Molecular Immunology 90 (2017) 27–32

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Activating transcription factor 3 protects mice against pseudomonas aeruginosa-induced acute lung injury by interacting with lipopolysaccharide binding protein

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ARTICLE INFO

Keywords:
Activating transcription factor 3
Acute lung injury
Pseudomonas aeruginosa
LPS-binding protein

ABSTRACT

Excessive inflammatory response is critical event in the pathogenesis of acute lung injury (ALI). Previous study has shown that activating transcription factor 3 (ATF3) plays a role in downregulate inflammatory responses including ventilation-induced ALI. We hypothesized that ATF3 have a protective effect in ALI induced by pseudomonas aeruginosa. PA was intra-tracheally administrated to ATF3 knock-out (KO) mice to establish ALI model. Inflammatory factors, BALF protein, lung wet to dry ratio, lung injury score and mortality were determined. The activation of NF-κB was detected by western blot and Co-immunoprecipitation (Co-ip) was used to determinate the binding of ATF3 to LBP. Peritoneal macrophages were isolated from ATF3 KO mice and stimulated by PA. PA increased the expression of ATF3 in the lung tissues in ATF3 wild type (WT) mice. ATF3 deficiency significantly increased the concentration of TNFα, IL-6 and IL-1β in the supernatant of peritoneal macrophages, lung tissue and BALF after PA stimulation and also enhanced the activity of NF-κB. ATF3 deficiency also enhanced the BALF protein concentration and increased the lung wet to dry ratio. The lung injury score and mortality were higher in ATF3 KO mice treated with PA. Moreover, ATF3 was demonstrated to bind to LBP These finding suggest ATF3 protect mice against ALI induced by PA partly due to the binding to LBP.

1. Introduction

The acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) is a common cause of acute respiratory failure and is often caused by pneumonia and sepsis (Watkins et al., 2012). Advances have been made in understanding the pathophysiology of ARDS, but therapies remain limited (Bein et al., 2016).

Activation of neutrophils can occur by recognition of structural features of microbes, including the bacterial cell wall component lipopolysaccharide (LPS) (Fujishima, 2014). LPS binds to a specific binding protein, known as LPS-binding Protein (LBP) (Imai et al., 2008). The binding of the LPS/LBP complex to CD14 and toll like receptors (TLRs) results in the transfer of a signal across the cell membrane and activation of NF-κB which results in cytokine production (Su et al., 1995). However, LBP also was shown to detoxify LPS by transferring LPS into high density lipoprotein (HDL).

ATF3, as an HDL-inducible target gene (Moore and Fisher, 2014), inhibited the expression of chemokine CCL4 in mouse macrophages. The ROS-mediated super induction of ATF3 caused high susceptibility to bacterial and fungal infections through the suppression of IL-6 (Hoetzenecker et al., 2011). Recent study showed that ATF3 Protects Pulmonary Resident Cells from Acute and Ventilator-Induced Lung Injury (Akram et al., 2010). These studies indicated that ATF3 acts as a brake on inflammatory response (De Nardo et al., 2014; Boespflug et al., 2014). We hypothesized that ATF3 may protects mice against pseudomonas aeruginosa-induced acute lung injury partly through binding to LBP.

Abbreviations: ALI, acute lung injury; ATF3, activating transcription factor 3; PA, pseudomonas aeruginosa; KO, knockout mice; WT, wild type; BALF, bronchoalveolar lavage fluid; IL, interleukin; TNF, αtumor necrosis factor α; NFκa, nuclear factor kappa B; Co-ip, Co-immunoprecipitation; TLR4, toll-like receptor 4; LPS, lipopolysaccharide; LBP, lipopolysaccharide binding protein; ROS, reactive oxygen species; HDL, high density lipoprotein; qRT-PCR, quantitative real time PCR; ELISA, enzyme linked immunosorbent assay; μL, microliter

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http://dx.doi.org/10.1016/j.molimm.2017.06.037
Received 10 April 2017; Received in revised form 14 June 2017; Accepted 19 June 2017

2. Materials and methods

2.1. Animals and PA-induced ALI model

The ATF3 knockout (KO) mice were developed on a C57BL/6 genetic background and were a gift from Dr. Tsonwin Hai (Ohio State University, Columbus, USA). Animals were maintained in 22 °C room with a 12 h light/dark cycle and received chow and drinking water ad libitum. The animal protocol was approved by the Animal Care Committee of Renji Hospital, School of Medicine, Shanghai Jiaotong University. To establish ALI mouse model, PA 50 μl (1.5×10^8 CFU/ml) dissolved in saline was administered intra-tracheally to mice after lightly anesthetized by ether inhalation. The mice were randomly divided into four groups: (1) wild type (WT, C57BL/6), (2) ATF3 KO, (3) WT + PA and (4) PA + ATF3 KO (KO + PA) mice. The mice were killed at 6 h after PA challenge (n = 5 in each group). Blood and lung samples were harvested aseptically for subsequent experiments. Right lung was divided into two parts, one of which was snap-frozen and stored in liquid nitrogen for RNA extraction, protein isolation, and tissue homogenate. The other part was fixed immediately for histological evaluation. Left lung was used to determine the wet to dry ratio. To evaluate the effect of ATF3 on PA-induced ALI, another 30 mice were divided into two equal groups: (1) WT + PA and (2) KO + PA mouse group and given the same treatments as above. For survival experiments, the survival time was recorded daily for 7 days.

2.2. Cell preparation and stimulation

Peritoneal macrophages were isolated from ATF3 KO and WT mice according to Pineda-Torra et al. (Pineda-Torra et al., 2015). Cells (1×10^6 cells) were grown in DMEM medium containing 10% fetal bovine serum, and maintained at 37 °C in an incubator containing 5% CO2 and treated with PA (1×10^5 CFU in a final volume of 250 μl in HBSS) or the same volume saline for 6 h. The cells were divided into four groups: (1) WT, (2) KO, (3) WT + PA and (4) KO + PA.

2.3. Quantitative real-time PCR

The lungs were dissected and immediately frozen in liquid nitrogen for RT-PCR. Total RNA was isolated using TRIzol and RT-PCR was performed according to the manufacturer's instructions (Takara, Dalian, China). Single-stranded cDNA was amplified by 35 cycles of PCR. The PCR profile was as follows: 30 s denaturation at 94 °C; 45 s annealing at 60 °C; and 60 s extension at 72 °C. Quantitative real-time PCR (qRT-PCR) was performed using iTaq Universal SYBR Green Supermix according to the manufacturer's instructions (Takara, Dalian, China). The reaction conditions were as follows: denaturation at 95 °C for 30 s; followed by 40 cycles at 95 °C for 5 s and 60 °C for 30 s. Data analysis was completed using the comparative ΔΔCT method with the mRNA levels of interest normalized to the levels of GAPDH. The primers are included in Table 1.

2.4. Western blotting

The proteins were loaded in SDS-PAGE and after electrophoresis, the proteins were transferred to PVDF membranes using a steady flow model (200 mA) over 60 min. After being blocked with non-fat milk in a TBST buffer containing 0.05% Tween-20, the membranes were incubated with primary antibody at 4 °C overnight. The next day, the primary antibody was detected by chemo-luminescence using an appropriate peroxidase-conjugated secondary antibody. The intensity of each band was analyzed by the Image J software (NIH, USA).

2.5. Cytokine measurements

TNFα, IL-6 and IL-1β secretion in BALF and peritoneal macrophages were quantified using a murine ELISA detection kit (Enzyme-linked Biotechnology, Shanghai, China) according to the manufacture's protocol.

2.6. Bronchoalveolar lavage (BAL) protein measurements

BAL was collected from mice followed by rapid centrifugation and collection of supernatants. The total protein was measured by BCA Protein Assay Kit (Beyotime, China) according to the manufacture's protocol.

2.7. Determination of the lung wet to dry ratio

The lung tissues were collected and measured with the wet weight and recorded. Then the lungs were desiccated in a 70 °C oven and the tissue dry weight was repeatedly measured until the weight was unchanged over 48–72 h to allow the calculation of the lung wet/dry ratio.

2.8. Histopathology

The lung were fixed in 4% paraformaldehyde, the tissues were embedded in paraffin and processed for standard hematoxylin-eosin staining and examination by a light microscope (Leica Microsystems).

2.9. Determination of the interaction of ATF3 with LBP by Co-immunoprecipitation

Lungs from ATF3 KO and WT mice treated with PA were cut off and lysed with cold protein lysis buffer (50 mM HEPES, pH 7.4, 250 mM NaCl, 0.1% NP-40, 2 mM EDTA, 10% glycerol, protease inhibitors cocktail and 1 mM PMSF) for 30 min and. were centrifuged at 12000 rpm for 10 min at 4 °C. Supernatant of tissue lysates were transferred into new tubes and mixed with primary antibodies and incubated at 4 °C with gentle agitation overnight. Then protein A/G beads were added to capture antigen antibody complex, which subsequently proceeded heat denaturing and immunoblotting.

2.10. Statistical analysis

All experimental results were analyzed using SPSS 13.0. The data are presented as the mean ± SEM. The one-way analysis of variance (ANOVA) was used to analyze the differences of the groups. The survival curves were determined by the Kaplan-Meier method and the log-

Table 1
Primer for PCR.

Primer	Forward	Reverse
ATF3	5′-CAGAGTGCCTGCAGAAAGAG-3′	5′- GGTGCAGGTTGAGCATGTAT-3′
GAPDH	5′-AACTTTGGCATTGTGGAAGG-3′	5′-ACACATTGGGGGTAGGAACA-3′
TNF-α	5′-TCTCTTCAAGGGACAAGGCT-3′	5′GGCAGAGAGGAGGTTGACTT-3′
IL-6	5′-CTCTGGGAAATCGTGGAAAT-3′	5′-CCAGTTTGGTAGCATCCATC-3′
IL-1β	5′-TCAGGCAGGCAGTATCACTC-3′	5′-CATGAGTCACAGGGATGGG-3′