

Protectin DX Exhibits Protective Effects in Mouse Model of Lipopolysaccharide-Induced Acute Lung Injury

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Abstract

Background: Acute lung injury (ALI) is a severe disease with high mortality and poor prognosis. Protectin DX (PDX), a pro-resolving lipid mediator, exhibits protective effects in ALI. Our experiment aimed to explore the effects and related mechanisms of PDX in mice with ALI induced by lipopolysaccharide (LPS).

Methods: BALB/c mice were randomly divided into five groups: sham, LPS, LPS plus 1 ng of PDX (LPS + PDX-1 ng), LPS plus 10 ng of PDX (LPS + PDX-10 ng), and LPS plus 100 ng of PDX (LPS + PDX-100 ng). Bronchoalveolar lavage fluids (BALFs) were collected after 24 h, and total cells, polymorphonuclear leukocytes, monocyte-macrophages, and lymphocytes in BALF were enumerated. The concentration of interleukin (IL)-1 β , IL-6, IL-10, tumor necrosis factor- α (TNF- α), macrophage inflammatory protein (MIP)-1 α , and MIP-2 in BALF was determined, and histopathological changes of the lung were observed. The concentration of protein in BALF and lung wet/dry weight ratios were detected to evaluate pulmonary edema. After determining the optimal dose of PDX, neutrophil-platelet interactions in whole blood were evaluated by flow cytometry.

Results: The highest dose of PDX (100 ng/mouse) failed to provide pulmonary protective effects, whereas lower doses of PDX (1 ng/mouse and 10 ng/mouse), especially 1 ng PDX, alleviated pulmonary histopathological changes, mitigated LPS-induced ALI and pulmonary edema, inhibited neutrophil infiltration, and reduced pro-inflammatory mediator (IL-1 β , IL-6, TNF- α , and MIP-1 α) levels. Meanwhile, 1 ng PDX exhibited pro-resolving functions in ALI including upregulation of monocyte-macrophage numbers and anti-inflammatory mediator IL-10 levels. The flow cytometry results showed that PDX could inhibit neutrophil-platelet interactions in ALI.

Conclusion: PDX exerts protective effects in LPS-induced ALI by mitigating pulmonary inflammation and abrogating neutrophil-platelet interactions.

Key words: Acute Lung Injury; Blood Platelets; Lipopolysaccharide; Neutrophils; Protectin DX

INTRODUCTION

Acute lung injury (ALI) can be triggered by many infectious and noninfectious factors including trauma, blood transfusion, and pancreatitis; however, researches had shown that bacterial infection is the leading cause of ALI.^[1,2] On average, 86.2 out of 100,000 people acquire ALI in the US.^[3]

The pathophysiological characteristics of ALI are the destruction of pulmonary and vascular endothelia, leading to increased pulmonary permeability, platelet activation, coagulation impairment, as well as neutrophil and platelet aggregation.^[4-8] Recent research has shown that platelets

exhibit critical functions in inflammation, especially at the early stage neutrophil-mediated inflammation.^[9] The platelets implanted at the site of damaged vascular

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endothelium interact with neutrophils, accelerating the aggravation of neutrophils and platelets, which leads to further neutrophil recruitment.^[10] In addition, a large number of neutrophil–platelet complexes were found in the circulation system during infections, resulting in multiple organ dysfunction.^[11] In transfusion-associated ALI, a lack of neutrophils or platelets can mitigate ALI.^[12] Therefore, neutrophil activation and neutrophil-platelet interactions play an important role in ALI.

It has been reported that specific pro-resolving mediators (SPMs) partly exert their anti-inflammatory and pro-resolving functions by regulating immune cells including neutrophils and platelets. Protectin DX (PDX), a member of SPM, has potential anti-inflammatory and inflammation pro-resolving effects.^[13] Liu *et al.* indicated that PDX mitigated reactive oxygen species production, inhibited degranulation of neutrophils, and reduced the release of myeloperoxidase induced by formyl-methionine-leucine-phenylalanine and phorbol ester.^[14] Recent studies have shown that PDX increased the survival rate of cecum ligation and puncture-induced sepsis in mice while promoting the transformation of M1 macrophages into M2 macrophages.^[15] In addition, in bleomycin-induced pulmonary fibrosis, PDX ameliorated lung dysfunction and fibrosis through epithelial–mesenchymal transition.^[16] However, research on PDX in ALI is limited, and the related mechanisms of PDX in pulmonary inflammation resolution remain elusive.

Therefore, we performed this study to investigate whether PDX inhibits pulmonary inflammation and exerts protective effects in lipopolysaccharide (LPS)-induced ALI and explored the potential mechanisms.

METHODS

Animal preparation and ethical approval

BALB/c mice were purchased from Hua Fu Kang Co. (Beijing, China). All mice were male, 6–8 weeks, weighed 20–25 g, and were raised in specific pathogen-free conditions for 3 days before experimentation. All animal experiments were authorized by the Animal Care and Use Committee of Tongji Medical College of Huazhong University of Science and Technology. All animal studies have been reported in accordance with the ARRIVE guidelines for reporting experiments involving animals.^[17,18]

Experiment protocol

The mice were randomly divided into five groups ($n = 6$ per group): sham, LPS, LPS plus 1 ng of PDX (LPS + PDX-1 ng), LPS plus 10 ng of PDX (LPS + PDX-10 ng), and LPS plus 100 ng of PDX (LPS + PDX-100 ng). All mice were anesthetized intraperitoneally with 45 mg/kg of 2% pentobarbital sodium. Then, the mice received an intratracheal instillation of LPS (from *Escherichia coli* serotype O55:B5; Sigma-Aldrich Co., St. Louis, MO, USA) at a dose of 3 mg/g. Mice in the sham group

received only saline (1.5 ml/kg). Two hours later, the mice were administered with saline or PDX (1 ng, 10 ng, or 100 ng) (Cayman Chemical, Ann Arbor, MI, USA) through tail vein. Mice were sacrificed 24 h after LPS instillation.

Histological analysis of lung tissues

The middle lobe of the right lung was fixed in 4% paraformaldehyde and completely embedded in paraffin. Lungs were cut into sections, stained with hematoxylin and eosin, and observed using optical microscopy. Lung injury pathological scores were measured according to previous study.^[5]

Total leukocyte counts and differential leukocyte counts in bronchoalveolar lavage fluid

In a segregated series of studies, bronchoalveolar lavage fluid (BALF) was collected by lavaging the left lung (0.5 ml \times 3 times). The BALF was centrifuged for 10 min at 1200 r/min. The supernatant was removed and stored at -80°C for further detection. Then, red blood cell lysis buffer was added to the pellet to wipe out the red blood cells. Total BALF cells were measured using a hemocytometer. The remaining BALF cells were stained with Wright-Giemsa staining. Differential leukocyte counts were quantified by optical microscopy. A total of 200 cells were counted.

Evaluation of pulmonary edema

Pulmonary edema was assessed by detecting the protein concentration in BALF and wet/dry (W/D) weight ratios. The protein concentration in BALF was determined using a BCA Protein Assay Kit according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, US). The upper lobes of the right lung were harvested, weighed, and placed in an oven at a temperature of 60°C for 5 days to evaluate the W/D weight ratios. The dry lungs were weighed, and the W/D weight ratio was calculated.

Inflammatory cytokines' analysis in bronchoalveolar lavage fluid

The concentrations of interleukin (IL)-1 β , IL-6, IL-10, tumor necrosis factor- α (TNF- α), macrophage inflammatory protein (MIP)-1 α , and MIP-2 in BALF were determined using an enzyme-linked immunosorbent assay (ELISA) kit (RayBiotech Inc., Norcross, GA, USA) to assess pulmonary inflammation.

Assessing neutrophil–platelet interactions

After an optimal dose of PDX was identified, the mice were randomly divided into three groups: (1) sham group: mice were instilled with 0.9% saline intratracheally and then administered 0.9% saline intravenously 2 h later; (2) LPS group: mice received intratracheal instillation with LPS (3 mg/kg) and were then administered 0.9% saline intravenously 2 h later; and (3) LPS + PDX group: mice received intratracheal instillation with LPS (3 mg/kg) and were then administered with 1 ng PDX intravenously 2 h later. Mice were anesthetized and sacrificed 24 h after LPS instillation. Whole blood was collected and processed, which

included red blood cell lysis and blocking with CD16/32 Fc, and the cells were then stained with GR-1, CD62P, or CD41 (eBioscience). Finally, neutrophil–platelet interactions were evaluated by flow cytometry.

Statistical analysis

All statistical analyses were performed using Excel (microsoft office, USA) and GraphPad Prism 6 (GraphPad Software, USA). Data were presented as the mean \pm standard deviation. All data were tested by normality tests. Data were analyzed using one-way analysis of variance with a Bonferroni *post hoc* test for multiple *t*-tests. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Protectin DX alleviated pulmonary histopathological changes during acute lung injury

The sham group showed normal lung tissue with a few macrophages in the alveolar space, and neither interstitial thickening nor the presence of leukocytes in alveolar space was observed under optical microscopy [Figure 1a]. Compared with the sham group, the other groups showed damaged lung tissue; in particular, LPS caused substantial changes in pulmonary architecture. There was a large number of neutrophils in the alveolar space and mesenchyme; in addition, protein debris and hyaline membranes were occasionally observed in the alveolar space [Figure 1b]. In the LPS + PDX-1 ng group, the infiltration of neutrophils in the alveolar space and mesenchyme was obviously decreased, and interstitial thickening was not observed [Figure 1c]. In the LPS + PDX-10 ng group, there was slight lung tissue damage and mildly decreased infiltration of neutrophils [Figure 1d]. In the LPS + PDX-100 ng group, there were no obvious histopathological changes compared with the LPS group [Figure 1e]. Lung injury scores were consistent with lung histopathological changes [Figure 1f]. Therefore, there

were significantly lower pathological scores in ALI mice administrated with 1 ng PDX.

Protectin DX inhibited infiltration of leukocytes, especially neutrophils, into the alveolar space in acute lung injury mice

The cells were almost exclusively alveolar macrophages in the sham group. When the mice received an intratracheal instillation of LPS, the total leukocyte counts in BALF increased significantly. After mice were treated with a lower dose of PDX (1 ng or 10 ng), total leukocyte counts decreased. However, the high dose of PDX (100 ng) failed to reduce the total leukocyte infiltration [Figure 2a]. As shown in Figure 2b and 2c, the neutrophil counts were higher and the number of monocytes/macrophages was lower in BALF in the LPS group than in the sham group. PDX, especially at 1 ng/mouse, significantly reduced neutrophil infiltration into the alveolar space and increased monocyte/macrophage counts in BALF.

Protectin DX stabilized pulmonary permeability in acute lung injury mice

The protein concentration in BALF and W/D ratios are two indicators of pulmonary permeability. As shown in Figure 3a, the protein concentration in BALF increased after the intratracheal instillation of LPS. When treated with PDX (1 ng or 10 ng), the protein concentration dramatically decreased. Figure 3b shows the variation of pulmonary edema in different groups. The W/D ratio in the LPS group increased in comparison to the sham group. The result showed that 1 ng PDX dramatically decreased the W/D ratio in mice with ALI.

Protectin DX facilitated the resolution of inflammation in acute lung injury mice

There are various cytokines involved in the process of acute inflammation. We used an ELISA to determine the concentration of different cytokines in each group to detect

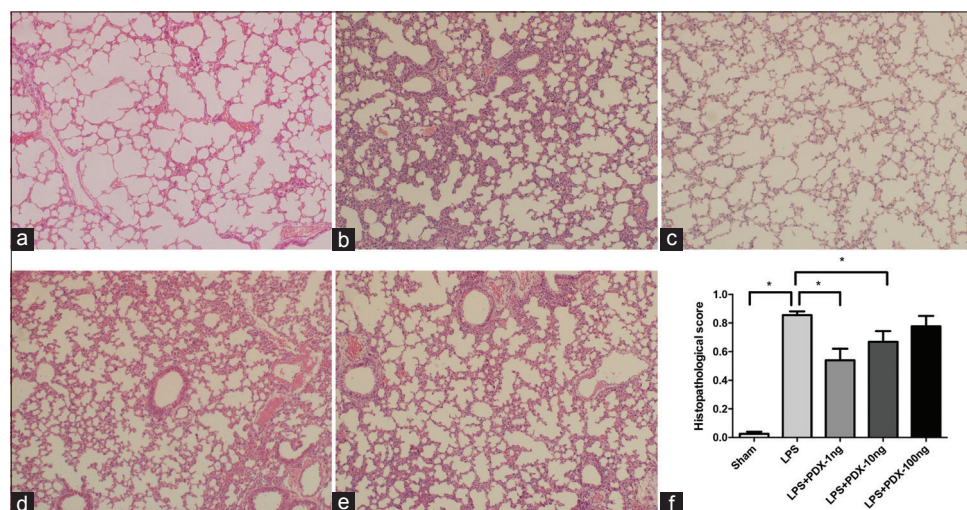


Figure 1: PDX alleviated pulmonary histopathological changes during ALI. (a) Sham group; (b) LPS group; (c) LPS + PDX-1 ng group; (d) LPS + PDX-10 ng group; (e) LPS + PDX-100 ng group; lung sections were stained with hematoxylin-eosin, $\times 100$. (f) Histopathological score. Data are mean \pm SD. $n = 6$. $*P < 0.0001$. SD: Standard deviation; PDX: Protectin DX; ALI: Acute lung injury; LPS: Lipopolysaccharide.

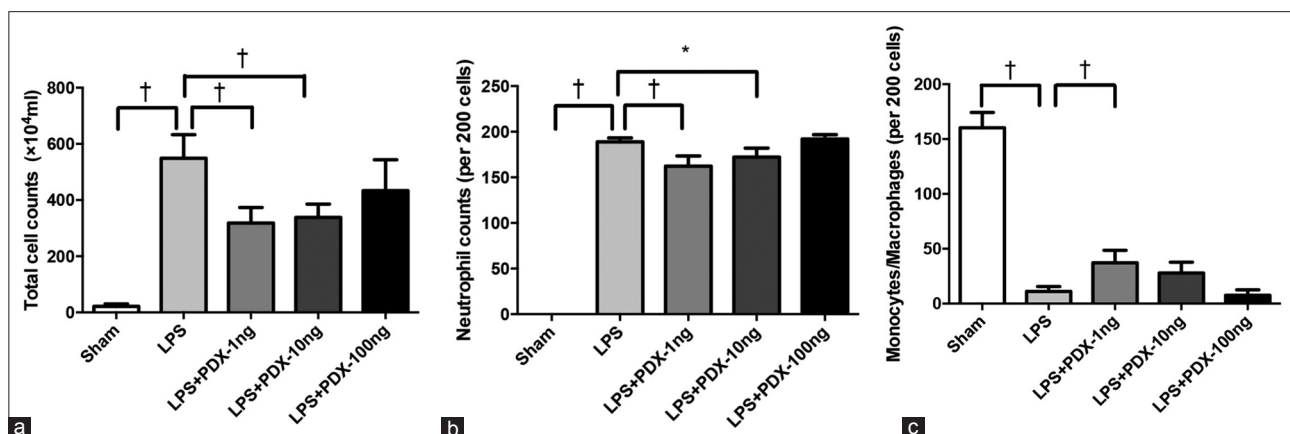


Figure 2: PDX inhibited leukocyte infiltration, especially neutrophil infiltration, into the alveolar space in ALI mice. (a) Total leukocyte counts in BALF; (b) neutrophil counts in BALF; (c) monocyte/macrophage counts in BALF. Data are mean \pm SD. $n = 6$. * $P < 0.01$, † $P < 0.0001$. SD: Standard deviation; BALF: Bronchoalveolar lavage fluid; PDX: Protectin DX; ALI: Acute lung injury.

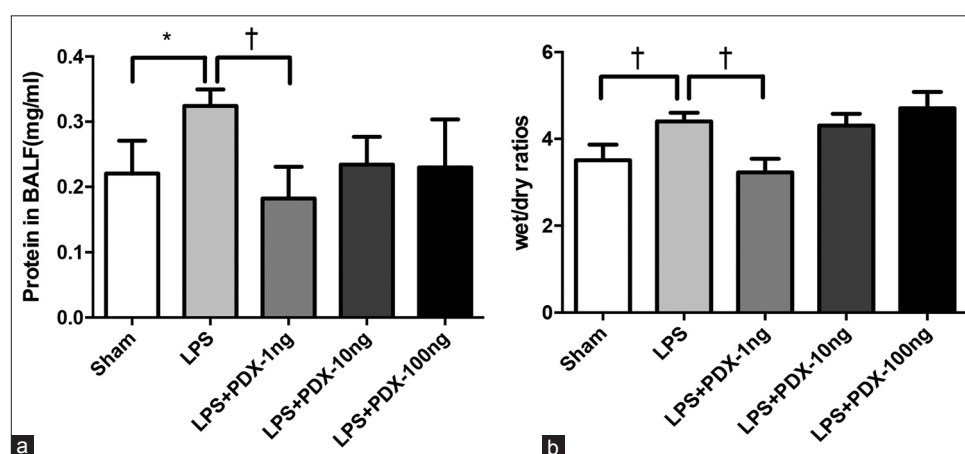


Figure 3: PDX stabilized pulmonary permeability in ALI mice. Only 1 ng/mouse of PDX mitigated the permeability. (a) Protein concentration in BALF; (b) wet/dry ratio. Data are mean \pm SD. $n = 6$. * $P < 0.01$, † $P < 0.0001$. SD: Standard deviation; BALF: Bronchoalveolar lavage fluid; PDX: Protectin DX; ALI: Acute lung injury.

the effects of PDX in ALI. In contrast to the sham group, the concentration of pro-inflammatory cytokines IL-1 β , TNF- α , IL-6, MIP-1 α , and MIP-2 in BALF were upregulated in the LPS group. When the mice were treated with 1 ng/mouse of PDX, the concentrations of IL-1 β , TNF- α , IL-6, and MIP-1 α in BALF were reduced, and the concentration of anti-inflammatory cytokine IL-10 was increased. However, intravenous injection of PDX (10 ng or 100 ng) did not change inflammatory cytokine secretion in comparison to secretion in the LPS group [Figure 4].

Protectin DX inhibited neutrophil–platelet interactions in acute lung injury

Neutrophils were stained with APC-Ly6G, and platelets were stained with FITC-CD41 to detect the interactions of neutrophils and platelets. We applied flow cytometry analysis to evaluate the proportion of Ly6G(+) CD41(+) cells and determine the interactions of neutrophils and platelets. As shown in Figure 5a, the proportion of Ly6G(+) CD41(+) cells in the LPS group was much higher than the proportion in the sham group. However, the LPS + PDX group showed a lower proportion of Ly6G(+) CD41(+) cells than the LPS

group. CD62P is mainly expressed on activated endothelium and platelets, playing a critical role in the interactions of neutrophils and platelets. Next, we used flow cytometry analysis to determine the proportion of Ly6G(+) CD62P(+) cells to evaluate the interactions between neutrophils and platelets. The result in Figure 5b indicated that the double positive cells were obviously increased in the LPS group compared to the cells in the sham group. After intravenous injection of PDX (1 ng/mouse), the Ly6G(+) CD62P(+) double positive cells in the LPS group were decreased. Figure 5c and 5d is the statistical graphs of Figure 5a and 5b.

DISCUSSION

In this study, we confirmed that PDX exerted dose-dependent pulmonary protection in ALI mice induced by LPS. PDX promoted inflammation resolution within a therapeutic window. It was found that 100 ng of PDX failed to provide protective effects, whereas lower doses of PDX (1 ng or 10 ng), especially a dose of 1 ng/mouse of PDX, mitigated pulmonary histopathological changes, inhibited neutrophil infiltration, alleviated permeability, reduced

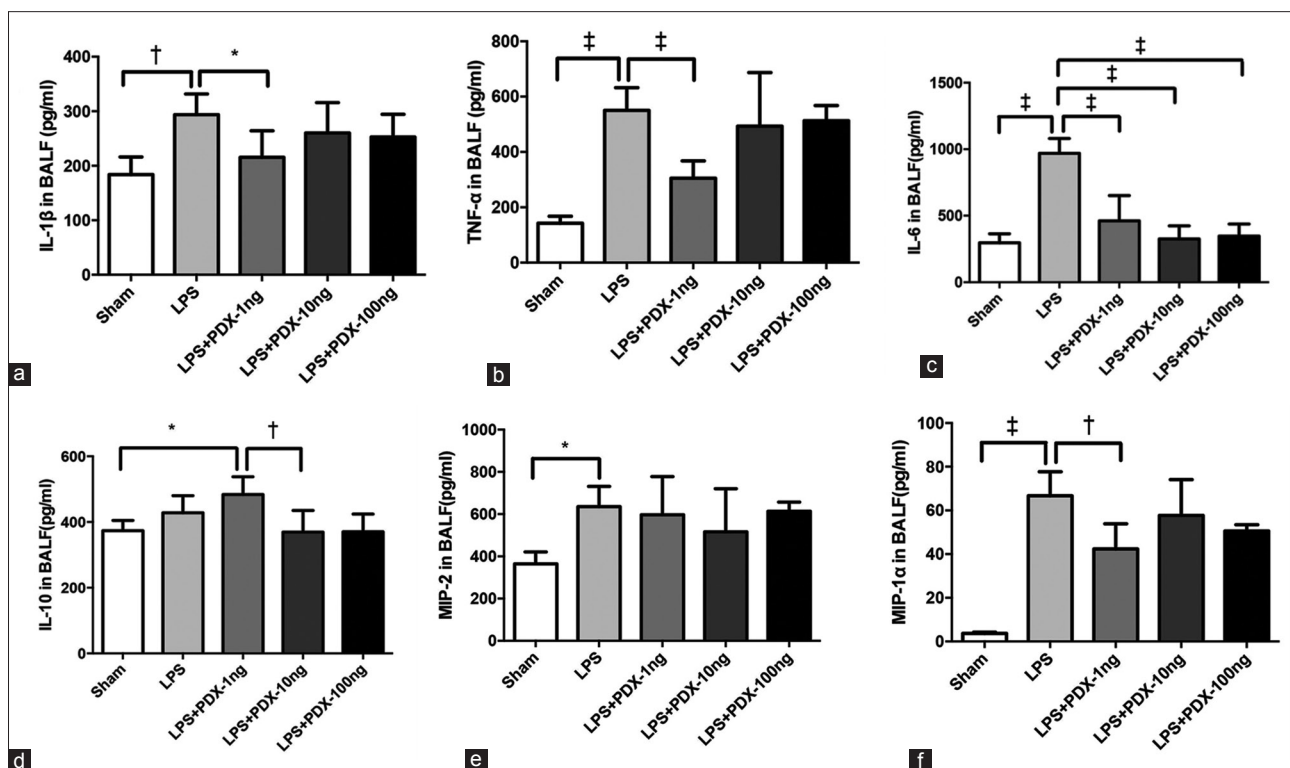


Figure 4: PDX facilitated inflammation resolution in ALI. (a) Concentration of IL-1 β ; (b) concentration of TNF- α ; (c) concentration of IL-6; (d) concentration of IL-10; (e) concentration of MIP-2; (f) concentration of MIP-1 α . Data are mean \pm SD. $n = 6$. * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.0001$. SD: Standard deviation; PDX: Protectin DX; ALI: Acute lung injury; IL: Interleukin; TNF- α : Tumor necrosis factor- α ; MIP-2: Macrophage inflammatory protein.

inflammatory cytokines, upregulated anti-inflammatory cytokines, and abrogated neutrophil–platelet interactions in LPS-induced ALI.

According to the previous study, PD1 inhibited the infiltration of neutrophils in LPS-induced peritonitis.^[19] Moreover, PD1 enhanced macrophage phagocytosis, increased lymphocyte filtration, and downregulated the release of various cytokines.^[20] PD1 was also reported to strikingly reduce allergic pulmonary inflammation when used in nanogram quantities (2–200 ng).^[21] As an isomer of PD1, PDX might exhibit similar protection in mice suffering from pulmonary inflammation. Therefore, in our study, we chose PDX administration at a concentration ranging from 1 to 100 ng/mouse.

The results of our research showed that LPS compromised the integrity of the pulmonary architecture and formed hyaline membranes, additionally lead to the formation of protein-rich edema and hemorrhage. The increased protein concentration in BALF combined with increased lung W/D ratio appropriately reflects the hyperpermeability of the pulmonary barrier. Our study also demonstrated that lower dose of PDX (1 ng or 10 ng) reduced total cell numbers in lung exudation, mitigated neutrophil infiltration and that the 1 ng PDX was more effective. These results were consistent with pulmonary histopathological changes. In addition, we confirmed that 1 ng/mouse of PDX alleviated pulmonary permeability by mitigating pulmonary edema. However, the

number of monocytes/macrophages showed a reversed trend. In mice treated with PDX (1 ng/mouse), we found that the number of monocytes/macrophages in the LPS + PDX-1 ng group was higher than that in the LPS group. Based on the elevated monocytes/macrophages counts, we speculate that monocytes/macrophages may participate in inflammation resolution. However, the corresponding mechanism warrants further investigation.

The analysis of cytokines' production in BALF suggested that 1 ng PDX inhibited the secretion of pro-inflammatory factors, such as IL-1 β , IL-6, TNF- α , and MIP-1 α , increased the release of anti-inflammatory cytokine IL-10 and had no influence on MIP-2. It was reported that MIP-1 α and MIP-2 belong to the superfamily of chemokines. They perform crucial effects on cell proliferation, neutrophil recruitment, and enhanced pro-inflammatory cytokines' release.^[22–24] In addition, MIP-1 α was mainly produced by hematopoietic cells (such as monocytes, macrophages, T lymphocytes, and B lymphocytes), and MIP-2 was secreted by both hematopoietic cells and nonhematopoietic cells.^[25] Therefore, a possible reason why PDX could not regulate the release of MIP-2 may be associated with its binding on selective or specific cells.

In a murine postoperative ileus (POI) model, PDX was released and modulated neutrophil extravasation, exhibiting anti-inflammation to cure POI.^[26] Although there is no report on the protective effect of PDX on ALI, a recent study

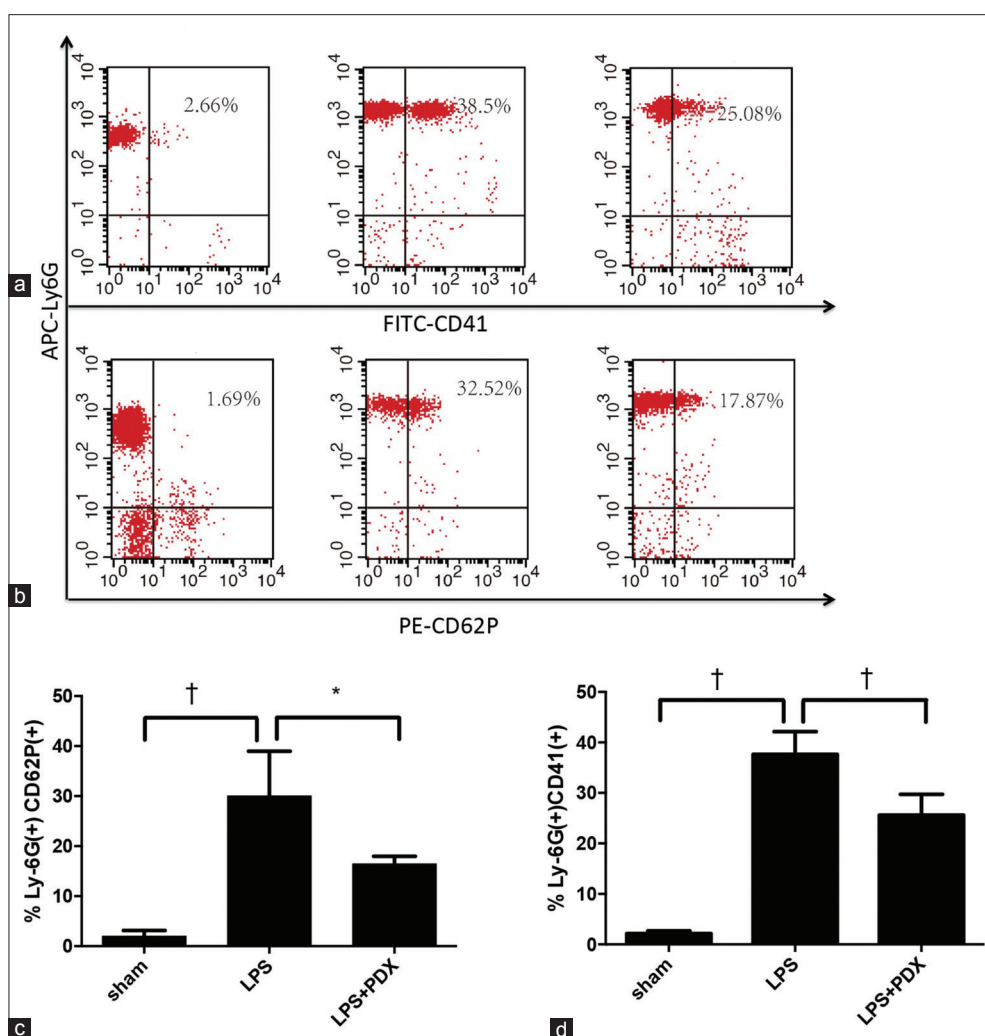


Figure 5: PDX inhibited neutrophil–platelet interactions in ALI. (a) Ly-6G and CD41; (b) Ly-6G and CD62P; (c) percentages of Ly-6G(+) CD41(+) cells; (d) percentages of Ly-6G(+) CD62P(+) cells. Data are mean \pm SD. $n = 6$. * $P < 0.01$, † $P < 0.0001$. SD: Standard deviation; PDX: Protectin DX; ALI: Acute lung injury.

has demonstrated that, in bleomycin-induced pulmonary fibrosis, PDX inhibited inflammatory cells' infiltration and extracellular matrix deposition and mitigated respiratory dysfunction.^[16] Some of these data are consistent with our results.

Neutrophils were reported to be key cells participating in the process of pulmonary inflammation,^[27,28] so the interactions between neutrophils and platelets may have a critical role in inflammation. As shown in the data above, we set the concentration of PDX at 1 ng/mouse as an optimal dose. CD41 is a specific membrane marker of platelets, whereas CD62P is mainly expressed on activated vascular endothelium or platelets and displays a significant impact on neutrophils–platelets or platelets–endothelium interactions.^[29] In our study, we showed that PDX could inhibit the interaction of neutrophils and platelets and restrain CD62P independent aggregation of neutrophils and platelets.

A limitation of our study is that we focused our studies on pro-inflammatory cytokines, inflammatory cells, and interaction of neutrophil and platelet, as the inflammation

resolution is a comprehensive process, we did not explore other mechanisms. In addition, we found that PDX promoted the macrophages' counts; however, the changes of function in macrophages and polarization of macrophages were not investigated, and they will be determined in our future studies.

In summary, PDX exhibits protective effects in ALI induced by LPS by mitigating pulmonary histopathological changes, inhibiting leukocyte infiltration, alleviating pulmonary epithelial–endothelial permeability, reducing inflammatory cytokines, and inhibiting neutrophil–platelet interactions.

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Conflicts of interest

There are no conflicts of interest.

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保护素DX对脂多糖诱导的急性肺损伤的保护效应

摘要

背景: 急性肺损伤 (ALI) 在临床上死亡率高预后差。保护素DX (PDX), 一种促炎症消退介质, 发挥着对急性肺损伤的保护效应。本实验主要探讨PDX在LPS诱导的急性肺损伤中的作用及其相关机制。

方法: BALB/c小鼠被随机分到5个组: sham组, LPS组, LPS+1ng PDX组 (LPS+PDX-1ng), LPS+10ng PDX组 (LPS+PDX-10ng), LPS+100ng PDX组 (LPS+PDX-100ng)。24小时后收集小鼠支气管肺泡灌洗液 (BALF), 计数BALF中总细胞数、中性粒细胞数、单核细胞-巨噬细胞数和淋巴细胞数。测定BALF中炎症因子IL-1 β , IL-6, TNF- α , IL-10, MIP-1a和MIP-2。观察肺病理学变化, 测定BALF中蛋白浓度和肺组织湿/干重比用于评价肺水肿。以此确定PDX的最佳剂量, 并用流式细胞术分析中性粒细胞-血小板相互作用。

结果: 高剂量PDX (100ng/只) 不能发挥对肺的保护效应, 然而低剂量的PDX (1 ng/只, 10 ng/只), 尤其是1 ng PDX, 能减轻肺病理改变, 减缓LPS诱导的急性肺损伤和肺水肿, 抑制中性粒细胞渗出, 下调促炎因子 (IL-1 β , IL-6, TNF- α 和MIP- α)。同时1 ng PDX在急性肺损伤中发挥促炎症消退的作用, 包括上调单核-巨噬细胞比例和抗炎因子IL-10。流式细胞术结果显示PDX能抑制急性肺损伤中性粒细胞-血小板相互作用。

结论: PDX通过减轻肺部炎症和中性粒细胞-血小板相互作用发挥对LPS诱导的急性肺损伤的保护效应。