Proteinase-Activated Receptor-1, CCL2, and CCL7 Regulate Acute Neutrophilic Lung Inflammation

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Abstract

PAR₁ plays a central role in mediating the interplay between coagulation and inflammation, but its role in regulating acute neutrophilic inflammation is unknown. We report that antagonism of PAR₁ was highly effective at reducing acute neutrophil accumulation in a mouse model of LPS-induced lung inflammation. PAR₁ antagonism also reduced alveolar–capillary barrier disruption in these mice. This protection was associated with a reduction in the expression of the chemokines, CCL2 and CCL7, but not the proinflammatory cytokines, TNF and IL-6, or the classic neutrophil chemoattractants, CXCL1 and CXCL2. Antibody neutralization of CCL2 and CCL7 significantly reduced LPS-induced total leukocyte and neutrophil accumulation, recovered from the bronchoalveolar lavage fluid of challenged mice. Immunohistochemical analysis revealed that CCL2 predominantly localized to alveolar macrophages and pulmonary epithelial cells, whereas CCL7 was restricted to the

pulmonary epithelium. In keeping with these observations, the intranasal administration of recombinant CCL2 (rCCL2) and rCCL7 led to the accumulation of neutrophils within the lung airspaces of naive mice in the absence of any underlying inflammation. Flow cytometry analysis further demonstrated an increase in Ly6G^{hi} neutrophils expressing the chemokine receptors, CCR1 and CCR2, isolated from mouse lungs compared with circulating neutrophils. Conversely, the expression of CXCR2 decreased on neutrophils isolated from the lung compared with circulating neutrophils. Furthermore, this switch in chemokine receptor expression was accentuated after acute LPS-induced lung inflammation. Collectively, these findings reveal a novel role for PAR₁ and the chemokines, CCL2 and CCL7, during the early events of acute neutrophilic inflammation.

Keywords: inflammation; lung; neutrophil; chemokine; proteinase-activated receptor-1

The early stage of acute inflammation is usually associated with an influx of neutrophils into the injured tissue. Although this rapid innate immune response provides immediate host protection against infectious micro-organisms, excessive leukocyte accumulation can lead to over-exuberant inflammation and tissue damage (1). Understanding the factors that control the early accumulation of neutrophils is critical in the context of host defense and for the

control of immunopathology associated with lung diseases, such as acute respiratory distress syndrome (ARDS).

The lung epithelium functions as a mucosal barrier that prevents the entry of noxious substances from the environment and performs the essential function of gaseous exchange. It is therefore vital that pulmonary epithelial integrity is maintained to sustain these essential physiological functions. If this barrier is compromised,

a complex series of pathways are initiated, involving the interplay between inflammation and coagulation, which provides host defense against infection and promotes wound healing and the maintenance of the endothelial–epithelial barrier (2). This process is highly complex and involves many factors, including cytokines, chemokines, growth factors, cell adhesion molecules, and coagulation proteins.

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Many of the cellular effects of coagulation proteinases are mediated via activation of the proteinase-activated receptors (PARs), which are a family of G protein-coupled receptors comprised of four members (PAR₁₋₄). Evidence obtained from biochemical studies and from knockout mice suggests a key role for the major high-affinity thrombin receptor, PAR₁, in mediating the complex interplay between coagulation and inflammation in response to lung injury (2–5). It is known that PAR₁ regulates vascular integrity to systemic inflammation and modulates the release of proinflammatory cytokines (6). This may involve complex interactions between various proteinases, such as thrombin and matrix metalloproteinases, as well as inflammatory mediators. Activation of PAR₁ on epithelial cells, monocytes/macrophages, and vascular endothelial cells leads to the release of proinflammatory mediators, including the cytokines, TNF, IL-1 β , IL-2, and IL-6, and the chemokines, CXCL8 (IL-8) and CCL2 (monocyte chemoattractant protein-1 [MCP-1]) (2), mediators that have been directly associated with neutrophil activation, migration, and the pathogenesis of ARDS (7, 8). Indeed, the alveolar-capillary barrier disruption associated with lung injury has been attributed to the immediate influx of neutrophils into the airspaces (9).

Neutrophils are constantly trafficking through the pulmonary circulation, and are rapidly recruited into the lung in response to multiple inflammatory mediators, of which the chemokine, CXCL8 (IL-8), and the functional mouse homologs, CXCL1 (KC) and CXCL2 (macrophage inflammatory protein-2 α [MIP-2 α]), are considered the most potent chemoattractants for neutrophil extravasation across endothelial and epithelial surfaces (1). Elevated levels of CXCL8 have also been associated with several inflammatory diseases, including ARDS (10). However, the contribution of other chemokines to neutrophil recruitment to the lung is less clear, although several studies have recently implicated CCL2 in this process (11, 12). CCL2, and the related chemokine, CCL7, are predominantly associated with the egress of monocytes out of the bone marrow in response to proinflammatory stimuli (13–15), and the recruitment of monocytes and macrophages into peripheral tissue during episodes of

inflammation or infectious disease (16, 17). However, it is uncertain if these CC chemokines contribute to the early migration of neutrophils, in response to direct lung injury, or whether the lung affords unconventional chemotactic properties on migrating neutrophils.

We now demonstrate that inhibiting PAR₁ using a selective PAR₁ antagonist dampens the accumulation of neutrophils into alveolar spaces and reduces alveolar leak after LPS-induced inflammation. Furthermore, antagonism of PAR₁ attenuated the expression of the CC chemokines, CCL2 and CCL7, rather than mediators classically associated with neutrophil migration. Importantly, neutrophil recruitment into inflamed lung airspaces was significantly decreased by antibody neutralization of CCL2 and CCL7, demonstrating that both these chemokines play a key role in the trafficking of neutrophils into the inflamed lung. Immunohistochemical analysis revealed that both CCL2 and CCL7 predominantly immunolocalized to the pulmonary epithelium, although CCL2 also localized to the endothelium and alveolar macrophages. The instillation of recombinant CCL2 (rCCL2) or rCCL7 further confirmed a role for these chemokines in the recruitment of neutrophils into the lung. Moreover, CCL7 had a preferential effect on neutrophil accumulation compared with CCL2. Indeed, neutrophils isolated from lung tissue were found to express the chemokine receptors CCR1, CCR2, and CCR3, whereas CCR1 and CCR2 expression was further enhanced during inflammation. Taken together, these findings have important implications for our understanding of the interaction between coagulation and CC chemokines during acute neutrophilic inflammation.

Materials and Methods

Pulmonary Inflammation Model

Experiments were conducted with local ethical approval in accordance with the Home Office, UK. Female Balb/c mice (6–8 wk old; Charles River, Margate, UK) were anesthetized (5% isoflurane) and challenged with LPS in sterile saline (125 μ g/kg, 50 μ l intranasally; *Escherichia coli* 0127:B8; Sigma Aldrich, Gillingham, UK). After 3 hours, animals were killed

(urethane, intraperitoneally, 20 g/kg), and bronchoalveolar lavage (BAL) performed (3 $\times$ 0.5 ml to a total of 1.5 ml PBS). Total and differential cell counts were quantified after cytopspin. Alternatively, BAL fluid was isolated and whole lungs were removed and homogenized.

Treatment Protocols

Mice were injected intraperitoneally with the specific PAR₁ antagonist, (α S)-N-[(1S)-3-amino-1-[[[(phenylmethyl)amino]carbonyl]propyl]- α -[[[1-(2,6-dichlorophenyl)methyl]-3-(1-pyrrolidinylmethyl)-1H-indazol-6-yl]amino]carbonyl]amino]-3,4-difluorobenzene propanamide RWJ 58259 (5 mg/kg; a kind gift from Claudia Derian, Johnson & Johnson Pharmaceutical Research and Development, Spring House, PA), 30 minutes after LPS administration. This antagonist is highly specific for PAR₁, and does not interact with other PARs or G protein-coupled receptors. Formulations were used as described previously (18, 19). The specificity, pharmacological characteristics, and optimal dosing regimen have been previously described (19–21), and the biological properties of the compound recently reviewed (22). In other experiments, mice received 10 μ g (intranasally) anti-CCL2 (R&D Systems, Abingdon, UK), anti-CCL7 (Peprotech, London, UK), anti-CXCL10 (R&D Systems), or anti-CXCL1 (R&D Systems)-specific neutralizing antibodies at the same time as LPS (intranasal). BAL fluid was analyzed at 3 hours. rCCL2 or rCCL7 (both from Peprotech) were administered to Balb/c mice (500 ng/mouse intranasally) in sterile PBS. BAL fluid was analyzed at 3 hours as described previously here.

Low Density Array PCR Analysis

Total RNA was extracted from pulverized, frozen lung using TRIzol (see manufacturer's protocol (Invitrogen, Life Technologies, Paisley, UK), DNase treated using a DNA free kit (Ambion, Life Technologies), and cDNA synthesized from 1 μ g RNA per sample using a Superscript kit (Invitrogen). Expression levels of known inflammatory mediators were analyzed in cDNA using Taqman low-density array PCR chips and normalized to 18s RNA. Relative differences in expression were calculated using the Δ CT method.

Myeloperoxidase, Chemokine, and Protease Measurement

Lung tissue homogenates were prepared as described previously (23). Briefly, frozen lung powder was mixed with PBS (10% wt/vol) containing protease inhibitors (Complete Mini; Roche Diagnostics, West Sussex, UK). Samples were homogenized on ice (Eppendorf, Stevenage, UK), centrifuged ($16,000 \times g$, 4°C , 15 min), and myeloperoxidase measured by ELISA (Hycult Biotechnology, Plymouth Meeting, PA). Serum albumin levels were measured by ELISA (Bethyl Laboratories, Montgomery, TX). Chemokine levels in supernatants were measured by Luminex. IFN- γ , CCL2, CCL7, and IL-8 were also measured by ELISA (PeproTech). Thrombin-anti-thrombin (TAT) levels were measured by ELISA (EnzymeResearch, South Bend, IN). Activated protein C (APC), matrix metalloproteinase (MMP)-1, MMP-2, and neutrophil elastase 2 were also measured by ELISA (Antibodies Online, Atlanta, GA). Receptor for advanced glycation endproducts was measured by ELISA (Abcam).

Immunohistochemical Detection of CCL2, CCL7, and PAR₁

Briefly, mouse lungs were inflated with 4% formaldehyde, dehydrated through an alcohol gradient, and embedded in paraffin wax. Serial sections ($4 \mu\text{m}$) were dewaxed and antigens unmasked by microwaving sections with 10 mM sodium citrate buffer (pH 6.0). Endogenous peroxide activity was blocked with 3% hydrogen peroxide, and sections were blocked with 3% horse serum with 1% BSA in PBS for goat primary antibodies, and with 3% goat serum with 1% BSA in PBS for rabbit to primary antibodies. Sections were incubated for 16 hours at 4°C with primary antibodies, anti-CCL2 (0.4 $\mu\text{g}/\text{ml}$; Santa Cruz Biotechnology, Dallas, TX), anti-CCL7 (1 $\mu\text{g}/\text{ml}$; PeproTech), and anti-PAR₁ (2 $\mu\text{g}/\text{ml}$; Santa Cruz Biotechnology). Goat and rabbit IgG controls (Vector Laboratories, Peterborough, UK) were included as negative control. Sections were incubated for 30 minutes with biotin-labeled anti-goat or anti-rabbit secondary antibodies (Vector Laboratories), followed by 30-minute incubation with ABC complex (Vector Laboratories). Color was developed with diaminobenzidine (DAB) chromogen

(BioGenex, Fremont, CA) for 5 minutes. Sections were counterstained with hematoxylin, dehydrated, and permanently mounted. Slides were digitally scanned using a NanoZoomer (Hamamatsu, Welwyn Garden City, UK) at the equivalent of $\times 200$ magnification.

Analysis of CCRs by Flow Cytometry

Blood was isolated in heparinized tubes from naive and LPS-challenged mice. Lung was isolated and homogenized as previously described. Both tissues underwent red blood cell lysis (Roche Diagnostics). Leukocytes were stained with anti-Ly6G-PE monoclonal antibody (clone IA8; BD Biosciences, Oxford, UK), anti-CCR1-FITC, anti-CCR2-APC, and anti-CCR3-PerCP, or anti-CXCR2-PerCP (all from AbDSetotec, Kidlington, UK). Neutrophils (Ly6G^{high}) were specifically gated on FSc-Ly6G^{high}, and the percentage of neutrophils expressing each chemokine receptor calculated. Isotype controls, rat anti-mouse IgG (BD Biosciences), and fluorescence minus one controls (antibodies as stated above) were used to set compensations and gating strategies.

Statistical Analysis

Statistical analysis was performed using Graphpad Prism software (Graphpad, La Jolla, CA). Comparison of more than two groups was evaluated by two-way ANOVA. Real-time PCR data were analyzed by the ΔCT method. Differences in cytokine or chemokine mRNA expression, protein release, or cell number between treatment groups was evaluated by one-way ANOVA with Newman-Keuls method for *post hoc* pair-wise comparisons, by Mann-Whitney *t* test, or unpaired *t* test with Welch's correction. A *P* value of less than 0.05 was considered significant.

Results

PAR₁ Signaling Contributes to Acute Lung Inflammation

To determine the role of PAR₁ in the early stages of acute lung inflammation, we evaluated the effect of a potent and highly selective PAR₁ antagonist (RWJ58259) after intranasal challenge with LPS (125 $\mu\text{g}/\text{kg}$). The PAR₁ antagonist was administered (5 mg/kg RWJ58259, intraperitoneally) 30 minutes after LPS challenge, the dose chosen on the basis of previous reports of

effective *in vivo* PAR₁ inhibition at this concentration (20, 21). As expected, LPS caused a significant increase in total cell and neutrophil recruitment into alveolar spaces after 3 hours (Figures 1A and 1B), events that are indicative of the early stages of acute inflammation (24). Antagonism of PAR₁ 30 minutes after the onset of lung inflammation significantly decreased total cell and neutrophil numbers recruited to the airspaces (Figures 1A and 1B). Macrophage numbers in BAL fluid remained unchanged in all treatment groups, indicating that PAR₁ antagonism did not affect early macrophage recruitment (Figure 1C). The effect of PAR₁ antagonism on LPS-induced neutrophil recruitment was independently verified by flow cytometry, which demonstrated a decrease in Ly6G⁺ neutrophils recovered from BAL fluid and from whole-lung homogenates (Figure 1D). Furthermore, PAR₁ antagonism resulted in decreased myeloperoxidase levels in lung homogenates (Figure 1E). To examine the effect of PAR₁ antagonism on LPS-induced disruption of the alveolar-capillary barrier, serum albumin levels were measured in BAL fluid recovered from saline- and LPS-challenged mice. Serum albumin levels were increased in the BAL fluid of LPS-challenged mice, and were significantly decreased (*P* = 0.04) after PAR₁ antagonism (Figure 1F). These data, therefore, demonstrate that PAR₁ signaling influences early neutrophilic inflammation and promotes alveolar-capillary barrier disruption in a model of acute lung inflammation.

To assess the effect of PAR₁ antagonism on the subsequent progression of neutrophilic inflammation, mice were challenged with LPS (125 $\mu\text{g}/\text{kg}$, intranasally) followed by RWJ58259 treatment 30 minutes thereafter. Mice were then killed at 6 and 24 hours after LPS challenge, and the number of BAL fluid neutrophils assessed. A single dose of PAR₁ antagonist, given 30 minutes after LPS challenge, reduced neutrophil accumulation at 6 and 24 hours (see Figure E1 in the online supplement). To determine the cellular and molecular mechanisms underlying this inflammatory process, we focused on events occurring 3 hours after LPS challenge.

The proinflammatory effects of PAR₁ signaling are mediated by the coagulation proteinase, thrombin, whereas

Figure 1. Proteinase-activated receptor (PAR) 1 antagonist treatment significantly attenuates LPS-induced lung injury. Mice were killed 3 hours after LPS (125 μ g/kg intranasally) or saline challenge with or without the highly selective PAR₁ antagonist, RWJ 58259 (5 mg/kg), dosed therapeutically (intraperitoneally) after 30 minutes. Lungs were lavaged (1.5 ml PBS total) or removed and homogenized for FACS analysis. Total (A) and differential bronchoalveolar lavage (BAL) fluid neutrophils (B) were quantified by hemocytometer and cytopsin counts. BAL fluid macrophages were also differentially counted from cytopsin preparations (C). Ly6G⁺ neutrophils (Ly6G^{high} F4/80^{low/neg}) isolated from BAL fluid or lung homogenates were further assessed by flow cytometry (D). Neutrophil myeloperoxidase (MPO) activity in lung homogenates was assessed by ELISA (E). Alveolar-capillary barrier permeability was measured as serum albumin in BAL fluid by ELISA (F). For each treatment a mean value of at least $n = 5$ per group, from three separate experiments, are shown. Data were analyzed by one-way ANOVA with Newman-Keuls *post hoc* test. *** $P < 0.0001$, ** $P < 0.01$, * $P < 0.05$.

cytoprotective effects are mediated by APC bound to its endothelial receptor, endothelial protein C receptor (25). To determine the relative influence of thrombin versus APC after LPS-induced lung injury, we measured TAT complexes and APC in the BAL fluid and lung homogenates of control and LPS-treated mice. A significant elevation of TAT was observed in the BAL fluid after LPS-challenge, but no increase in APC was observed (Figure E2). To rule out any effects of alternative proteinases on PAR₁ activation, the matrix metalloproteinases, MMP-1 and MMP-2, and neutrophil elastase 2 were measured. No differences in the expression levels of any of these proteinases were detected after LPS challenge (Figure E2). In addition, no difference in the levels of the receptor for advanced glycation endproducts was detected, suggesting that epithelial cell death does not contribute to the inflammatory response during the acute phase (Figure E2).

PAR₁ Signaling Influences the Expression of Multiple Inflammatory Mediators

To investigate the mechanism by which PAR₁ signaling influences acute lung inflammation, we next examined the effect of LPS challenge and subsequent PAR₁ antagonism on proinflammatory cytokine and neutrophil-specific chemokine levels in lung homogenates. To identify potential PAR₁-regulated cytokines and chemokines, we used a low-density RT-PCR array to enable the profiling of 91 inflammatory mediators (Table E1). Of the 91 genes analyzed, 31 genes were differentially expressed in lung tissue after challenge with LPS compared with saline treatment. The differential gene expression profile included the up-regulation of several genes known to be important for the generation of inflammatory responses, such as TNF, ILs, CXC chemokines, and CC chemokines. Further analysis revealed that 13 genes exhibited decreased expression after PAR₁ antagonism (Table E1).

However, PAR₁ antagonism did not attenuate the expression of the conventional proinflammatory cytokines, TNF and IL-6 (Figures 2A and 2B), or the CXCR2 ligands, CXCL1 and CXCL2 (Figures 2C and 2D), suggesting that these cytokines/chemokines are not regulated downstream of PAR₁ signaling in this model of LPS-induced lung inflammation.

Of those genes up-regulated after LPS challenge, PAR₁ antagonist treatment decreased the expression of several CC and CXC chemokines, ILs, and TNF-related factors. Importantly, two closely related CC chemokines, CCL2 and CCL7, were decreased after treatment with the PAR₁ antagonist (Figures 2E and 2F). These chemokines are known to induce the egress of monocytes from the bone marrow and recruit monocytes/macrophages into inflamed tissue (14, 15). However, these chemokines are not generally considered to be major chemoattractants for neutrophils.

To confirm the low density array (LDA) expression analysis, we measured

Figure 2. Low-density array analysis of inflammatory markers after PAR1 antagonism. Mice were killed 3 hours after LPS (125 μ g/kg intranasally) or saline challenge with or without the highly selective PAR₁ antagonist, RWJ 58259 (5 mg/kg), dosed therapeutically (intraperitoneally) after 30 minutes. Lungs were removed, snap frozen, and homogenized under liquid nitrogen before RNA was isolated and run on a low-density gene array consisting of 91 inflammatory markers (Table E1). Gene expression after LPS treatment revealed 25 markers that exhibited reduced expression after PAR₁ antagonism. Of these genes, the proinflammatory cytokines, TNF (A) and IL-6 (B), and the neutrophil chemoattractants, CXCL1 (C) and CXCL2 (D), are depicted. In addition, the chemokines, CCL2 (E) and CCL7 (F), were measured. For each treatment a mean value of at least $n = 5$ per group, from three separate experiments, are shown. Data were analyzed by one-way ANOVA with Newman-Keuls *post hoc* test. * $P < 0.05$.

protein levels in lung homogenates. Treatment with LPS significantly increased the expression of TNF, IL-6, CXCL1, and CXCL2 (Figures 3A–3D), but the expression of these proteins was not affected by PAR₁ antagonist treatment. Similarly, LPS challenge increased the expression of CCL2 and CCL7 (Figures 3E and 3F), and, as observed in the LDA RT-PCR analysis, PAR₁ antagonist treatment also decreased the expression of these

chemokines at the protein level. These data provide strong support for the notion that PAR₁ plays a role in regulating CCL2 and CCL7 expression after LPS-induced lung inflammation.

CCL2 and CCL7 Contribute to Leukocyte Accumulation after LPS Challenge

To examine the potential roles of CCL2 and CCL7 in LPS-induced lung inflammation,

we used specific neutralizing antibodies to block these chemokines. Treatment with the CCL2- or CCL7-neutralizing antibodies reduced respective chemokines to basal levels at 3 hours (Figures 4A and 4B), and therefore confirmed effective target engagement. Administration of anti-CCL2 antibody significantly decreased both the total cell number and the number of neutrophils isolated from BAL fluid after challenge with LPS (Figures 4C and 4D).

Figure 3. PAR₁ antagonist treatment decreases CCL2 and CCL7 expression. Mice were killed 3 hours after LPS (125 μ g/kg intranasally) or saline challenge with or without the PAR₁ antagonist, RWJ 58259 (5 mg/kg), dosed intraperitoneally after 30 minutes. Lungs were removed and homogenized. Levels of TNF (A), IL-6 (B), CXCL1 (C), and CXCL2 (D) protein were measured in lung homogenates using a Luminex bead array. Protein levels of CCL2 (E) and CCL7 (F) were measured from lung homogenates by ELISA. For each treatment a mean value of at least $n = 5$ per group, from three separate experiments, are shown. Data were analyzed by one-way ANOVA with Newman-Keuls *post hoc* test. *** $P < 0.001$, ** $P < 0.01$.

Neutralization of CCL2 did not decrease the number of macrophages isolated from the BAL fluid after LPS challenge (Figure 4E). Administration of anti-CCL7 antibody also significantly reduced total cell and neutrophil accumulation into airspaces after LPS challenge (Figures 4F and 4G). However, neutralization of CCL7 did not affect the number of macrophages (Figure 4H). Taken together, these data led us to conclude that both CCL2 and CCL7

influence early neutrophil accumulation into the inflamed lung.

Because LDA PCR analysis indicated that the LPS-induced chemokines, CXCL10 and CX3CL1, may be responsive to treatment with the PAR₁ antagonist (Table E1), *in vivo* neutralization experiments were also performed with antibodies to CXCL10 or CX3CL1. No decrease in neutrophil accumulation was observed after neutralization of these

chemokines (Figure E3), suggesting that these chemokines are not directly involved in neutrophil migration into the LPS-inflamed lung.

CCL2, CCL7, and PAR₁ Immunolocalization in the Inflamed Lung

The observation that neutrophil migration downstream of PAR₁ activation was mediated by the nonclassical neutrophil

Figure 4. CC chemokines influence early leukocyte accumulation in response to LPS challenge. Mice were killed 3 hours after LPS (125 μ g/kg intranasally) or saline challenge. Mice were administered anti-CCL2- or anti-CCL7-neutralizing antibody (10 μ g/mouse), or control IgG, within the nasal challenge volume. Lungs were removed, homogenized, and CCL2 (A) and CCL7 (B) levels measured by ELISA after anti-CCL2 or anti-CCL7 antibody treatment. Lungs were lavaged and BAL fluid total cells (C), neutrophils (D), and macrophages (E) quantified after administration of anti-CCL2. After anti-CCL7 treatment, lungs were lavaged and BAL fluid total cells (F), neutrophils (G), and macrophages (H) quantified. Mean and SEM values of at least $n = 5$ per group for two separate experiments are shown for each treatment. Data were analyzed by one-way ANOVA with Newman-Keuls *post hoc* test. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. n.s., not significant.

chemokines, CCL2 and CCL7, was unexpected. To determine the cellular source of these chemokines, we next examined the immunolocalization of CCL2 and CCL7 in serial lung sections from saline-treated and LPS-challenged mice. Weak CCL2 staining was detected in lung sections from saline-treated animals (Figure 5A). However, after LPS treatment, there was increased immunolocalization of CCL2 in bronchial epithelial cells, alveolar macrophages, and endothelial cells, and, to

a lesser extent, alveolar epithelial cells (Figures 5B and 5C). Similarly, there was weak CCL7 staining in saline-treated control lung, which was mainly restricted to the bronchial epithelium (Figure 5D), whereas CCL7 immunostaining of bronchial epithelial cells was markedly increased in response to LPS injury (Figures 5E and 5F). However, little immunolocalization of CCL7 was detected in endothelial cells, alveolar epithelial cells, or alveolar macrophages, suggesting that

CCL2 and CCL7 are expressed within different lung microcompartments. Immunolocalization of PAR₁ demonstrated differential expression in lung microenvironments, including the bronchial epithelium and alveolar macrophages in saline-treated mouse lung (Figure 5G). An increase in PAR₁ staining was detected in bronchial epithelial cells, alveolar macrophages, and endothelial cells after LPS challenge (Figures 5H and 5I).

Figure 5. Immunohistochemical analysis of CCL2, CCL7, and PAR₁ expression in the lungs of LPS-treated mice. Mice were killed 3 hours after LPS (125 μ g/kg intranasally) or saline challenge. Lungs were inflated with 4% formaldehyde and serial paraffin embedded sections (4 μ m) cut. Immunohistochemistry was performed on each section to detect CCL2 (A–C), CCL7 (D–F), or PAR₁ (G–I). Images were digitally captured using a NanoZoomer (Hamamatsu) at the equivalent of $\times 200$ magnification. High-magnification images were taken of LPS-treated lungs equivalent to $\times 400$ magnification (C, F, and I). Images are representative of five mice per treatment group.

To gain an understanding of the relative contributions of PAR₁ and LPS signaling to the release of CCL2 and CCL7, bronchial epithelial cells (Beas2Bs) and monocytes (THP-1) were stimulated with thrombin, LPS, or a combination of the two. Thrombin and LPS induced the release of CCL2 from bronchial epithelial cells, whereas only LPS stimulated the release of CCL2 from THP-1 cells. Neither thrombin nor LPS stimulated the release of CCL7 from either bronchial epithelial or monocytic cell lines (Figure E4). In contrast, only thrombin stimulation induced the release of IL-8 from epithelial cells, whereas only LPS induced the expression of IL-8 in monocytes (Figure E4). Considering that thrombin and LPS were unable to stimulate CCL7 release, whereas CCL7 immunolocalized to the pulmonary epithelium, we next tested the effects of the classical inflammatory mediators, TNF, IFN- γ , or a combination

of TNF and IFN- γ (cytomix) on CCL2 and CCL7 release from epithelial cells. Both TNF and IFN- γ readily induced the release of CCL2 (Figure E5). In contrast, only a combination of TNF and IFN- γ stimulation caused a substantial release of CCL7, whereas IFN- γ alone induced the release of a small, but significant, amount of CCL7 (Figure E5). To assess the role of IFN- γ downstream of PAR₁ *in vivo*, mice were challenged with LPS and treated with the PAR₁ antagonist, RWJ58259, 30 minutes later. However, PAR₁ antagonism had no effect on IFN- γ expression in either BAL fluid or whole-lung tissues.

Neutrophils Migrate into Lung Airspaces in Response to CCL2 and CCL7

To determine whether CCL2 and CCL7 are able to directly recruit leukocytes into the lung, we next administered rCCL2 or rCCL7 into the lungs of naive mice and sampled the

BAL fluid after 3 hours. Direct instillation of either rCCL2 or rCCL7 increased the total cell number recovered from BAL fluid compared with saline-treated controls (Figure 6A). The magnitude of the response was greater for rCCL2 compared with rCCL7. Differential cell counts revealed that administration of rCCL2 or rCCL7 resulted in the recruitment of neutrophils into lung airspaces (Figure 6B). When expressed as a percentage of total cells recovered from BAL fluid, the data revealed that rCCL7 promoted a preferential accumulation of neutrophils compared with rCCL2 (Figure 6C), although total neutrophil numbers were similar. Furthermore, rCCL2 induced the recruitment of greater numbers of monocytes/macrophages into the BAL fluid compared with rCCL7 (Figures 6D and 6E). Taken together, these data reveal that CCL2 and CCL7 attract neutrophils into the lung in the absence of any underlying inflammation, and that CCL7

Figure 6. CCL2 and CCL7 induce an influx of leukocytes into the BAL fluid. Naive mice were administered with either rCCL2 or rCCL7 (500 ng/mouse, intranasally) and 3 hours later BAL fluid was recovered. BAL fluid total cell counts (A) were measured after rCCL2 and rCCL7 treatment. Differential cell counts were performed on isolated BALF by cytopsin. The total number of neutrophils (B) and the percentage of neutrophils (C) in BAL fluid were calculated. In addition, the total number of macrophages (D) and the percentage of macrophages (E) in BAL fluid were calculated. Data were analyzed by one-way ANOVA with Newman-Keuls *post hoc* test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

preferentially promotes the recruitment of neutrophils compared with CCL2, whereas CCL2 recruits high levels of both macrophages and neutrophils.

Neutrophils Express CC Chemokine Receptors in the Inflamed Lung

To assess the capacity of neutrophils to respond to CC chemokines, we next assessed the expression of the known CC chemokine receptors, CCR1, CCR2, and CCR3, by neutrophils isolated from the

blood and lungs of naive and LPS-challenged mice, and compared the expression of these receptors with the major neutrophil chemoattractant receptor, CXCR2, by flow cytometry. Minimal expression of CCR1, CCR2, or CCR3 was observed on neutrophils isolated from the blood of naive and LPS-challenged mice (Figures 7A and 7B). In comparison, nearly all neutrophils isolated from the blood expressed CXCR2 (Figures 7A and 7B). A small percentage of neutrophils isolated

from naive lung expressed CCR1, CCR2, and CCR3 (Figure 7C), compared with over 95% of neutrophils that expressed CXCR2 (Figure 7C). After LPS challenge, the number of neutrophils expressing CCR1 and CCR2 isolated from lung tissue increased (Figure 7D). The percentage of CCR2-expressing neutrophils, in particular, increased from roughly 10% in naive lung to greater than 35% after LPS challenge (Figure 7D). There was no change in neutrophils expressing CCR3 isolated from

Figure 7. CC chemokine receptor expression of neutrophils isolated from the blood and lung. Mice were administered with LPS (125 μ g/kg intranasally) or received no LPS (naïve), blood and lungs isolated, and single-cell suspensions prepared. Cells were stained for Ly6G and the neutrophil population

inflamed lung tissue compared with naive lung (Figure 7D). Interestingly, concurrent with increased CCR1 and CCR2 expression, the percentage of CXCR2-expressing neutrophils decreased after challenge with LPS (Figure 7E). In a naive lung, greater than 95% of neutrophils expressed CXCR2, whereas less than 60% of neutrophils expressed CXCR2 after LPS challenge, indicating that the chemokine receptor repertoire of neutrophils changes when migrating into lung tissue during an inflammatory response.

Discussion

Neutrophil accumulation at sites of inflammation is essential for the early innate immune response to infection, but is also associated with sterile inflammatory events. Although an influx of neutrophils is important for the control of infection and clearance of pathogens and damaged cells, excessive neutrophilia can lead to further tissue damage and immunopathology (26). In the context of lung injury, the potentially fatal lung condition, ARDS, is characterized by disruption of the alveolar–capillary interface, resulting in leakage of fluid into the interstitium and alveolar compartment, and the accumulation of neutrophils within airspaces (27). Current evidence suggests that excessive neutrophil accumulation contributes to tissue damage and alveolar–capillary barrier disruption (28), and is directly associated with the severity of disease (29–31). Furthermore, alveolar–capillary barrier disruption has been directly associated with the immediate influx of neutrophils (3 h), rather than peak of influx (24 h) or secondary events of neutrophil migration (9). However, the endogenous factors and pathways that influence the rapid recruitment of neutrophils into the inflamed lung remain poorly defined. We now report that neutrophil recruitment into the lung airspaces is attenuated by antagonism of the high-affinity thrombin receptor, PAR₁, in a mouse model of LPS-induced lung inflammation.

PAR₁ antagonists have been investigated extensively as potential antiplatelet agents for the treatment of thrombotic diseases. For example, RWJ

58259 significantly reduced thrombus platelet aggregation in a cynomolgus monkey model of vascular injury–induced thrombosis, by specifically targeting PAR₁ and not PAR_{2–4} (21). The same antagonist also attenuated vascular injury in a vascular restenosis model involving balloon angioplasty in rats (20). Importantly, our *in vivo* studies used a dose of RWJ 58259 that was consistent with these previous reports. It is now recognized that the vascular barrier–modulatory effects of the PAR₁ signaling axis are much more complex, and are highly dependent on the nature and concentration of the activating proteinase. For example, at high concentrations, thrombin promotes endothelial cell contraction and vascular leak via activation of PAR₁ in association with the sphingosine-1-phosphate receptor (S1P), S1P3. In contrast, both low concentrations of thrombin and APC mediate barrier-protective effects by signaling via PAR₁ in association with the endothelial protein C receptor and S1P1 (25). PAR₁ is now considered to be a receptor for danger signals, influencing the release of several proinflammatory mediators. Our data suggest that thrombin is the key proteinase that activates PAR₁ in this model of lung injury. Although other proteinases, such as MMP-1 and neutrophil elastase, can activate PAR₁ under certain inflammatory conditions (6, 32), these proteinases are unchanged and therefore unlikely to play a major role during the acute phase of lung inflammation in our model. Furthermore, apoptotic signals and mediators released from damaged epithelial cells are also likely to modulate the inflammatory response and contribute to the complex signaling processes that occur during lung injury (32).

Previous studies have demonstrated that PAR₁ signaling can lead to the release of certain proinflammatory cytokines, such as TNF, IL-6, and CXCL8 (IL-8) (33–36). In the present study, we now show that PAR₁ signaling influences the expression of CCL2 and CCL7, which, in turn, contribute to the accumulation of neutrophils into inflamed lung tissue. It is recognized that chemokines (particularly CXC chemokines)

play a key role in regulating neutrophil migration into lung tissue (37, 38), and animal models of ARDS have been instrumental in revealing a central role for CXCL8 in neutrophil migration into the lung (1). There is compelling evidence that CXCL8, and the functional mouse homologs, CXCL1 and CXCL2, are released by alveolar macrophages and lung epithelial cells in response to key inflammatory mediators, such as LPS, TNF, and IL-1 β (39). Release of CXCL8 from these cells establishes a chemokine gradient that not only provides neutrophils with directional movement, but also delivers an activation signal that primes these cells during the process of transendothelial migration. Although it is clear that CXCL8 is important for the regulation of neutrophil migration (40), it is far from clear as to the extent to which other chemokines contribute to neutrophilic inflammation.

Previous studies have shown that neutralization of CXCL8 in the BAL fluid of patients with mild ARDS (acute lung injury) does not completely abolish the neutrophil chemotactic activity of this fluid (10), suggesting that other chemotactic factors are present that contribute to neutrophil migration. Indeed, several other chemokines have been implicated in neutrophil migration, including CXCL10 (IP-10) (41), CXCL5 (ENA-78) (42), and CCL2 (11, 12). Taken together, these observations suggest that, although classical chemoattractants, such as CXCL8 (or murine CXCL1 and CXCL2), likely play key roles, neutrophils are capable of responding to a variety of chemoattractants when migrating into inflamed tissue. However, the influence that individual chemokines have on neutrophil migration is likely to be dependent on the inflammatory stimulus and the tissue in which the chemokine is acting. In the case of the lung, we would reason that tissue-specific signals within unique microenvironments may be required for effective neutrophil migration across the pulmonary capillary endothelium and alveolar epithelium.

In the present study, we now demonstrate that neutrophil migration into inflamed lung tissue after LPS challenge is

Figure 7. (Continued.) specifically gated (Ly6G^{high} against forward scatter [FSc]). The expression of CCR1, CCR2, CCR3, and CXCR2 on neutrophils was calculated and represented as *dot plots*. Neutrophils isolated from naive blood (A), LPS-treated blood (B), naive lungs (C), and LPS-treated lungs (D) were analyzed, and the percentage of chemokine receptor–positive cells calculated (E). Data are representative of five mice per group. Data were analyzed by one-way ANOVA with Newman-Keuls *post hoc* test. **P* < 0.05 compared with naive blood.

mediated by CCL2 and CCL7. Indeed, we found that PAR₁ antagonism was not associated with a reduction in the expression of the classic murine neutrophil chemoattractants, CXCL1/CXCL2, after LPS-induced inflammation, but rather influenced CCL2 and CCL7 levels. The observation that PAR₁ signaling contributes to CCL2 expression in the inflamed lung is consistent with our previous findings reported in the context of fibrotic lung injury, where we demonstrated that PAR₁ deficiency is associated with an attenuated CCL2 response in the mouse model of bleomycin-induced lung fibrosis (23, 36, 43). We now show that PAR₁ antagonism is also associated with a reduction in CCL2 and CCL7 levels, indicating that PAR₁ signaling influences the expression of two CC chemokines not commonly recognized as classical neutrophil chemoattractants. Antibody neutralization studies further revealed that CCL2 and CCL7 contribute to neutrophil accumulation in LPS-induced acute lung inflammation. It is clear from previous findings (43) that CCL2 is directly regulated by PAR₁ signaling. Immunohistochemical analysis of sections from mouse lungs challenged with LPS support this notion, as CCL2 and PAR₁ colocalized primarily to the bronchial epithelium, endothelium, and alveolar macrophages. Furthermore, stimulation of bronchial epithelial cells *in vitro* with the PAR₁-activating proteinase, thrombin, led to increased CCL2 production. However, compared with wide-spread immunostaining observed for CCL2, CCL7 immunoreactivity was primarily restricted to the bronchial epithelium, indicating that these two chemokines are differentially expressed within specific lung microcompartments. Furthermore, in our *in vitro* studies, we were unable to demonstrate a direct functional link between PAR₁ signaling and CCL7 release in either bronchial epithelial cells or monocytic cells,

further supporting the notion that CCL2 and CCL7 are regulated by different signaling pathways. In contrast, a combination of TNF and IFN- γ was effective at inducing the release of CCL7, suggesting that CCL7 expression may require highly proinflammatory signals, the complexity of which is not easily mimicked in an *in vitro* setting. Direct instillation of rCCL2 and rCCL7 further showed that these chemokines alone can elicit neutrophil recruitment into naive lungs. In particular, rCCL7 preferentially promoted the recruitment of neutrophils compared with CCL2, although absolute numbers recovered from BAL fluid were similar. We propose that this effect may be explained by the greater promiscuity of CCL7, which can bind several receptors, including CCR1, CCR2, and CCR3, whereas CCL2 only signals through CCR2.

CCL2 is widely regarded as a major monocyte chemoattractant, whereas CCL7 has previously been shown to act as a chemoattractant for several leukocyte populations, including basophils, mast cells, eosinophils, DCs, T lymphocytes, and monocytes/macrophages (44, 45). In particular, CCL7 has been shown to act as a monocyte chemoattractant during bone marrow egress in conjunction with CCL2, a process that is dependent on the expression of CCR2 (15). However, the function of CCL2 and CCL7 remain poorly understood during leukocyte migration into peripheral tissue, and may be critically dependent on the type of inflammatory stimulus and the site of injury (28, 46, 47).

We now demonstrate that neutrophils migrating into the lung in response to LPS also up-regulate CCR1 and CCR2. Conversely, CXCR2 expression was down-regulated on neutrophils from inflamed lung. These studies highlight the potential of neutrophils to respond to CC chemokines, which, in turn, may have important implications for the therapeutic inhibition of these receptors, and the inhibition of

CXCL8, in the control of neutrophilic inflammation. Studies that aim to further our understanding of neutrophil CC chemokine receptor usage during lung inflammation are currently in progress, as it will be important to fully elucidate the dynamics of neutrophil recruitment with respect to the acquisition of novel chemokine receptors within different tissue microenvironments.

In conclusion, we provide strong support that PAR₁ signaling contributes to alveolar-capillary barrier disruption and neutrophil accumulation after LPS-induced lung inflammation by influencing the levels of the CC chemokines, CCL2 and CCL7. Our findings further suggest that CCL2 and CCL7 are not only differentially regulated by disparate signaling pathways within specific lung microenvironments, but that they also differentially influence the recruitment of inflammatory leukocytes, albeit with overlapping functional modalities. Achieving the appropriate balance of reducing excessive neutrophil accumulation without impacting host innate immune function represents a major challenge for the treatment of acute and chronic inflammatory conditions. Modulating this CC chemokine response via antagonism of PAR₁ signaling, or by blocking these chemokines directly, may represent novel opportunities for interfering with excessive neutrophilia and tissue damage in diseases such as ARDS, and potentially other conditions associated with acute neutrophilic inflammation. In terms of strategies aimed at inhibiting PAR₁ signaling, PAR₁ antagonists are currently being evaluated as novel antithrombotic agents in several large multicenter trials (48, 49). Our findings provide support for extending the evaluation of these agents in the context of ARDS and, potentially, other acute neutrophilic inflammatory conditions. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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