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RESEARCH PAPER

Platelet secretion of CXCL4 is Rac1-dependent and regulates neutrophil infiltration and tissue damage in septic lung damage

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BACKGROUND AND PURPOSE

Platelets are potent regulators of neutrophil accumulation in septic lung damage. We hypothesized that platelet-derived CXCL4 might support pulmonary neutrophilia in a murine model of abdominal sepsis.

EXPERIMENTAL APPROACH

Polymicrobial sepsis was triggered by coecal ligation and puncture (CLP) in C57BL/6 mice. Platelet secretion of CXCL4 was studied by using confocal microscopy. Plasma and lung levels of CXCL4, CXCL1 and CXCL2 were determined by ELISA. Flow cytometry was used to examine surface expression of Mac-1 on neutrophils.

KEY RESULTS

CLP increased CXCL4 levels in plasma, and platelet depletion reduced plasma levels of CXCL4 in septic animals. Rac1 inhibitor NSC23766 decreased the CLP-enhanced CXCL4 in plasma by 77%. NSC23766 also abolished PAR4 agonist-induced secretion of CXCL4 from isolated platelets. Inhibition of CXCL4 reduced CLP-evoked neutrophil recruitment, oedema formation and tissue damage in the lung. However, immunoneutralization of CXCL4 had no effect on CLP-induced expression of Mac-1 on neutrophils. Targeting CXCL4 attenuated plasma and lung levels of CXCL1 and CXCL2 in septic mice. CXCL4 had no effect on neutrophil chemotaxis *in vitro*, indicating it has an indirect effect on pulmonary neutrophilia. Intratracheal CXCL4 enhanced infiltration of neutrophils and formation of CXCL2 in the lung. CXCR2 antagonist SB225002 markedly reduced CXCL4-provoked neutrophil accumulation in the lung. CXCL4 caused secretion of CXCL2 from isolated alveolar macrophages.

CONCLUSIONS AND IMPLICATIONS

Rac1 controls platelet secretion of CXCL4 and CXCL4 is a potent stimulator of neutrophil accumulation in septic lungs via generation of CXCL2 in alveolar macrophages. Platelet-derived CXCL4 plays an important role in lung inflammation and tissue damage in polymicrobial sepsis.

Abbreviations

BALF, bronchoalveolar lavage fluid; CLP, coecal ligation and puncture; PE, phycoerythrin; PMNL, polymorphonuclear leukocytes

Tables of Links

TARGETS	
GPCRs[a]	**Catalytic receptors**[b]
CXCR2	Mac-1 (integrin, alpha M subunit)
CXCR4	**Enzymes**[c]
PAR4	MPO

LIGANDS	
AYPGKF	CXCL4
CCL5	GTP
CD40 ligand (CD40L)	PGE1
CXCL1	Rac1
CXCL2	SB225002

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2006) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b,c]Alexander *et al.*, 2006 a,b,c).

Introduction

Management of patients with sepsis poses a significant challenge to clinicians. One reason is related to the lack of a comprehensive understanding of the underlying pathophysiology in sepsis. It is well known that the lung is a very sensitive and critical target organ in sepsis (Parrillo, 1993). Although neutrophil recruitment constitutes a central component in the host response to bacterial infections (Gorbach and Bartlett, 1974; Reutershan *et al.*, 2005), excessive neutrophil responses are known to cause organ damage in sepsis (Issekutz and Issekutz, 1992; Reutershan *et al.*, 2005; Basit *et al.*, 2006; Asaduzzaman *et al.*, 2008). Based on experiments blocking neutrophil recruitment, several studies have shown that neutrophil accumulation constitutes a rate-limiting step in septic lung damage (Asaduzzaman *et al.*, 2008, 2009b; Hasan *et al.*, 2011). It is interesting to note that accumulating data implicates platelets in the development of septic lung injury. For example, several studies have shown that platelets promote neutrophil activation and recruitment to the lung in sepsis (Zarbock *et al.*, 2007; Asaduzzaman *et al.*, 2009a). One study has shown that platelet-derived CD40L is a potent inducer of neutrophil infiltration in septic lung injury (Rahman *et al.*, 2009). However, platelets harbour a wide spectrum of different pro-inflammatory compounds, such as chemokines, which could mediate platelet-dependent accumulation of neutrophils in septic lung damage.

CXCL4 is one of the most abundant chemokines in platelets and belongs to the CXC chemokine family although it lacks an ELR sequence needed for binding to chemotactic CXCRs on neutrophils (Clark-Lewis *et al.*, 1993). The literature on the chemotactic activity of CXCL4 is complex and contradictory. For example, one early study reported that CXCL4 exerts chemotactic activity toward neutrophils (Deuel *et al.*, 1981). However, these findings could not be confirmed in later studies, and the early findings may have been caused by contamination with other chemokines (Petersen *et al.*, 1996). The studies on the role of CXCL4 have also been complicated due to the absence of clearly defined CXCL4 receptors. CXCL4 binds avidly to glycosaminoglycans, such as a chondroitin sulphate proteoglycan on neutrophils (Petersen *et al.*, 1998). In addition, CXCL4 has been shown to bind to CXCR3B, which is a splice variant of CXCR3 expressed in humans but not in mice (Lasagni *et al.*, 2003; Kowalska *et al.*, 2010). Another study has reported that CXCL4 also can bind to CXCR3A on human T-lymphocytes (Mueller *et al.*, 2008). Nonetheless, experimental evidence has shown that CXCL4 plays a role in regulating neutrophil recruitment and tissue damage in complex inflammatory disease models, such as liver fibrosis (Zaldivar *et al.*, 2010) and intestinal reperfusion injury (Lapchak *et al.*, 2012). One study reported that plasma levels of CXCL4 are elevated in patients with sepsis (Lorenz and Brauer, 1988), raising the question of whether CXCL4 can regulate neutrophil-dependent tissue damage in abdominal sepsis.

The intracellular signalling cascades triggering platelet secretion of CXCL4 are not well understood. We recently reported that Rac1, a member of the Ras-homologus (Rho) family, not only plays a key function septic lung damage (Hwaiz *et al.*, 2013) but also controls platelet secretion of CD40L in sepsis (Hwaiz *et al.*, 2014). Other studies have identified an essential role of Rac1 in lamellipodia formation, phospholipase Cγ2 activation, granule secretion and clot retraction in platelets (McCarty *et al.*, 2005; Akbar *et al.*, 2007; Pleines *et al.*, 2009). Thus, we hypothesized that Rac1 might be involved in the secretion of CXCL4 from platelets in the present study.

Thus, one aim of the present study was to examine the function of Rac1 in regulating platelet secretion of CXCL4. Moreover, we investigated the role of CXCL4 in regulating pulmonary neutrophilia and tissue injury in polymicrobial sepsis.

Methods

Animals and materials

All experiments were performed using male C57BL/6 mice (Taconic Bioscience, Lille Skensved, Denmark) 8–9 weeks old (20–25 g) 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. Animals were kept in a pathogen-free facility on a 12–12 h light–dark cycle and had free access to food (R3 breeding food for rats and mice; Lantmännen, Kemstad, Sweden) and tap water. Mice were housed for 1 week before use in the experiments. Animals were kept at a maximum of

seven mice per cage with environment enrichment, such as a little house and toys. Animals were anaesthetized by i.p. administration of 75 mg ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 25 mg xylazine (Janssen Pharmaceutica, Beerse, Belgium) ·kg^{-1} body weight. Animals were evaluated every 6 h during the experiments. At each inspection, mice that appeared moribund, such as lack of response to stimulation or no movement, and animals with respiratory distress or agonal breathing were killed by CO_2 inhalation and cervical dislocation. The ARRIVE guidelines (Kilkenny *et al.*, 2010) and an editorial on the application of ARRIVE guidelines to pharmacological studies (McGrath *et al.*, 2010) were consulted for all *in vivo* studies involving animals.

Experimental model of sepsis

Abdominal sepsis was induced in anaesthetized mice by puncture of the coecum as previously described in detail (Wichterman *et al.*, 1980). First, the abdomen was opened, and the coecum was filled with faeces by milking stool backward from the ascending colon. Then a ligature was placed below the ileocoecal valve, and the coecum was soaked with PBS (pH 7.4). The coecum was punctured twice with a 21-gauge needle, and a small amount of bowel contents was extruded. The coecum was then returned into the peritoneal cavity, and the abdominal wall was closed. Animals were treated i.p. with vehicle (dH_2O) or with 5 mg·kg^{-1} of the Rac1 inhibitor, NSC23766 (N6-[2-[[4-(Diethylamino)-1-methylbutyl] amino]-6-methyl- 4-pyrimidinyl]-2 methyl-4, 6-quinolinediamine trihydrochloride, Tocris Bioscience, Bristol, UK). This selection of dose of NSC23766 was based on previous publications (Hwaiz *et al.*, 2013, 2014). NSC23766 is a specific and reversible Rac1 inhibitor that competitively inhibits the interaction between Rac1 and Rac-specific guanine nucleotide exchange factors. NSC23766 does not affect the activity of related Rho GTPases, including Cdc42 or RhoA *in vitro* or *in vivo* (Gao *et al.*, 2004; Akbar *et al.*, 2006). A control antibody (clone 141945 R&D Systems, Minneapolis, MN, USA) or a monoclonal antibody against murine CXCL4 (clone 140910, R&D Systems) was injected i.p. (10 μg per mouse) 30 min before coecal ligation and puncture (CLP) induction. In order to study the role of platelets, a platelet-depleting antibody directed against murine CD42b (GP1bα, 1.0 mg·kg^{-1}, Emfret Analytics GmbH & Co. KG, Wurzburg, Germany) or an isotype control antibody (clone R3–34, 1.0 mg·kg^{-1}, BD Biosciences Pharmingen, San Jose, CA, USA) was given i.p. 2 h before the CLP. Sham mice underwent the same surgical procedures, that is, laparotomy and resuscitation, but the coecum was neither ligated nor punctured. The animals were then returned to their cages and provided food and water *ad libitum*. Animals were re-anaesthetized 6 and 24 h after CLP induction. The left lung was ligated and excised after lung perfusion for oedema measurement. The right lung was used for collecting bronchoalveolar lavage fluid (BALF) in which neutrophils were counted. Next, the lung was perfused via the heart with PBS, and one part of the right lung was fixed in formaldehyde for histology, and the remaining lung tissue was snap-frozen in liquid nitrogen and stored at −80°C for later ELISA and myeloperoxidase (MPO) assays as described subsequently. Anaesthetized animals were killed by cervical dislocation.

MPO assay

Lung tissue was thawed and homogenized in 0.02 M phosphate buffer (pH 7.4). Supernatant was discarded after centrifugation for 10 min at 8770 *g* at room temperature, and then the pellet was dissolved by adding 1 mL of 0.5% hexadecyltrimethylammonium bromide. Samples were stored at −20°C overnight and then thawed and kept at in a 60°C water bath for 2 h followed by 90 s sonication. Supernatant was collected after 5 min centrifugation at 8770 *g*, and the MPO activity in the supernatant was determined spectrophotometrically 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) as described previously (Krawisz *et al.*, 1984). Values were expressed as MPO U·g^{-1} tissue.

BALF and lung oedema

Animals were placed supine, and the trachea was exposed by dissection. A catheter was inserted into the trachea. BALF was collected by five washes of 1 mL of PBS containing 5 mM EDTA. The number of polymorphonuclear leukocytes (PMNLs) was counted in a Burker chamber. The left lung was excised and then weighed. The left lung was then dried at 60°C for 72 h and re-weighed. The change in the ratio of wet weight to dry weight was used as indicator of lung oedema formation.

Intratracheal challenge with CXCL4

Intratracheal administration of CXCL4 was performed via a skin incision over the trachea and injection of murine CXCL4 (1 μg, PeproTech Nordic, Stockholm, Sweden) into the trachea with a 27G needle in anaesthetized animals. Negative control mice underwent the same surgical procedures but received only PBS intratracheally. Four hours after CXCL4 challenge, BALF was collected as described previously for analysis of CXCL2 levels and number of neutrophils. In certain experiments, a CXCR2 antagonist (SB225002, 4 mg·kg^{-1}, Calbiochem, Merck, Darmstadt, Germany) or vehicle (PBS) was administered i.p. 10 min before intratracheal injection of CXCL4.

Histology

Lung tissue was fixed in 4% formaldehyde phosphate buffer overnight and then dehydrated and paraffin embedded. Six micrometre sections were stained with haematoxylin and eosin. Lung injury was quantified in a blinded manner by use of a modified scoring system (Carraway *et al.*, 2003; Borzone *et al.*, 2007), including size of alveoli, thickness of alveolar septae, alveolar fibrin deposition and neutrophil scoring system infiltration graded on a zero (absent) to four (extensive) scale. In each tissue sample, five random areas were scored, and the mean value was calculated. The histology score is the sum of all four parameters.

Flow cytometry

For analysis of surface expression of Mac-1 on circulating neutrophils, blood was collected (1:10 acid citrate dextrose) 6 h after CLP induction and incubated (10 min at room temperature) with an anti-CD16/CD32 antibody-blocking Fcγ III/II receptors to reduce non-specific labelling and then incubated with phycoerythrin (PE)-conjugated anti-Ly6G (clone

RB6-8C5, rat IgG2b, eBioscience, Frankfurt, Germany) and FITC-conjugated anti-Mac-1 (clone M1/70, integrin αM China, rat IgG2b κ, BD Biosciences Pharmingen) antibodies. Alveolar macrophages were isolated as described later and incubated with an anti-CD16/CD32 antibody-blocking Fcγ III/II receptors and a peridininchlorophyll protein-Cy5.5-conjugated anti-mouse F4/80 antibody (clone BM8, eBioscience, Frankfurt, Germany) and a FITC-conjugated anti-Ly6G (clone 1A8, BD Biosciences) antibody. Cells were fixed, and erythrocytes were lysed; neutrophils were recovered following centrifugation. Flow-cytometric analysis was performed according to standard settings on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) and analysed with CellQuest Pro software (BD Biosciences).

ELISA

CXCL1, CXCL2 and CXCL4 levels in lung tissue and plasma were analysed by use of double antibody Quantikine ELISA kits (R&D Systems, Europe, Abingdon, Oxon, UK) using recombinant murine CXCL1, CXCL2 and CXCL4 as standards.

Neutrophil chemotaxis

Neutrophil chemotaxis was performed as previously described (Smith *et al.*, 1991). Neutrophils were isolated from bone marrow by use of Ficoll-Paque™ as previously described in detail (Swamydas and Lionakis, 2013). Briefly, 1.5×10^6 neutrophils were placed in the upper chamber of the transwell inserts (5 μm pore size; Corning Costar, Corning, NY, USA). Inserts were placed in wells containing medium alone (control) or medium plus murine CXCL2 (0.1 $\mu g \cdot mL^{-1}$, R&D Systems) or murine CXCL4 (0.1, 0.5 and 1 $\mu g \cdot mL^{-1}$, R&D Systems). After 120 min, inserts were removed, and migrated neutrophils were stained with Turks solution. Chemotaxis was determined by counting the number of migrated neutrophils in a Burker chamber.

Alveolar macrophage secretion of CXCL2

Bronchoalveolar lavage fluid was collected as described previously from healthy mice. Cells were isolated by centrifugation (450 *g*, 10 min) and resuspended in RPMI1644. Next, cells were washed three times and then allowed to rest and adhere for 4 h. Adherent cells were resuspended in RPMI1644 media, and purity of alveolar macrophages was determined by staining cells with anti-F4/80 and anti-Ly6G antibodies as described previously. Macrophages were defined as $F4/80^+/Ly6G^-$ cells. The purity of alveolar macrophages was more than 97% (not shown). Alveolar macrophages were co-incubated with recombinant murine CXCL4 (0.2, 0.3, 0.4 and 0.5 $\mu g \cdot mL^{-1}$) for 4 h (37°C) and then CXCL2 levels were measured by ELISA.

Platelet isolation and CXCL4 secretion

Blood collected in syringes containing 0.1 mL of acid–citrate–dextrose was immediately diluted with equal volumes of modified Tyrode solution (1 $\mu g \cdot mL^{-1}$ PGE_1 and 0.1 $U \cdot mL^{-1}$ apyrase) and centrifuged (200 *g*, 5 min). Platelet-rich plasma was collected and centrifuged (800 *g*, 15 min), and pellets were resuspended in modified Tyrode solution. After being washed once more (10 000 *g*, 5 min), 0.5×10^6 platelets were seeded on a chamber slide coated with fibrinogen (20 $\mu g \cdot mL^{-1}$). Adherent platelets were stimulated with the PAR4 agonist (AYPGKF, 200 μM, 37°C) with and without NSC23766 (100 μM). Platelets were fixed with 2% paraformaldehyde for 5 min and washed and blocked with 1% goat serum for 45 min. Platelets were then permeabilized with 0.15% Triton X-100 for 15 min followed by washing and incubation with an anti-CD16/CD32 antibody (10 min) to block Fcγ III/II receptors and reduce non-specific labelling and a rabbit polyclonal primary antibody against CXCL4 (bs-2548R, Bioss, Boston, MA, USA) for 2 h. Chamber slides were washed and incubated with FITC-conjugated anti-rabbit secondary antibody (Cell Signaling Technology, Beverly, MA, USA) and platelet-specific PE-conjugated anti-CD41 (clone MWReg30, eBioscience, San Diego, CA, USA) for 1 h. Chamber slides were washed three times, and confocal microscopy was performed using Meta 510 confocal microscopy (Carl Zeiss, Jena, Germany). FITC and PE were excited by 488 and 543 nm laser lines, and corresponding emission wavelengths of FITC and PE were collected by the filters of 500–530 and 560–590 nm respectively. The pinhole was ~1 airy unit, and the scanning frame was 512 × 512 pixels. The fluorescent intensity was calculated by use of ZEN2009 software.

Pull-down assay and Western blotting

Rac1 activity was determined in platelets from sham and CLP mice pretreated with vehicle (dH_2O) or NSC23766 by active Rac1 pull-down and detection kit using the protein-binding domain of GST-PAK1, which binds with the GTP-bound form of Rac1 (Pierce Biotechnology, Rockford, IL, USA). Briefly, platelets were suspended in lysis buffer on ice and centrifuged (16 000 *g*, 15 min). Ten microlitres from each lysate were removed to measure protein content using Pierce BCA Protein Assay Reagent (Pierce Biotechnology), and the rest was used for the pull-down assay. Supernatant containing equal amount of proteins was then diluted with 2× SDS sample buffer and boiled for 5 min. Proteins were separated using SDS-PAGE (10–12% gel). After being transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA), blots were blocked with Tris buffer saline/Tween20 containing 3% BSA at room temperature for 1 h, followed by incubation with an anti-Rac1 antibody (1:1000) at 4°C overnight. Binding of the antibody was detected using peroxidase-conjugated anti-mouse antibody (1:100 000, Pierce Biotechnology) at room temperature for 2 h and developed by Immun-Star WesternC Chemiluminescence Kit (Bio-Rad). Total Rac1 and β-actin were used as loading controls.

Statistics

Sample size calculations were based on a previous study (Asaduzzaman *et al.*, 2009a) with an effect size of 40% (mean value 100 in sham and 60 in treated animals) with SD of 20. This, together with a power of 0.8 and a *P*-value of 0.05, required a sample size of at least four animals per group. Data were presented as mean values ± SEM. Statistical evaluations were performed using Kruskal–Wallis one-way ANOVA on ranks followed by multiple comparisons versus control group (Dunnett's method). $P < 0.05$ was considered significant, and *n* represents the total number of mice in each group.

Statistical analysis was performed by using SigmaPlot® 10.0 software (Systat Software, Chicago, IL, USA).

Results

Rac1 regulates platelet secretion of CXCL4

Plasma levels of CXCL4 were low but detectable in sham animals (Figure 1A). CLP increased CXCL4 levels in plasma by 41-fold (Figure 1A). To deplete animals of platelets, an anti-CD41 antibody was administered before CLP induction. It was observed that platelet depletion reduced plasma levels of CXCL4 by 87% in septic animals (Figure 1A), indicating that platelets are the dominating source of CXCL4 in abdominal sepsis. Treatment with the Rac1 inhibitor NSC23766 had no effect on CXCL4 levels in the plasma of control animals (Figure 1A). However, administration of NSC23766 reduced plasma levels of CXCL4 by 77% in CLP animals (Figure 1A). Moreover, we found that CLP increased Rac1-GTP levels in platelets, indicating that Rac1 is activated in platelets in septic animals (Figure 1B and 1C). Administration of NSC23766 abolished CLP-evoked Rac1 activation in platelets (Figure 1B and 1C), demonstrating that NSC23766 is an effective inhibitor of Rac1 activation. We stimulated isolated platelets with a PAR4 agonist (AYPGKF) in order to determine the direct role of Rac1 in regulating platelet secretion of CXCL4. It was found that CXCL4 was present in unstimulated platelets and that co-incubation with the PAR4 agonist reduced intracellular levels of CXCL4 in platelets (Figure 2A and B). Notably, co-incubation with NSC23766 markedly decreased the PAR4 agonist-induced secretion of CXCL4 from the platelets (Figure 2A and 2B).

CXCL4 regulates septic lung injury

It was found that CLP increased lung oedema and that immunoneutralization of CXCL4 reduced oedema formation in the lung by 65% in septic animals (Figure 3A). The lung injury in septic mice was characterized by severe destruction of tissue microarchitecture, extensive oedema of and massive infiltration of neutrophils (Figure 3B). Inhibition of CXCL4 attenuated CLP-induced tissue damage and neutrophil accumulation in the lung (Figure 3B). Quantification of the morphological damage revealed that CLP significantly enhanced

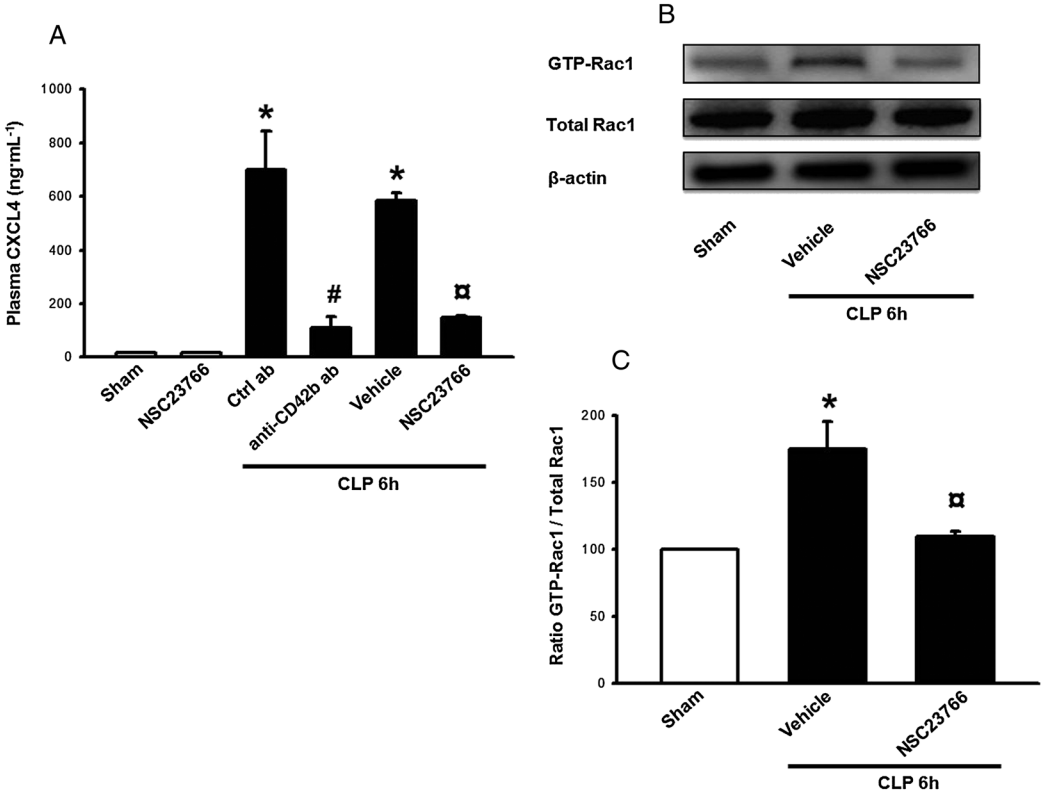

Figure 1

Rac1 regulates platelet secretion of CXCL4 in sepsis. Animals were treated with vehicle, NSC23766 (5 mg·kg^{-1}), a control ab (Ctrl ab) or an anti-CD42b ab before CLP induction. (A) ELISA was used to quantify the levels of CXCL4 in the plasma 6 h after CLP induction. (B) Rac1-GTP was determined by Western blotting using GST-PAK pull-down beads 6 h after induction of CLP. (C) Band intensities were quantified in isolated platelets by densitometry and normalized to total Rac1. Western blots are representative of five independent experiments. Mice were treated with the Rac1 inhibitor NSC23766 (5 mg·kg^{-1}) or vehicle before the induction of CLP. Sham-operated mice served as negative controls. Data represent mean ± SEM and $n = 5$. *$P < 0.05$ versus Sham; $^{\#}P < 0.05$ versus Ctrl ab + CLP; and $^{¤}P < 0.05$ versus Vehicle + CLP. Statistical evaluations were performed using Kruskal–Wallis one-way ANOVA on ranks followed by multiple comparisons versus Sham, versus Ctrl ab and versus Vehicle + CLP (Dunnett's method). All experiments in the sham group in (C) received a value of 100 for the statistical analysis.

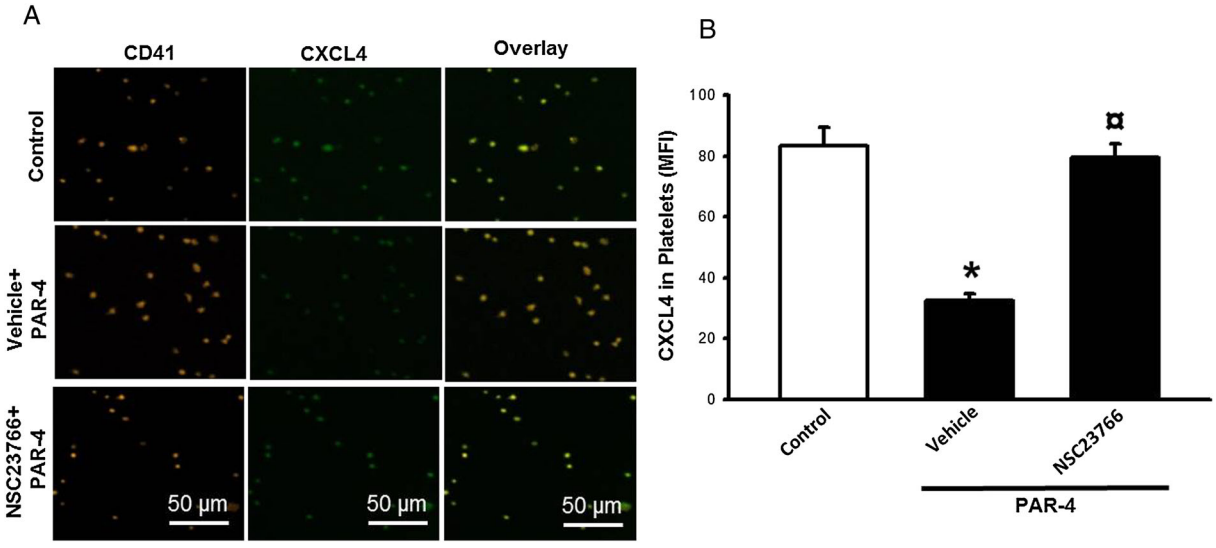

Figure 2

Rac1 regulates agonist-induced secretion of CXCL4 in platelets. (A) Isolated platelets were incubated with or without NSC23766 (10 μM) and then stimulated with recombinant PAR4 (200 μM), and the level of CXCL4 in permeabilized CD41$^+$ platelets was determined by confocal microscopy. (B) Aggregate data showing mean fluorescence intensity of CXCL4 in platelets. Non-stimulated platelets served as control. Data represent mean ± SEM and $n = 5$. *$P < 0.05$ versus control and $^{¤}P < 0.05$ versus vehicle + PAR4. Statistical evaluations were performed using Kruskal–Wallis one-way ANOVA on ranks followed by multiple comparisons versus control and versus vehicle + PAR4 (Dunnett's method).

lung damage score and that immunoneutralization of CXCL4 decreased CLP-evoked tissue injury in the lung by 67% (Figure 3C).

CXCL4 regulates neutrophil infiltration in septic lung injury

CLP increased pulmonary levels of MPO by 24-fold, and immunoneutralization of CXCL4 reduced MPO activity in the lung by 57% in septic mice (Figure 4A). CLP increased the number of alveolar neutrophils by 19-fold, and administration of the anti-CXCL4 antibody decreased the number of alveolar neutrophils by 42% in the inflamed lungs (Figure 4B). Neutrophil expression of Mac-1 was increased in septic mice (Figure 4C). Inhibition of CXCL4 function had no effect on neutrophil expression of Mac-1 (Figure 4C). *In vitro* CXCL2 triggered robust migration of isolated neutrophils, whereas CXCL4 exerted no chemotactic effect on neutrophils (Figure 4D), suggesting that the CXCL4-dependent accumulation of neutrophils is not a direct action on neutrophils but rather an indirect effect of CXCL4.

CXCL4 regulates CXC chemokine formation in sepsis

Plasma and lung levels of CXCL1 and CXCL2 were low but detectable in sham mice (Figure 5). We found that CXCL1 and CXCL2 levels in the plasma increased by 1183-fold and 1153-fold, respectively, in septic animals (Figure 5A and 5B). Inhibition of CXCL4 decreased CLP-induced plasma levels of CXCL1 by 94% and CXCL2 by 95% (Figure 5A and 5B). Moreover, CLP increased lung levels of CXCL1 by 144-fold and CXCL2 by 581-fold (Figure 5C and 5D). Immunoneutralization of CXCL4 decreased CLP-induced increases of CXCL1 and CXCL2 by 85% and 95% respectively (Figure 5C and 5D). We next studied direct effects of CXCL4 on neutrophil accumulation in the lung. Intratracheal administration of CXCL4 enhanced pulmonary levels of CXCL2 (Figure 6A) and the number of alveolar neutrophils (Figure 6B). In addition, treatment with the CXCR2 antagonist SB225002 reduced CXCL4-triggered recruitment of neutrophils by 82% in the lung (Figure 6C). We next isolated alveolar macrophages and co-incubated them with CXCL4 and found that CXCL4 increased CXCL2 formation (Figure 6D). This finding was repeated in RAW264.7 cells showing that CXCL4 enhanced secretion of CXCL2 from macrophages (not shown).

Discussion

Our present findings indicate an important function of Rac1-mediated secretion of CXCL4 from platelets in sepsis. In addition, this study reveals key mechanisms controlling CXCL4-dependent pulmonary accumulation of neutrophils in abdominal sepsis. These results point to an important role of platelets in sepsis and suggest that inhibition of Rac1 signalling and/or CXCL4 function might be useful strategies to ameliorate septic lung damage.

Platelets are not only critical in wound healing and thrombosis but also exert numerous pro-inflammatory functions in the host response to bacterial invasion (Rahman *et al.*, 2009, 2013; Hwaiz *et al.*, 2014). For example, data have shown that platelets regulate numerous aspects of leukocyte responses to severe infections (Asaduzzaman *et al.*, 2008, 2009a). One such key aspect of the inflammatory response is neutrophil activation and accumulation at sites of microbial invasion. For example, platelet-derived CD40L has been reported to regulate sepsis-evoked neutrophil activation and

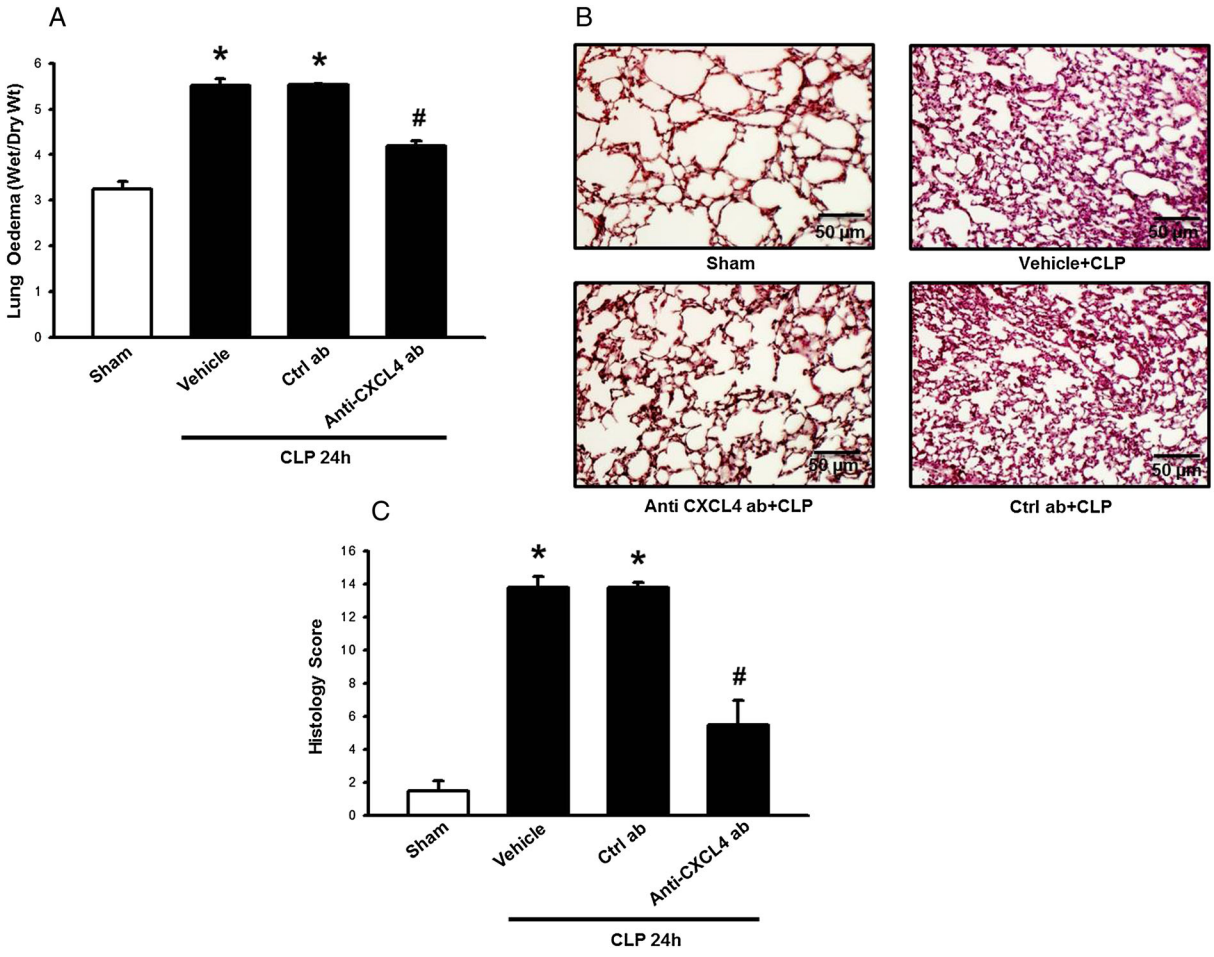

Figure 3

CXCL4 regulates lung damage in sepsis. (A) Oedema formation in the lung. (B) Representative haematoxylin and eosin sections of lung are shown. Animals were treated with vehicle, a control ab (Ctrl ab) or an anti-CXCL4 ab before CLP induction. (C) Lung injury scores as described in the Methods section 24 h after CLP induction. Sham-operated animals served as negative controls. Data represent mean ± SEM and $n = 5$. $^{*}P < 0.05$ versus sham and $^{\#}P < 0.05$ versus Ctrl ab + CLP. Statistical evaluations were performed using Kruskal–Wallis one-way ANOVA on ranks followed by multiple comparisons versus Sham and versus Ctrl ab + CLP (Dunnett's method).

tissue recruitment (Rahman *et al.*, 2009, 2013). Notably, platelets harbour several other pro-inflammatory substances, including a plethora of different chemokines (Yan *et al.*, 1994; Flad and Brandt, 2010). The most abundant chemokines in platelets are CCL5 and CXCL4, but these chemokines are poor stimulators of neutrophil migration (Petersen *et al.*, 1996; Hartl *et al.*, 2008). Instead, CCL5 and CXCL4 are potent activators lymphocytes, macrophages and eosinophils (McColl *et al.*, 1993; Hartl *et al.*, 2008). Furthermore, studies in the literature have reported that targeting CXCL4 can decrease neutrophil recruitment in models of inflammatory diseases (Zaldivar *et al.*, 2010; Grommes *et al.*, 2012; Lapchak *et al.*, 2012). Interestingly, we found that platelet depletion greatly reduced plasma levels of CXCL4 in CLP animals, suggesting that platelets are a dominating source of circulating CXCL4 in polymicrobial sepsis. Because the mechanisms of platelet secretion of CXCL4 are not known, we wanted first to examine the signalling mechanisms controlling platelet secretion of CXCL4. We observed that platelets from septic mice exhibited increased Rac1 activity. In addition, treatment with the Rac1 inhibitor NSC23766 prior to CLP induction markedly decreased plasma levels of CXCL4 in septic animals, indicating that Rac1 signalling is a critical component in regulating circulating levels of CXCL4 in abdominal sepsis. Next, we wanted to determine the direct role of Rac1 in controlling platelet secretion of CXCL4. It was found that co-incubation of platelets with NSC23766 abolished the PAR4 agonist-induced platelet secretion of CXCL4 *in vitro*, showing for the first time that Rac1 regulates CXCL4 secretion from platelets. In this context, it is interesting to note that Rac1 was demonstrated to be involved in agonist-evoked mobilization of P-selectin and platelet-derived growth factor in platelets (Akbar *et al.*, 2007; Dwivedi *et al.*, 2010), and considering that P-selectin, platelet-derived growth factor and CXCL4 are localized in platelet α-granules (Akbar *et al.*, 2007; Galkina and Ley, 2007; Gleissner *et al.*, 2008; Blair and Flaumenhaft, 2009), these present findings suggest that Rac1 is a potential regulator of α-granule secretion in platelets.

Activation of innate immune cells is a landmark in the host response to severe infections and is an important cause

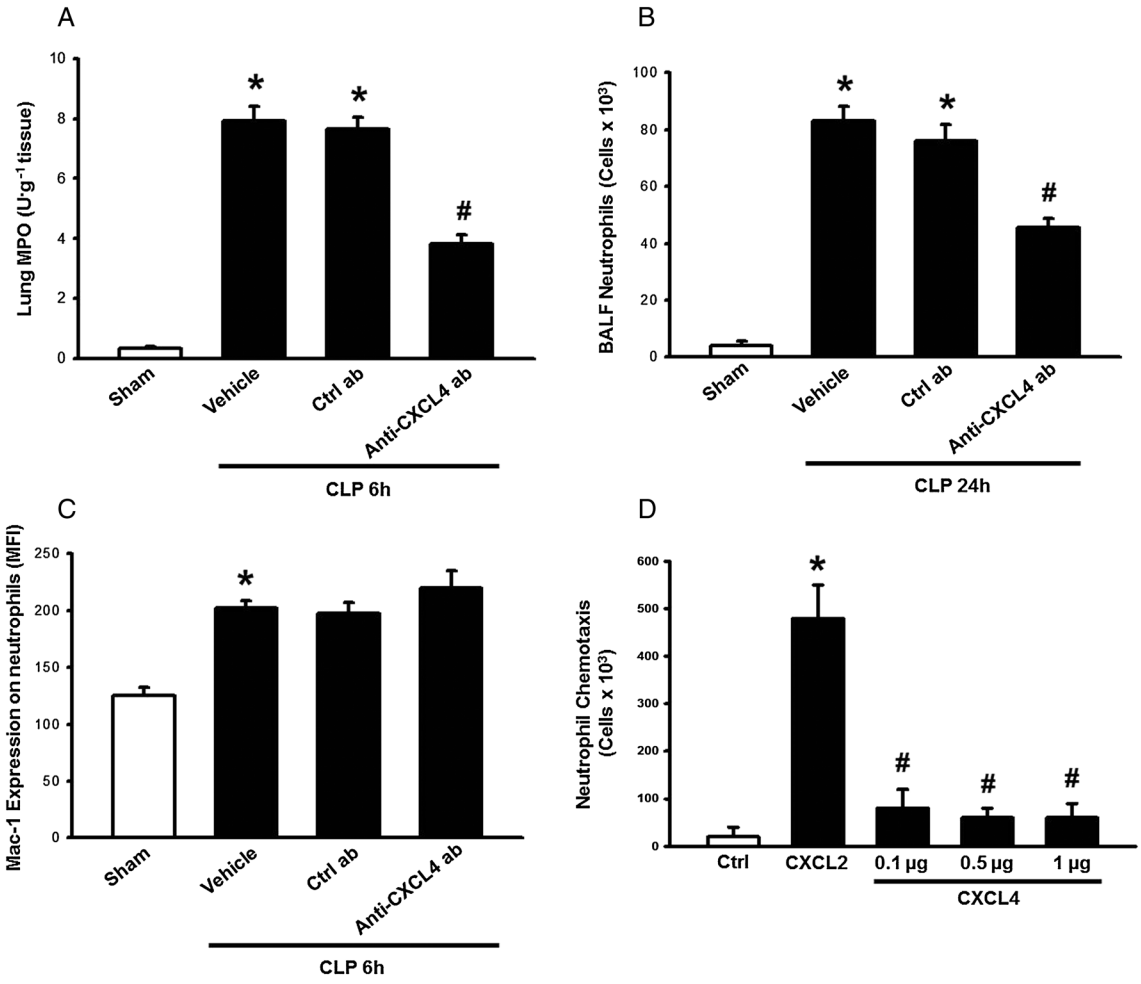

Figure 4

CXCL4 regulates pulmonary recruitment of neutrophils in sepsis. (A) Lung MPO levels at 6 h post-CLP. (B) Number of BALF neutrophils 24 h after CLP induction. (C) Mac-1 expression on circulating neutrophils 6 h after CLP induction. Animals were treated with vehicle, a control antibody (Ctrl ab) or an anti-CXCL4 ab before CLP induction. Sham-operated animals served as negative controls. (D) Neutrophils isolated from bone marrow were analysed for their migration in response to PBS (Control), CXCL2 (0.1 $\mu g \cdot mL^{-1}$) or CXCL4 (0.1, 0.5 or 1 $\mu g \cdot mL^{-1}$). Non-stimulated neutrophils served as negative control. Data represent mean ± SEM and $n = 5$. $^{*}P < 0.05$ versus Sham or Control and $^{\#}P < 0.05$ versus Ctrl ab + CLP or CXCL2. Statistical evaluations were performed using Kruskal–Wallis one-way ANOVA on ranks followed by multiple comparisons versus sham and versus Ctrl ab + CLP (Dunnett's method).

of acute lung injury characterized by disturbed gaseous exchange (Remick, 2007; Asaduzzaman *et al.*, 2009a). In the present study, we demonstrate that inhibition of CXCL4 reduces oedema formation and tissue damage in the lungs of septic mice, suggesting that CXCL4 exerts a causative role in the development of acute lung injury in polymicrobial sepsis. In this context, it should be noted that one study reported that overexpression of CXCL4 protects against endotoxin-induced acute lung injury (Kowalska *et al.*, 2007). In contrast, another study showed that inhibition of CXCL4 protects against endotoxin-induced acute lung injury (Grommes *et al.*, 2012). The reason for these discrepant findings is not known, but it is well known that administration of endotoxin alone is not an optimal substitute for studying polymicrobial sepsis. Nonetheless, our present findings suggest that CXCL4 plays a pro-inflammatory role in abdominal sepsis. This notion is in line with recent observations reporting a pro-inflammatory role of CXCL4 in complex diseases, such as atherosclerosis and liver fibrosis (Zaldivar *et al.*, 2010; Karshovska *et al.*, 2014). However, administration of the anti-CXCL4 antibody 2 h after induction of CLP had no effect on inflammation and tissue damage in the septic lung (not shown). Our data showed that immunoneutralization of CXCL4 reduced pulmonary MPO activity, a marker of neutrophil accumulation, by 57% in septic animals, which correlated well with the 42% reduction of neutrophil numbers in the bronchoalveolar space. These findings suggest that CXCL4 is an important regulator of pulmonary neutrophilia in polymicrobial sepsis. This notion is also supported by a recent study showing that neutrophil accumulation in the lung in a model of mesenteric ischemia and reperfusion is decreased in mice lacking CXCL4 (Lapchak *et al.*, 2012). Knowing that neutrophil infiltration is a rate-limiting step in septic lung injury, our data suggest that the protective effect of inhibiting CXCL4 is related to the reduction in neutrophil recruitment in the lung.

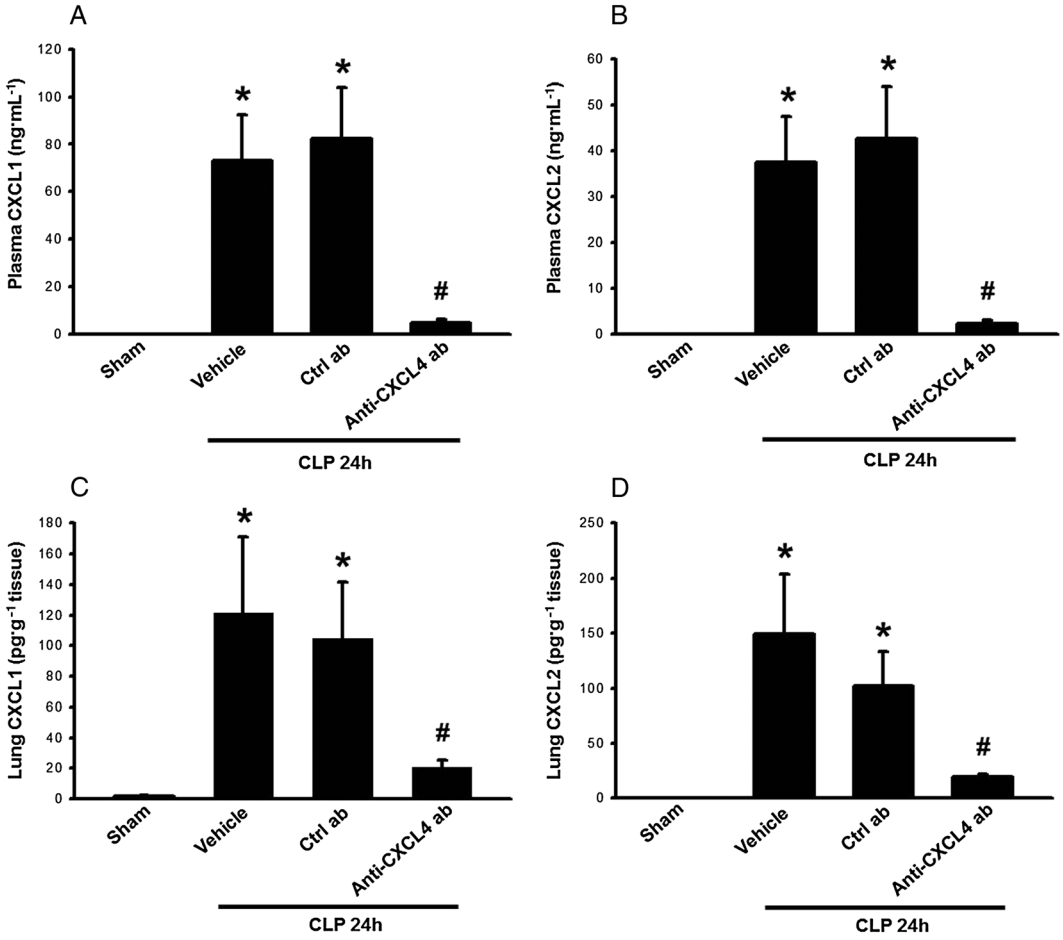

Figure 5

CXCL4 controls CXC chemokine formation in sepsis. Plasma levels of (A) CXCL1 and (B) CXCL2 and lung levels of (C) CXCL1 and (D) CXCL2 determined 24 h after CLP induction. Animals were treated with vehicle, a control antibody (Ctrl ab) or an anti-CXCL4 ab before CLP. Sham-operated animals served as negative controls. Data represent mean ± SEM and $n = 5$. $^{*}P < 0.05$ versus sham and $^{\#}P < 0.05$ versus Ctrl ab + CLP. Statistical evaluations were performed using Kruskal–Wallis one-way ANOVA on ranks followed by multiple comparisons versus sham and versus Ctrl ab + CLP (Dunnett's method).

Neutrophil accumulation at extravascular sites of tissue injury and inflammation is a multistep process mediated by specific adhesion molecules on neutrophils, such as CD162 and Mac-1 (Asaduzzaman *et al.*, 2008; Zhang *et al.*, 2013). Herein, we studied whether targeting of CXCL4 could reduce neutrophil up-regulation of Mac-1. However, inhibition of CXCL4 had no effect on neutrophil expression of Mac-1 in septic mice, indicating that CXCL4 is not involved in Mac-1 expression on neutrophils. Tissue trafficking of neutrophils is coordinated by secreted CXC chemokines, including CXCL1 and CXCL2 (Tekamp-Olson *et al.*, 1990). Previous reports have shown that CXC chemokines play an important role in septic lung injury (Hasan *et al.*, 2013; Hwaiz *et al.*, 2013). Herein, it was observed that immunoneutralization of CXCL4 abolished plasma and lung levels of CXC chemokines in CLP animals. These results indicate that CXCL4 might control neutrophil accumulation indirectly via generation of CXC chemokines in the septic lung. This conclusion is also in line with our present results demonstrating that local intratracheal challenge with CXCL4 enhanced generation of CXCL2 and neutrophil recruitment in the lung. That local injection of CXCL4 can provoke neutrophil infiltration in the lung is supported by a previous study showing that local administration of CXCL4 triggers accumulation of neutrophils in the skin (Sharpe *et al.*, 1991). Moreover, we observed that blocking CXCR2 function markedly decreased pulmonary neutrophilia induced by intratracheal administration of CXCL4, which further supports the conclusion that CXCL4 triggers neutrophil accumulation in the lung via generation of CXC chemokines in the inflamed lung. We next asked if alveolar macrophages might be a target cell of CXCL4. We found that co-incubation of isolated alveolar macrophages with CXCL4 dose-dependently increased CXCL2 secretion, suggesting that CXCL4 is a potent inducer of CXCL2 secretion in alveolar macrophages. In fact, this is the first time that CXCL4 has been demonstrated to promote macrophage secretion of CXCL2 although this observation is in line with findings showing that CXCL4 stimulates macrophage phagocytosis and oxidative burst (Pervushina *et al.*, 2004). It is important to note that although these findings point to a role of alveolar macrophages in mediating CXCL4-induced pulmonary accumulation

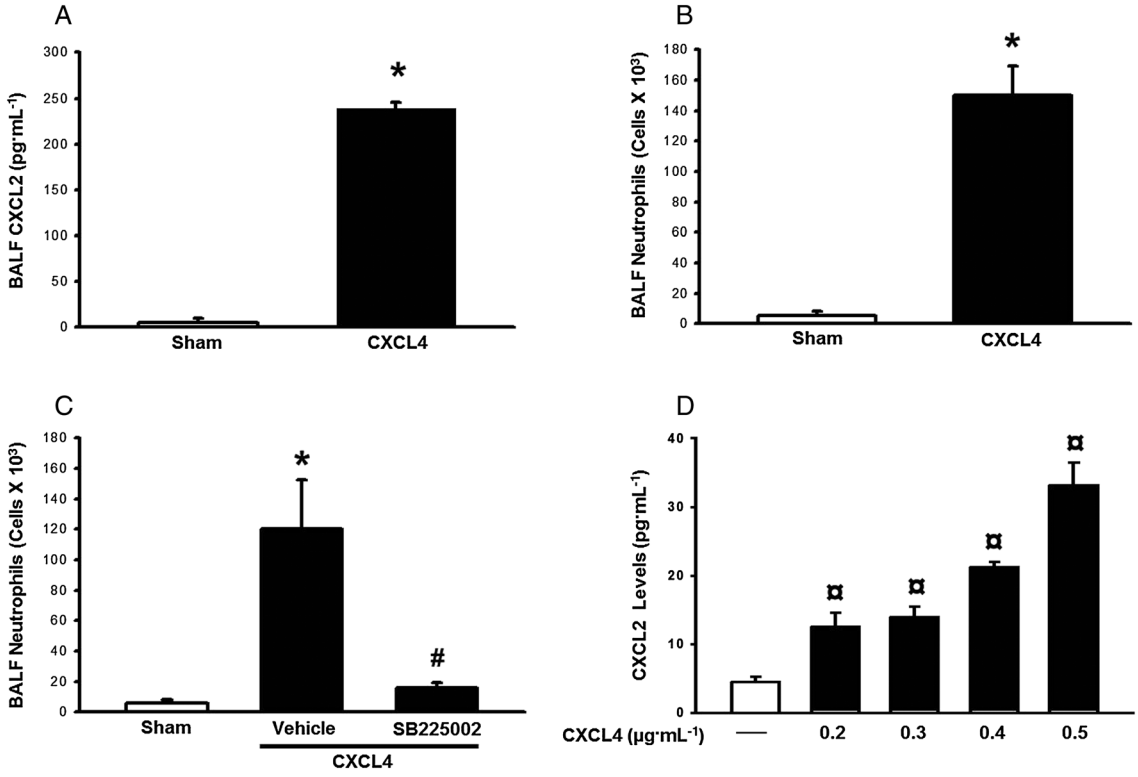

Figure 6

CXCL4-induced neutrophil recruitment is dependent on CXCL2 formation. Levels of (A) CXCL2 and (B) number of neutrophils in the lung after intratracheal challenge with CXCL4. (C) Neutrophil accumulation in the lungs of animals treated with vehicle or the CXCR2 antagonist SB225002 before intratracheal challenge with CXCL4. (D) Isolated alveolar macrophages were stimulated with the doses of CXCL4 indicated; then CXCL2 levels were determined by use of ELISA. Data represent mean ± SEM and $n = 5$. $^{*}P < 0.05$ versus Sham, $^{\#}P < 0.05$ versus vehicle + CXCL4 and $^{¤}P < 0.05$ versus vehicle (-). Statistical evaluations were performed using Kruskal–Wallis one-way ANOVA on ranks followed by multiple comparisons versus sham, versus Ctrl ab + CLP and versus vehicle (Dunnett's method).

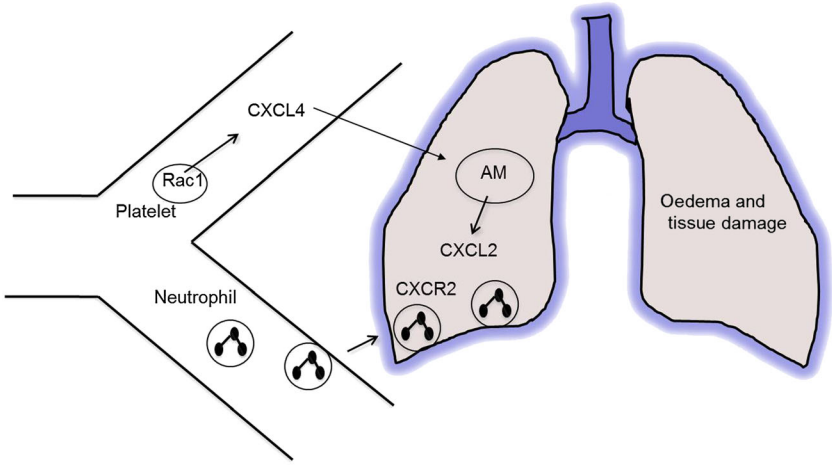

Figure 7

Proposed model for neutrophil recruitment in septic lung damage mediated by platelet-derived CXCL4. Abdominal sepsis triggers Rac1 activation and Rac1-dependent secretion of CXCL4 from platelets. CXCL4 activates alveolar macrophages (AM) leading to CXCL2 secretion in the lung. Increased local concentrations of CXCL2 stimulate neutrophil recruitment to the lung where they cause tissue oedema and injury.

of neutrophils in sepsis, our results do not exclude that CXCL4 also targets other cells, such as endothelial or epithelial cells in the lung.

Figure 7 shows a schematic representation of how platelet-derived CXCL4 causes neutrophil accumulation in septic lung injury. Thus, our findings suggest that Rac1 signalling is

enhanced in platelets and regulates platelet secretion of CXCL4 in polymicrobial sepsis. Moreover, our results indicate that CXCL4 controls neutrophil accumulation via secretion of CXCL2 from alveolar macrophages in septic lung injury. Thus, these findings not only delineate complex mechanisms of neutrophil trafficking in sepsis but also suggest that platelet-derived CXCL4 plays a key role in regulating inflammation and tissue damage in septic lung injury.

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Author contributions

R. H., M. R. and E. Z. performed the experiments, analysed data and wrote the manuscript. H. T. supervised the project, designed the experiments and wrote the manuscript.

Conflict of interest

Authors have no financial conflicts of interest.

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