International Immunopharmacology 72 (2019) 243–251

Contents lists available at ScienceDirect

International Immunopharmacology

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

Doxycycline alleviates paraquat-induced acute lung injury by inhibiting neutrophil-derived matrix metalloproteinase 9

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ARTICLE INFO

Keywords:
Doxycycline
Paraquat
Acute lung injury
Neutrophil
Matrix metalloproteinase 9

ABSTRACT

Paraquat (PQ), a highly toxic herbicide, selectively accumulates in the lungs and causes pulmonary damage through oxidative and inflammatory processes after intentional or accidental poisoning. The resulting acute lung injury (ALI) is characterized by neutrophil infiltration and extensive inflammation with rapid respiratory failure. However, effective therapies are lacking. We tested the hypothesis that suppressing neutrophil-derived matrix metalloproteinase 9 (MMP9) would ameliorate the inflammatory milieu and alleviate PQ-induced ALI. Lung injury was assessed in mice intratracheally injected with PQ aerosol by measuring the lung static compliance, cell count and neutrophil percentage of the bronchoalveolar lavage fluid (BALF) and lung, alveolar-capillary permeability, and histopathological lung injury scores. MMP9/2 activity was assessed by gelatin zymography, and the location of neutrophils and MMP9 in the lung was evaluated by immunofluorescence costaining. In the neutrophil depletion experiment, mice received anti-Ly6G antibody intraperitoneally; for the MMP inhibition experiment, an MMP inhibitor, doxycycline (DOX), was administered by gavage. In PQ-induced ALI, the activity of neutrophil-derived MMP9 but not MMP2 increased significantly. Neutrophil depletion reduced the inflammatory burden, improved pulmonary edema, and reduced the PQ-induced overexpression of MMP9. Consistently, oral delivery of DOX to mice decreased the overexpression of MMP9 that was activated by PQ and phenocopied the resolution of PQ-induced ALI observed after neutrophil depletion. Taken together, our results show for the first time that DOX is involved in the resolution of PQ-induced ALI via a mechanism involving reducing the activity of neutrophil-derived MMP9. We speculate that DOX may represent a novel therapeutic strategy for PQ-induced ALI.

1. Introduction

Paraquat (PQ) is a nonselective herbicide that has been widely used in over 120 countries since the 1960s due to its high efficiency and low residues in crops [1]. However, PQ is highly toxic to mammals. Although the exact mechanisms underlying PQ toxicity remain unclear, extensive studies have indicated that PQ-induced toxicity is due to sustained redox cycling and the subsequent generation of reactive oxygen-nitrogen species (RONS), which results in oxidative stress-related inflammation [1,2]. In particular, PQ tends to efficiently accumulate in lung tissues, where the concentration can be 6–10 times higher than that in plasma, and the compound is retained in the lung even when the levels in blood and other organs start to decrease [1]. Thus, acute lung injury (ALI) characterized by interstitial inflammation and severe pneumonia at the early phase of intoxication followed by respiratory failure may induce high mortality clinically.

Many investigators have indicated that the early stage of PQ poisoning includes a severe systemic inflammatory response represented by an increase in the proportion of neutrophils and a marked elevation in the total number of white blood cells. Moreover, nonsurvivors have significantly higher leukocyte and neutrophil counts, whereas there is no significant difference in lymphocyte and platelet counts [3,4]. Accordingly, white blood cell count at admission is emphasized as an index of predicting outcomes in PQ poisoning [5]. In the experimental animal model of PQ intoxication, the inflammatory response that arises during the early destructive phase, which is maintained throughout the

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https://doi.org/10.1016/j.intimp.2019.04.015
Received 14 November 2018; Received in revised form 20 March 2019; Accepted 5 April 2019
Available online 16 April 2019
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subsequent proliferative phase, involves a rapid and extensive influx of inflammatory cells, mainly neutrophils and macrophages, into the interstitium and alveolar spaces [6–9]. Infiltration by a significant number of neutrophils accompanies the early pathological changes of PQ poisoning. This finding suggests that neutrophilia may play a significant role in the toxicity of acute PQ poisoning [4]. However, conventional antioxidant and anti-inflammatory treatments are not sufficiently effective, which raises the question of whether targeting neutrophils and their microenvironment rather than targeting other leukocytes can reduce their capacity to support inflammation and potentially resolve lung injury.

Matrix metalloproteinases (MMPs), a group of zinc-dependent endopeptidases that are produced by a variety of cell types and that can influence a number of processes, have been found to play an important role in a variety of pulmonary diseases [10–13]. MMP2 (gelatinase A) and MMP9 (gelatinase B), are considered as two of the most extensively studied MMPs [14–16]. In addition to degrading matrix components, MMP2 and MMP9 are capable of regulating chemokines and cytokines and are therefore important in the regulation of the inflammatory process and cell migration to the inflammatory site [17–19]. Some studies have observed that MMPs play an important role in the process of PQ-induced pulmonary fibrosis, which has drawn our attention [20,21]. In another classic process of RONS generation, stroke-induced ischemia-reperfusion injury, neutrophils, which are the most important source of elevated MMP9 in the early injury stage, can be rapidly recruited to the local lesion and are involved in blood-brain barrier breakdown [22,23]. Similarly, in the pathogenesis of PQ intoxication, both RONS activation and vascular leakage are involved in the mechanism of lung injury.

Thus, we hypothesize that neutrophils may be quickly recruited into the lungs and release MMPs to contribute to ALI. Depleting neutrophils might represent an effective way to reverse PQ-induced ALI. We also screened a clinical drug, doxycycline (DOX), which has therapeutic effects, including both anti-infection and MMP inhibitory effects [24,25]. We hypothesize that the inhibition of neutrophil-derived MMPs by DOX would phenocopy the improvement in PQ-induced ALI that resulted from neutrophil depletion.

2. Materials and methods

2.1. Animals

Male C57BL/6J mice (8–9 wk. old) were purchased from Charles River Laboratories (Beijing, China) and studied as approved by the Institutional Animal Care and Use Committee of Nanjing Medical University (NMU; Jiangsu, China) (Permit Number: IACUC-1712010). All experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of NMU.

2.2. Induction and measurement of ALI

As described in our previous study (Hao S, et al. Am J Respir Crit Care Med 193;2016:A4144.), mice were administered PQ (Sigma-Aldrich, MO, USA) at a single dose of 0.02 mg per mouse (PQ diluted in 50 μl of sterile saline buffer) via intratracheal aerosolization (Model IA-IC microsprayer, High Pressure Syringe Model FMJ-250, Penn-Century, PA, USA) (Fig. 1A). Control animals received an equal volume of sterile saline. Mice were sacrificed 3 days or 7 days post-PQ administration (Fig. 1B). Referring to a previous study [26], lung injury was assessed by measuring the static compliance, cell count and neutrophil percentage of bronchoalveolar lavage fluid (BALF) and lung, alveolar-capillary permeability and lung injury scores from hematoxylin and eosin (HE) staining. For the time-dependent MMP9/2 activity assay, ALI was induced in mice as described above and the lung tissues were harvested at 6 h, 24 h, 3 days or 7 days after PQ exposure, respectively.

2.3. Neutrophil depletion

Following the reference method [27], neutrophils were depleted by intraperitoneal injection of 400 μg of anti-Ly6G (1A8 clone, Biolegend, CA, USA) per mouse one day before PQ exposure and every other day post PQ exposure until harvest day. Control groups received 400 μg of rat isotype control (RatIg, Biolegend, CA, USA) at the same time points (Fig. 1C).

2.4. MMP inhibition

As other investigators have reported [28] and we previously described [29], DOX can act as an efficacious MMP inhibitor. For the MMP9 inhibition study, DOX (Sigma-Aldrich, MO, USA) treatment (20 mg/kg in 0.3 ml of water) was administered daily by gavage 3 days before PQ exposure and for 6 consecutive days until samples were harvested. Control groups were intragastrically administered an equal volume of water (Fig. 1D).

We also validated our findings using another potent and competitive inhibitor of MMP9, SB-3CT [30,31]. SB-3CT (Medchem Express, NJ, USA) was freshly dissolved in vehicle consisting of 10% dimethyl sulfoxide (DMSO) in PBS. According to previous studies [31,32], SB-3CT (25 mg/kg) was administered intraperitoneally five times: 30 min before PQ exposure, 3, 6, 24 and 48 h after PQ exposure.

2.5. Sample preparation and routine blood test

Mice were anesthetized with 1% pentobarbital intraperitoneal injection. Blood was drawn from the right ventricle and 100 μl of blood was collected in a BD microtainer-anticoagulant tube and analyzed using an automatic blood cell analyzer for animals (Mindray, BC-2800vet, Shenzhen, China) to detect the neutrophil ratio. The remaining of blood was collected in a BD microtainer chemistry tube, and the serum was extracted for the subsequent ELISA assay.

2.6. Sample preparation and flow cytometry

Referring to our previous study [33], bronchoalveolar lavage (BAL) samples (lavaged three times with 0.5 ml of PBS containing 5 mM ethylenediaminetetraacetic acid) were collected after centrifugation and lysed with red blood cell lysis buffer (Sigma-Aldrich, MO, USA). Then, 10 μl of the cell resuspension solution was counted on a hemocytometer, and the remaining fluid was used for flow cytometry analysis. The right lung lower lobe was homogenized and filtered for single-cell suspensions for flow cytometry. The percentage of neutrophils present in the airways or lung was analyzed by flow cytometry. Recovered cells were stained with monoclonal anti-mouse antibodies: Ly6G-APC (1A8) and Gr1-PE (RB6–8C5) (Biolegend, CA, USA). Cells were stained for 15 min on ice before being washed and analyzed on a FACSVerse flow cytometer (BD, NJ, USA) using FlowJo V10.0.7 (Tree Star, Ashland, USA). Gr-1^{high} Ly6G+ cells were considered neutrophils.

2.7. Measurement of total protein levels in BALF and lung tissue

To evaluate alveolar-capillary permeability in the lung, the BALF supernatants were collected, and the total protein level (μg/ml BALF) was measured by the BCA method (Thermo Scientific, MA, USA). The upper part of the left lung lobe was homogenized in 1% NP40 lysis buffer (Beyotime, Shanghai, China) using an Atpio homogenizer (xianou-24, Nanjing, China). After centrifugation (4 °C, 12000 rpm, for 15 min), the supernatants were aspirated for the BCA assay.

2.8. Determination of myeloperoxidase (MPO) activity in BALF

To quantify the extent of neutrophil infiltration into the lung, the MPO activity was measured in BALF by a colorimetric assay using an

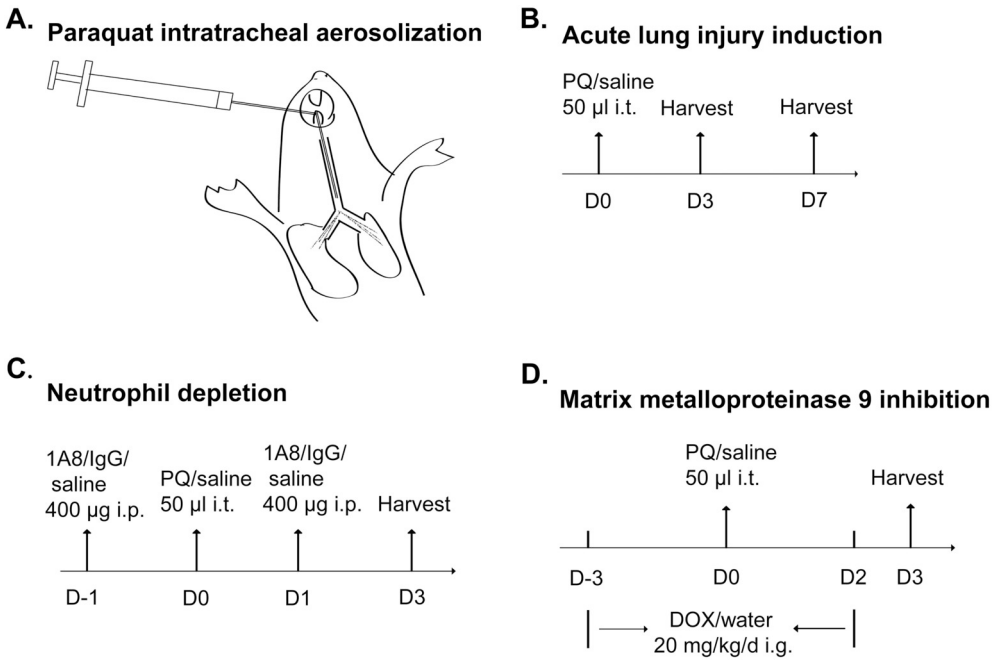

Fig. 1. Study design. (A) Schematic representation of paraquat (PQ) aerosol administered by intratracheal instillation. (B) Timeline of the animal model experimental treatments: 50 μl of PQ (0.02 mg) or saline per mouse was administered on day 0, and mice were sacrificed on day 3 or day 7. (C) Protocol performed in the neutrophil depletion experiment: mice received anti-Ly6G (1A8 clone), isotype control IgG or saline intraperitoneally one day before (day −1) and the day (day 1) after the animal model was established, and samples were harvested on day 3. (D) Protocol performed in the matrix metalloproteinase 9 (MMP9) inhibition experiment: the MMP inhibitor doxycycline (DOX) or sterile water was administered daily by gavage three days before (day −3) the animal model was established, the treatment was continued until day 2, and samples were harvested on day 3.

MPO activity test kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

2.9. ELISA for cytokines

The levels of the cytokines IL-1β and IL-6 in both BALF and serum were measured using mouse ELISA kits from R&D (MN, USA) according to the manufacturer's instructions.

2.10. Gelatin zymography for MMP9/2

The preparation procedures for BALF and lung tissue homogenates are both described above. BALF supernatants (20 μl) and lung homogenates of equal protein content (20 μl) were treated under nonreducing conditions and subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 8% polyacrylamide gels containing 0.1% gelatin (Amresco, PA, USA) at a constant voltage of 80 V at 0 °C. Mouse spinal cord sample was used as positive control (Fig. S1A) since its representative bands of MMP9/2 in gelatin zymogram which has been identified in our previous study [34–36]. After electrophoresis, the gels were washed four times with renaturing buffer (Novex, CA, USA) for 15 min to remove SDS, and then incubated for 48 h at 37 °C in developing buffer (Novex, CA, USA). Then, the gels were stained with Coomassie brilliant blue R-250 (1%, with 10% acetic acid, 10% isopropyl alcohol, diluted with dd H_2O) for 40 min. After destaining, the gels were scanned immediately using a Molecular Imager (ChemiDoc XRS+, Bio-Rad Laboratories, CA, USA). MMP activity was determined by densitometry using Quantity One 1-D Analysis Software (Bio-Rad Laboratories, CA, USA).

2.11. Histological analysis

The lower part of the left lung lobe was fixed with 4% paraformaldehyde, embedded into paraffin, and prepared into sections of 4 μm thickness. After staining with HE, the slides were evaluated under a light microscope (Nikon Eclipse C1, Tokyo, Japan) at 100–400× magnification by a board-certified pathologist who was blind to the treatments the mice received. The severity of lung damage was scored according to the animal model lung injury scoring system of the American Thoracic Society, which had five histological parameters: neutrophils in the alveolar space, neutrophils in the interstitial space, hyaline membranes, proteinaceous debris filling the airspaces and alveolar septal thickening [26]. The resulting injury score is a continuous value between zero and one (inclusive).

2.12. Immunofluorescence for colocalization of MMP9 and neutrophils

For formalin-fixed paraffin-embedded tissues, mounted sections were dewaxed by washing with xylene, rehydrated and blocked. The sections were then incubated with primary antibodies for Ly6G (R&D, MN, USA) and MMP9 (Abcam, MA, USA) overnight at 4 °C. Then, the sections were washed and stained with fluorophore-conjugated secondary antibodies for 1 h at room temperature. Cover slips were mounted using mounting medium (Servicebio, Wuhan, China), allowed to harden overnight at 4 °C and then sealed. Slides were observed under a Nikon Eclipse C1 fluorescence microscope (Nikon, Tokyo, Japan). Fluorescent image panoramic scanning and acquisition were performed by a Pannoramic 250/MIDI scanner (3D HISTECH, Budapest, Hungary) using Caseviewer2.0 software (3D HISTECH, Budapest, Hungary).

2.13. Statistics

All data were analyzed with GraphPad Prism 5 (GraphPad Software Inc., CA, USA). Data are presented as the mean ± S.D. for all experiments. Differences between groups were assessed by Student's *t*-test or one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. All tests of statistical significance were two-sided, and the statistical significance was set at $P < 0.05$.

3. Results

3.1. PQ intratracheal aerosolization induces ALI and neutrophil burst in mice

We first observed that the behavior of PQ-induced lung injury changed with time, as indicated in Fig. 2. We used the single-dose model of intratracheal PQ aerosol in mice and harvested samples on days 3 and 7. Compared with the saline group, the group with this model exhibited severe alveolar protein leakage (Fig. 2A), airspace inflammation (Fig. 2B), and an increase in inflammatory cytokines

(Fig. 2C) and MPO activity in BALF (Fig. 2D); decreased static compliance and elevated resistance of lung (Fig. 2E); and significantly higher lung injury scores measured with HE staining compared with saline group (Fig. 2F–G) at day 3. Additionally, PQ also exerted a systemic effect, as reflected by the decreased body weight (Fig. 2H) and increased neutrophil proportion in blood (Fig. 2I) on day 3. More importantly, neutrophil proportions measured by flow cytometry in both BALF and lung tissues showed significantly increased levels compared

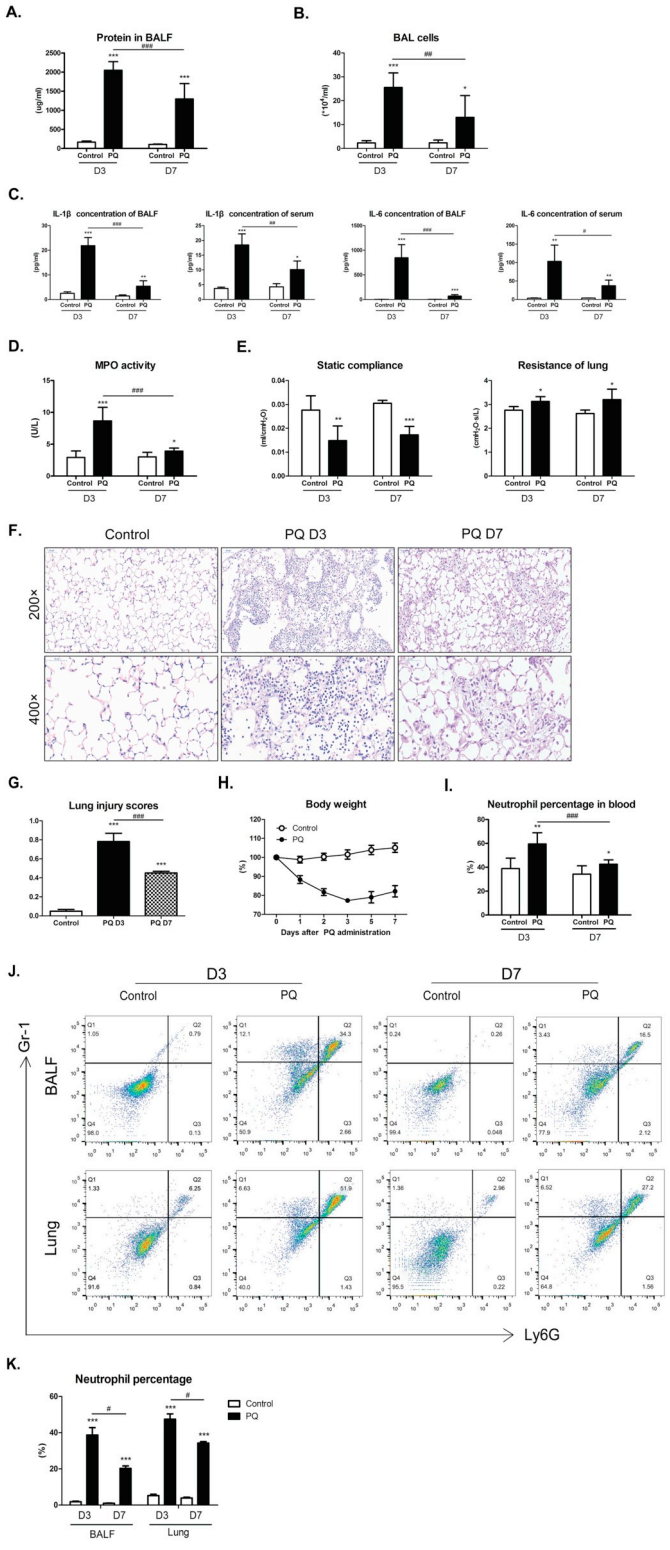

Fig. 2. Establishing a mouse model of PQ-induced acute lung injury (ALI). (A) Total protein concentration in bronchoalveolar lavage fluid (BALF). (B) Quantification of lavaged bronchoalveolar lavage (BAL) cells after PQ instillation. (C) Levels of the proinflammatory cytokines IL-1β and IL-6 in both BALF and serum. (D) MPO activity in BALF. (E) Static compliance and resistance of the lung. (F) Mouse lung sections stained with hematoxylin and eosin (HE). Original magnification: top ×200, bottom ×400. (G) Histological lung injury scores measured with HE-stained slides. (H) Daily measurement of mouse weight following saline or PQ treatment. (I) Neutrophil proportion in blood. (J) Neutrophil proportion in lung homogenate and BALF assessed by flow cytometric analysis. (K) Quantitative data of the neutrophil proportion analyzed by flow cytometry. *P < 0.05, **P < 0.01, and ***P < 0.001 compared to the saline control. #P < 0.05, ##P < 0.01, and ###P < 0.001 PQ groups compared between day 3 and day 7. Saline group ($n = 6$), PQ group ($n = 8$) in both day 3 and day 7 experiments.

to those in the saline group (Fig. 2J–K). By contrast, on day 7, all the injury and inflammatory parameters, especially the neutrophil proportion index, were gradually restore but still did not approach that of the saline control mice. All the above data suggested that PQ-induced ALI and the peak period of neutrophil burst presented on day 3 in this mouse model.

3.2. PQ mainly induces MMP9 secretion in mouse lungs during the acute phase

To determine the gelatinases (both MMP9 and MMP2) expression in the PQ-treated mouse lung, we evaluated the total gelatinases activity in this animal model by gelatin zymography at the following time points: hour 6, hour 24, hour 72, and day 7 after PQ challenge (Fig. 3). The activity of MMP9 was significantly elevated at hour 24 after PQ challenge compared to that in the saline group and significantly increased, reaching a peak corresponding to a > 7-fold increase relative to the level in the control group at hour 72; the activity then showed a decreasing trend on day 7, which was consistent with the changes in neutrophil levels. Additionally, the activity of another gelatinase, MMP2, showed a different trend; the activity increased gradually after PQ administration and then reached the peak value on day 7.

We considered that MMP9 is involved mainly in the early inflammatory phase of PQ-induced ALI, whereas MMP2 participates in

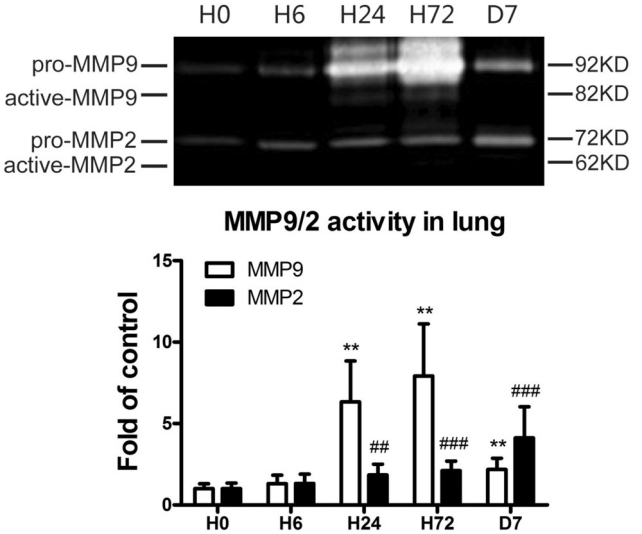

Fig. 3. Time course of MMP9/2 development in mouse lungs after PQ administration. The activities of the gelatinases MMP9 and MMP2 in lung tissues were determined by gelatin zymography at the following time points: H0 as the control, H6, H24, H72 and D7 after PQ administration (each $n = 5$). **P < 0.01, MMP9 level compared to that at H0. ##P < 0.01 and ###P < 0.001, MMP2 level relative to that at H0.

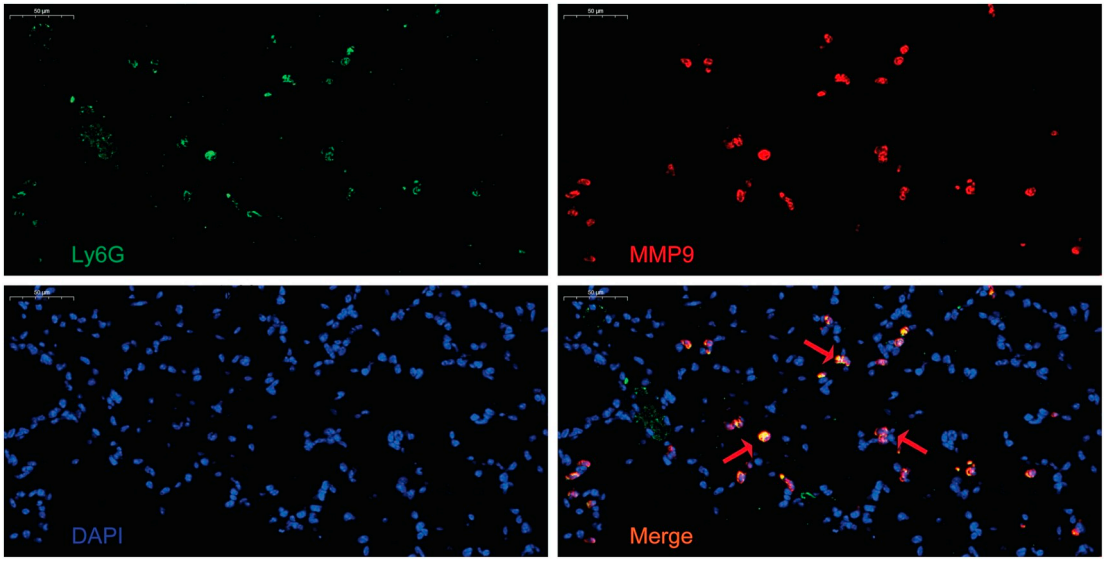

Fig. 4. MMP9 in lung tissues is mainly produced by neutrophils in PQ-induced ALI. Immunofluorescence images show staining of lung tissues from PQ-treated mice with DAPI (blue) or antibodies against neutrophils (green) or MMP9 (red). Red arrowheads indicate representative cells positively stained for both neutrophils and MMP9 (merge). Images were taken at a magnification of ×400. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the late repair and fibrosis phase. MMP9 rather than MMP2 may represent the target for further investigation of the resolution of PQ-induced ALI.

3.3. MMP9 is mainly produced by neutrophils in PQ-induced ALI

Previous studies concluded that during the inflammatory process, a significant portion of MMP9 results from neutrophils [27,37]. To confirm these findings in our PQ-induced ALI model, we performed immunofluorescence costaining of MMP9 and the neutrophil-specific marker Ly6G. In PQ-treated lung tissues, we observed that most of the cells were stained positively for both markers, suggesting that neutrophils were the predominant cellular source of MMP9 produced after PQ administration (Fig. 4).

3.4. Depletion of neutrophils decreases MMP9 activity and alleviates PQ-induced ALI

Ly6G is exclusively expressed on neutrophils and has been reported to deplete neutrophils independently in several studies [27,38]. Using this specific antibody (1A8 clone), we observed an effective depletion of neutrophils in both BALF samples and lung tissues on day 3 in the 1A8-treated PQ group compared to the isotype IgG treatment group (Fig. 5A–B). Additionally, the cell count in BALF was significantly decreased after neutrophil depletion (Fig. 5C). Notably, after neutrophil depletion, the MMP9 activities in both BALF and lung tissues were significantly decreased in the PQ-challenged mice (Fig. 6A). Moreover, compared with IgG treatment, 1A8 treatment reduced the MPO activity (Fig. 6B) and the total protein content in BALF (Fig. 6C), inhibited the increase in inflammatory cytokines (Fig. 6D), and relieved the airspace inflammation measured by the pathological lung injury scores (Fig. 6E).

Taken together, our data suggest that neutrophil depletion decreases MMP9 activity and ameliorates PQ-induced ALI. Thus, neutrophils are pathogenic after PQ administration and contribute to ALI.

3.5. Doxycycline inhibits the excessive expression of MMP9 induced by PQ and ameliorates ALI

We next tested the hypothesis that inhibiting neutrophil-derived MMP9 would phenocopy the improvement in PQ-induced ALI that resulted from neutrophil depletion. DOX, one of the most potent non-specific MMP inhibitor, which acts by degrading pro-MMP zymogen and inhibiting MMP mRNA transcription [39,40], was used in the present work. In the DOX-treated PQ-challenged group, MMP9 activities in both BALF and lung homogenate were significantly lower than those of the nontreatment group, which suggested that DOX is an effective inhibitor of MMP9 in this animal model (Fig. 7A). Interestingly, flow cytometry analysis indicated that the elevated neutrophil proportions in both BALF and lung tissues of the PQ-challenged group were significantly reduced by DOX treatment (Fig. 7B–C). Furthermore, the DOX-treated PQ group also showed effects similar to those of neutrophil depletion, as reflected by the decreased protein concentration of BALF (Fig. 7D), decreased cell number in BALF (Fig. 7E), decreased MPO activity in BALF (Fig. 7F), improvement in inflammatory cytokines in both BALF and serum (Fig. 7G) and finally relieved the airspace inflammation, which was quantified by the pathological lung injury scores (Fig. 7H).

As DOX is a non-specific MMP inhibitor, we also validated our findings using another MMP inhibitor, SB-3CT (Fig. S1). SB-3CT treatment significantly inhibited MMP9 activity and alleviated PQ-induced ALI.

In summary, neutrophil-derived MMP9 plays an important role in the pathogenesis of PQ-induced ALI. Our data show that the MMP inhibitor DOX could also ameliorate PQ-induced ALI.

4. Discussion

In this study, we identified, for the first time, that neutrophils and their product, MMP9, play a critical role in the pathogenesis of PQ-induced ALI. Neutrophil depletion in this animal model effectively reduced the overexpression of MMP9 and alleviated the PQ-induced ALI. Furthermore, we found that treatment with the MMP inhibitor DOX and SB-3CT could also phenocopy the resolution of PQ-induced ALI observed after neutrophil depletion.

The involvement of neutrophils in ALI of different etiology (including sepsis, trauma, multiple transfusion, ischemia-reperfusion, and pancreatitis) has been demonstrated. Neutrophils are considered to play a key role in the progression of ALI and acute respiratory distress syndrome (ARDS). Activation and transmigration of neutrophils is a hallmark event in these respiratory diseases [41]. Consistent with

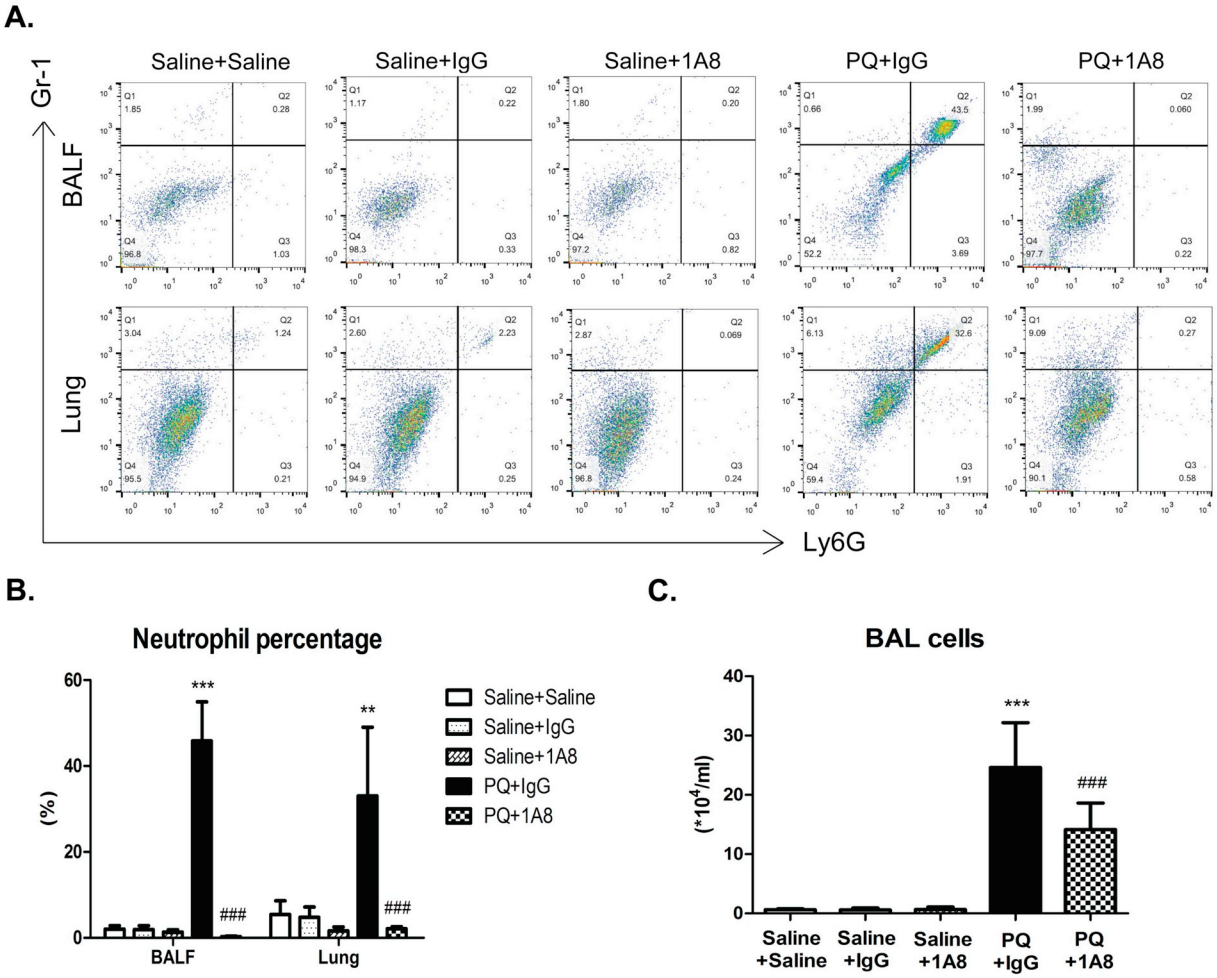

Fig. 5. Ly6G antibody significantly depletes neutrophils in both BALF and lung tissues of PQ-instilled mice. Neutrophils were depleted by intraperitoneal injection of 400 μg of anti-Ly6G antibody (1A8 clone) per mouse one day before PQ instillation and the day after the animal model was established. The control group received 400 μg of rat isotype control IgG at the same time points. (A–B) Depletion of neutrophils in 1A8-treated mice compared with IgG-treated mice was verified by flow cytometric analysis. (C) Lavaged cells were significantly decreased after neutrophil depletion. **P < 0.01 and ***P < 0.001 compared to the Saline + IgG group. ###P < 0.001 compared to the PQ + IgG group. Saline + Saline group: $n = 5$; Saline + IgG group: $n = 5$; Saline + 1A8 group: $n = 5$; PQ + IgG group: $n = 7$; PQ + 1A8 group: $n = 7$.

previous findings [42,43], we observed significantly increased neutrophil proportions in the blood, BALF and lung tissues of our PQ intratracheal aerosolization mouse model (Fig. 2I–K). Additionally, neutrophil depletion in this animal model effectively reduced the percentage of neutrophils induced by PQ almost to the level of the control group in both lung and BALF and alleviated the corresponding ALI.

Recently, a class of substances derived from neutrophils during the inflammatory process was found to play an important role in ALI/ARDS and therefore attracted our attention. Gelatinases, which are known to degrade almost all basement membrane constituents, can change the alveolar-capillary permeability and can also regulate chemokines and cytokines and mediate neutrophil transmigration into the alveolar space. In particular, activated neutrophils secrete significant amounts of MMP9, which is a major elastolytic MMP [44], resulting in a vicious cycle [27,45]. In this study, we found that, consistent with the expression trend of neutrophils, MMP9 but not MMP2 activity increased during the early phase (24 h) of the PQ-induced ALI model and dramatically increased to the peak level at 72 h (Fig. 3). Moreover, neutrophil depletion could also significantly decrease MMP9 but not MMP2 activities in both BALF and lung tissues (Fig. 6A). This finding indicated that MMP9 is involved in the early destructive stage of PQ-induced ALI, resulting in an important positive feedback loop for neutrophil activation and chemotaxis [19].

To validate our findings, we treated mice with the MMP inhibitor SB-3CT and a drug used in clinical practice, DOX [18,24]. DOX is a tetracycline antibiotic widely used in the treatment of infections caused by gram-positive organisms. Consistently, reports suggest that DOX can serve as a small molecule inhibitor of MMP9 [25,40,46,47], independent of its antimicrobial effect [48]. What's more, DOX is the only MMP inhibitor approved by the Food and Drug Administration (FDA) for use in periodontal disease [49]. The inhibition of MMP9 occurs at lower doses than needed for antimicrobial effects and has been clinically utilized in several disease states [50]. Our study indicated that DOX could significantly inhibit the overexpression of MMP9 induced by PQ administration in both lung tissues and BALF. Additionally, DOX reduced neutrophil infiltration and markedly suppressed the levels of proinflammatory cytokines, as well as MPO release. Moreover, DOX also improved the histological characteristics of the lung. Above all, we successfully found that the MMP inhibitor DOX could attenuate PQ-induced ALI in a mouse model.

In conclusion, our findings demonstrate that neutrophils and their product, MMP9, play a crucial role in the pathogenesis of PQ-induced ALI. Therefore, neutrophils and MMP9 might represent novel pharmacological targets for the treatment of PQ-induced ALI. Direct neutrophil depletion therapy has not yet been developed at the clinical level due to

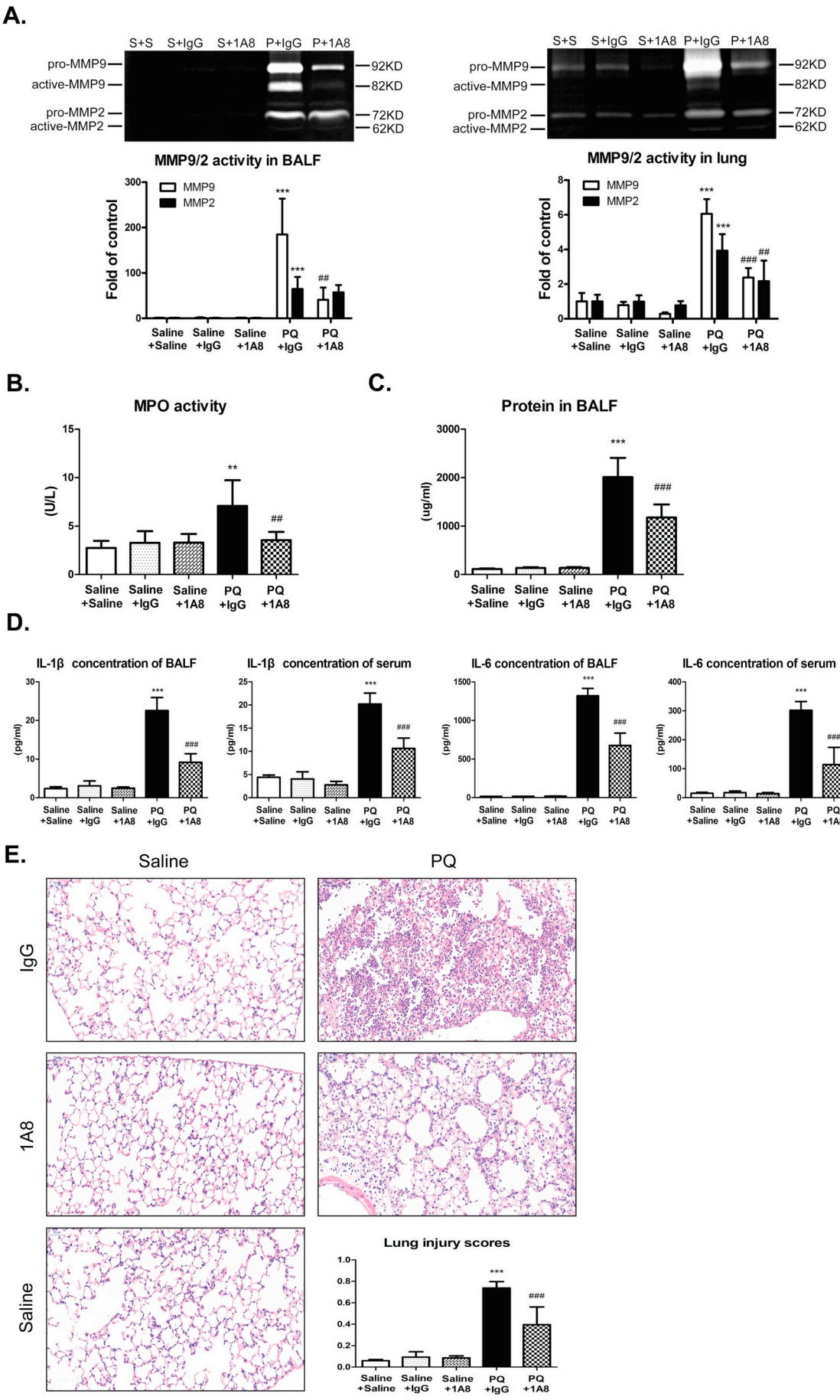

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Fig. 6. Depletion of neutrophils decreases MMP9 activity and alleviates PQ-induced ALI. Neutrophils were depleted by anti-Ly6G antibody, and the following parameters indicating lung injury were evaluated. (A) Neutrophil depletion significantly reduced MMP9 activities in both BALF and lung tissues. (B) The MPO activity in BALF and (C) total protein concentration in BALF were significantly decreased after neutrophil depletion. (D) The levels of the proinflammatory cytokines IL-1β and IL-6 in both BALF and serum were reduced significantly. (E) After neutrophil depletion, the mouse lung sections had less airspace inflammation, interstitial edema was alleviated, and the lung injury scores were significantly lower than those of the IgG-treated PQ group. Original magnification: ×200. **P < 0.01 and ***P < 0.001 compared to the Saline + IgG group. ##P < 0.01 and ###P < 0.001 compared to the PQ + IgG group.

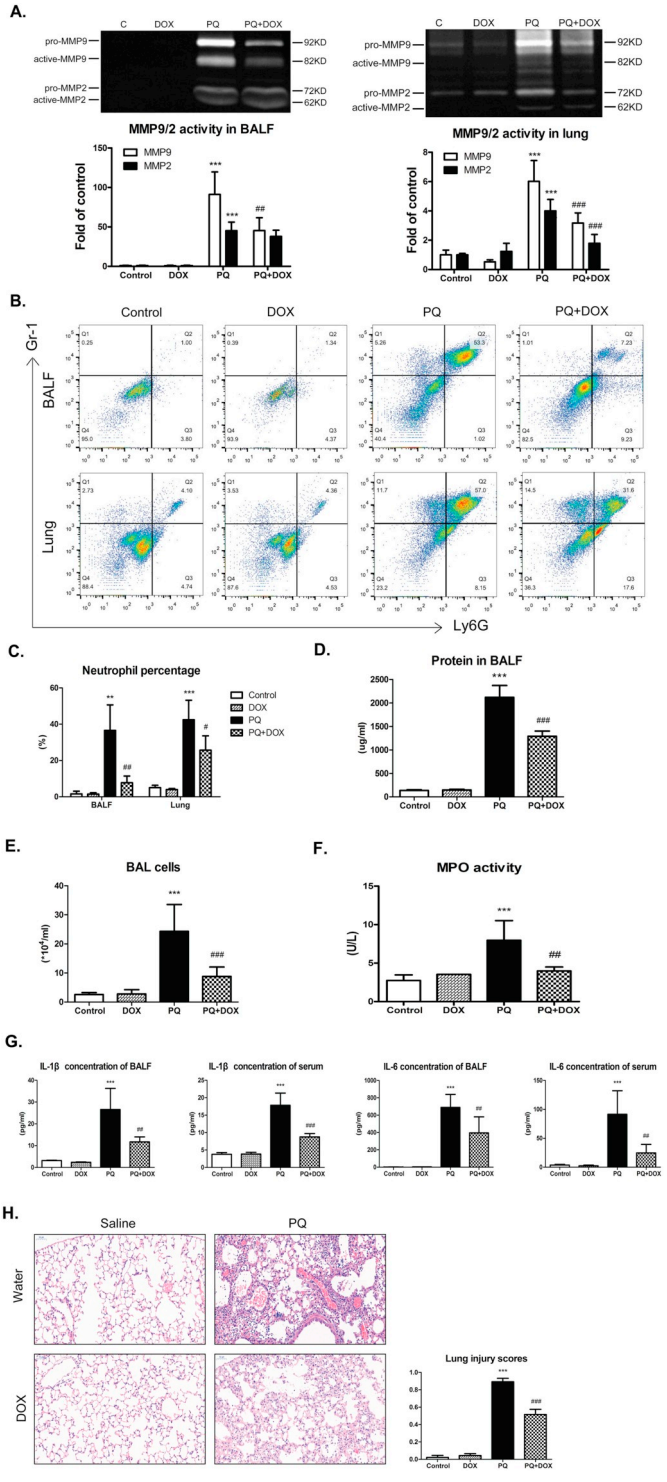

Fig. 7. The MMP inhibitor DOX ameliorates PQ-induced ALI. Mice were gavaged with DOX (20 mg/kg/day) three days before PQ administration, and the treatment continued daily until the day before harvest day. The control group was gavaged with the same volume of water. MMP9 activity and parameters indicating lung injury were evaluated. (A) MMP9 activities in both BALF and lung tissues were significantly reduced in DOX-treated mice compared with PQ-instilled mice. (B) Neutrophil proportions after DOX treatment in both BALF and lung were assessed by flow cytometric analysis, and the quantitative data (C) showed significantly lower values than those in PQ-instilled mice. (D) Total BALF protein levels and (E) lavaged cells in the BALF were significantly decreased after DOX treatment. (F) The MPO activity in BALF and (G) the levels of proinflammatory cytokines were also significantly lower after DOX treatment. (H) HE-stained lung sections showed the resolution of airspace inflammation and the decreased histological lung injury scores in DOX-treated PQ-instilled mice compared with PQ-instilled mice. Original magnification: ×200. **P < 0.01 and ***P < 0.001 compared to the Saline control group. #P < 0.05, ##P < 0.01, and ###P < 0.001 compared to the PQ group. Control group: $n = 5$; DOX group: $n = 5$; PQ group: $n = 7$; PQ + DOX group: $n = 7$.

the high risk of infection caused by immunodeficiency. We expect that the MMP inhibitor DOX will be an effective therapeutic strategy that can address neutrophil pathogenicity in PQ poisoning patients. Moreover, since current therapies for PQ poisoning, including antioxidant, anti-inflammatory and glucocorticoid treatments, have been demonstrated to be less effective than desired, combination therapy with novel MMP9 inhibitors based on DOX may provide new research directions or future therapeutic strategies.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.04.015.

Acknowledgements

This work was supported by National Natural Science Foundation of China (81772057, 81671899), Natural Science Found of Jiangsu Province (BK20171500), Program for Innovative Medical Research Team of Jiangsu Province (CXTDA2017007), Jiangsu Province's key provincial talents program (QNRC2016597).

Declaration of interest

The authors declare that there are no conflicts of interest.

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