Original Article
The inflammatory response and T lymphocytes with SLAM expression in macrophages of mice infected with mycobacterium tuberculosis

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Abstract: Objective: This study aims to analyze the levels of inflammatory cytokines, T lymphocytes and signaling lymphocytic activation molecule (SLAM) in a mouse model of mycobacterium tuberculosis (M.tb). Methods: A total of 40 mice were randomly and equally divided into the strain group receiving a tail vein injection of M.tb and the control group with normal saline. At 2 weeks (w), 4 w, 6 w and 8 w after infection, the body weight, survival rate, and bacteria count in the lungs of the mice were observed. Tumor necrosis factor-alpha (TNF-α), interleukin-12 (IL-12), IL-10, cluster of differentiation (CD)\textsuperscript{3}CD\textsuperscript{4} and CD\textsuperscript{3}CD\textsuperscript{8} cells were measured by flow cytometry. SLAM expression was detected by qPCR. Results: Compared with the normal saline control group, the body weight in the strain group was significantly decreased at 4 w and 8 w after infection, with statistical elevation of CD\textsuperscript{3}CD\textsuperscript{4} T lymphocytes and CD\textsuperscript{3}CD\textsuperscript{8} T lymphocytes at 2 w and 4 w (P<0.05). As time extended after 8 W, CD\textsuperscript{3}CD\textsuperscript{4} T lymphocytes began to decline whereas CD\textsuperscript{3}CD\textsuperscript{8} T lymphocytes began to be increased. At 2 weeks after infection, the expression level of TNF-α, IL-12p40 in the lung MΦ of mice in the strain group was markedly increased compared with that in the control group, (P<0.05). SLAM and IL-10 levels were rapidly increased at 2 w and 4 w, while they began to slightly decline at 6 w and 8 w (P<0.05). Conclusion: M.tb infection induces the increase of SLAM levels in the lungs of mice, while causing the alternation of TNF-α, IL-12, IL-10, CD\textsuperscript{3}CD\textsuperscript{4} and CD\textsuperscript{3}CD\textsuperscript{8} cells, which provided new insights for the diagnosis and evaluation of TB in clinical practice.

Keywords: Tuberculosis, mycobacterium tuberculosis, tumor necrosis factor-α, interleukin, signaling lymphocytic activation molecule

Introduction
Tuberculosis (TB), caused by intracellular pathogenic mycobacterium tuberculosis (M.tb), represents a serious infectious disease, second only to AIDS. According to WHO statistics, there are more than 8 million new cases of TB every year, in which more than 1.5 million people died of this disease [1]. Macrophages (MΦ) are not only the main effector cells killing M.tb but also the sites where M.tb survives and reproduces in the body [2], and the balance between the two functions determines the final outcome of M.tb infection. Hence, MΦ play a key role in the study on TB. M.tb can induce the body’s cellular immune responses, so it has been developed as a method for detecting M. tb-specific T lymphocytes [3-5]. According to previous studies, cytokines such as interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) [6, 7] exert crucial effects on M.tb infection. The signaling lymphocytic activation molecule (SLAM) plays vital roles in the activation of lymphocytes, the secretion of cytokines, and the differentiation of T helper 1 (Th1)/Th2 cells in the immune process. The indicators of the inflammatory response and T lymphocytes with SLAM expression in lung MΦ of mice infected with M.tb are rarely reported. This study aims to investigate the changes of the inflammatory response and T lymphocytes with SLAM expression in MΦ of mice infected with M.tb in order to provide basis for the diagnosis of TB in the future.

Materials and methods
Laboratory materials

Laboratory animals and strains: A total of 40 clean-grade Kunming mice aged 6-8 weeks (w)
old, with a body weight of 18-22 g, including 20 males and 20 females were provided by The First People's Hospital of Wenling. The international standard M.tb virulent H37Rv strain was purchased from China's National Institute for the Control of Pharmaceutical and Biological Products. The laboratory mice were divided into the M.tb virulent H37Rv strain model group (n=20) and the normal saline control group (n=20) according to a random number method. At 2 w, 4 w, 6 w and 8 w after infection, the mice were sacrificed for detection, and 5 mice were set at each time point. The study was approved by The First People's Hospital of Wenling.

Main instruments: LPS was purchased from Sigma, St. Louis, MO, USA, TRIzol reagent from Invitrogen, Carlsbad, CA, USA, the total micro ribonucleic acid (miRNA) extraction kit from (Cat no: AM1560, Ambion, Austin, TX, USA), DAB kit from Vector Laboratories, Burlingame, CA, and flow cytometer from Biovision, USA.

Establishment of the rat model of infection: M.tb colonies in a good growth state on the medium were added with a small amount of normal saline containing 0.05% Tween-20 for bacterial suspension. The amount of bacteria in each mouse was approximately \(2 \times 10^6\) cells/mL. In total 0.2 mL bacterial suspension was taken and injected into each mouse of the experimental group via the tail vein. The control group received the tail vein injection of an equal volume of normal saline. The infected mice were raised in IVC and with given ad libitum access to ordinary chow and water in the Biosafety level 3 laboratory.

Observation of the body weight and survival rate of mice: The body weight of each mouse was measured every day after injection, and the body weight (g) and survival rate of the mice at 2 w, 4 w, 6 w and 8 w after infection were recorded.

Proliferation of M.tb in the lung tissues: At 2 w, 4 w, 6 w and 8 w after infection, the mice in the control group and the strain group were executed, after which the lung tissues of the mice in each group were taken, ground, diluted, smeared on a medium plate and cultured for 5-10 days. Finally, the number of bacteria in each group was counted.

Expression of TNF-α, IL-12p40 and IL-10 in the lung Mφ of the two groups of mice

Sampling of cell suspensions culture was prepared and cells were washed. Fluorescence-labelled antibody was added for further incubation and fixation. Then the expression was detected via flow cytometry.

Numbers of CD3^+CD4^+ and CD3^+CD8^+ cells in the lungs of the two groups of mice

Mice at different time points were taken, executed via cervical dislocation method and dissected. The lung tissues were collected, digested, filtered, and lysed to prepare single-cell lung suspension. The proportions of CD3^+CD4^+ and CD3^+CD8^+ cells in the lungs at 2 w, 4 w, 6 w and 8 w after infection were measured via flow cytometry.

Determination of the level of SLAM in the lung tissues of the two groups of mice

The mice in the control group and the experimental group at 2 w, 4 w, 6 w and 8 w after infection were executed, respectively. After the mice were executed, the lung tissues of each group of mice were taken to be ground so as to extract the total RNA and identify the quality. An ultraviolet spectrophotometer was applied to calculate the ratio of the absorbance at 260 nm to that at 280 nm (A_{260}/A_{280}) of RNA (1.8-2.1) and detect its purity. Then electrophoresis was carried out to verify the integrity of RNA.

RNA isolation and qRT-PCR

Cells were mixed with Trizol (Invitrogen, Carlsbad, CA, USA) at ratio of 1:3. Then the solution was moved to an Ep tube and mixed with 200 μL chloroform. After being vibrated for 15 sec, the upper aqueous phase was mixed with 500 μL isopropanol for 10 min. After being centrifuged at 12,000 g for 10 min, the precipitate was mixed with 1 mL ethanol (75%). After being centrifuged at 4°C and 7,500 g for 5 min, the supernatant was removed and the tube was dried for 10 min. Next, the RNA was dissolved in DEPC water and purity was determined by ultraviolet spectrophotometer. After RNA concentration was determined, reverse transcription (TOYOBO, Osaka, Japan) was performed in a 20 μL system including 4 μL 25 mM MgCl_2, 2 μL reverse transcription 10× Buffer, 2
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μL 10 mM dNTP mixture, 0.5 μL ribonuclease inhibitor, 0.5 μL AMV reverse transcriptase, 1 μL random primers, 1 μg total RNA and nuclease-free water to a final volume of 20 μL under 42°C for 15 min and 85°C denature (Promega, Fitchburg, WI, USA). Real-time PCR was then performed by using SYBR Premix Ex Taq GC kit (Takara, Kusatsu, Shiga, Japan) (7.5 μL 2× pre-mix, 10 mM forward and reverse primers, dH2O to a final volume of 15 μL) in the following condition: 94°C denature for 30 sec, followed by 40 cycles each containing 94°C denature for 5 sec, and 60°C annealing for 30 sec with LightCycler 480 (Roche Diagnostics, Indianapolis, IN, US). The experiment was repeated 3 times to reduce errors and biases, and β-actin was selected as an internal reference. The built-in software of the real-time detector (ABI-7500, New York, New York State, USA) was adopted to analyze data, and the relative expression level of SLAM messenger RNA (mRNA) was expressed as 2−ΔΔCt.

Statistical methods

Statistical Product and Service Solutions (SPSS) 20.0 software (SPSS Inc., Chicago, IL, USA) was adopted for analysis. Data were recorded using (X ± s), and the t-test or χ2 test was used for comparison between groups. P<0.05 represented that the difference was statistically significant. The figures were made with GraphPad software (La Jolla, CA, USA).

Results

Observation of the body weight and survival rate of mice

The mice in the control group were in good status and active with an enormous appetite and glossy hair, while the mice in the strain group were in bad spirits, somnolent and inactive with a poor appetite and tousy hair. The average body weight of each group of mice was compared weekly. The body weights (g) of mice in the strain group at 0 w, 2 w, 4 w, 6 w and 8 w after infection were (18.74±2.05), (20.07±1.86), (24.97±1.90), (23.46±0.99) and (21.37±1.24), respectively, while those in the normal saline control group were (18.61±1.93), (22.94±2.38), (29.78±1.95), (31.67±1.38) and (34.56±2.28), respectively. There were no significant differences in the body weights between the two groups before infection and at 2 w after infection (P>0.05). After 4 w of infection, as time went on, the body weight in the infection group exhibited a downtrend compared with that in the normal saline control group (P<0.05). At 8 w after infection, the difference was the most obvious (P<0.05). The above results indicate that M.tb infection poses severe damage to the body of mice (Figure 1).

Observation of the proliferation of M.tb in the lung tissues

Six mice in each group were executed at 2 w, 4 w, 6 w and 8 w, and the bacterial colony counts in the lung tissues of the mice were compared. Detection revealed that in the strain group, colony counts (log10 CFU) at 2 w, 4 w, 6 w and 8 w after infection were (6.18±0.54), (8.25±0.39), (7.58±0.42) and (5.83±0.46), respectively, but there were no colonies detected in the control group. The differences at each time point were statistically significant (P<0.05). At 8 w after infection, the difference was the most obvious (P<0.05). The above results indicate that M.tb infection poses severe damage to the body of mice (Figure 1).

Comparisons of CD3+CD4+ and CD3+CD8+ cells in the lungs of mice between the strain group and the control group

Detection via flow cytometry revealed that the proportions of CD3+CD4+ cells in the lungs of mice in the strain group at 2 w, 4 w, 6 w and 8 w after infection were (60.15±2.34), (64.21±
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Figure 2. Comparisons of colony counts in the lung tissues of mice between the two groups at each time point. Note: *P<0.05 vs. control group.

1.98), (61.97±2.03) and (58.46±2.19), respectively, while those in the control group were (57.13±2.46), (56.92±1.84), (56.34±2.17) and (55.96±2.23), respectively. At 2 w after infection, the expression level of CD3^+CD4^+ T lymphocytes in the lungs of mice in the strain group was remarkably up-regulated compared with that in the control group (P<0.05), and the increase reached the peak at 4 w (P<0.01). As time passed, the expression level of CD3^+CD4^+ T lymphocytes began to decline. However, at 8 w after infection, this expression level remained high in the strain group (Figure 3A).

The expression level of CD3^+CD8^+ T lymphocytes in the strain group was down-regulated at 2 w, and reached the lowest at 4 w (P<0.01). As time passed, this expression level began to be increased, and at 8 w, there was no significant difference between the strain group and the control group (P>0.05) (Figure 3B).

Comparisons of various inflammatory indexes at different time points of infection between the two groups of mice

Flow cytometry was adopted to measure the changes in the expression of cytokines, TNF-α, IL-10 and IL-12p40 in the lung Mφ of mice. At 2 weeks after infection, the expression level of TNF-α in the strain group was markedly increased compared with that in the control group (P<0.05), and the expression level remained high at 8 w (P<0.05) (Figure 4A).

Similar to the expression of TNF-α, the expression level of IL-12p40 in the lung Mφ in the strain group began to be elevated at 2 w after infection, and the difference in comparison with that in the control group was statistically significant (P<0.05). The level reached the peak at 4 w (P<0.05), and slightly declined at 6 w and 8 w (Figure 4B).

Different from the expression levels of TNF-α and IL-12p40, the expression level of IL-10 in the strain group reached the peak at 4 w after infection, but showed a downtrend at the later stage (P>0.05) (Figure 4C).

Determination of the level of SLAM in the lung tissues of the two groups of mice

Lung tissues at different time points were collected, and fluorescence qPCR was applied to detect the changes in the expression of SLAM in the lung tissues of the mice. The results manifested that the level of SLAM in the strain group was rapidly increased at 2 w after infection and peaked at 4 w, showing significant differences compared with those in the normal saline control group (P<0.05). The level began to slightly decline from 6 w and 8 w (Figure 5).

Discussion

M.tb infects and replicates in cells, and Mφ functions as a double-edged sword that it not only plays roles in the survival and proliferation of pathogens, but also exerts effects on the inhibition and elimination of pathogens [8, 9]. The infection of M.tb will stimulate the secretion of a variety of crucial cytokines by Mφ, including such inflammatory cytokines as TNF-α and IL-12 as well as such inhibitory cytokines as IL-10 and TGF-β [10, 11], in which TNF-α and IL-10 exert crucial effects on M.tb infection [12]. It was found in this study that at 2 weeks after infection, the expression of TNF-α in the lung Mφ of mice in the strain group was rapidly increased compared with that in the control group, and this level remained high at 8 w. The level of IL-12p40 in the lung Mφ in the strain group was elevated at 2 w after infection, reached the peak at 4 w (P<0.05), and slightly declined at 6 w and 8 w. Different from the expression levels of TNF-α and IL-12p40, the expression level of IL-10 in the strain group reached the peak at 4 w after infection, but...
showed a notable downturn at the later stage. Consistent with our result, Mφ infected with M.tb can stimulate an increase in the expression of TNF-α, which is conducive to the production of granuloma in the body to prevent infection and diffusion and induce the body to initiate protective immunity [13]. IL-12 induces the body to initiate protective Th1 response, so as to resist against the infection by pathogens [14, 15]. IL-10 inhibits antigen presentation by decreasing the expression of Mφ co-stimulatory cytokines, and mainly suppresses the immunization of cells infected with M.tb [16]. Other studies have reported that IL-10 benefits the survival and reproduction of pathogens in the lungs of TB patients and reduces the production of TNF-α, IL-12, IFN-γ and other protective cytokines [17].

The balance between CD4+ T cells and CD8+ T cells is of great significance in maintaining cellular immunity in the body. In this experiment, detection results of flow cytometry manifested that compared with those in the control group, the expression of CD3+CD4+ T lymphocytes in the lungs of mice in the strain group at 2 w after infection was remarkably up-regulated and peaked at 4 w. At 2 w after infection, the expres-
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It was also found from this study that detection of the level of SLAM in the lung tissues of mice via fluorescence qPCR revealed that the expression level of SLAM in the strain group was rapidly increased at 2 w after infection and peaked at 4 w, showing significant differences compared with those in the normal saline control group. The level began to slightly decline at 6 w and 8 w. SLAM family receptors not only exist on the surface of a variety of mature hematopoietic cells as adhesion molecules, but also are involved in innate immune and adaptive immune responses to a certain extent [20-23]. For instance, after binding to ligands, SLAM family receptors can regulate the proliferation of T lymphocytes, the cytotoxic effect and production of cytokines, and regulate the cytolytic effect of NK cells, secretion of cytokines, inhibition of MHC-independent cells, activation of B cells, humoral immune memory, killing of neutrophils and Mφ, and aggregation of platelets as well. The regulatory role of SLAM-related receptors in immune cells was first evidenced by the mediation and activation effects of anti-SLAM antibodies on the proliferation of activated CD4+ T cells and the secretion of IFN-γ, which is a process independent of T-cell receptors (TCRs) [24]. Nevertheless, SLAM can only mediate the proliferation of CD4+ T cells activated by TCRs and the secretion of IFN-γ [25]. The cross linking of SLAM can enhance the cytotoxic effect of cells mediated by TCRs and the release of granules on activated human CD8+ T cells [26]. Similarly, the overexpression of SLAM on CD8+ T cells enhances the secretion of IFN-γ and the cytotoxic effect of cells [27]. However, its specific mechanism needs further experimental research.

Conclusion

M.t.b infection results in the up regulation of SLAM in the lungs of mice, while causes the alternation of TNF-α, IL-12, IL-10, CD3+CD4+ and CD3+CD8+ cells. Our data has provided basis for the further detection of TB in clinical practice.

Disclosure of conflict of interest

None.

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