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Rac1-dependent secretion of platelet-derived CCL5 regulates neutrophil recruitment via activation of alveolar macrophages in septic lung injury

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ABSTRACT

Accumulating evidence suggest that platelets play an important role in regulating neutrophil recruitment in septic lung injury. Herein, we hypothesized that platelet-derived CCL5 might facilitate sepsis-induced neutrophil accumulation in the lung. Abdominal sepsis was induced by CLP in C57BL/6 mice. CLP increased plasma levels of CCL5. Platelet depletion and treatment with the Rac1 inhibitor NSC23766 markedly reduced CCL5 in the plasma of septic mice. Moreover, Rac1 inhibition completely inhibited proteasePAR4-induced secretion of CCL5 in isolated platelets. Immunoneutralization of CCL5 decreased CLP-induced neutrophil infiltration, edema formation, and tissue injury in the lung. However, inhibition of CCL5 function had no effect on CLP-induced expression of Mac-1 on neutrophils. The blocking of CCL5 decreased plasma and lung levels of CXCL1 and CXCL2 in septic animals. CCL5 had no effect on neutrophil chemotaxis in vitro, suggesting an indirect effect of CCL5 on neutrophil recruitment. Intratracheal challenge with CCL5 increased accumulation of neutrophils and formation of CXCL2 in the lung. Administration of the CXCR2 antagonist SB225002 abolished CCL5-induced pulmonary recruitment of neutrophils. Isolated alveolar macrophages expressed significant levels of the CCL5 receptors CCR1 and CCR5. In addition, CCL5 triggered significant secretion of CXCL2 from isolated alveolar macrophages. Notably, intratracheal administration of clodronate not only depleted mice of alveolar macrophages but also abolished CCL5-induced formation of CXCL2 in the lung. Taken together, our findings suggest that Rac1 regulates platelet secretion of CCL5 and that CCL5 is a potent inducer of neutrophil recruitment in septic lung injury via formation of CXCL2 in alveolar macrophages. *J. Leukoc. Biol.* 97: 975–984; 2015.

Abbreviations: BALF = bronchoalveolar lavage fluid, CD40L = cluster of differentiation 40 ligand, CLP = cecal ligation and puncture, Mac-1 = macrophage-1 antigen, MPO = myeloperoxidase, NSC23766 = N6-[2-[[4-(diethylamino)-1-methylbutyl] amino]-6-methyl- 4-pyrimidinyl]-2 methyl-4, 6-quinolinediamine trihydrochloride, PAK = p21-activated kinase 1 protein, PAR4 = protease-activated receptor 4, Rac1 = Ras-related C3 botulinum toxin substrate 1, Rho = Ras homologus

Introduction

Neutrophil activation and recruitment constitute key features in the host response to systemic bacterial infections [1, 2]. Neutrophils are needed for microbial defense, but excessive tissue accumulation of neutrophils can cause organ damage in sepsis. The lung is the most sensitive and critical target organ in sepsis, and neutrophil recruitment constitutes a rate-limiting step in septic lung injury [3–5]. For example, the targeting of specific adhesion molecules, including CD11a, CD44, and CD162, not only decreases pulmonary infiltration of neutrophils but also protects against septic lung damage [3–5]. Interestingly, apart from their well-known role in hemostasis and wound healing [6, 7], a growing body of evidence suggests that platelets exert proinflammatory actions, such as supporting tissue infiltration of leukocytes in septic lung injury [8, 9]. For example, it has been reported that platelet-derived CD40L is a potent inducer of neutrophil infiltration in septic lung injury [10]. However, platelets contain a plethora of potential mediators, including chemokines, capable of stimulating leukocyte activation and recruitment [8–10].

One of the most prevalent chemokine in platelets is CCL5 (RANTES), which belongs to the CC chemokine family and is a potent stimulator of T cells, macrophages, and eosinophils [11–14]. Neutrophils do not normally express the CCL5 receptors, including CCR1 and CCR5 [15–17]. However, it has been reported that activated neutrophils under certain circumstances can up-regulate CCR1 [18, 19]. Moreover, several studies have reported that high CCL5 expression correlates with neutrophil activation in lung disease [20, 21]. Inhibition of CCL5 function has been reported to reduce neutrophil activation and accumulation in models of encephalitis, endotoxemia, stroke, and coronary ischemia, raising the question whether CCL5 might play a potential role in abdominal sepsis [21–24]. The intracellular signaling cascades triggering platelet secretion of CCL5 are not well understood. We have recently observed that Rac1, a member of the Rho family, not only plays an important

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role in septic lung injury [25] but also regulates platelet secretion of CD40L in sepsis [26]. Moreover, it has been reported that Rac1 is essential for lamellipodia formation, granule secretion, clot retraction, and phospholipase Cγ2 activation in platelets [27–30]. Thus, we hypothesized herein that Rac1 might be involved in the regulation of platelet secretion of CCL5 in abdominal sepsis.

Based on the above considerations, we studied the role of Rac1 in regulating platelet secretion of CCL5, as well as the function of CCL5 in controlling neutrophil recruitment and lung damage in abdominal sepsis. For this purpose, we used a model based on CLP.

MATERIALS AND METHODS

Animals

Experiments were performed with the use of male C57BL/6 mice (20–25 g). 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). Animals were anesthetized by i.p. administration of 75 mg ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 25 mg xylazine (Janssen Pharmaceutica, Beerse, Belgium)/kg body weight.

Experimental model of sepsis

Polymicrobial sepsis was induced by puncture of the cecum in anesthetized mice. Through a midline incision the cecum was exposed and filled with feces by milking stool backward from the ascending colon, and a ligature was placed below the ileocecal valve. The cecum was soaked with PBS and punctured twice with a 21 gauge needle, and a small amount of bowel contents was extruded. The cecum was then returned into the peritoneal cavity, and the abdominal wall was closed. Animals were treated with vehicle (dH_2O) or with 5 mg/kg of the Rac1 inhibitor, NSC23766 (Tocris Bioscience, Bristol, United Kingdom). A control antibody (clone 54447; R&D Systems, Minneapolis, MN, USA) or a mAb against murine CCL5 (clone 53405; R&D Systems) was injected i.p. (10 μg/mouse), 30 min before CLP induction. Sham mice underwent the same surgical procedures, that is, laparotomy and resuscitation, but the cecum was neither ligated nor punctured. The animals were then returned to their cages and provided food and water ad libitum. Animals were reanesthetized 6 or 24 h after CLP induction. The left lung was ligated and excised for edema measurement. The right lung was used for collecting BALF, in which neutrophils were counted. Next, the lung was perfused with PBS, 1 part was fixed in formaldehyde for histology, and the remaining lung tissue was snap frozen in liquid nitrogen and stored at −80°C for later MPO assays and ELISA, as described subsequently.

MPO assay

Lung tissue was thawed and homogenized in 1 ml 0.5% hexadecyltrimethylammonium bromide. Samples were freeze thawed, after which the MPO activity of the supernatant was determined spectrophotometrically as the MPO-catalyzed 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 U/g tissue.

BALF

Animals were placed supine, and the trachea was exposed by dissection. An angiocatheter was inserted into the trachea. BALF was collected by 5 washes of 1 ml PBS containing 5 mM EDTA. The number of neutrophils was counted in a Burker chamber.

Histology

Lung samples were fixed by immersion in 4% formaldehyde phosphate buffer overnight and then dehydrated and paraffin embedded. Sections (6 μm) were stained with H&E. Lung injury was quantified in a blinded manner by adoption of a modified scoring system [31, 32], including size of alveoli spaces, thickness of alveolar septae, alveolar fibrin deposition, and neutrophil scoring system infiltration graded on a 0 (absent)–4 (extensive) scale. In each tissue sample, 5 random areas were scored, and the mean value was calculated. The histology score is the sum of all 4 parameters.

Lung edema

The left lung was excised and then weighed. The tissue was then dried at 60°C for 72 h and reweighed. The change in the ratio of wet weight:dry weight was used as an indicator of lung edema formation.

ELISA

CXCL1, CXCL2, and CCL5 levels in lung tissue and plasma were analyzed by use of double antibody Quantikine ELISA kits (R&D Systems). Murine rCXCL1, rCXCL2, and rCCL5 were used as standards.

Flow cytometry

For analysis of surface expression of Mac-1, CCR1, CCR5, and CXCR2 on circulating neutrophils, blood was collected (1:10 acid citrate dextrose), 6 h after CLP induction, and incubated with an anti-CD16/CD32 antibody blocking FcγIII/IIRs to reduce nonspecific labeling. Samples were then incubated with PE-conjugated anti-Gr-1 (clone RB6-8C5; eBioscience, Frankfurt, Germany) and FITC-conjugated anti-Mac-1 (clone M1/70; BD Biosciences PharMingen, San Jose, CA, USA) antibodies. Samples were also incubated with a PerCP-Cy5.5-conjugated anti-mouse CXCR2 antibody (clone TG11/CXCR2, rat IgG2a; BioLegend, San Diego, CA, USA), a PE-conjugated anti-CCR1 antibody (clone CTC5; R&D Systems), or a PE-conjugated anti-CCR5 antibody (clone 643854; R&D Systems). Cells were fixed, erythrocytes were lysed, and neutrophils were recovered following centrifugation. Alveolar macrophages were isolated as described below and incubated with an anti-CD16/CD32 antibody blocking FcγIII/IIRs, a PerCP-Cy5.5-conjugated anti-mouse F4/80 antibody (clone BM8; eBioscience), and a FITC-conjugated anti-Ly6G antibody (clone 1A8; BD Biosciences PharMingen), as well as a PE-conjugated anti-CCR1 antibody (clone CTC5) or a PE-conjugated anti-CCR5 antibody (clone 643854). Flow cytometric analysis was performed according to standard settings on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) and analyzed with CellQuest Pro software (BD Biosciences PharMingen). A viable gate was used to exclude dead and fragmented cells.

Alveolar macrophage secretion of CXCL2

BALF was collected as above from healthy mice, and cells were isolated by centrifugation (450 g, 10 min), as described previously [33]. Cells were resuspended in RPMI (Invitrogen, Carlsbad, CA, USA) and adjusted to a concentration of 2×10^5/ml. Cells were then stained with a PE-conjugated anti-F4/80 and a FITC-conjugated anti-mouse Ly-6G antibody, as described above, and the purity of isolated macrophages was determined by flow cytometry. Macrophages were identified as $F4/80^+/Ly6G^-$ cells. Macrophages were coincubated with murine rCCL5 (500 ng/ml) for 4 h (37°C) and then were CXCL2 levels measured by ELISA.

Intratracheal challenge with CCL5

Through an intratracheal catheter, murine rCCL5 (1 μg; PeproTech, Neuilly-Sur-Seine, France) or vehicle was administered into the lungs and neutrophils, and CXCL2 levels were quantified in BALF, 4 h later. Animals were treated i.p. with vehicle or a CXCR2 antagonist (SB225002, 4 mg/kg; Calbiochem, Merck, Darmstadt, Germany) before intratracheal challenge with CCL5. In separate experiments, 100 μl clodronate liposomes or PBS liposomes was administered intratracheally, 24 h before subsequent intratracheal challenge with CCL5. Liposomes were purchased from Encapsula NanoSciences (Brentwood, TN, USA). BALF was collected 4 h after CCL5 administration for quantification of neutrophils and CXCL2 levels.

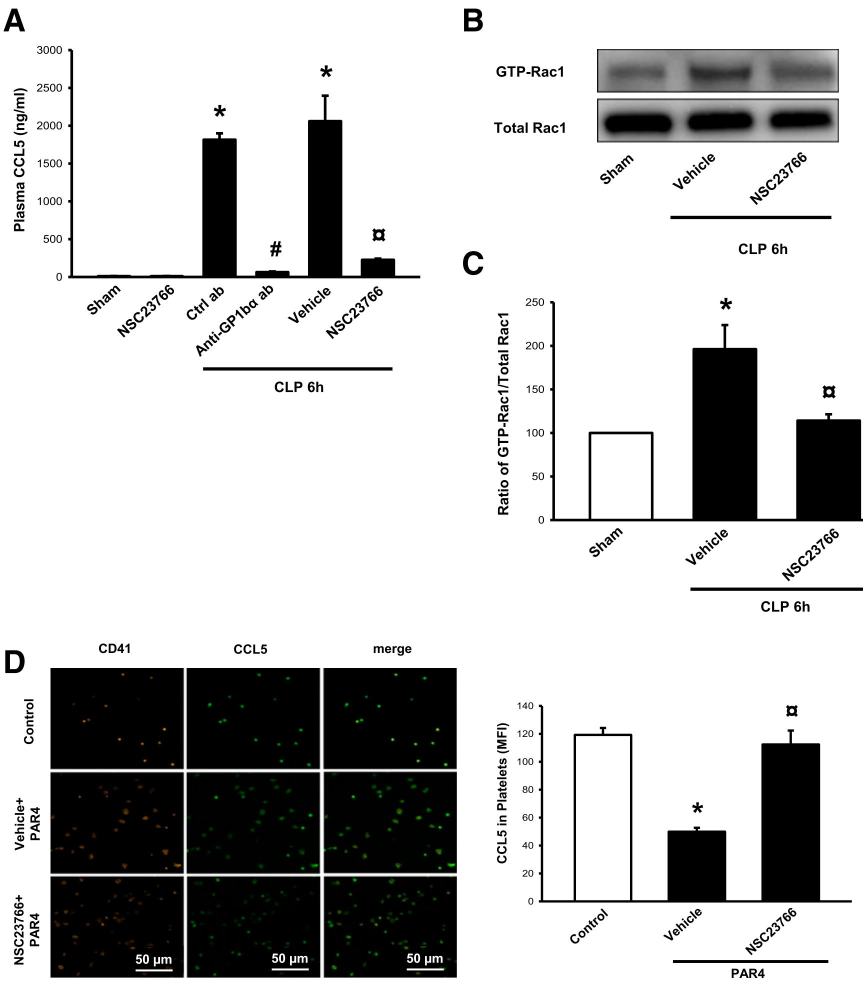

Figure 1. Rac1 regulates platelet secretion of CCL5 in sepsis. Animals were treated with vehicle, NSC23766 (5 mg/kg), a control antibody (Ctrl ab), or an anti-glycoprotein 1bα antibody (GP1bα ab) before CLP induction. (A) ELISA was used to quantify the levels of CCL5 in the plasma, 6 h after CLP induction. (B) Rac1-GTP was determined by Western blotting by use of 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 5 independent experiments. Mice were treated with the Rac1 inhibitor NSC23766 (5 mg/kg) or vehicle before CLP induction. Sham-operated mice served as negative controls. (D) Left, Isolated platelets were incubated with or without NSC23766 (10 μM) and then stimulated with rPAR4 (200 μM), and the level of CCL5 in permeabilized $CD41^+$ platelets was determined by confocal microscopy; Right, Aggregate data showing mean fluorescence intensity (MFI) of CCL5 in platelets. Nonstimulated platelets served as control. Data represent mean ± SEM, and $n = 5$. *$P < 0.05$ versus Sham or Control; #$P < 0.05$ versus Ctrl ab + CLP; open circle in box symbol, $P < 0.05$ versus Vehicle + CLP or Vehicle + PAR4.

Neutrophil chemotaxis

Neutrophils were isolated from bone marrow by use of Ficoll-Paque. Neutrophils (1.5×10^6) were placed in the upper chamber of the Transwell inserts with a pore size of 5 μm (Corning Costar, Corning, NY, USA). Inserts were placed in wells containing medium alone (control) or medium plus CXCL2 (100 ng/ml; R&D Systems) or CCL5 (500 ng/ml). 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.

Platelet isolation and CCL5 secretion

Blood was collected in syringes containing 0.1 ml acid–citrate–dextrose, diluted immediately with equal volumes of modified Tyrode solution (1 μg/ml PGE_1 and 0.1 U/ml 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 1 more time (10,000 g, 5 min) 0.5×10^6 platelets were seeded on a chamber slide coated with fibrinogen (20 μg/ml). Adherent platelets were stimulated with PAR4 (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. Then, platelets were permeabilized with 0.15% Triton X-100 for 15 min, followed by washing and incubation with an anti-CD16/CD32 antibody (10 min) blocking FcγIII/IIRs to reduce nonspecific labeling and a rabbit polyclonal primary antibody against CCL5 (bs-1324R; Bioss, Boston, MA, USA) for 2 h. Chamber slides were washed and incubated with a FITC-conjugated anti-rabbit secondary antibody (Cell Signaling Technology, Beverly, MA, USA) and a platelet-specific, PE-conjugated anti-CD41 antibody (clone MWReg30; eBioscience) for 1 h. Chamber slides are washed 3 times, and confocal microscopy was performed by use of Meta 510 confocal microscopy (Carl Zeiss, Jena, Germany). FITC and PE were excited by 488 nm and 543 nm laser lines, and corresponding emission wavelengths of FITC and PE were collected by the filters of 500–530 nm 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 ZEN 2009 software.

Pull-down assay and Western blotting

Rac1 activity was determined in platelets from sham and CLP mice pretreated with vehicle or NSC23766 by active Rac1 pull-down and detection kit by use of the protein-binding domain of GST-PAK1, which binds with the GTP-bound form of Rac1 (Pierce Biotechnology, Rockford, IL, USA). In brief, platelets were suspended in lysis buffer on ice and centrifuged (16,000 g, 15 min). Ten microliters from each lysate was removed to measure protein content by use of Pierce BCA Protein Assay Reagent (Pierce Biotechnology), and the rest was used for the pull-down assay. Supernatants containing equal amount of proteins were then diluted with 2× SDS sample buffer and boiled for 5 min. Proteins were separated by use of SDS-PAGE (10–12% gel). After transferring to a nitrocellulose membrane (BioRad Laboratories, Hercules, CA, USA), blots were blocked with TBS/Tween 20 containing 3% BSA at room temperature for 1 h, followed by incubation with an anti-Rac1 antibody

(1:1000) at 4°C overnight. The binding of the antibody was detected by use of 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 Laboratories). Total Rac1 was used as a loading control.

Statistics

Data were presented as mean values ± SEM. Statistical evaluations were performed by use of nonparametrical test (Mann-Whitney). $P < 0.05$ was considered significant, and *n* represents the total number of mice in each group. Statistical analysis was performed by use of SigmaPlot 10.0 software (Systat Software, Chicago, IL, USA).

RESULTS

Rac1 regulates platelet secretion of CCL5 in sepsis

CLP increased plasma levels of CCL5 from 12.3 ng/ml in sham mice up to 2075 ng/ml, corresponding to a 169-fold increase (**Fig. 1A**). We found that depletion of platelets abolished the CLP-induced increase in plasma levels of CCL5 (Fig. 1A), suggesting that platelets are the main source of CCL5 in abdominal sepsis. CLP increased Rac1-GTP levels in platelets, indicating that Rac1 is activated in platelets in septic animals (Fig. 1B and C). Notably, administration of the Rac1 inhibitor NSC23766 completely inhibited CLP-evoked Rac1 activation in platelets (Fig. 1B and C), showing that NSC23766 is an effective inhibitor of Rac1 activation. Administration of NSC23766 in control mice had no effect on plasma levels of CCL5 (Fig. 1A). In contrast, treatment with NSC23766 decreased plasma levels of CCL5 in septic mice from 2075 to 236 ng/ml, corresponding to an 89% reduction (Fig. 1A). To determine the direct role of Rac1 in regulating platelet secretion of CCL5, isolated platelets were stimulated with PAR4 in vitro. We observed that CCL5 was present in resting platelets and that stimulation with PAR4 decreased intracellular levels of CCL5 in platelets (Fig. 1D). Notably, coincubation of platelets with NSC23766 prevented PAR4-induced secretion of CCL5 from platelets (Fig. 1D).

CCL5 regulates lung damage in sepsis

Pulmonary edema was determined as changes in lung wet:dry ratio. It was found that the lung wet:dry ratio increased after CLP (**Fig. 2A**). Notably, treatment with an antibody directed against CCL5 decreased the CLP-induced increase in lung wet:dry ratio by >65% (Fig. 2A). CLP caused significant lung damage, typified by severe destruction of pulmonary tissue microstructure, extensive edema of interstitial tissue, and massive infiltration of neutrophils (Fig. 2B). Immunoneutralization of CCL5 reduced CLP-evoked tissue destruction and neutrophil infiltration in the lung (Fig. 2B). Quantification of the morphologic damage showed that CLP markedly increased lung injury score and that inhibition of CCL5 significantly decreased CLP-induced tissue damage in the lung (Fig. 2C).

CCL5 regulates pulmonary recruitment of neutrophils in sepsis

MPO is a useful marker of neutrophils. It was observed that CLP increased pulmonary levels of MPO by 24-fold (**Fig. 3A**). Notably, we found that inhibition of CCL5 function decreased CLP-induced MPO activity in the lung by >47% (Fig. 3A). In addition, CLP induction increased the number of alveolar neutrophils by 21-fold (Fig. 3B). Immunoneutralization of CCL5 reduced the number of alveolar neutrophils by 42% in septic animals (Fig. 3B). Mac-1 is an important adhesion molecule regulating neutrophil adhesion and trafficking [4]. Mac-1 expression was increased on the surface of circulating neutrophils in septic mice (Fig. 3C). However, administration of the anti-CCL5 antibody had no effect of Mac-1 expression on septic neutrophils (Fig. 3C). In contrast to CXCL2, CCL5 exerted no chemotactic effect of isolated neutrophils (Fig. 3D). These findings suggest that CCL5-dependent recruitment of neutrophils is not a direct effect on neutrophils but rather an indirect effect of CCL5. Neutrophil expression of CXCR2 was apparent on neutrophils from sham mice and down-regulated after CLP induction (Fig. 3E). However, we observed that neutrophils from both sham and CLP animals did not express CCR1 nor CCR5 (Fig. 3E).

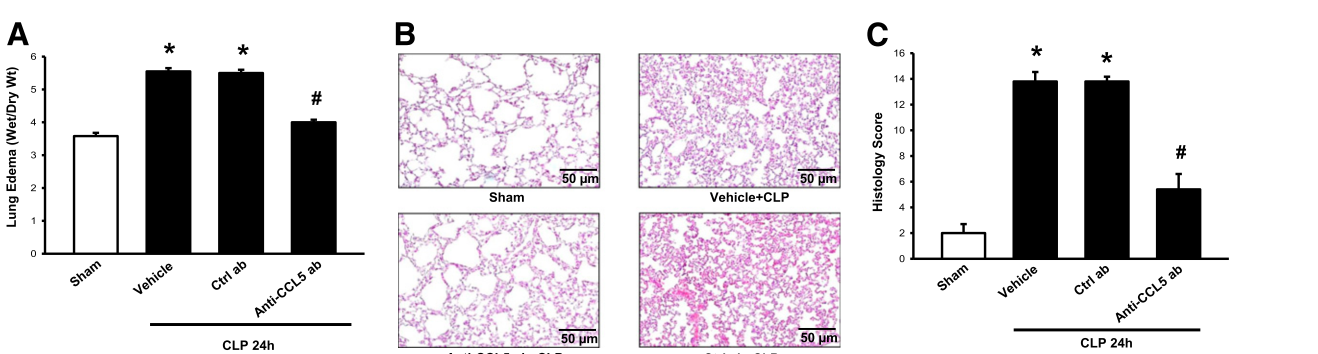

Figure 2. CCL5 regulates lung damage in sepsis. (A) Edema formation in the lung. (B) Representative H&E sections of the lung are shown. Animals were treated with vehicle, a control antibody, or an anti-CCL5 antibody before CLP induction. (C) Lung injury scores, as described in Materials and Methods, 24 h after CLP induction. Sham-operated animals served as negative controls. Data represent mean ± SEM, and $n = 5$. *$P < 0.05$ versus Sham; #$P < 0.05$ versus Ctrl ab + CLP.

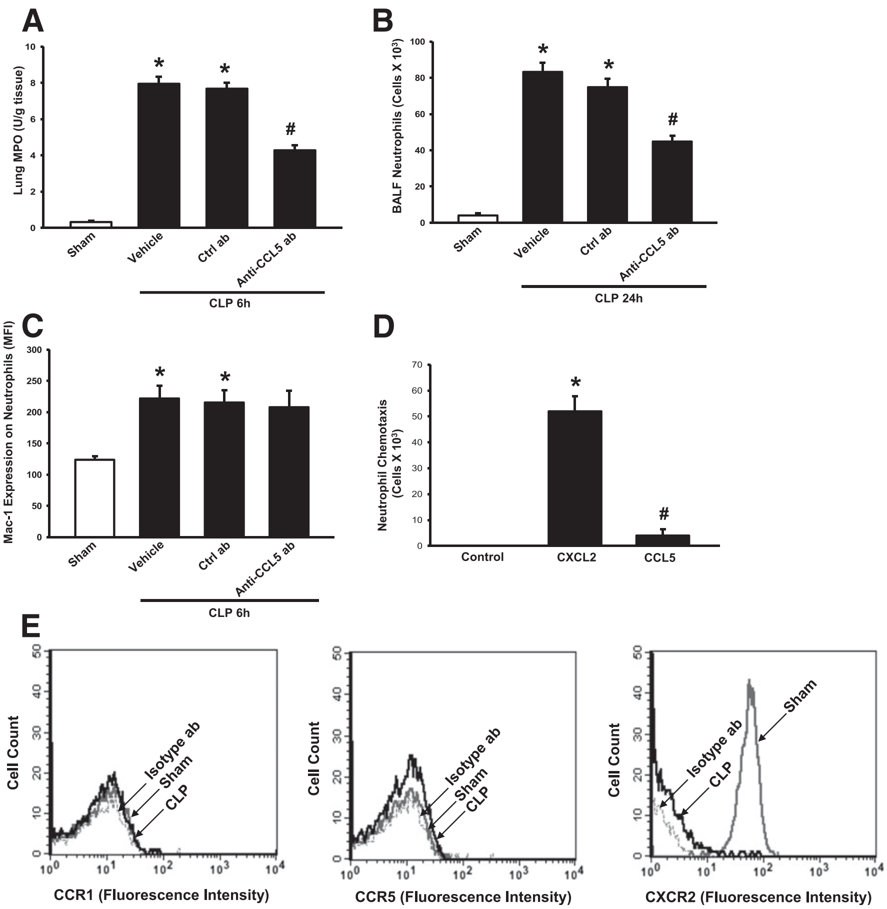

Figure 3. CCL5 regulates pulmonary recruitment of neutrophils in sepsis. (A) Lung MPO levels at 6 h post-CLP. (B) Number of BALF neutrophils, 24 h after CLP induction. (C) Mac-1 expression on circulating neutrophils, 6 h after CLP induction. Animals were treated with vehicle, a control antibody, or an anti-CCL5 antibody before CLP induction. Sham-operated animals served as negative controls. (D) Neutrophils isolated from bone marrow were analyzed for their migration in response to PBS (Control), CXCL2 (100 ng/ml), or CCL5 (500 ng/ml). Nonstimulated neutrophils served as negative control. (E) CCR1, CCR5, and CXCR2 expression on circulating neutrophils in sham and CLP animals. Cells were also stained with an isotype antibody (Isotype ab). Data represent mean ± SEM, and $n = 5$. *$P < 0.05$ versus Sham or Control; #$P < 0.05$ versus Ctrl ab + CLP or CXCL2.

CCL5 regulates CXC chemokine formation in sepsis

CXCL1 and CXCL2 levels in the plasma and lung were low in sham animals (**Fig. 4**). CLP markedly increased CXCL1 and CXCL2 levels in the plasma (Fig. 4A and B). Immunoneutralization of CCL5 reduced CLP-evoked plasma levels of CXCL1 by 81% and CXCL2 by 85% (Fig 4A and B). In addition, CLP enhanced pulmonary levels of CXCL1 and CXCL2 by 76- and 542-fold, respectively (Fig. 4C and D). Inhibition of CCL5 attenuated lung levels of CXCL1 by 87% and CXCL2 by 93% in septic animals (Fig. 4C and D). We next asked whether lung macrophages might be a link between platelet-derived CCL5 and neutrophil recruitment in abdominal sepsis. First, we administered CCL5 locally in the lung by intratracheal infusion and found that local CCL5 challenge significantly increased pulmonary levels of CXCL2 (**Fig. 5A**) and the number of alveolar neutrophils (Fig. 5B). Moreover, administration of the CXCR2 antagonist SB225002 abolished CCL5-induced neutrophil accumulation in the lung (Fig. 5C). Then, we isolated alveolar macrophages and observed that these cells express the CCL5 receptors, i.e., CCR1 and CCR5 (**Fig. 6A**). In addition, we found that coincubation of alveolar macrophages with CCL5 triggered a clear-cut increase in CXCL2 formation (Fig. 6B). This finding was repeated in RAW264.7 macrophages showing that CCL5 challenge caused a significant increase in macrophage secretion of CXCL2 (not shown). Finally, intratracheal administration of clodronate not only depleted animals of alveolar macrophages (Fig. 6C) but also significantly decreased CCL5-induced formation of CXCL2 in the lung (Fig. 6D).

DISCUSSION

Patients with abdominal sepsis pose a significant challenge to clinicians, which is partly a result of an incomplete understanding of the pathophysiology. This study documents an important role of Rac1-dependent secretion of CCL5 from platelets in sepsis. Moreover, our data also delineate the mechanisms regulating CCL5-mediated neutrophil recruitment in septic lung injury. These novel findings help to clarify the role of platelets in sepsis and suggest that the targeting of Rac1 signaling and/or the function of CCL5 might be useful ways to protect lung function in abdominal sepsis.

Numerous studies have pointed to a functional role of platelets in regulating pathologic aspects of the inflammatory response in severe infections [10, 26, 34]. For example, there is evidence in the literature showing that platelets are important for the development of dysfunctional coagulation in sepsis [35]. Moreover, accumulating evidence has demonstrated that platelets are potent regulators of neutrophil accumulation in septic lung damage [10, 26]. One apparent key mechanism is secretion of potent proinflammatory mediators, such as CD40L, harboring

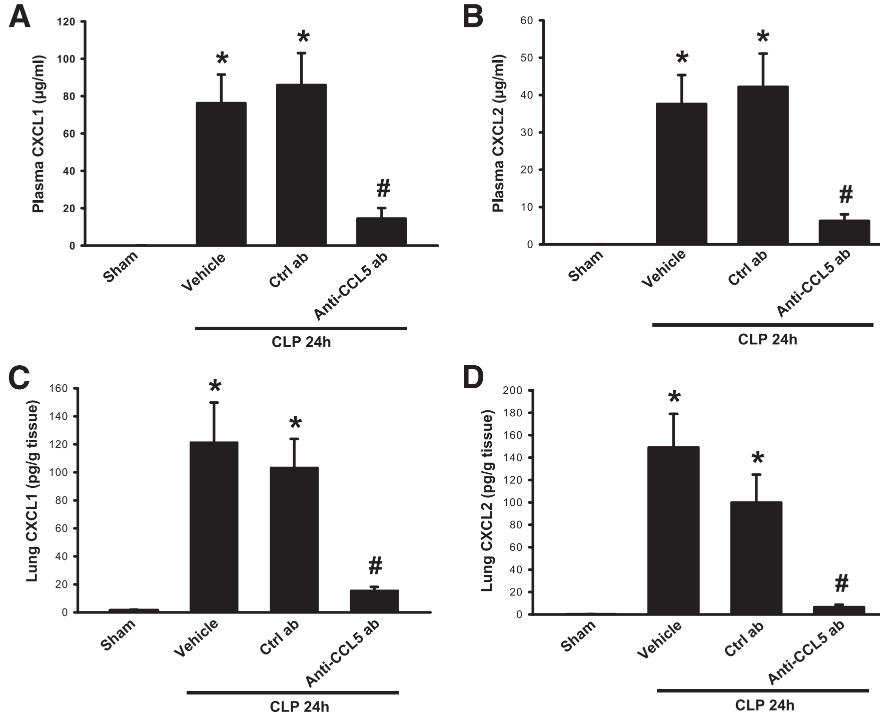

Figure 4. CCL5 regulates CXC chemokine formation in sepsis. Plasma levels of (A) CXCL1 and (B) CXCL2 and lung levels of (C) CXCL1 and (D) CXCL2 determined 24 h after CLP induction. Animals were treated with vehicle, a control antibody, or an anti-CCL5 antibody before CLP. Sham-operated animals served as negative controls. Data represents mean ± SEM, and $n = 5$. *$P < 0.05$ versus Sham; and #$P < 0.05$ versus Ctrl ab + CLP.

in platelets [10, 26]. In this context, it is interesting to note that platelets contain numerous other proinflammatory compounds, such as chemokines [36, 37]. However, the most prevalent chemokines in platelets, i.e., CCL5 and CXCL4, mainly activate lymphocytes, macrophages, and eosinophils and have a low or no direct chemotactic effect on neutrophils [15, 17]. Nonetheless, there are reports in the literature indicating that the targeting of CCL5 can decrease neutrophil recruitment in models of inflammatory diseases [21]. Thus, we first wanted to study the signaling mechanisms regulating platelet secretion of CCL5. It was found that Rac1 activity was enhanced in septic platelets. We next asked whether Rac1 activity might control platelet secretion of CCL5. It was observed that depletion of platelets markedly decreased the sepsis-evoked enhancement of CCL5 levels in plasma, indicating that platelets are the dominating source of circulating CCL5 in abdominal sepsis. Notably, administration of the Rac1 inhibitor NSC23766 abolished the sepsis-induced increase of plasma levels of CCL5, suggesting that Rac1 is a key regulator of circulating levels of CCL5 in sepsis. In addition, NSC23766 completely inhibited PAR4-triggered secretion of CCL5 in isolated platelets in vitro, supporting the conclusion that Rac1 regulates CCL5 secretion from platelets. With the consideration that NSC23766 was recently shown to inhibit agonist-induced mobilization of P-selectin in platelets [28, 38] and that P-selectin and CCL5 are localized in the platelet α-granules [28, 39–41], our results indicate that Rac1 could be involved in the mobilization and secretion of α-granules in platelets. In this context, it is interesting to note that we have recently reported that simvastatin treatment decreases sepsis-provoked pulmonary neutrophilia and tissue injury [42]. With the knowledge that statins prevent isoprenylation of Rho proteins, such as Rac1, which is necessary for their function [43], our present findings

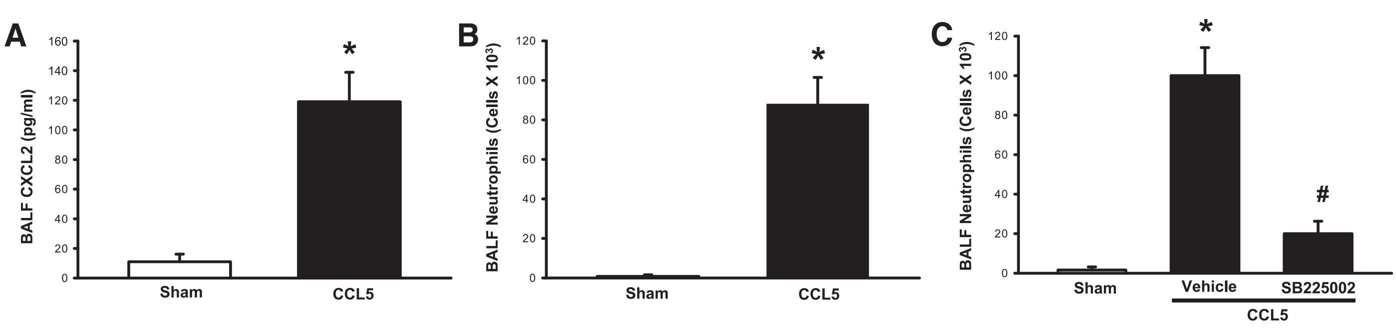

Figure 5. CCL5-induced neutrophil recruitment is dependent on CXCL2 formation. Levels of (A) CXCL2 and (B) number of neutrophils in the lung after intratracheal challenge with CCL5. (C) Neutrophil accumulation in the lungs of animals treated with vehicle or the CXCR2 antagonist SB225002 before intratracheal challenge with CCL5. Data represent mean ± SEM, and $n = 5$. *$P < 0.05$ versus Sham; #$P < 0.05$ versus Vehicle + CCL5.

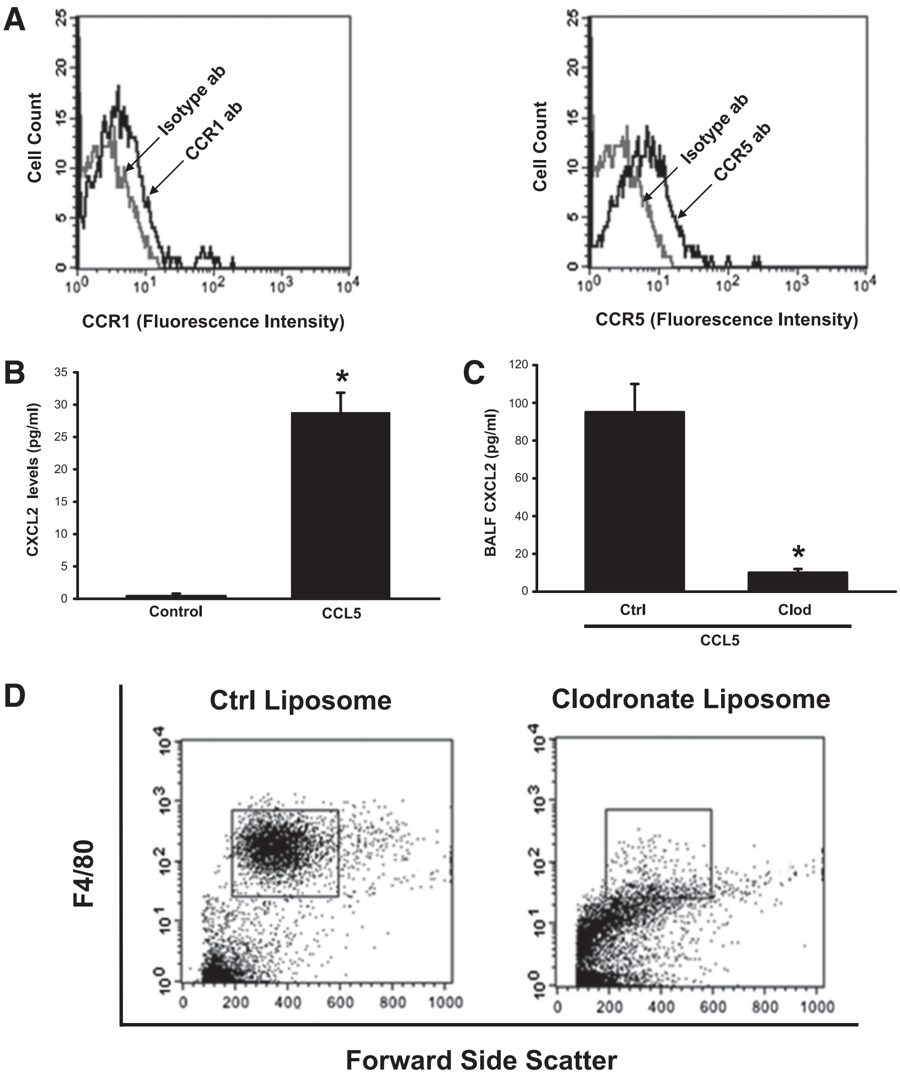

Figure 6. (A) Alveolar macrophage surface expression of CCR1 and CCR5. Isolated alveolar macrophages were stained with antibodies against CCR1 and CCR5, as described in Materials and Methods. (B) Isolated alveolar macrophages were stimulated with CCL5 (500 ng/ml), and the CXCL2 levels in supernatants were determined by use of ELISA. (C) Lung levels of CXCL2 and (D) alveolar macrophages in animals treated intratracheally with a control liposome (Ctrl) or a liposome containing clodronate, as described in Materials and Methods. Data represent mean ± SEM, and $n = 5$. *$P < 0.05$ versus Control or Vehicle + CCL5.

on the role of Rac1 might help explain the protective effects of simvastatin on lung injury in abdominal sepsis.

Sepsis is typified by a generalized activation of the host innate immune system, including neutrophils and macrophages, causing acute lung injury with impaired gaseous exchange, which is the most insidious feature in patients with abdominal sepsis [9, 44]. Herein, we show that immunoneutralization of CCL5 protects against pulmonary edema and tissue damage in septic animals, indicating that CCL5 plays an important role in septic lung injury. This finding extends on previous studies reporting that CCL5 appears to be critical in diseases, such as encephalitis, endotoxemia, stroke, and coronary ischemia [21–24]. Herein, we could show that the targeting of CCL5 function decreased lung levels of MPO, a marker of neutrophils, by >47% in septic mice. This inhibitory effect on MPO correlated well with our finding that immunoneutralization of CCL5 reduced sepsis-induced neutrophil infiltration in the bronchoalveolar space by 42%, suggesting that CCL5 is a potent regulator of neutrophil accumulation in septic lung damage. With the consideration of the close relationship between neutrophil recruitment and pulmonary damage, it might be assumed that the protective effect of targeting CCL5 is a result of the inhibition of pulmonary neutrophilia. Several previous studies have reported that inhibition of CCL5 can decrease neutrophil accumulation in the lung [20, 21], heart [45], colon [46], liver [47], and brain [23], suggesting that CCL5 might control extravascular trafficking of neutrophils in multiple organs. Neutrophils are normally unresponsive to CC chemokines [12, 48]. In this context, it is interesting to note that some previous studies reported that neutrophils stimulated with GM-CSF, TNF-α, and IFN-γ can, under certain circumstances, up-regulate CC chemokine receptors, including CCR1 [18, 19, 49]. Thus, we next asked whether neutrophils up-regulate the CCL5 receptors CCR1 and CCR5 in abdominal sepsis. However, we found that neither CCR1 nor CCR5 was expressed on neutrophils in sham or CLP animals, suggesting that CCL5 regulates neutrophil trafficking in septic lung injury in an indirect manner. This notion is also supported by our findings showing that in contrast to CXCL2, CCL5 exerts no direct chemotactic effect on neutrophils.

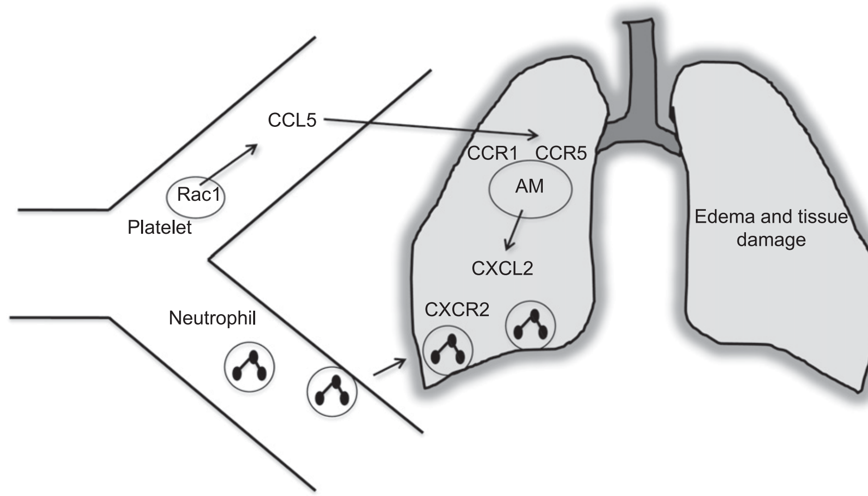

Figure 7. Proposed model for neutrophil recruitment in septic lung damage mediated by platelet-derived CCL5. Abdominal sepsis triggers Rac1 activation and Rac1-dependent secretion of CCL5 from platelets. CCL5 activates alveolar macrophages (AM) expressing CCR1 and CCR5, leading to CXCL2 secretion in the lung. Increased local concentrations of CXCL2 stimulate neutrophil recruitment to the lung, where they cause tissue edema and injury.

Accumulation of neutrophils at extravascular sites of inflammation is a multistep process facilitated by specific adhesion molecules expressed on neutrophils, including CD162 and Mac-1 [4, 50]. Therefore, we examined whether inhibition of CCL5 might control neutrophil activation and expression of Mac-1. However, immunoneutralization of CCL5 had no effect on Mac-1 up-regulation on neutrophils in septic animals, suggesting that CCL5 is not a regulator of Mac-1 expression on neutrophils. Neutrophil trafficking in the extravascular space is orchestrated by secreted CXC chemokines, such as CXCL1 and CXCL2, which are murine homologs of human IL-8 [51]. Indeed, previous studies have documented a functional role of CXC chemokines in abdominal infections [25, 52]. In the present study, we found that the targeting of CCL5 markedly decreased plasma and pulmonary levels of CXC chemokines in septic mice. These findings suggest that CCL5 might regulate neutrophil recruitment in septic lung injury indirectly via formation of CXC chemokines in the lung. This notion is also supported by our findings showing that local intratracheal administration of CCL5 increased formation of CXCL2 and neutrophil infiltration in the lung. In addition, we observed that inhibition of CXCR2 abolished neutrophil accumulation in the lung, triggered by local intratracheal challenge with CCL5, further supporting the concept that CCL5 promotes sepsis-induced neutrophil recruitment in the lung via formation of CXC chemokines. We next asked if alveolar macrophages might be a target cell of CCL5 in the formation of CXCL2 in the lung. We isolated alveolar macrophages from the murine lung and found that these cells express the CCL5 receptors CCR1 and CCR5, which is in line with previous reports [53–55]. Interestingly, we observed that coincubation of alveolar macrophages with CCL5 caused significant secretion of CXCL2, indicating that CCL5 is a potent stimulator of CXCL2 formation in alveolar macrophages. This notion is in line with our observation, demonstrating that intratracheal administration of clodronate not only depleted lungs of alveolar macrophages but also abolished CCL5-evoked generation of CXCL2 in the lung, suggesting that alveolar macrophages are an important target cell of CCL5 in mediating pulmonary formation of CXCL2. Thus, these findings demonstrate how CCL5 indirectly triggers neutrophil recruitment in the lung via alveolar macrophage secretion of CXCL2 in abdominal sepsis.

A schematic representation of the proposed model for neutrophil recruitment in septic lung damage mediated by platelet-derived CCL5 is shown in **Fig. 7**. In summary, these results indicate that Rac1 activity is increased in platelets and regulates platelet secretion of CCL5 in abdominal sepsis. In addition, our findings show that CCL5 regulates neutrophil recruitment in septic lung injury via activation of alveolar macrophages, leading to local secretion of CXCL2. Thus, our novel data not only elucidate complex mechanisms regulating pulmonary neutrophil trafficking in sepsis but also suggest that the targeting of Rac1 signaling and platelet-derived CCL5 might be a useful way to control pathologic inflammation and tissue damage in the lung in abdominal sepsis.

AUTHORSHIP

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

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DISCLOSURES

The authors have no financial conflicts of interest.

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