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The role of PSGL-1 in pathogenesis of systemic inflammatory response and coagulopathy in endotoxemic mice

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ABSTRACT

Introduction: Endotoxemia often results in systemic inflammatory response syndrome (SIRS), coagulation disturbance and acute lung injury (ALI), and such a condition is associated with the activation of platelets, leukocytes and vascular endothelial cells (VECs). P-selectin glycoprotein ligand 1 (PSGL-1) is a key regulatory molecule in the activation of platelets, leukocytes and VECs. However, it still remains largely unexplored whether PSGL-1 plays an important role in SIRS, coagulation dysfunction and ALI of endotoxemia. In the present study, we aimed to study the role of PSGL-1 in above-mentioned situations using endotoxemic mice.

Materials and methods: An endotoxemia model was established in BALB/c mice via lipopolysaccharide (LPS) administration. Moreover, the mice were simultaneously injected with PSGL-1 antibody for intervention. The survival rate, morphologic changes of lung tissues, platelet-leukocyte adhesion, tissue factor expression on leukocytes, fibrinogen deposition in lung tissues, serum levels of inflammatory factors and the activation of VECs were determined.

Results: The results showed that the aggregation and recruitment of platelets and leukocytes in lung tissues, the expression of tissue factor on leukocytes, the serum levels of inflammatory factors, the activation of VECs, and the fibrinogen deposition in lung tissues were increased in endotoxemic mice, which were significantly alleviated by administration of PSGL-1 antibody. Moreover, blockade of PSGL-1 markedly increased survival rate, and alleviated coagulation disturbance and lung injury in endotoxemic mice.

Conclusions: Taken together, PSGL-1 played an important role in pathogenesis of SIRS and coagulation dysfunction and ALI in endotoxemic mice.

1. Introduction

In the pathogenesis of lipopolysaccharide (LPS)-induced endotoxemia, systemic inflammatory response syndrome (SIRS), coagulation disturbance and acute lung injury (ALI) play a pivotal role, leading to impaired microcirculation, disseminated intravascular coagulation (DIC) and multiple organ failure. In our previous research, we have found that activated platelets can activate leukocytes and vascular endothelial cells (VECs), and promote systemic inflammatory responses and coagulation disturbance in endotoxemic mice [1]. Activation and interaction of platelets, leukocytes and VECs play an important role in inflammation and coagulation disorders in sepsis. Inflammation triggers the synthesis of interleukins (IL-6) and tumor necrosis factor (TNF)-α [2], which activate the coagulation pathway via expression of tissue factor (TF). TF and fibrin regulate the inflammatory response via altering cytokine expression, inflammatory cell migration and activation [3]. Fibrinogen and D-dimer are associated with coagulation. Analysis of the inflammation and coagulation would provide targets for therapeutic intervention in endotoxemia.

P-selectin glycoprotein ligand 1 (PSGL-1) is a key regulatory molecule in the activation of platelets, leukocytes and VECs in inflammatory disease [4–6]. PSGL-1 is expressed on the leukocyte membrane and it can mediate leukocyte adhesion [7,8]. It binds to activated platelets and P-selectin on the surface of endothelial cells [9,10]. It can also bind to activated E-selectin on endothelial cell surface [11]. Interaction of PSGL-1 with P-selectin on platelets promotes the formation of platelet-leukocyte aggregation and enhances the adhesion of inflammatory cells to VECs, leading to infiltration of

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leukocytes in tissues [12]. PSGL-1 can interact with P-selectin expressed on endothelial cells to promote leukocyte-endothelial cell adhesion and participate in vascular injury [13], atherosclerosis [14] and thrombus [15]. It has shown that blockade of P-selectin can improve oxygenation and reduce infiltration of leukocytes in lung tissue in ALI [16]. PSGL-1 is associated with the accumulation of neutrophils in the septic lungs [4]. Inhibition of PSGL-1 can reduce leukocyte adhesion in arterioles [17].

Based on the above-mentioned findings, it is still unclear whether PSGL-1 promotes SIRS, coagulation dysfunction and ALI in endotoxemia. In the present study, we aimed to investigate the effects of PSGL-1 on inflammation, coagulation disturbance and organ damage in a murine endotoxemic model induced by LPS administration.

2. Materials and methods

2.1. Animals and treatments

BALB/c mice [6–8 weeks old; 20–25 g, approval number SYXK (Xiang) 2011-0001] were purchased from Hunan Slikejingda Experimental Animal Co., Ltd. (Changsha, China). After a 1-week acclimatization period, mice were randomly divided into four groups as follows: (1) NS (normal saline, NS) + anti-control group, (2) NS + anti-PSGL-1 group, (3) LPS + anti-control group and (4) LPS + anti-PSGL-1 group. Mice were intraperitoneally administered with 6 mg/kg LPS (E-coli, O111:B4, L2630, Sigma-Aldrich, St. Louis, MO, USA) or sham treated with NS. Immediately following LPS stimulation, mice were intraperitoneally injected with a single dose of 50 μg [18] anti-PSGL-1 mAb (4RA10) (BD Biosciences, San Jose, CA, USA) or equal amount of isotype-matched control antibody (BD Biosciences, San Jose, CA,USA). For survival analysis, 20 sex- and age-matched mice in each group were used. Survival was monitored every 12 h during the first 2 days and daily thereafter. Mice (n = 10) were sacrificed and used in the assays described below. The effects of different treatments were evaluated at 24 h after the injections. All experiments were approved by the local animal experimental and care committees.

2.2. Histological analysis

At 24 h after administration of anti-PSGL-1 mAb (4RA10) or isotype-matched control antibody, mice were anesthetized and sacrificed by cardiac puncture and exsanguination. Lung tissues were collected and fixed in 4% formaldehyde overnight. Then fixed specimens were dehydrated and paraffin-embedded. Subsequently, 4-μm sections were prepared and stained with hematoxylin and eosin (H&E).

2.3. Blood examination

Blood samples were collected from mice at 24 h after administration of anti-PSGL-1 mAb (4RA10) or isotype-matched control antibody. Platelet counts were determined in EDTA-anticoagulated blood using routine analytical methods (Sysmex XE-5000, the Netherlands).

2.4. Blood protein analysis

After 24 h of administration of anti-PSGL-1 mAb (4RA10) or isotype-matched control antibody, serum was prepared from venous blood, and then all samples were stored at −80 °C prior to further analysis. The levels of TNF-α, IL-6, D-dimer, sVCAM-1 and VEGF in serum were determined using enzyme-linked immunosorbent assay (ELISA) kits (Boster Biological Technology Co., Ltd., Wuhan, China). Absorbance at a wavelength of 450 nm was determined according to the manufacturers' instructions using a microplate reader (1510, Thermo Fisher Scientific, VANTAA, Finland). All specimens were tested at the same time.

2.5. Immunofluorescence analysis

At 24 h after administration of anti-PSGL-1 mAb (4RA10) or isotype-matched control antibody, lung tissues were collected and fixed. The collected samples were stained with antibodies against CD41 (platelet marker), Ly6G (neutrophil marker), CD11b (leukocyte marker), TF and fibrinogen. How PSGL-1 affected platelet-leukocyte interaction was analyzed, and the expression of TF on leukocytes and the deposition of fibrinogen in the lung tissues were also examined. Lung tissue sections were deparaffinized, and antigen retrieval was achieved by microwaving the tissue slides for 4 min in citrate buffer (pH 6.0). The slides were incubated with PE-conjugated rat anti-CD41 antibody (1:50, Abcam, Cambridge, UK) and FITC-conjugated rabbit anti-Ly6G antibody (1:50, Abcam, Cambridge, UK) or FITC-conjugated rat anti-CD11b antibody (1:100, Abcam, Cambridge, UK) and PE-conjugated rabbit anti-TF antibody (1:50, Abcam, Cambridge, UK) or FITC-conjugated rat anti-CD31 antibody (1:100, Abcam, Cambridge, UK) and PE-conjugated rabbit anti-TF antibody (1:50, Abcam, Cambridge, UK). Subsequently, the sections were incubated with FITC-conjugated rabbit anti-mouse anti-fibrinogen antibody (1:100, Abcam, Cambridge, UK). After a final wash, the sections were mounted in Vectashield with 4′,6-diamidino-2-phenylindole (DAPI), cover slipped and visualized using fluorescence microscope.

2.6. Western blotting analysis

Proteins were extracted from individual frozen tissues, and the lung tissues were homogenized in RIPA lysis buffer supplemented with appropriate protease inhibitors. Soluble proteins were recovered after centrifugation at 12,000 rpm for 10 min at 4 °C. The protein concentrations were determined using a BCA protein assay kit according to the manufacturer's instructions. The proteins were denatured at 100 °C for 20 min. Equal amounts of proteins were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene fluoride (PVDF) membranes. The blots were blocked with 5% nonfat dry milk in Tris buffered saline (TBS) containing 0.5% Tween-20 (TBST) at room temperature for 1 h. Blots were then incubated with rabbit anti-fibrinogen antibody (1:1000, Abcam, Cambridge, MA, USA) and anti-β-actin antibody (1:2000, Abcam, Cambridge, MA, USA) in blocking buffer at 4 °C overnight. Subsequently, the blots were washed with 0.5% TBST for three times and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG at room temperature for 1 h. Immunoreactive bands were visualized with ECL reagents (Amersham Pharmacia Biotech, Basel, Switzerland).

2.7. Statistical analysis

Data were expressed as mean ± SEM for each group. Differences among groups were statistically analyzed using one-way ANOVA. Bonferroni's multiple comparisons were used as post-test analyses. Survival data were analyzed.

3. Results

3.1. Blockade of PSGL-1 increases the survival rate and attenuates the lung damage in endotoxemic mice

To assess the effect of PSGL-1 during endotoxemia, the mice were intraperitoneally injected with 6 mg/kg LPS. The mortality in the LPS group was significantly increased at 24 h post-injection, with a total mortality rate of 50%. In contrast, all mice in the NS + anti-control group and NS + anti-PSGL-1 group survived during the observation period. Kaplan-Meier analysis revealed a significant difference in survivals between the groups ($P < 0.01$), suggesting that blockade of PSGL-1 significantly reduced the mortality during endotoxemia (Fig. 1).

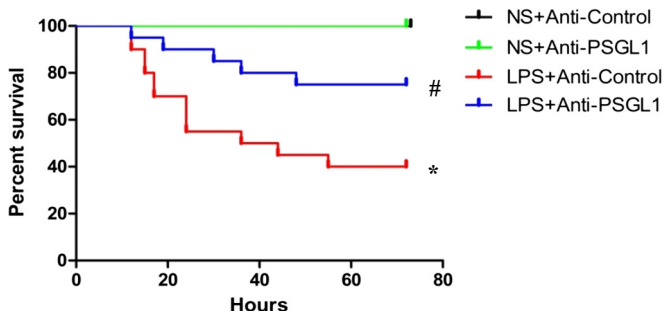

Fig. 1. Effect of PSGL-1 antibody on the survival rate of endotoxemic mice. $^{*}P < 0.01$, vs. NS + anti-control group; $^{\#}P < 0.01$, vs. LPS + anti-control group (n = 20).

As endotoxemia causes a life-threatening inflammatory condition that involves multiple organ injury, we examined the effect of PSGL-1 on LPS-induced lung injury. The lung architecture was disrupted, and increased cell infiltration was observed in the LPS group (Fig. 2). However, blockade of PSGL-1 could attenuate LPS-induced histological changes of lung. The results indicated that blockade of PSGL-1 could reduce the lung damage caused by endotoxemia (Fig. 2). Taken together, these results suggested that blockade of PSGL-1 played a protective role during endotoxemia.

3.2. PSGL-1 promotes the infiltration of platelet-neutrophil in the lung tissues of endotoxemic mice

Recent studies have shown that platelet-leukocyte aggregation in tissues can mediate inflammatory reactions and coagulation dysfunction [19,20]. We investigated the effect of PSGL-1 on the formation of platelet-neutrophil infiltration by staining lung tissues with antibodies against CD41 and Ly6G (Fig. 3). By using immunofluorescence analysis, we found the formation of platelet-neutrophil infiltration in the lung tissues during endotoxemia (Fig. 3). The lungs of control mice did not show significant cell infiltration, while infiltration of both platelets and neutrophils was observed LPS-challenged animals. In contrast, blockade of PSGL-1 decreased the platelet-neutrophil aggregation in lung tissues. These results suggested that PSGL-1 could mediate inflammatory reactions and coagulation dysfunction by promoting the infiltration of platelet-neutrophil during endotoxemia.

3.3. PSGL-1 promotes the expression of TF on white blood cells (WBCs) in endotoxemic mice

To study whether PSGL-1 affected the TF expression on WBCs, we analyzed TF expression on leukocytes in lung tissues. The expression of TF on leukocytes in lung tissue was detected by antibodies against TF (red) and CD11b (green). The results showed that the expression of TF on leukocyte was significantly increased in the LPS + anti-control group. Co-localization (yellow) was observed in the lungs. The expression of TF on leukocytes in the LPS + anti-PSGL-1 group was significantly reduced. These results indicated that PSGL-1 promoted the expression of TF on leukocyte and increased the occurrence of coagulation disorders in endotoxemic mice (Fig. 4).

3.4. PSGL-1 increases the levels of TNF-α and IL-6 in serum of endotoxemic mice

To assess the effect of PSGL-1 on systemic inflammatory responses, we examined the levels of TNF-α and IL-6 in serum. Fig. 5 shows that the levels of TNF-α and IL-6 were detected in all samples. In the LPS + anti-control group, the levels of TNF-α and IL-6 in the serum were higher compared with the NS + anti-control group ($P < 0.01$). In addition, the levels of TNF-α and IL-6 in serum of the LPS + anti-PSGL-1 group were decreased compared with the LPS + anti-control group ($P < 0.01$). These results indicated that PSGL-1 played an important role in promoting systemic inflammatory responses in endotoxemic mice (Fig. 5A and B).

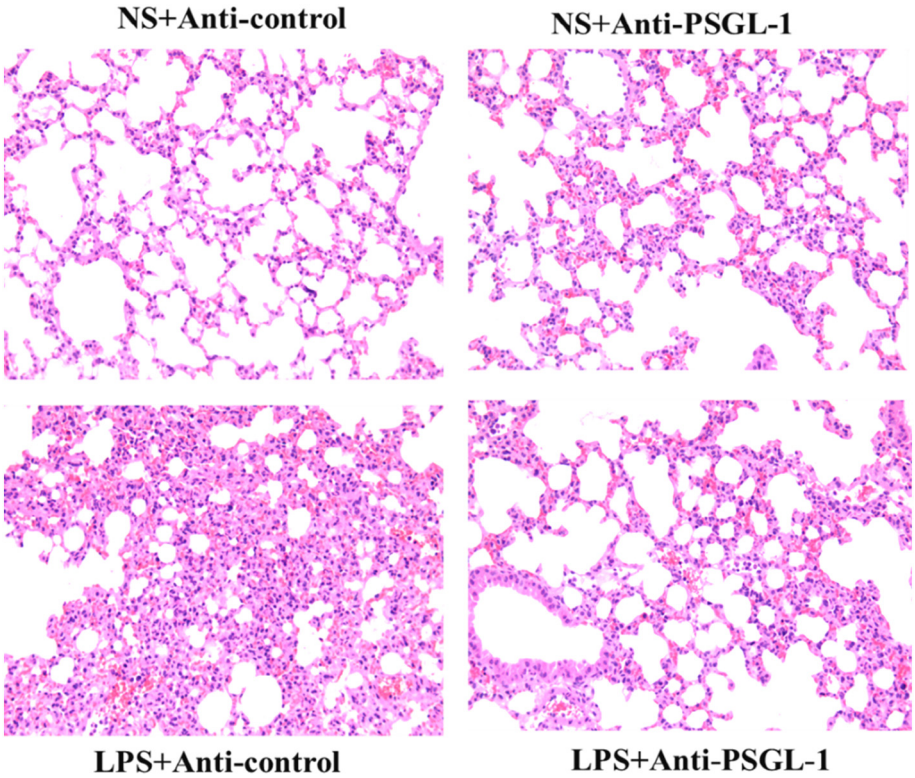

Fig. 2. Effect of PSGL-1 antibody on lung damage in endotoxemic mice (HE staining, magnification 20×; n = 5).

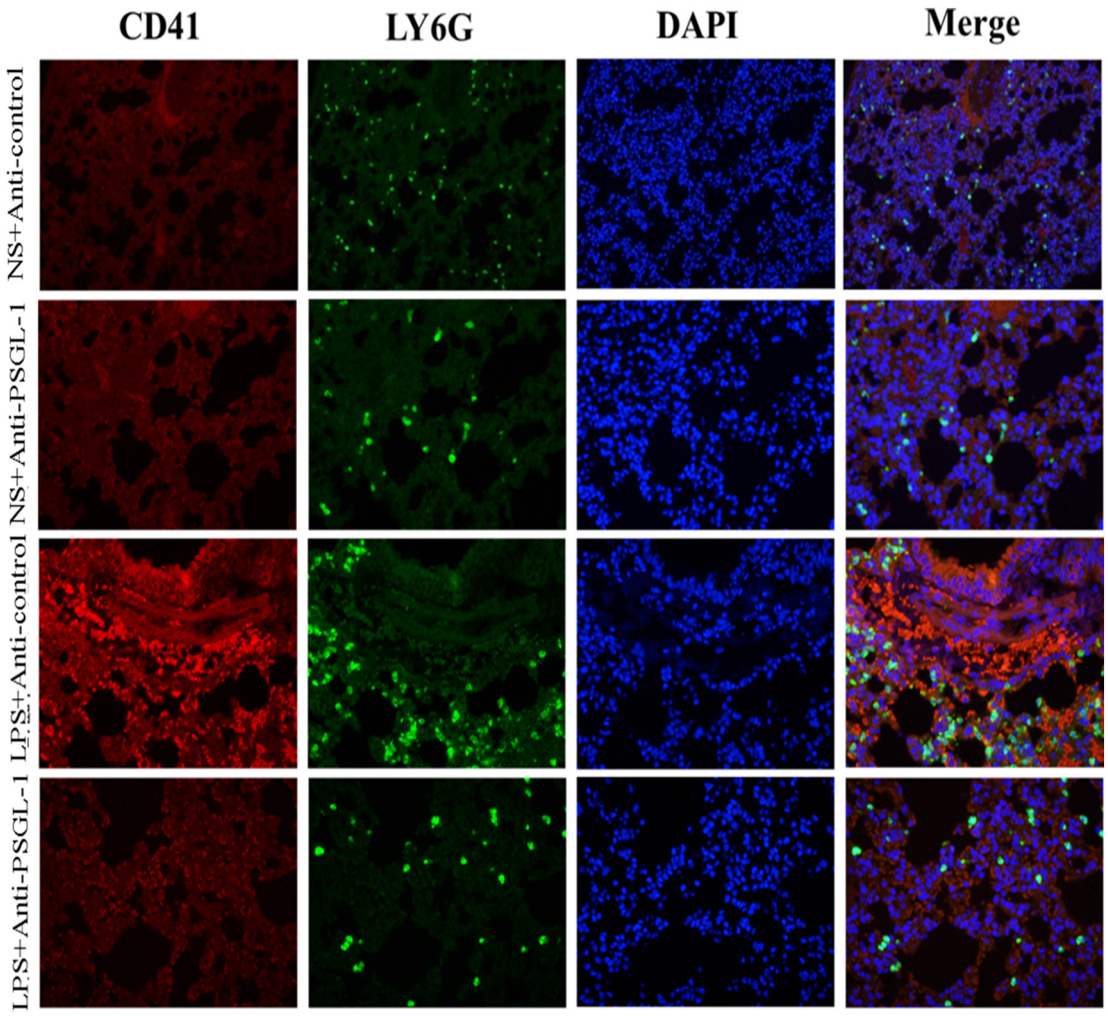

Fig. 3. Effect of PSGL-1 antibody on platelet-leukocyte aggregation in the lung tissues of endotoxemic mice.
Representative immunohistofluorescence images of CD41 and Ly6G staining (CD41, red; Ly6G, green; nucleus, blue; scale bar: 20 μm) in lung tissues. Images are representative of three different experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. PSGL-1 promotes LPS-induced endothelial activation and increases the vascular permeability of endotoxaemic mice

As an important cellular adhesion molecule, PSGL-1 is expressed on the surface of endothelial cells, especially in the damaged endothelium [21]. To assess the effect of PSGL-1 on endothelial cells, we examined levels of sVCAM-1 and VEGF in the serum. At 24 h after LPS challenge, the levels of sVCAM-1 and VEGF in the LPS group were significantly increased compared with the control group. In contrast, blockade of PSGL-1 could decrease the levels of sVCAM-1 and VEGF compared with the LPS group (Fig. 6A and B). These data suggested that PSGL-1 could promote LPS-induced endothelial activation and increase the vascular permeability.

3.6. PSGL-1 decreases the platelet count and increases the D-dimer level of endotoxemic mice

We investigated the effect of PSGL-1 on platelet count in animal models of endotoxemia. The results showed that blockade of PSGL-1 significantly attenuated the LPS-induced reduction in platelet counts (Fig. 7A). These data suggested that PSGL-1 played a role in mitigating LPS-induced thrombocytopenia.

To investigate whether PSGL-1 affected LPS-induced DIC, we assessed the plasma level of D-dimer (diagnostic marker of DIC). The D-dimer level in the LPS + anti-PSGL-1 group was significantly decreased compared with the LPS + anti-control group (Fig. 7B). Therefore, our data indicated that PSGL-1 played a critical role in LPS-induced DIC.

3.7. PSGL-1 promotes the deposition of fibrinogen in lung tissues of endotoxemic mice

It is well established that fibrinogen) increases the interaction of inflammatory cells with endothelium, leading to strongly increased inflammatory responses [22]. Fibrinogen plays a major role during inflammation and is associated with coagulation. We examined the fibrinogen deposition in the lung tissues by using immunofluorescence and Western blotting analysis. The results showed that LPS-induced fibrinogen deposition was significantly reduced in the LPS + anti-PSGL-1 group in lung tissues (Fig. 8A and B), while blockage of PSGL-1 significantly increased the fibrinogen deposition. These results suggest that PSGL-1 promoted the fibrinogen deposition in the lungs of endotoxemic mice. Therefore, our data indicated that PSGL-1 played a critical role in LPS-induced inflammation and coagulation disorders.

4. Discussion

In the present study, endotoxemic mice were injected with the anti-PSGL-1 mAb (4RA10). These data suggested that PSGL-1 played a role

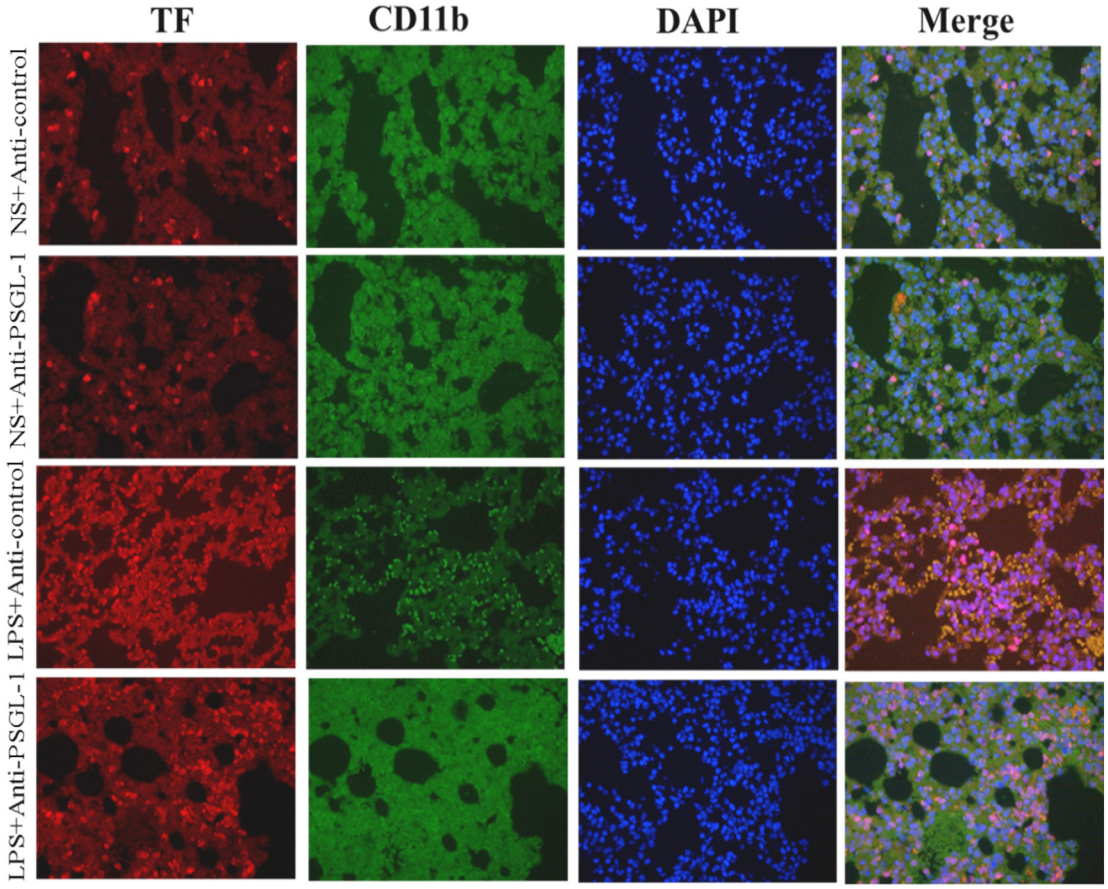

Fig. 4. Effect of PSGL-1 antibody on the expression of TF on WBCs in the lung tissues of endotoxemic mice.
Representative immunohistofluorescence images of lung tissues with CD11b and TF staining (TF, red; CD11b, green; nucleus, blue; scale bar: 20 μm). Images are representative of three different experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in inflammation, coagulation disorders and ALI through affecting the activation of platelets, leukocytes and endothelial cells in endotoxemic mice.

To investigate the effect of PSGL-1, we established an endotoxemic model by LPS injection and found that LPS challenge resulted in higher mortality in mice. Blockade of PSGL-1 significantly reduced the morphological damage, and the infiltration of inflammatory cells in alveolar and interstitial spaces of lung tissue induced by LPS. These results demonstrated that PSGL-1 played an important role in organ damage and death in endotoxemia.

Platelet-leukocyte interactions are critical steps for leukocyte recruitment and activation, playing an important role in the pathogenesis

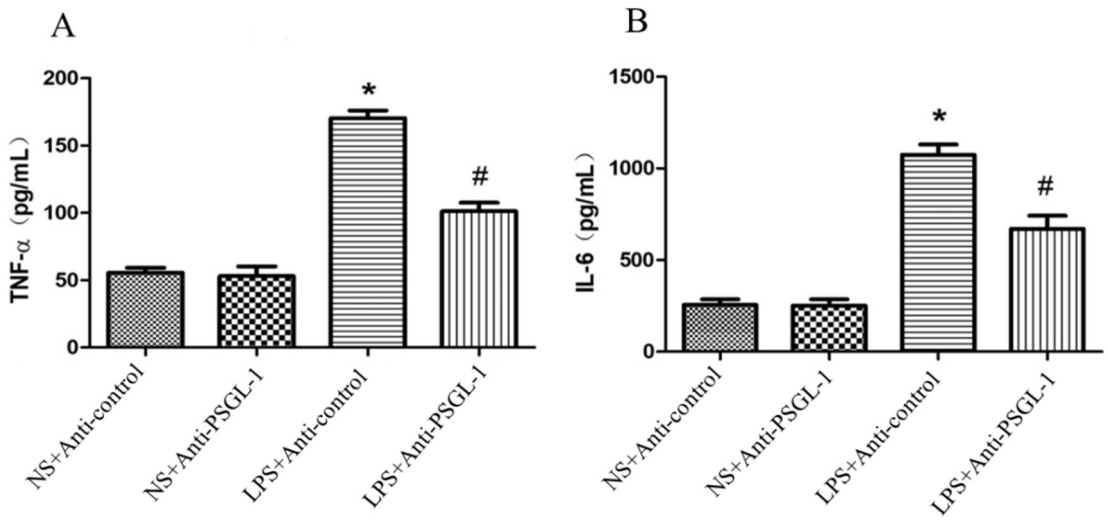

Fig. 5. Effect of PSGL-1 antibody on the serum levels of TNF-α and IL-6 in endotoxemic mice.
A) TNF-α. B) IL-6. Values are expressed as mean ± SEM ($^{*}P < 0.01$, vs. control; $^{\#}P < 0.01$, vs. LPS, n = 10).

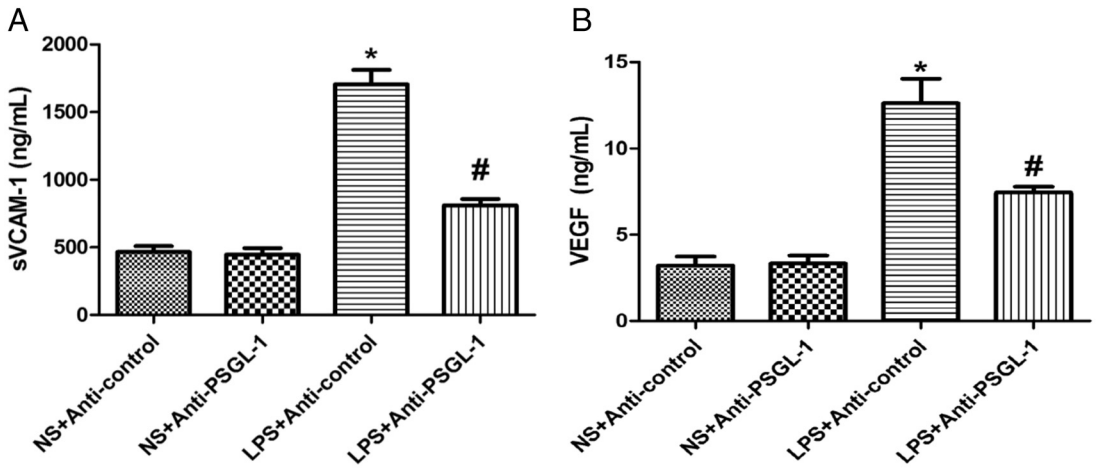

Fig. 6. PSGL-1 promotes LPS-induced endothelial activation and decreases the vascular permeability of endotoxaemic mice.
Expression levels of sVCAM-1 (A) and VEGF (B) in serum. Values are expressed as mean ± SEM. (*$P < 0.01$, vs. control; #$P < 0.01$, vs. LPS, n = 10).

of inflammation and thrombosis [23]. Previous studies have found that in acute ischemic stroke, blockade of PSGL-1 can significantly reduce platelet-leukocyte aggregation in mice [24,25] and attenuate inflammation. Our results indicated that PSGL-1 promoted platelet-leukocyte accumulation in lung tissue and participated in inflammatory reactions and coagulation disorders during endotoxemia.

Increasing levels of pro-inflammatory cytokines, including TNF-α and IL-6, contribute to the inflammation [26]. Our results indicated that PSGL-1 could significantly promote systemic inflammatory responses in endotoxemic mice through decreasing the levels of TNF-α and IL-6.

The endothelial activation is associated with vascular permeability and inflammation, leading to increased expressions of VEGF and sVCAM-1 in endotoxemia [27]. PSGL-1 triggers inflammation by interacting with P-selectin and E-selectin expressed by endothelial cells [28], increases ICAM-1 expression, and enhances adhesion of neutrophils to endothelial cells, resulting in increased lung neutrophil infiltration [29]. VEGF induces the expression of VCAM-1 in endothelial cells and promotes the adhesion of leukocytes [30]. VEGF signaling upregulates the TF expression as well as its procoagulant activity [31]. Our data showed that blockade of PSGL-1 could inhibit the activation of endothelial cells and decrease the vascular permeability through reducing the levels of sVCAM-1 and VEGF in endotoxemia. It indicated that PSGL-1 promoted inflammatory reactions and coagulation disorders through affecting endothelial cells during endotoxemia.

The D-dimer measurement has become one of the most valuable indicators to diagnose and monitor thrombotic disorders and DIC [32]. Platelets are recognized for their ability to modulate immune responses and homeostasis [33]. We found that the platelet count was decreased and the level of D-dimer was increased in LPS-challenged mice. In contrast, when animals were pretreated with PSGL-1 antibody, the platelet count and the level of D-dimer were alleviated. Therefore, PSGL-1 could affect the platelet count and alleviate DIC caused by LPS.

TF is strongly induced during inflammation in leukocytes [34]. TF-dependent activation of coagulation enhances inflammation in endotoxemia [35]. Endotoxin-induced activation of TF appears to be mediated by cytokines, such as TNF-α and IL-6 [36]. Fibrinogen is known as coagulation factor I, and it is associated with hyper-coagulability [37]. Fibrinogen acts as a bridging molecule enhancing leukocyte-endothelium interaction [38]. Fibrinogen alters inflammation not only by affecting leukocyte migration, but also by directly modulating the inflammatory response of leukocytes and endothelial cells [22]. PSGL-1 can recruit leukocytes, affect the reduction of vascular TF [39] and fibrin deposition [39,40] and impair the TF expression on platelet [41]. TF is a key clotting factor [42] and an important mediator of thrombosis [43]. The coagulation cascade is initiated by the expression

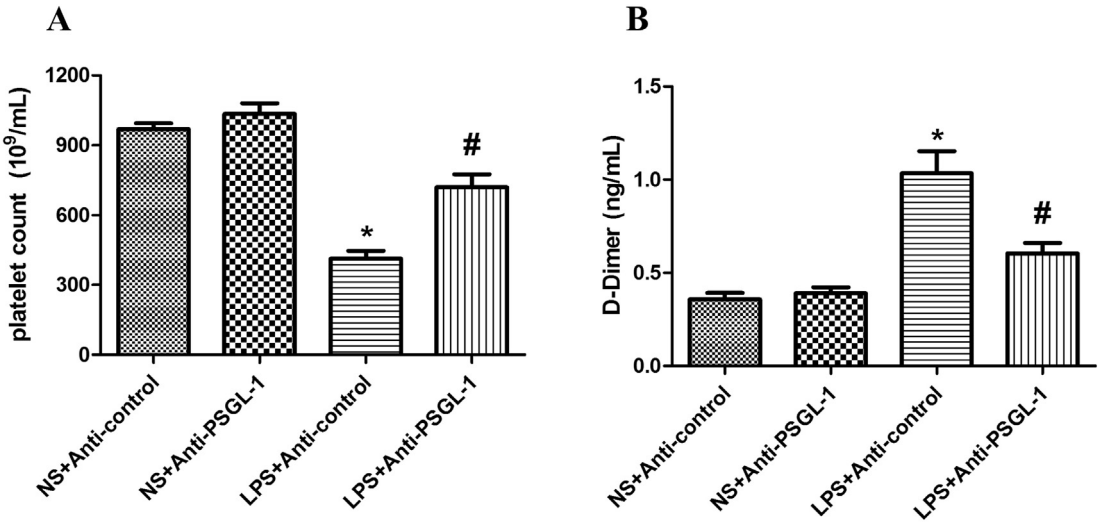

Fig. 7. PSGL-1 promotes the LPS-induced coagulation disturbance at 24 h after LPS challenge.
A. Platelet count. B. Plasma D-dimer level. Values are expressed as mean ± SEM (*$P < 0.01$, vs. control; #$P < 0.01$, vs. LPS, n = 10).

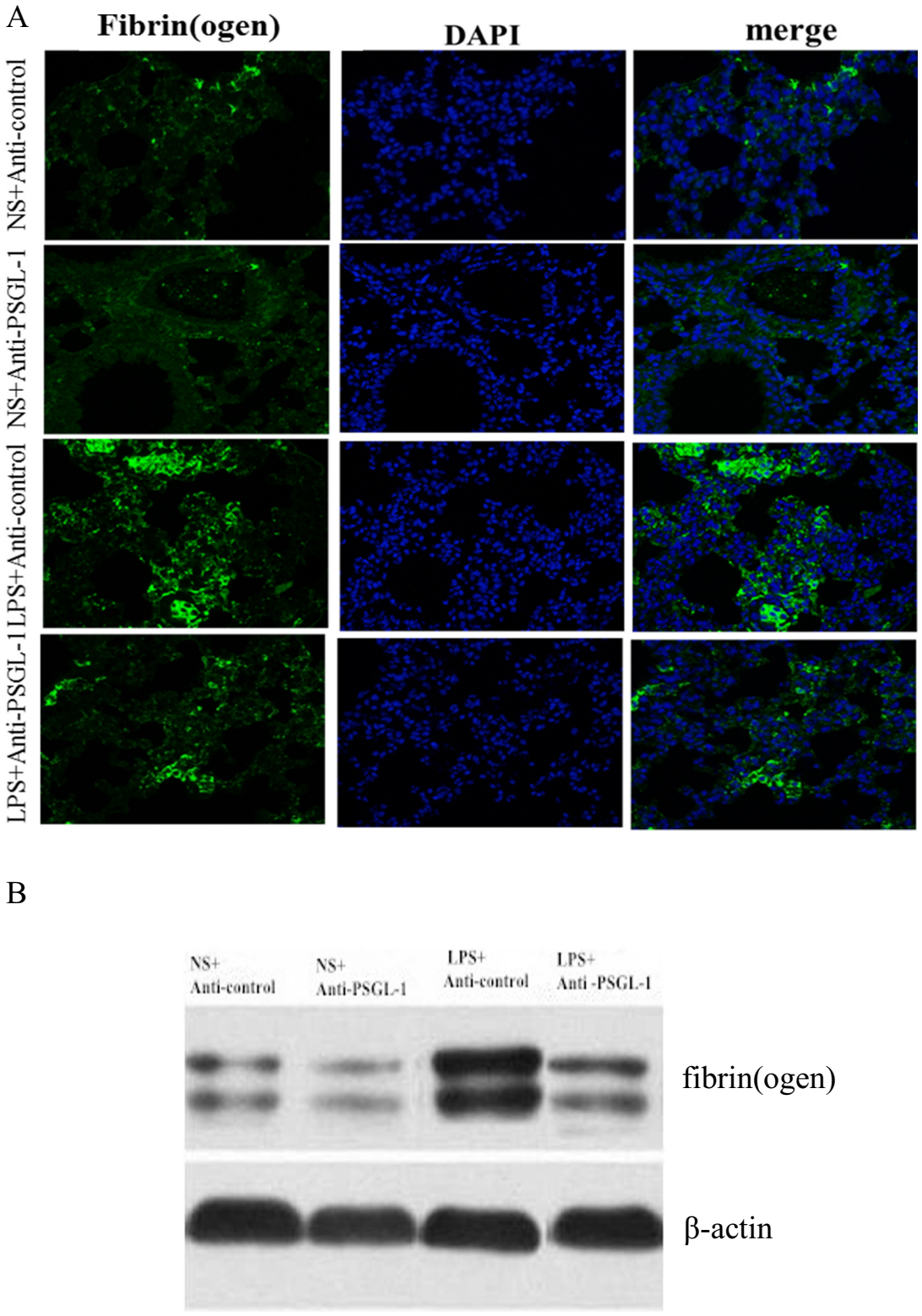

Fig. 8. PSGL-1 promotes the LPS-induced inflammation and coagulation disorders in the lung tissue at 24 h after LPS challenge.
A. Representative images of fibrinogen staining (fibrinogen: green; nucleus: blue; scale bar: 20 μm) for mice. B. The expression of fibrinogen was determined by the Western blotting analysis. The expression levels were normalized relative to β-actin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of TF by neutrophils [44]. These data confirmed that blockade of PSGL-1 could improve inflammation and coagulation disorders through decreasing leukocyte-endothelium interaction induced by LPS.

Collectively, PSGL-1 played an important role in the activation and interaction of platelets-leukocytes-endothelial cells. It promoted the infiltration of platelet-leukocyte, the expression of TF on leukocytes, the activation of endothelial cells, and the deposition of fibrinogen, resulting in enhanced systemic inflammatory response and coagulation

disturbance in endotoxemia.

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Declaration of competing interest

The author declares that there are no conflicts of interest.

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