Role of IL-10 and TNF-α during \textit{Mycobacterium tuberculosis} infection in murine alveolar macrophages

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**ABSTRACT.** \textit{Mycobacterium tuberculosis} (\textit{Mtb}) is known to be responsible for tuberculosis (TB), but the pathogenesis of this disease and the host defense mechanisms involved are, for the most part, poorly understood. In this study, we divided 30 male C57BL/6 mice into control and infection groups, and following injection with physiological saline or \textit{Mtb}, respectively, euthanized five mice from each group on days 1, 3, and 7. TNF-α and IL-10 levels were measured by enzyme-linked immunosorbent assay and flow cytometry, with the latter also being performed to assess apoptosis rates. Protein expression of STAT3 and its phosphorylated form (p-STAT3) was analyzed by western blotting. After \textit{Mtb} infection, TNF-α and IL-10 levels, alveolar macrophage apoptosis, and STAT3 and p-STAT3 expression increased significantly on days 1, 3, and 7 (P < 0.05), with maximum values on day 3. Furthermore, the Pearson correlation test showed that production of the cytokines TNF-α and IL-10 correlated strongly with expression...
of STAT3 and p-STAT3 proteins (P < 0.05). Taken together, our results suggest that the STAT3 signaling pathway might play a key role in the regulation of cell proliferation and alveolar macrophage apoptosis in response to Mtb. This provides a theoretical mechanism behind TB pathogenesis and host defense against Mtb, and contributes towards development of an effective treatment.

**Keywords:** Mycobacterium tuberculosis; Mouse alveolar macrophages; TNF-α; IL-10; STAT3

**INTRODUCTION**

*Mycobacterium tuberculosis* (Mtb), one of the earliest described bacterial pathogens, can infect and cause morbidity and mortality in humans, as well as several veterinary species. Although the literature describing this organism is vast, pulmonary tuberculosis (TB) caused by Mtb remains one of the most common fatal diseases, affecting millions of people worldwide every year (Oxlade et al., 2009; Russell, 2011; Cooper and Torrado, 2012). Recently, alveolar macrophages (AMs) from patients with a history of TB have been shown to have a reduced ability to control Mtb infection *in vitro* compared to those harvested from “non-TB” subjects (Persson et al., 2013). Further evidence has demonstrated that macrophages play an important role in Mtb infection. During early infection stages, Mtb is phagocytosed by these cells, whose initial innate response can affect intracellular bacterial survival and proliferation. The potential molecular mechanism behind macrophage control of Mtb infection may involve the production of reactive nitrogen (RNI) and oxygen intermediates (ROI) and changes in phagolysosomal pH inducing apoptosis, among other factors (Cooper and Torrado, 2012; Sakamoto, 2012). In addition, the first cells to be infected release pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-10, IL-12, and IL-18, which exert various effects on macrophage functions and T cell responses (Cooper et al., 2011; Redford et al., 2011). For example, macrophages can be activated by cytokines such as IFN-γ and TNF-α. In response to infection, such molecules may reduce mycobacterial proliferation by stimulation of apoptosis and ROI/RNI production. In contrast, immunosuppressive cytokines such as IL-10 and transforming growth factor (TGF)-β can inhibit macrophage functions, resulting in enhanced intracellular bacterial growth and reduction of nitric oxide production (Redford et al., 2011). Thus, the balance between TNF-α and IL-10 produced by macrophages against Mtb is thought to modulate induction of apoptosis during infection (Cooper et al., 2011; Sakamoto, 2012). As a transcription factor, signal transducer and activator of transcription 3 (STAT3) participates in many cytokine-related signaling pathways regulated by the suppressor of cytokine signaling family, in various cells and organs. Recent data regarding the activation and function of STAT3 in the lung during the acute inflammatory response suggest that these molecules are potential targets in the regulation of pulmonary inflammation (Gao and Ward, 2007; Tang et al., 2011). However, whether STAT3 can increase cytokine production or induce AM apoptosis in countering Mtb remains unknown.

In this study, we used an Mtb-infected mouse model to investigate the effect of this microorganism on production of TNF-α and IL-10 in AMs, and assess the relationship between the STAT3 pathway and cytokine production. This provides a theoretical basis for understanding the immune process during Mtb infection and developing a relevant clinical treatment.
MATERIAL AND METHODS

Animals

All the procedures in the present study were performed in accordance with the animal research guidelines of the Cangzhou Central Hospital Ethics Committee. Thirty male C57BL/6 mice, aged 6-8 weeks and weighing 18-24 g, were purchased from the Shanghai Laboratory Animal Center (Shanghai, China). The mice were divided randomly into control and infection groups. Animals in the latter (N = 15) were given an intravenous injection of 400 µL Mtb at 1.0 x 10⁷ cells/mL in the tail (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China). Control mice (N = 15) received an equal amount of physiological saline. Five mice from each group were euthanized on days 1, 3, and 7 following injection, to collect AMs.

Cell culture

Alveolar spaces were washed with 1 mL sterile phosphate-buffered saline (PBS), and the collected fluid centrifuged for 5 min at 350 g to harvest alveolar cells. These cells were re-suspended in complete Dulbecco’s modified Eagle’s medium containing 10% normal human serum, 100 U/mL penicillin, and 100 µg/mL streptomycin, and cultured for 6 h at 37°C in 5% CO₂. Attached AMs were collected by digestion with PBS containing 0.25% trypsin and centrifuged for 5 min at 350 g.

Measurement of TNF-α and IL-10 cytokine production

AM culture supernatant was collected to determine TNF-α and IL-10 levels by commercially available enzyme-linked immunosorbent assay (ELISA; Shanghai Weixiong Bio-Tech Inc., Shanghai, China) and measuring optical density at 450 nm. AMs were incubated with 10 µg/mL monensin (Sigma, St. Louis, MO, USA) for 6 h, centrifuged at 350 g for 5 min, and re-suspended at 1 x 10⁶ cells per tube in PBS binding buffer. After penetration of the fixation solution, AMs were blocked with rat serum for 4 h, then incubated in the dark for 30 min with 0.2 µg phycoerythrin-conjugated anti-mouse TNF-α, 1 µg fluorescein isothiocyanate (FITC)-conjugated anti-mouse IL-10, or an antibody of equivalent isotype (Beijing Dakewe Biotechnology Co., Beijing, China). The cells were then washed twice with PBS and centrifuged at 350 g for 5 min. The supernatant was re-suspended in 100 µL PBS and used for flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA) with FlowJo version 7.6.2 (Tree Star Inc., San Carlos, CA, USA).

Flow cytometric analysis of AM apoptosis

To investigate the cytotoxic effect of Mtb, the early apoptosis of AMs was assessed using an annexin V-FITC/propidium iodide (PI) kit (Beijing Solarbio Co., Beijing, China). AMs were re-suspended in binding buffer at 1 x 10⁶ cells per tube, and incubated with 5 µL annexin V-FITC in the dark for 15 min at 4°C. Subsequently, cells were exposed to 10 µL PI dye in the dark for 5 min at 4°C, and detected within 1 h by flow cytometry using FlowJo version 7.6.2. Each sample was tested in triplicate.
Western blotting analysis of STAT3 and phosphorylated STAT3 (p-STAT3)

Expression of STAT3 and p-STAT3 proteins was analyzed by western blotting. Cell lysate was used to extract total protein from both alveolar cells and AMs, and supernatants were collected and quantified with a bicinchoninic acid protein assay kit (Bio-Rad Laboratories Ltd., Hercules, CA, USA). Equal amounts of protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). Following blocking with 5% nonfat milk in Tris-buffered saline-Tween 20, membranes were incubated for 2 h at room temperature with primary antibodies against β-actin (diluted 1:500), STAT3 (1:500), and p-STAT3 (1:200). After being washed, membranes were exposed to horseradish peroxidase-conjugated secondary goat anti-rabbit STAT3 and goat anti-mouse p-STAT3 polyclonal antibodies (1:2000; Beijing Zhongshan Biotechnology Co., Beijing, China) for 1 h at room temperature. Enhanced chemiluminescence reagent (Millipore) was used for protein visualization, and STAT3 and p-STAT3 levels were normalized to those of β-actin.

Statistical analysis

Statistical analyses were performed with the SPSS 19.0 software package (SPSS Inc., Chicago, IL, USA). Data are reported as means ± SD. The paired t-test was performed to assess differences between the control and infected groups, and the Pearson correlation test was employed to analyze the relationship between cytokine production and protein expression. The threshold for statistical significance was set at P < 0.05.

RESULTS

TNF-α and IL-10 levels increased following Mtb infection of alveolar cells

After Mtb infection, alveolar cell culture supernatant was collected to measure TNF-α and IL-10 concentration by ELISA. As shown in Figure 1A and B, Mtb infection caused a significant increase in TNF-α levels on days 1 (37.32 ± 4.28 vs 14.65 ± 3.22 pg/mL, P < 0.05), 3 (62.44 ± 5.02 vs 13.86 ± 3.51 pg/mL, P < 0.05), and 7 (42.34 ± 6.85 vs 14.52 ± 2.65 pg/mL, P < 0.05), with the highest value recorded on day 3. Similarly, after Mtb infection, IL-10 production increased dramatically on days 1 (73.86 ± 2.66 vs 48.62 ± 1.05 pg/mL, P < 0.05), 3 (104.72 ± 3.87 vs 40.28 ± 0.83 pg/mL, P < 0.05), and 7 (85.16 ± 4.57 vs 41.89 ± 0.27 pg/mL, P < 0.05), peaking on day 3.

TNF-α and IL-10 levels increased following Mtb infection of AMs

Alveolar cell culture supernatant was harvested after Mtb infection to test TNF-α and IL-10 levels by flow cytometry. As depicted in Figure 2A and B, Mtb infection resulted in a significant increase in TNF-α on days 1 (2.83 vs 0.27, P < 0.05), 3 (5.63 vs 0.30, P < 0.05), and 7 (3.45 vs 0.08, P < 0.05), with the most elevated expression observed on day 3. Similarly, in response to Mtb infection, production of IL-10 increased markedly on days 1 (2.36 vs 0.08, P < 0.05), 3 (4.97 vs 0.51, P < 0.05), and 7 (3.32 vs 0.88, P < 0.05), culminating on day 3 (Figure 2C and D).
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Figure 1. A. TNF-α and B. IL-10 levels increased following Mycobacterium tuberculosis infection in alveolar cells, as measured by enzyme-linked immunosorbent assay (N = 5). *P < 0.05.

Figure 2. A. and B. TNF-α and C. and D. IL-10 levels increased following Mycobacterium tuberculosis infection in alveolar cells, as measured by flow cytometry (N = 5). *P < 0.05.

Early apoptosis of AMs increased after Mtb infection

The early apoptosis of AMs infected with Mtb was assessed by the annexin V-FITC/PI double-staining method. As shown in Figure 3A and B, the AM apoptosis rate increased significantly on days 1 (3.25 vs 0.87, P < 0.05), 3 (8.84 vs 0.79, P < 0.05), and 7 (5.63 vs 0.66, P < 0.05) following Mtb infection. The maximum rate was measured on day 3, consistent
with the production of TNF-α and IL-10. This indicates that levels of these cytokines in AMs directly induce the early apoptosis of these cells.

**Figure 3. A. and B.** Early apoptosis rate of alveolar macrophages infected with *Mycobacterium tuberculosis*, as measured by flow cytometry (N = 5). PI = propidium iodide. *P < 0.05; **P < 0.01.

**STAT3 and p-STAT3 protein expression increased in AMs after *Mtbc* infection**

Expression of STAT3 and p-STAT3 proteins was analyzed by western blotting. As demonstrated in Figure 4A and B, *Mtbc* infection induced a significant increase in STAT3 expression on days 1 (0.68 ± 0.27 vs 0.14 ± 0.08, P < 0.05), 3 (0.78 ± 0.04 vs 0.09 ± 0.11, P < 0.05), and 7 (0.55 ± 0.13 vs 0.17 ± 0.06, P < 0.05), with the highest levels on day 3. Likewise, p-STAT3 production rose substantially after exposure to *Mtbc* on days 1 (0.43 ± 0.16 vs 0.06 ± 0.10, P < 0.05), 3 (0.67 ± 0.05 vs 0.18 ± 0.12, P < 0.05), and 7 (0.34 ± 0.16 vs 0.12 ± 0.08, P < 0.05), peaking on day 3. Furthermore, the Pearson correlation test showed production of both TNF-α and IL-10 cytokines to be highly correlated with expression of STAT3 and p-STAT3 proteins (Table 1; P < 0.05). This suggests that *Mtbc* infection results in heightened TNF-α and IL-10 levels, subsequently inducing AM apoptosis by activation of the STAT3 pathway.
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DISCUSSION

Although Mtb causes pulmonary tuberculosis, one of the most common deadly diseases, the pathogenic and host defense mechanisms operating during Mtb infection are poorly understood (Oxlade et al., 2009; Russell, 2011). Macrophages are critical in both permitting the survival of Mtb and in the innate and adaptive immune responses of the host (Russell, 2011; Sakamoto, 2012). They promote the T cell activation and recruitment crucial for containing Mtb within granulomas in the lung (Oxlade et al., 2009; Macdonald et al., 2012; Sakamoto, 2012). Macrophages can also produce cytokines and lipid mediators, which have been firmly established as key players in adaptive immunity, as both effectors and regulators, during Mtb infection (Oxlade et al., 2009; Russell, 2011).

TNF-α and IL-10 perform important functions in response to Mtb infection, which have been well documented in both mouse models and infected humans. Rapid Mtb growth and the death of the host have been clearly demonstrated following TNF-α blockade in vitro, in vivo, and in knockout mouse models, revealing the protective role of this cytokine in Mtb infection (Bruns et al., 2009; Jozefowski et al., 2011; Bourigault et al., 2013; Roh et al., 2013). Conversely, IL-10 has been found at increased levels in tuberculosis patients, and elevated innate production of IL-10 is associated with greater susceptibility to this disease (Cooper et al., 2011; Sakamoto, 2012). IL-10 reduces the protective response to Mtb in animal models.
only when strongly induced, e.g., in CBA mice, in which IL-10 is produced by phagocytes within the lung lesion, reducing expression of TNF-α and IL-12 (Turner et al., 2002). IL-10 has also recently been shown to block phagosome maturation in human AMs (O’Leary et al., 2011). The importance of IL-10 and TNF-α in macrophages during Mtb infection may be double edged. The balance between these cytokines produced by macrophages in response to Mtb or the network of which they are a part may modulate the induction of apoptosis during infection (Mootoo et al., 2009; Meng et al., 2015; Prabhavathi et al., 2015). In the present study, Mtb infection caused significant increases in TNF-α and IL-10 levels in both alveolar cells and AMs on days 1, 3, and 7 (P < 0.05), with the highest values measured on day 3. The apoptosis rate of AMs exposed to Mtb varied in a similar manner, indicating that TNF-α and IL-10 levels in these cells may directly induce their early apoptosis.

STAT3 is a member of the cytoplasmic protein family, and is activated by a large number of extracellular stimuli, including IL-6, IL-10, various other cytokines, granulocyte-colony stimulating factor, and epidermal growth factor (Stepkowski et al., 2008; Braun et al., 2013; Hutchins et al., 2013). Activation of STAT3 signaling has been implicated in the regulation of cell proliferation, differentiation, transformation, and apoptosis (Yang et al., 2007; Braun et al., 2013). Recently, the role of this pathway in lipopolysaccharide (LPS)-induced TNF-α and IL-10 production has been investigated in cultured murine AMs (MH-S cells; Meng et al., 2014). In MH-S cell culture supernatant, TNF-α and p-STAT3 concentration decreases following addition of the p38 inhibitor SB203580, suggesting a relationship between cytokine production and STAT3 (Meng et al., 2014). Knocking down STAT3 expression in AMs significantly reduces levels of TNF-α, IL-6, macrophage inflammatory protein (MIP)-1α, MIP-1β, and C5a upon immunoglobulin G immune complex stimulation. Over-expression of a dominant-negative STAT3 protein in primary human monocyte-derived macrophages prevents induction of IL-10 mRNA by IL-10, but not by LPS, indicating a close correlation between this cytokine and STAT3 (Staples et al., 2007). However, whether STAT3 can increase cytokine production or induce AM apoptosis to combat Mtb infection remains unknown. In the present study, STAT3 and p-STAT3 expression was significantly increased on days 1, 3, and 7 (P < 0.05) after exposure to Mtb, with maximum values on day 3. Furthermore, the Pearson correlation test showed that levels of both TNF-α and IL-10 were highly correlated with expression of STAT3 and p-STAT3 proteins (P < 0.05). This indicates that Mtb infection may result in elevated production of these cytokines, and subsequently induce AM apoptosis by activating the STAT3 pathway.

In conclusion, Mtb infection can induce early apoptosis and heightened production of the cytokines TNF-α and IL-10, as well as increased expression of STAT3 and p-STAT3 proteins in AMs. Taking these observations together, we postulate that STAT3 signaling may play a key role in regulating AM proliferation and apoptosis in the control of Mtb. This provides a theoretical basis to improve our understanding of Mtb pathogenesis and corresponding host defense mechanisms, and contributes to the development of clinical treatment options.

Conflicts of interest

The authors declare no conflict of interest.
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