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Original Article

PirB protects rat against hyperoxia-induced acute lung injury through inhibiting inflammation

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Abstract: PirB plays an important role in the immune system and is associated with bacterial infections and TNF-α expression. However, the contribution of pulmonary PirB to hyperoxic-induced acute lung injury (HALI) remains undefined. In this study, we established hyperoxic-induced acute lung injury model in rats and used hematoxylin and eosin (H&E) staining for lung injury assessment. Pulmonary PirB expression was determined by Western blotting and immunochemistry staining at different time points after hyperoxic treatment. Intravenously injection of p-PirB/cationic liposome complex was employed to over-expression of PirB in the lungs with HALI in rats. The lung injury and related cytokine expression was assessed by H&E staining and ELISA separately. Our results indicated that pulmonary PirB expression was significantly suppressed in the rats with HALI. Intravenously injection of p-PirB/cationic liposome complex dramatically increased PirB expression in the lungs, prolonged the survival time and ameliorated lung injury of rats with HALI. The related pro-inflammatory cytokine IL-6 and TNF-α expression in the lungs was significantly down-regulated by PirB. Collectively, our study suggested that PirB has a protective role against HALI and provided a novel target for HALI therapy.

Keywords: PirB, lung injury, hyperoxia

Introduction

Oxygen therapy with oxygen concentration >60% is a common medical intervention to critical care patients in order to improve the arterial partial pressure of oxygen and satisfy tissue oxygen demands [1, 2]. However, overexposure to high oxygen concentrations may result in serious lung damages due to the oxidative stress [3]. Hyperoxic-induced acute lung injury (HALI), a major subtype of lung damages resulting from oxygen poisoning, can develop severe respiratory failure and death [4]. Thus, it is necessary and significance to provide novel target for HALI therapy.

Paired immunoglobulin-like receptors A (PirA) and paired immunoglobulin-like receptors B (PirB) comprise paired immunoglobulin-like receptors (PIRs) family, which are predominantly expressed by myeloid cells endowing them with the potential to discriminate self from non-self [5]. PIRs are orthologues of the human immunoglobulin-like transcript (ILT)/leukocyte Ig-like receptor (LIR) family of receptors, which are expressed predominantly by myeloid cells in a pair-wise fashion [6, 7]. Comprehensive structural and biochemical analyses have shown that the amino acid sequences of the PirA and PirB ectodomains are over 92% identical and bind the same MHC-I ligands [8, 9]. While PirB has an important role in the immune system [10], it was also known to be expressed by neurons, present in neuronal growth cones, and associated with synapses and other diseases [11-14]. Meanwhile, recent data suggest an intricate link between PirB and TNF-α in bacterial infections [6, 15]. However, the contribution of pulmonary PirB to HALI remains undefined.

In this study, we report that pulmonary PirB expression was significantly reduced in the lungs of a hyperoxia-induced acute lung injury rat model. Over-expression of PirB in the lungs after injection with p-PirB/lipoplexes complex significantly prolonged survival and attenuated

the severity of lung injury in HALI rats. Furthermore, we found that PirB may exert its effects by inhibiting the secretion of pro-inflammatory cytokine TNF-α and IL-6.

Material and methods

Animal study

Animal care and experimental manipulation were approved by the Institutional Committee on Animal Care and Use of Sichuan University. Sprague-Dawley rats with a weight range from 200 to 230 g were obtained from Animal Center of Sichuan University (Chengdu, China) and allowed to acclimate to new environment for 2 weeks. The rats were given free access to standard rodent food and tap water. After acclimation, the animals were exposed to high levels of oxygen (hyperoxia group; n=5). Another group served as control exposed to a normal level of oxygen (normoxia group). Exposure to hyperoxia was performed in an airtight plastic chamber with flow rates of oxygen around 5.0 l/min that maintained ≥95% oxygen over the course. The oxygen level was constantly monitored with an oxygen sensor. To control the exhaled carbon dioxide levels lower than 0.5%, granular soda lime was used in the chamber.

Histology

Rat lungs were embedded in paraffin and processed for histological analysis. Lung sections were stained with hematoxylin and eosin (H&E) and subjected to lung injury assessment. The existence of alveolar edema/exudates, hemorrhage, and interstitial/alveolar cellular infiltration were determined. PirB expression was examined by immunohistochemistry using a rabbit anti-rat PirB antibody (1:50 dilution; Merck Millipore, MA, USA). The slides were subsequently incubated with a 1:200 dilution of biotin-conjugated goat anti-rabbit secondary antibody for 15 min at 37°C and streptavidin-biotin complex at 37°C for 15 min (IHC kit SP9001, Zsbio, Beijing China). The immunoreaction was visualized by using diaminobenzidine (DAB, Maixin Bio, Fuzhou, China) peroxide solution and cellular nuclei were counterstained with hematoxylin (Beyotime, Beijing, China). All specimens were evaluated using Olympus B × 600 microscope and Spot Fiex camera.

Cytokines measurement

The frozen lungs were dissolved and boiled in 1 ml RIPA lysis buffer (Beyotime, Beijing, China) with 10 μl protease inhibitor cocktail (Merck Millipore, MA, USA) for 30 min. Then the lysis buffer was collected after centrifugation (12000 g) for 15 min at 4°C. The protein level was determined with BCA kit (Beyotime, Beijing, China). The levels of tumor necrosis factor-α (TNF-α) and Interleukin-6 (IL-6) in the lung of rat were determined by ELISA (NeoBioscience, Beijing, China) following standard protocols.

Western blotting

The frozen lungs were dissolved and boiled in 1 ml RIPA lysis buffer (Beyotime, Beijing, China) with 10 μl protease inhibitor cocktail (Merck Millipore, MA, USA) for 30 min. Then the lysis buffer was collected after centrifugation (12000 g) for 15 min at 4°C. The protein level was determined with BCA kit (Beyotime, Beijing, China). 20 μg of protein was subjected to electrophoresis on 8% SDS-PAGE gels and transferred to nitrocellulose membranes. The membranes were blocked in PBS containing 5% skimmed milk for 1 h at room temperature and then reacted with a primary antibody against PirB (1:200 dilution; Merck Millipore, MA, USA). The quantity of the sample was normalized based on the level of GAPDH (Abcam, UK).

Animal treatment

DOTAP (1, 2 dioleoyl-3-trimethylammonium-propoane) (Alabaster, AL, USA) and cholesterol (Sigma-Aldrich, St. Louis, MO) were mixed in a 1:1 molar ratio, dried down in round-bottom tubes, then rehydrated in 5% glucose solution by heating at 50°C for 6 h. For *in vivo* injection, pDNA/lipoplexes were prepared immediately before injection by gently mixing cationic liposome with plasmid DNA at a ratio of 50 μg total cationic liposome to 12.5 μg plasmid DNA in a 300 μl sterile solution of 5% glucose in water. The pDNA/lipoplexes were intravenously injected. The plasmid-based PirB expression system (p-PirB) was injected as the treatment group and the vehicle pVax was used as the negative control group. n=10 for each group.

Statistical analysis

All values are expressed as the mean ± standard deviation of n observations (n≥3). Stu-

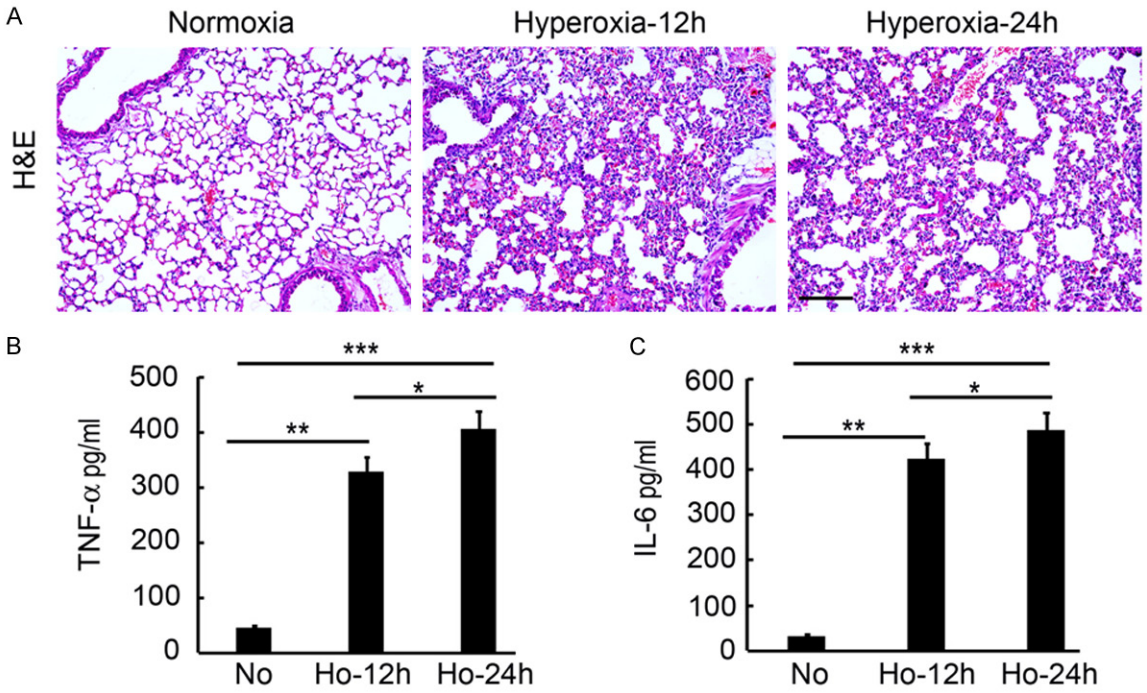

Figure 1. Histopathologic changes in the lung of rat with HALI. The rats were treated at hyperoxia condition for 12 and 24 h and executed. The lungs of rat were used for H&E staining and ELISA analysis. A. Representative images of H&E stained tissues showing the extent of lung injury. (Scale bar =100 μm). B&C. ELISA analysis of the TNF-α and IL-6 expression in the lungs of rat after hyperoxia or normoxia treatment. (n=4; *, p<0.01; **, p<0.01; ***, p<0.001).

dent's t test was used to assess differences between the two groups with SPSS version 19.0 (IBM SPSS, Armonk, NY, USA). Log-rank tests were performed using Kaplan-Meier survival curves. A two-tailed *P* value of less than 0.05 was considered to be statistically significant.

Results

Induction of lung injury in HALI rats

To investigate the hyperoxia-induced histopathologic changes, the rat lung after hyperoxia treatment for 12 and 24 h were collected for further H&E staining and TNF-α and IL-6 measurement. As shown in **Figure 1A**, hyperoxia induced lung injury was observed in the hyperoxia-treated rats, manifesting as hemorrhage, the infiltration of inflammatory cells into alveoli and lung parenchyma, and alveolar wall thickening. Meanwhile, no observed histopathologic change was found in the normoxia-treated group. Furthermore, ELISA measurement indicated that the pro-inflammatory cytokine TNF-α and IL-6 expression was significant increase after hyperoxia treatment for 12 h and 24 h (**Figure 1B** and **1C**). These results demonstrated the lung injury-induced by hyperoxia in rats.

Reduction of PirB in the lung of rat with HALI

To investigate the role of PirB in HALI, we determined the PirB expression in the lung of rats with HALI. At 12 h and 24 h after hyperoxia treatment, the lungs of HALI rats were collected for further IHC staining and western blotting. IHC staining indicated that PirB was normally expressed in bronchial epithelial cells, vascular endothelial cells and alveolar epithelial cells in normoxia treatment rats. At 12 h and 24 h after hyperoxia treatment, less PirB positive bronchial epithelial, vascular endothelial cells and alveolar epithelium were found, compared with the normoxia-treated group (**Figure 2A**). Furthermore, Western blotting was employed to detect PirB expression in the lungs of rat with HALI. As shown in **Figure 2B**, the PirB expression was significant reduced at 12 h after hypoxia treatment with 55% inhibition. Meanwhile, the PirB expression was dramatically reduced at 24 h after hypoxia treatment

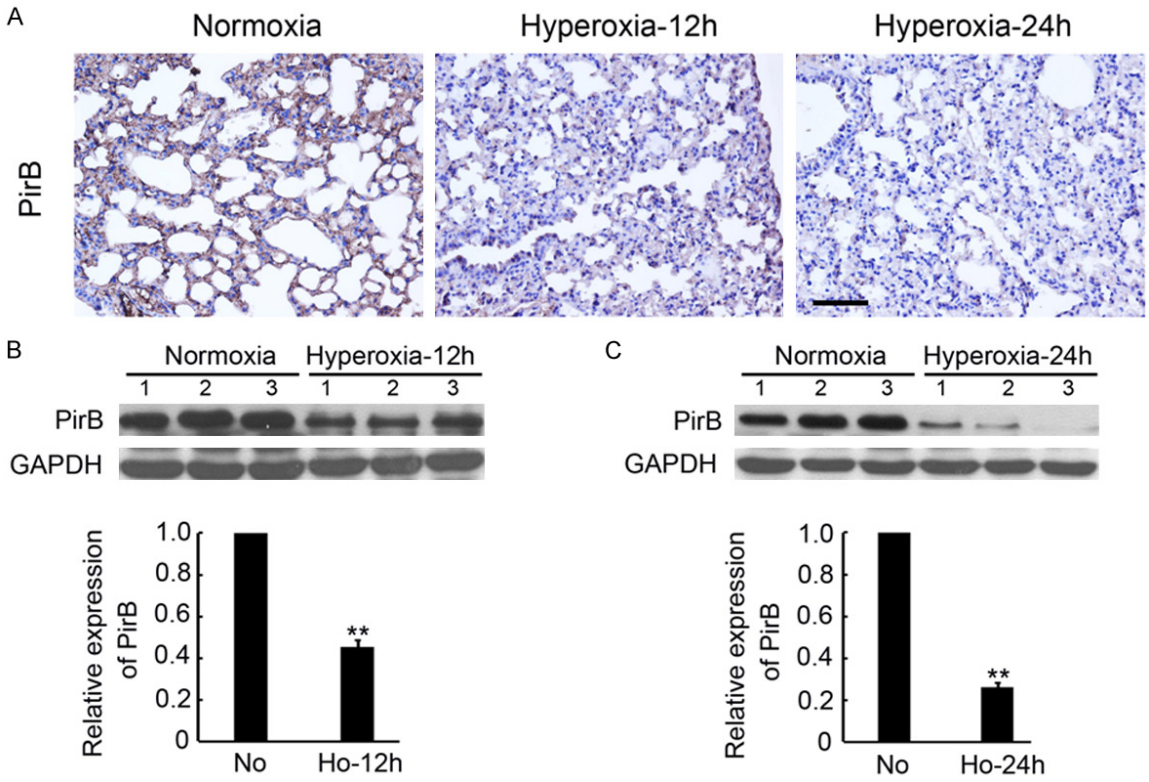

Figure 2. The down-regulation of PirB in the lungs of rat with HALI. The rats were treatment at hyperoxia condition for 12 and 24 h and executed. The lungs of rat were used for PirB staining and western blotting analysis. (A) Representative images of PirB immunostaining for lung tissues. (Scale bar=100 μm). (B&C) Western blotting analysis of PirB expression in the lungs of rat after hyperoxia for 12 h (B) and 24 h (C) or normoxia treatment. (n=3; **, p<0.01).

with 74% inhibition (**Figure 2C**). These results strongly suggest that PirB participates in the development of HALI.

PirB overexpression prolonged the survival time of rat with HALI

To determine the biological function of PirB repression in HALI, plasmid-based PirB expression was used to prevent the physiological down-regulation of this protein after hyperoxia treatment. The plasmid DNA (pVax or p-PirB) and cationic liposome, in 300 μl of 5% glucose was intravenously injected into the lung of rats with HALI. At 24 h after injection, the lungs were collected and used for further analysis. In the paraffin embedding lung tissues, the PirB protein was identified in the alveolar wall, confirming the over-expression of PirB in lung tissues with HALI (**Figure 3A**). Western blotting analysis also revealed that the PirB protein was present in the lung tissues of PirB injection group (**Figure 3B**). These results suggest that plasmid-based PirB/cationic liposome complex can effectively up-regulate pulmonary PirB levels in the rats with HALI.

Next, the pVax/cationic liposome complex and p-PirB/cationic liposome complex injected rats were used for hyperoxia treatment. The pVax/cationic liposome complex-injected rats died at 24 h after hyperoxia treatment, and only two rats (20%) were survived at 7 days after hyperoxia treatment (**Figure 4**). However, in the p-PirB injected group, seven rats (70%) were survived at 7 days after hyperoxia treatment (**Figure 4**). These results indicated that PirB overexpression significantly prolonged the survival time of rat with HALI.

PirB overexpression ameliorated lung injury of rat with HALI

To assess the effect of PirB on lung injury of rat with HALI, lung tissues in rats injected with pVax/cationic liposome complex or p-PirB/cationic liposome complex were collected for further analysis. As expected, hyperoxia-induced

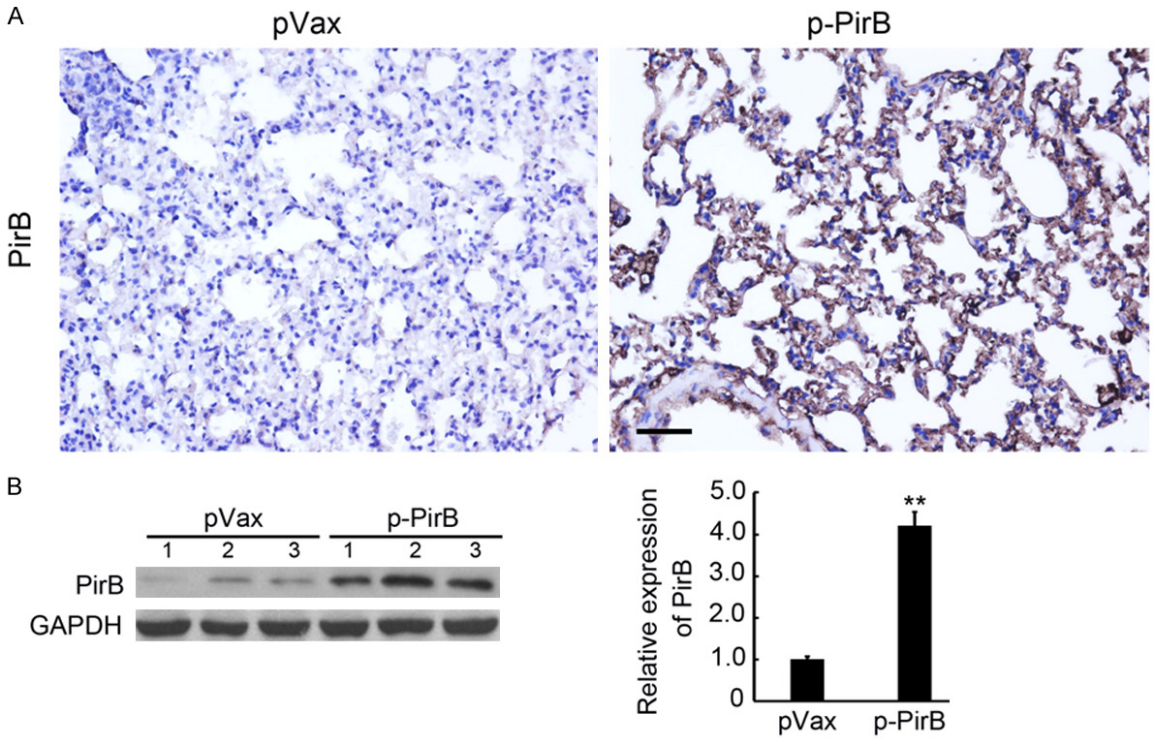

Figure 3. Overexpression of PirB in the lungs of rat. The rats were intravenously injected with the plasmid DNA (pVax or p-PirB) and cationic liposome in 300 μl of 5% glucose. At 24 h after injection, the lungs were collected for further PirB staining and western blotting analysis. A. Representative images of PirB immunostaining for lung tissues. (Scale bar =100 μm). B. Western blotting analysis of PirB expression in the lungs of rat after plasmid injection. (n=3; **, P<0.01).

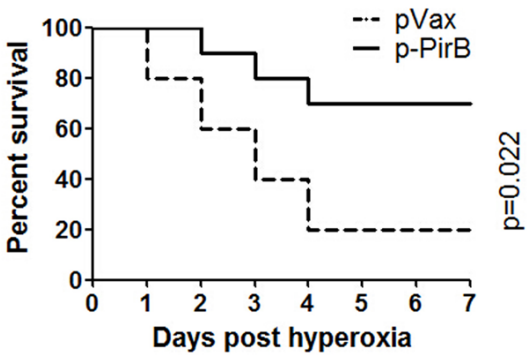

Figure 4. PirB overexpression prolonged the survival time of rat with HALI. The survival time was recorded over 7 d. n=10 for each group; P=0.022.

lung injury in pVax injected rats was shown as hemorrhage, the infiltration of inflammatory cells, and alveolar wall thickening. However, less hemorrhage area and less inflammatory cells that infiltrated into alveoli and lung parenchyma were found in the p-PirB-treated rat, compared with the pVax-treated group (**Figure 5A**). Meanwhile, alveolar wall of p-PirB-treated rat was thinner than pVax-treated rat (**Figure 5A**). Further ELISA analysis demonstrated that PirB overexpression can effectively prevent TNF-α and IL-6 upregulation induced by hyperoxia (**Figure 5B** and **5C**). These results suggested that PirB overexpression ameliorated lung injury of rat with HALI and the pro-inflammatory cytokine TNF-α and IL-6 upregulation.

Discussion

In the present study, we aimed to determine whether and how pulmonary PirB affect HALI in rats. We first found that PirB expression in the lungs was significantly suppressed in rats with HALI, suggesting that PirB might be involved in the development of acute inflammation and the related lung injury. Furthermore, the overexpression of pulmonary PirB prolonged the survival time of rat, alleviated lung injury and prevented TNF-α and IL-6 upregulation induced by hyperoxia. Our results suggested that PirB has a protective role against HALI.

Various studies indicated that PirB plays an important role in the immune system, expressed by several different cells and associated with

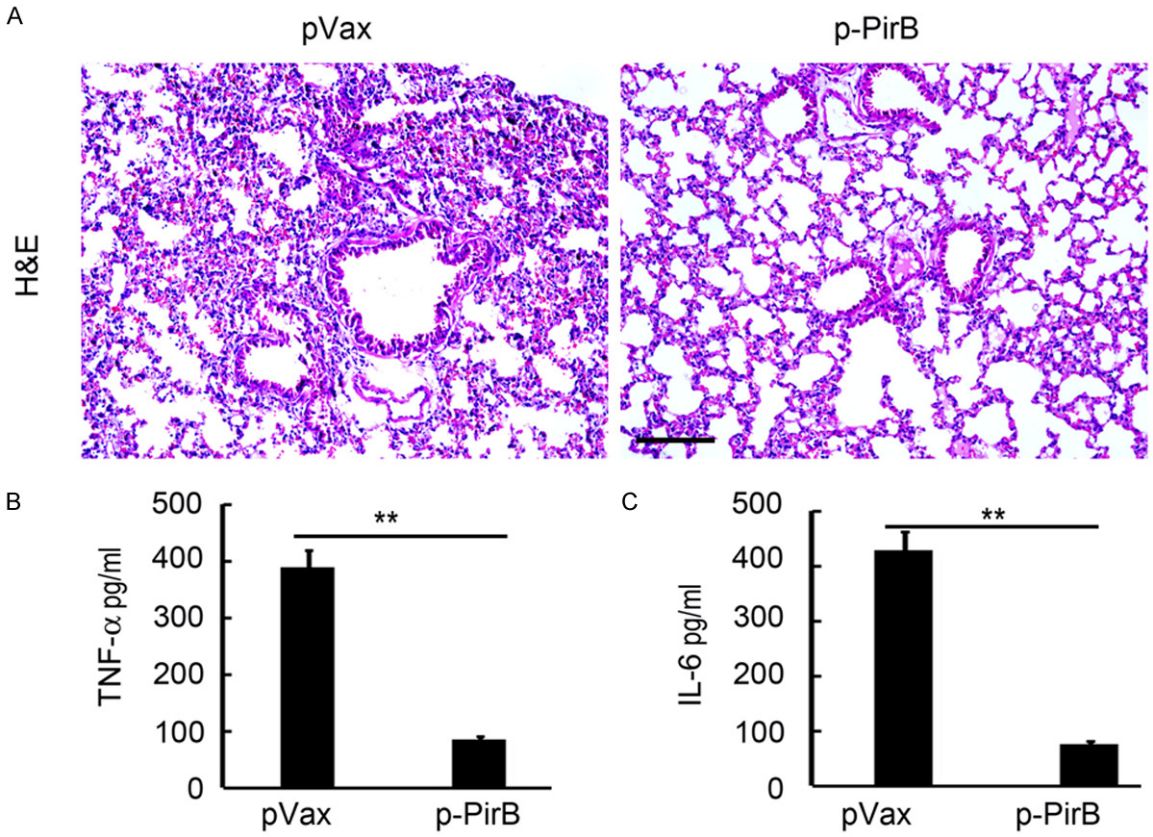

Figure 5. PirB overexpression ameliorated lung injury of rat with HALI. The lung of rat after p-PirB or pVax injection and hyperoxia treatment were collected for further H&E staining and ELISA analysis. A. Representative images of H&E stained tissues showing the extent of lung injury. (Scale bar =100 μm). B&C. ELISA analysis of the TNF-α and IL-6 expression in the lungs of rat after p-PirB or pVax injection. (n=4; **, p<0.01).

the pathogenic process of several diseases. PirB was demonstrated to express in neurons and PirB KO mice have smaller infarcts and enhanced motor recovery after ischemic stroke induced by middle cerebral artery occlusion (MCAO) [16]. Furthermore, in PirB KO mice, corticospinal projections from the motor cortex are enhanced, and the reactive astrocytic response is dampened after MCAO [16]. Therefore, PirB-involved molecules that function in the immune system act not only to limit synaptic plasticity in healthy neurons, but also to exacerbate brain injury after ischemia [16]. In Alzheimer's disease, PirB not only contributed to memory deficits present in adult mice, but also mediated loss of synaptic plasticity in juvenile visual cortex [17]. These studies demonstrated that the PirB contributes to neuropathology and suggest therapeutic uses of blocking PirB function in brain diseases. In our previous study, we found that the mRNA and protein levels of PirB increased in newborn rat cortical neurons following HI damage [13]. Treatment with PirB antibodies is able to improve axonal regeneration following HI damage compared with normal axonal growth [13]. In the current study, we investigated the contribution of pulmonary PirB to HALI in rats. Our results firstly demonstrated the reducing expression of PirB in the lungs of rats with HALI (**Figure 2**) and indicated that intravenously injection of p-PirB/cationic liposome complex dramatically increased PirB expression in the lungs of rats with HALI (**Figure 3**), prolonged the survival time (**Figure 4**) and ameliorated lung injury (**Figure 5**) of rats with HALI. Collectively, the present study suggested that PirB has a protective role against HALI and provided a novel target for HALI therapy.

Several signaling pathways were regulated by PirB. In macrophages, PirB deficiency increased the production of pro-inflammatory cytokines (IL-6, IL-1β and TNF-α) and activation of MAPK and NF-κB following bacterial activation [14]. In

plasmacytoid dendritic cells (PDCs), PirB suppressed Fms-like tyrosine kinase3 ligand-induced PDC differentiation in BM cells, as well as Toll-like receptor 9-mediated IFN-α production by PDCs, through the dephosphorylation of STAT1/STAT2 [18]. We previously indicated that inhibition of PirB promote the regeneration of HI-damaged axons through regulating the Rho-ROCK signaling pathway [13]. Furthermore, PirB silencing markedly decreased hyperoxia-induced apoptosis, increased cell viability and decreased the expression of caspases 3 and 8, and Fas in oligodendrocyte precursor cells (OPCs) [12]. In the present study, we demonstrated the prevention role of PirB in hyperoxia-induced TNF-α and IL-6 upregulation (**Figure 5**). These results indicated that TNF-α and IL-6 maybe the downstream targets that involved in PirB ameliorating HALI. Meanwhile, various studies found oxidative stress is purported to play an important role in the pathogenesis of HALI [4, 19, 20]. We also have determined the expression of ROS and SOD in the lungs of rats with HALI. But the results demonstrated that there was no significant difference between the pVax/cationic liposome complex injection group and p-PirB/cationic liposome complex injection group (data no shown). Collectively, the present study suggested that PirB has a protective role against HALI through inhibiting the pulmonary inflammation, but not the oxidative stress. Notably, PirB is widely expressed in macrophages [14], DCs [18] and other immune cells [10, 21] and play a crucial role in immune regulation [7]. In the present study, we only demonstrated the general reduction of PirB protein expression in the lungs of rats with HALI, however, whether PirB has more expression and thus dominant function in infiltrated inflammatory cells that causing lung injury in HALI rats is still unclear. It is needed to clarify this point with further *in vivo* and *in vitro* study.

Conclusions

In sum, our results demonstrated the reduction of pulmonary PirB expression in the rats with HALI and over-expression of pulmonary PirB prolonged the survival time of rat, alleviated lung injury. We further showed that hyperoxia-induced TNF-α and IL-6 upregulation in the lungs of rats was significantly inhibited by pulmonary PirB. However, the direct targets of PirB during this process should be identified in the future study. The present study suggested that PirB is involved in the development of acute inflammation and the related lung injury and thus PirB has a protective role against HALI.

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Disclosure of conflict of interest

None.

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