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RESEARCH PAPER

Maresin 1 mitigates LPS-induced acute lung injury in mice

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BACKGROUND AND PURPOSE

Acute lung injury (ALI) is a severe illness with a high rate of mortality. Maresin 1 (MaR1) was recently reported to regulate inflammatory responses. We used a LPS-induced ALI model to determine whether MaR1 can mitigate lung injury.

EXPERIMENTAL APPROACH

Male BALB/c mice were injected, intratracheally, with either LPS (3 mg·kg^{-1}) or normal saline (1.5 mL·kg^{-1}). After this, normal saline, a low dose of MaR1 (0.1 ng per mouse) or a high dose of MaR1 (1 ng per mouse) was given i.v. Lung injury was evaluated by detecting arterial blood gas, pathohistological examination, pulmonary oedema, inflammatory cell infiltration, inflammatory cytokines in the bronchoalveolar lavage fluid and neutrophil–platelet interactions.

KEY RESULTS

The high dose of MaR1 significantly inhibited LPS-induced ALI by restoring oxygenation, attenuating pulmonary oedema and mitigating pathohistological changes. A combination of ELISA and immunohistochemistry showed that high-dose MaR1 attenuated LPS-induced increases in pro-inflammatory cytokines (TNF-α, IL-1β and IL-6), chemokines [keratinocyte chemokine, monocyte chemoattractant protein-5, macrophage inflammatory protein (MIP)-1α and MIP-1γ], pulmonary myeloperoxidase activity and neutrophil infiltration in the lung tissues. Consistent with these observations, flow cytometry and Western blotting indicated that MaR1 down-regulated LPS-induced neutrophil adhesions and suppressed the expression of intercellular adhesion molecule (ICAM)-1, P-selection and CD24.

CONCLUSIONS AND IMPLICATIONS

High-dose MaR1 mitigated LPS-induced lung injury in mice by inhibiting neutrophil adhesions and decreasing the levels of pro-inflammatory cytokines.

Abbreviations

AA, arachidonic acid; ALI, acute lung injury; ARDS, acute respiratory distress syndrome; ATLs, aspirin-triggered lipoxins; BALF, bronchoalveolar lavage fluid; DHA, docosahexaenoic acid; ICAM-1, intercellular adhesion molecules; KC, keratinocyte chemokine; MaR1, maresin 1; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; MPO, myeloperoxidase; NS, normal saline; PUFAs, polyunsaturated fatty acids

Introduction

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are life-threatening conditions that contribute significantly to critical illness (Matthay *et al.*, 2012). Despite advances in understanding the pathophysiology of ALI/ARDS, there is still no effective treatment for ALI/ARDS (Wheeler and Bernard, 2007; Eickmeier *et al.*, 2013). Although different mechanisms are involved in the pathogenesis of ALI, inflammation is one of the leading causes. ALI is the extreme inflammatory process characterized by extensive neutrophil influx into the lungs, the expression of pro-inflammatory mediators, and lung epithelium and endothelium damages, which results in pulmonary oedema and gas exchange deterioration (Jin *et al.*, 2007). Blocking various aspects of the inflammatory cascades has been reported to attenuate ALI (Shang *et al.*, 2010; Gong *et al.*, 2012), indicating that inflammatory modulating agents may offer a potential preventative or therapeutic approach for ALI.

Inflammation is an appropriate response for effective host defence (Serhan, 2007; Maderna and Godson, 2009). However, excessive inflammatory responses can lead to tissue destruction, fibrosis and eventual organ failure (Maderna and Godson, 2009). To limit excessive inflammation, organisms have developed different protective regulatory mechanisms to dampen inflammation, such as anti-inflammatory cytokines and antioxidant agents (Lawrence *et al.*, 2002). Recent findings have highlighted the role of pro-resolving mediators derived from polyunsaturated fatty acids (PUFAs) in regulating the inflammatory response (Serhan *et al.*, 2002; Decker *et al.*, 2009). Lipoxin A_4, lipoxin B_4 and aspirin-triggered lipoxins (ATLs) are the first mediators biosynthesized from arachidonic acid (AA) (Chiang *et al.*, 2005; Alessandri *et al.*, 2013; for nomenclature see Alexander *et al.*, 2013). Besides producing AA-derived mediators, PUFAs generate omega-3 fatty acids, including eicosapentaenoic acid and docosahexaenoic acid (DHA) (Alessandri *et al.*, 2013). These, in turn, are enzymatically transformed to generate resolvins, protectins and the most recently identified macrophage-derived pro-resolving mediators, denoted as the maresins (Serhan *et al.*, 2009). These new DHA-derived mediators are biosynthesized in macrophages by 14-lipoxygenation of DHA, producing the hydroperoxy-containing intermediate 14*S*-hydroperoxydocosa-4*Z*,7*Z*,10*Z*,12*E*,16*Z*, 19*Z*-hexanoic acid (14*S*-HpDHA). This intermediate undergoes further conversion via 13(14)-epoxidation, which is an important process in the biosynthesis of potent mediators such as 7*R*,14*S*-dihydroxydocosa-4*Z*,8*E*,10*E*,12*Z*,16*Z*,19*Z*-hexanoic acid, named as maresin 1 (MaR1) (Dalli *et al.*, 2013). MaR1 is the first member identified from this family of macrophage-derived pro-resolving mediators (Serhan *et al.*, 2012).

There is increasing evidence that these mediators can moderate the inflammatory response and provide protective effects against ALI/ARDS. In our previous studies, post-treatment with an ATL analogue induced the expression of haeme oxygenase-1 to attenuate LPS-induced ALI in mice (Jin *et al.*, 2007). In addition, Xie *et al.* (2013) demonstrated that resolvin D1 alleviates pulmonary oedema, restores pulmonary capillary permeability and reduces tight junction disruption in mice with LPS-induced ALI. However, it is not yet known whether MaR1 could have protective effects in LPS-induced ALI.

MaR1 has displayed both pro-resolving and anti-inflammatory activities in zymosan-induced peritonitis (Serhan *et al.*, 2009). In addition, MaR1 has been reported to stimulate tissue regeneration and control pain *in vivo* and *in vitro* (Serhan *et al.*, 2012). In the current study, we investigated the effect of MaR1 on LPS-induced ALI in mice, as well as the possible mechanisms involved in these processes. Our data suggest that MaR1 can attenuate LPS-induced lung injury by inhibiting neutrophil adhesion and the production of pro-inflammatory mediators.

Methods

Animals

All animal experiments were approved 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 (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). Ten-week-old male BALB/c mice (Hua Fu Kang Company, Beijing, China), weighing 20–25 g, were housed four per cage in a specific pathogen-free room. The animals were given standard laboratory chow and water *ad libitum*, and housed in a room with temperature of 22–24°C and humidity of 60–65%, with 12 h light/dark cycles. A total of 128 mice were used in this study, and the mice were acclimatized to the environment for 14 days before experiments.

Experimental procedures

Before surgery, all animals were anaesthetized with 80 mg·kg^{-1} of 2% sodium pentobarbital i.p. (Sigma-Aldrich Co, St. Louis, MO, USA). Heart rate, body temperature and toe pinch were consistently monitored to detect the depth of anaesthesia. After successful endotracheal intubation, mice were instilled with LPS (from *Escherichia coli* serotype O55:B5; Sigma-Aldrich Co) at a dosage of 3 mg·kg^{-1} (Xu *et al.*, 2011) or normal saline (NS, 1.5 mL·kg^{-1}). One hour after LPS or NS administration, NS, a low dose (0.1 ng) or high dose (1 ng) of MaR1 (Cayman Chemical, Ann Arbor, MI, USA) diluted in NS to the same volume (0.1 mL per mouse) was injected via the tail vein (Serhan *et al.*, 2012).

The mice were randomly divided into four groups: the sham group (sham), which were administered intratracheal and i.v. NS; the LPS group (LPS), which included intratracheal LPS and i.v. NS; the LD-MaR1 group (LD-MaR1), which included intratracheal LPS and i.v. low-dose MaR1; the HD-MaR1 group (HD-MaR1), which included intratracheal LPS and i.v. high-dose MaR1.

After 24 h following LPS administration, the mice were killed by an overdose of sodium pentobarbital.

Histological analysis of lung tissues

The left lungs were inflated to 15 cm H_2O with 4% paraformaldehyde and removed for paraffin embedding. Sections were subsequently stained with haematoxylin and eosin. Lung

injury scores were quantified by an investigator blinded to the treatment groups using recently published criteria (Matute-Bello *et al.*, 2011).

Immunohistochemistry

After deparaffinization, sections were incubated in 3% hydrogen peroxide for 30 min and then blocked with 5% goat serum albumin for 20 min. The sections were incubated with anti-mouse ly-6G (Gr-1) purified antibodies (1:100, clone RB6-8CS; eBioscience, Inc., San Diego, CA, USA) overnight at 4°C. Then, the secondary antibody (ChemMateTMEn Vision+/HRP) was added for 50 min at 4°C. Finally, the sections were stained with diaminobenzidine followed by haematoxylin.

Differential leukocyte counts and evaluation of pulmonary oedema

In a separate series of experiments, mice were killed and bronchoalveolar lavage fluid (BALF) was collected by flushing the left lung (0.4 mL × 3 times). The lower lobes of the right lung from each group were assessed for dry/wet weight ratios. The remaining lung tissues were harvested for further analysis and stored at –80°C.

Total BALF cells were measured using a haematocytometer. One part of the BALF was centrifuged for 5 min at 92 *g* using cytospins (Thermo Fisher Scientific, Waltham, MA, USA) on a microscopic slide, and BALF cells were stained by Giemsa. The rest of the BALF was filtrated through a 0.22 μm pore-size filter and then stored at –80°C for protein and cytokine detection. We evaluated pulmonary oedema using the BALF protein and pulmonary wet/dry weight ratios. The BALF protein concentration was assessed with the BCA Protein Assay Kit (Thermo Fisher Scientific). For the wet/dry weight ratios, wet lung tissues were weighed, placed in an oven for 24 h and weighed again after they were dried. The wet/dry weight ratio was calculated using these measurements.

BALF cytokine and pulmonary myeloperoxidase (MPO) activity

The TNF-α, IL-1β, IL-10, IL-6 and keratinocyte chemokine (KC) levels in the BALF were measured using ELISA kits (Neo-Bioscience Technology Co, Shanghai, China). BALF levels of monocyte chemoattractant protein (MCP)-5, macrophage inflammatory protein (MIP)-1α and MIP-1γ were measured using the ELISA kits (RayBiotech, Inc., Norcross, GA, USA). Lung tissues were homogenized with isotonic sodium chloride to detect MPO activity (Shang *et al.*, 2009). Pulmonary MPO activity was measured using the MPO kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Western blot analysis

Protein was isolated from the lung tissue homogenates according to the protocol provided by the Protein Extraction Reagents Kit (KeyGEN BioTECH, Nanjing, China). Proteins were separated by electrophoresis on 10% polyacrylamide SDS gels and transferred to a PVDF membrane. The membranes were blocked with 5% non-fat milk for 60 min, and probed with antibodies against P-selectin, CD24 and intercellular adhesion molecule-1 (ICAM-1) (1:500; all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) and β-actin (1:1000; Antgene Biotechnology, Wuhan, China). After an overnight incubation, the membranes were incubated with goat-anti-rabbit or goat-anti-mouse antibodies (1:3000; Antgene Biotechnology) for 1 h. The protein was detected with chemiluminescence reagents (Beyotime Institute of Biotechnology, Shanghai, China). Images were scanned with the UVP imaging system and analysed using Image J software (version 1.45s; NIH, Bethesda, MD, USA).

Analysis of arterial blood gas

To analyse arterial blood gas, a separate set of mice was randomly allocated into four groups: sham, LPS, LD-MaR1 and HD-MaR1. Mice were subjected to the same LPS, MaR1 or NS administrations as described earlier. Twenty-three hours after LPS administration, mice were anaesthetized, tracheostomized and ventilated using a custom-made ventilator. After an initial lung recruitment manoeuvre (30 cm H_2O for 5 s), animals were ventilated for 30 min (tidal volume 8–9 mL·kg^{-1}, positive end-expiratory pressure 2.5 cm H_2O, respiratory rate 120 breaths per min and FiO_2 0.21) to standardize the lung volume history (Patel *et al.*, 2012). At the end of the ventilation, the arterial blood was drawn through the left carotid artery. Arterial blood gas was immediately analysed with a blood gas analyser (Radiometer, Copenhagen, Denmark).

Assessment of neutrophil–platelet interactions

To assess neutrophil–platelet interactions, a separate set of mice was randomly allocated into four groups: sham, LPS, LD-MaR1 and HD-MaR1. The mice were subjected to the same LPS, MaR1 or NS administration as described earlier. Twenty-four hours after LPS administration, whole blood was collected from the right ventricle. Blood was lysed with the red blood cell lysis buffer and centrifuged. The supernatant was discarded, and the cell pellet was resuspended in 100 μL of PBS. The cell suspension was blocked with CD16/32 Fc block (eBioscience, Inc.) for 30 min then labelled with either APC-conjugated anti-mouse ly-6G (Gr-1) (clone RB6-8C5), FITC-conjugated anti-mouse CD41 (clone eBioMWReg30) or

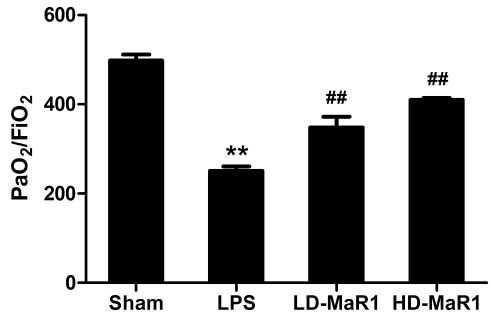

Figure 1

MaR1 mitigated LPS-induced ALI. Arterial blood gas showed that MaR1 attenuated the arterial oxygenation in ALI. LPS (3 mg·kg^{-1}) or NS (1.5 mL·kg^{-1}) was administered intratracheally 60 min before i.v. injection of NS, LD-MaR1 (0.1 ng per mouse) or HD-MaR1 (1 ng per mouse). Data are shown as means ± SEM, $n = 8$. $^{**}P < 0.01$ versus the sham group; $^{\#\#}P < 0.01$ versus the LPS group.

PE-conjugated anti-mouse P-selectin antibodies (clone Psel.KO2.3) (eBioscience, Inc.) for 30 min. The ly-6G$^+$CD41$^+$ and ly-6G$^+$P-selectin$^+$ populations were assessed by flow cytometry.

Statistical analysis

All data are expressed as mean ± SEM and were analysed by SPSS software (version 16.0; SPSS, Inc., Chicago, IL, USA). Intergroup differences were analysed by one-way ANOVA followed by the least significant difference *post hoc* test. A *P*-value of less than 0.05 was considered statistically significant.

Results

MaR1 mitigated LPS-induced ALI

As shown in Figure 1, arterial oxygenation significantly deteriorated after intratracheal LPS administration. Mean arterial oxygen tension (PaO_2/FiO_2) in the LPS group was significantly lower than that in the sham group ($P < 0.01$), and the PaO_2/FiO_2 in the LPS group achieved clinical ALI criteria (<300). However, treatment with MaR1 effectively mitigated the change in arterial oxygenation. The PaO_2/FiO_2 in the LD-MaR1 and HD-MaR1 groups recovered to normal levels and was higher than in the LPS group ($P < 0.01$).

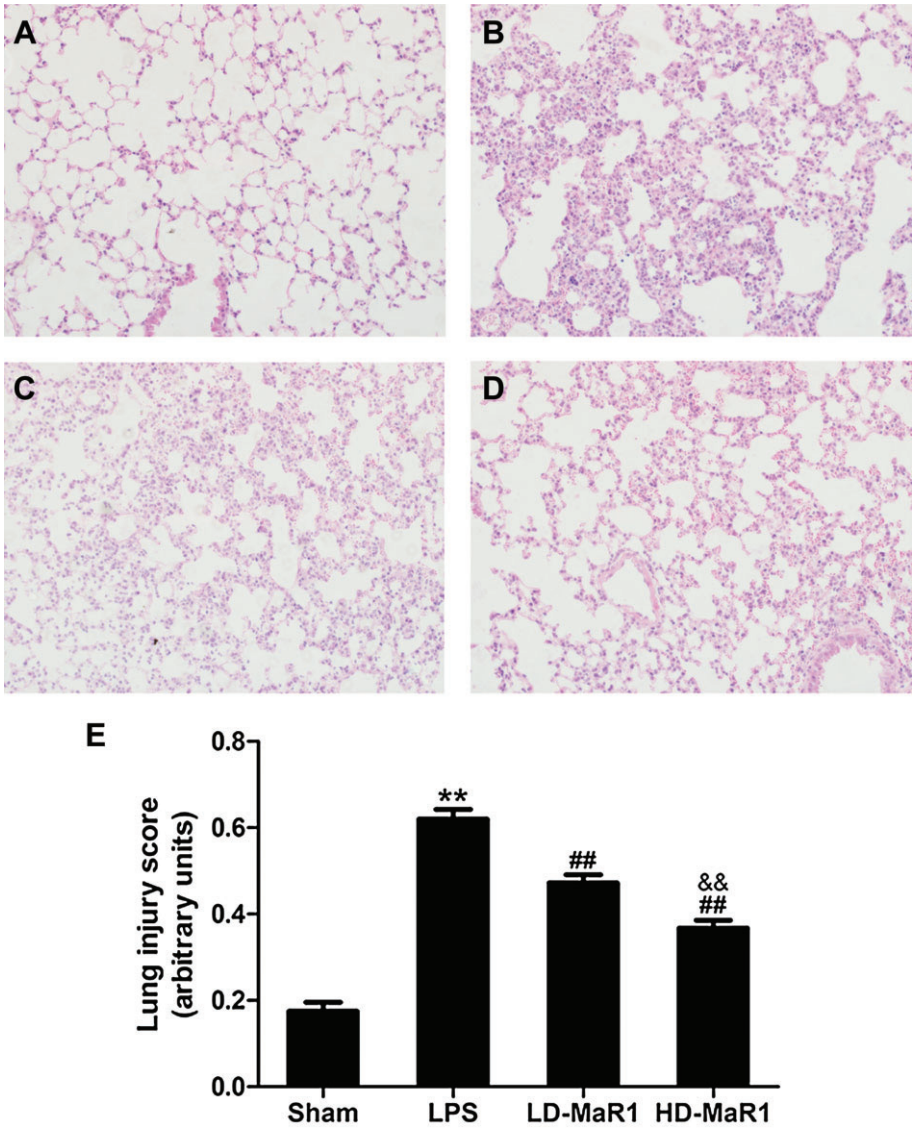

Figure 2

MaR1 preserved lung architecture in LPS-induced ALI. Representative photomicrographs of pulmonary histology (haematoxylin and eosin, magnification 200×). LPS (3 mg·kg^{-1}) or NS (1.5 mL·kg^{-1}) was administered intratracheally 60 min before i.v. injection of NS, LD-MaR1 (0.1 ng per mouse) or HD-MaR1 (1 ng per mouse). (A) The sham group. (B) The LPS group. (C) The LD-MaR1 group. (D) The HD-MaR1 group. (E) Lung injury score. Data are shown as means ± SEM, $n = 8$. $^{**}P < 0.01$ versus the sham group; $^{\#\#}P < 0.01$ versus the LPS group; $^{\&\&}P < 0.01$ versus the LD-MaR1 group.

As shown in Figure 2A, the sham group had normal lung parenchyma, and fewer macrophages were observed in the alveolar space under light microscopy. In contrast, interstitial oedema, neutrophil infiltration, haemorrhage, alveolar disarray and thickness of the alveolar septum were observed in the LPS group (Figure 2B). The LD-MaR1 group showed slight amelioration of the lung injury (Figure 2C). Furthermore, the HD-MaR1 group showed significant attenuation of lung injury compared with the LD-MaR1 group (Figure 2D). Lung injury scores were assessed in parallel with the pathohistological changes (Figure 2E).

MaR1 attenuated pulmonary oedema and microvascular permeability

We examined pulmonary oedema by measuring lung wet/dry weight ratios. Figure 3A shows a significant increase in the wet/dry weight ratio in the LPS group compared with the sham group ($P < 0.01$). In contrast, the wet/dry ratios in the LD-MaR1 and HD-MaR1 groups were lower than the LPS group ($P < 0.01$). BALF protein is used to determine the index of microvascular permeability (Chatterjee *et al.*, 2007). As shown in Figure 3B, LPS induced a significant disruption in the pulmonary microvascular barrier compared to the sham group ($P < 0.01$). MaR1 treatment markedly reduced the BALF protein level ($P < 0.01$) compared to that of the LPS group, indicating less microvascular permeability. In addition, HD-MaR1 dramatically mitigated disruption in the pulmonary microvascular barrier compared to the LD-MaR1 group ($P < 0.01$).

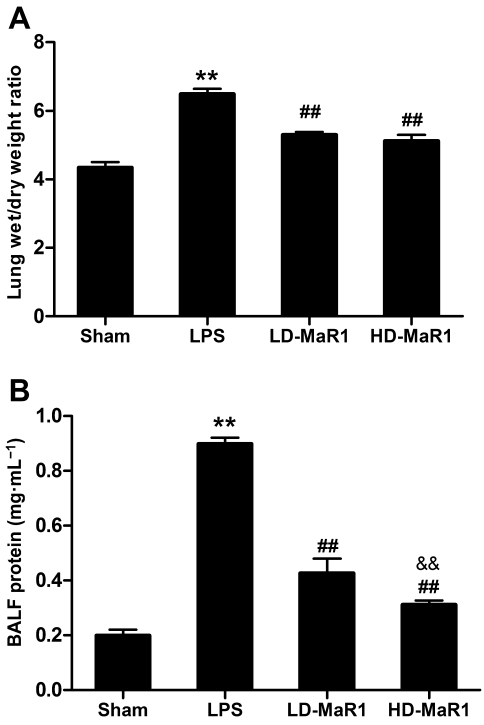

Figure 3

MaR1 attenuated pulmonary oedema and microvascular permeability. LPS (3 mg·kg^{-1}) or NS (1.5 mL·kg^{-1}) was administered intratracheally 60 min before i.v. injection of NS, LD-MaR1 (0.1 ng per mouse) or HD-MaR1 (1 ng per mouse). (A) Lung wet/dry weight ratio. (B) BALF protein. Data are shown as means ± SEM, $n = 8$. $^{**}P < 0.01$ versus the sham group; $^{\#\#}P < 0.01$ versus the LPS group; $^{\&\&}P < 0.01$ versus the LD-MaR1 group.

MaR1 altered leukocyte recruitment in the lungs

BALF cytology suggested a dramatic change in the alveoli after ALI. In the sham group, the cells were almost entirely alveolar macrophages. After LPS administration, the numbers of total cells, neutrophils and macrophages increased, and the proportion of neutrophils increased compared with those in the sham group ($P < 0.01$). However, MaR1 led to significant and dose-dependent reductions in total BALF cell numbers relative to the LPS group ($P < 0.01$) (Figure 4A). In addition, MaR1 significantly decreased BALF polymorphonuclear leukocytes compared with the LPS group ($P < 0.01$) (Figure 4B). However, administration of MaR1 had no effect on BALF macrophages (Figure 4C).

MaR1 inhibited lung neutrophil infiltration

As shown in Figure 4D, MPO activity in the LPS group was higher than the sham group ($P < 0.01$). In contrast, there was significant reduction of MPO activity in the HD-MaR1 group compared with the LPS group ($P < 0.01$). To investigate neutrophil infiltration in the lung tissues, neutrophils were identified with ly-6G immunostaining. As shown in Figure 5, LPS-induced ALI led to increased numbers of ly-6G cells relative to the sham group (Figure 5A and B). Furthermore, MaR1 decreased the numbers of ly-6G positive cells in a dose-dependent manner (Figure 5C and D).

MaR1 regulated LPS-induced inflammatory cytokines

The inflammatory cytokines were reported to be involved in the neutrophil recruitment and propagation of the inflammatory response. To investigate the impacts of inflammatory cytokines, we detected these particular cytokines using the ELISA. At the end of the experiment, the LPS group showed high levels of pro-inflammatory cytokines in the BALF, such as TNF-α, IL-1β, IL-6, KC, MCP-5, MIP-1α and MIP-1γ. Administration of MaR1 effectively decreased the levels of pro-inflammatory cytokines compared with the LPS group, including TNF-α, IL-1β, IL-6, KC, MCP-5, MIP-1α, MIP-1γ ($P < 0.05$, $P < 0.01$; Figure 6). Several inflammatory cytokines in the HD-MaR1 group, such as IL-6, KC, MCP-5 and MIP-1α, were lower than that in the LD-MaR1 group ($P < 0.05$, $P < 0.01$; Figure 6D–G). In addition, the anti-inflammatory cytokine IL-10 was slightly increased in the MaR1 group compared to the LPS group ($P < 0.01$; Figure 6C).

MaR1 suppressed neutrophil adhesions

Vascular inflammation with heterotypic interactions between neutrophils, platelets and endothelial cells are important early events in ALI (Zarbock *et al.*, 2006; Looney *et al.*, 2009). To investigate neutrophil–platelet interactions, whole blood from the right ventricle was collected and analysed by flow cytometry. The neutrophil–platelet interactions were identified as the ly-6G$^+$CD41$^+$ population. LPS led to a significant increase in the % of ly-6G$^+$CD41$^+$ cells relative to the sham

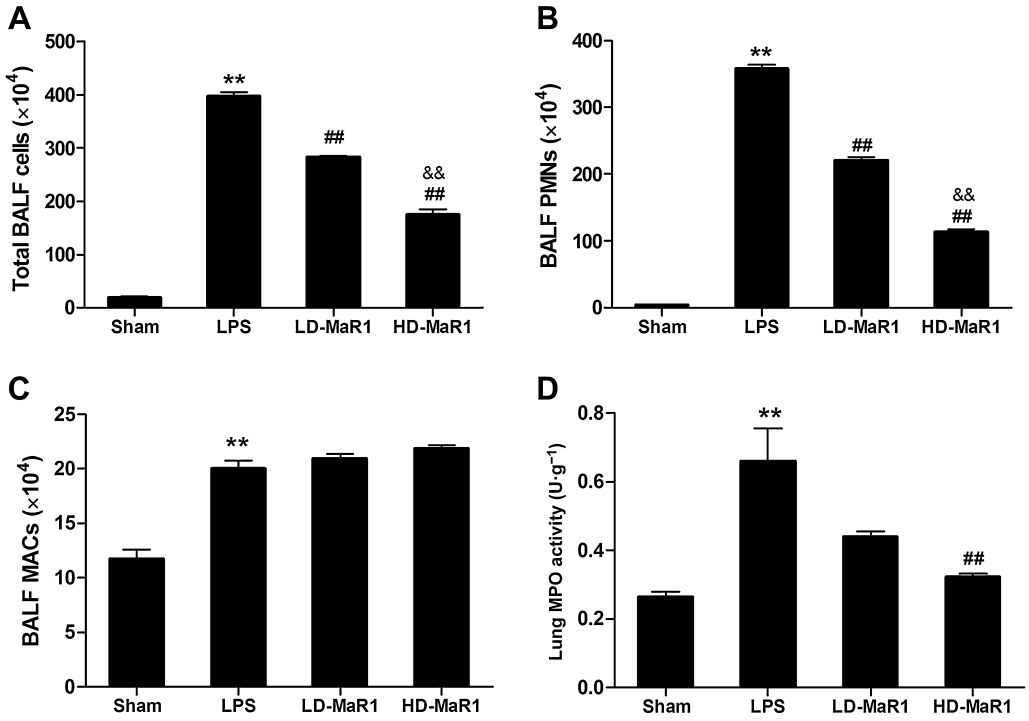

Figure 4

MaR1 affected leukocyte recruitment and neutrophil infiltration. LPS (3 mg·kg^{-1}) or NS (1.5 mL·kg^{-1}) was administered intratracheally 60 min before i.v. injection of NS, LD-MaR1 (0.1 ng per mouse) or HD-MaR1 (1 ng per mouse). (A) Total BALF cells. (B) BALF polymorphonuclear leukocytes (PMNs). (C) BALF macrophages (MACs). (D) Pulmonary MPO activity. Data are shown as means ± SEM, $n = 8$. $^{**}P < 0.01$ versus the sham group; $^{\#\#}P < 0.01$ versus the LPS group; $^{\&\&}P < 0.01$ versus the LD-MaR1 group.

group ($P < 0.01$). In contrast, MaR1 decreased the % of ly-6G$^+$CD41$^+$ cells in a dose-dependent manner (Figure 7A and C). P-selectin plays an important role in neutrophil–platelet interactions in ALI (Zarbock *et al.*, 2006; Looney *et al.*, 2009). LPS administration led to a dramatic increase in the % of ly-6G$^+$ P-selectin$^+$. A reduction in the ly-6G$^+$ P-selectin$^+$ population was observed in the HD-MaR1 group (Figure 7B and D). Furthermore, Western blot analysis showed that MaR1 suppressed the LPS-induced expression of P-selectin, its neutrophil ligand CD24 and ICAM-1 (Figure 8).

Discussion

Dysregulated inflammation, inappropriate accumulation and activities of leukocytes and platelets, uncontrolled activation of coagulation pathways, and altered permeability of alveolar endothelial and epithelial barriers are the central pathophysiological characteristics of ALI and ARDS (Matthay *et al.*, 2003; 2012; Matthay and Zimmerman, 2005). LPS, a glycolipid of the outer membrane of gram-negative bacteria, is a common cause of ALI/ARDS (Moon *et al.*, 2009). Lower respiratory tract exposure to LPS by intratracheal instillation is a well-recognized model for ALI, as it mimics the pathological aspects of clinical development of ALI induced by gram-negative pulmonary infection (Wang *et al.*, 2011). Clinical strategies for ALI/ARDS are restricted to mechanical ventilation and supportive treatments (Shang and Yao, 2014). Omega-3 fatty acid supplements are widely used in animals and humans because they are believed to have therapeutic potential. Indeed, convincing evidence from clinical trials supports their effectiveness in treating inflammatory disorders (Dwyer *et al.*, 2004; Serhan *et al.*, 2009).

MaR1 is known to play a role in tissue homeostasis, inflammation resolution, would healing and host defence. MaR1 shows remarkable anti-inflammatory properties, including regulating neutrophil infiltration in zymosan-induced peritonitis and macrophage phagocytosis when directly compared with other omega-3 fatty acid-derived mediators such as neuroprotectin/protectin D1 and resolvin E1 (Serhan *et al.*, 2009). Apart from leukocyte-directed actions, MaR1 demonstrates other potent activities (*in vivo* and *in vitro*) where it is produced by macrophages. MaR1 studies in *Planaria* have provided new pathways in the DHA metabolome that can potentially link organ regenerative responses and wound healing with the resolution of local acute inflammation and pain (Serhan *et al.*, 2012). Macrophages possess a striking functional and phenotypic plasticity that becomes apparent during the resolution phase of inflammation (Ortega-Gomez *et al.*, 2013). Dalli *et al.* (2013) speculated that the novel 13*S*,14*S*-epoxy-maresin is converted by human macrophages to MaR1, which inhibits leukotriene A_4 hydrolase and shifts macrophage phenotype. Consistent with previous reports, MaR1 protects mice against a model of experimental colitis, possibly by inhibiting the NF-κB pathway and enhancing the macrophage M2 phenotype

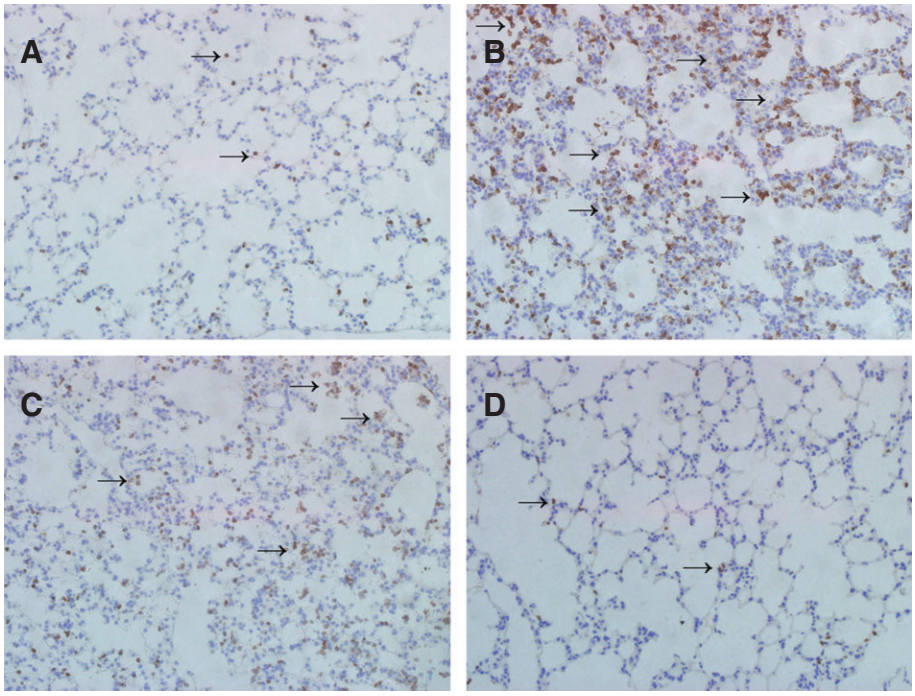

Figure 5

MaR1 inhibited neutrophil infiltration into the lung tissues (magnification 200×). LPS (3 mg·kg^{-1}) or NS (1.5 mL·kg^{-1}) was administered intratracheally 60 min before i.v. injection of NS, LD-MaR1 (0.1 ng per mouse) or HD-MaR1 (1 ng per mouse). (A) The sham group. (B) The LPS group. (C) The LD-MaR1 group. (D) The HD-MaR1 group.

(Marcon *et al.*, 2013). Furthermore, MaR1 attenuates the pro-inflammatory response of bronchial epithelial cells to organic dust through a mechanism that does not appear to depend on reduced NF-κB, AP-1 or SP-1-related signalling. Instead, it may be mediated partly through SRE-associated signalling. However, the direct effect of MaR1 on ALI has not been previously addressed in any studies (Nordgren *et al.*, 2013).

Our study provided the first evidence that MaR1 induces anti-inflammatory effects, decreasing the severity of the LPS-induced ALI in a dose-dependent manner. Our present data showed that a high dose of MaR1 significantly improved the arterial oxygenation, mitigated the severity of the pathohistological changes and restored the increased pulmonary permeability. As demonstrated by ELISA analysis, MaR1 reduced LPS-induced production of pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6. In the present study, inhibition of heterotypic interactions between neutrophils, platelets and endothelial cells was at least partly involved in the anti-inflammatory mechanisms of MaR1 on LPS-induced ALI.

Neutrophil recruitment and activation can cause tissue damage that contributes to the pathogenesis of ALI/ARDS (Abraham, 2003). With an appropriate stimulus (e.g. infection, mechanical trauma, ischaemia, toxins, chemicals and antigens), neutrophils exit the circulation through a well-characterized series of events involving rolling, adhesion and transmigration across the vascular endothelium into the interstitial space. P-selectin is expressed by activated endothelial cells and platelets as a molecular regulator of heterotypic neutrophil–platelet and platelet–endothelial cell interactions (Frenette *et al.*, 1998). The neutrophil rolling process is mediated predominantly by P-selectin and its neutrophil ligand CD24. In tissue inflammation and ALI, platelet depletion leads to diminished neutrophil recruitment (Zarbock *et al.*, 2006). As a measure of vascular inflammation, neutrophil–platelet interactions were detected by the whole blood from the right ventricle, which subsequently flows into the pulmonary vasculature. Our flow cytometry data revealed that MaR1 suppressed LPS-induced neutrophil–platelet interactions by reducing the percentage of ly-6G$^+$CD41$^+$ and ly-6G$^+$P-selectin$^+$ cells. In addition, our Western blot data showed that MaR1 down-regulated the LPS-induced expression of P-selectin and its neutrophil ligand CD24. Previous studies have also shown that aspirin-triggered resolvin D1 and lipoxin A_4 inhibit neutrophil–platelet interactions in acid-induced ALI in mice (Eickmeier *et al.*, 2013) as well as in *Porphyromonas gingivalis*-induced periodontal disease (Borgeson *et al.*, 2011).

During secondary capture, neutrophils interact with circulating or endothelial adherent platelets to augment neutrophil–endothelial interactions in inflamed vascular beds for neutrophil tissue recruitment (Zarbock and Ley, 2009; Eickmeier *et al.*, 2013). ICAM-1, a known CD11b/CD18 ligand (Diamond *et al.*, 1990), is critical for neutrophil adhesion to and migration across the endothelium (Sakamoto *et al.*, 1997). It has been reported that ICAM-1 inhibition may have a protective effect in lung injury (Kumasaka *et al.*, 1996) and sepsis (Xu *et al.*, 1994). As a measurement of neutrophil adhesion, cell–cell interactions between neutrophils and the endothelium were monitored by Western blot for ICAM-1 in lung tissues. Our study showed that MaR1 treatment

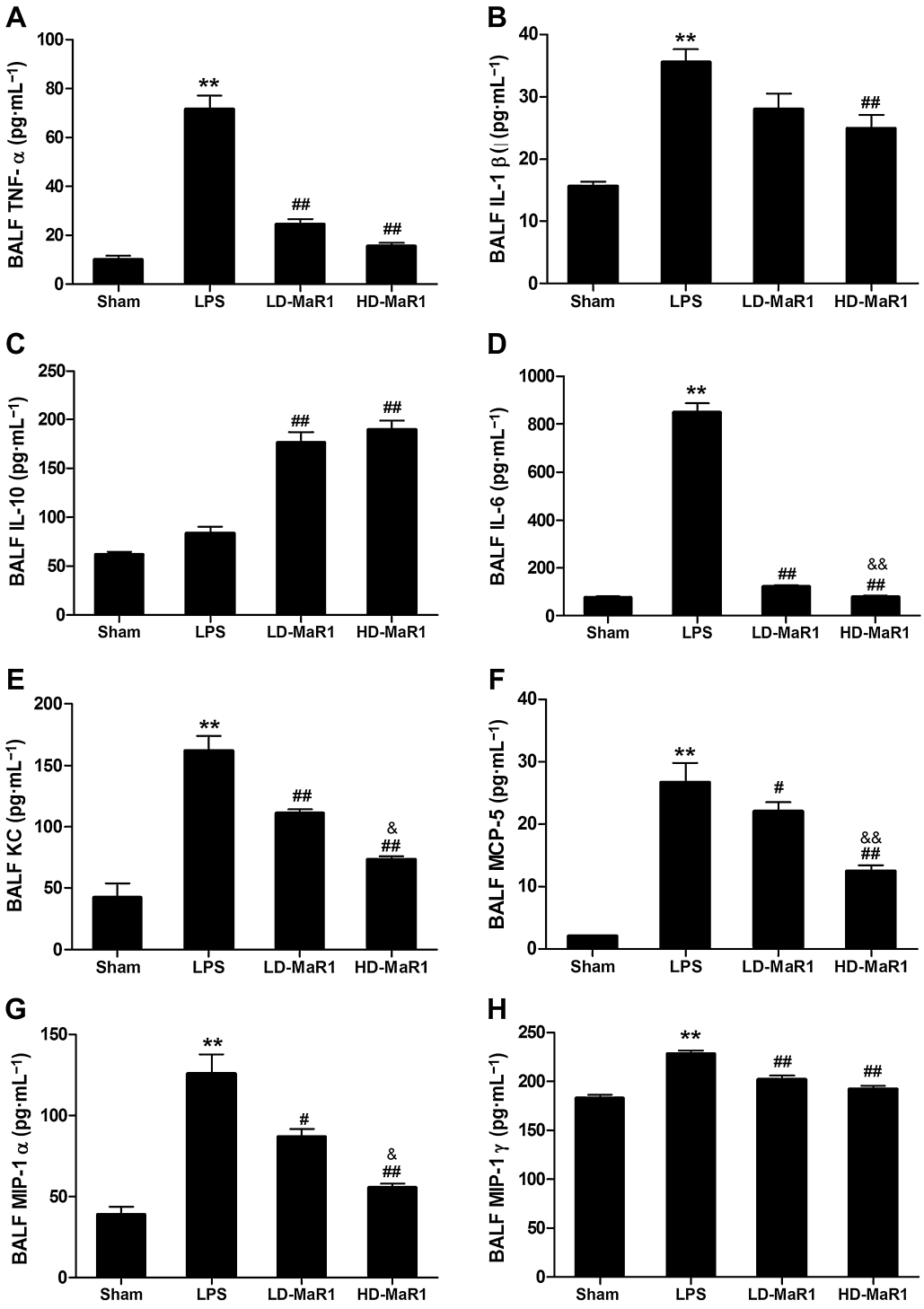

Figure 6

MaR1 affected LPS-induced inflammatory cytokines. LPS (3 mg·kg^{-1}) or NS (1.5 mL·kg^{-1}) was administered intratracheally 60 min before i.v. injection of NS, LD-MaR1 (0.1 ng per mouse) or HD-MaR1 (1 ng per mouse). (A) TNF-α. (B) IL-1β. (C) IL-10. (D) IL-6. (E) KC. (F) MCP-5. (G) MIP-1α. (H) MIP-1γ. Data are shown as means ± SEM, $n = 8$. $^{**}P < 0.01$ versus the sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus the LPS group; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ versus the LD-MaR1 group.

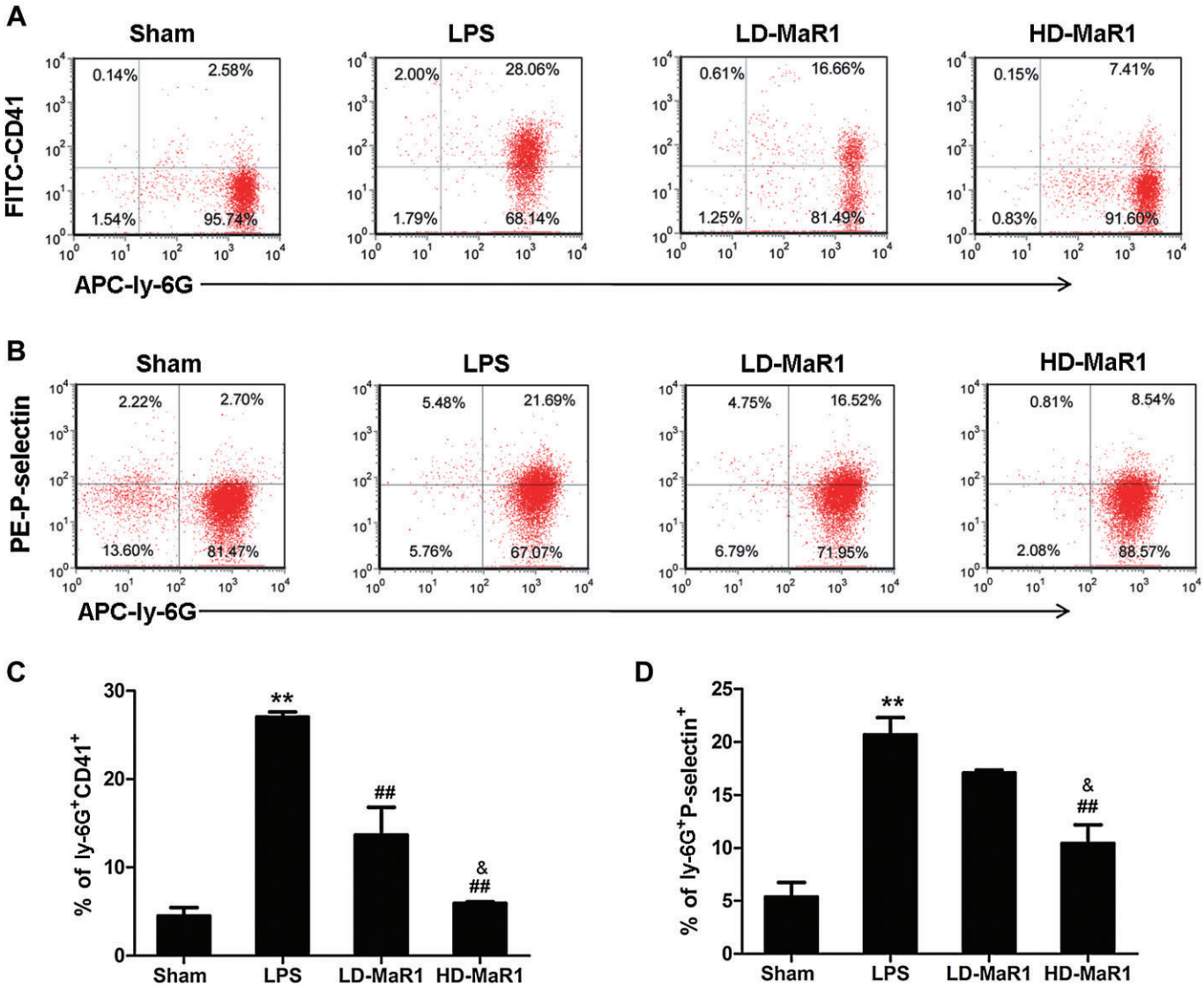

Figure 7

MaR1 suppressed neutrophil–platelet interactions. LPS (3 mg·kg^{-1}) or NS (1.5 mL·kg^{-1}) was administered intratracheally 60 min before i.v. injection of NS, LD-MaR1 (0.1 ng per mouse) or HD-MaR1 (1 ng per mouse). Whole blood was collected through the right ventricle and lysed with red blood cell lysis buffer. Neutrophil–platelet interactions were monitored by flow cytometry. (A) Represents ly-6G$^+$CD41$^+$ cells detected in murine blood 24 h after ALI induction. (B) Represents ly-6G$^+$P-selectin$^+$ cells detected in murine blood 24 h after ALI induction. (C) The % of ly-6G$^+$CD41$^+$ cells. (D) The % of ly-6G$^+$P-selectin$^+$ cells. Data are shown as means ± SEM, $n = 8$. $^{**}P < 0.01$ versus the sham group; $^{\#\#}P < 0.01$ versus the LPS group; $^{\&}P < 0.05$ versus the LD-MaR1 group.

down-regulated the expression of ICAM-1, suggesting that MaR1 attenuated neutrophil adhesion. Consistent with our findings, similar studies have shown that lipoxin A_4 and resolvin D1 inhibited ICAM-1 expression in 1321N1 human astrocytoma (Decker *et al.*, 2009) and LPS-induced lung injury (Wang *et al.*, 2011).

Subsequently, neutrophils crawl on endothelial cells until they find appropriate spots for migration into the interstitium (Phillipson *et al.*, 2006; Alessandri *et al.*, 2013). Pulmonary MPO activity is considered to be an indicator of neutrophil infiltration. In the present study, a combination of the pulmonary MPO activity and immunochemical staining for ly-6G was used to evaluate the presence of neutrophils in the interstitium. We found that MaR1 mitigated the neutrophil infiltration in addition to alleviating LPS-induced lung damage. Furthermore, BALF cytology indicated that neutrophils were present in the alveolar spaces. Taken together, these findings suggested that MaR1-mediated protection in LPS-induced ALI resulted, in part, from the suppression of neutrophil–platelet interactions, which inhibited the adhesion of neutrophils to the endothelium effectively decreasing neutrophil transmigration.

The overexpression of pro-inflammatory cytokines is another important component of the inflammatory response. Animal studies have identified multiple inflammatory mediators that attenuate injury if they are inhibited (Uhlig and

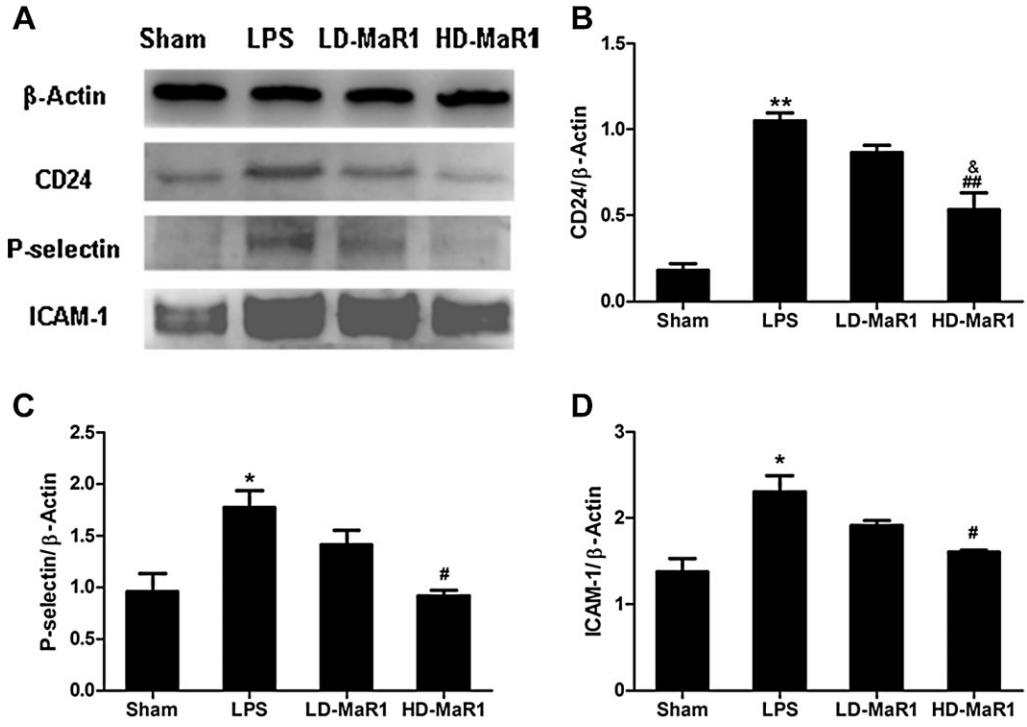

Figure 8

MaR1 decreased the expression of CD24, P-selectin and ICAM-1. LPS (3 mg·kg^{-1}) or NS (1.5 mL·kg^{-1}) was administered intratracheally 60 min before i.v. injection of NS, LD-MaR1 (0.1 ng per mouse) or HD-MaR1 (1 ng per mouse). (A) Western blot bands illustrate the MaR1-mediated inhibition of CD24, P-selectin and ICAM-1. β-Actin was used as a loading control. Results shown are representative of three independent experiments. Density analysis revealed that LPS up-regulated the levels of (B) CD24, (C) P-selectin and (D) ICAM-1, and this effect was inhibited by MaR1 treatment. Data are shown as means ± SEM, $n = 8$. *$P < 0.05$,**$P < 0.01$ versus the sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus the LPS group; $^{\&}P < 0.05$ versus the LD-MaR1 group.

Uhlig, 2004), whereas clinical studies have shown a strong correlation between various mediators and mortality (Ranieri *et al.*, 1999). Inflammatory cytokines can amplify the inflammatory response by stimulating the release of chemoattractant factors by alveolar macrophages and airway epithelial cells, as well as the expression of adhesion molecules by leukocytes and epithelial cells (Keane and Strieter, 2002). IL-1β and TNF-α can trigger the expression of other cytokines and activate the NF-κB signalling pathway (Li *et al.*, 2013; 2014). Moreover, pro-inflammatory chemokines such as MCP-5, MIP-1α and MIP-1γ recruit more leukocytes and enhance cytokine secretion. KC (murine functional analogue of human IL-8) is a potent neutrophil chemoattractant and activator (Seki *et al.*, 2010). Thus, inhibiting the production of pro-inflammatory cytokines is an important role for controlling lung injury. In our model of LPS-induced ALI, MaR1 decreased the production of several pro-inflammatory cytokines and chemokines, including TNF-α, IL-1β, IL-6, KC, MCP-5, MIP-1α and MIP-1γ. In addition, MaR1 promoted the generation of the anti-inflammatory cytokine IL-10. IL-10 is a major anti-inflammatory cytokine that limits the induction of a variety of pro-inflammatory mediators (Grutz, 2005). Moreover, MaR1 inhibited the production of pro-inflammatory cytokines in a dose-dependent manner. The effects of MaR1 on cytokines and chemokines were in agreement with the observed inhibition of neutrophil infiltration in our model. It has also been reported that aspirin-triggered resolvin D1 and resolvin E1 reduce the production of pro-inflammatory cytokines in acid-induced lung injury and bacterial pneumonia (Seki *et al.*, 2010; Eickmeier *et al.*, 2013).

In summary, our findings showed that the macrophage DHA-derived mediator MaR1 mediated anti-inflammatory effects in a model of LPS-induced ALI. In our study, MaR1 mitigated lung injury by inhibiting neutrophil adhesion and pro-inflammatory cytokine production. Taken together, these data suggest that MaR1 may represent a potential new therapeutic approach for the treatment of ALI/ARDS.

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Author contributions

J. G. and Z-Y. W. wrote this article, analysed the data and carried out the experiments. H. Q., B. L., H-B. L. and L. C.

established the animal models and collected samples. C-Y. Y., Y-X. W., J.W. and S-Y. Y. performed the histology and molecular biological techniques. Y. S. and S-L. Y. designed the experimental programme and revised the article.

Conflict of interest

None.

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