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IMMUNOLOGICAL ASPECTS

CD4$^+$FoxP3$^+$ T regulatory cells in drug-susceptible and multidrug-resistant tuberculosis

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SUMMARY

Regulatory T cells (T_{reg}) increase in active tuberculosis (TB). However, whether T_{reg}-mediated immune suppression affect the susceptibility to active TB or development of multidrug-resistant (MDR) TB is not yet clear. We compared circulatory T_{reg} frequencies in drug susceptible (DS) and MDR TB before and after anti-TB treatment.

Circulatory T_{reg} frequencies were measured in blood samples from 33 DS TB, 7 mycobacterial culture-positive active MDR TB, 16 stable MDR TB who had been culture negative for at least 6 months, and 14 healthy controls before and after treatment. T_{reg} frequency was measured by flow cytometry using cell-surface marker CD4 and intracellular marker FoxP3.

T_{reg} frequency was higher in DS TB and active MDR TB patients than in healthy controls ($p < 0.05$), with no significant difference between the former. T_{reg} frequency was higher in patients with sputum acid-fast bacilli smear-positive TB than in patients with smear-negative TB, but the increase did not correlate with the radiologic extent of TB or presence of a cavity. After successful treatment, T_{reg} decreased to control levels in DS TB and MDR TB patents.

The pattern of change, in which T_{reg} frequency increased during active infection and normalized to control levels after successful treatment, was similar in DS and MDR TB patients.

1. Introduction

Although tuberculosis (TB) mortality and incidence rates are decreasing worldwide, there were an estimated 8.7 million new cases of TB and 1.4 million people died from TB, including almost one million deaths in 2011 [1]. The development of TB is a consequence of *Mycobacterium tuberculosis* infection and the host immune response to this pathogen. T cell-mediated immunity is the main response mechanism against TB and is mediated mainly by a Th1-type immune response with high levels of IL-12 and interferon-gamma (IFN-γ) production by antigen-specific T cells [2]. Although this immune response is essential for eradication of the invading bacilli, an intense, unregulated immune reaction and the consequent excess inflammatory response can cause immunologic damage to host tissues. A balanced, adequate immune response is essential. There is continuing interest in the role of regulatory T cells (T_{reg}), a subset of CD4$^+$ T cells, in the regulation of effector T cells in TB [3,4]. T_{reg} can inhibit IFN-γ production by T cells through the production of IL-10 and transforming growth factor-beta (TGF-β), as well as through mechanisms that depend on cell-to-cell contact [5]. However, the exact role of T_{reg} in TB is not certain. Many previous studies reported T_{reg} cell expansion in the blood, lung, or other tissues of patients with active TB [3,6], suggesting that T_{reg}, by suppressing T cell immunity, are a susceptibility factor in the development of TB [7,8]. In contrast, other studies showed that T_{reg} were not elevated in TB [9,10]. In addition, recent work performed in non-human primates suggested the increase of T_{reg} represents a compensatory anti-inflammatory response rather than immune suppression [11].

Multidrug-resistant TB (MDR TB) is a special form of drug resistant TB that differs from drug-susceptible TB (DS TB) in clinical

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and radiological aspects [12]. Several studies showed impaired T cell immunity or dysregulated cytokine responses in MDR TB [13,14]. However, there are only a few reports about T_{reg} in MDR TB [15,16]. Most studies reported increased circulating T_{reg} in MDR TB, suggesting that the increased frequency of T_{reg} might play an important role in the development of MDR TB. We hypothesized that if T_{reg} play an important role in the susceptibility to active TB or in the development of MDR TB, the frequency of circulating T_{reg} may be increased even after treatment. The present study was conducted to evaluate whether the frequency of circulatory T_{reg} is higher in DS TB and MDR TB patients than in healthy controls even after successful treatment. We also investigated whether the frequency of circulatory T_{reg} differs between DS TB and MDR TB patients.

2. Materials and methods

2.1. Study design and subjects

Patients who were diagnosed with active pulmonary TB in one tertiary hospital between April 2008 and December 2009 were prospectively recruited. Pulmonary TB (PTB) was defined when *M. tuberculosis* was isolated from cultures of respiratory specimens (sputum, bronchial washing, bronchoalveolar lavage fluid). Patients with extrapulmonary TB were excluded. Patients with uncontrolled diabetes, malignancy, connective tissues disease, interstitial lung disease including idiopathic pulmonary fibrosis, recent or current immunosuppressive drug use, or HIV infection were excluded.

The study population was categorized into four groups: DS TB, active MDR TB, stable MDR TB, and normal healthy controls. DS TB was defined as susceptible to isoniazid and rifampicin according to the drug susceptibility test (DST) results. MDR TB was defined as resistant to at least isoniazid and rifampin. DSTs were performed at the Korean Institute of Tuberculosis, the Supranational TB Reference Laboratory, Seoul, South Korea. The proportion method and the pyrazinamidase test were used for DST at the Korean Institute of Tuberculosis [17]. The MDR TB group was subclassified as active MDR or stable MDR. The active MDR TB group included MDR TB patients with sputum samples that were culture-positive for *M. tuberculosis*, irrespective of treatment duration. The stable MDR TB group included patients who responded successfully to susceptible anti-TB drugs and/or surgery and maintained sputum culture conversion to negative for at least 6 months. Blood was sampled twice, at the time of diagnosis and after 6 months of anti-TB treatment. Fourteen healthy volunteers with no history of TB and no significant past medical history were included as a control group; blood sampling for T_{reg} frequency was performed once. The study protocol was approved by the institutional review board.

2.2. Blood sample preparation and flow cytometry measurement

We defined T_{reg} cells as $CD4^+FoxP3^+$ T cells. After informed consent was obtained, 8 mL of peripheral blood was drawn from all participants. Blood samples were collected in BD Vacutainer Sodium Citrate Cell Preparation Tubes (CPT). Peripheral mononuclear cells (PBMCs) were isolated from heparinized blood in CPTs following the manufacturer's instructions. Briefly, CPTs containing blood samples were centrifuged for 28 min at 1600 $\times$ *g* and 18 °C. The PBMC layer from the CPTs was removed and suspended in RPMI-1640 (Caisson Laboratories, Logan, UT) after washing with PBS. Cells were analyzed by flow cytometry using FITC conjugated anti-CD4 antibody (clone RPA-T4, eBioscience, San Diego, CA) for surface phenotyping.

Surface-stained cells were labeled for intracellular FoxP3 using an anti-FoxP3 staining kit (eBioscience, San Diego, CA) according to the manufacturer's recommendations. Cells were run in a BD FACSCalibur (BD Biosciences, San Jose, CA) and subsequently analyzed by BD CellQuest Pro software (BD Biosciences, San Jose, CA). After patients completed treatment, we collected blood samples from the patients again and analyzed the samples following the same process.

2.3. Disease severity according to chest CT criteria and sputum acid-fast bacilli (AFB) smear

Disease severity in TB patients was judged by radiologic and microbiologic criteria. All but 5 patients who were diagnosed with active pulmonary TB underwent a chest computerized tomography (CT) scan. Their chest CT images were evaluated by a board-certified pulmonary radiologist, who was blinded to the patients' clinical and laboratory records. The radiologic disease severity of each TB patient was classified according to the extent of parenchymal lesion: grade 1 was defined as TB involvement of less than 25% of the total lung parenchyma; grade 2 was 25–50%; grade 3 was 50–75%; and grade 4 was more than 75% of the lung parenchyma [18]. The presence or absence of a cavity in CT scans was also recorded. The microbiologic severity of TB was classified according to the results of a sputum AFB smear, and the AFB smear was categorized as trace to grade 4 according to the criteria of the American Thoracic Society (ATS)/Centers for Disease Control and Prevention (CDC) [19].

2.4. Statistical analysis

Data are presented as the mean $\pm$ standard deviation. The two-tailed unpaired Student's *t*-test was used to analyze continuous variables, and the Mann–Whitney *U* test was used if the assumption of normality was not met. Serial changes in T_{reg} frequencies were analyzed with a paired *t*-test; $p < 0.05$ was considered statistically significant. For categorical variables, intergroup comparisons were performed using the ANOVA F-test and χ^2 test. Fisher's exact test was used if the expected cell count for a 2×2 table was <5. Correlations were calculated by means of the Pearson's correlation test. All results were analyzed using SPSS v.18.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Number and clinical characteristics of the patients in each group

In this study, 14 healthy controls, 40 active TB patients with positive AFB sputum cultures, and 16 stable MDR TB patients were enrolled. From the drug susceptibility test results, 33 patients were diagnosed as DS TB and 7 patients as MDR TB among the 40 active TB patients. Table 1 summarizes the demographic and clinical characteristics of the participants. Age, body mass index, hemoglobin, and cholesterol level were not different between the groups. The serum albumin level was lower in the DS TB group (Table 1).

3.2. Comparison of the proportion of $CD4^+FoxP3^+$ T cells in patients with DS TB and MDR TB

The frequency of $CD4^+$ T cells was not different between the groups. Figure 1A presents representative fluorescence-activated cell sorter (FACS) plots, showing the frequency of peripheral $CD4^+FoxP3^+$ T cells in patients with TB at the time of diagnosis (left) and after the completion of treatment (right).