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Tim-3 signaling pathway as a novel negative mediator in lipopolysaccharide-induced endotoxic shock

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ABSTRACT

Sepsis is a complex clinical condition caused by a dysregulated immune response to an infection. However, the mechanism by which our immune system controls this amplified inflammation is largely unknown. In this study, we investigated whether Tim-3 pathway could serve as a negative mediator in lipopolysaccharide (LPS)-induced endotoxic shock. Our results showed that Tim-3 was expressed on $CD4^+$ T cells, $CD8^+$ T cells, and NK cells, and was significantly increased in the peritoneal cavity of septic mice. Tim-3 acted as a marker of immune exhaustion and Tim-3-positive T cells and NK cells had a lower interferon (IFN)-γ production. Furthermore, blockade of Tim-3 pathway significantly accelerated mortality in septic mice, while activation of this pathway prolonged survival time. *In vitro* administration of Tim-3 blocking antibody restored the release of IFN-γ from splenocytes and decreased splenocyte apoptosis, and increased levels of IFN-γ and tumor necrosis factor (TNF)-α were also detected in septic mice at 24 h post *in vivo* administration of the antibody. In contrast, activation of Tim-3 pathway prevented cell proliferation. Thus, Tim-3 signaling pathway acts as a novel negative mediator in LPS-induced endotoxic shock and could be a potential therapeutic target for the treatment of sepsis.

1. Introduction

Sepsis is a complex clinical condition caused by a dysregulated immune response to an infection, resulting in multi-organ failure with a fatal outcome [1]. A recent epidemiological study found that the incidence of sepsis was approximately 3.0 cases per 1000 population and that the overall mortality was approximately 30–50% or even greater in patients with a more severe syndrome [2]. The development of this severe syndrome was mostly caused by lipopolysaccharide (LPS) of Gram-negative bacteria. Current concepts in understanding this dramatic clinical condition suggest that an overwhelming innate inflammatory response associated with early release of factors such as interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α is involved [1]. For achieving appropriate immune response, our immune system needs some negative regulators to limit this over-amplified inflammatory response. However, the mechanism by which our immune system controls this amplified inflammation is largely unknown.

T-cell immunoglobulin and mucin domain (Tim)-3 is a Th1-specific type 1 membrane protein that is not expressed on the surface of naive T cells, but emerges on the surface of fully differentiated $CD4^+$ Th1 cells. Galectin-9 has been identified as a ligand for Tim-3. Binding of galectin-9 to Tim-3 causes an inhibitory signal, resulting in apoptosis of Th1 cells and down-regulation of Th1-type immunity [3]. Previous studies have shown that Tim-3 pathway plays important roles in regulating the process of autoimmune diseases such as multiple sclerosis [4] and type 1 diabetes [5]. Besides autoimmune diseases, Tim-3 pathway has been shown to be involved with other disease models such as transplant tolerance [6–8], antitumor immunity [9–11], and limiting antiviral immune responses [12–14]. Moreover, a recent study has shown that Tim-3 pathway promotes homeostasis of sepsis by negatively regulating the TLR response [15]. However, the effect of the Tim-3 pathway on LPS-induced endotoxic shock is still largely unknown.

Tim-3 signaling pathway has been shown as a negative mediator in many chronic inflammatory diseases such as multiple sclerosis [16], colitis [17], and tumor cell dissemination [10,18]. Tim-3 also negatively regulates antiviral responses, and $Tim\text{-}3^+$ $CD8^+$ T cells exhibit proliferative senescence phenotypes and

Abbreviations: LPS, lipopolysaccharide; IL, interleukin; TNF, tumor necrosis factor; Tim-3, the T-cell immunoglobulin and mucin domain-3; IFN, interferon; PHA, phytohemagglutinin.

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decreased cytokine production upon antigen challenge [12]. Furthermore, previous studies have also shown that Tim-3 serves divergent functions in NK cells. At least two studies have shown that Tim-3 is an inducible human NK cell receptor that enhances interferon (IFN)-γ production in response to galectin-9 [19,20]. Thus, current concepts suggest that Tim-3 may be able to influence a range of inflammatory conditions through promoting or terminating Th1 immunity, by virtue of differential expression on innate versus adaptive immune cells [21]. In this study, we addressed the specific role of Tim-3 pathway in an acute inflammatory model and found that Tim-3 pathway acted as a novel negative mediator in LPS-induced endotoxic shock.

2. Methods

2.1. Mice

BALB/c mice were maintained separately at the Animal Facility of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, under controlled conditions (specific-pathogen free, 22 °C, 55% humidity, and 12 h light/dark cycle). Male mice (6–8 weeks of age, weight 20–25 g) were selected for the study. All experimental procedures on animals used in this study were performed according to the protocol approved by the Institutional Animal Care and Use Committee at the Tongji Medical College.

2.2. LPS-induced endotoxemia model

For LPS-mediated endotoxemia model, mice were injected i.p. with 15 mg/kg of LPS (*E. coli* O55:B5, Sigma–Aldrich) dissolved in PBS. The viability of mice was examined every 4 h. The general conditions and mortality were recorded for up to 6 d after injection to ensure that no additional late deaths occurred. In some experiments, 100 μg Tim-3 blocking antibody (clone 8B.2C12; eBioScience, San Diego, CA), 100 μg recombinant mouse galectin-9 (clone 3535-GA; R&D Systems, Minneapolis, MN) or 100 μg IFN-γ neutralizing antibody (clone H22; eBioScience, San Diego, CA) was administered i.p. 30 min after LPS injection. PBS containing mouse IgG was used as a control.

2.3. Flow cytometric analysis

Experimental mice were sacrificed 0, 4, 12, and 24 h after LPS injection. Peritoneal cells were obtained by lavage of the peritoneal cavity with 10 ml PBS. Cell surface staining was performed on freshly prepared peritoneal cells or splenocytes after erythrocyte lysis. All cells were pre-incubated for 10 min at room temperature in 10% fetal calf serum (FCS) to block Fc receptors and non-specific binding. After washing three times, antibodies against the following antigens were added to the cell suspensions: anti-CD3-FITC (11-0031), anti-CD4-FITC (11-0042), anti-CD8-FITC (11-0081), anti-CD8-APC (53-6.7), anti-CD11b-FITC (11-0112), anti-F4/80-FITC (11-4801), anti-CD44-FITC (IM7), anti-NKp46-APC (29A1.4), and anti-Tim-3-PE (12–5870) (eBioscience). Isotype controls with irrelevant specificities were included as negative controls. All of these cell suspensions were incubated for 30 min on ice. After two washes, the pellets were resuspended in 500 μl cold staining buffer and followed by analysis with FACScan flow cytometer (Becton Dickinson). Data analysis was performed using FlowJo version 7.6.1 software (TreeStar).

2.4. Intracellular cytokine staining

For intracellular staining, the cells were collected after surface staining. After washings, the cells were fixed and permeabilized with Fixation and Permeabilization Buffer (BD Pharmingen) and stained with anti-IFN-γ-FITC or anti-IFN-γ-PE (XMG1.2, eBioscience) for 30 min in dark. After washings, the cells suspended in cold staining buffer were analyzed by flow cytometry.

2.5. Cell apoptosis analysis

Experimental mice were sacrificed at 24 h after LPS injection, and peritoneal cells were harvested. $CD4^+$ T cells were purified from the spleens of septic mice by using anti-CD4 microbeads (Miltenyi Biotech). Purified $CD4^+$ T cells were stimulated with 10 μg/mL phytohemagglutinin (PHA) in the presence of 1 μg/mL Tim-3 blocking antibody or PBS control. Then, the Annexin V-APC Apoptosis Detection Kit (17-8007, eBioscience) was used for detection of apoptosis in peritoneal cells or stimulated $CD4^+$ T cells. All procedures were performed according to the manufacturer's instructions.

2.6. Cell proliferation analysis

Purified splenic $CD4^+$ T cells derived from LPS-sensitized mice were labeled with CFSE (Invitrogen Life Technologies) at 2.5 μM in PBS for 15 min at 37 °C. The unconjugated CFSE was eliminated by washing the cells with RPMI 1640 containing 10% FCS. The labeled cells were resuspended in culture medium and co-cultured with 5 μg/mL anti-CD3 and 2 μg/mL anti-CD28 in 24-well flat-bottom plates. To measure the effect of galectin-9 on cell proliferation, mouse IgG control or different concentrations (5 μg/ml, 10 μg/ml) of recombinant galectin-9 were added to the medium. After 5 days of culture, cells from 24-well plates were harvested and analyzed for CFSE intensities.

2.7. ELISA for determination of IFN-γ, TNF-α, and IL-4

Experimental mice were sacrificed 0, 4, 12, and 24 h post LPS injection. Blood samples were collected by heart puncture. Peritoneal fluids were collected by lavage of the peritoneal cavity with 5 mL of PBS. Cytokine productions in serum and peritoneal lavage fluid were measured by a standard sandwich cytokine ELISA procedure according to the instructions of mouse IFN-γ, TNF-α, and IL-4 detection kits (R&D Systems, Minneapolis, MN).

2.8. Statistical analysis

Statistical analysis of survival curves was performed by Kaplan–Meier test and log-rank test. Comparisons were made using two-tailed Student's *t*-test. GraphPad Prism (version 5.01, GraphPad) software was used for statistical analysis. Statistical significance was determined as $p < 0.05$.

3. Results

3.1. Tim-3 is present on $CD4^+$ T cells, $CD8^+$ T cells, and NK cells after LPS infection

Tim-3 signaling mediates many disease models. However, whether Tim-3 is involved in the development of LPS-induced endotoxic shock is uncertain. In this study, the expression of Tim-3 on peritoneal lavage cells was investigated at 24 h after LPS injection. We found that Tim-3 was not expressed on neutrophils and macrophages (Fig. 1A), although neutrophils and macrophages were the most abundant cells in the peritoneal cavity. However, Tim-3 was expressed on $CD4^+$ T cells, $CD8^+$ T cells (Fig. 1A) and NK cells (Fig. 1B). The percentages of Tim-3-positive $CD4^+$ T cells, $CD8^+$ T cells and NK cells significantly increased up to 6% (6.44 ± 0.28%), 24% (23.63 ± 3.48%), and 28% (27.81 ± 1.49%)