

Effects of Th17/Treg cell imbalance on HIV replication in patients with AIDS complicated with tuberculosis

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Abstract. The purpose of this study was to determine the effect of Th17/Treg cell imbalance on HIV replication in patients with AIDS complicated with tuberculosis (TB). We selected 32 patients with AIDS combined with TB infection in our hospital and 30 healthy individual as controls. The Th17/Treg ratio in peripheral blood lymphocytes was detected by flow cytometry. Compared with healthy subjects, Th17 cells first declined in HIV patients with TB, but gradually increased over the course of the disease. Treg showed an increasing trend in HIV patients with TB. The Th17/Treg ratio was significantly altered as the condition gradually deteriorated. ELISA showed that interleukin (IL)-17, IL-6 and IL-10 in patients with HIV complicated with TB were significantly lower than in healthy subjects. The imbalance of Th17/Treg cells can promote HIV virus replication in AIDS patients with TB infection, which can aggravate the condition.

Introduction

The human immunodeficiency virus (HIV) is the main cause of the acquired immune deficiency syndrome (AIDS) (1). HIV is an RNA retrovirus that infects immune cells and uses the host cell transcriptional and translational machineries to proliferate and, at the same time, undermine the health of the human host. By 2015 there were nearly 40 million AIDS patients worldwide (2), with an increasing trend and a shift towards the younger population. By the end of 2015, ~1 million people had AIDS in China, with the population below 45 years accounting for 86.4% of all patients. Therefore, strengthening the diagnosis and treatment of AIDS is critical to control this global epidemic. Clinical research shows that *Mycobacterium tuberculosis* (*M. tuberculosis*) is the main pathogen causing AIDS mortality (3). *M. tuberculosis* can

proliferate in AIDS patients and destruct the respiratory system and other organs. Studies have shown that the main clinical features of AIDS are CD4⁺ T cell reduction, excessive immune activation, and rapid increase in HIV (4). As the main target of HIV/AIDS, CD4⁺ T cells play an important role in the occurrence and development of AIDS. In recent years, research has shown that differentiation and maturation of T helper 17 (Th17) and T regulatory (Treg) cells from CD4⁺ T cells play key roles in resistance to amplification and invasion of the HIV virus.

Interleukin-17 (IL-17) promotes the expression of cytokines and plays an important role in the inflammatory immune response. Th17 cells are thought to have a significant inhibitory effect on the replication and amplification of the HIV. Research shows that Th17 in the late stage of infection and the early stage of inflammation can promote the expression of immune factors, link innate and adaptive immunity, and improve overall immunity (5). The main role of Treg is to inhibit the effect of T lymphocytes to prevent excessive autoimmune symptoms, thereby reducing the body resistance to external pathogens. Under normal circumstances, the Th17/Treg ratio is relatively stable, but inflammation and other immune conditions disturb this balance (6). For example, during inflammation, TGF- β can promote Treg cell production and promote the differentiation of Th17 and other cells. In the later stages of the inflammatory reaction, TGF- β can inhibit the immune response by inhibiting the proliferation of Treg cells, so the Th17/Treg balance is critical to maintain the normal immune function. There are few reports about the effect of Th17/Treg cell immune imbalance on HIV replication in patients with AIDS complicated with tuberculosis (TB) (7). Here, we studied for the first time the Th17/Treg cell immune imbalance effect on virus replication in AIDS patients with TB. Overall, we want to provide the theoretical and experimental basis to diagnose and treat patients with AIDS complicated with TB.

Materials and methods

Patient information. We recruited 32 patients treated for AIDS combined with TB infection in Xiangya Hospital from January 2011 to March 2015. Among them, 18 were male and 14 were female. The average age was 35.3 \pm 12.5 years. Thirty healthy individuals were also recruited as controls, including 18 males and 14 females, with an average age of 32.7 \pm 13.2 years. The study was approved by the Ethics

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Committee of Xiangya Hospital and informed consents were signed by the patients and/or guardians.

Inclusion criteria: i) HIV patients with TB; and ii) ages between 20 and 50 years. Exclusion criteria: i) Other inflammatory diseases; ii) suffering from other immune system diseases; and iii) ages below 20 years and over 50 years.

Reagents and instruments. Main reagents: Fetal bovine serum (FBS) and L-glutamic acid (both from Hyclone, Logan, UT, USA), PBS buffer solution (Alfa, Suzhou, China), monoclonal first antibody of IL-17, IL-6 and IL-10, and HRP-labeled polyclonal second antibody (both from Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Main instruments: Flow cytometry, biological safety cabinet and nitrogen canister (all from Thermo Fisher Scientific, Inc.), protein electrophoresis apparatus (Beijing 61 Instrument Factory, Beijing, China), low-temperature high-speed centrifuge (Hitachi, Tokyo, Japan), and cell counter (Thermo Fisher Scientific, Inc.).

Sample collection. We collected 5 ml of elbow vein blood through the EDTA anticoagulation (Applied Biosystems, Foster City, CA, USA) vacuum collected blood vessels, and carried on the examination in 12 h.

Cell count. We used flow cytometry to carry out technical statistics on different cells. The method was first to take the 15 μ l CD4-FITC monoclonal antibody (Acris Antibodies, Inc., San Diego, CA, USA) into the sample tube of the flow cytometry, and then add 50 μ l of each blood sample, gently mix evenly, and preservation avoiding light and store it at room temperature for 10 min. Then, the percentage of CD4⁺ T cells and the total cells were detected by flow cytometry (8).

Enzyme-linked immune response. Total protein samples were obtained and the expression of IL-17, IL-6 and IL-10 were determined by ELISA, and the specific operation was performed in accordance with the ELISA kit (Qiagen GmbH, Hilden, Germany) instructions. The standard curve was made according to the ELISA standard curve step and the protein samples were diluted in elution buffer with a ratio of 1:100. After diluting the samples with sterilized PBS (pH 7.2) with a ratio of 1:200, 100 μ l were added to a 96-well plate, and then 50 μ l were added to each well. After incubation at room temperature for 2 h, the TMB substrate was added, with the determination of absorbance at 495 nm. Then, the IL-17, IL-6 and IL-10 concentration in each sample were calculated according to the standard curve (9).

Treg cell expression level detection. Two flow type tubes were used as the test and control tubes. The liquid transfer gun was used to add 100 μ l cells at 10⁶/ml and 10 μ l CD4-PE/CD25-APC (Becton-Dickinson, San Jose, CA, USA), placed at 4°C for 30 min, 1 ml fixative solution (BD Biosciences, Franklin Lakes, NJ, USA) was added and mixed well, incubated at 4°C avoiding light for 30 min, and centrifuge washing with permeabilization buffer. Then, 1 μ l 1 rat serum was added with 4°C incubation for 15 min, 2.5 μ l PE anti-mouse/rat Foxp3 antibody (BD Biosciences) and 2.5 μ l PE rat IgG2a homotype were added with 4°C incubation

Table I. Baseline expression of Th17 cells in peripheral blood lymphocytes.

Groups	No. of cases	CD4 ⁺ (cells/ μ l)	Th17 cells (%)
Healthy population	30	912 \pm 254	4.24 \pm 0.36
HIV complicated with tuberculosis infection	32	145 \pm 38	1.21 \pm 0.13

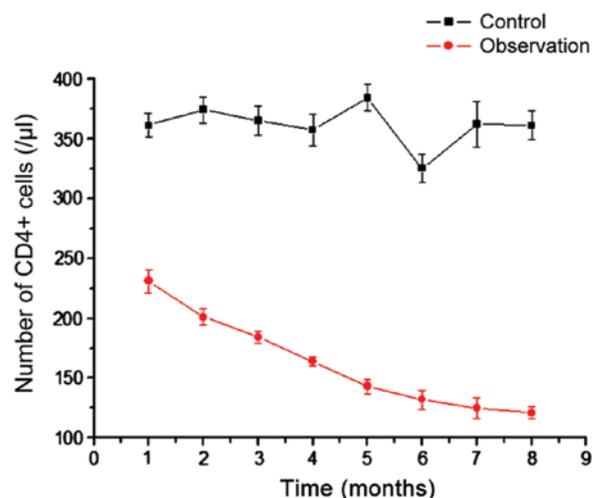


Figure 1. CD4⁺ T cell count in peripheral blood lymphocytes in the observation and the control groups.

in the dark for 30 min. After three washes, 500 μ l of Flow Cytometry Staining Buffer (BD Biosciences) was added. Finally, flow cytometry was used to analyze the expression level of Treg on CD4⁺ T lymphocytes (10).

Th7 cell expression detection. Peripheral venous blood (10 ml) was collected, peripheral mononuclear cells (PBMC) were separated by Ficoll density gradient centrifugation, PBMC density was adjusted to 2 \times 10⁶/ml, and resuspended in RPMI-1640 culture medium (Invitrogen Life Technologies, Carlsbad, CA, USA). PBMC suspension 2 \times 10⁶/ml was added into the 24-well culture plates, 1 ml/well, 20 ng/ml phorbol myristate acetate (PMA) and 1 μ g/ml ionomycin (both from Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), were cultivated for 2 h in the culture box (37°C, 5% CO₂), then the monensin 2 nmol/ml (Sigma-Aldrich; Merck KGaA), was added and cultured for 2 h. The cells were collected, washed 3 times in PBS and divided into control and test tubes. Each tube received 10 μ l CD4-FITC (Becton-Dickinson), then incubated for 15 min at room temperature, adding 100 μ l fixative with mixing at room temperature for 15 min, after PBS washing 3 times, 100 μ l of film developing agent was added, gently mixed evenly, and placed at room temperature in the dark for 5 min. In the experimental tube, we added 5 μ l of anti-human IL-17 mAb (BD Biosciences). Into the control tubes, 5 μ l of control antibody IgG-PE was added. The tubes were mixed at room temperature for 30 min in the dark. After

Table II. Baseline expression of Treg cells in peripheral blood lymphocytes.

Groups	No. of cases	CD4 ⁺ (cells/ μ l)	Treg cells (%)
Healthy population	30	912 \pm 254	4.24 \pm 0.36
HIV complicated with tuberculosis infection	32	1.832 \pm 316	9.14 \pm 1.31

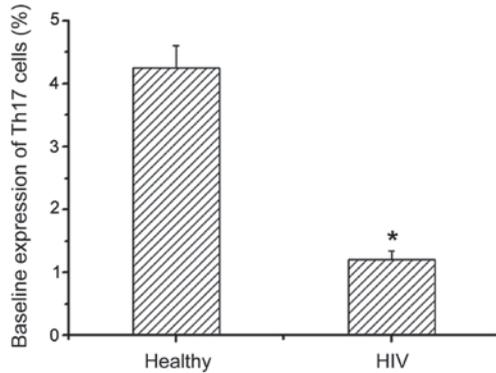


Figure 2. Baseline expressions of Th17 cells in peripheral blood lymphocytes in the observation and the control groups. *Statistically significant difference between groups at P<0.05.

3 PBS washes, the cells were detected by flow cytometry with 500 μ l PBS suspension cells, and the expression level of Th17 cells was analyzed by CD4⁺ T lymphocytes (10).

Viral load measurement. Viral loads in different blood samples were measured by COBAS kit (Roche Diagnostics, Indianapolis, IN, USA). The kit could be used for quantitative determination of viral load (fluorescent quantitative PCR), and the process was carried out following the instructions of the manufacturer.

Statistical analysis. The experimental data were processed with SPSS 20.0 (SPSS, Inc., Chicago, IL, USA) statistical software. The experimental data are expressed as mean \pm SD. Single factor analysis method was used to analyze the data between different groups. P<0.05 was considered to indicate a statistically significant difference.

Results

CD4⁺ T cell count in peripheral blood lymphocytes. We analyzed the number of CD4⁺ T cells in the control and observation groups (Fig. 1). Compared with CD4⁺ T cells in the blood of healthy people, the CD4⁺ T cells in the observation group were significantly reduced, and the difference was more significant with time (Fig. 1). This shows that AIDS with TB infection can reduce the number of CD4⁺ T cells. As the main immune cells infected by HIV, the gradual decrease in the number of CD4⁺ T cells can explain the increasing number of viral particles.

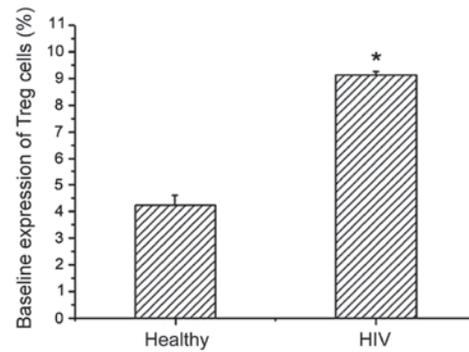


Figure 3. Baseline expressions of Treg cells in peripheral blood lymphocytes in the observation and control groups. *Statistically significant difference between groups at P<0.05.

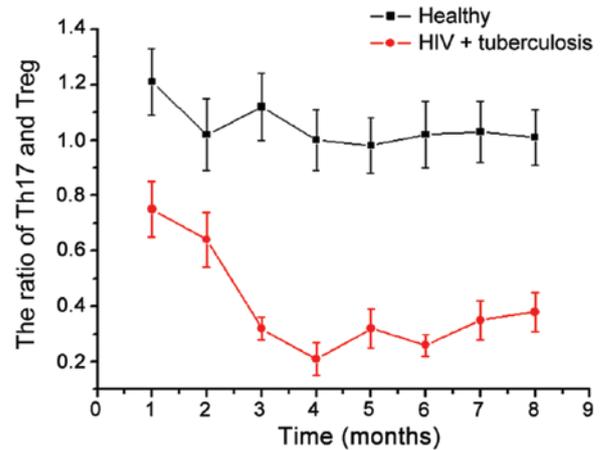


Figure 4. Changes of Th17/Treg ratio in peripheral blood lymphocytes in the observation and control groups.

Baseline expression of Th17 cells in peripheral blood lymphocytes. We next examined the expression levels of Th17 in healthy people and patients with AIDS complicated with TB infection by flow cytometry (Table I). Compared with healthy people, Th17 expression levels in AIDS patients with TB infection were significantly decreased, and there was a significant difference between them (Fig. 2). This suggests that the HIV virus can reduce the level of Th17 cells and thereby disrupt the immune system.

Expression of Treg in peripheral blood lymphocytes. Treg expression levels in healthy people and AIDS patients with TB infection were detected by flow cytometry (Table II). Compared with healthy people, the Treg level in AIDS patients with TB infection showed an increasing trend, and there was a significant difference between the two (Fig. 3). This shows that the immune system can enhance immunity by increasing the amount of Treg expression, thereby enhancing the ability to remove HIV.

Changes of Th17/Treg ratio in peripheral blood lymphocytes. We then examined the changes in Th17/Treg in healthy people and patients with HIV (Fig. 4). Compared with stable ratio of Th17/Treg in the healthy population, the Th17/Treg ratio in patients with AIDS complicated with TB infection had

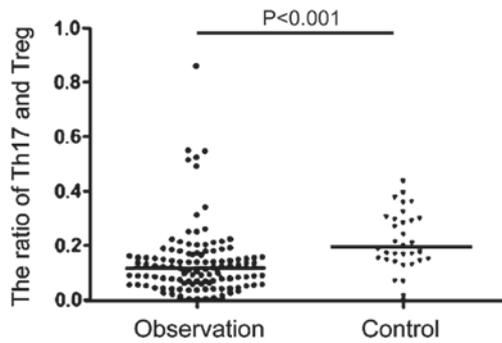


Figure 5. Correlation between Th17/Treg ratio and viral load in the observation and control groups.

greater variability. In the initial period of HIV infection, there was no significant change in the proportion of Th17/Treg, but over time, the Th17/Treg ratio showed a gradual downward trend. In the late stage, the Th17/Treg ratio showed a gradual upward trend; the Th17/Treg ratio strongly correlates with the stability of the collective immune system. With the Th17/Treg immune imbalance gradually increasing in the observation group, the difference in viral load was even more remarkable, which indicates that there is significant correlation between Th17/Treg immune imbalance and AIDS complicated with TB infection (Fig. 5).

Changes of IL-17, IL-6 and IL-10 in peripheral blood lymphocytes. Finally, we detected the changes of IL-17, IL-6 and IL-10 by ELISA (Fig. 6). Compared with the levels of IL-17, IL-6 and IL-10 in healthy subjects (3.02 ± 0.48 , 3.13 ± 0.76 , 2.89 ± 0.83 pg/ml), patients with AIDS complicated with TB infection showed significantly higher levels, and the differences were significant (Fig. 6). With the aggravation of the disease, IL expression decreased gradually, with the later stage showing significantly lower levels of IL-17, IL-6 and IL-10 than in the healthy population.

Discussion

TB infection is the main cause of death in AIDS patients and it also leads to many diseases following HIV infection (11). HIV can also promote the progression of TB in lung degeneration (12). Our study showed that co-infection of HIV with *M. tuberculosis* can accelerate the differentiation of immune cells. In this process, T cells induced by *M. tuberculosis* can activate virus replication to promote HIV transformation of non-infectious into infectious, and finally promote the transition to AIDS and the subsequent deterioration. In recent years, research has shown that HIV associated with TB infection decreases the number of CD4⁺ T cells and leads to the lack of related functions (13). The number of CD4⁺ T cells is often used as an indicator to measure the immune system function. CD4⁺ T cells can be divided into Th17 cells and Treg cells (14). The number of Th17 and Treg cells and the proportion of CD4⁺ T cells are related to the development of the disease. For instance, in patients with acute respiratory distress syndrome (15), the CD4⁺ T cells gradually decreased as the condition gradually deteriorated and the Th17/Treg ratio gradually decreased (16). Here, we found that the Th17/Treg ratio

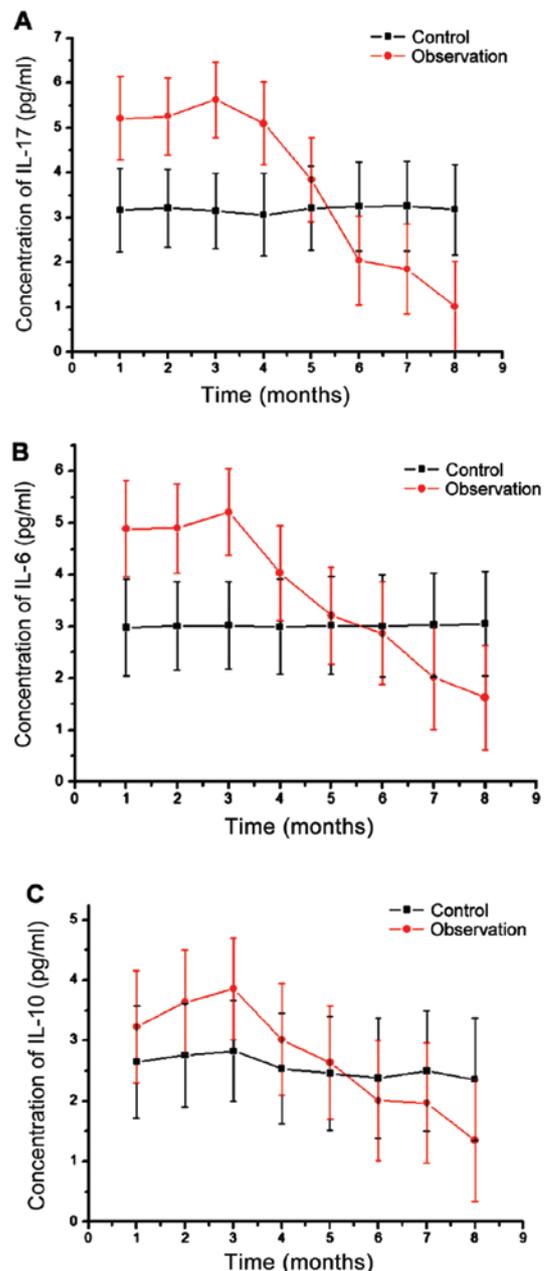


Figure 6. Changes of IL-17, IL-6 and IL-10 in peripheral blood lymphocytes in the observation and control groups. (A) Changes of IL-17 in peripheral blood lymphocytes over time. (B) Changes of IL-6 in peripheral blood lymphocytes over time. (C) Changes of IL-10 in peripheral blood lymphocytes over time. IL, interleukin.

also showed a tendency to decrease as AIDS/TB progressed. Study on Treg cells has shown that TGF- β 1 can promote the differentiation of CD4⁺ T cells into Treg cells, whereas Treg cells can block and inhibit viral replication, and thus play a therapeutic role in the treatment of disease (17). At the same time, Th17 cells are mainly involved in the inflammatory reaction (7), the abnormal increase of Th17 cells can induce autoimmune diseases (18,19). Here, we measured Treg, Th17, and CD4⁺ T cell changes and found that CD4⁺ T cells showed a gradual downward trend as AIDS with TB progressed. Also, the Treg/Th17 ratio was significantly reduced compared to the healthy group. This indicates that Treg/Th17 is imbalanced in patients with HIV combined with *M. tuberculosis*, and this

immune imbalance leads to increased HIV replication. HIV mainly destroys the immune system and immune cells, so it eventually leads to a decrease in the number of major immune cells, such as CD4⁺ T cells, showing that the imbalance of Treg/Th17 can largely lead to aggravation of AIDS with TB infection.

Competing interests

The authors declare that they have no competing interests.

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