

Ashish K. Sharma, Lucas G. Fernandez, Alaa S. Awad, Irving L. Kron and Victor E. Laubach

Am J Physiol Lung Cell Mol Physiol 293:105-113, 2007. First published Apr 6, 2007;
doi:10.1152/ajplung.00470.2006

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Proinflammatory response of alveolar epithelial cells is enhanced by alveolar macrophage-produced TNF- α during pulmonary ischemia-reperfusion injury

Ashish K. Sharma,¹ Lucas G. Fernandez,¹ Alaa S. Awad,² Irving L. Kron,¹ and Victor E. Laubach¹

Departments of ¹Surgery and ²Medicine, University of Virginia Health System, Charlottesville, Virginia

Submitted 7 December 2006; accepted in final form 4 April 2007

Sharma AK, Fernandez LG, Awad AS, Kron IL, Laubach VE. Proinflammatory response of alveolar epithelial cells is enhanced by alveolar macrophage-produced TNF- α during pulmonary ischemia-reperfusion injury. *Am J Physiol Lung Cell Mol Physiol* 293: L105–L113, 2007. First published April 6, 2007; doi:10.1152/ajplung.00470.2006.—Pulmonary ischemia-reperfusion (IR) injury entails acute activation of alveolar macrophages followed by neutrophil sequestration. Although proinflammatory cytokines and chemokines such as TNF- α and monocyte chemoattractant protein-1 (MCP-1) from macrophages are known to modulate acute IR injury, the contribution of alveolar epithelial cells to IR injury and their intercellular interactions with other cell types such as alveolar macrophages and neutrophils remain unclear. In this study, we tested the hypothesis that following IR, alveolar macrophage-produced TNF- α further induces alveolar epithelial cells to produce key chemokines that could then contribute to subsequent lung injury through the recruitment of neutrophils. Cultured RAW264.7 macrophages and MLE-12 alveolar epithelial cells were subjected to acute hypoxia-reoxygenation (H/R) as an in vitro model of pulmonary IR. H/R (3 h/1 h) significantly induced KC, MCP-1, macrophage inflammatory protein-2 (MIP-2), RANTES, and IL-6 (but not TNF- α) by MLE-12 cells, whereas H/R induced TNF- α , MCP-1, RANTES, MIP-1 α , and MIP-2 (but not KC) by RAW264.7 cells. These results were confirmed using primary murine alveolar macrophages and primary alveolar type II cells. Importantly, using macrophage and epithelial coculture methods, the specific production of TNF- α by H/R-exposed RAW264.7 cells significantly induced proinflammatory cytokine/chemokine expression (KC, MCP-1, MIP-2, RANTES, and IL-6) by MLE-12 cells. Collectively, these results demonstrate that alveolar type II cells, in conjunction with alveolar macrophage-produced TNF- α , contribute to the initiation of acute pulmonary IR injury via a proinflammatory cascade. The release of key chemokines, such as KC and MIP-2, by activated type II cells may thus significantly contribute to neutrophil sequestration during IR injury.

cytokines; chemokines

LUNG ISCHEMIA-REPERFUSION (IR) injury is a major complication in many patients after lung transplantation resulting in significant mortality and morbidity (9, 21). IR injury has also been shown to predispose the lung allograft to rejection (12). Previous studies have demonstrated IR injury to be biphasic, with an acute macrophage-dependent phase followed by a later phase characterized by neutrophil recruitment and activation (4, 5). There is ample evidence supporting a prominent role for alveolar macrophages in the initiation and propagation of acute lung IR injury (3, 15). Proinflammatory cytokines and chemokines, such as TNF- α and monocyte chemoattractant protein-1 (MCP-1), respectively, are secreted early by alveolar macrophages after IR and are postulated to contribute to lung IR

injury (10, 12, 13, 22). Depletion of alveolar macrophages dramatically reduces IR injury, lung dysfunction, and induction of cytokines/chemokines such as TNF- α , macrophage inflammatory protein-2 (MIP-2), and MCP-1 (22). Work by Eppinger et al. (3) strengthens the role of alveolar macrophages in acute IR injury by demonstrating a requirement for TNF- α , IFN- γ , and MCP-1 through the use of specific neutralizing antibodies. One likely mechanism for decreased injury after cytokine neutralization is the suppression of macrophage function, since TNF- α and IFN- γ are involved in respiratory burst activity and other inflammatory functions of macrophages (8, 13, 17). Together, these observations suggest that alveolar macrophages are activated early by IR and initiate a cascade of events leading to the activation of the recipient inflammatory system.

Although the central role of alveolar macrophages and neutrophils in IR injury is becoming better understood, the importance of other resident lung cell populations such as alveolar epithelial cells, and their cross talk with alveolar macrophages and neutrophils, remains unclear. There is increasing evidence suggesting that the alveolar epithelium, traditionally regarded as the target of an inflammatory response, may contribute significantly to the development and resolution of the inflammatory reaction (2). The proinflammatory role of type II epithelial cells via secretion of chemokines has been shown in other models of inflammatory lung injury (2, 20). The apparent parallels between lung IR injury and other forms of acute inflammatory injury point to the speculation that type II epithelial cells could significantly contribute to pulmonary IR injury. Synthesis of chemokines such as MCP-1 from pulmonary epithelial-like cells and its induction by macrophage-derived mediators such as TNF- α have been demonstrated (19).

The current study focuses on the effect of IR-activated alveolar macrophages via TNF- α on subsequent activation of alveolar epithelial cells. We hypothesized that following IR, alveolar macrophage-produced TNF- α further activates alveolar epithelial cells to subsequently produce key chemokines that could lead to neutrophil recruitment and lung injury. To specifically focus on alveolar macrophages and epithelial cells, we utilized an in vitro model encompassing a murine macrophage cell line, RAW264.7, and a murine alveolar type II epithelial cell line, MLE-12. These cells were exposed to periods of acute hypoxia and reoxygenation (H/R) to model in vivo lung IR injury. H/R resulted in the induction of proinflammatory cytokines and chemokines by alveolar macrophages as well as epithelial cells. Moreover, macrophage-

Address for reprint requests and other correspondence: V. E. Laubach, Dept. of Surgery, Univ. of Virginia Health System, PO Box 801359, Charlottesville, VA 22908 (e-mail: laubach@virginia.edu).

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derived TNF- α was found to specifically augment chemokine induction by alveolar epithelial cells demonstrating that 1) epithelial cells produce specific proinflammatory chemokines and 2) a cross talk exists between alveolar macrophages and epithelial cells via TNF- α following IR injury. These events likely result in enhanced neutrophil recruitment and activation, thereby further exacerbating pulmonary IR injury.

MATERIALS AND METHODS

Reagents. Recombinant murine TNF- α was purchased from R&D Systems (Minneapolis, MN). Anti-TNF- α antibody was purchased from Calbiochem (San Diego, CA). The murine macrophage (RAW264.7) and alveolar type II epithelial (MLE-12) cell lines were obtained from American Type Culture Collection (Manassas, VA). Cell culture components were obtained as follows: matrigel and rat tail collagen was from BD Biosciences (Franklin Lakes, NJ); keratinocyte growth factor was from Peprotech (Rocky Hill, NJ); and bronchial epithelial cell growth medium was from Clonetics (Walkersville, MD).

Animals. For the purpose of isolating primary alveolar epithelial cells and alveolar macrophages, we utilized 6-wk-old male C57BL/6 and Swiss-Webster mice, respectively (The Jackson Laboratory, Bar Harbor, ME). Animal acquisition and use was under the supervision of the Center for Comparative Medicine and a licensed veterinarian. All animals received humane care in compliance with the "Principles of Laboratory Animal Care," formulated by the National Society for Medical Research, and "The Guide for the Care and Use of Laboratory Animals," prepared by the National Academy of Science and published by the National Institutes of Health. The animal protocol was approved by the Institutional Animal Care and Use Committee.

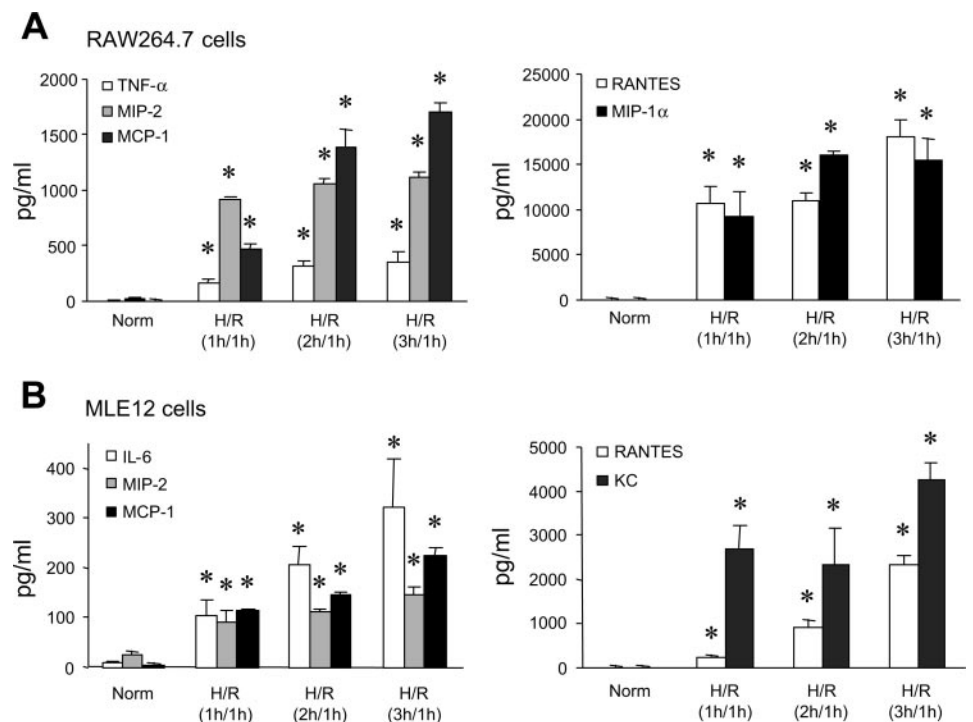
Cell culture. RAW264.7 cells were grown in complete medium consisting of DMEM with 4.5 g/l glucose containing 10% FBS and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA) at 37°C in a humidified atmosphere of 5% CO₂ and 95% O₂. MLE-12 cells were grown in DMEM with 15 mM HEPES under similar conditions as described above. For exposure to H/R, culture dishes were placed in a humidified, sealed hypoxic chamber (Billups-Rothenberg, Del Mar,

CA) that was purged with 95% N₂ and 5% CO₂ for 25 min to establish hypoxia. The chamber was then placed in a cell culture incubator for the desired time period after which it was opened and the culture media was immediately analyzed for O₂ concentration using a blood-gas analyzer (Chiron Diagnostics). Reoxygenation was achieved by removing the plates from the hypoxic chamber and placing them in a normoxic, humidified incubator (37°C, 5% CO₂ and 95% O₂) for 1 h. The partial percentage of O₂ in the culture media after hypoxia exposure was consistently found to be 5% compared with the normoxic PO₂, which was 21%. The culture media reached atmospheric PO₂ (21%) within 5 min of removal from the hypoxic chamber. All experiments (both normoxic and hypoxic) were performed at 37°C. Pretreatment of RAW264.7 cells with anti-TNF- α -neutralizing antibody (1 μ g/ml) was done 1 h before the hypoxic induction, wherever indicated. The optimum dose for anti-TNF- α antibody (1 μ g/ml) was selected based on a dose-response experiment wherein the selected dose completely inhibited TNF- α .

Coculture experiments. MLE-12 cells were cultured in a 12-well culture plate (5×10^5 cells/well) on a Transwell membrane insert (Corning Costar), and RAW264.7 cells (5×10^5 cells/well) were cultured in a 12-well plate. After normoxia (4 h) or H/R (3 h/1 h), the MLE-12 culture insert was washed once with PBS and placed into the well containing the H/R-activated RAW264.7 cells (with or without anti-TNF- α -neutralizing antibody) for an additional 4-h period. MLE-12 and RAW264.7 cells were also exposed simultaneously (in the same wells) to H/R for 3 h/1 h with or without anti-TNF- α -neutralizing antibody. Conversely, H/R-activated (3 h/1 h) epithelial cells on Transwell inserts (and accompanying media) were placed into wells containing normoxic macrophages (prewashed with PBS) for 4 h. For the mixed cocultures, MLE-12 and RAW264.7 cells were cultured together (in the same monolayer) in a 12-well plate at a density of 2.5×10^5 cells (of each cell type) per well for 15 h and washed once with PBS before being subjected to either normoxia (4 h) or H/R (3 h/1 h). The culture media was then collected and analyzed for cytokine/chemokine analysis as described below.

Cytokine and chemokine protein analysis. Cell culture media was collected and centrifuged at 1,000 g for 2 min, and the supernatant was frozen at -80°C. The cytokine and chemokine protein content

Fig. 1. Hypoxia-reoxygenation (H/R)-induced cytokine/chemokine induction in RAW264.7 and MLE-12 cells. Expression of proinflammatory cytokines and chemokines was measured in cell culture supernatants after the indicated times of hypoxia and reoxygenation. **A:** H/R-exposed RAW264.7 cells showed significant, multifold induction of TNF- α , macrophage inflammatory protein-2 (MIP-2), monocyte chemoattractant protein-1 (MCP-1), RANTES, and MIP-1 α compared with normoxic controls (Norm). **B:** H/R-exposed MLE-12 cells showed significant induction of IL-6, MIP-2, MCP-1, RANTES, and KC compared with normoxic controls. Separate graphs are depicted because of differences in the scale of the y-axis. All experiments are $n = 5$. * $P < 0.05$ vs. normoxia.



was quantified using the Bioplex Bead Array technique using a multiplex cytokine panel assay (Bio-Rad Laboratories, Hercules, CA). The samples were analyzed as instructed by the Bioplex array reader, which is a fluorescent-based flow cytometer employing a bead-based multiplex technology, each of which is conjugated with a reactant specific for a different target molecule.

Isolation and culture of primary alveolar type II cells. Alveolar type II cells were isolated from 6-wk-old C57BL/6 mice and cultured as described by Rice et al. (18). Mice were anesthetized with ketamine and xylazine and exsanguinated, and the trachea was isolated and cannulated. The diaphragm was cut, and the sternum and thymus were removed. A 21-gauge needle with a 10-ml syringe was used to perfuse the lungs via the pulmonary artery with 0.9% saline. Dispase (1 ml) was rapidly instilled through the tracheal cannula and was followed by 1 ml of 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. Subsequently, lungs were 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 μ m followed by 40 μ m) and centrifuged at 130 g for 8 min at 4°C and placed on prewashed 100-mm culture dishes that had been coated for 24 h at 4°C with 42 μ g of anti-CD45 and 16 μ g of anti-CD32 antibodies (BD Biosciences, San Jose, CA) in PBS. After incubation for 1 h at 37°C, type II cells were collected by centrifugation and resuspended in DMEM + 5% charcoal-stripped FBS and keratinocyte growth factor (10 ng/ml). Cells were plated at a density of 5×10^5 per 25-mm culture dish coated with 70% matrigel and 30% rat tail collagen. Cells were cultured for 5 days before being used for the experiments. With the use of this technique, the purity of isolated type II cells was >95% as determined by immunostaining for pro-surfactant protein C using pro-SP-C antibody (Chemicon International, Billerica, MA).

Isolation and culture of primary alveolar macrophages. Primary alveolar macrophages were isolated from the bronchoalveolar lavage (BAL) fluid of 6-wk-old Swiss-Webster mice and subsequently cultured. Briefly, the mice were anesthetized with ketamine and xylazine and exsanguinated, and the trachea was isolated and cannulated. The lungs were then perfused three times with 1 ml of PBS, and the BAL fluid was retrieved. For each experiment, BAL fluid from three to five mice was pooled and centrifuged at 330 g for 10 min, and the pellet was resuspended in DMEM containing 10% FBS. The cells were plated on 25-mm culture dishes (5×10^5 cells/dish) and incubated at 37°C overnight before being used for the experiments. Differential cell counts of BAL fluid using Diff-Quik (Baxter Healthcare, Compton, UK) demonstrated ~97% pure population of alveolar macrophages.

Statistical analysis. All experiments were performed with $n = 5$. Values are presented as means \pm SEM. Statistical significance was determined using a two-tailed Student's *t*-test accepting a significance level of $P < 0.05$.

RESULTS

H/R induces proinflammatory cytokine/chemokine activation in RAW264.7 and MLE-12 cells. RAW264.7 macrophages and MLE-12 epithelial cells were exposed to various time periods (1, 2, or 3 h) of hypoxia (5% O₂) followed by 1 h of reoxygenation (21% O₂). Analysis of the media from H/R-exposed RAW264.7 cells showed a markedly significant induction of TNF- α , MIP-2, MCP-1, RANTES, and MIP-1 α at all time points compared with normoxic controls (Fig. 1A). No induction of CXCL1/keratinocyte-derived chemokine (KC), IL-6, IFN- γ , or IL-1 β was observed in RAW264.7 cells after H/R (data not shown).

Exposure of MLE-12 cells to H/R resulted in significant induction of IL-6, MIP-2, MCP-1, RANTES, and KC (Fig. 1B). In contrast to RAW264.7 cells, no induction of TNF- α was observed by MLE-12 cells after H/R (data not shown). In addition, there was no induction of IFN- γ or IL-1 β by MLE-12 cells after H/R at any time point (data not shown). Since the most potent activation of proinflammatory cytokines/chemokines in both RAW264.7 and MLE-12 cells was observed after 3 h of hypoxia and 1 h of reoxygenation, this specific time period was chosen for all subsequent experiments.

H/R induces proinflammatory cytokine/chemokine activation in primary alveolar macrophages and type II epithelial cells. To confirm that the cytokine/chemokine induction observed in RAW264.7 and MLE-12 cells after H/R is not limited to these immortalized cell lines, primary murine alveolar macrophages and type II epithelial cells were exposed to 3 h of hypoxia and 1 h of reoxygenation. Similar to RAW264.7 cells, H/R-exposed primary alveolar macrophages resulted in significant induction of TNF- α , RANTES, MIP-1 α , MIP-2, and MCP-1 compared with normoxic controls (Fig. 2A). No induction of KC, IL-6, IFN- γ , or IL-1 β was observed in primary alveolar

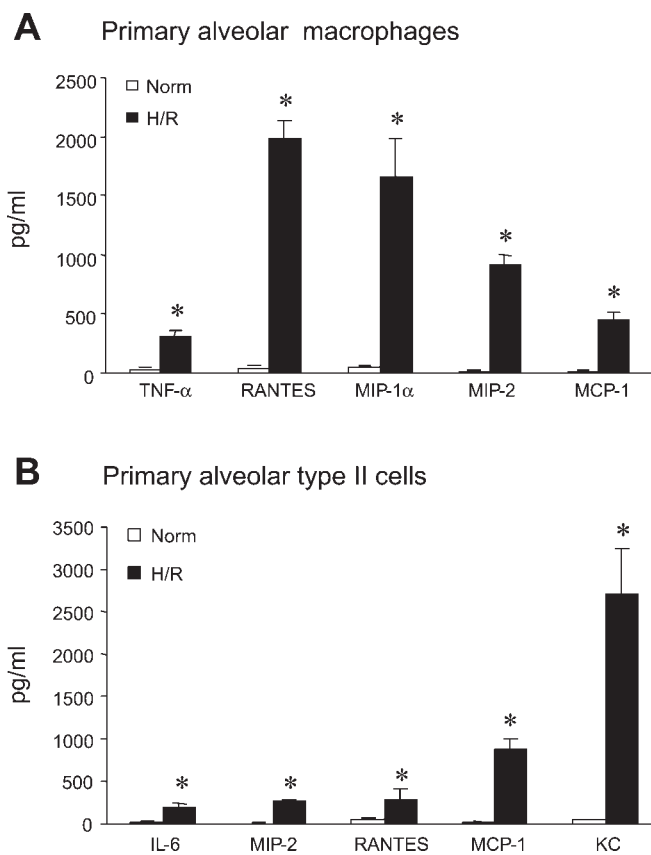


Fig. 2. H/R-induced cytokine/chemokine induction in primary alveolar macrophages and type II cells. Primary alveolar macrophages and type II cells were isolated and cultured as described in MATERIALS AND METHODS. The cells were exposed to H/R (3 h/1 h), after which the culture media was analyzed for the induction of cytokines and chemokines. A: H/R-exposed primary alveolar macrophages showed a significant, multifold induction of TNF- α , RANTES, MIP-1 α , MIP-2, and MCP-1 and compared with normoxic controls. B: H/R-exposed primary type II cells showed a significant induction of IL-6, MIP-2, RANTES, MCP-1, and KC compared with normoxic controls. All experiments are $n = 5$. * $P < 0.05$ vs. normoxia.

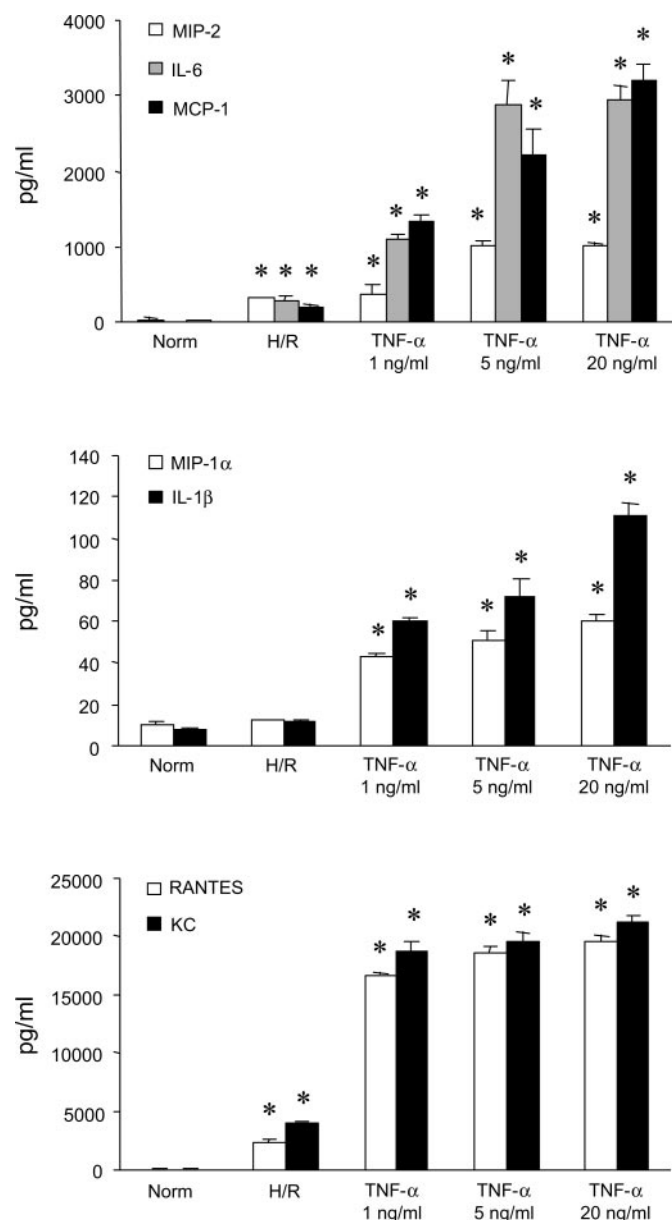


Fig. 3. Induction of proinflammatory cytokines/chemokines by TNF- α in MLE-12 cells. The induction of cytokines/chemokines in normoxic MLE-12 cells after treatment with recombinant TNF- α (1, 5, or 20 ng/ml) for 4 h was measured in the culture supernatants. TNF- α induced a significant, dose-dependent expression of MIP-2, IL-6, MCP-1, MIP-1 α , IL-1 β , RANTES, and KC compared with normoxic controls. Cytokine induction in MLE-12 cells after H/R (3 h/1 h) is also shown for comparison. Separate graphs are depicted because of differences in the scale of the y-axis. All experiments are $n = 5$. * $P < 0.05$ vs. normoxia.

macrophages after H/R (data not shown). Similar to MLE-12 cells, primary alveolar type II cells exhibited a markedly significant increase in the expression of IL-6, MIP-2, RANTES, MCP-1, and KC after H/R (Fig. 2B). No induction of TNF- α , IFN- γ , or IL-1 β was observed in primary alveolar epithelial cells after H/R (data not shown).

TNF- α activates type II epithelial cells. Recombinant TNF- α was used to test the hypothesis that MLE-12 cells can be activated by TNF- α , a prominent proinflammatory cytokine rapidly produced by alveolar macrophages after IR. Normoxic

MLE-12 cells were exposed to recombinant TNF- α (1, 5, or 20 ng/ml) for 4 h, and the resultant cytokine/chemokine induction was measured. TNF- α significantly induced the activation of MIP-2, IL-6, MCP-1, MIP-1 α , IL-1 β , RANTES, and KC in MLE-12 cells compared with unstimulated, normoxic controls (Fig. 3). Significant induction of IL-1 β , IL-6, RANTES, MIP-2, MCP-1, and KC was also observed in primary alveolar type II epithelial cells after TNF- α (20 ng/ml) treatment (Fig. 4). Here we used a dose of 20 ng/ml TNF- α because this dose resulted in the highest induction of cytokine expression in MLE-12 cells (Fig. 3).

TNF- α from alveolar macrophages modulates the proinflammatory activation of type II epithelial cells. Since H/R induced TNF- α production in alveolar macrophages but not in type II epithelial cells, we then investigated the role of macrophage-derived TNF- α in type II cell activation. To do this, we utilized a coculture technique, as described in MATERIALS AND METHODS, in which MLE-12 epithelial cells (preexposed to either 4 h of normoxia or to 3 h/1 h H/R) were cocultured with RAW264.7 macrophages (preexposed to either 4 h of normoxia or to 3 h/1 h H/R) for an additional 4 h. Coculture of normoxic epithelial cells with normoxic macrophages did not induce cytokine/chemokine expression (Fig. 5, A-F, lane 1). Coculture of normoxic epithelial cells with H/R-activated macrophages resulted in significantly augmented induction of IL-6, MCP-1, KC, RANTES, and MIP-2 (lane 3) compared with H/R-activated macrophages alone (Fig. 5, A-E, lane 2). This

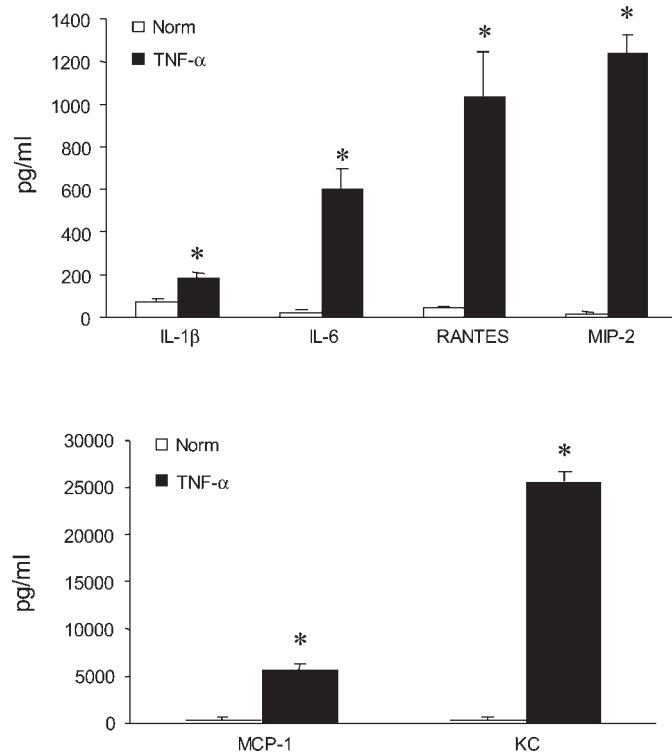


Fig. 4. Induction of proinflammatory cytokines/chemokines by TNF- α in primary type II cells. Primary alveolar type II cells were isolated and cultured as described in MATERIALS AND METHODS. Treatment of normoxic, primary type II cells with recombinant TNF- α (4 h, 20 ng/ml) resulted in significant, multifold induction of IL-1 β , IL-6, RANTES, MIP-2, MCP-1, and KC compared with corresponding normoxic controls. Separate graphs are depicted because of differences in the scale of the y-axis. All experiments are $n = 5$. * $P < 0.05$ vs. normoxia.

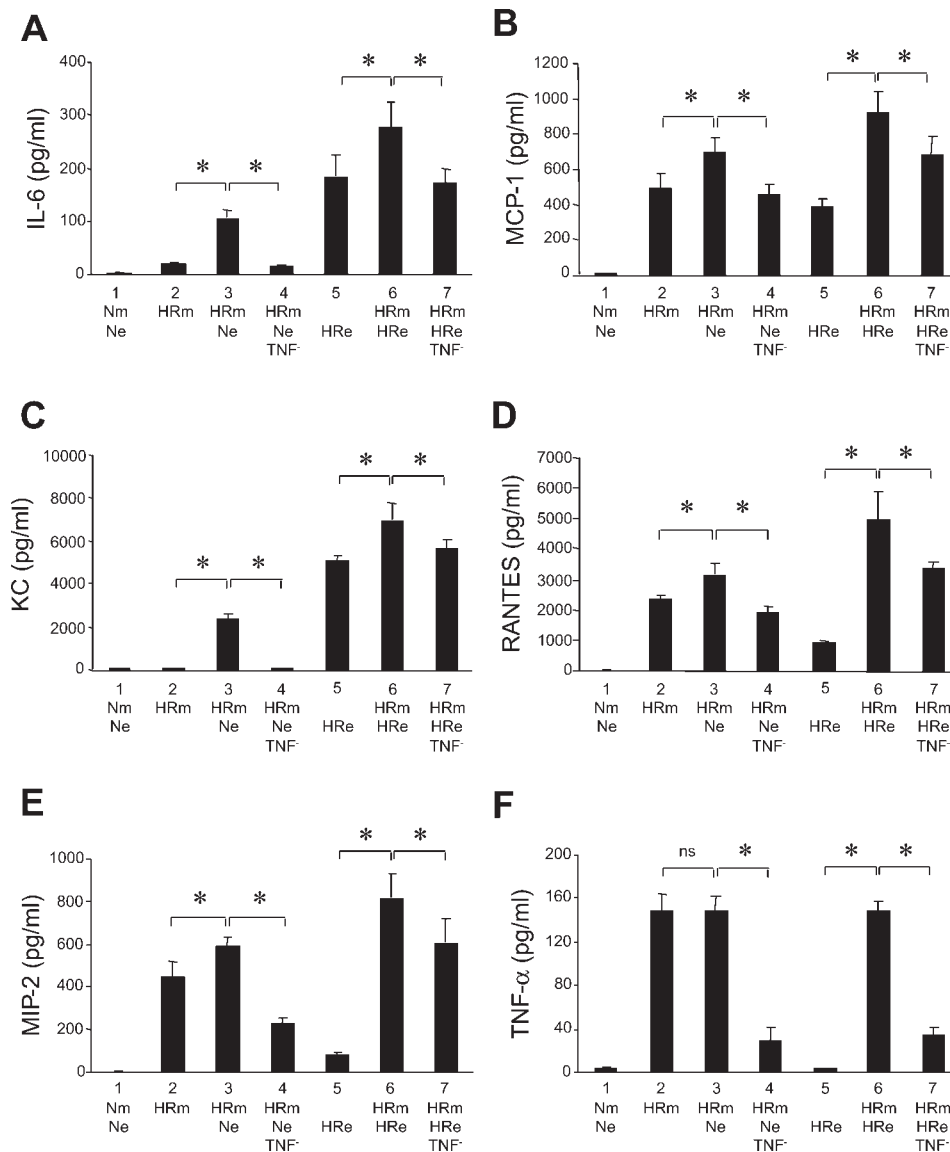


Fig. 5. A–F: H/R-exposed macrophages activate type II epithelial cells via TNF- α . Cocultures of RAW264.7 macrophages and MLE-12 epithelial cells that were exposed to either 4 h of normoxia or 3 h/1 h of H/R were evaluated for expression of the indicated cytokines/chemokines as described in MATERIALS AND METHODS. Coculture of normoxic epithelial cells with H/R-activated macrophages (lane 3) resulted in significant augmentation of IL-6, MCP-1, KC, RANTES, and MIP-2 induction compared with H/R-activated macrophages alone (lane 2). This augmentation was completely attenuated by anti-TNF- α antibody (lane 4). Coculture of H/R-activated epithelial cells with H/R-activated macrophages also resulted in significant augmentation of cytokine/chemokine induction (lane 6) compared with H/R-activated macrophages alone (lane 5). This augmentation was significantly attenuated by anti-TNF- α antibody (lane 7). Lane 1, normoxic macrophages (Nm) cocultured with normoxic epithelial cells (Ne); lane 2, H/R-activated macrophages (HRm) only; lane 3, normoxic epithelial cells cocultured with H/R-activated macrophages; lane 4, same as lane 3 but with the inclusion of anti-TNF- α antibody (TNF $^{-}$); lane 5, H/R-exposed epithelial cells (HRe) only; lane 6, H/R-exposed epithelial cells cocultured with H/R-exposed macrophages; lane 7, same as lane 6 but with the inclusion of anti-TNF- α antibody (TNF $^{-}$). All experiments were $n = 5$; * $P < 0.05$; ns, not significant.

augmentation in cytokine/chemokine production was prevented by anti-TNF- α antibody treatment (Fig. 5, A–E, lane 4). In addition, H/R-activated macrophages significantly augmented cytokine/chemokine production in H/R-activated MLE-12 cells (Fig. 5, A–E, lane 6) compared with H/R-activated MLE-12 cells alone (lane 5). This augmentation was also significantly attenuated by anti-TNF- α antibody (lane 7). Compared with H/R-activated macrophages alone (lane 2), the level of TNF- α expression did not differ after coculture with either normoxic (lane 3) or H/R-activated (lane 6) epithelial cells and was almost completely blocked by anti-TNF- α antibody (Fig. 5F, lanes 4 and 7).

Type II epithelial cells do not modulate the proinflammatory activation of alveolar macrophages. To investigate the possibility of bidirectional communication wherein type II epithelial cells may modulate the production of proinflammatory cytokines/chemokines by alveolar macrophages, normoxic RAW264.7 cells were cocultured with H/R-activated MLE-12 cells for 4 h. The analysis of cytokine/chemokine protein induction indicated no change in IL-6, MCP-1, KC, RANTES, MIP-2, or TNF- α

expression between the H/R-activated epithelial cells alone (lane 3) and the normoxic macrophages cocultured with H/R-activated epithelial cells (lane 2) (Fig. 6). These results suggest that the alveolar macrophages modulate the proinflammatory activation of epithelial cells but not vice-versa.

Direct contact interaction between alveolar macrophages and epithelial cells modulates the expression of key chemokines after H/R. To investigate the potential involvement of direct contact interactions between alveolar macrophages and epithelial cells as an additional mechanism contributing to H/R injury, RAW264.7 and MLE-12 cells were cultured together in a mixed monolayer at a 1:1 ratio and subsequently exposed to either normoxia (4 h) or H/R (3 h/1 h). Mixed cultures of normoxic macrophages and epithelial cells did not show any cytokine/chemokine induction (Fig. 7). Exposure to H/R induced significant activation of IL-6, MCP-1, KC, RANTES, MIP-2, and TNF- α in the mixed cultures, all of which were significantly reduced by anti-TNF- α -neutralizing antibody (Fig. 7). Interestingly, despite the lower number of cells, the activation of MCP-1 and KC in the mixed cultures (Fig. 7) was

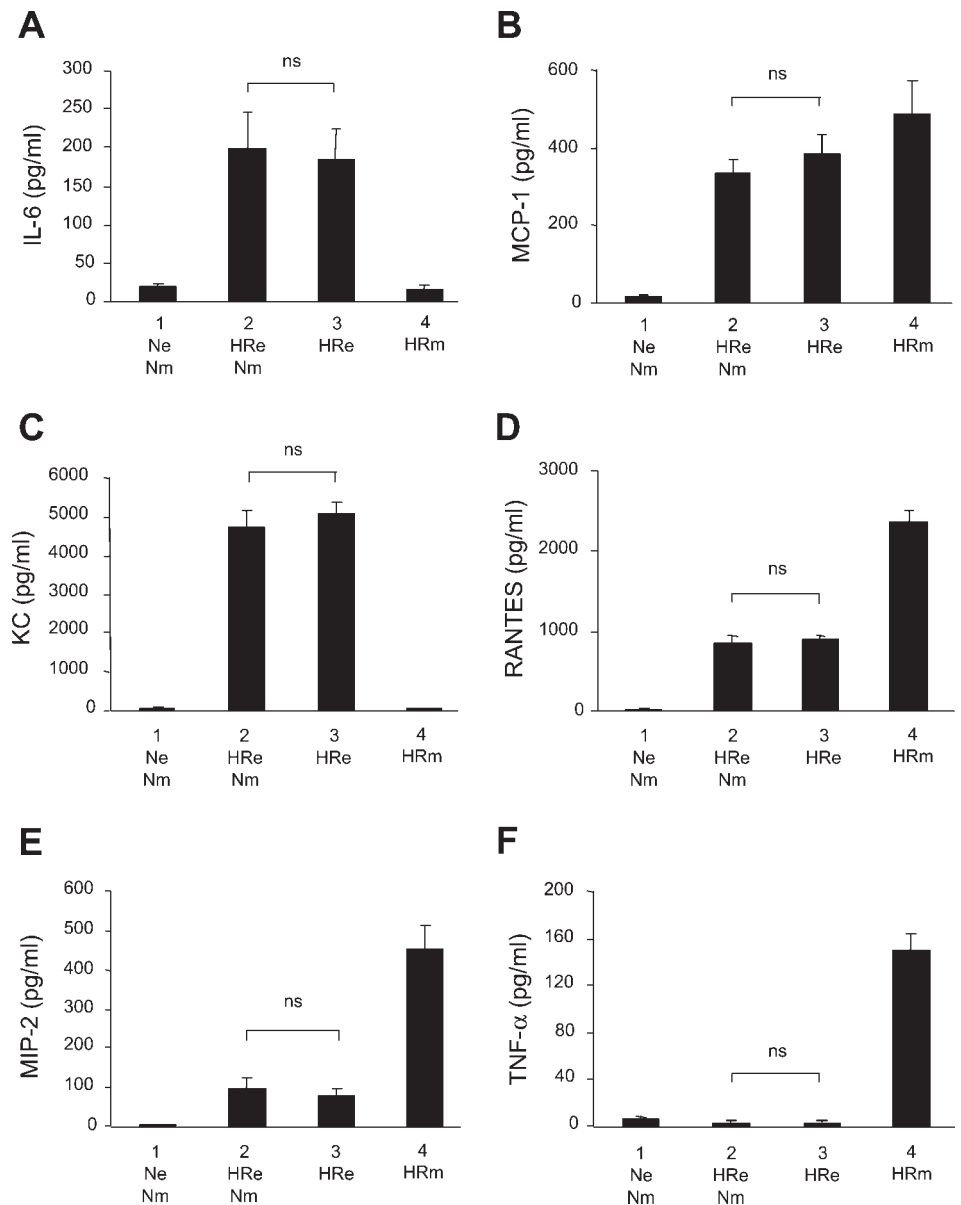


Fig. 6. A–F: H/R-exposed type II epithelial cells do not modulate cytokine/chemokine activation by macrophages. Cocultures of RAW264.7 cells and MLE-12 cells that were exposed to either 4 h of normoxia or 3 h/1 h of H/R were evaluated for expression of the indicated cytokines/chemokines as described in MATERIALS AND METHODS. Coculture of normoxic macrophages and epithelial cells showed no cytokine/chemokine induction (lane 1). Coculture of normoxic macrophages with H/R-activated epithelial cells (lane 2) showed no significant change in cytokine/chemokine induction from H/R-activated epithelial cells alone (lane 3), indicating that type II epithelial cells do not modulate cytokine/chemokine activation by macrophages. The level of cytokine/chemokine induction by H/R-exposed macrophages only are shown for comparison (lane 4). All experiments were $n = 5$; ns = not significant.

fourfold and twofold higher, respectively, than the corresponding activation of the cocultured monolayers (Fig. 5, lane 6). This suggests that cell-cell interaction between macrophages and epithelial cells may augment the induction of certain chemokines after H/R.

DISCUSSION

Although there have been various studies employing different techniques to study the pathophysiology associated with pulmonary IR injury, the specific cellular mechanisms remain unclear. Studies from our laboratory have demonstrated the biphasic nature of IR injury, initiated by resident alveolar macrophages followed by a response from infiltrating neutrophils (5, 22). Intercellular communication between immune and nonimmune cells is a vital process in the initiation, maintenance, and progression of any inflammatory response. In the lung, alveolar macrophages and alveolar epithelium are in close proximity to each other at the alveolar septal junction

(16), making it a logical assumption that these cell types may interact during proinflammatory responses after IR. However, there is very little evidence suggesting an active role of alveolar epithelial cells in acute lung IR injury and specifically in response to alveolar macrophage-dependent factors such as TNF- α . The current study demonstrates potent induction of key chemotactic factors such as KC and MIP-2 by type II epithelial cells after H/R, an induction that is significantly augmented by alveolar macrophage-produced TNF- α . Hence, the activation of alveolar epithelial cells may provide a vital link between the initial macrophage-dependent response and the subsequent recruitment and activation of circulating neutrophils.

In the present study, we utilized an *in vitro* model to mimic pulmonary IR injury, by subjecting alveolar macrophages and epithelial cells to acute hypoxia followed by reoxygenation (normoxia). Since Atochina et al. (1) have demonstrated that pulmonary tissue oxygenation can occur through pulmonary

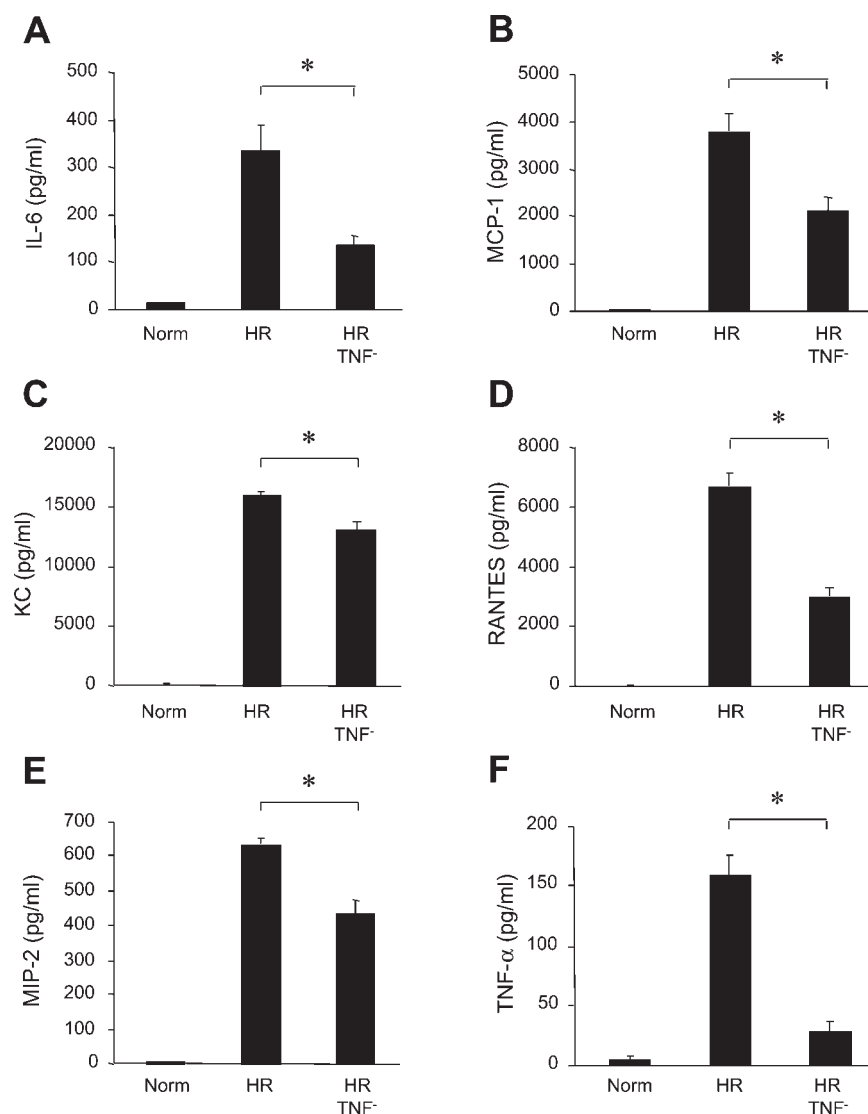


Fig. 7. *A–F*: direct contact interaction between macrophages and epithelial cells modulates activation of cytokines/chemokines after H/R. RAW264.7 and MLE-12 cells were cocultured as a mixed monolayer at a ratio of 1:1 as described in MATERIALS AND METHODS. The mixed cocultures during normoxic exposure did not show induction of any cytokines/chemokines. Exposure of mixed cocultures to H/R induced significant expression of IL-6, MCP-1, KC, RANTES, MIP-2, and TNF- α . Treatment with TNF- α antibody (HR TNF⁻) significantly attenuated the H/R-induced expression of each cytokine/chemokine. All experiments were $n = 5$; * $P < 0.05$.

vasculature or alveolar oxygen diffusion, lung ischemia may not necessarily lead to tissue anoxia. Hence, our model employed a constant hypoxic condition (5% O₂) for all culture experiments. We recognize that a limiting factor with this strategy is that 5% O₂ is not anoxia (ischemia), and some may consider this to be mild hypoxia. Nevertheless, we observed very potent cellular responses to acute H/R, responses that are similar to what we observed using an isolated mouse lung model of IR injury (22). The potent proinflammatory response of RAW264.7 and primary alveolar macrophages, specifically secretion of TNF- α , points to an initial source of IR injury. More importantly, the activation of alveolar type II cells and release of specific chemokines such as KC, IL-6, MIP-2, and MCP-1 in response to H/R and macrophage-produced TNF- α suitably demonstrate that 1) alveolar type II cells are involved in the initial response of acute lung IR injury, 2) this response is at least partially mediated by macrophage-produced TNF- α , and 3) specific chemotactic factors released by type II cells may promote the inflammatory cascade by contributing to neutrophil sequestration.

Elevated chemokine expression such as KC, MIP-2, and MCP-1 are an essential event for leukocyte infiltration and

accumulation at sites of inflammation, and the potent chemoattractant properties of these chemokines for monocytes (MCP-1), neutrophils (MIP-2, KC), or eosinophils and basophils (RANTES) are well documented (4, 14). Previous studies have shown that IL-1 β from alveolar macrophages plays an important role in epithelial cell mediator production in other models of acute lung injury (7). However, in our model we did not observe any induction of IL-1 β or IFN- γ in H/R-induced alveolar macrophages or epithelial cells. This important observation further pinpoints TNF- α as a key mediator in the initiation and progression of IR injury. IL-1 β has also been postulated to play a role in neutrophil-dependent lung injury (11), and since we did not observe significant IL-1 β activation, it further accentuates the role of KC and MIP-2 as important mediators of neutrophil sequestration in IR injury. Although TNF- α treatment of primary epithelial cells did induce IL-1 β production (Fig. 4), this was quantitatively much smaller than the activation of other cytokines/chemokines.

In a previous study, we observed a significant induction of TNF- α , MCP-1, and MIP-2 in an isolated, buffer-perfused mouse model of lung IR injury (22). Here, macrophage deple-

tion prevented the induction of TNF- α and MCP-1, whereas MIP-2 expression was only intermediately reduced, thereby suggesting another source of MIP-2 production. The present study suggests that this source could be alveolar type II cells, which not only produce MIP-2 but also KC and MCP-1 in response to either H/R or TNF- α . The augmented induction of KC and MCP-1 in the mixed cocultures after H/R (Fig. 7) suggests another possible mechanism involving intercellular interactions in IR injury, i.e., direct contact interaction between alveolar macrophages and epithelial cells. This could be due to added contribution of cell-cell interactive factors such as adhesion molecules (e.g., ICAM-1), surfactant protein production, lipoxin production, nitric oxide, or other cell-cell interactive mediators. In fact, alveolar macrophages and epithelial cells were shown to interact in response to particulate matter, and this interaction is implicated in the enhancement of a variety of proinflammatory cytokines (6).

When comparing the primary cells to the immortalized cell lines, the magnitude of expression was found to be quantitatively different for some cytokines/chemokines (Fig. 1 vs. Fig. 2). For example, RANTES and MIP-1 α were induced nearly 10-fold higher in the cell lines, and MCP-1 was induced higher in primary macrophages but lower in primary epithelial cells vs. the corresponding cell lines. These quantitative differences in cytokine/chemokine expression are likely due to inherent characteristic differences between the primary cells and immortalized cells. More importantly, however, the pattern of H/R-induced activation of proinflammatory mediators by primary alveolar macrophages and type II cells was similar to the RAW264.7 and MLE-12 cells, respectively, suggesting that, overall, RAW264.7 and MLE-12 cells are closely representative of the responses of the primary cells.

In summary, the results of this study suggest that alveolar type II cells have an active contribution to acute lung IR injury, which is at least partly in response to alveolar macrophage-produced TNF- α , and thus may augment neutrophil recruitment via secretion of chemoattractants such as KC and MIP-2. Although these results suggest that TNF- α is a key mediator of acute lung IR injury, TNF- α may be only transiently expressed after IR and thus may not be the only mediator important in IR injury mediated by neutrophil activation in sequestration. However, these results support our previous study, which demonstrated that TNF- α deficiency, via use of TNF- α knockout mice, significantly attenuates acute lung IR injury (12). Importantly, the expression of KC by type II cells appears to be largely dependent on the communication between alveolar macrophages and type II cells via TNF- α and not solely on H/R. The dynamic contribution of alveolar type II cells may account for a substantial portion of the pathophysiological events resulting in acute pulmonary IR injury. The elucidation of cell-specific proinflammatory patterns and the resultant cytokine/chemokine activation involving alveolar epithelial cells illustrated in this study, subsequent to conditions of H/R, is novel. The putative signaling mechanisms in alveolar epithelium, involving proinflammatory cytokines/chemokines, transcription factors, and oxidative stress mechanisms, in conjunction with the interaction with other pulmonary cell types, may help delineate the specific targets for future therapeutic intervention strategies.

GRANTS

This work was funded by National Heart, Lung, and Blood Institute Grant RO1-HL-077301 (V. E. Laubach).

REFERENCES

1. **Atochina EN, Muzykantov VR, Al-Mehdi AB, Danilov SM, Fisher AB.** Normoxic lung ischemia/reperfusion accelerates shedding of angiotensin converting enzyme from the pulmonary endothelium. *Am J Respir Crit Care Med* 156: 1114–1119, 1997.
2. **Crestani B, Aubier M.** Inflammatory role of alveolar epithelial cells. *Kidney Int Suppl* 65: S88–S93, 1998.
3. **Eppinger MJ, Deeb GM, Bolling SF, Ward PA.** Mediators of ischemia-reperfusion injury of rat lung. *Am J Pathol* 150: 1773–1784, 1997.
4. **Eppinger MJ, Jones ML, Deeb GM, Bolling SF, Ward PA.** Pattern of injury and the role of neutrophils in reperfusion injury of rat lung. *J Surg Res* 58: 713–718, 1995.
5. **Fiser SM, Tribble CG, Long SM, Kaza AK, Cope JT, Laubach VE, Kern JA, Kron IL.** Lung transplant reperfusion injury involves pulmonary macrophages and circulating leukocytes in a biphasic response. *J Thorac Cardiovasc Surg* 121: 1069–1075, 2001.
6. **Fujii T, Hayashi S, Hogg JC, Mukae H, Suwa T, Goto Y, Vincent R, van Eeden SF.** Interaction of alveolar macrophages and airway epithelial cells following exposure to particulate matter produces mediators that stimulate the bone marrow. *Am J Respir Cell Mol Biol* 27: 34–41, 2002.
7. **Ishii H, Fujii T, Hogg JC, Hayashi S, Mukae H, Vincent R, van Eeden SF.** Contribution of IL-1 β and TNF- α to the initiation of the peripheral lung response to atmospheric particulates (PM₁₀). *Am J Physiol Lung Cell Mol Physiol* 287: L176–L183, 2004.
8. **Issekutz AC, Issekutz TB.** Quantitation and kinetics of blood monocyte migration to acute inflammatory reactions, and IL-1 alpha, tumor necrosis factor-alpha, and IFN-gamma. *J Immunol* 151: 2105–2115, 1993.
9. **King RC, Binns OA, Rodriguez F, Kanithanon RC, Daniel TM, Spotnitz WD, Tribble CG, Kron IL.** Reperfusion injury significantly impacts clinical outcome after pulmonary transplantation. *Ann Thorac Surg* 69: 1681–1685, 2000.
10. **Krishnadasan B, Naidu BV, Byrne K, Fraga C, Verrier ED, Mulligan MS.** The role of proinflammatory cytokines in lung ischemia-reperfusion injury. *J Thorac Cardiovasc Surg* 125: 261–272, 2003.
11. **Laffon M, Lu LN, Modelska K, Matthay MA, Pittet JF.** α -Adrenergic blockade restores normal fluid transport capacity of alveolar epithelium after hemorrhagic shock. *Am J Physiol Lung Cell Mol Physiol* 277: L760–L768, 1999.
12. **Maxey TS, Enelow RI, Gaston B, Kron IL, Laubach VE, Doctor A.** Tumor necrosis factor-alpha from resident lung cells is a key initiating factor in pulmonary ischemia-reperfusion injury. *J Thorac Cardiovasc Surg* 127: 541–547, 2004.
13. **Mayer AM, Pittner RA, Lipscomb GE, Spitzer JA.** Effect of in vivo TNF administration on superoxide production and PKC activity of rat alveolar macrophages. *Am J Physiol Lung Cell Mol Physiol* 264: L43–L52, 1993.
14. **Miller EJ, Nagao S, Carr FK, Noble JM, Cohen AB.** Interleukin-8 (IL-8) is a major neutrophil chemotaxin from human alveolar macrophages stimulated with staphylococcal enterotoxin A (SEA). *Inflamm Res* 45: 386–392, 1996.
15. **Naidu BV, Krishnadasan B, Farivar AS, Woolley SM, Thomas R, Van Rooijen N, Verrier ED, Mulligan MS.** Early activation of the alveolar macrophage is critical to the development of lung ischemia-reperfusion injury. *J Thorac Cardiovasc Surg* 126: 200–207, 2003.
16. **Parra SC, Burnette R, Price HP, Takaro T.** Zonal distribution of alveolar macrophages, type II pneumocytes, and alveolar septal connective tissue gaps in adult human lungs. *Am Rev Respir Dis* 133: 908–912, 1986.
17. **Phillips WA, Croatto M, Hamilton JA.** Priming the macrophage respiratory burst with IL-4: enhancement with TNF-alpha but inhibition by IFN-gamma. *Immunology* 70: 498–503, 1990.
18. **Rice WR, Conkright JJ, Na CL, Ikegami M, Shannon JM, Weaver TE.** Maintenance of the mouse type II cell phenotype in vitro. *Am J Physiol Lung Cell Mol Physiol* 283: L256–L264, 2002.

19. **Standiford TJ, Kunkel SL, Phan SH, Rollins BJ, Strieter RM.** Alveolar macrophage-derived cytokines induce monocyte chemoattractant protein-1 expression from human pulmonary type II-like epithelial cells. *J Biol Chem* 266: 9912–9918, 1991.
20. **Wang S, Young RS, Sun NN, Witten ML.** In vitro cytokine release from rat type II pneumocytes and alveolar macrophages following exposure to JP-8 jet fuel in co-culture. *Toxicology* 173: 211–219, 2002.
21. **Zenati M, Yousem SA, Dowling RD, Stein KL, Griffith BP.** Primary graft failure following pulmonary transplantation. *Transplantation* 50: 165–167, 1990.
22. **Zhao M, Fernandez LG, Doctor A, Sharma AK, Zarbock A, Tribble CG, Kron IL, Laubach VE.** Alveolar macrophage activation is a key initiation signal for acute lung ischemia-reperfusion injury. *Am J Physiol Lung Cell Mol Physiol* 291: L1018–L1026, 2006.

