Regulation of inflammation by Rac2 in immune complex-mediated acute lung injury

James L. Dooley,1 Dalia Abdel-Latif,1 Chris D. St. Laurent,1 Lakshmi Puttagunta,2 Dean Befus,1 and Paige Lacy1

1Department of Medicine, and 2Department of Laboratory Medicine and Pathology, Pulmonary Research Group, University of Alberta, Edmonton, Alberta, Canada

Submitted 4 September 2008; accepted in final form 29 September 2009

Dooley JL, Abdel-Latif D, St. Laurent CD, Puttagunta L, Befus D, Lacy P. Regulation of inflammation by Rac2 in immune complex-mediated acute lung injury. Am J Physiol Lung Cell Mol Physiol 297: L1091–L1102, 2009. First published October 2, 2009; doi:10.1152/ajplung.90471.2008.—Acute lung injury (ALI) is an inflammatory disorder associated with recruitment and activation of neutrophils in lungs. Rac2, a member of the Rho GTPase subfamily, is an essential regulator of neutrophil degranulation, superoxide release, and chemotaxis. Here, we hypothesized that Rac2 is important in mediating lung injury. Using a model of IgG immune complex-mediated ALI, we showed that injury was attenuated in rac2−/− mice compared with wild-type (WT) mice undergoing ALI, with significant decreases in alveolar leukocyte numbers, vascular leakage, and the inflammatory mediators, myeloperoxidase (MPO) and matrix metalloproteinases (MMPs). Reduced injury in rac2−/− mice was not associated with diminished cytokine and chemokine production, since bronchoalveolar lavage (BAL) levels of IL-17, TNF, CCL3, CXCL1, and CXCL2 were similarly increased in WT and rac2−/− mice with ALI compared with sham-treated mice (no ALI). BAL levels of MMP-2 and MMP-9 were significantly decreased in the airways of rac2−/− mice with ALI. Immunohistochemical analysis revealed that MMP-2 and MMP-9 expression was evident in alveolar macrophages and interstitial neutrophils in WT ALI. In contrast, MMP-positive cells were less prominent in rac2−/− mice with ALI. Chimeric mice showed that Rac2-mediated lung injury was dependent on hematopoietic cells derived from bone marrow. We propose that lung injury in response to immune complex deposition is dependent on Rac2 in alveolar macrophages and neutrophils. Neutrophils; macrophages; bronchoalveolar lavage; cytokines; bone marrow chimeras; matrix metalloproteinases

ACUTE LUNG INJURY (ALI) is a common clinical disorder involving acute respiratory failure caused by injury to alveolar epithelial and endothelial barriers in the lung (4, 42). Its severe form is acute respiratory distress syndrome (ARDS), which is accompanied by a high rate of morbidity and mortality (20–50%; Refs. 3, 36, 42). A current medical intervention for ALI is to maintain patients on a low tidal volume respiratory ventilation protocol (17). Several drug intervention strategies have been tested for ALI but have failed to reduce mortality associated with the disease (17, 50).

Neutrophil recruitment and activation are major causative factors in ALI and lead to tissue-damaging degranulation and superoxide release. Regulation of mediator release from neutrophils occurs through ligand-receptor stimulation of Rho GTPases. Of these, all three Rac proteins are expressed in neutrophils (Rac1, Rac2, and Rac3), whereas Rac1 and Rac2 have been investigated more extensively than Rac3 in these cells (9, 24, 30). Rac1 and Rac2 exhibit 92% amino acid identity and are functionally interchangeable in their ability to regulate superoxide generation and chemotaxis, although human neutrophils mainly express Rac2 (9). However, Rac1 and Rac2 serve distinct, nonredundant functions in neutrophils. Whereas Rac1 controls cell spreading, Rac2 is critical for migration as well as superoxide release and exocytosis of azurophilic granules (2, 22). Specifically, Rac2 is required for F-actin formation in neutrophils, which is essential for numerous cellular activities including chemotaxis, superoxide release, and degranulation (19, 41). Primary granule exocytosis in neutrophils is dependent on Rac2-dependent actin remodeling, demonstrated by the inability of Rac2−/− neutrophils to translocate primary granules to the cell membrane for their release (1, 2).

The function of Rac2 in a neutrophil-mediated inflammatory model has not been investigated. Our aim was to elucidate the role of Rac2 in experimental ALI induced by IgG immune complex deposition in the airways (reverse passive Arthus reaction), a model of lung inflammation and injury. This model of injury is specifically dependent on neutrophil recruitment to the airways and their activation based on experiments in rats (27). In this study, we examined the effects of gene deletion of Rac2 in immune complex-mediated ALI in mice (41).

MATERIALS AND METHODS

Mice. Rac2-deficient mice (rac2−/−) were established by gene disruption (41). All gene-deleted mice were backcrossed against the C57BL/6 strain background for ≥11 generations. Animals were bred on-site and housed under specific virus antigen-free conditions on a 12:12-h light-dark cycle and fed autoclaved food and water as needed. All experiments used male mice at 8–12 wk of age. Wild-type (WT) animals were C57BL/6 mice housed and maintained in the same manner as for rac2−/− mice. Animal experiments were approved for ethics by the University of Alberta Animal Policy and Welfare Committee.

Immune complex-mediated ALI. Isoflurane inhalation using a precision vaporizer instrument (Ohio Medical, Gurnee, IL), and intraperitoneal injections of ketamine and xylazine (1.5 mg and 150 μg/mouse, respectively) were used for anesthesia and analgesia. The reverse passive Arthus reaction was induced in the airways of the animal by intratracheal injection of 40 μl containing 16, 40, or 160 μg of rabbit anti-ovalbumin IgG (Valent Pharmaceuticals International, Costa Mesa, CA) in water, followed by an intravenous tail vein injection of 100–μl low endotoxin (<0.1 IU/ml) 4 mg/ml ovalbumin (Sigma-Aldrich, Oakville, Ontario, Canada) dissolved in saline (5, 11, 35). Sham-treated mice were C57BL/6 WT and rac2−/− animals that were subjected to intratracheal injection of 40-μl saline followed by intravenous injection of 400-μg ovalbumin. Anti-ovalbumin was...
administered via tracheal injection in anesthetized animals following surgical dissection of the skin surrounding the throat and upper thoracic cavity and separation of the underlying musculature. The antibody was injected by needle and syringe through the second and third tracheal cartilage rings, and immediately following this, the animals were sutured and allowed to recover. Mice were killed 6 h after injections by an intraperitoneal overdose of ketamine and xylazine (15 and 1.5 mg per mouse, respectively). In agreement with previous findings, we determined that maximal lung injury was evident at 6 h past injection of anti-ovalbumin and ovalbumin (23), and significant albumin leakage into the alveoli was obtained at a dose of 160-µg anti-ovalbumin. These procedures were performed in a sterile environment. We chose 160-µg anti-ovalbumin as the optimal dose to detect lung inflammatory events that are dependent on Rac2 expression.

Bronchoalveolar lavage fluid. Collection of bronchoalveolar lavage (BAL) fluid occurred 6 h following injection of intravenous ovalbumin (1-ml PBS × 6). After BAL fluid was centrifuged at 300 g for 5 min, the total and differential cell counts of the BAL fluid were determined from the cell pellet using Diff-Quik (Fisher Scientific, Nepean, Ontario, Canada) on cell cytospins. For analysis of markers in BAL supernatants, 1 ml of PBS was used for BAL collection to ensure an adequate concentration could be measured.

Marker assays for BAL supernatants. Myeloperoxidase (MPO) was assayed using tetramethylbenzidine (TMB) as the substrate, as previously described (2, 32). Briefly, 150 µl of TMB substrate solution was added to 50 µl of BAL in a 96-well microplate and incubated at room temperature for 15 min before termination of the reaction with 50 µl of 1 M H₃PO₄. Microplates were read spectrophotometrically at 450 nm using a Power Wave XS plate reader (BioTek Instruments, Winooski, VT). Absorbance values for background (PBS only) were subtracted from sample MPO values. Lactoferrin was assayed in BAL and cell supernatants by ELISA measurement based on cross-reactivity of human anti-lactoferrin (Sigma-Aldrich) for murine lactoferrin (2). Murine albumin was determined by ELISA (Bethyl Laboratories, Montgomery, TX). Cytokines (IL-1β, IL-17, and TNF), chemokines (CCL3, CXCL1, and CXCL2), and matrix metalloproteinase (MMP-2 and MMP-9) were determined by a customized Pierce SearchLight multiplex ELISA assay system (Pierce, Rockford, IL) or 20 µg/ml mouse anti-MMP-2 (cross-reactive for mouse MMP-2; clone 42-5D11; Millipore, Billerica, MA) or 20 µg/ml rat anti-mouse MMP-9 (clone 116134; R&D Systems, Minneapolis, MN) was detected by biotinylated anti-mouse IgG that was cross-reactive for rat anti-MMP-9, following a mouse-on-mouse block step using the manufacturer’s protocol (Vector M.O.M. Basic Kit; Vector Laboratories, Burlingame, CA). Mouse IgG1 (BD Biosciences Canada, Mississauga, Ontario, Canada) was used at 40 µg/ml in place of primary antibody for isotype controls.

Measurement of O₂•⁻ release from neutrophils. Generation of extracellular O₂•⁻ from cells in suspension was measured as previously described (31). SOD-inhibitable absorbance was calculated using 2.11 × 10⁷ M⁻¹ cm⁻¹ for reduced cytochrome c.

Chimera generation. Mice were irradiated with a single dose of ~10 Gy from a 137Cs source. Following irradiation (18–24 h), mice were reconstituted with freshly prepared bone marrow cells (1 × 10⁶ cells per animal) from donor animals by intravenous injection using a previously reported technique (16). Bone marrow cells were prepared by flushing femurs and tibias by syringe and 26-gauge needles in a laminar flow hood and placing cells in sterile HBSS using a mortar and pestle with femurs and tibias in fetal calf serum and HBSS. Bone marrow cells were counted and resuspended in fresh HBSS at 10 × 10⁶ per milliliter. A volume of 100-µl bone marrow cell suspension was injected through the tail vein 18–24 h after irradiation. Mice were then housed in clean conventional housing for 6–8 wk to allow full bone marrow reconstitution before experimentation.

RESULTS

Rac2 mediates airway neutrophil infiltration and lung injury. Lung injury was induced in alveolar spaces using the reverse passive Arthus reaction. Six hours after intratracheal administration of anti-ovalbumin IgG (16–160 µg) followed by intravenous tail vein injection of ovalbumin (400 µg), mice were euthanized and subjected to BAL. In WT mice, increased BAL leukocyte infiltration occurred, the majority of which were neutrophils (>90% at doses ≥40 µg of antibody; Fig. 1, A and B). In contrast, Rac2-deficient mice showed significantly less cellular inflammation and fewer alveolar neutrophils in
Rac2 is required for leukocyte infiltration and protein leakage in immune complex-mediated acute lung injury (ALI). Mice were treated with increasing doses of intratracheal anti-ovalbumin (40 μl) together with intravenous ovalbumin (400 μg). Control mice were subjected to sham surgery with intratracheally administered saline (40 μl) followed by intravenous ovalbumin (400 μg). Bronchoalveolar lavage (BAL) samples were analyzed from mice euthanized at 6 h postinjection for total cell counts (A), neutrophil counts (B), and albumin (C) in wild-type (WT) and rac2−/− mice (n = 11–17). *P < 0.05; **P < 0.01; ***P < 0.001. Ab, antibody.

Fig. 1. Rac2 is required for leukocyte infiltration and protein leakage in immune complex-mediated acute lung injury (ALI). BAL samples from WT ALI contained a mixture of airway and systemic blood cells as well as structural cells. Thus multiple mechanisms unrelated to Rac2 may become activated when large doses are used, which would complicate our interpretations of the findings. Therefore, we chose to use the lower dose of 160 μg of anti-ovalbumin (with 400 μg of ovalbumin) in the following experiments to determine the specific role of Rac2 in lung inflammation and injury.

Lung injury was measured by determining albumin concentrations in BAL fluid as an indication of plasma leakage. Albumin levels were increased in BAL from WT controls at the highest dose of anti-ovalbumin tested (160 μg; Fig. 1C). However, in Rac2-deficient animals, albumin did not increase over control (ovalbumin-injected) animals, indicating that injury was attenuated. These parameters remained similar at 24 h postinduction of ALI (data not shown).

Lung parenchyma in WT and rac2−/− ALI was examined by histology (Fig. 2A). Quantitative histology on lung sections indicated that WT ALI consistently showed a patchy distribution of inflammatory foci, with focal hemorrhage, intraalveolar neutrophils, as well as neutrophilic inflammation in interstitial spaces, septal walls, and airway walls (Fig. 2B). These were significantly reduced in rac2−/− ALI, although some parenchymal neutrophilic accumulation was evident in these animals.

Neutrophils are recruited to lung interstitium in Rac2-deficient mice. Neutrophils from Rac2-deficient mice have been shown to have defective chemotaxis related to altered F-actin dynamics while expressing normal levels of adhesion molecules CD11a, CD11b, CD11c, CD18, and CD61 (19, 22, 41). To determine the extent of neutrophil recruitment to lungs in rac2−/− ALI, immunofluorescence of neutrophils (Gr-1+ cells) was determined in lung sections from mice with ALI. Neutrophil recruitment to the lung interstitium was observed in both WT and Rac2-deficient ALI (Fig. 2C). More neutrophils were found in the lung parenchyma of Rac2-deficient ALI than in control animals injected with ovalbumin only, as determined by the percentage of Gr-1+ immunofluorescence averaged from 10 fields (at ×200 magnification) randomly chosen from mid-sagittal lung sections (Fig. 2D). At higher magnifications, Gr-1+ neutrophils were evident in WT ALI, but were rare in rac2−/− ALI, neutrophils were less evident in alveolar spaces (Fig. 2E).

Cytokines and chemokines in airways of Rac2-deficient mice are elevated during lung injury. To investigate the possibility that reduction of neutrophil egression into the airways in Rac2-deficient mice may be related to reduced cytokine or chemokine release, we measured BAL levels of a selected group of immunomodulatory cytokines. IL-1β, TNF, and the murine homolog of human IL-8, CXCL1 (formerly keratinocyte-derived cytokine; KC) are critical for recruitment of neutrophils and initiating injury in this model (39). IL-17 released from T cells is a potent stimulus for stromal endothelial cells, leading to IL-8-dependent neutrophil recruitment (29). CXCL1 is required to establish a chemotactic gradient for neutrophils from damaged epithelium by binding to syndecan-1, generated by matrilysin-induced cleavage of this heparin sulphate proteoglycan bound to epithelial cells (33). In addition, CCL3 (formerly macrophage inflammatory pro-
neutrophils (arrows) into alveolar spaces in WT but not $\text{rac}^{2-/-}$ mice. Sections were stained for the neutrophil marker Gr-1 (green) shown in combination with the epithelial cell marker surfactant protein C (SP-C; red) and counterstained with nuclear marker 4'-diamidino-2-phenylindole (DAPI; blue). Scale bar, 100 μm.

Fig. 3. Cytokine and chemokines profiles in immune complex-mediated ALI from WT and $\text{rac}^{2-/-}$ mice. Values are shown for cytokines (IL-1β, IL-17, and TNF) and chemokines (CCL3, CXCL1, and CXCL2) in BAL samples from mice treated with increasing doses of anti-ovalbumin and 400 mg ovalbumin ($n = 5$). **$P < 0.01$. KO, knockout.

Rac2 is required for release of MMP-2 and MMP-9 in lung injury. We next determined the levels of granule-derived mediators MPO, lactoferrin, and MMP-2/-9 in BAL samples obtained from WT and Rac2-deficient mice with ALI. In $\text{rac}^{2-/-}$ animals with ALI, MPO was reduced in BAL samples (Fig. 4A). In contrast, BAL levels of lactoferrin were elevated in both WT and Rac2-deficient mice during lung injury (Fig. 4B). These findings suggest that Rac2$^{2-/-}$ neutrophils recruited to lung interstitial spaces release secondary granules, enriched in lactoferrin, into parenchymal tissues and alveolar spaces.

Interestingly, MMP-2 and MMP-9 were also significantly diminished in BAL from Rac2-deficient ALI (Fig. 4 C and D). Reduced activity of MMP-2/-9 in BAL samples from Rac2-deficient mice was confirmed by gelatin zymography (data not shown). In vitro experiments with Rac2$^{2-/-}$ neutrophils demonstrated decreased MMP-2 and MMP-9 release on immune complex stimulation (P. Lacy, unpublished observations).

However, MMP-2 and MMP-9 expression was not specific to neutrophils, as determined by immunohistochemical analysis of lung sections (Fig. 4, E and F). Staining for MMP-2/-9 was not strongly evident in control WT or $\text{rac}^{2-/-}$ animals, although there was faint staining in bronchial epithelial cells. In WT ALI, elevated MMP-2 and MMP-9 expression was present in tissue inflammatory cells, predominantly in alveolar macrophages, with weaker staining associated with interstitial neutrophils (Fig. 4E, circle, and 4F, magnified inset). Although some staining was evident in airway epithelial cells in WT control and ALI lung sections, there was no marked increase in MMP-2 or MMP-9 in epithelial cells from ALI lungs. In contrast, lung sections from $\text{rac}^{2-/-}$ ALI showed greatly diminished levels of all cells positive for MMP-2 and MMP-9.
AJP-Lung Cell Mol Physiol • VOL 297 • DECEMBER 2009 • www.ajplung.org

A

B

C

D

E

F
particular alveolar macrophages, in correlation with reduced numbers of MMP-2/-9-positive neutrophils.

Since IgG-immune complex-mediated ALI has been shown to be dependent on neutrophil-derived oxidants (27), we tested the possibility that production of oxygen radicals may also be reduced in rac2⁻/⁻ ALI. However, WT and rac2⁻/⁻ ALI lung sections did not show increased antibody staining of the oxidant, nitrotyrosine, over control lungs, even at 160-μg antibody (data not shown). In addition, we tested for oxidative stress markers (using commercial assays from Hycult for nitrotyrosine and from OxisResearch for lipid peroxidation markers and oxidized glutathione) in BAL samples and found no increases in WT or rac2⁻/⁻ ALI mice over control animals. Specifically, each of the nitrotyrosine, lipid peroxidation, and kinetic GSH/GSSG assays demonstrated robust standard curve responses, but none of the BAL samples from sham-treated and injured WT and rac2⁻/⁻ mice showed significant increases above baseline absorbance values (data not shown). However, when bone marrow neutrophils were isolated from WT and rac2⁻/⁻ mice and stimulated in vitro to generate superoxide by measurement of cytochrome c reduction, significant attenuation in superoxide production was detected in Rac2⁻/⁻ neutrophils (Fig. 5). These data suggest that neutrophils derived from rac2⁻/⁻ bone marrow were deficient in generating oxidants, although we did not find significant changes in the levels of products of oxidant-related damage in the airways.

Defect in transepithelial migration is associated with hematopoietic cells. Transepithelial migration of neutrophils in Rac2-deficient mice may be related to a defect in Rac2 expression in hematopoietic and/or nonhematopoietic tissues. To investigate this, we utilized adoptive transfer of marrow cells from Rac2-deficient mice into lethally irradiated WT mice to generate chimeras. These were compared with irradiated rac2⁻/⁻ mice reconstituted with CD45.1⁺ WT bone marrow. As controls, we reconstituted irradiated WT mice with CD45.1⁺ WT bone marrow and Rac2-deficient recipient mice with Rac2-deficient marrow. This chimeric model has been shown to reconstitute 99% of peripheral blood neutrophils (16). After bone marrow reconstitution (6–8 wk), chimeras were subjected to ALI using 160-μg anti-ovalbumin and 400-μg ovalbumin and compared with ovalbumin-only controls. This dose of antibody was chosen for its ability to increase vascular leakage in WT mice (Fig. 1C). To confirm marrow replacement with donor cells in the airways during ALI, we determined the percentage of CD45.1⁺/Gr-1⁺ cells in BAL fluid from CD45.1⁺/CD45.1⁻ chimeras. We found that 82 ± 5% of Gr-1⁺ BAL neutrophils were from donor animals (CD45.1⁺/Gr-1⁺) in WT/WT chimeras with ALI (n = 8). This did not differ significantly from WT/Rac2⁻/⁻, which had 79 ± 8% CD45.1⁺/Gr-1⁺ neutrophils in their BAL fluid following ALI (n = 5). There were insignificant levels of CD45.1⁺/Gr-1⁺ neutrophils detected in BAL fluid from WT/WT or WT/Rac2⁻/⁻ control animals or from chimeras that had received rac2⁻/⁻ bone marrow transplants. These observations indicate that the majority of neutrophils in the airways of WT/WT and WT/Rac2⁻/⁻ chimeras with ALI were derived from CD45.1⁺ donor bone marrow, confirming marrow engraftment along with neutrophil proliferation and recruitment to the airways during ALI. In addition, engraftment and recruitment of WT bone marrow in rac2⁻/⁻ recipients appeared to be similar to WT/WT chimeras, indicating that there were no defects in WT donor neutrophil reconstitution or accumulation in the lungs of WT/Rac2⁻/⁻ animals with ALI.

In bone marrow chimeras, WT mice reconstituted with WT bone marrow (WT/WT) exhibited significant increases in total cell numbers in BAL samples in ALI, most of which were neutrophils (Fig. 6, A and B). The numbers of alveolar macrophages did not vary between any of the control and ALI chimeras. This suggests that the numbers of macrophages recruited to the airways were unaffected by immune complex activation in bone marrow chimeras. In contrast, irradiated WT mice injected with Rac2-deficient marrow (Rac2⁻/⁻/WT) had reduced BAL total cell numbers compared with WT/WT ALI, which was largely the result of decreased neutrophil numbers, with no significant changes in alveolar macrophages, dendritic cells, or lymphocytes. Irradiated Rac2-deficient mice reconstituted with WT bone marrow (WT/Rac2⁻/⁻) did not significantly differ in BAL cell numbers from WT/WT ALI, suggesting that Rac2-dependent inflammation and injury in this model was specifically dependent on hematopoietically derived cells. Rac2-deficient mice reconstituted with Rac2-deficient marrow (Rac2⁻/⁻/Rac2⁻/⁻) exhibited reduced inflammation similar to that found in WT mice given Rac2-deficient bone marrow (Rac2⁻/⁻/WT).

![Fig. 5](http://ajplung.physiology.org/) Superoxide generation from bone marrow neutrophils obtained from WT and rac2⁻/⁻ mice. Freshly isolated bone marrow neutrophils were stimulated with PMA (500 ng/ml), and O₂⁻ production was measured using cytochrome c reduction by spectrophotometry. *P < 0.05 (n = 4).

![Fig. 4](http://ajplung.physiology.org/) Proinflammatory mediator release in lung injury responses to immune complexes. WT and rac2⁻/⁻ mice were treated with increasing doses of immune complexes, and levels of myeloperoxidase (MPO; A), lactoferrin (B), matrix metalloproteinase-2 (MMP-2; C), and MMP-9 (D) were assayed in BAL samples (n = 5). **P < 0.05; ***P < 0.01; ****P < 0.001; n.s., not significant. E: immunohistochemistry of paraffin-embedded lung sections from WT and rac2⁻/⁻ animals following control treatment (ovalbumin alone) and ALI showing MMP-2 and MMP-9 immunoreactivity indicated by the red/brown diaminobenzidine (DAB) stain. Sections were counterstained by hematoxylin (blue) to show lung morphology. Arrows indicate alveolar macrophages that were positive for MMP-2 or MMP-9. F: magnified section of lung from WT ALI showing MMP-2 immunoreactivity in alveolar macrophages. Circle, region showing neutrophilic inflammation showing staining for MMP-9. Scale bar, 100 μm.
Similar trends of vascular leakage (albumin) and MPO release were observed in WT/WT compared with WT/Rac2−/−/H11002/H11002 chimeras, which were significantly reduced in Rac2−/−/H11002/H11002/Rac2−/− and Rac2−/−/Rac2−/−/H11002/Rac2−/− chimeras (Fig. 6, C–E). Levels of albumin (C), MPO (D), and lactoferrin (E) are also shown from the same samples. *P < 0.05; **P < 0.01; ***P < 0.001.

DISCUSSION

In this study, we show that the Rho GTPase, Rac2, has an essential role in the pathogenesis of ALI. Disruption of the gene encoding Rac2 significantly attenuated lung inflammation and injury induced by immune complex deposition. Neutrophils were observed to accumulate in interstitial lung tissues during ALI in rac2−/−/H11002/H11002 animals, but few transmigrated into alveolar spaces. The levels of alveolar MPO and MMP-2/9 were attenuated in rac2−/−/H11002/H11002 mice with ALI; however, this was not significant based on ANOVA and post hoc analysis.

Levels of IL-1β in BAL samples were increased in WT/WT ALI over Rac2−/−/H11002/H11002, although TNF secretion was similar across all chimeras (Fig. 7, A and B). MMP-2/9 levels were also reconstituted in WT/WT mice compared with Rac2−/−/H11002/H11002 ALI (Fig. 7, C and D). These findings suggest that alveolar neutrophil recruitment and subsequent lung injury in response to immune complex-mediated ALI and release of MPO, IL-1β, and MMP-2/9 are dependent on Rac2-expressing hematopoietic cells.
as neutrophil migration into alveolar spaces, resulting in lung injury. Although disruption of the gene encoding Rac2 attenuated immune complex-mediated lung injury, it did not abolish the pathology. At higher doses of antibody in rac2−/− mice with ALI, increased alveolar neutrophil counts were observed, suggesting that other pathways may compensate for Rac2-mediated cell transmigration in this model, such as Rac1, Rac3, Cdc42, and Rho.

ALI and ARDS are characterized by interstitial lung inflammation and injury. Inflammation in these disorders is associated with infiltration of leukocytes including monocytes/macrophages and neutrophils and involves endothelial and epithelial barrier dysfunction. Recruited leukocytes generate proinflammatory mediators, leading to edema and alveolar hemorrhage, a process associated with clinical findings of ALI defined by rapid-onset bilateral pulmonary infiltrates and hypoxemia (4, 36). Immune complex-mediated ALI is an established model that most closely resembles transfusion-related ALI (TRALI), which is dependent on Fcγ receptor activation by IgG complexed to antigen or cellular receptors (34, 45). Lung injury in this model has previously been shown to be dependent on activated neutrophils releasing reactive oxygen species and toxic granule-derived mediators including MPO and lactoferrin (7, 23, 26–28).

In murine models of IgG immune complex-mediated ALI, the inflammatory cascade begins with cross-linking of FcγRIII receptors (CD16) on macrophages by immune complexes along with immune complex-mediated production of complement C5a (25, 46). Alveolar macrophages are activated and quickly release proinflammatory cytokines TNF and IL-1β (23), upregulating selectins and ICAM-1 on vascular endothelial cells to facilitate adhesion of neutrophils and their transmigration to sites of immune complex deposition (47). In addition, activated alveolar macrophages generate a range of chemokines to attract neutrophils, including CCL3, CXCL1, and CXCL2 (6, 18, 23). Neutrophils are recruited from the bloodstream and are activated to release reactive oxygen species and toxic granule-derived mediators including MPO and lactoferrin (7, 23, 26–28). Other mediators released from a variety of lung cells that contribute to injury include MMP-2 and MMP-9 (21).

Lung injury induced by immune complexes is dependent on MMP-2/-9, released by many cells, including macrophages, fibroblasts, smooth muscle cells, and neutrophils (14, 21). Macrophages are the main cell types that express MMP-2/-9 during immune complex-mediated ALI (21, 38). We found elevated levels of MMP-2/9-positive cells in lungs from WT ALI, with the majority of these cells being morphologically similar to alveolar macrophages and interstitial neutrophils. In contrast, MMP-2/-9-positive cells were infrequently observed in rac2−/− ALI. These findings suggest that Rac2 has a previously unrecognized role in generating MMP-2 and MMP-9 (21).

Fig. 7. Release of cytokines and metalloproteases in BAL samples from chimeras. IL-1β (A), TNF (B), MMP-2 (C), and MMP-9 (D) release was determined in BAL samples obtained from WT and rac2−/− chimeras (n = 6–9). *P < 0.05; **P < 0.01; ***P < 0.001.
There are several possible mechanisms by which gene deletion of Rac2 could lead to reduced injury in ALI. The restricted expression of Rac2 to hematopoietic tissues and our finding regarding the reconstitution of injury in WT/Rac2\(^{-/-}\) chimeras with ALI suggest that Rac2 mainly functions in macrophages, neutrophils, and potentially other cells derived from bone marrow. Based on our immunohistochemistry for MMP-2/9 in lung sections (Fig. 4F), we speculate that Rac2 may be a regulatory GTPase for exocytosis of secretory products including MMP-2/9 from macrophages and neutrophils. Other possible mechanisms for Rac2 in ALI include F-actin-mediated neutrophil chemotaxis and recruitment as well as NADPH oxidase-associated oxidant production from neutrophils. All of these events may collectively serve to diminish recruitment and activation of inflammatory cells to the airways in response to immune complex.

In neutrophils, the function of Rac2 is related to adhesion, chemotaxis, activation of NADPH oxidase, and primary granule release (2, 9, 10, 41). In the present study, neutrophil numbers were increased in lung interstitium, although they were not elevated in the alveolar spaces in rac2\(^{-/-}\) mice with ALI. Since rac2\(^{-/-}\) mice have a 2.5- to 3-fold higher level of circulating neutrophils than their WT counterparts (41), reduced neutrophil numbers in BAL samples in rac2\(^{-/-}\) mice are not likely related to lower circulation numbers. It is conceivable that altered neutrophil actin remodeling, an expected phenotype of Rac2 deficiency, might cause dysregulated neutrophil retention in pulmonary capillaries (48). There are three possible stages that could be affected in transepithelial migration of neutrophils: adhesion, transmigration, and migration. Rac2\(^{-/-}\) neutrophil adhesion has been shown to be reduced to glycosylation-dependent cell adhesion molecule 1 (GlyCAM-1) and P-selectin, and Rac2\(^{-/-}\) cells show defective chemotactic responses to formyl-Met-Leu-Phe and IL-8 (41). A limitation of the findings in this study is that we could not determine where the defect in the recruitment cascade occurred in rac2\(^{-/-}\) mice with ALI.

Similar levels of proinflammatory cytokines (TNF and IL-17) and chemokines (CCL3, CXCL1, and CXCL2) were found in the BAL fluid of immune complex-treated WT and rac2\(^{-/-}\) mice, with the exception of IL-1β. Neutrophil retention in the lung parenchyma of rac2\(^{-/-}\) mice was not likely to be caused by deficient IL-1β production, since antibody to IL-1β prevented neutrophil accumulation in the lungs, thereby producing a different phenotype from that of rac2\(^{-/-}\) mice with ALI (39).

Rac2 is an essential regulatory protein for NADPH oxidase activation in neutrophils (41). The model of IgG immune complex-mediated ALI used in this study is specifically dependent on production of toxic oxygen metabolites from neutrophils (27). Application of intratracheal catalase to scavenge H\(_2\)O\(_2\) from alveolar spaces prevented alveolar hemorrhage and edema in ALI but did not reduce neutrophil recruitment to interstitial tissues. In contrast, SOD, a highly specific scavenger for superoxide, did not reduce lung injury in this model (27). We could not detect oxygen metabolites in lung tissue from animals with ALI by nitrotyrosine labeling (generated from peroxynitrite reaction with tyrosine residues) or assay for oxidative stress products. Evidence from our study suggests that bone marrow neutrophils from rac2\(^{-/-}\) mice were deficient in generating superoxide, in agreement with previous reports (40, 41). Therefore, it is possible that reduced injury in rac2\(^{-/-}\) mice with ALI is related to the defect in superoxide release from neutrophils, although we could not detect products of oxidant-related activity in the lungs to confirm this. The inability to detect oxidant-related injury in this mouse model differs from previous studies using a rat model, on which this study was based, by Gao and colleagues (20). These findings suggest that there may be a species difference in the dependency of lung injury on the production of toxic oxygen metabolites, which has not been reported before. Of interest, clinical findings demonstrate that antioxidants fail to reverse or attenuate ALI (17).

We found significantly diminished levels of MPO in BAL from rac2\(^{-/-}\) ALI compared with ALI in WT mice. In contrast, secondary granule lactoferrin was elevated in WT and rac2\(^{-/-}\) mice with ALI, suggesting that Rac2\(^{-/-}\) neutrophils in the parenchyma were releasing secondary granules in response to immune complex stimulation. Although it is possible that MPO and MMP levels were diminished due to fewer neutrophils in the BAL, our finding of high levels of lactoferrin suggests that this mediator diffused from activated tissue neutrophils into alveolar spaces where it was detected in BAL samples. These observations correlate with in vitro findings showing a dependency of primary granule exocytosis on Rac2, whereas secondary granule release of lactoferrin was independent of Rac2 function (2).

Bone marrow chimera data generated from WT and rac2\(^{-/-}\) mice with ALI demonstrated that reduced lung injury was associated with bone marrow-derived, Rac2-expressing hematopoietic cells. The number of BAL cells in WT/Rac2\(^{-/-}\) chimeras, compared with WT/WT ALI, was significantly diminished during lung injury. However, these chimera do not distinguish between the various hematopoietic cell types that express Rac2, including macrophages and neutrophils, which are fully reconstituted by donor cells in bone marrow chimeras by 6–8 wk after irradiation (37). With regard to the apparent increase in BAL MPO in Rac2\(^{-/-}\)/Rac2\(^{-/-}\) chimeras with ALI, it is important to note that total cells and neutrophils were also slightly elevated in rac2\(^{-/-}\) mice with ALI at the same dose of antibody used for the chimera experiments (160 μg).

In chimeras using irradiated animals, WT/WT and WT/Rac2\(^{-/-}\) animals with ALI showed an increased density of total cells and neutrophils, along with elevated albumin and cytokine levels in BAL, compared with nonirradiated WT and rac2\(^{-/-}\) mice with ALI. It is speculated that these increased levels were related to radiation treatment, which systemically enhances proinflammatory cytokine expression.

In summary, Rac2 gene deletion significantly attenuated lung inflammation and injury induced by immune complex deposition. A salient finding in this study is that Rac2 may be important for MMP-2 and MMP-9 generation by alveolar macrophages and neutrophils as well as for neutrophil transmigration into the alveolar spaces during injury. Based on our observations, we have shown that Rac2-mediated injury in this model may not be dependent on the production of toxic oxygen metabolites in the lungs. These findings suggest a novel function for Rac2 in acute inflammatory processes and provide potential targets for therapeutic intervention in ALI and its associated complications.
ACKNOWLEDGMENTS

We thank Robin Stocks and Tom Turner in the Department of Pathology for their assistance with immunohistochemistry and generating images of lung sections.

GRANTS

This study was supported by Canada Foundation for Innovation, the Canadian Institutes of Health Research, SickKids Foundation, Alberta Heritage Foundation for Medical Research, Natural Sciences and Engineering Research Council of Canada, National Institutes of Health Grant HL-027068, and the Canadian Lung Association.

DISCLOSURES

The authors have no conflict of interest to declare for this study.

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