

rPSGL-1-Ig, a recombinant PSGL-1-Ig fusion protein, ameliorates LPS-induced acute lung injury in mice by inhibiting neutrophil migration

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Abstract

The binding of selectin to P-selectin glycoprotein ligand-1 (PSGL-1) mediates the tethering and rolling of leukocytes on the endothelium during leukocyte migration and inflammation. Recombinant human PSGL-1-Ig fusion protein (rPSGL-1-Ig) is a widely used selectin inhibitor that prevents neutrophil entry into inflamed or reperfused tissues. We hypothesized that rPSGL-1-Ig could be used to as a drug for the treatment of acute lung injury (ALI). We induced murine ALI by injecting mice with lipopolysaccharide (LPS) and then treated the mice with rPSGL-1-Ig. We determined the lung injury index, wet/dry ratio, and inflammatory cytokine level in differentially treated mice. The symptoms of LPS-induced lung injury were alleviated by rPSGL-1-Ig treatment. The histopathological index of LPS-induced lung injury improved after rPSGL-1-Ig treatment. rPSGL-1-Ig treatment also reduced the recruitment of inflammatory cells, including neutrophils, into the lung, as well as reducing the level of inflammatory cytokines. These data suggest that rPSGL-1-Ig protein has a therapeutic effect on LPS-induced lung injury.

Key words: Recombinant PSGL-1-Ig fusion protein, LPS-induced acute lung injury, neutrophil migration.

Introduction

Acute lung injury (ALI) is a syndrome that consists of acute hypoxemic respiratory failure with bilateral pulmonary infiltrates. It is associated with both pulmonary and non-pulmonary risk factors and is not primarily due to left atrial hypertension (1). Recent studies have advanced our understanding of the epidemiology, pathogenesis, and treatment of this disease. However, more progress is needed in order to reduce mortality and morbidity from ALI and acute respiratory distress syndrome (2-4).

All leukocytes that participate in innate or adaptive immunity can migrate to sites of inflammation or tissue injury by crossing the endothelial barrier between blood and tissue (5, 6). The process of leukocyte recruitment involves a tightly regulated cascade of adhesive interactions between leukocytes and endothelial cells: (i) capture of leukocytes by activated endothelium, (ii) rolling of leukocytes on the endothelium, (iii) slow rolling of leukocytes on the endothelium, (iv) activation of leukocytes by chemokines on the endothelium, (v) firm adhesion of leukocytes onto the endothelium, (vi) post-adhesion crawling or locomotion of leukocytes, and (vii) transendothelial migration or diapedesis. Distinct families of adhesion molecules, such as P-selectin and L-selectin, control the recruitment of the cells (7, 8).

Leukocyte rolling is mediated by the selectin family of adhesion molecules (P-, E-, and L-selectin) via interactions with glycoprotein counter-ligands (9, 10). P-selectin glycoprotein ligand-1 (PSGL-1) is a mucin-like selectin counter-receptor that binds preferentially to P-selectin but also with low affinity to E-selectin (2). Numerous studies have demonstrated that inhibition of the PSGL-1 signaling pathway effectively inhibits

leukocyte rolling in models of inflammation (11-14). Previous studies have demonstrated that engagement of PSGL-1 triggers intracellular signaling events, including the activation of signaling molecules and integrins (15).

Soluble recombinant PSGL-1 fused to the Fc domain of human IgG1 (rPSGL-1-Ig) is a widely used selectin inhibitor. PSGL-1 acts by preventing neutrophil entry into inflamed or reperfused tissues. We asked if rPSGL-1-Ig could be used to treat ALI. We injected lipopolysaccharide (LPS)-induced ALI mice via the tail vein with rPSGL-1-Ig or with human IgG as a negative control. rPSGL-1-Ig reduced the lung injury index and neutrophil infiltration in the bronchoalveolar lavage fluid (BALF) in mice with LPS-induced ALI. In addition, the expression of proinflammatory cytokines, such as TNF- α and IL-6, in the BALF was lower in rPSGL-1-Ig-treated mice than in IgG-treated mice. Our data demonstrate that rPSGL-1-Ig has potential as a therapeutic drug for ALI.

Materials and methods

Mice

Forty C57BL/6 mice were purchased from the SLRC Laboratory (Shanghai, China). The mice were randomized into a PBS-treated control group (n = 10, PBS-treated), an LPS-induced ALI group (n = 10, LPS-induced), an LPS-induced human IgG-treated group (n = 10, hIgG-treated), and an LPS-induced rPSGL-1-treated group (n = 10, rPSGL-1-Ig-treated). Mice were sacrificed 24 h after the administration of LPS and protein.

rPSGL-1-Ig was purchased from Y's Therapeutics Company (Burlingame, CA, USA). rPSGL-1-Ig was systemically administered to mice via tail vein injection.

tion at a concentration of 5 mg/kg body weight. As a control for protein administration, mice received isotype-matched human IgG1 (Invitrogen, San Francisco, CA, USA).

Establishment of the acute lung injury model in mice

Female C57BL/6 mice (8 to 10 weeks old) were treated with 20 mg/kg LPS (Sigma-Aldrich, St. Louis, MO, USA) from *Escherichia coli* (serotype 0111:B4) in 100 μ L of PBS or with an equal volume of PBS, as vehicle control, via intraperitoneal injection.

Histopathological analysis and lung injury scores

Following sacrifice at 24 h after LPS and protein administration, the whole lower lobe of the left lung was fixed in a 4% formaldehyde neutral buffer solution for 24 h, dehydrated in a graded ethanol series, embedded in paraffin, and sliced at 5- μ m thickness. Paraffin sections were stained with hematoxylin & eosin (H&E) for histopathological analysis.

In order to evaluate the severity of lung injury, we used a semi-quantitative histological index of quantitative assessment (IQA) for lung injury (3). Eight sections were randomly selected for each group of mice, and 10 fields from each section were examined with microscopy (40 \times). A pathologist who was blinded to the study evaluated all of the sections. The average values were considered a semi-quantitative histological IQA of lung injury.

Lung wet/dry weight ratio

The superior lobe of the right lung was cleansed and weighed to obtain the wet weight and then placed in an oven at 80°C for 48 h for measurement of the dry weight. The ratio of the wet weight to dry weight was calculated to assess tissue edema.

Bronchoalveolar lavage (BAL) examination

The trachea was exposed and cannulated with a catheter. The left lung was lavaged three times with sterile PBS (0.5 mL/wash). The fluid recovered after lavage was greater than 90% on average. The BAL fluid (BALF) was centrifuged at 2000 rpm for 10 min at 4°C. The supernatant was stored at -80°C for cytokine and protein analysis, and the cell pellet was resuspended in PBS for neutrophil counting.

ELISA

TNF- α , IL-6, IL-1 α , and MIP-2 were detected using ELISA, which was performed according to the protocol recommended by the manufacturer of the ELISA kit used (R&D, USA). The experiment was repeated three times, and the mean value was calculated.

Neutrophil count in the BALF

The BALF was collected from differentially treated mice 24 h after injury to determine the total cell count, percentage of neutrophils, and myeloperoxidase (MPO) activity. The MPO activity was assessed using previously described methods (16).

Measurement of protein concentration in the BALF

The concentration of protein in the BALF was measured using Bradford reagent (Protein Assay kit; Bio-Rad, Hercules, CA, USA). Briefly, 160 μ L of each standard and sample solution was pipetted into separate microtiter plate wells, and 40 μ L of the dye reagent was added to each well and mixed thoroughly. The mixture was incubated at room temperature for at least 5 min before measuring the optical density (OD) at 595 nm. Comparison to a standard curve provided a relative measurement of the protein concentration.

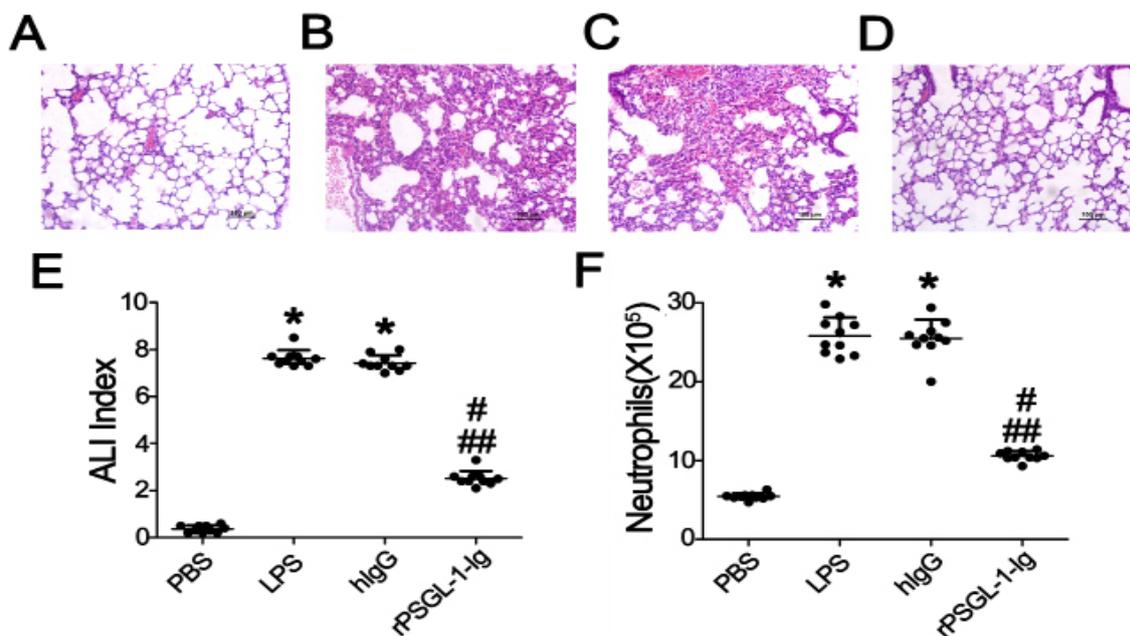


Figure 1. Histopathological index of injured murine lungs. H&E-stained lung sections from (A) PBS-treated mice, (B) LPS-induced ALI mice, (C) human IgG-treated mice, and (D) rPSGL-1-Ig-treated mice. (E) The lung injury index of the differentially treated mice. (F) The neutrophil count in the BALF of differentially treated mice.

*P < 0.05 compared with the PBS-treated group; #P < 0.05 compared with the LPS-induced ALI group; ##P < 0.05 compared with the human IgG-treated ALI group.

ALI, acute lung injury; PBS, phosphate-buffered saline; LPS, lipopolysaccharide; H&E, hematoxylin and eosin; BALF, bronchoalveolar lavage fluid; hIgG, human IgG; rPSGL-1-Ig, recombinant P-selectin glycoprotein ligand-1-IgG fusion protein; Ctr, control.

Myeloperoxidase activity assay

MPO activity in the homogenized lung tissue was measured as described by Gray et al. (17). The MPO concentration was determined using an MPO ELISA kit (BlueGene, China). Briefly, the lung tissue was homogenized and centrifuged at 15,000 rpm for 20 min at 4°C. The supernatants and standard samples were added to a microtiter plate (100 μ L/well) that had been precoated with a murine anti-MPO mAb. After incubation for 1 h at 37°C, the plate was washed six times. Substrate and stop solution were added, and the OD at 450 nm was measured using a microplate reader. All samples were assayed in triplicate.

Neutrophil purification and adhesion

Peripheral blood was collected from C57BL/6 mice. Neutrophils were isolated as previously described by Luo and Dorf (18) and resuspended in Hank's balanced salt solution (HBSS)-H (HBSS supplemented with 10 mM HEPES (pH 7.4)) containing 1.3 mM Ca^{2+} and 0.8 mM Mg^{2+} . The purity and cell viability of the neutrophil preparations were consistently >98%, as assessed with acetic blue staining and trypan blue exclusion, respectively.

Neutrophils were resuspended at 5×10^6 cells/mL in HBSS-H containing 1.3 mM Ca^{2+} and 0.8 mM Mg^{2+} and labeled with 5 μ M calcein-AM, an intracellular fluorescent dye, for 30 min at 37°C. Labeled cells were washed and resuspended at 5×10^6 cells/mL in HBSS-H containing 1.3 mM Ca^{2+} and 0.8 mM Mg^{2+} , and 50 μ L of cells was added to Costar 96-well plates, which had been coated overnight with 20 μ g/mL recombinant mouse L-selectin (100 μ L/well; 50045-M03H; Sino Biological Inc.). Thereafter, 50 μ L of rPSGL-1-Ig at increasing concentrations (2 \times) was added to the wells, using equal volumes of human IgG as the control. Neutrophils were allowed to adhere for 30 min at 37°C. They were then washed three times by immersion in cold PBS. The adherent cells were lysed by the addition of ddH₂O, and fluorescence was measured using a 96-well plate fluorescence reader.

Statistical analysis

All data were analyzed using SPSS13.0 software and expressed as the mean \pm SD. Significant differences were assessed by one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference test. A probability value of less than 0.05 was considered to indicate a statistically significant difference.

Results

Therapeutic effect of rPSGL-1-Ig on LPS-induced murine acute lung injury

Previous studies have demonstrated that PSGL-1, a mucin-like selectin counter-receptor, binds preferentially to P-selectin and with low affinity to E selectin (2). In addition, studies have shown that inhibition of the PSGL-1 signaling pathway inhibits the rolling of leukocytes in models of inflammation (11-14). Moreover, rPSGL-1-Ig, a widely used selectin inhibitor, could prevent neutrophil entry into inflamed or reperfused tissues. Therefore, we asked if rPSGL-1-Ig had a therapeutic effect on LPS-induced ALI.

To answer this question, we induced ALI by injecting mice intraperitoneally with LPS. One hour after LPS injection, the mice were treated with human IgG and rPSGL-1-Ig via tail vein injection. As shown in Fig. 1, PSGL-1-Ig-treated mice showed less infiltration of inflammatory cells and better lung histology 24 h after injection (Fig. 1D) than the LPS-induced ALI mice (Fig. 1B) or human IgG-treated ALI mice did (Fig. 1C). To further assess the therapeutic effect of rPSGL-1-Ig on lung injury, we scored injury in the left lung in mice from each group (Fig. 1E). The average score of the rPSGL-1-Ig-treated group was significantly lower than that of the LPS-induced ALI group ($P < 0.05$) and human IgG-treated ALI group ($P < 0.05$).

The neutrophil count in the BALF is shown in Fig. 1F. Compared with that of the LPS-induced ALI group and human IgG-treated ALI group, the neutrophil count of the rPSGL-1-Ig-treated group was substantially re-

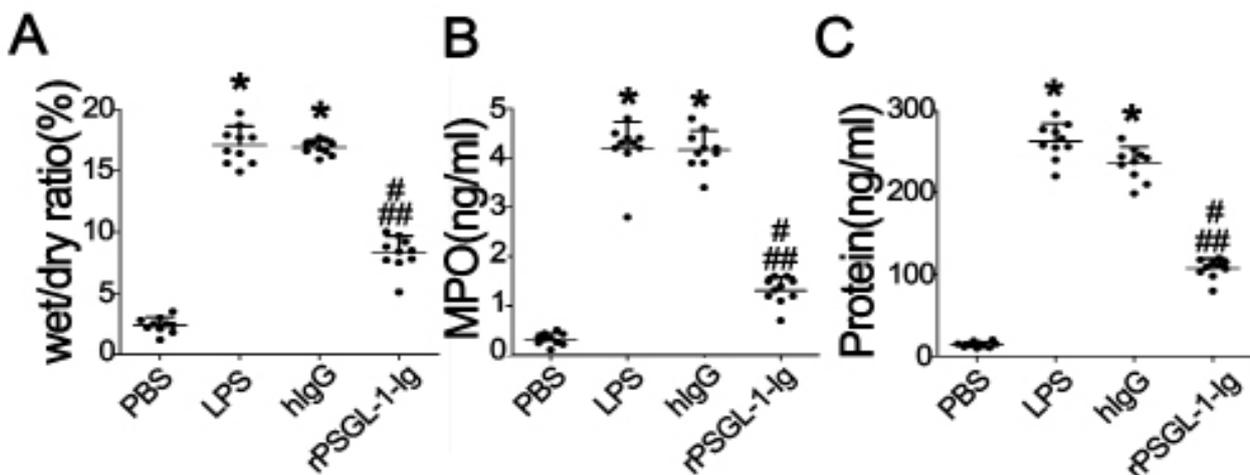


Figure 2. Lung wet/dry ratio, protein concentration, and MPO activity in the murine lung. Lung wet/dry ratio (A), MPO activity in lung homogenates (B), protein concentration in the BALF (C) of differentially treated ALI mice.

* $P < 0.05$ compared with the PBS-treated group; # $P < 0.05$ compared with the LPS-induced ALI group; ## $P < 0.05$ compared with the human IgG-treated ALI group.

ALI, acute lung injury; PBS, phosphate-buffered saline; LPS, lipopolysaccharide; H&E, hematoxylin and eosin; BALF, bronchoalveolar lavage fluid; hIgG, human IgG; rPSGL-1-Ig, recombinant P-selectin glycoprotein ligand-1-IgG fusion protein; Ctr, control.

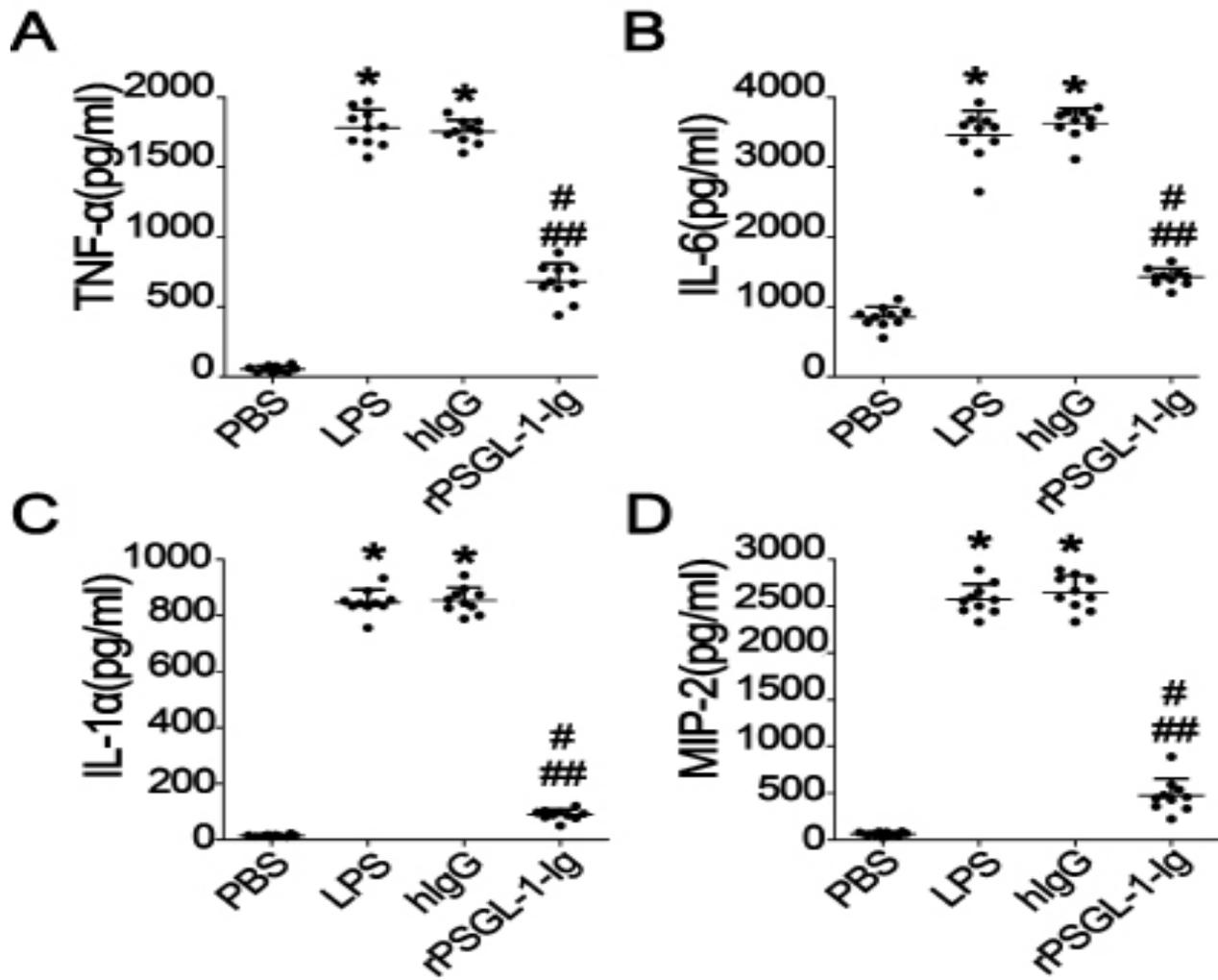


Figure 3. Proinflammatory cytokines in the BALF of ALI mice. TNF- α (A), IL-6 (B), IL-1 α (C), and MIP-2 (D) levels in the BALF of ALI mice following different treatments.

* $P < 0.05$ compared with the PBS-treated group; # $P < 0.05$ compared with the LPS-induced ALI group; ## $P < 0.05$ compared with the human IgG-treated ALI group.

ALI, acute lung injury; PBS, phosphate-buffered saline; LPS, lipopolysaccharide; H&E, hematoxylin and eosin; BALF, bronchoalveolar lavage fluid; hIgG, human IgG; rPSGL-1-Ig, recombinant P-selectin glycoprotein ligand-1-IgG fusion protein; Ctr, control.

duced. These data suggest that rPSGL-1-Ig has a therapeutic effect on LPS-induced lung injury by inhibiting the infiltration of neutrophils in lung tissue.

The lung wet/dry ratio, protein concentration in the BALF, and MPO in lung homogenates

LPS-induced mice treated with rPSGL-1-Ig showed lower lung wet/dry ratio than that shown by mice treated with human IgG (Fig. 2A). The difference in the wet/dry ratio between the rPSGL-1-Ig-treated group and the human IgG-treated group or LPS-induced group was significant ($P < 0.05$).

Additionally, we measured the MPO activity in lung homogenates and protein concentration in the BALF in the differentially treated groups. MPO activity in lung homogenates (Fig. 2B) and protein concentration in the BALF (Fig. 2C) were lower in the group treated with rPSGL-1-Ig than in the LPS-induced ALI group or human IgG-treated ALI groups ($P < 0.05$). These data are consistent with the effects of rPSGL-1-Ig on lung injury and neutrophil infiltration.

Expression of proinflammatory cytokines in the lung

To determine the effects of rPSGL-1-Ig on local inflammatory milieu in the lung, we measured the ex-

pression of several inflammatory cytokines in the lung BALF with ELISA (Fig. 3). Exposure to LPS increased the levels of inflammatory cytokines such as TNF- α , IL-6, IL-1 α , and MIP-2 (Fig. 3). The administration of rPSGL-1-Ig attenuated the LPS-induced increase in inflammatory cytokines. As shown in Fig. 3, the levels of TNF- α , IL-6, IL-1 α , and MIP-2 in mice from the rPSGL-1-Ig-treated group mice were lower than those in mice from the LPS-induced ALI groups and human IgG-treated groups. These results are consistent with the effects of rPSGL-1-Ig on lung injury.

rPSGL-1-Ig blocks neutrophil adhesion to L-selectin

To elucidate the effect of rPSGL-1-Ig on the L-selectin-mediated adhesion of neutrophils, we isolated neutrophils from the peripheral blood of mice and measured the adhesion of neutrophils to L-selectin in the presence of different concentrations of rPSGL-1-Ig. As shown in Fig. 4, the adhesion of neutrophils was blocked when the concentration of rPSGL-1-Ig was >10 $\mu\text{g/mL}$. These results are consistent with the decreased infiltration of neutrophils into damaged lung tissue described above.

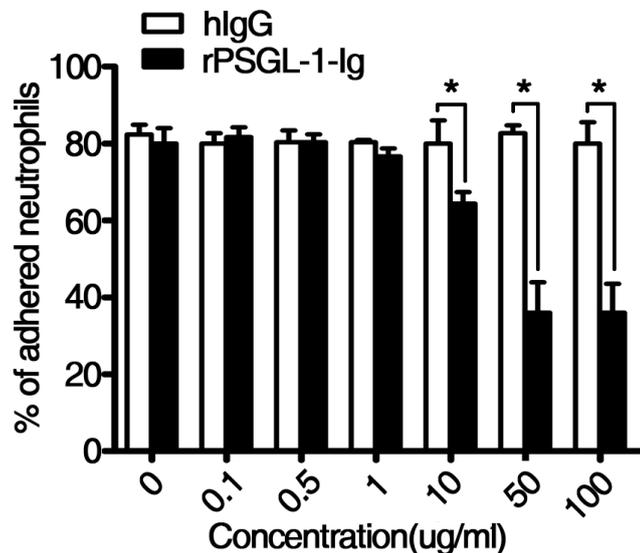


Figure 4. rPSGL-1-Ig blocks the adhesion of neutrophils to L-selectin. Neutrophils were incubated with rPSGL-1-Ig or human IgG, and the mixture was added to Costar 96-well plates coated with L-selectin recombinant protein. Neutrophils were allowed to adhere for 30 min at 37°C, and the number of adhered neutrophils was determined as described. The results show the percentage of neutrophils that adhered to L-selectin. Data are presented as the mean \pm SD of three experiments.

* $P < 0.05$ compared to the human IgG-treated group. ALI, acute lung injury; PBS, phosphate-buffered saline; LPS, lipopolysaccharide; H&E, hematoxylin and eosin; BALF, bronchoalveolar lavage fluid; hIgG, human IgG; rPSGL-1-Ig, recombinant P-selectin glycoprotein ligand-1-IgG fusion protein; Ctr, control.

Discussion

Neutrophils are recruited from the blood to sites of inflammation, where they contribute to immune defense, but can also cause tissue damage. During inflammation, neutrophils roll along the microvascular endothelium before arrest and transmigration. Arrest requires conformational activation of the integrin lymphocyte function-associated antigen 1, which can be induced by selectin engagement. In neutrophil migration, selectins play a critical role in the adhesion of neutrophils to the microvascular endothelium (19). In addition, the migration of neutrophils from the blood to sites of inflammation plays an important role in the pathogenesis of ALI (20-22).

As an important proinflammatory cytokine, TNF- α contributes to the occurrence and persistence of ALI (6). TNF- α can induce the production and release of inflammatory cytokines, including IL-6, during ALI. IL-1 β and MIP-2 also play important roles in ALI.

PSGL-1 is a mucin-like glycoprotein that binds P-selectin, as well as L- and E-selectin. It is expressed on certain leukocytes, such as neutrophils, as well as on inflamed endothelial cells. Previous studies have demonstrated that PSGL-1 interacts with P-selectin in addition to E- and L-selectin. PSGL-1 also promotes the migration of neutrophils into inflammatory tissues (23-25). Therefore, blockage of this migration-promoting signaling pathway can reduce the tissue damage caused by inflammatory lymphocyte infiltration and subsequent inflammatory responses. Therefore, in this study, we examined whether rPSGL-1-Ig had a thera-

peutic effect on LPS-induced lung injury.

In this study, we treated LPS-induced ALI mice with rPSGL-1-Ig protein via tail vein injection. The rPSGL-1-Ig-treated mice showed better lung histology and less infiltration of inflammatory cells 24 h after injection (Fig. 1) than the LPS-induced ALI mice (Fig. 1B) and human IgG-treated mice did (Fig. 1C). The infiltration of neutrophils into lung tissue is an important process in ALI. Therefore, we determined the neutrophil count in the BALF. The number of neutrophils in the BALF was lower in the rPSGL-1-treated group than in the LPS-induced ALI group and human IgG-treated group. Taken together, our data suggested that rPSGL-1 had a therapeutic effect on LPS-induced ALI in mice. To confirm the beneficial effect of rPSGL-1-Ig on lung injury, we measured the protein concentration in the BALF and MPO activity in lung homogenates. Our results showed that treatment of LPS-induced ALI mice with rPSGL-1-Ig not only reduced the protein concentration in the BALF, but also decreased MPO activity in lung homogenates (Fig. 2B and 2C). Furthermore, the lung wet/dry ratio was lower in the rPSGL-1-Ig-treated mice than in the LPS-induced ALI mice and human IgG-treated mice (Fig. 2A). We also measured proinflammatory cytokine levels in the BALF of differentially treated ALI mice. We found that the levels of proinflammatory cytokines such as IL-6, TNF- α , IL-1 β , and MIP-2 were lower in ALI mice treated with rPSGL-1-Ig than in mice treated with human IgG (Fig. 3). Adhesion assays (Fig. 4) supported the conclusion that rPSGL-1-Ig inhibited the infiltration of neutrophils into lung tissue and attenuated lung injury.

In summary, our data suggest that rPSGL-1-Ig has beneficial effects on ALI. As a selectin ligand, rPSGL-1-Ig interacts with PSGL-1 receptor to block the infiltration of inflammatory lymphocytes into damaged lung tissue in ALI. Thus, rPSGL-1-Ig has potential as a new drug candidate for the treatment of LPS-induced lung injury that acts by inhibiting neutrophil migration from the blood into damaged lung tissue.

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