Toxicology Letters 269 (2017) 41–46

Contents lists available at ScienceDirect

Toxicology Letters

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

MyD88 gene knockout attenuates paraquat-induced acute lung injury

Haitao Shen[a], Na Wu[b], Yu Wang[a], Feng Guo[a], Lianghong Chen[a], Zhigang Zhang[a], Dong Jia[a], Min Zhao[a,*]

[a] Department of Emergency Medicine, Shengjing Hospital of China Medical University, Shenyang 110004, People's Republic of China
[b] Department of Endocrinology, Shengjing Hospital of China Medical University, Shenyang 110004, People's Republic of China

HIGHLIGHTS

- Paraquat poisoning activates the MyD88-dependent pathway causing ALI.
- *MyD88* gene knockout attenuates paraquat-induced ALI.
- *MyD88* gene knockout reduces the levels of serum inflammatory cytokines in paraquat poisoning.

ARTICLE INFO

Article history:
Received 23 November 2016
Received in revised form 17 January 2017
Accepted 21 January 2017
Available online 25 January 2017

Keywords:
Paraquat
Acute lung injury
MyD88
Proinflammatory cytokines

ABSTRACT

Objective: This study investigated the role of myeloid differentiation factor 88 (MyD88) in paraquat-induced acute lung injury (ALI).

Methods: C57BL mice were divided into the control group, paraquat group, *MyD88* knockout (KO) group, and *MyD88* KO plus paraquat group. At 48 h after paraquat poisoning, serum and lung tissues were collected. ELISA was employed to detect tumor necrosis factor-α (TNF-α) and interleukine-1β (IL-1β) contents in serum. Lung tissues were processed for hematoxylin-eosin staining, followed by histological scoring. PCR was performed to detect the mRNA expression of MyD88, TNF-α, and IL-1β in the lungs. Immunofluorescence staining was done to evaluate the expression and distribution of MyD88 and nuclear factor κB (NF-κB) in the lungs. Western blotting was conducted to detect the protein level of toll-like receptor (TLR) 4, TLR9, MyD88, and NF-κB in the lungs.

Results: Paraquat poisoning significantly increased serum inflammatory cytokines, as well as MyD88, TLR4, TLR9, and NF-κB, and resulted in ALI. After *MyD88* KO, the levels of inflammatory cytokines and NF-κB decreased markedly, and ALI was also attenuated although TLR4 and TLR9 expression continued at an elevated level.

Conclusion: MyD88 mediates paraquat-induced ALI, and *MyD88* gene knockout may attenuate paraquat-induced ALI and reduce the production of proinflammatory cytokines.

1. Introduction

Paraquat is a quick-acting organic heterocyclic herbicide that can kill green plant tissue on contact and has been widely used worldwide. Although lots of measures have been taken to treat paraquat poisoning, the mortality is as high as 70%–80%. Death is usually caused by multiple organ failure based on acute lung injury (ALI) for the lungs are target organs of paraquat. Alveolar epithelial cells may actively transport paraquat into cells, which elevates the paraquat concentration in lung tissues 10–90 times than in plasma. Thus, paraquat causes more severe lung injury than other organs, so ALI become the major cause of death in cases of paraquat poisoning (Kan et al., 2014). Although a large number of studies have investigated paraquat poisoning, the molecular mechanism underlying the pathogenesis of paraquat-induced ALI is still poorly understood.

Myeloid differentiation factor 88 (MyD88) is a key adaptor in the Toll-like receptor/interleukin-1 (TLR/IL-1) receptor (TIR) pathway, and TLRs are important components in the innate immune system that are responsible for the recognition of

* Corresponding author at: Department of Emergency Medicine, Shengjing Hospital of China Medical University, 36 Sanhao Street, Shenyang 110004, People's Republic of China.
E-mail address: zhaom@sj-hospital.org (M. Zhao).

http://dx.doi.org/10.1016/j.toxlet.2017.01.015
0378-4274/

pathogen-associated molecular patterns and the subsequent activation of the innate immune system. IL-1 is a representative inflammatory cytokine in the acquired immune response and plays a key role in the specific immune response and inflammatory reaction (Dunne and O'Neill, 2003). In recent years, evidence has shown that TIR is able to facilitate the progression of ALI (Wang et al., 2016; Madathilparambil et al., 2016; Ferreira et al., 2016). At least two TIR pathways have been identified: the MyD88-dependent and the MyD88-independent pathways. All TIR-related signal transduction depends on MyD88 except for the TLR3-mediated one (Rhee, 2011). MyD88-dependent TIR activation may induce the activation of the downstream molecule nuclear factor κB (NF-κB) via MyD88, leading to the release of inflammatory cytokines that promote lung inflammation and cause ALI (Herold et al., 2013; Hemmi et al., 2000). Inhibition of TLR activation attenuates ALI and suppresses the production of inflammatory cytokines (Ma et al., 2009; Nadigel et al., 2011). The severity of paraquat-induced ALI is positively related to the IL-1 level (Erroi et al., 1992), indicating that interventions targeting the TIR/MyD88 pathway may relieve ALI after paraquat poisoning, and the key adaptor in the MyD88-dependent TIR pathway may become a new target in the therapy of paraquat-induced ALI. Thus, we undertook this study to investigate lung injury and inflammatory cytokines in paraquat-poisoned mice with the *MyD88* gene knockout in order to elucidate the role of MyD88 in the pathogenesis of paraquat-induced ALI.

2. Materials and methods

2.1. Materials

Paraquat (Sigma-Aldrich, St. Louis, MO, USA), tumor necrosis factor-α (TNF-α) and interleukine-1β (IL-1β) ELISA kits (R&D Systems, Minneapolis, MN, USA), antibodies against TLR4, TLR9, MyD88, and NF-κB p-p65 (Novus, Littleton, CO, USA), and other reagents (analytically pure grade) were used in the present study.

2.2. Animals

Specific-pathogen-free, 8-week-old wild-type and *MyD88*$^{-/-}$ C57BL/6J mice were purchased from the Animal Institute of Nanjing University and were housed in the Central Animal Center of the Affiliated Shengjing Hospital of China Medical University at a constant temperature (20–25 °C) and a relative humidity of 40%–70% with a 12 h/12 h light/dark cycle. Animals were given ad libitum access to water and food. This study was approved by the Ethics Committee of the Affiliated Shengjing Hospital of China Medical University (2015PS302 K).

2.3. Animal model and grouping

Wild-type mice were randomly divided into the control group (A group, n = 8) and paraquat group (B group, n = 8); *MyD88*$^{-/-}$ mice were randomly divided into the *MyD88* KO plus paraquat group (C group, n = 6) and *MyD88* KO control group (D group, n = 6). In Groups B and C, mice were intraperitoneally injected with 30 mg/kg paraquat (10 mg/mL paraquat in saline). In Group A and Group D, mice were injected with an equal volume of normal saline.

2.4. Sample collection and storage

At 48 h after paraquat poisoning, animals were intraperitoneally anesthetized with 10% chloral hydrate at 300 mg/kg. Mice were placed in a supine position, and thoracotomy was performed after sterilization. A 1-mL syringe was used to puncture the right ventricle, followed by blood collection. Blood was transferred to a 1.5-mL tube and centrifuged at 3000 rpm for 10 min at 4 °C. The supernatant was collected in a 1.5-mL tube (0.2 mL/tube) and stored at −80 °C. After blood collection, the precipitate was removed, and normal saline was used to flush the lung tissues. When the fluid flowing from the body was clear, both lungs were harvested. The color, nature, and pathological changes in the lungs were observed. After being washed with normal saline, the left lung was fixed in 4% paraformaldehyde at 4 °C overnight. On the second day, tissues were dehydrated in 30% sucrose at 4 °C, then embedded in optimum cutting temperature compound and stored at −80 °C. The right lung was stored in liquid nitrogen at −80 °C for further study.

2.5. Detection of serum TNF-α and IL-1β

Serum was centrifuged at 10,000 rpm for 10 min at 4 °C, and the supernatant was collected. Serum IL-1β and TNF-α levels were measured with the appropriate ELISA kits according to the manufacturer's instructions (R&D Systems).

2.6. Measurement of wet-to-dry weight ratio

The middle lobe of the right lung was washed with normal saline, water was removed with a filter, and the lung tissues were weighed as wet weight. Then, the lung tissues were placed in an oven at 80 °C and weighed 48 h later as dry weight. The wet-to-dry weight ratio was calculated to evaluate lung edema: wet-to-dry weight ratio (W/D) = wet weight/dry weight (both in mg).

2.7. Scoring of lung injury

The left lung was cut into blocks (about 0.5 cm in thickness) and then subjected to dehydration in alcohol, transparentization, embedding, sectioning, and H&E staining. Lung pathology was evaluated under a light microscope, and lung injury was scored according to the method proposed by Mikawa et al. (2003) based on (1) alveolar congestion, (2) hemorrhage, (3) neutrophil infiltration or aggregation in the alveolar space or vascular wall, and (4) alveolar wall thickening or hyaline membrane formation. Each was graded 0–4: 0, no lesion or very mild injury; 1, mild injury; 2, moderate injury; 3, severe injury; and 4, extremely severe injury. The sum of each score was used as the final lung injury score.

2.8. Detection of mRNA expression of MyD88, TNF-α, and IL-1β by RT-PCR

The lower lobe of the right lung was homogenized in Trizol reagent for subsequent extraction of total RNA according to the manufacturer's instructions. RNA was reversely transcribed into cDNA. The primers used in PCR were as follows: MyD88: 5′-GTGCCGTCGGATGGTAGT-3′ (forward), 5′-CAGTGATGAACCGCAGGAT-3′ (reverse); TNF-α: 5′-GCAAGCTTCGCTCTTCTGTCTACTGAACTTCGG-3′ (forward), 5′-GCTCTAGAATGAGATAGCAAATCGGCTGACGG-3′ (reverse); IL-1β: 5′-CGCAGCAGCACATCAACAAGAGC-3′ (forward), 5′-TGTCCTCATCCTGGAAGGTCCACG-3′ (reverse). PCR was performed in a Model 7500 Thermal Cycler (Applied Biosystems, Foster City, CA, USA), and the results were subsequently analyzed.

2.9. Detection of MyD88 and p-p65 expression by immunofluorescence staining

The section prepared from the upper lobe of the right lung was washed in 0.1 mol/L phosphate buffered saline (PBS) twice, followed by antigen retrieval in 0.01 mol/L sodium citrate (pH =