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ORIGINAL ARTICLE
Iran J AllergyAsthmaImmunol
December 2017; 16(6):511-519.

Imbalance of Th17/Treg in the Pathogenesis of Mice with Paraquat-induced Acute Lung Injury

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Received: 6 April 2017; Received in revised form: 23 May 2017; Accepted: 4 July 2017

ABSTRACT

Recent studies suggest that imbalances in the ratios of CD4+ T helper cell subsets, T helper-17 (Th17) and regulatory T (Treg) cells play a crucial role in the pathogenesis of acute lung injury (ALI). However, studies of the imbalance of Th17/Treg in paraquat (PQ)-induced ALI have not been reported. Therefore, we investigated whether the ratio of Th17/Treg cells in a mouse model of PQ-induced ALI contributes to pathogenesis of ALI.

Male Kunming mice were randomly treated with saline (control group) or PQ (PQ-poisoned (PQP) group); mice were sacrificed at either 12 hours (PQP-12h) or 24 hours (PQP-24h and control) post-treatment. Hematoxylin-eosin and TUNEL staining procedures were performed to examine inflammation and apoptosis. The presence of Th17 and Treg cells was measured by flow cytometry; the expression of putative Th17 cytokines and transcription factors was measured by ELISA and western blot analysis.

Compared with control mice, lung inflammation and apoptosis were dramatically increased in PQP mice at 12 and 24 hours after poisoning. In addition, poisoned mice displayed significant increases in the presence of CD4+IL-17+ T cells (Th17) and in the expression of IL-17A and IL-17, as measured by flow cytometry and western blot assays. This increase was most notable after 24 hours of PQ exposure. Furthermore, poisoned mice displayed marked decreases in the presence of CD4+CD25+Foxp3+ T cells (Treg) and in the expression of IL-35 and the transcription factor Foxp3.

These results suggest that an imbalanced ratio of Th17/Treg cells may contribute to the pathogenesis of PQ-induced ALI.

Keywords: Acute lung injury; Paraquat; Poisoning; Th17/Treg imbalance

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INTRODUCTION

Paraquat (PQ), one of the most widely-used organic heterocyclic herbicides in agriculture, is highly effective at weeding and is not injurious to crops. However, PQ is highly toxic to humans and animals.[1]There is a high mortality rate associated with PQ exposure, which has been attributed to the lack of an effective treatment to counteract the toxic effects of the poison. A prior study found that the mortality rate after PQ exposure can reach 90%.[2] PQ can be absorbed through multiple body surfaces, including the skin, membrane, and respiratory tract; subsequently, it rapidly distributes into tissues and causes functional damage to multiple organs.[3] Recently, a clinical study has shown that paraquat poisoning (PQP) can cause severe damage to multiple organs – the most notable of these is acute lung injury (ALI), since PQ accumulates in the lung tissue at concentrations that can reach 10 to 90-fold higher than those found in the blood.[4]Early-stage lung damage from PQ exposure is mainly composed of ALI; later-stage lung damage manifests as irreversible pulmonary fibrosis, which is the primary cause of death in patients with PQP.[5] Despite robust clinical studies and experimental animal models, the pathogenesis of this devastating disease remains poorly defined. However, some studies have indicated that activation of multiple inflammatory cells, and the release of inflammatory mediators, may play a crucial role in the development and outcome of PQP.

Recent studies have suggested that CD4+ T cells are active in the pathogenesis of ALI. As important immunomodulatory cells, CD4+ T cells can strengthen or suppress the activation of other immune cells. The balance between T helper-17 (Th17) cells and CD4+CD25+Foxp3+Regulatory T (Treg) cells is known to function in the pathogenesis of a number of inflammatory and autoimmune diseases.[6-8] Th17 cells play a key pro-inflammatory role in a variety of immune inflammatory diseases through the secretion of IL-17, which can prompt the release of a large number of downstream inflammatory factors.[9,10]Treg cells can indirectly antagonize Th17 cells, thereby suppressing inflammation and inhibiting the immune response.[11,12] Thus, there is a reciprocal relationship between Th17 cells and Treg cells, both in development and function.

Emerging data suggests that Th17 and Treg cells play a crucial role in ALI.[13,14] However, studies on the imbalance of Th17/Treg in PQ-induced ALI have not been reported. Therefore, the present study aimed to investigate the putative imbalance of Th17/Treg and related changes in cytokine expression in the pathogenesis of PQ-induced ALI induced and to further characterize possible mechanisms of tissue injury.

MATERIALS AND METHODS

Experimental Animals and Protocols

Kunming mice (male, 4-6 weeks old, 18–22 g body weight) were obtained from the Laboratory Animal Center of Guangxi Medical University (Nanning, Guangxi, China) and maintained under specific pathogen-free conditions. Room temperature and humidity were set at 23±3°Cand 55.5±10%, respectively. All the mice were provided food and water ad libitum with a standard laboratory diet. Animal experimental protocols were approved by the Ethical Principles in Animal Research adopted by the Guangxi Medical University for Animal Experimentation (No. 2014-KY-161).

Experimental Design

Mice were randomly assigned to either the normal control group or the PQP group. A single dose of PQ (20 mg/kg, dissolved in saline, Sigma, St. Louis, MO, USA) was administrated to the PQP mice by intraperitoneal injection. The control group was treated with normal saline instead of PQ at volumes equivalent to the PQ dosage. Mice were sacrificed at 12 hours (PQP-12h; n=8) or 24 hours post-treatment (PQP-24h, n=8; and control) and specimens were harvested, including bronchoalveolar lavage fluid (BALF), lung and spleen. The collection of BALF was performed as previously described[15] and the supernatant was stored at -80°C until measurement of cytokine levels. The left lung tissue was fixed with 4% paraformaldehyde for H&E and TUNEL staining, while the right lung tissue was snap-frozen by immersion in liquid nitrogen, and then stored until western blot was performed. The spleen tissue was analyzed by flow cytometry.

Hematoxylin-eosin (H&E) for the Examination of Inflammation

The left lung tissue was isolated from the mice,

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fixed with 4% paraformaldehyde, and embedded in paraffin, then cut into 4-μm thick micro-sections. The micro-sections were stained with H&E for the examination of inflammation by microscopy (Olympus, Tokyo, Japan). The examiners were blinded to the origin of the stained tissue.

TUNEL for the Examination of Apoptosis

Terminal deoxynucleotidyl transferase-mediated dUPT nick end labeling (TUNEL) staining was performed to observe apoptosis. The protocol was carried out according to the manufacturer's instructions for the kit (In Situ Cell Death Detection kit, POD, Roche, Germany). The expression of positive cells was observed by computer pathological image analysis system under the high magnification view (400×).

Cytokine Measurement Using ELISA

The cytokine concentrations of IL-17A and IL-35 in BALF were measured by commercial ELISA according to the manufacturer's instructions (Cusabio Biotech CO. Ltd, China). The absorbance was measured at 450 nm by a micro-plate ELISA reader (Bio-Rad Laboratories, CA, USA).

Flow Cytometry Analysis: Quantification of Th17 and Regulatory T (Treg) Cells

The percentages of Th17 and Treg cells from the spleen tissue were determined by flow cytometry. Single cell suspensions from spleens were prepared at a concentration of 1×106 cells/mL. Cells were washed and re-suspended in phosphate-buffered saline (PBS). For quantification of Th17 cells, cytokine intracellular staining of IL-17 was performed. Cells were incubated in Roswell Park Memorial Institute (RPMI) medium with 2% fetal bovine serum (FBS) and with 30 ng/mLphorbolmyristate acetate (PMA) (Sigma-Aldrich, USA), 1 mg/mL of ionomycin (Sigma-Aldrich, USA) and 2 μL monensin (eBioscience, San Diego, CA, USA) for 4 h at 37°C. Percp-Cy5.5-labeled anti-mouse CD4 and PE-labeled anti-mouse IL-17 were used to detect Th17 cells (eBioscience, San Diego, CA, USA). For Treg quantification, cells were stained with Percp-anti-CD4 (eBioscience, San Diego, CA, USA), PE-anti-CD25 (eBioscience, San Diego, CA, USA). Cells were separated using a cell sorter (BD FACSCalibur, BD Biosciences, California, USA) and data were analyzed with FCS Express Software (BD Biosciences, San Diego, CA, USA).

Western Blot Analysis of IL-17 and Foxp3 Expression

Total protein was isolated from the right lung by homogenization with a radioimmunoprecipitation assay (RIPA) buffer (Beyotime, China). The samples were centrifuged at 12000 rpm for 15 min to remove debris and intact cells, then the supernatant was collected. Whole proteins in the lysate were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto nitrocellulose membranes. The membranes were blocked for 1 h with Tris-buffered saline containing 0.05% Tween (TBST) and5% powder milk, then probed overnight at 4°C with antibodies against Foxp3 (Santa Cruz Biotechnology, Inc.) and IL-17 (Santa Cruz Biotechnology, Inc.), respectively. After washing with TBST, membranes were incubated with a fluorophore-labeled goat anti-rabbit antibody (1:800 dilution, EarthOX, LLC, San Francisco, CA, USA) diluted in secondary antibody dilution buffer. Signals of each sample were detected using a sweep membrane apparatus (LI-COR, Inc., USA).

Statistical Analysis

Statistical analyses were performed using the SPSS statistical software (version 17.0, SPSS, Inc., Chicago, USA). Graphs were constructed within GraphPad Prism v5.0 (GraphPad Software, San Diego, California, USA). The data are presented as mean±standard deviation (SD). Multiple groups were analyzed via one-way analysis of variance (ANOVA) followed by post-testing with Fisher's LSD for multiple comparison of means. Pearson product-moment correlation coefficients were calculated. For all analyses, a p of <0.05 was considered to indicate a statistically significant difference.

RESULTS

Histopathology Analyses of Mice Lungs Treated with PQ by H&E Staining

Pathological changes were investigated by H&E staining. Serial lung sections from PQ-induced mice (harvested at 12 and 24 hours after PQ administration) were analyzed to determine the effects of PQ treatment on lung tissue. As illustrated in Figure 1A: the control group exhibited normal lung tissue

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structure, with no signs of inflammation or destructive changes. However, clear pathological changes were observed in PQP-12h and PQP-24h mice. The PQP-12h group exhibited slight alveolar edema and moderate infiltration of inflammatory cells. The PQP-24h group exhibited obvious changes in lung tissue, including engorgement of alveolar wall blood capillaries and infiltration of inflammatory cells into alveolar spaces (these cells were predominantly polymorphonuclear leukocytes). These results demonstrated that PQ administration induced ALI. (Figure 1A).

Cell Apoptosis of Mice Lungs Treated with PQ by TUNEL

TUNEL staining was performed to measure apoptosis. Brown-stained nuclei were regarded as TUNEL-positive cells. Positive cells were located in the nucleus and scattered in the distribution. Representative sections from mouse lungs in each experimental group are shown in Figure 1B: the control group exhibited very few apoptotic cells and cell nuclei were stained blue. The apoptotic cells in the PQP-12h and PQP-24h mouse tissues were significantly more abundant than in the control group, and this abundance increased over time (Figure 1B).

The Levels of Cytokines (IL-17A and IL-35) in BALF of PQ-Induced ALI Mice Model

The BALF-specific expression of Th17-related cytokine IL-17A and Treg-related cytokine IL-35 were determined by ELISA as indicators of cytokine production. As shown in Figure 2, IL-17 expression was significantly higher in the PQP-12h group than in the control group (control group: 246.66±111.312 pg/mL, PQP-12h group: 469.01±221.588 pg/mL, p=0.034). Additionally, IL-17 expression was significantly higher in the PQP-24h group than in the control group (control group: 246.66±111.312 pg/mL, PQP-24h group: 680.38±59.569 pg/mL, p=0.001), and IL-17 expression was significantly higher in the PQP-24h group than in the PQP-12h group (PQP-12h group: 469.01±221.588 pg/mL, PQP-24h group: 680.38±59.569 pg/mL, p=0.042). However, IL-35 expression was significantly decreased in the PQP-12h group, compared with the control group (control group: 146.42±38.42 pg/mL, PQP-12h group: 92.896±52.899 pg/mL, p=0.047). Furthermore, IL-35 expression was significantly decreased in the PQP-24h group, compared with the control group (control group: 146.42 ±38.42 pg/mL, PQP-24h group: 35.03±10.365 pg/mL, p=0.001), and IL-35 expression was significantly increased in PQP-24h group (PQP-24h group: 92.896±52.899 pg/mL, PQP-24h group: 35.03±10.365 pg/mL, p=0.034). These data indicate that changes in the expression of IL-17 and IL-35 contribute to the IL-17/Treg imbalance (Figure 2).

The Percentage of Th17 Cells and Treg Cells in the Spleen of the ALI Models

Flow cytometry was used to examine the percentage of Th17 cells and CD4+CD25+Foxp3 cells among the CD4+ T cells in the spleen. As shown in Figure 3, the PQP-12h group exhibited an increase in Th17 cells (4.702±0.752%), compared with the control group (2.04±0.142%); this was significantly different from PQP-12h group (p<0.01). PQP-24h group exhibited a Th17 cell percentage of 7.446±1.53% of total splenic CD4+ T cells. However, the normal control group exhibited a population of CD4+CD25+Foxp3 cells of 12.42±1.32%. Compared with the control group, PQ-induced mice showed a significantly lower proportion of CD4+CD25+Foxp3 cells (PQ-12h group: 10.042±0.888, PQ-24h group: 6.82±0.342, p<0.01). These findings indicate that PQ could affect the percentage of Th17 cells and CD4+CD25+Foxp3 cells in the spleen of affected animals (Figure 3).

The Protein Expression of IL-17 and Foxp3 in Lung tissue of PQ-Treated Mice

Western blotting was used to measure the expression of IL-17 and Foxp3 in lung tissue; these proteins are respective Th17 and Treg cell markers. As shown in Figure 4, compared with the normal control group, IL-17 expression was significantly increased in the PQP-12h and PQP-24h groups and PQ-treated animals exhibited a time-dependent increase in IL-17 protein levels. Conversely, the expression of Foxp3 decreased at 12 and 24 hours after PQ administration, compared with the control group. (Figure 4).

Correlation between IL-35 Expression and the Percentage of CD4+CD25+Foxp3 Cells in PQ-Treated Mouse Lungs

As shown in Figure 5, IL-35 expression in BALF was significantly positively correlated with the percentage of CD4+CD25+Foxp3 cells (r=0.907,

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$p<0.05$). This result suggests that IL-35 expression is closely related to the proportion of CD4+CD25+Foxp3 cells in the lungs of mice with PQ-associated ALI (Figure 5).

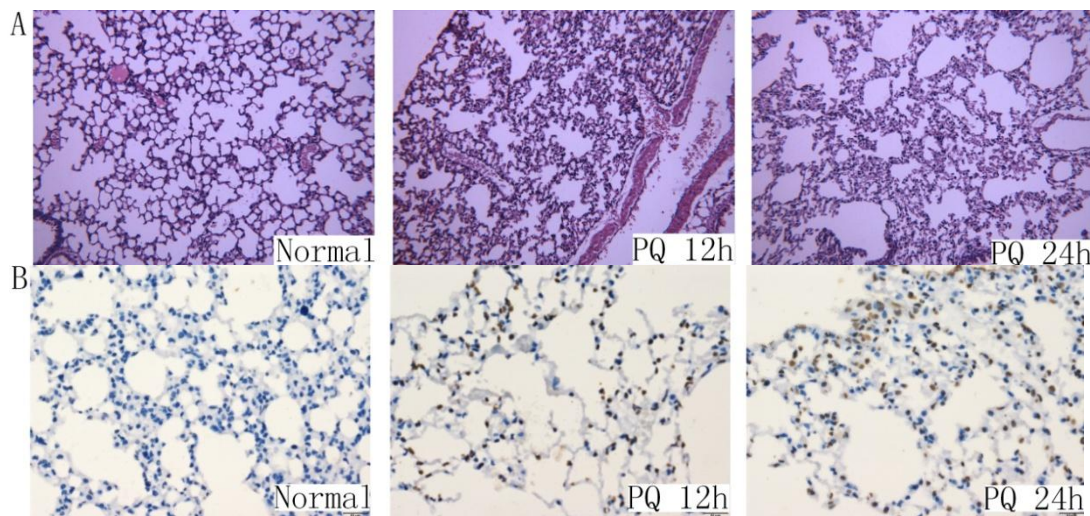

Figure 1. Histologic findings with HE and TUNEL staining of lung tissues. A H&E stained histological evaluation of mouse lungs in the normal control group, the PQP12h group and the PQP 24h group (x200 magnification). The normal control group shown the normal lung tissue structure, The PQP 12h group exhibited slighter alveolar edema and less inflammation cells infiltration. The PQP 24h group revealed the obvious changes of lung tissue, exhibited the blood capillary of alveolar wall engorgement and the infiltration of inflammatory cells in alveolar spaces (predominantly polymorhponuclear leukocyte).B To observe the apoptosis of pulmonary histocyte of mice in each group under the optical microscope (x400 magnification). Brown positive nuclei were indicated with the TUNEL-positive cells. Positive cells were located in the nucleus and scattered in the distribution. The normal control group demonstrated very little apoptotic cells and nucleus was stained blue. The apoptosis cells in the PQP 12h group and the PQP 24h group were significantly higher than that in the normal control group, which was increased with time-dependence.

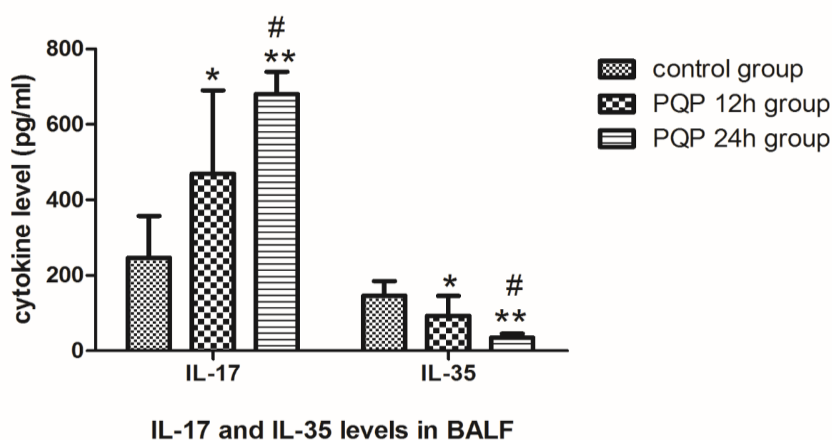

Figure 2. The levels of IL-17 and IL-35 in BALF were measured by ELISA. Cytokines (IL-17 and IL-35) levels in bronchoalveolar lavage fluid. In PQP 12h group and PQP 24h group, the levels of IL-17 in BALF were largely increased compared with the normal control group ($p<0.05$). In PQP 12h group and PQP 24h group, IL-35 levels were significantly reduced compared with the normal control group ($p<0.05$). Data are presented as mean±SD. *$p<0.05$ and **$p<0.01$ versus normal control group; #$p<0.05$ versus PQP 12 h group.

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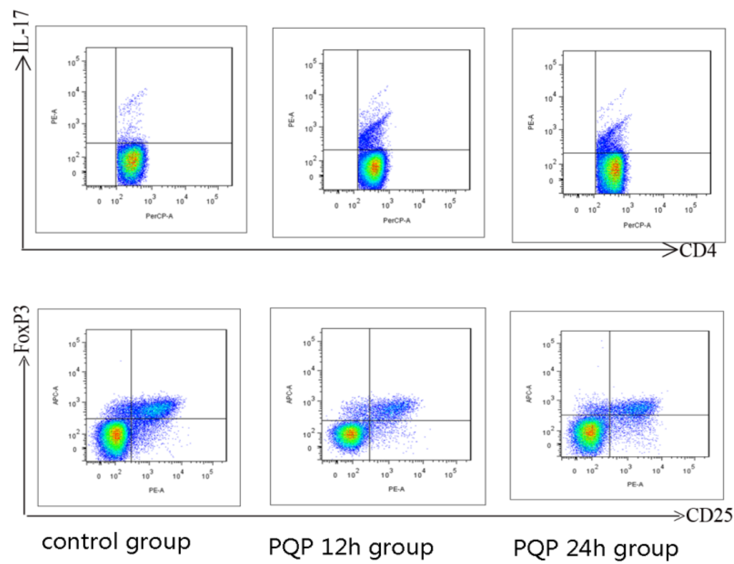

Figure 3. The percentage of Th17 Cells and Treg Cells in mice with PQ. Spleen from paraquat(PQ) -treated mice (12 and 24 hours after PQ administration) and the normal control group were investigated the percentage of Th17 cells and CD4$^+$CD25$^+$Foxp3 cells among the CD4$^+$ T cells by flow cytometry.

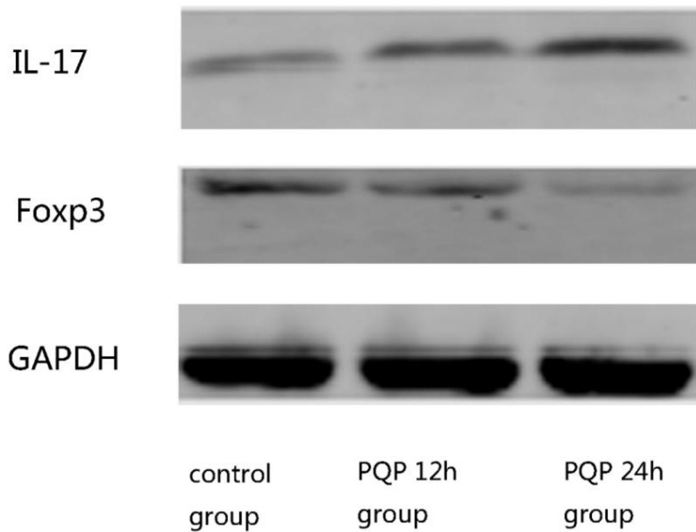

Figure 4. IL-17 and Foxp3 protein expression in lung tissue. IL-17 and Foxp3 protein expression in lung tissues were determined by western blots. Expression levels were normalized with that of GAPDH. IL-17 and Foxp3 were expressed in both normal control group and the poisoned groups (PQP 12h group and PQP 24h group). IL-17 was strongly expressed and Foxp3 was weakly expressed in poisoned groups compare to the control group.

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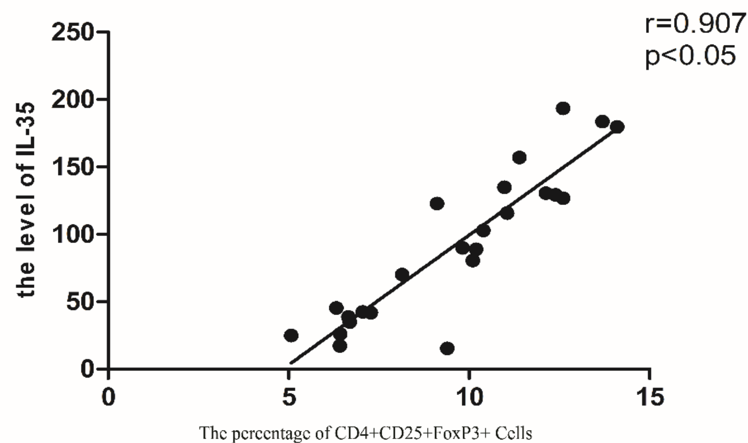

Figure 5. The correlation between IL-35 and CD4+CD25+Foxp3 cells. IL-35 expression in bronchoalveolar lavage fluid was significantly positively correlated with the percentage of CD4+CD25+Foxp3 cells (r=0.907, *p*<0.05).

DISCUSSION

To determine the effects of PQ on the balance of Th17/Treg cells, we used classical methods to prepare murine models of ALI through intraperitoneal injection of PQ solution (20 mg/kg). The results of our study demonstrated that the poisoned group (PQP-12h and PQP-24h) had the following histological characteristics: the PQP-12h group exhibited slight alveolar edema and moderate infiltration of inflammatory cells; the PQP-24h group exhibited obvious changes in lung tissue, including engorgement of blood capillaries in the alveolar wall and infiltration of inflammatory cells into alveolar spaces (predominantly polymorphonuclear leukocyte). Similar pathological changes also were reported in the study of Yang and colleagues.[16]

Furthermore, TUNEL staining was performed to observe apoptosis. The results discovered that there are very few TUNEL-positive cells in the control group, whereas there were abundant apoptotic cells in the poisoned group – this abundance increased with time. Taken together, these results indicate that the poisoned models were successful.

During the development of PQP, the lung is the primary target organ to suffer injury; the pathogenesis involves inflammation and oxidative stress injury. Concomitantly, inflammatory cells release a variety of substances that cause inflammatory cascade reactions[17,18.] It has recently been shown that immune regulation disorders can become a significant factor in stimulation of inflammation. The newly-identified CD4+ T cell subsets, Th17 and Treg cells, attract much attention and play an important role in the occurrence and development of ALI,[13,14,19] but the specific mechanisms of PQ-induced ALI remain poorly defined. Th17 cells are regarded as having a role in immune inflammatory diseases that are characterized by the secretion of IL-17A and the release of a large number of downstream inflammatory factors.[20] In our study, the percentage of CD4+IL-17+ T cell in the spleen tissue, Th17-related expression of cytokine IL-17A in BALF, and the protein expression of IL-17 in the lung tissue were all dramatically increased in the PQP-12h group and the PQP-24h group. More importantly, these results increased with time. These findings demonstrate that Th17 cells may be involved in the pathogenesis of PQ-induced ALI in mice, through the secretion of IL-17A, and may promote inflammatory responses.

However, Treg cells have drawn wide attention as immune-regulatory cells, characterized by production of IL-10 and IL-35. These play a key anti-inflammatory role in a variety of immune inflammatory diseases and suppression of the immune response.[21]The balance between Th17 and Treg cells plays an important role in the maintenance of immune homeostasis. Once the balance is broken, it may lead to a series of inflammatory reactions[22-24]. The results of the present study showed that the frequency of CD4+CD25+Foxp3+ T cell, the level of IL-35 and the protein expression of the transcription factor Foxp3 were conspicuously decreased in the poisoned group. Importantly, there is a positive correlation between the level of IL-35 and the percentage of

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CD4+CD25+Foxp3+ T cell. The above data suggest that Treg cells may be contributed to the improvement of the disease.

Prior studies have shown that the imbalance of Th17/Treg cells may be involved in the pathogenesis of ALI. Specifically, higher expression of Th17 cells and lower expression of CD4+CD25+Treg cells has been shown in the poisoned group compared with the control group, similar to our results in the current study. Meanwhile, we further found that the level of IL-17A was increased in BALF after poisoning, which is consistent with higher percentage of Th17 cells after poisoning. However, the expression of IL-35 and the percentage of Treg cells also followed the above synchronous change. Moreover, the protein expression of IL-17 and Foxp3 was observed in the lung tissue, our results showed the followed changes: the protein expression of IL-17 was significantly higher in the PQP 12h group and the PQP 24h group than that in the normal control group, which increased with time-dependence. Whereas, compared with the normal control group, the protein expression of Foxp3 was decreased in the PQP-12h group and the PQP-24h group. Consequently, the above experimental results demonstrated that an imbalance of Th17/Treg cells may be involved in the pathogenesis of ALI induced by PQ. There is a possibility that the Th17-related immune reaction became unbalanced although more Th17 cells were involved. In contrast, Treg cell frequency was significantly negatively associated with the process. On the one hand, the organism can release more inflammatory factors through increasing the proportion of Th17 cells, which promote immune responses and inflammatory reactions, further aggravating the damage to the body. On the other hand, the decrease of Treg cells can affect the function of immune regulation. It is not enough to fight against the pro-inflammatory effect of Th17 cells and expand inflammatory response. It is for these reasons that the inflammatory cascade reaction is augmented that can promote the development of the disease. It is consistent with the correlation[25]. Accordingly, we suggested that Th17/Treg imbalance may be an important feature of the pathogenesis of PQ-associated ALI.

In conclusion, the current study proves that the imbalance between Th17 and Treg cells may be related to pathogenesis of PQ-induced ALI. Inflammation and immune injury induced by PQ in mice can lead to the imbalance of Th17/Treg cells and the changes of related cytokines, which play a key role in the immune status and the progression of disease. Nevertheless, further studies are required to validate these conclusions by using in vitro cell culture methods, to deeper reveal the mechanics of Th17/Treg imbalance in ALI induced by PQ.

ACKNOWLEDGEMENTS

The research received funding from the Talents Highland of Emergency and Medical Rescue of Guangxi Province in China (Nos: GXJZ201503). The research received funding from the National Natural Science Foundation of China (Nos: 81360290).

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