NADPH oxidase mediates synergistic effects of IL-17 and TNF-α on CXCL1 expression by epithelial cells after lung ischemia-reperfusion

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Sharma AK, Mulloy DP, Le LT, Laubach VE. NADPH oxidase mediates synergistic effects of IL-17 and TNF-α on CXCL1 expression by epithelial cells after lung ischemia-reperfusion. Am J Physiol Lung Cell Mol Physiol 306: L69–L79, 2014. First published November 1, 2013; doi:10.1152/ajplung.00205.2013.—Ischemia-reperfusion (I/R) injury leads to increased mortality and morbidity in lung transplant patients. Lung I/R injury involves inflammation contributed by innate immune responses. IL-17 and TNF-α, from iNKT cells and alveolar macrophages, respectively, contribute importantly to lung I/R injury. This study tests the hypothesis that IL-17 and TNF-α synergistically mediate CXCL1 (a potent neutrophil chemokine) production by alveolar type II epithelial (ATII) cells via an NADPH oxidase-dependent mechanism during lung I/R. Using a hilar clamp model, wild-type and p47phox−/− (NADPH oxidase-deficient) mice underwent left lung I/R, with or without recombinant IL-17 and/or TNF-α treatment. Wild-type mice undergoing I/R treated with combined IL-17 and TNF-α had significantly enhanced lung dysfunction, edema, CXCL1 production, and neutrophil infiltration compared with treatment with IL-17 or TNF-α alone. However, p47phox−/− mice had significantly less pulmonary dysfunction, CXCL1 production, and lung injury after I/R that was not enhanced by combined IL-17-TNF-α treatment. Moreover, in an acute in vitro hypoxia-reoxygenation model, murine ATII cells showed a multifold synergistic increase in CXCL1 expression after combined IL-17-TNF-α treatment compared with treatment with either cytokine alone, which was significantly attenuated by an NADPH oxidase inhibitor. Conditioned media transfer from hypoxia-reoxygenation-exposed iNKT cells and macrophages, major sources of IL-17 and TNF-α, respectively, to ATII cells significantly enhanced CXCL1 production, which was blocked by NADPH oxidase inhibitor. These results demonstrate that IL-17 and TNF-α synergistically mediate CXCL1 production by ATII cells after I/R, via an NADPH oxidase-dependent mechanism, to induce neutrophil infiltration and lung I/R injury.

Lung transplant; alveolar epithelial cells; ischemia-reperfusion injury; primary graft dysfunction

LUNG TRANSPLANTATION CONTINUES to be the primary treatment strategy for many end-stage pulmonary diseases. However, the long-term prognosis of patients undergoing this procedure remains dismal because of development of chronic rejection leading to ~50% mortality five years after transplant (15, 39). Lung ischemia-reperfusion (I/R) injury is the major cause of primary graft dysfunction after lung transplantation, which increases the risk for the development of chronic rejection in the form of bronchiolitis obliterans in these patients (6, 40). We have previously reported that iNKT cell-produced IL-17 and alveolar macrophage-produced TNF-α are critical initiators of lung inflammation and injury after I/R (33, 43). Although iNKT cells and alveolar macrophages play a critical role in the innate immune responses after lung I/R, alveolar type II epithelial (ATII) cells also contribute to lung injury and recruitment of neutrophils via the production of potent chemokines such as CXCL1 (2). Therefore, the present study investigates the effects of IL-17 and TNF-α on ATII cell activation and production of CXCL1 and subsequent neutrophil infiltration during lung I/R injury.

IL-17 is a proinflammatory cytokine involved in the pathogenesis of certain diseases via the coordination of local tissue inflammation through the upregulation of proinflammatory cytokines and chemokines (e.g., TNF-α, CXCL1, CXCL2, and CCL2). In the setting of lung I/R, we have previously documented that iNKT cells can rapidly produce IL-17, which then orchestrates lung inflammation and injury via neutrophil recruitment (33). A potent, one-way relationship exists between macrophages and ATII cells wherein macrophage-produced TNF-α directly stimulates CXCL1 production from ATII cells (32). However, relatively little information is known as to the precise mechanistic action of cellular cross talk between iNKT cells, alveolar macrophages, and ATII cells. Therefore, our first hypothesis was that IL-17 can induce CXCL1 production from ATII cells. Previous reports have also indicated that IL-17, in concert with TNF-α can synergistically increase the expression of chemokines (16, 24, 36). Therefore, our second hypothesis was that IL-17 and TNF-α synergistically effect ATII cell-dependent CXCL1 production after I/R.

It is well established that production of reactive oxygen species (ROS) plays an important role in lung I/R injury (20, 27). The potential enzymatic sources of ROS after I/R include NADPH oxidase, nitric oxide synthase, xanthine oxidase, and the mitochondrial respiratory chain (31, 44). During hypoxia or ischemia, it has been shown that pulmonary epithelial or endothelial cell-derived ROS from NADPH oxidase is a crucial factor in mediating inflammation and tissue injury (11, 28). In the present study, we used p47phox−/− mice (deficient in the p47phox subunit of NADPH oxidase) and apocynin (an NADPH oxidase inhibitor) to test our third hypothesis: that a mechanism of CXCL1 generation by ATII cells in response to IL-17 and TNF-α after I/R entails NADPH oxidase activation.

With the use of a murine in vivo hilar clamp model of lung I/R and an in vitro hypoxia-reoxygenation model, the current study demonstrates that IL-17 and TNF-α synergistically activate ATII cells to produce CXCL1. This synergistic activation is attenuated by blockade of NADPH oxidase-dependent superoxide generation, resulting in decreased CXCL1 production, neutrophil infiltration, and subsequent lung inflammation and injury. The results of this study offer new insight into the molecular mechanisms of IL-17- and TNF-α-mediated signaling pathways contributing to lung I/R injury and signify that NADPH oxidase in ATII cells may be a novel therapeutic

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target to prevent or treat lung I/R injury and primary graft dysfunction in lung transplant patients.

MATERIALS AND METHODS

Animals. This study used 8- to 12-wk-old male C57BL/6 wild-type mice (Jackson Laboratory, Bar Harbor, ME) and p47phox−/− mice (C57BL/6-J-Ncf1m/m; Jackson Laboratory). The p47phox−/− mice, which are congenic to C57BL/6 mice, are deficient in the p47phox subunit of NADPH oxidase, thus preventing NADPH oxidase-mediated ROS production. Mice were randomly assigned to different groups that underwent either sham surgery or lung I/R. This study conformed to the National Institutes of Health guidelines and was conducted under animal protocols approved by the University of Virginia’s Institutional Animal Care and Use Committee.

Lung I/R model. An in vivo hilar clamp model of lung I/R was used wherein mice undergoing I/R were subjected to 1 h left lung ischemia (via left hilar occlusion) followed by 2 h of reperfusion as previously described (41). Sham animals received the same surgery but without hilar occlusion. Mice were anesthetized with inhaled isoflurane, intubated with PE-60 tubing, and connected to a pressure-controlled ventilator (Harvard Apparatus, South Natick, MA). Mechanical ventilation with room air was performed at 150 strokes/min, 0.5 ml stroke volume, and peak inspiratory pressure <20 cmH2O. Heparin (20 U/kg) was given immediately preceding the ischemic period via external jugular injection to minimize thrombosis in the pulmonary vasculature during ischemia. Left thoracotomy was performed by dividing the left third rib, and the left hilum was exposed. A 6–0 prolene suture was passed around the left hilum facilitated by a tip-curved (22-G) gavage needle. Both ends of the suture were threaded through a 5-mm-long PE-50 tube. Hilar occlusion was achieved by synching down the hilar suture and securing the PE-50 tube with a small surgical clip to maintain consistent tension against the hilum. The thoracotomy was then closed with surgical clips, and the mouse was extubated and allowed to recover during the entire 1-h hilar occlusion period. Mice were extubated during the ischemic and reperfusion periods to minimize injury due to mechanical ventilation. The average total time on the ventilator for each animal was <7 min. Five minutes before reperfusion, the mouse was reanesthetized and reintubated. Reperfusion was achieved by removing the clip, tube, and hilar suture. Again, the chest was closed with surgical clips. The mouse was extubated and placed back in the cage during the 2-h reperfusion period. To minimize pain and discomfort, an analgesic (0.2 mg/kg buprenorphine) was administered to all animals at the beginning of surgical intervention.

Cytokine treatment of mice. Treatment of mice with recombinant TNF-α (625 ng/ml) or IL-17 (500 ng/ml) (R&D Systems, Minneapolis, MN) was performed intratracheally 5 min before ischemia. These doses were based on a previous study by Liu et al. (24). Following inhalational anesthesia with isoflurane, the trachea was exposed in the cervical midline and circumferentially controlled with a silk-suture tie. Orotracheal intubation with PE-60 tubing permitted mechanical ventilation, confirming appropriate placement before injection. The silk suture was then tied to secure the endotracheal tube and to limit leakage of the tracheal treatment. Saline alone or saline plus cytokine(s) (50 μl total volume) was then injected intratracheally in each mouse. The endotracheal tube was then reconnected to the ventilator to promote distal passage of the treatment into the lungs.

Pulmonary function. At the end of the 2-h reperfusion period, pulmonary function was evaluated using an isolated, buffer-perfused mouse lung system (Hugo Sachs Elektronik, March-Hugstetten, Germany) as previously described (41). Mice were anesthetized with ketamine and xylazine. A tracheostomy was performed, and animals were ventilated with room air at 100 breaths/min at a tidal volume of 7 μl/g body wt with a positive end-expiratory pressure of 2 cmH2O using a MINIVENT mouse ventilator (Hugo Sachs Elektronik). Animals were exsanguinated by inferior vena cava transection. The subdiaphragmatic portion of the animal was excised and discarded. The anterior chest plate was removed, exposing the lungs and heart. A 4–0 silk suture was passed behind the pulmonary artery (PA) and the aortic root. A partial half-knot was created with the suture, leaving room for the cannula to be passed into the PA. A small curvilinear
incision was made in the right ventricular outflow tract with the perfusate flowing at 0.2 ml/min, and the PA cannula was passed through the pulmonary valve into the PA. The partial half-knot was then tightened. The left ventricle was immediately vented with a small incision at the apex of the heart. The mitral apparatus was carefully dilated, and the left atrial cannula was passed through the mitral valve into the left atrium. The placement of the left atrial and the PA cannulas was further confirmed by pressure tracings generated by the PULMODYN data acquisition system (Hugo Sachs Elektronik). The airway resistance describes the resistive forces to airflow in the airways and is calculated from the relation between transpulmonary pressure and velocity of airflow. Lung compliance is an index of the functional stiffness of the lung and is calculated from the relation between tidal volume and transpulmonary pressure.

Myeloperoxidase measurement. Myeloperoxidase (MPO) levels were measured in BAL fluid using a mouse MPO ELISA kit (Hycult Biotech, Uden, The Netherlands). MPO is abundant in the azurophilic granules of polymorphonuclear neutrophils and was used as an indicator of neutrophil activation and infiltration into alveolar airspaces.

Lung wet/dry weight. Lungs were weighed and then desiccated until a stable dry weight was achieved. Lung wet-to-dry weight ratio was then calculated as an indicator of edema. Separate groups of animals that did not undergo BAL were used to measure lung wet/dry weight.

Immunohistochemistry. Immunostaining to identify neutrophils was performed as described previously (41). Lungs were inflation-fixed at 20 cmH2O with 4% paraformaldehyde and paraffin embedded. Immunostaining of lung sections was performed with rat anti-mouse neutrophil antibody (GR1.1; Santa Cruz Biotechnology) using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Purified normal rat immunoglobulin G (eBioscience, San Diego, CA) was used as a negative control. Alkaline phosphatase-conjugated anti-rat immunoglobulin G (Sigma Aldrich) was used as the secondary antibody, and signals were detected with Fast-Red (Sigma Aldrich). Sections were counterstained with hematoxylin. For each lung section, neutrophils were counted in five random fields at 40× magnification and averaged. These counts did not distinguish between cells in various compartments of the lung (e.g., airspace, interstitial, or vascular) but included all cells in peripheral lung.

In vitro hypoxia-reoxygenation. Primary murine iNKT cells and primary alveolar macrophages were isolated as previously described (32, 33) and cultured overnight in RPMI media containing 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA) at 37°C and 5% CO2. For exposure to hypoxia-reoxygenation (HR), 24-well culture plates were placed in a humidified, sealed hypoxia chamber (Billups-Rothenberg, Del Mar, CA) that was purged with 95% N2 and 5% CO2 for 25 min to establish hypoxia as described previously (32). The chamber was then placed in a cell culture incubator for 3 h after which it was opened, and the culture media was immediately analyzed for O2 concentration using a blood-gas analyzer (Chiron Diagnostics). The partial percentage of O2 in the culture media after hypoxia exposure was consistently found to be 5% vs. 21% in normoxic cultures. Reoxygenation was achieved by removing the plates from the hypoxia chamber and placing them in a normoxic, humidified incubator (37°C, 5% CO2) for 1 h. Conditioned media transfer (CMT) experiments were performed by exposing iNKT cells or alveolar macrophages to HR followed by transfer of this conditioned media (1:1 ratio) to washed ATII cells (MLE12 murine ATII cell line; ATCC, Manassas, VA), which were then exposed to HR. CXCL1 in culture media was measured by ELISA (R&D Systems).

ROS measurement. For confocal microscopy, ATII cells grown on chamber slides were treated under different conditions as described.
followed by incubation with 10 μM dichlorofluorescein (DCF dye; Molecular Probes, Grand Island, NY) for 5 min and washed with PBS. The images were captured using a Zeiss LSM510 confocal microscope and analyzed by LSM510 image browser software. Quantification of DCF dye in ATII cell lysates was performed using an OxiSelect Intracellular ROS assay kit as instructed (Cell Biolabs, San Diego, CA). All DCF solutions were protected from light to prevent light-induced auto-oxidation. Fluorescence intensity was measured by a fluorometric plate reader at 480/530 nm and expressed as relative fluorescence units.

**NADPH oxidase activity assay.** NADPH oxidase activity was measured in cells using a Lumimax Superoxide Anion Detection Kit (Agilent Technologies, Santa Clara, CA) as per the manufacturer’s instructions.

**Isolation and culture of primary ATII cells.** Primary ATII cells were isolated from C57BL/6 and p47phox−/− mice and cultured as described previously (32). Mice were anesthetized with ketamine and xylazine, the trachea was cannulated, and lungs were removed and perfused via the PA with 0.9% saline to remove blood. Dispersed (1 ml) was rapidly instilled through the tracheal cannula followed by 1 ml agarose, after which the lungs were covered with ice for 2 min to gel the agarose. Lungs were harvested and placed in 1 ml of dispase at room temperature for 45 min. Lungs were then transferred to a 60-mm culture dish containing 7 ml of HEPES-buffered DMEM and 100 U/ml DNase I, and lung tissue was gently teased from the bronchi. The cell suspension was filtered through two cell strainers (100 μm) and centrifuged at 130 g for 8 min at 4°C and placed on prewashed 100-mm culture dishes that had been coated for collagen. Cells were cultured for 5 days before being used for the experiments.

**Pulmonary dysfunction after I/R is exacerbated by IL-17 and TNF-α via NADPH oxidase.** To investigate the effects of exogenous IL-17 and TNF-α on lung dysfunction after I/R, pulmonary function was measured after I/R or sham surgery in WT and p47phox−/− mice pretreated with recombinant IL-17 and/or TNF-α (Fig. 1). WT mice displayed significant pulmonary dysfunction after I/R as indicated by increased airway resistance and PA pressure as well as decreased pulmonary compliance. Lung dysfunction was significantly exacerbated in WT mice undergoing I/R after combined treatment with IL-17 and TNF-α compared with I/R alone. However, there was no difference in lung function of WT mice undergoing I/R after treatment with either IL-17 or TNF-α alone. Furthermore, pulmonary dysfunction after I/R was significantly attenuated in p47phox−/− mice compared with WT mice (Fig. 1). Combined

![Graph](http://ajplung.physiology.org/)

**Fig. 3.** IL-17 and TNF-α enhance neutrophil infiltration and edema after I/R via NADPH oxidase. A: immunohistochemical staining of lung sections was performed to assess neutrophil infiltration after I/R in WT and p47phox−/− mice (p47−/−). Representative images of immunostaining are depicted wherein neutrophils are stained red, sections are counterstained with hematoxylin, and images are at ×40 magnification (bar = 50 μm). The number of neutrophils per high-power field (neutrophils/HPF) (B) and myeloperoxidase (MPO) levels in BAL fluid (C) were significantly increased after I/R in WT mice compared with sham, which were further enhanced by combined treatment with IL-17 and TNF-α. However, treatment of p47phox−/− mice undergoing lung I/R with IL-17 and TNF-α failed to enhance neutrophil numbers or MPO compared with untreated p47phox−/− mice. D: a significant increase in pulmonary edema (wet/dry weight) occurred after I/R in WT mice vs. sham, which was significantly enhanced after treatment with IL-17 and TNF-α. Edema was significantly attenuated in p47phox−/− mice compared with WT mice and remained unchanged after treatment with IL-17 and TNF-α. *P < 0.05 vs. WT sham; **P < 0.05 vs. all; ***P < 0.05 vs. WT IR; n = 5/group.
Fig. 4. Hypoxia-reoxygenation (HR) or TNF-α induces reactive oxygen species (ROS) generation by alveolar type II epithelial (ATII) cells. ROS production was assessed in ATII epithelial cells by immunofluorescence (A) and quantification of fluorescence intensity (B and C) in cells treated with dichlorofluorescein (DCF) dye. A: compared with normoxic conditions (Norm), HR or TNF-α (TNF) treatment, but not IL-17 treatment, markedly increased ROS production in ATII cells, which was attenuated by apocynin (APO) pretreatment. B: quantification of DCF dye in normoxic cells. A significant increase in fluorometric intensity occurred in normoxic cells treated with TNF-α, but not IL-17, compared with normoxia alone. Combined treatment of TNF-α and IL-17 did not further enhance ROS production compared with TNF-α treatment alone. *P < 0.05 vs. Norm; #P < 0.05 vs. Norm+TNF; §§P < 0.05 vs. Norm+IL-17+TNF; n = 5/group. C: quantification of DCF dye in cells exposed to HR (with a normoxic control for comparison). A significant increase in DCF dye fluorescence intensity occurred after HR in ATII cells. TNF-α, but not IL-17, significantly enhanced HR-induced ROS production. However, IL-17 treatment did not contribute to ROS generation in HR-exposed cells with or without TNF-α treatment. *P < 0.05 vs. Norm; #P < 0.05 vs. HR; §§P < 0.05 vs. HR+IL-17; §§§P < 0.05 vs. HR+TNF; **P < 0.05 vs. HR+IL-17+TNF; n = 5/group.
treatment with IL-17 and TNF-α failed to worsen lung function in p47phox−/− mice. There was no difference in lung function of WT and p47phox−/− mice undergoing sham surgeries, and lung function also remained unchanged in p47phox−/− mice undergoing I/R after treatment with IL-17 or TNF-α compared with I/R alone (data not shown). These results demonstrate that a combined treatment with IL-17 and TNF-α exacerbates lung dysfunction after I/R in WT mice, which is dependent on NADPH oxidase activity.

CXCL1 production is synergistically increased after I/R by IL-17 and TNF-α via an NADPH oxidase-dependent pathway. The expression of proinflammatory cytokines and chemokines was measured in BAL fluid to assess pulmonary inflammation. A significant induction of CXCL1 (KC), IL-6, CCL2 (MCP-1), and CCL5 (RANTES) occurred after I/R in WT mice compared with sham (Fig. 2). Treatment of WT mice with IL-17 or TNF-α significantly enhanced CXCL1 production after I/R, only TNF-α enhanced IL-6 production, and neither enhanced CCL2 or CCL5 production. However, combined treatment of WT mice undergoing I/R with IL-17 and TNF-α significantly enhanced production of all four cytokines compared with I/R alone. Importantly, CXCL1 production was synergistically exacerbated to multifold levels (nearly 10-fold) by combined treatment with IL-17 and TNF-α in WT mice undergoing I/R compared with I/R alone. Moreover, production of CXCL1, IL-6, CCL2, and CCL5 was significantly attenuated in p47phox−/− mice after I/R compared with WT mice after I/R (Fig. 2). Treatment of p47phox−/− mice undergoing I/R with combined IL-17 and TNF-α failed to enhance cytokine production. Also, there was no difference in CXCL1 production in WT and p47phox−/− mice undergoing sham surgeries, and CXCL1 levels remained unchanged in p47phox−/− mice undergoing I/R after treatment with IL-17 or TNF-α alone compared with I/R alone (data not shown). These results demonstrate that combined treatment with IL-17 and TNF-α synergistically induces CXCL1 production via an NADPH oxidase-dependent manner.

Neutrophil infiltration and pulmonary edema after I/R are enhanced by IL-17 and TNF-α via NADPH oxidase. To evaluate a potential synergistic role for IL-17 and TNF-α in neutrophil infiltration after I/R, neutrophils were quantified in lung sections by immunohistochemistry (Fig. 3A). Neutrophil numbers were similar between WT and p47phox−/− sham mice (data not shown). Significant infiltration of neutrophils oc-
occurred in WT mice after I/R, which was further enhanced by combined IL-17 and TNF-α treatment (Fig. 3, A and B). Neutrophil infiltration after I/R was significantly attenuated in p47phox−/− mice compared with WT mice and was not significantly elevated by combined IL-17 and TNF-α treatment.

MPO, an enzyme abundantly present in neutrophil azurophilic granules, was measured in BAL fluid as an indicator of neutrophil activation and infiltration into alveolar airspaces. There was no difference in MPO levels between WT and p47phox−/− sham mice (data not shown). MPO levels were significantly increased in WT mice after I/R, which was further enhanced by combined IL-17 and TNF-α treatment (Fig. 3C). MPO levels were significantly attenuated after I/R in p47phox−/− mice, and this was not further enhanced by combined IL-17 and TNF-α treatment.

A marked increase in pulmonary edema (lung wet/dry weight) occurred in WT mice after I/R compared with sham, which was blocked in p47phox−/− mice after I/R (Fig. 3D). Treatment with combined IL-17 and TNF-α significantly enhanced edema after I/R in WT mice but not in p47phox−/− mice. These results provide evidence that IL-17 and TNF-α can significantly enhance neutrophil infiltration and activation and pulmonary edema via an NADPH oxidase-dependent mechanism.

**Hydroxyl reoxygenation and TNF-α induce NADPH oxidase-dependent superoxide generation by ATII cells.** Because IL-17 and TNF-α enhanced lung dysfunction, injury, and CXCL1 production via an NADPH oxidase-dependent mechanism in the in vivo I/R model, we further investigated the role of this signaling pathway in murine ATII cells using a HR model as a surrogate for lung I/R. ROS generation was assessed (via DCF dye, which displays increased fluorescence intensity in the presence of ROS) by immunofluorescence microscopy (Fig. 4A) and fluorometric quantification (Fig. 4, B and C). Treatment of normoxic ATII cells with TNF-α (50 ng/ml for 4 h) but not IL-17 (50 ng/ml for 4 h) markedly increased ROS generation, which was attenuated by apocynin (600 μM; an NADPH oxidase inhibitor). ROS production was also markedly elevated by HR alone, which was attenuated by apocynin. HR-exposed ATII cells treated with TNF-α alone had a significant increase in ROS production compared with HR alone (Fig. 4C). Similarly, ROS generation was markedly elevated in HR-exposed ATII cells treated with combined IL-17 and TNF-α, which was attenuated by apocynin (Fig. 4C).

Superoxide anion generation in ATII cells was then measured by a chemiluminescence-based assay as a specific indicator of NADPH oxidase activity. A significant increase in superoxide anion generation occurred in normoxic ATII cells after treatment with TNF-α, but not IL-17, which was blocked by apocynin pretreatment (Fig. 5A). There was no significant difference in superoxide anion generation by normoxic ATII cells after combined treatment with IL-17 and TNF-α compared with either treatment alone, which is significantly attenuated by apocynin. The small graph in the inset depicts an enlargement of the first three data values of the larger graph to better view their relationship. *P < 0.05 vs. Norm; *P < 0.05 vs. Norm +TNF; **P < 0.05 vs. Norm +IL-17 +TNF; ND = not detected; n = 8/group. B: HR-exposed ATII cells significantly increased CXCL1 production compared with normoxia, which was significantly attenuated by apocynin pretreatment. Treatment with TNF-α but not IL-17, significantly enhanced CXCL1 production by ATII cells compared with HR alone, which was significantly attenuated by apocynin. Combined treatment of HR-exposed ATII cells with IL-17 and TNF-α synergistically increased CXCL1 production compared with treatment with HR +TNF, which was significantly attenuated by apocynin pretreatment. *P < 0.05 vs. Norm; *P < 0.05 vs. HR; **P < 0.05 vs. HR +IL-17; §P < 0.05 vs. HR +TNF; §§P < 0.05 vs. HR +IL-17 +TNF; n = 8/group. C: HR significantly increased CXCL1 production by primary WT ATII cells compared with normoxia, which was significantly attenuated in HR-exposed primary ATII cells from p47phox−/− mice. *P < 0.05 vs. WT Norm; **P < 0.05 WT HR.
pared with treatment with TNF-α alone. Exposure of ATII cells to HR resulted in significant superoxide anion generation, which was attenuated by apocynin (Fig. 5B). Treatment of HR-exposed ATII cells with TNF-α or combined IL-17-TNF-α, but not IL-17 alone, significantly enhanced superoxide anion generation compared with HR alone, and this was also blocked by apocynin. These results demonstrate that ROS generation in ATII cells after HR or TNF-α treatment is primarily due to NADPH oxidase-dependent superoxide anion production. Furthermore, IL-17 does not induce or enhance HR- or TNF-α-dependent superoxide anion generation.

**IL-17/TNF-α-dependent synergistic enhancement of CXCL1 production by ATII cells is mediated by NADPH oxidase.** To determine if the IL-17/TNF-α-dependent synergistic enhancement of CXCL1 in vivo could be because of the activation of ATII cells, we measured CXCL1 production by ATII cells in vitro after HR. Treatment of normoxic ATII cells with TNF-α significantly increased CXCL1 production, which was significantly abrogated by apocynin pretreatment (Fig. 6A). A very small increase in CXCL1 production by normoxic ATII cells occurred after IL-17 treatment, which was not decreased by apocynin. A multifold (≈8-fold), synergistic exacerbation of CXCL1 production by ATII cells occurred after combined treatment with IL-17 and TNF-α compared with TNF-α alone, and this was also significantly attenuated by apocynin (Fig. 6A).

HR alone induced significant CXCL1 production by ATII cells compared with normoxia, which was attenuated by apocynin (Fig. 6B). Treatment with TNF-α, but not IL-17, significantly enhanced CXCL1 production by HR-exposed ATII cells compared with HR alone, and this enhancement was significantly attenuated by apocynin. A synergistic (≈8-fold) exacerbation of CXCL1 production by ATII cells after HR occurred after combined treatment with IL-17 and TNF-α compared with TNF-α alone, and this was also significantly attenuated by apocynin (Fig. 6B). Furthermore, the role of NADPH oxidase in CXCL1 production was confirmed using primary ATII cells after HR, where a multifold increase in CXCL1 was observed by HR-exposed primary WT cells compared with normoxia, which was significantly attenuated in HR-exposed ATII cells from p47phox−/− mice (Fig. 6C). These results suggest that TNF-α induces CXCL1 production by

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**Fig. 7.** iNKT cell-produced IL-17 and alveolar macrophage-produced TNF-α mediate CXCL1 production by ATII cells via NADPH oxidase. An in vitro model of HR was used to evaluate the effect of primary iNKT cells and alveolar macrophages on ATII cells. A: after HR, iNKT cells produce IL-17, macrophages (Mac) produce TNF-α, and ATII cells produce CXCL1 vs. normoxic (Norm) controls. *P < 0.05 vs. Norm; n = 6–8/group. B: CXCL1 production was significantly enhanced upon conditioned media transfer (CMT) from HR-exposed macrophages (MacHR) to HR-exposed ATII cells (ATIIHR) compared with ATIIHR alone. No change in CXCL1 production was observed by CMT from HR-exposed iNKT cells (iNKTHR) to HR-exposed ATII cells (ATIIHR) compared with ATIIHR alone. However, a synergistic increase in CXCL1 production was observed upon combined CMT from iNKTHR and MacHR to ATIIHR, which was significantly blocked by apocynin (Apo) pretreatment of ATIIHR cells. *P < 0.05 vs. ATIIHR; **P < 0.05 vs. ATIIHR; ***P < 0.05 vs. MacHR (CMT) ATIIHR; ##P < 0.05 vs. iNKTHR/MacHR (CMT) ATIIHR; n = 8/group.
ATII cells via an NADPH oxidase-dependent pathway and that IL-17 can also induce a small increase in CXCL1 production independent of NADPH oxidase. Importantly, the multifold, synergistic enhancement of CXCL1 production by combined IL-17-TNF-α treatment is dependent on NADPH oxidase activity.

*iNKT cell-produced IL-17 and alveolar macrophage-produced TNF-α synergistically increase CXCL1 production by ATII cells.* CMT experiments were performed to evaluate potential cross talk between iNKT cells (the major source of IL-17 after I/R), alveolar macrophages (the major source of TNF-α after I/R), and ATII cells. Primary iNKT cells, but not alveolar macrophages or ATII cells, display significantly increased IL-17 production after HR (Fig. 7A). Primary alveolar macrophages, but not iNKT cells or ATII cells, show significantly increased TNF-α production after HR (Fig. 7A). ATII cells, but not iNKT cells or alveolar macrophages, exhibit significantly increased CXCL1 production after HR (Fig. 7A).

CMT from HR-exposed alveolar macrophages (which contains TNF-α) to HR-exposed ATII cells significantly enhanced CXCL1 production compared with HR-exposed ATII cells alone (Fig. 7B). CMT from HR-exposed iNKT cells (which contains IL-17) did not enhance CXCL1 production in HR-exposed ATII cells. However, CMT from both HR-exposed iNKT cells and alveolar macrophages (1:1 ratio) to HR-exposed ATII cells resulted in a synergistic, 2.1-fold increase in CXCL1 production that was blocked by apocynin pretreatment (Fig. 7B). Taken together, these results confirm that iNKT cell-derived IL-17 and alveolar macrophage-derived TNF-α synergistically enhance CXCL1 production by ATII cells after HR, which is blocked by inhibition of NADPH oxidase.

**DISCUSSION**

The present study demonstrates that lung dysfunction, inflammation, and injury after I/R is mediated by a specific cross talk between iNKT cells, alveolar macrophages, and ATII cells. Our results demonstrate that IL-17 and TNF-α have the distinct ability to exacerbate lung dysfunction and injury after I/R in a NADPH oxidase-dependent manner. Moreover, a synergistic amplification of CXCL1, a potent neutrophil chemokine, as well as enhanced neutrophil infiltration and activation occur after combined treatment with IL-17 and TNF-α in WT mice after lung I/R, which is prevented in p47phox−/− mice. Furthermore, using an in vitro model, we demonstrated that ATII cells are a major source of CXCL1 in response to HR and that the synergistic effects of IL-17 and TNF-α on ATII cell-generated CXCL1 are mediated by NADPH oxidase-dependent superoxide anion generation. Taken together, these results demonstrate that iNKT cell-produced IL-17 and alveolar macrophage-produced TNF-α activate ATII cells after I/R to synergistically augment CXCL1 production via an NADPH oxidase-dependent pathway, resulting in subsequent neutrophil infiltration and lung I/R injury (as summarized in Fig. 8).

Previous studies from our laboratory and others have demonstrated the upstream initiatory role of iNKT cells and alveolar macrophages in mediating the proinflammatory signaling cascade leading to lung inflammation and injury after I/R (18, 32–34, 43). In lung I/R injury, parenchymal cells such as ATII cells also play a crucial role, since they are a major source of CXCL1, which can be secreted in response to specific proinflammatory cytokines such as TNF-α (32). This signaling cascade, involving expression of potent chemokines such as CXCL1, ultimately leads to neutrophil infiltration and activation and subsequent lung injury after I/R. Therefore, the molecular mechanisms of ATII cell-produced CXCL1 production, in response to IL-17 and TNF-α by iNKT cells and macrophages, respectively, represent a critical signaling axis during lung I/R injury.

In the current study, we identify a novel role of IL-17 in amplifying the TNF-α-mediated inflammatory effects on ATII cells during lung I/R injury. Although previous studies have demonstrated the ability of IL-17 and TNF-α to synergistically induce cytokines (16, 24), the present study represents the first demonstration, to our knowledge, of a critical role for NADPH oxidase in mediating the synergistic production of CXCL1 after lung I/R. It has been shown that, although IL-17 treatment offers a mild stimulus for proinflammatory gene expression, IL-17 has the ability to generate a synergistic response in conjunction with other cytokines such as TNF-α. Our present study supports these studies by showing that IL-17 treatment leads to mild production of CXCL1 in both in vivo and in vitro models of I/R while TNF-α treatment leads to a robust induction of CXCL1 production. Importantly, coadministration of IL-17 and TNF-α results in a remarkable, multifold amplification of CXCL1 production as well as enhanced neutrophil infiltration, edema, and lung dysfunction. The ability of TNF-α, but not IL-17, to induce NADPH oxidase-dependent superoxide anion generation in ATII cells and the attenuation of CXCL1 production by apocynin treatment suggests that the NADPH oxidase pathway is an important signaling mechanism for the generation of CXCL1. These results also indicate that activation of NADPH oxidase by I/R alone or TNF-α alone is a critical factor for ATII-mediated CXCL1 production and that IL-17 plays a prominent role in the amplification of this
signaling pathway to result in lung inflammation and injury after I/R.

It is well known that NADPH oxidase plays a critical role in lung I/R injury (5, 7, 29, 42). The contributory role of NADPH oxidase to lung injury has been postulated to be largely attributed to endothelial cells and neutrophils, whereas the role of NADPH oxidase in ATII cells in the setting of lung I/R has not been defined. Insight was provided in a study by Leverence et al. that showed that modulation of NADPH oxidase-dependent oxidative stress alters the expression of CXCL1 in lipopolysaccharide-treated ATII cells, wherein the phosphorylation, activation, and translocation of p47phox, a key activation event for the NOX2 isoform of NADPH oxidase, was shown to be a critical event (23). Our data also support these findings, since p47phox−/− mice displayed reduced CXCL1 production, which is largely produced by ATII cells after lung I/R. In addition, treatment of p47phox−/− mice with exogenous IL-17 and TNF-α failed to induce lung dysfunction and CXCL1 production, thereby suggesting a critical role for NOX2 in the IL-17/TNF-α synergistic production of CXCL1 after I/R. Depending on the type of stimuli used to activate NADPH oxidase, a variety of protein kinases, including PKC, AKT, p38MAP kinase, ERK1/2, and IRAK-4, have been shown to phosphorylate p47phox in vitro and in vivo (8, 19, 23). Recent studies also implicate dual oxidases DUOX1 and DUOX2 as potential H2O2-producing isoforms of NADPH oxidase found in airway and ATII epithelial cells (9, 10, 12). Although a detailed assessment of the various isoforms of NADPH oxidase was beyond the scope of the current study, our data suggest that it is the NOX2 isoform that is primarily responsible for I/R- and TNF-α-induced CXCL1 production by ATII cells. This is evidenced by the fact that 1) p47phox−/− mice are protected from I/R injury and that recombinant TNF-α does not augment this injury and 2) CXCL1 production after I/R or TNF-α treatment is blocked by apocynin, which inhibits NADPH oxidase activation by impairing the translocation to the membrane of the cytosolic component p47phox of the NADPH complex (35).

IL-17 is known to promote the expression of various chemokines and cytokines via gene transcription or mRNA stabilization (3, 13, 17, 26, 37). In particular, IL-17 has been shown to stabilize CXCL1 mRNA via Akt1 and TRAF protein pathways (30, 36). On the other hand, NF-κB activation by TNF-α has been shown to be a major transcriptional regulator of CXC chemokine gene expression (1, 24). Previous studies have demonstrated that TNF-α induces NADPH oxidase activation in various cells, including alveolar epithelial cells (21, 22, 38). It is likely that a similar mechanism is involved in the synergistic effect of IL-17/TNF-α on CXCL1 production by ATII cells after lung I/R, wherein macrophage-derived TNF-α induces CXCL1 gene transcription via activation of NOX2 (with subsequent NF-κB activation), and iNKT cell-derived IL-17 stabilizes CXCL1 mRNA, leading to amplified levels of CXCL1 secretion after I/R (see Fig. 8).

This study does have some limitations. One limitation is that the mouse hilar clamp model of I/R injury does not completely represent a clinical lung transplant scenario, since the mouse lungs are not ventilated during surgery or reperfusion. Rather than using continuous ventilation in our model, the ventilation time was kept to a minimum to minimize the effect of ventilator-induced lung injury. The clinical relevance of this model is supported by the fact that there is remarkable synergy between the inflammatory markers observed in lung-transplant patients (e.g., CXCL1, IL-17, and TNF-α) (2, 18) and the mouse hilar clamp model (33, 43). A second limitation is that use of the p47phox−/− mice in our study does not eliminate the possibility that NOX1 could also be playing a role because p47phox is an important subunit for activation of both NOX1 and NOX2 isoforms of NADPH oxidase. However, we believe that it is predominantly the NOX2 isoform that is modulating CXCL1 production in ATII cells after I/R.

In summary, the current study unites several distinct, cellular pathways involved in CXCL1 production after lung I/R. Evidence is provided for a pivotal role of the NADPH oxidase pathway in the modulation of lung inflammation and injury after I/R via mediating the synergistic effects of IL-17 (from iNKT cells) and TNF-α (from alveolar macrophages) on ATII cell-produced CXCL1. These results identify novel therapeutic targets for the prevention or treatment of primary graft dysfunction after lung transplantation.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES

10. Fischer H, Gonzales LK, Kolla V, Schwarzer C, Miot F, Illek B, Ballard PL. Developmental regulation of DUOX1 expression and func-


