Role of PAR2 in murine pulmonary pseudomonal infection

Theo J. Moraes, Raiza Martin, Jonathan D. Plumb, Eric Vachon, Cheryl M. Cameron, Ali Danesh, David J. Kelvin, Wolfram Ruf, and Gregory P. Downey

1Department of Immunology and 2Division of Respiratory, Department of Medicine, University of Toronto, 3Toronto General Hospital Research Institute of University Health Network, and 4Division of Respiratory Medicine, Department of Pediatrics, Hospital for Sick Children, Toronto, Canada; and 5Department of Immunology, Scripps Research Institute, La Jolla, California

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Moraes TJ, Martin R, Plumb JD, Vachon E, Cameron CM, Danesh A, Kelvin DJ, Ruf W, Downey GP. Role of PAR2 in murine pulmonary pseudomonal infection. Am J Physiol Lung Cell Mol Physiol 294: L368–L377, 2008. First published December 14, 2007; doi:10.1152/ajplung.00036.2007.—Proteinases can influence lung inflammation by various mechanisms, including via cleavage and activation of protease-activated receptors (PAR) such as PAR2. In addition, proteinases such as neutrophil and/or Pseudomonas-derived elastase can disarm PAR2 resulting in loss of PAR2 signaling. Currently, the role of PAR2 in host defense against bacterial infection is not known. Using a murine model of acute Pseudomonas aeruginosa pneumonia, we examined differences in the pulmonary inflammatory response between wild-type and PAR2−/− mice. Compared with wild-type mice, PAR2−/− mice displayed more severe lung inflammation and injury in response to P. aeruginosa infection as indicated by higher bronchoalveolar lavage fluid neutrophil numbers, protein concentration, and TNF-α levels. By contrast, IFN-γ levels were markedly reduced in PAR2−/− compared with wild-type mice. Importantly, clearance of P. aeruginosa was diminished in PAR2−/− mice. In vitro testing revealed that PAR2−/− neutrophils killed significantly less bacteria than wild-type murine neutrophils. Further, both neutrophils and macrophages from PAR2−/− mice displayed significantly reduced phagocytic efficiency compared with wild-type phagocytes. Stimulation of PAR2 on macrophages using a PAR2-activating peptide resulted in enhanced phagocytosis directly implicating PAR2 signaling in the phagocytic process. We conclude that genetic deletion of PAR2 is associated with decreased clearance of P. aeruginosa. Our data suggest that a deficiency in IFN-γ production and impaired bacterial phagocytosis are two potential mechanisms responsible for this defect.

neutrophil; phagocytosis; proteinase; innate immunity; bacterial killing; inflammation

GRAM-NEGATIVE PNEUMONIA often complicates the course of hospitalized or immunocompromised patients, although increasingly these organisms are the cause of community-acquired pneumonias (20). What factors predispose individuals to infection with these organisms is not completely understood but is important clinically because of the considerable morbidity and mortality associated with established infection (2). Pseudomonas aeruginosa is a gram-negative bacterium that accounts for a significant proportion of these life-threatening pneumonias. P. aeruginosa can infect multiple organ systems including the lung. However, despite a number of virulence factors and a propensity for resistance to multiple antibiotics, P. aeruginosa pneumonia remains largely confined to a select group of susceptible individuals, indicating that under most circumstances host defenses are able to thwart invasive infection with this pathogen.

Bacterial proteinases such as the elastolytic metalloproteinase LasB (EC 3.4.24.26), also known as pseudolysin or P. aeruginosa elastase (32), are thought to contribute to the pathogenicity of the microbe and can injure host tissues in a variety of ways (13). In addition to exogenous proteinases (released from microbes or inhaled from the environment), host-derived proteinases (from inflammatory cells or the vasculature) in the lung can also act in a nondegradative fashion and influence cellular processes through the activation of protease-activated receptors (PARs). There are currently four known PARs, and all are expressed by various cells in the lung. Signaling through PAR2 has been shown to modulate inflammation in the lung, with both pro- and anti-inflammatory effects reported (6). Recently, it is has also been shown that both P. aeruginosa-derived and neutrophil elastase can disarm PAR2 (12). Thus loss of PAR2 signaling may be an important factor in disease states in which excess elastase is found in the airways, such as acute lung injury/adult respiratory distress syndrome and cystic fibrosis (CF). Although these conditions are associated with a predisposition to P. aeruginosa pneumonia, the function of PAR2 in this context is not known.

In the current study, we sought to examine the role of PAR2 in an in vivo model of P. aeruginosa pneumonia. We demonstrate that absence of PAR2 is associated with enhanced inflammation and altered bronchoalveolar lavage cytokine profile in response to intratracheal administration of P. aeruginosa. Importantly, PAR2−/− mice displayed reduced bacterial clearance, an observation that may be attributable to reduced IFN-γ levels and a primary defect in phagocytosis. These observations are relevant to conditions, such as CF, acute lung injury/adult respiratory distress syndrome, and chronic obstructive pulmonary disease, that are associated with increased pulmonary levels of extracellular proteinases such as elastase.

MATERIALS AND METHODS

Chemicals and materials. Endotoxin-free reagents and plastics were used in all experiments unless otherwise indicated. PBS was purchased from University of Toronto Media Services. PAR2-activating peptide (AP) (SLIGRL) and inactive peptide (IP) (LSIGRL) were purchased from the Alberta Peptide Institute (Edmonton, Canada). Needles, syringes, and catheters were from BD Sciences (Franklin Lakes, NJ). N-formyl-Met-Leu-Phe, cytochalasin D, amastatin, BSA, N-acetyl cysteine, and amiloride were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were of the highest grade commercially available.

Address for reprint requests and other correspondence: G. P. Downey, Academic Affairs, K701b, National Jewish Medical and Research Center, 1400 Jackson St., Denver, CO 80206 (e-mail: downeyg@njc.org).

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and PMA were from Sigma-Aldrich (St. Louis, MO). Sheep red blood cells were from ICN/Cappel (Aurora, OH). Dihydrothoradamine and FITC were from Molecular Probes (Eugene, OR). Paraformaldehyde (16%) and glutaraldehyde (EM-grade) were from Canemco (St. Laurent, Canada). Trypan blue stain was from Life Technologies (Grand Island, NY). Glass coverslips (25 mm) were from VWR Scientific (West Chester, PA).

Antibodies. Rabbit anti-sheep IgG was from ICN/Cappel. FITC-labeled mouse monoclonal antibody to the human CD11b (Mac-1) antigen (VIM12) was from Caltag Laboratories (Burlingame, CA). Alexa Fluor 488-fluorescent goat anti-mouse and Alexa Fluor 488-labeled goat anti-rabbit IgG secondary antibodies were from Molecular Probes.

Bacterial culture. A clinical isolate of \(P.\) aeruginosa was obtained from the Clinical Microbiology Laboratory, Hospital for Sick Children, Toronto (courtesy of L. Burrows and J. Kus). PAO1, a nonpiliated strain of \(P.\) aeruginosa (courtesy Joseph Lam, University of Guelph) the complete genomic sequence of which is known (43), was also utilized in selected experiments. Bacteria were maintained at \(-80^\circ\text{C}\) as glycerol stocks and streaked onto Luria-Bertani (LB) agar plates or \(P.\)seudomonas isolation agar plates (PIA; Difco) to obtain single colonies before use. One colony was grown overnight in LB medium and then subcultured for 1 h the next day. Bacteria were then washed once with sterile PBS, sedimented at 6,000 g for 10 min at 22°C, and resuspended in PBS. Measurement of optical density at 600 nm allowed for estimation of bacterial concentration [based on previously generated standard curve: colony-forming units (CFU) vs. optical density at 600 nm]. Each mouse received 1

Bacterial isolation. Murine bone marrow cells were collected from C57BL/6 and PAR2/- mice femurs and isolated using discontinuous Percoll gradients as described previously (21).

Bacterial killing assay. To assess neutrophil killing activity, neutrophils were washed twice and then resuspended in HBSS. PAO1 (subcultured as described in Bacterial culture) was opsonized with human serum (20% solution in PBS) for 1 h at room temperature. The PAO1 bacteria (\(5 \times 10^5\)) were then added to the neutrophils (\(1 \times 10^8\)) in a 96-well plate, and human serum (final concentration of 20%) was added to the assay (final volume was 120 \(\mu\)l). Control wells consisted of PAO1 added to HBSS and human serum alone. The plate was then incubated at 37°C for 90 min. Forty microliters from each well were removed at 0 and 90 min; lysed in 0.1% Triton X-100 for 10 min; and, after serial dilutions, plated on PIA agar plates for 24 h. Percent bacterial survival was determined as follows: 100 \(\times\) (CFU/\(\text{final}\) CFU/\(\text{initial}\)). Survival was normalized to control for each day and then bacterial killing was calculated as 100 - percent survival (thus control was 0% killing for each experiment day).

Phagocytosis. Two methods were used to assess the phagocytic ability of neutrophils. First, \(1 - 2 \times 10^6\) \(P.\) aeruginosa were labeled with 1 mg/ml FITC in PBS for 30 min. Bacteria were fixed with 2% EM-grade glutaraldehyde and then washed extensively. Bacteria and neutrophils were combined at a multiplicity of infection of 20:1, sedimented briefly for 5 min at 37°C to facilitate bacteria-neutrophil contact, gently resuspended, and then incubated for an additional 20 min, after which they were cooled to 4°C to stop phagocytosis. Five microliters of this cell suspension were added to 5 \(\mu\)l 0.5% trypan blue (to quench extracellular fluorescence) on a glass slide with a glass coverslip, visualized with a Leica DMRBI inverted fluorescence microscope, and scored for phagocytic index (number of bacteria in 100 neutrophils). The investigator was blinded to neutrophil genotype. In the second method, 50 \(\mu\)l of a 2-\(\mu\)m fluorescent bead suspension (Ex/Em 441/486 nm, Polysciences, Warrington, PA) were opsonized with human IgG (50\ mg/ml, Sigma) in a 1:1 ratio in 1 ml HBSS for 1 h at 37°C. The beads were washed twice and then placed on ice. Neutrophils (\(1 \times 10^6\)) were placed in 100 \(\mu\)l HBSS in an eppendorf tube. Ten microliters of the bead suspension were added, and the solution was briefly sedimented, incubated for 25 min at 37°C, and then cooled to 4°C. Experimental tubes also contained 100 \(\mu\)l PAR2 AP, PAR2 IP, or PBS vehicle. Control tubes contained 5 \(\mu\)l cytchalasin B and were kept on ice. Phagocytosis was quantified by measuring cell-associated fluorescence of neutrophils using a FACScan flow cytometer. Bacterial internalization was confirmed in a subset of samples using fluorescence microscopy.

To assess the phagocytic ability of macrophages, peritoneal macrophages were harvested from mice by lavage and cultured on 25-mm acid-washed glass coverslips and allowed to adhere overnight. One-hundred microliters of a 10% sheep erythrocyte solution were washed twice with PBS, incubated with 1 \(\mu\)l of rabbit anti-sheep erythrocyte IgG for 1 h, and washed again. Approximately 0.5–1.0 \(\times\) 10\(^6\) erythrocytes (50 \(\mu\)l) were allowed to sediment by gravity onto the macrophages and incubated at 37°C for 25 min. After incubation, hypotonic lysis of the extracellular erythrocytes was achieved by addition of water for 30 s, followed by immediate replacement with calcium and magnesium-free PBS. The coverslips were mounted on Attofluor cell chambers (Invitrogen), and quantification of phagocytosis was conducted using an inverted microscope (Leica DM-IRB).

To assess the effect of PAR2 activation, macrophages were incubated in 100 \(\mu\)M PAR2 AP and 100 \(\mu\)M PAR2 IP or equivalent PBS vehicle during incubation with erythrocytes. Ten micromoles of amastatin were used in all conditions to prevent peptide degradation.

Neutrophil oxidative burst and CD11b expression. Neutrophils (0.5–1 \(\times\) 10\(^6\)) from PAR2-/- and wild-type mice were isolated and resuspended in HBSS. For assessment of oxidant production, 10 \(\mu\)M
dihydrorhodamine (final concentration) were added to each tube followed by 1 μl of 10^{-6} M PMA or DMSO control and incubated for 15 min at 37°C in the dark. Cells were then sedimented, resuspended in 10 μl PBS, added to 400 μl PBS containing 2% paraformaldehyde, and analyzed by flow cytometry. For CD11b expression, neutrophils suspended in HBSS were pretreated with 1 μM cytochalasin D for 5 min at 37°C before stimulation with 10^{-6} M N-formyl-Met-Leu-Phe for 5 min. Cells were washed once and incubated with FITC anti-CD11b in HBSS with 0.2% (wt/vol) sodium azide for 45 min at 4°C. Cells were then fixed with 2% (vol/vol) paraformaldehyde and analyzed by flow cytometry. The relative fluorescence intensity represents the geometric mean of 10,000 cells gated on light scattering properties. Results are presented as relative fluorescence intensity normalized to unstimulated cells.

**Statistics.** Data analyzed with Graphpad Prism using an unpaired t-test when two groups were compared (BAL CFU and BAL neutrophils) or by ANOVA with Tukey’s multiple comparison test (MCT) if three groups were compared (BAL protein). The data from the phagocytosis assays (see Fig. 6) were analyzed using a paired t-test with samples matched between experimental days. Data are reported as means ± SD with P values. Significant differences were reported when P < 0.05.

**RESULTS**

**Intratracheal delivery of *P. aeruginosa* results in lung inflammation after 24 h.** We first sought to develop a model of gram-negative pneumonia that would reflect certain aspects of the situation in humans. Preliminary experiments were conducted examining the effects of intratracheal instillation of increasing numbers of bacteria into the lungs of mice. We chose a dose of bacteria (1 × 10^6 CFU of *P. aeruginosa* mouse) that resulted in a brisk inflammatory response associated with systemic symptoms (moderate weight loss, lethargy, and diminished grooming) but that was not fatal. The effects of instillation of bacteria were compared with that of PBS (vehicle) alone. Mice were euthanized 24 h after instillation of PBS or bacteria. Bronchoalveolar lavage was conducted, the cellular composition of the fluid was assessed on cytopsin preparations, and the bacterial load was quantified by direct bacterial culture. We observed a significant alveolar influx of leukocytes in the mice exposed to bacteria indicating a vigorous pulmonary inflammatory response (Fig. 1A). In addition, mice infected with *P. aeruginosa* lost more weight [8.2% (SD 2.7) vs. 2.1% (SD 3.9); P = 0.002] after 24 h than the control group exposed to PBS (Fig. 1B). Importantly, viable *P. aeruginosa* were detected in the lavage fluid of mice 24 h after instillation (Fig. 1C). As expected, no bacteria were cultured from the lungs of the control (PBS) group.

**PAR2−/− mice demonstrate an enhanced inflammatory response to *P. aeruginosa.*** After establishing a model of acute bacterial pneumonia, we examined differences in responses between wild-type and PAR2−/− mice. It should be noted that in vivo comparisons between wild-type and PAR2−/− mice infected with *P. aeruginosa* involved 15 mice in each group, and experiments were conducted on 6 separate days. One or more pairs of PAR2−/− and wild-type mice were studied on each day. No differences were noted between wild-type and PAR2−/− mice in response to PBS instillation alone as assessed by total cell numbers, differential cell counts, protein concentration, cytokine concentrations, and bacterial counts in BAL fluid (data not shown). Results from wild-type mice exposed to PBS are shown as a baseline (Fig. 2, black bars). There was no mortality in either group in response to instillation of live *P. aeruginosa* in the immediate 24 h postinfection. As an index of cellular inflammation, we measured neutrophil and macrophage numbers in the BAL 24 h after *P. aeruginosa* instillation. There were significantly more neutrophils in the BAL fluid from PAR2−/− compared with wild-type mice [2.64 × 10^6 neutrophils/ml (SD 1.0) vs. 1.92 × 10^6 neutrophils/ml (SD 0.68); P = 0.036; Fig. 2A]. Macrophage numbers were not different between the two groups of mice (Fig. 2B).

As an additional indicator of the degree of lung injury, we examined protein levels in the BAL fluid. Total protein levels in the BAL were significantly higher in the PAR2−/− than in wild-type mice [2,093 μg/ml (SD 486) vs. 1,151 μg/ml (SD 443); P = 0.001; Fig. 2C].

**PAR2−/− mice demonstrate reduced bacterial clearance of *P. aeruginosa.*** To quantify bacterial clearance, we cultured blood and BAL fluid from the mice 24 h after *P. aeruginosa* instillation. There was no evidence of bacteremia in any of the mice tested (n = 12). Importantly, significantly more bacteria were recovered in the BAL fluid from the PAR2−/− compared with wild-type mice. As bacterial growth is exponential, the results are expressed as the log of the CFU recovered in the BAL [2.68 log CFU/ml BAL (SD 0.78) vs. 1.91 log CFU/ml BAL (SD 1.10); P = 0.039; Fig. 2D].

**PAR2−/− mice demonstrate an altered BAL cytokine profile in response to *P. aeruginosa.*** We determined the BAL concentrations of nine separate cytokines thought to be important in regulation of lung inflammation. PAR2−/− mice had increased TNF-α levels [425 pg/ml (SD 279) vs. 168 pg/ml (SD 264); P = 0.05] and reduced IFN-γ levels [1.9 pg/ml (SD 1.6) vs. 28.6 pg/ml (SD 52.8); P = 0.045] compared with wild-type mice. There was a trend to higher IL-6 and lower IL-12 levels.

![Fig. 1](http://ajplung.physiology.org/). *Pseudomonas* administration results in objective evidence of disease. In A–C, intratracheal *P. aeruginosa* (clinical isolate) infected mice are indicated with open bars (n = 15) and PBS control are indicated with gray bars (n = 4). Bars are means ± SD. A: total cells in bronchoalveolar lavage (BAL) fluid after 24 h. B: percent weight loss after 24 h. C: log colony forming units (CFU) bacteria/ml BAL fluid are shown. ND, not detected. *P < 0.05.
in the PAR2−/− mice, but these differences did not reach statistical significance (P = 0.07 and 0.06, respectively). There were no differences in the levels of the other cytokines measured including IL-2, IL-4, IL-5, IL-10, and monocyte chemotactic protein-1 between PAR2−/− and wild-type mice (Fig. 3).

**PAR2−/− mice demonstrate an enhanced inflammatory response to and reduced bacterial clearance of PAO1.** To expand on the observations in PAR2−/− mice infected with the *P. aeruginosa* clinical isolate, we repeated experiments using a well-characterized laboratory strain of *P. aeruginosa*, PAO1. Consistent with the studies with the *P. aeruginosa* clinical isolate, we observed that PAR2−/− mice had more protein [1,033 µg/ml (SD 343) vs. 644 µg/ml (SD 200); P = 0.038], higher neutrophil numbers [2.50 × 10^6 neutrophils/ml (SD 0.64) vs. 1.58 × 10^6 neutrophils/ml (SD 0.60); P = 0.028], and more viable bacteria in the BAL fluid compared with wild-type mice [1.66 log CFU/ml BAL (SD...
PAR2−/− neutrophils display defective bacterial killing. To further investigate the importance of PAR2 in neutrophil function, we examined killing of *P. aeruginosa* PAO1 by wild-type and PAR2−/− neutrophils. We observed that PAR2−/− neutrophils killed significantly fewer bacteria compared with wild-type neutrophils [bacterial killing normalized to control 21.5% (SD 23.1) for PAR2−/− vs. 63.6% (SD 17.8) for wild type; *P* = 0.028; Fig. 5A].

PAR2−/− neutrophils have reduced phagocytic ability. To examine further the defect in neutrophil microbicidal function associated with PAR2 deficiency, we assessed selected aspects of neutrophil activation. Initially we measured surface expression of CD11b, an adhesion molecule and complement receptor (CR3) found in neutrophil granules and secretory vesicles, by flow cytometry. Basal surface expression of CD11b was similar between PAR2−/− and wild-type neutrophils whether isolated from the BAL fluid (Fig. 5B) or bone marrow (Fig. 5B). In response to stimulation with the potent activating agent PMA, neutrophils isolated from either BAL fluid (Fig. 5C) or bone marrow (Fig. 5D) displayed an increase in surface expression of CD11b. However, no differences were noted between wild-type and PAR2−/− neutrophils in this response.

Next, oxidant production was measured by flow cytometry using dihydrorhodamine. Oxidant production in quiescent cells, after stimulation with PMA or after exposure to bacteria, did not differ between wild-type and PAR2−/− neutrophils. This result was true of neutrophils isolated from BAL fluid (Fig. 5, B and D) or from bone marrow (Fig. 5, C, E, and F).

By contrast PAR2−/− neutrophils exhibited a significantly reduced phagocytic ability compared with control neutrophils. The reduced phagocytosis was observed when phagocytosis was tested with *P. aeruginosa* as prey [phagocytic index: 50.5 (SD 23.0) vs. 13.3 (SD 4.0); *P* = 0.001; Fig. 6A] or with IgG opsonized latex beads as prey [42.9 arbitrary fluorescence units (SD 13.6) vs. 30.4 arbitrary fluorescence units (SD 9.0); *P* = 0.014; Fig. 6B]. Both neutrophil assays are reported as the mean of experiments performed on 3 separate days. These results suggest that the absence of PAR2 impairs phagocytosis. Conversely, to test if PAR2 signaling could enhance phagocytosis, neutrophils were stimulated with PAR2 AP. In this assay, neither wild-type nor PAR2−/− neutrophils increased ingestion of latex beads when PAR2 AP was added compared with PBS control (Fig. 6C). No effect was noted when the inactive peptide was used as a control.

PAR2 activation enhances phagocytosis by macrophages. In addition to neutrophils, macrophages are also professional phagocytes involved in host defense, and therefore, we tested whether PAR2 signaling influenced macrophage phagocytosis. Consistent with the neutrophil data, PAR2−/− macrophages displayed diminished phagocytosis compared with wild-type macrophages [phagocytic index: 30.0 (SD 8.6) vs. 89.1 (SD 48.3); *P* = 0.032; Fig. 6D, PBS groups]. However, unlike neutrophils, when wild-type macrophages were stimulated with PAR2 AP, an increase in the phagocytic index was noted compared with addition of PBS control [110 (SD 65) vs. 89 (SD 48); *P* < 0.05] or IP control [76 (SD 49); *P* < 0.001]. The phagocytic efficiency of wild-type macrophages treated with either inactive peptide or PBS was not significantly different (Fig. 6E). As expected, addition of PAR2 AP did not enhance phagocytosis in PAR2−/− macrophages [40 (SD 17) vs. 30 (SD 8.6); Fig. 6D] providing evidence that the AP mediated its effects through PAR2 and not via nonspecific cell activation. These experiments were performed on 3 separate days with consistent results.

**DISCUSSION**

Proteinases play key roles in lung injury often through modulation of specific receptors including PARs. While proteinases have detrimental effects and contribute to tissue injury, they also have beneficial effects. In this study, we demonstrate that when *P. aeruginosa* is instilled into the lungs of mice, absence of PAR2 is associated with an increased inflammatory response as manifest by enhanced alveolar protein leak, neutrophil infiltration, and altered cytokine production. We also provide evidence that PAR2−/− mice have a defect in clearance of *P. aeruginosa* that may be attributable to a defect in phagocytosis by neutrophils and macrophages and/or a defect in IFN-γ production.

Proteinases influence lung inflammation through nondegradative mechanisms, including activation of PARs. All four known PARs (PAR1, 2, 3 and 4) are expressed in the pulmonary epithelium and airway smooth muscle (3, 19, 37). Neutrophils express PAR1 and 2 but not PAR3 or 4 (17, 50) and macrophages express PAR1, 2, and 3 (8). Proteinases activate these receptors through cleavage of the extracellular amino terminus that functions as a “tethered ligand” that auto-activates the receptor triggering a variety of downstream effects. There is evidence for both pro- and anti-inflammatory roles for PAR2 in the lung (11, 14, 28, 36, 44, 45). To reconcile these apparently opposing actions of PAR2 in vivo, it has been
suggested that the intensity and duration of PAR2 activation may determine the predominant effect in inflammation; low magnitude or short duration signals are proinflammatory while stronger signals are anti-inflammatory (6). Protective effects of PAR2 have been attributed to PGE release (7, 22, 28, 29), whereas proinflammatory effects may be mediated by neurokinins.

Disarming of PAR2 by proteinases occurs when amino terminus receptor cleavage occurs at an alternate site removing the tethered ligand rather than “revealing” it. Neutrophil-derived serine proteinases (elastase and cathepsin G; Ref. 12) and bacterial proteinases such as Pseudomonas-derived elastase (13) and thermolysin (46) can disarm PAR2 resulting in reduced signaling by the receptor. The responses in PAR2−/− mice mimic the situation in which PAR2 is disarmed by exogenous proteinases. Thus the observation that Pseudomonas infection in PAR2−/− mice results in enhanced protein leak, infiltration of inflammatory cells, and decreased bacterial clearance may be especially relevant to conditions where increased elastase levels are present, such as CF. Disarming of PAR2 by bacterial elastase in this context might partly explain the predisposition to bacterial infection seen in CF patients. A study (52) in which aerosolized elastase inhibitor decreased bacterial proliferation in a chronic Pseudomonas infection model provides support for this notion.

The role of PAR2 in bacterial infection is incompletely understood. During the course of an infection, bacteria and host cells release proteinases (25) that in turn may activate or disarm PAR2 on host cells. That PAR2 plays a protective role in this milieu is supported by our observations. However, untoward effects of PAR2 have also been reported. Porphyromonas gingivalis, a causative agent in periodontitis, produces proteinases that activate PAR2 resulting in increased inflammation (16). Further, activation of PAR2 in a murine model of colitis using Citrobacter rodentium infection also results in enhanced inflammation in the colon (15). While
Inflammation is an essential physiological response to injury and infection, if excessive or unremitting, inflammation may be pathological and result in alveolar bone loss (in the P. pneumoniae model) and bacterial translocation from the gastrointestinal tract (in the C. rodentium model).

In our model of Pseudomonas pneumonia, we observed reduced bacterial clearance in the absence of PAR2. Given the different cell types that express PAR2, there are multiple potential mechanisms that may explain this protective role of PAR2 in vivo. One possibility is a defect in the induction of host defense responses in PAR2−/− mice. We observed a marked reduction in IFN-γ levels and a trend towards reduced IL-12 levels in the BAL fluid of PAR2−/− mice. Both of these cytokines are key components of a Th1-weighted immune response, although at 24 h it is likely too early for this adaptive response to be fully manifest. Nevertheless, IFN-γ is thought to be critical in defense against Pseudomonas infection and may help direct the delayed adaptive response (5, 23, 53). Interestingly, PAR2 activation on dendritic cells promotes T-cell production of IFN-γ (9). Further, in a murine model of pancreatitis, cerulein injection led to IFN-γ production in a PAR2-dependent fashion within 12 h (26). Thus there is evidence both that PAR2 activation promotes IFN-γ production and that IFN-γ is an important factor in clearing pseudomonal infection. This may partly explain the reduced bacterial clearance seen in vivo in our studies. However, other studies question the importance of IFN-γ in pseudomonal infection (4, 30). Some of these discrepancies may be related to the specifics of the model system used, to the location of the infection (lung or other organ), and to the ability of the bacteria to produce alginate. Alginate production, often associated with chronic pseudomonal infections, may confer resistance to killing by IFN-γ (40).

In addition to IFN-γ, PAR2 stimulation can directly lead to the production of a variety of cytokines including IL-6, IL-8, PGE2, matrix metalloproteinase-9, granulocyte-monocyte colony-stimulating factor, and eotaxin (3, 42, 48, 49) from a variety of cell types. There is also evidence that loss of PAR2 alters cytokine secretion (33). We observed higher levels of both IL-6 and TNF-α in the lavage fluid from PAR2−/− mice, although only the TNF-α levels reached statistical significance. These cytokines may reflect an enhanced inflammatory process in the PAR2−/− mice as is supported by the elevated cell counts and protein concentration. Alternatively, Pseudomonas is known to directly stimulate TNF-α production from leukocytes (41). Interestingly, excess TNF-α in the absence of functional PAR2 has been shown to promote cell death (34) and thus in this setting may be more harmful than protective.

The measurement of multiple mediators in studies such as the current one poses some statistical issues. In this study, we used a multiplex assay to measure the levels of nine separate cytokines and chemokines in BAL fluid. This raises the question of whether or not to use a statistical method for correction for multiple comparisons. However, the use of a correction factor under these conditions may result in a type II (β) error,
an issue that has become increasingly important with the analysis of large scale microarray and proteomic data sets.

We examined phagocyte function directly as we observed decreased bacterial clearance in PAR2−/− mice despite increased numbers of neutrophils and, in vitro, a reduction in bacterial killing. We detected a significant phagocytic defect in PAR2−/− neutrophils and macrophages. These findings suggest that PAR2 activation in these cells contributes to enhanced phagocytosis. In support of this hypothesis, we observed increased phagocytosis in macrophages exposed to PAR2 AP. In a different in vitro assay, uptake of microspheres or *Escherichia coli* bioparticles over 4–18 h by keratinocytes was enhanced by PAR2 AP (38, 39). PAR2 AP also enhanced microsphere uptake by murine IC-21 macrophages and murine 3T3 fibroblasts over 16 h (39). This suggests that the role of PAR2 is more generalized and may not be confined to professional phagocytes. The observation that PAR2 participates in neutrophil phagocytosis is novel. However, while PAR2−/− neutrophils exhibited defective phagocytosis, PAR2 AP did not induce enhanced phagocytosis in these cells. There are several potential explanations for this observation. First, PAR2 may be maximally stimulated in neutrophils and hence additional stimulation may not provide an additive effect. Second, despite the use of amastatin to prevent peptide degradation, release of proteolytic enzymes by neutrophils may overwhelm the capacity of amastatin and result in degradation of PAR2 AP. Nonetheless, the enhanced phagocytosis seen in the PAR2 AP treated macrophages, combined with the reduction of phagocytosis in PAR2−/− macrophages and neutrophils, provides strong evidence that PAR2 signaling modulates phagocytosis.

The concept that proteinases can influence phagocytosis is well established; however, both positive and negative effects have been reported (27, 31, 35, 51). *Pseudomonas* elastase has previously been shown to reduce phagocytosis by leukocytes (18). Further, neutrophils isolated from the lungs of CF patients also display reduced phagocytosis; an effect mimicked in non-CF neutrophils by elastase exposure (1). In both of these studies, the mechanism of elastase-induced reduced-phagocytosis was not defined. Our data suggest disarming of PAR2 as a likely possibility, it is also possible that as the plasma membrane invaginates during phagocytosis, PAR2 incorporates into the phagosome. Thus additional signaling could occur as PAR2 “samples” the contents of the phagolysosome as has been suggested for Toll-like receptors (47).

The mechanisms by which PAR2 promotes phagocytosis remain unknown. Through its cytosolic tail, PAR2 could recruit signaling molecules to aid the phagocytic process, such as MAP kinases, Rho-GTPases, calcium, or actin (42). PAR2 may upregulate surface expression of conventional phagocytic receptors such as FcyRII and III or CR3. It is also possible that PAR2-induced cytokine secretion may enhance phagocytosis. The contribution of this in vitro defect to the in vivo situation is difficult to quantify as several mechanisms may contribute to the reduced bacterial clearance in the PAR2−/− mice. As an example, activation of PAR2 upregulates P-selectin-mediated leukocyte rolling (24). In the absence of PAR2, inflammation is delayed and thus bacterial clearance may be altered.

This work suggests that promotion of PAR2 signaling and/or prevention of PAR2 disarming would improve outcomes in pseudomonal pneumonia. This remains to be confirmed in future studies.

In summary, we show that absence of PAR2 is associated with enhanced inflammation and reduced bacterial clearance in a murine model of *P. aeruginosa* pneumonia. One potential explanation for this in vivo finding is defective IFN-γ production in the lung of PAR2−/− mice. In addition, loss of PAR2 signaling results in reduced phagocytosis by neutrophils and macrophages in vitro. These observations are important as they describe a novel influence of PAR2 on phagocytosis and have implications for disease states in which elevated elastase levels in the lung may lead to a predisposition to bacterial infection.

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