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RESEARCH PAPER

P-selectin glycoprotein-ligand-1 regulates pulmonary recruitment of neutrophils in a platelet-independent manner in abdominal sepsis

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Background and purpose: Neutrophil-mediated lung injury is an insidious feature in sepsis although the mechanisms regulating pulmonary recruitment of neutrophils remain elusive. Here, we investigated the role of P-selectin glycoprotein-ligand-1 (PSGL-1) in sepsis-induced neutrophil recruitment and tissue injury in the lung.
Experimental approach: Bronchoalveolar infiltration of neutrophils, levels of myeloperoxidase, oedema formation and CXC chemokines were determined 24 h after caecal ligation and puncture (CLP) in mice. Animals were pretreated with a control antibody, monoclonal antibodies directed against PSGL-1 and P-selectin as well as a platelet-depleting antibody directed against GP1bα.
Key results: CLP caused pulmonary damage characterized by oedema formation, neutrophil infiltration and increased levels of CXC chemokines in the lung. Immunoneutralization of PSGL-1 or P-selectin reduced CLP-induced neutrophil recruitment in the bronchoalveolar space by more than 56% and lung myeloperoxidase activity by 62%. Notably, the inhibitory effect of the anti-PSGL-1 antibody on sepsis-induced neutrophil infiltration was also observed in platelet-depleted mice. Moreover, inhibition of PSGL-1 and P-selectin abolished CLP-induced oedema formation and tissue damage in the lung. CLP-induced formation of CXC chemokines was not changed in mice pretreated with the anti-PSGL-1 and anti-P-selectin antibodies.
Conclusions and implications: These data demonstrate that PSGL-1 plays a key role in pulmonary infiltration of neutrophils as well as lung oedema associated with abdominal sepsis. Moreover, our findings suggest that PSGL-1-dependent neutrophil recruitment is independent of circulating platelets. Thus, these novel findings indicate that PSGL-1 may be a useful target to protect against sepsis-induced accumulation of neutrophils and tissue damage in the lung.
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Keywords: neutrophil recruitment; lung injury; sepsis

Abbreviations: PSGL-1, P-selectin glycoprotein-ligand-1; CLP, cecal ligation and puncture; MIP-2, macrophage inflammatory protein-2; KC, cytokine-induced neutrophil chemoattractant; Mac-1, membrane-activated complex-1; BALF, bronchoalveolar lavage fluid; MPO, myeloperoxidase; PBS, phosphate buffered saline

Introduction

Despite aggressive surgical interventions, antibiotic therapies and immunoneutralization of tumour necrosis factor-α (TNF-α), sepsis and subsequent multiple organ failure remains the major cause of morbidity and mortality in intensive care units (Cohen 2002; Yano *et al.*, 2006). Intestinal perforation is a feared condition in abdominal sepsis in which toxic and polymicrobial contents of the bowel contaminate the abdominal cavity (Gorbach and Bartlett, 1974; Simon and Gorbach, 1984). It is well recognized that the lung is the most sensitive and critical organ affected in patients with sepsis (Babayigit *et al.*, 2005). In general, neutrophils are considered as the first line of defence against invading microorganisms. However, excessive accumulation of neutrophils is also considered to be a rate-limiting step in the pathophysiology of septic lung injury. For example, inhibition of specific adhesion molecules, such as membrane-activated complex-1 (Mac-1) and LFA-1, not only decrease pulmonary accumulation of neutrophils but also reduce oedema formation and protect against lung damage in abdominal sepsis (Asaduzzaman *et al.*, 2008).

It is widely held that tissue recruitment of leukocytes from the circulation is a multi-step process, in which initial leukocyte rolling is a precondition for subsequent firm adhesion along activated endothelial cells (Springer, 1994). Leukocyte rolling is mediated by the selectin family of adhesion molecules (P-, E- and L-selectins) by interacting with their glycoprotein counter-ligands (Carlos and Harlan, 1994; Vestweber

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and Blanks, 1999). 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 (Yang *et al.*, 1999). Numerous studies have shown that inhibition of PSGL-1 effectively inhibit leukocyte rolling in models of inflammation (Hicks *et al.*, 2003; Rijcken *et al.*, 2004; Mangan *et al.*, 2005; Santen *et al.*, 2007). However, the lung is unique in that initial arrest of neutrophils may be due to decreased deformability of activated neutrophils, which mechanically trap in narrow segments of the lung microvasculature (Worthen *et al.*, 1989; Yoshida *et al.*, 2006). This has raised questions related to the role of selectins and a rolling adhesive interaction for the recruitment of neutrophils in the lung. The literature on this topic is complex and partly contradictory. For example, some studies have reported that bacteria-provoked leukocyte accumulation in the lung is intact in mice deficient in P- and E-selectin (Mizgerd *et al.*, 1996; Wickel *et al.*, 1998). In contrast, others have shown that inhibition of P-, L- and E-selectin effectively inhibits leukocyte accumulation in models of acid-induced lung damage (Zarbock *et al.*, 2006), sepsis-induced lung injury (Ridings *et al.*, 1995) and allergen-induced asthma (Pitchford *et al.*, 2005). In this context, it may be important to note that platelets not only express P-selectin and PSGL-1 but also have the capacity to support accumulation of leukocytes (Frenette *et al.*, 2000; Burger and Wagner, 2003; Pitchford *et al.*, 2005; Laschke *et al.*, 2008). Thus, one explanation behind the conflicting data on the role of selectins may be that the contribution of platelet to neutrophil recruitment varies in the different disease models used. At present, the potential role of PSGL-1 in mediating neutrophil accumulation and lung injury in abdominal sepsis is not known.

Based on the above considerations, the aim of the present study was to determine the role of PSGL-1 in sepsis-induced neutrophil recruitment and lung damage in abdominal sepsis. Moreover, we wanted to determine the potential role of platelets in PSGL-1-dependent accumulation of neutrophils in sepsis-induced lung injury. For this purpose, we used the caecal ligation and puncture (CLP) model in which neutrophil-dependent lung injury is a prominent feature.

Methods

Animals

All experimental procedures were performed in accordance with the legislation on the protection of animals and were approved by the Regional Ethical Committee for Animal Experimentation at Lund University, Sweden. Experiments were performed by using male C57BL/6 mice weighing 20–25 g. Animals were anaesthetized by intraperitoneal (i.p.) administration of 7.5 mg Ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 2.5 mg xylazine (Janssen Pharmaceutica, Beerse, Belgium) per 100 g body weight.

Experimental model of sepsis

Polymicrobial sepsis in mice was induced by CLP as previously described in detail (Asaduzzaman *et al.*, 2008). In brief, animals were anesthetized and the abdomen was opened to exteriorize the caecum that was filled with feces by milking stool backwards from the ascending colon and a ligature was placed below the ileocaecal valve. The caecum was soaked with phosphate buffered saline (PBS; pH 7.4) and was then punctured twice with a 21-gauge needle. The caecum was then returned into the peritoneal cavity, and the abdominal wall was closed with a suture. To evaluate the functional importance of PSGL-1 and P-selectin monoclonal antibodies directed against murine CD162 (PSGL-1, clone 2PH1, rat IgG_1), CD62P (P-selectin, clone RB40.34, rat IgG_1) and an isotype-matched control antibody (clone R3-34, rat IgG_1) at a concentration of 1.6 $mg \cdot kg^{-1}$ (all antibodies from BD Biosciences Pharmingen, San Jose, CA, USA) were administered intravenously (i.v.) prior to CLP induction. To study the contribution of platelets, 1.0 $mg \cdot kg^{-1}$ of a monoclonal antibody directed against murine CD42b (GP1bα, rat IgG, Emfret Analytics GmbH & Co. KG, Wurzburg, Germany) was administered i.p. 2 h before induction of CLP. In another set of experiments, a combination of anti-GP1bα and anti-PSGL-1 antibodies were administered prior to CLP. The nomenclature of all antibodies or reagents mentioned above follows the format as previously described in detail (Alexander *et al.*, 2008). The same surgical procedures, that is, laparotomy and resuscitation, were performed in 'sham' mice, but the caecum was not ligated or punctured. The mice were then returned to their cages and provided food and water *ad libitum*. Animals were anesthetized 6 and 24 h after CLP induction. The left lung was ligated and excised for oedema measurement. The right lung was used for collecting bronchoalveolar lavage fluid (BALF) by lavage with 1 mL PBS (containing 5 $mmol \cdot L^{-1}$ EDTA) five times, and the number of neutrophils was quantified after mixing BALF with Turks solution (0.2 mg gentian violet in 1 mL glacial acetic acid, 6.25% v/v) in a Burker chamber. Next, the lung was perfused with PBS, one part was fixed in formaldehyde for histology, and the remaining lung tissue was weighed, snap-frozen in liquid nitrogen and stored at –80°C for later ELISA and myeloperoxidase (MPO) assays as described below.

Systemic leukocyte count

Blood was collected from tail vein and was mixed with Turks solution in a 1:20 dilution. Leukocytes were identified as monomorphonuclear (MNLs) and polymorphonuclear (PMNLs) cells in a Burker chamber.

Lung oedema

The left lung was excised, washed in PBS, gently dried by using blotting paper and weighed. The tissue was then dried at 60°C for 72 h and re-weighed. The change in the ratio of wet to dry weight was used as an indicator of lung oedema formation.

MPO activity

Frozen lung tissue was thawed and homogenized in 1 mL of 0.5% hexadecyltrimethylammonium bromide. Next, the sample was freeze-thawed, after which the MPO activity of the supernatant was measured as described by Krawisz *et al.* (1984). The enzyme activity was determined spectrophoto-

metrically as the MPO-catalysed change in absorbance in the redox reaction of H_2O_2 (450 nm, with a reference filter 540 nm, 25°C). Values were expressed as MPO unit·g^{-1} tissue.

ELISA

Pulmonary levels of chemokines [macrophage inflammatory protein-2 (MIP-2) and cytokine-induced neutrophil chemoattractant (KC)] were determined in lung samples, which were thawed and homogenized in PBS. Circulating levels of TNF-α were also determined in plasma samples. MIP-2, KC and TNF-α were analysed by using double antibody Quantikine ELISA kits (R & D Systems) using recombinant murine MIP-2, KC and TNF-α as standards. The lower limit of the assay was 0.5 pg·mL^{-1}.

Histology

Lung samples were fixed in 4% formaldehyde phosphate buffer overnight and then dehydrated and paraffin-embedded. Six μm sections were stained with haematoxylin and eosin (H & E).

Flow cytometry

For analysis of PSGL-1 expression on circulating neutrophils, blood was collected into heparinized syringes at 6 h post CLP induction. Immediately after collection, blood cells were incubated with an anti-CD16/CD32 antibody blocking Fcγ III/II receptors in order to reduce non-specific labelling for 10 min at room temperature and then incubated with APC-conjugated anti-Gr-1 (clone RB6-8C5, rat IgG_{2b}), PE-conjugated anti-CD162 (clone 2PH1, IgG_1) and FITC-conjugated anti-CD41 (clone MWReg30, Integrin α_{IIb} chain, IgG_1) antibodies (all antibodies from BD Biosciences Pharmingen, San Jose, CA, USA). Cells were fixed with 1% formaldehyde solution; erythrocytes were lysed by using red blood cell lysing buffer (Sigma Chemical Co., St. Louis, MO, USA), and neutrophils were recovered following centrifugation. Flow-cytometric analysis of PSGL-1 and CD41 (platelet marker) expression on neutrophil and Gr-1^+ cells was performed by first gating the neutrophil population of cells based on forward and side scatter characteristics on a FACSort flow cytometer (Becton Dickinson, Mountain View, CA, USA). A viable gate was used to exclude dead and fragmented cells.

Statistics

Data are presented as mean values ± SEM (standard error of the mean). Statistical evaluations were performed by using Kruskal-Wallis one-way analysis of variance on ranks followed by multiple comparisons versus control group (Dunnett's method). $P < 0.05$ was considered significant, and n represents the number of animals.

Results

PSGL-1 regulates sepsis-induced neutrophil recruitment in the lung

To study the role of PSGL-1 and P-selectin in septic lung injury, we assayed both levels of MPO and the number of neutrophils in BALF. MPO levels and BALF neutrophils in the lung represent early and late phases of neutrophil accumulation of neutrophils, and they peak at 6 h and 24 h respectively, in this CLP model (data not shown). We observed that MPO levels increased by more than 10-fold after induction of CLP (Figure 1A, $P < 0.05$ vs. Sham, $n = 5$). Notably, pretreatment with the anti-PSGL-1 and anti-P-selectin antibodies significantly decreased MPO levels by more than 62% in septic mice (Figure 1A, $P < 0.05$ vs. Control ab + CLP, $n = 5$). Analysis of BALF showed a clear-cut increase (53-fold) in the number of neutrophils 24 h after CLP (Figure 1B, $P < 0.05$ vs. Sham, $n = 5$). Interestingly, administration of the anti-PSGL-1 and the anti-P-selectin antibody markedly reduced CLP-induced recruitment of neutrophils into the bronchoalveolar compartment, corresponding to about 56% reduction (Figure 1B, $P < 0.05$ vs. Control ab + CLP, $n = 5$). As expected, we observed that systemic leukocyte counts decreased after induction of CLP (Table 1). Administration of the antibodies against PSGL-1 and P-selectin significantly increased the number of circulating leukocytes in septic mice (Table 1). In addition, it was found that the lung content of CXC chemokines was low but detectable in sham controls (Table 2). CLP significantly increased the tissue levels of MIP-2 and KC in the lung (Table 2, $P < 0.05$ vs. Sham, $n = 5$). However, functional inhibition of PSGL-1 and P-selectin had no effect on pulmonary production of the CXC chemokines in septic mice (Table 2, $P > 0.05$ vs. Control ab + CLP, $n = 5$). In addition, CLP-induced plasma levels of TNF-α in CLP mice were 20.2 ± 1.4 pg·mL^{-1} and 20.0 ± 2.5 pg·mL^{-1} receiving the control antibody and the anti-PSGL-1 antibody respectively ($P > 0.05$ vs. Control ab + CLP, $n = 5$).

Inhibition of PSGL-1 protects against sepsis-induced lung damage

Next, we asked whether inhibition of PSGL-1 and P-selectin protects against septic lung injury. Pulmonary oedema was determined as changes in lung wet : dry ratio, and the lung wet : dry ratio was clearly increased after CLP induction (Figure 1C). Notably, immunoneutralization of PSGL-1 and P-selectin decreased the CLP-induced increase in lung wet : dry ratio, corresponding to more than 71% reduction in oedema formation (Figure 1C, $P < 0.05$ vs. Control ab + CLP, $n = 5$). Histological examination of the lung tissue showed normal microarchitecture in sham-operated animals (Figure 2A), whereas CLP caused a severe destruction of the pulmonary tissue structure characterized by extensive oedema of the interstitial tissue and massive infiltration of neutrophils (Figure 2B,C). In line with the above data on lung oedema, it was found that administration of the anti-PSGL-1 antibody protected against CLP-induced destruction of tissue structure and neutrophil recruitment in the lung (Figure 2D).

PGSL-1-dependent pulmonary recruitment of neutrophils is independent of platelets

Considering that PSGL-1 is expressed on both neutrophils and platelets and that platelets are known to support pulmonary recruitment of neutrophils in abdominal sepsis (Asaduzzaman *et al.*, unpubl. obs.), it was of interest to

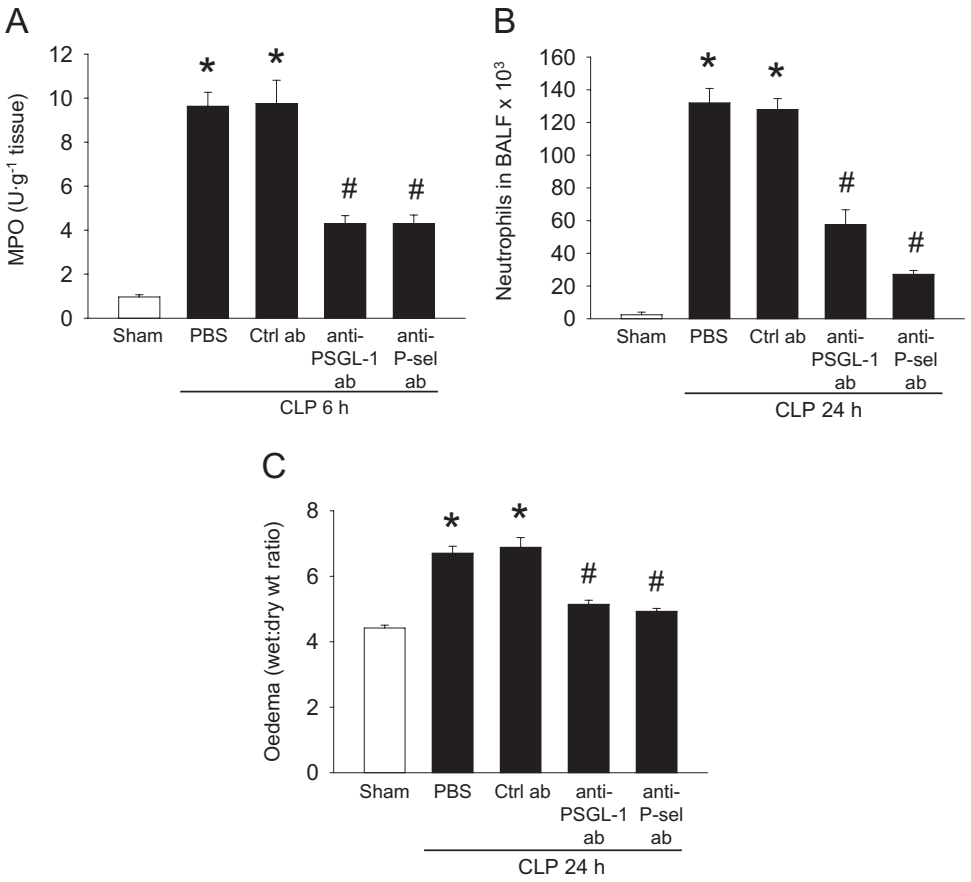

Figure 1 Role of PSGL-1 and P-selectin in CLP-induced pulmonary inflammation. (A) Lung MPO levels at 6 h post CLP, (B) number of BALF neutrophils, and (C) oedema formation in the lung 24 h following CLP induction. Mice were treated with an antibody directed against PSGL-1, P-selectin (P-sel), a control antibody (Ctrl ab) and PBS prior to induction of CLP. Sham-operated animals served as negative controls. Data represent mean ± SEM and $n = 5$. $^{*}P < 0.05$ vs. Sham and $^{\#}P < 0.05$ vs. Control ab + CLP. BALF, bronchoalveolar lavage fluid; CLP, caecal ligation and puncture; MPO, myeloperoxidase; PSGL-1, P-selectin glycoprotein-ligand-1; PBS, phosphate buffered saline.

Table 1 Differential counts of circulating leukocytes

	MNL	*PMNL*	*Total*
Sham	5.2 ± 0.4	1.7 ± 0.3	7.0 ± 0.6
PBS + CLP	1.0 ± 0.2*	0.6 ± 0.1*	1.6 ± 0.3*
Control antibody + CLP	1.0 ± 0.3*	0.5 ± 0.1*	1.5 ± 0.4*
Anti-PSGL-1 antibody + CLP	2.8 ± 0.3*,#	1.6 ± 0.2#	4.3 ± 0.3*,#
Anti-P-selectin antibody + CLP	2.2 ± 0.2*,#	1.7 ± 0.1#	3.9 ± 0.2*,#

Blood was collected from PBS-, control antibody-, anti-PSGL-1 antibody- and anti-P-selectin antibody-treated mice exposed to CLP for 24 h as well as from sham-operated animals. Cells were identified as MNLs and PMNLs. Data represent mean ± SEM, 10^6 cells·mL^{-1} and $n = 5$. $^{*}P < 0.05$ vs. Sham and $^{\#}P < 0.05$ vs. Control ab + CLP.

CLP, caecal ligation and puncture; MNLs, monomorphonuclear leukocytes; PBS, phosphate buffered saline; PMNLs, polymorphonuclear leukocytes; PSGL-1, P-selectin glycoprotein-ligand-1.

determine whether the PSGL-1-mediated neutrophil accumulation is dependent on circulating platelets in abdominal sepsis. First, we confirmed that neutrophils express PSGL-1 on their surface, and we also observed that PSGL-1 expression on neutrophil did not change after CLP induction (Figure 3). We next depleted mice of platelets in order to better define the role of platelets in relation to PSGL-1. Administration of an anti-GP1bα antibody reduced platelet counts in the blood from $640 \pm 34 \times 10^6$ mL^{-1} down to $120 \pm 5 \times 10^6$ mL^{-1}, corresponding to an 81% reduction. Moreover, platelet depletion reduced the CLP-induced increase of lung MPO levels by 53% (Figure 4, $P < 0.05$ vs. Control ab + CLP, $n = 5$). Interestingly, immunoneutralization of PSGL-1 in platelet-depleted mice reduced pulmonary levels of MPO by 92% in CLP mice (Figure 4, $P < 0.05$ vs. Control ab + CLP, $n = 5$), which is similar to the effect of the anti-PSGL-1 antibody on sepsis-induced neutrophil recruitment in mice with normal platelet counts.

Discussion and conclusions

The present study demonstrates a key role of PSGL-1 in the pulmonary accumulation of neutrophils in septic lung injury. Moreover, we provide evidence showing that functional inhibition of PSGL-1 reduces pulmonary oedema and tissue destruction in abdominal sepsis. Notably, our data show that this PGSL-1-regulated recruitment of neutrophils in septic lung injury is independent of platelets. Taken together, these novel findings suggest that targeting PSGL-1 function may be an effective approach to decrease lung damage in abdominal sepsis.

Table 2 CXC chemokines in lung (ng·g^{-1} tissue)

	MIP-2	*KC*
Sham	1.6 ± 0.3	5.8 ± 0.7
PBS + CLP	96.4 ± 11.8*	87.7 ± 9.6*
Control antibody + CLP	96.2 ± 15.8*	92.8 ± 2.8*
Anti-PSGL-1 antibody + CLP	85.9 ± 4.9*	96.3 ± 13.1*
Anti-P-selectin antibody + CLP	99.1 ± 11.6*	97.7 ± 5.4*

Lung tissue was collected from PBS-, control antibody-, anti-PSGL-1 antibody- and anti-P-selectin antibody-pretreated mice exposed to CLP for 24 h as well as from sham-operated animals. Lung levels of MIP-2 and KC were determined by ELISA. Data represent mean ± SEM and $n = 5$. *$P < 0.05$ vs. Sham.
CLP, caecal ligation and puncture; KC, cytokine-induced neutrophil chemoattractant; MIP-2, macrophage inflammatory protein-2; PBS, phosphate buffered saline; PSGL-1, P-selectin glycoprotein-ligand-1.

Sepsis is characterized by generalized activation of the host immune system in which the most insidious feature is lung injury and consequently impaired gas exchange (Matsuda *et al.*, 2005; Asaduzzaman *et al.*, 2008). It is widely held that neutrophil recruitment is a critical component in the pathophysiology of septic lung injury (Lomas-Neira *et al.*, 2004; 2006; Asaduzzaman *et al.*, 2008), which makes our understanding of the mechanisms regulating pulmonary accumulation of neutrophils, an important issue in developing novel and more effective sepsis therapies. In the present study, we show for the first time that inhibition of PSGL-1 and P-selectin functions effectively reduce neutrophil recruitment in the lung in polymicrobial sepsis. PSGL-1 is the main counter-ligand for P-selectin, which together constitute dominating molecules in mediating leukocyte rolling in most extra-lymphoid tissues (Sako *et al.*, 1993; Thorlacius *et al.*, 1994; 1997; Battistini *et al.*, 2003; Santen *et al.*, 2007). However, the role of P-selectin and a rolling adhesive

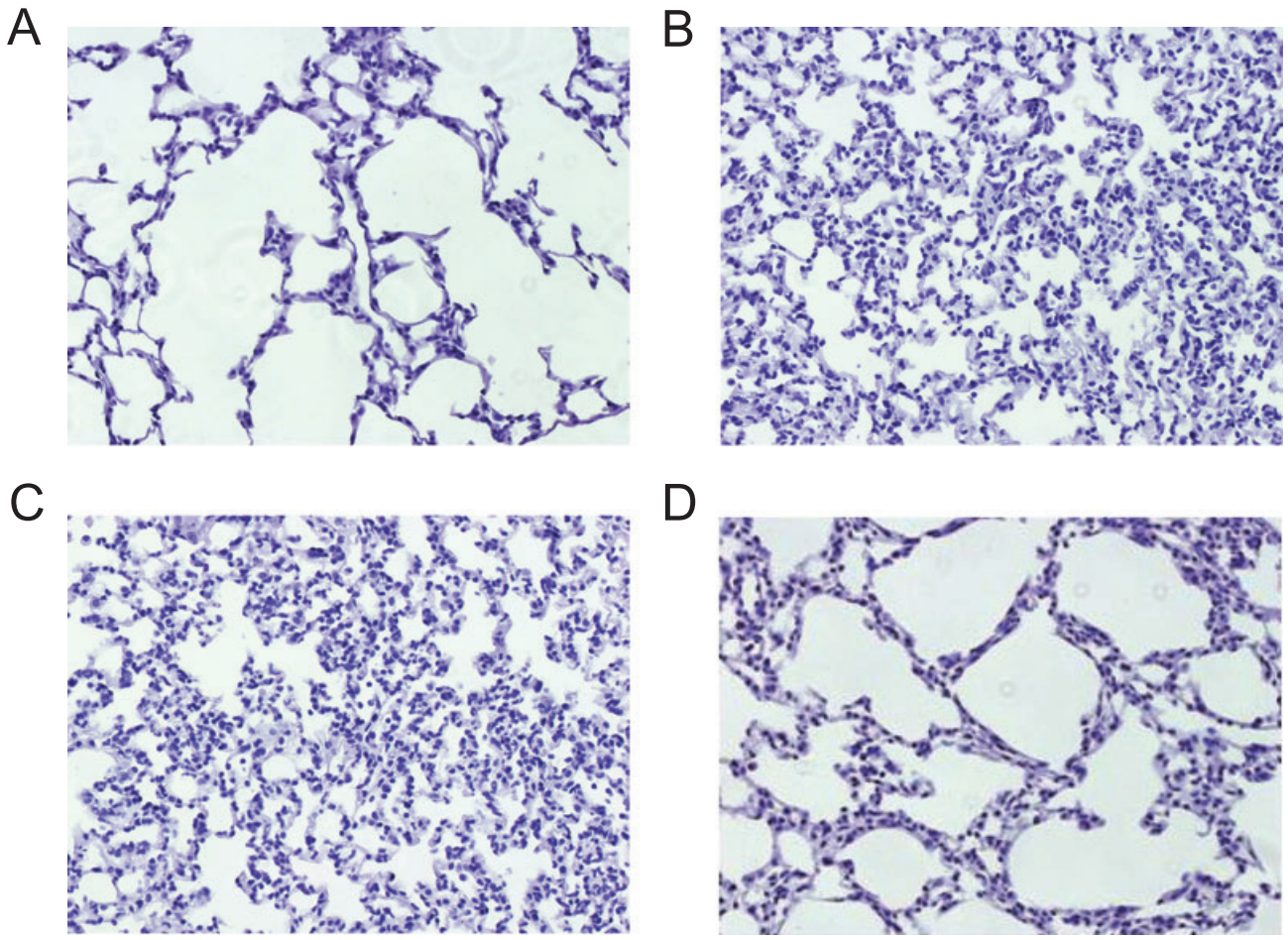

Figure 2 Representative sections of lung tissue, stained with haematoxylin & eosin. (A) Sham-operated animals served as negative controls. Mice were treated with (B) phosphate buffered saline, (C) a control antibody and (D) an antibody against P-selectin glycoprotein-ligand-1 prior to induction of caecal ligation and puncture, and samples were taken 24 h later. Original magnification ×200.

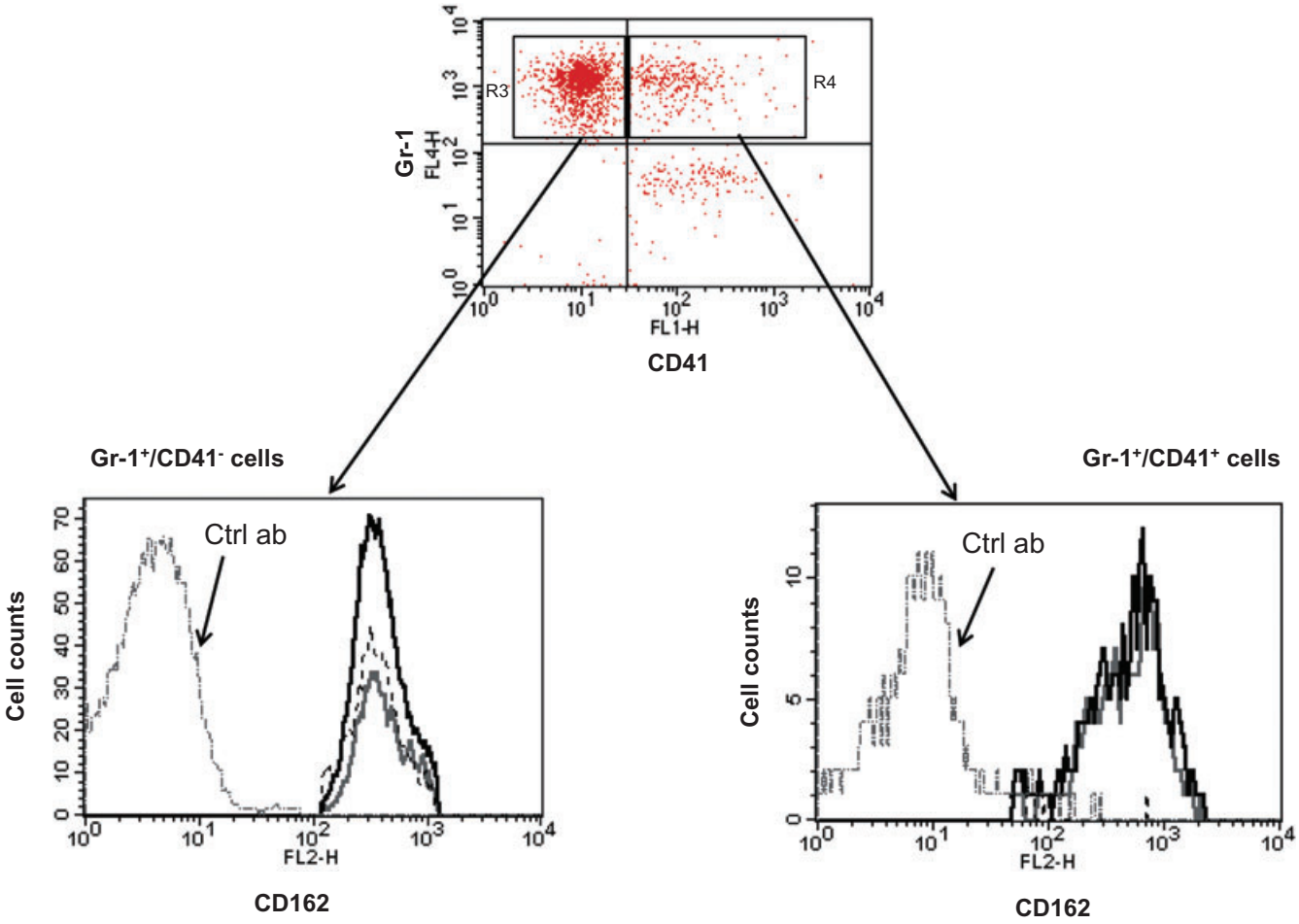

Figure 3 Expression of P-selectin glycoprotein-ligand-1 (PSGL-1) (CD162) on circulating neutrophils (Gr-1$^+$/CD41$^-$cells) and neutrophil-platelet aggregates (Gr-1$^+$/CD41$^+$cells) in sham mice (gray line) and in caecal ligation and puncture mice pretreated with a control antibody (dark line) and the platelet-depleting antibody (anti-GP1bα antibody, dashed line). Cells stained with an isotype control antibody are indicated in the figure (Ctrl ab). Neutrophils were first gated from standard settings on a FACSort flow cytometer based on forward and side scattering characteristics, and then Gr-1$^+$ cells were analysed for PSGL-1 expression. Data are representative of four experiments.

interaction in the lung has been questioned and is a controversial topic in the literature. On one hand, early studies reported that pulmonary accumulation of neutrophils is independent of P-selectin (Mizgerd *et al.*, 1996; Wickel *et al.*, 1998; Chandra *et al.*, 2003). On the other hand, more recent studies suggest that P-selectin is an important regulator of leukocyte recruitment in the lung in different experimental models (Kuligowski *et al.*, 2006; Zarbock *et al.*, 2006). The reason behind these discrepant findings is not known at present. Nonetheless, we observed, herein, that functional inhibition of PSGL-1 and P-selectin decreased pulmonary levels of MPO, a marker of neutrophil recruitment, by more than 62% in our model of abdominal sepsis. This effect correlated well with our observation that pretreatment with anti-PSGL-1 and anti-P-selectin antibody reduced sepsis-induced neutrophil infiltration in the bronchoalveolar space by 56%, indicating that both PSGL-1 and P-selectin are important adhesion molecules supporting neutrophil infiltration in septic lung damage. This notion is also in line with studies reporting that pulmonary accumulation of neutrophils is dependent on PSGL-1 function in models of acid-induced tissue injury and allergic inflammation in the lung (Kyriakides *et al.*, 2000, Borchers *et al.*, 2001). Secretion of CXC chemokines is known to regulate neutrophil accumulation in the lung parenchyma (Guo *et al.*, 2006). However, we found that CLP-induced formation of MIP-2 and KC was maintained in mice passively immunized against PSGL-1 and P-selectin, suggesting that the inhibitory effect of these adhesion molecules on neutrophil infiltration is not related to changes in the pulmonary production of CXC chemokines. This is also compatible with a recent study reporting that local tissue macrophages are the main producers of CXC chemokines, MIP-2 and KC, at sites of inflammation (De Filippo *et al.*, 2008). Moreover, it was found that blocking PSGL-1-mediated recruitment of neutrophils greatly protected against sepsis-induced lung oedema and tissue injury. Thus, these results suggest that targeting PSGL-1 may be a useful way to ameliorate septic lung injury.

Accumulating experimental data suggest that platelets exert numerous pro-inflammatory effects beyond their well-known functions in haemostasis and thrombosis (von

Hundelshausen and Weber, 2007). In particular, several recent studies have shown that platelets have the capacity to promote leukocyte recruitment in models of inflammation (Singbartl *et al.*, 2001; Pitchford *et al.*, 2005; von Hundelshausen and Weber, 2007; Laschke *et al.*, 2008). In this context, it is important to note that both platelets and neutrophils express PSGL-1 on their surface (Frenette *et al.*, 2000, this study). Therefore, we asked in this study whether the PSGL-1-mediated recruitment of neutrophils in the lung, was dependent on circulating platelets. Indeed, we observed that pulmonary infiltration of neutrophils was significantly reduced in platelet-depleted mice, confirming our previous observation that platelets support neutrophil accumulation in septic lung injury (Asaduzzaman *et al.*, unpubl. obs.). Notably, we observed that the inhibitory effects of the anti-PSGL-1 antibody were still observed in mice depleted of platelets. In fact, immunoneutralization of PSGL-1 reduced sepsis-induced neutrophil accumulation by 62% in mice with normal platelet counts and by 92% in platelet-depleted mice, suggesting that the reduction in neutrophil recruitment exerted by the anti-PSGL-1 antibody is independent of circulating platelets. In these experiments, we found that application of the anti-GP1bα antibody caused a marked reduction in platelet numbers (81% reduction) and whether more extensive platelet depletion could modulate the effect of the anti-PSGL-1 antibody on pulmonary infiltration of neutrophils is uncertain. However, considering that the impact of immunoneutralization of PSGL-1 on CLP-provoked neutrophil accumulation was even more pronounced in platelet-depleted animals (92% reduction) compared with that in control mice (62% reduction), the major conclusions in this study are not likely to be affected by a more extensive depletion of platelets. At this stage, it should be mentioned that we have previously found that platelet-dependent neutrophil infiltration in septic lung injury is mediated via up-regulation of Mac-1 on neutrophils (Asaduzzaman *et al.*, unpubl. obs.). However, we found also that inhibition of PSGL-1 had no effect on this platelet-mediated increase in Mac-1 expression on circulating neutrophils (not shown), which further supports the notion that PSGL-1-dependent pulmonary recruitment is not dependent on platelet functions.

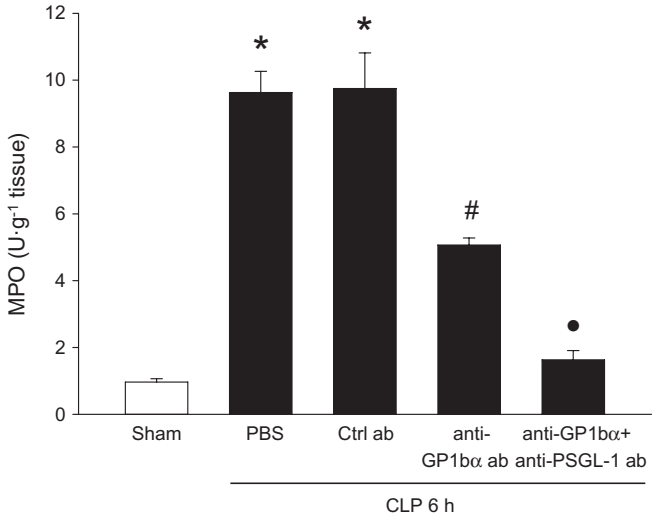

Figure 4 Role of platelets and PSGL-1 in CLP-induced MPO activity, a marker of neutrophil accumulation, in the lung. Mice were treated with an antibody directed against GP1bα, a combination of anti-GP1bα and anti-PSGL-1 antibodies as well as a control antibody (Ctrl ab) and PBS prior to induction of CLP. Sham-operated animals served as negative controls. Data represent mean ± SEM and $n = 5$. $^{*}P < 0.05$ vs. Sham, $^{\#}P < 0.05$ vs. Control ab + CLP and $^{\bullet}P < 0.05$ vs. anti-GP1bα ab + CLP. CLP, caecal ligation and puncture; MPO, myeloperoxidase; PSGL-1, P-selectin glycoprotein-ligand-1; PBS, phosphate buffered saline.

In conclusion, our novel data show that PSGL-1 supports neutrophil recruitment in the lung in abdominal sepsis (Figure 5). Moreover, we provide evidence showing that inhibition of PSGL-1 also protects against sepsis-induced oedema formation and tissue destruction in the lung. Interestingly, we found that this PSGL-1-mediated neutrophil infiltration in the lung is independent of circulating platelets in polymicrobial sepsis. Thus, these findings suggest that PSGL-1 may be a useful target in order to ameliorate sepsis-induced inflammation and tissue damage in the lung.

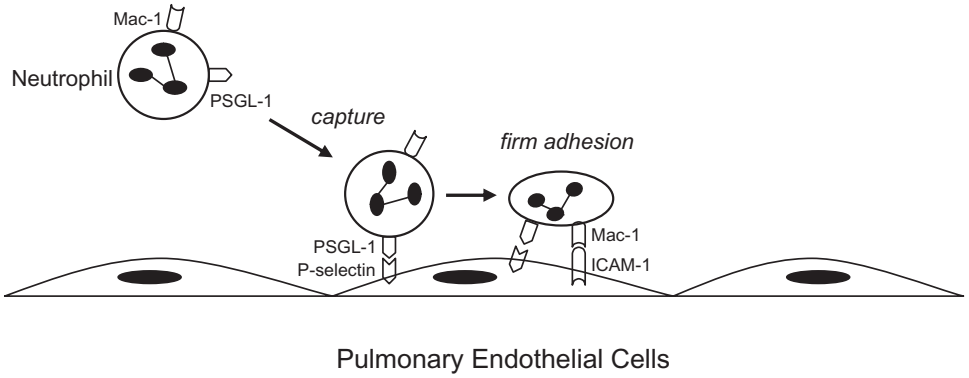

Figure 5 The schematic diagram summarizes the proposed hypothesis that PSGL-1 on neutrophils functions as a capturing adhesion molecule onto the pulmonary endothelial cells in CLP-induced septic lung injury. PSGL-1 mediates neutrophil capture onto the endothelium in a platelet-independent manner. Whether PSGL-1 also mediates neutrophil rolling in the pulmonary microvasculature remains unclear. In mice with CLP, Mac-1 is up-regulated on the surface of circulating neutrophils (Asaduzzaman *et al.*, 2008), which subsequently mediates firm adhesion onto activated endothelial cells, presumably via binding to ICAM-1 (Laudes *et al.*, 2004). CLP, caecal ligation and puncture; Mac-1, membrane-activated complex-1; PSGL-1, P-selectin glycoprotein-ligand-1.

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Conflict of interest

The authors state no conflict of interest.

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