL, 7.8 pg/mL and 9.375 pg/mL, respectively.

2.5. Cell surface and intracellular cytokine staining

The expression of surface markers was evaluated on PFMCs by staining 0.5×10^6 cells for 30 min at 4 °C with the respective mAbs in 100 μL volume of PBS buffer containing 0.1% BSA and 0.05% sodium azide. The cells were thereafter washed twice and fixed in 1% paraformaldehyde before acquisition. For the detection of intracellular cytokines, cells were incubated with peptides or BCG plus anti-CD28 for 12 h. Brefeldin A (Sigma–Aldrich, USA) was added to cells at a final concentration of 10 μg/mL. After stimulation, cells were washed twice with PBS, fixed with 4% paraformaldehyde and permeabilized in PBS buffer containing 0.1% saponin (Sigma), 0.1% BSA and 0.05% NaN_3 overnight at 4 °C. The cells were then stained with conjugated mAbs for intracellular cytokines (IFN-γ, IL-2 and TNF-α) for 30 min at 4 °C in dark. The stained cells were analyzed using a flow cytometer (FACSCalibur; BD Biosciences). Lymphocytes were gated on forward and side scatter profiles and analyzed using FlowJo software (Treestar, San Carlos, CA, USA).

2.6. CFSE labeling

PFMCs were resuspended in complete RPMI 1640 medium at 10^7cells/mL. Carboxyfluorescein diacetate succinimidyl ester