

Abstract

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 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 which could then contribute to subsequent lung injury through the recruitment of neutrophils. Cultured RAW264.7 macrophages and MLE12 alveolar epithelial cells were subjected to acute hypoxia-reoxygenation (H/R) as an *in vitro* model of pulmonary IR. H/R (3h/1h) significantly induced KC, MCP-1, MIP-2, RANTES and IL-6 (but not TNF- α) production by MLE12 cells whereas H/R induced TNF- α , MCP-1, RANTES and MIP-2 (but not KC) production by RAW264.7 cells. These results were confirmed using primary murine alveolar macrophages and primary alveolar type II cells. Importantly, using macrophage and epithelial co-culture 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 MLE12 cells. Collectively, these results demonstrate that alveolar type II cells, in conjunction with alveolar macrophages, 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.

Methods

Cell Culture: RAW264.7 and MLE12 cells grown in 100mm culture dishes containing 2×10^6 cells were placed in a humidified, sealed hypoxic chamber (Billups-Rothenberg Inc., Del Mar, CA) which was then 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, and reoxygenation was achieved by removing the plates from the hypoxic chamber and placing them in a normoxic, humidified incubator for 1h. The partial percentage of O₂ in the culture media after hypoxia exposure was consistently found to be 5% compared to the normoxic pO₂, which was 21%. Pretreatment of RAW264.7 cells with anti-TNF- α neutralizing antibody (1 μ g/ml) was done 1h prior to the hypoxic induction, where indicated.

Co-culture experiments: MLE12 cells were cultured in a 12-well cell culture plate (5×10^5 cells/well) on a semi-permeable support membrane insert (Transwell insert; Corning Costar, NY, USA), and the RAW264.7 cells (5×10^5 cells/well) were cultured in a 12-well plate. After 4h normoxia or H/R (3h/1h), the MLE12 culture insert was washed once with PBS and placed into the well containing the H/R-activated RAW264.7 macrophages (with or without anti-TNF- α neutralizing antibody) for an additional 4h period. MLE12 and RAW264.7 cells were also exposed simultaneously to H/R for 3h/1h, with or without anti-TNF- α neutralizing antibody. In the mixed co-cultures, MLE12 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, before being subjected to normoxia for 4h or hypoxia/reoxygenation for 3h/1h. Subsequently, in each condition, the culture media was collected and analyzed for cytokine/chemokine analysis using the Bioplex Read Array technique using a multiplex cytokine panel assay (Bio-Rad Laboratories, Hercules, CA).

Results

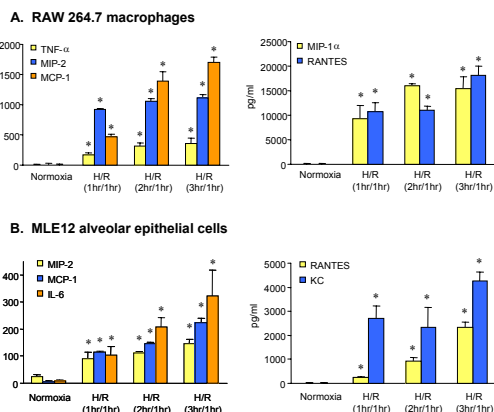


Figure 1. H/R-induced cytokine/chemokine induction in RAW264.7 and MLE12 cells. A) Hypoxia/reoxygenation (H/R)-exposed RAW264.7 cells showed significant, multifold induction of TNF- α , MIP-2, MCP-1, MIP-1 α and RANTES compared to normoxic controls. B) H/R-exposed MLE12 cells showed significant induction of MIP-2, MCP-1, IL-6, RANTES and KC compared to normoxic controls. All experiments are n=5. *p<0.05 versus normoxia

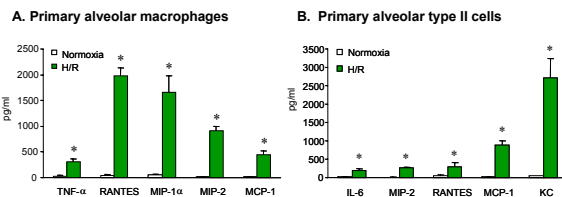


Figure 2. H/R-induced cytokine/chemokine induction in primary alveolar macrophages and type II cells. Primary cells were exposed to hypoxia (3h) followed by reoxygenation (1h) after which the culture media was analyzed for the induction of cytokines and chemokines. A) H/R-exposed primary alveolar macrophages showed a significant, multifold induction of TNF- α , MIP-2, MCP-1, and RANTES compared to normoxic controls. B) H/R-exposed primary type II cells showed a significant induction of KC, RANTES, MCP-1, MIP-2 and IL-6 compared to normoxic controls. All experiments are n=5. *p<0.05 versus normoxia.

Results

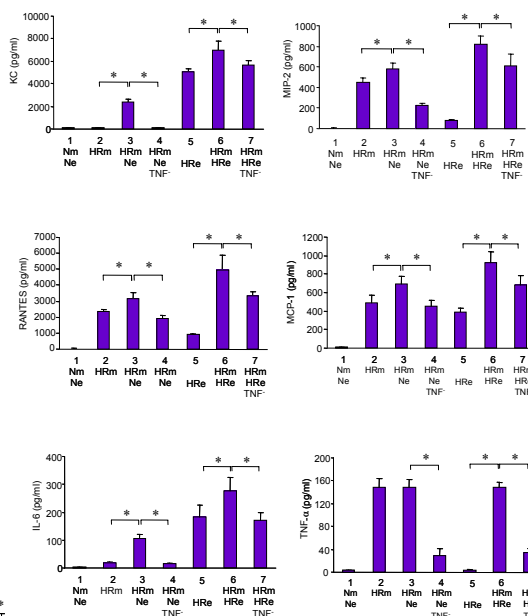


Figure 3. H/R-exposed macrophages activate type II epithelial cells via TNF- α . Co-cultures of RAW264.7 cells (m) and MLE12 cells (e) which were exposed to either 4h normoxia (N) or 3h/1h H/R (HR) were evaluated for cytokines/chemokine production.

- Lane 1,** normoxic macrophages co-cultured with normoxic epithelial cells;
- Lane 2,** H/R-activated macrophages only;
- Lane 3,** normoxic epithelial cells co-cultured with H/R-activated macrophages;
- Lane 4,** same as lane 3 but with the inclusion of anti-TNF- α antibody;
- Lane 5,** H/R-exposed epithelial cells only;
- Lane 6,** H/R-exposed epithelial cells co-cultured with H/R-exposed macrophages;
- Lane 7,** same as lane 6 but with the inclusion of anti-TNF- α antibody.

All experiments were n=5; * p < 0.05.

Results

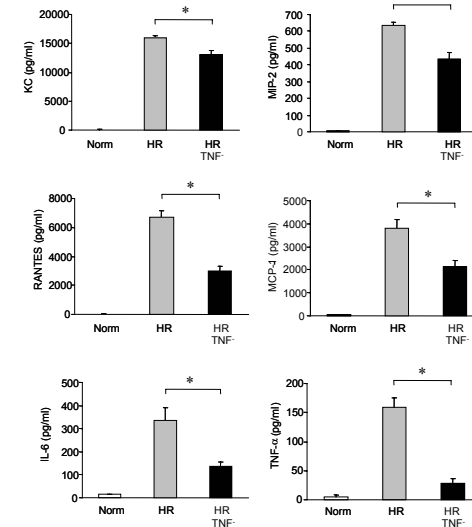


Figure 4. Direct contact interaction between macrophages and epithelial cells modulates activation of cytokines/chemokines after H/R. RAW264.7 and MLE12 cells were co-cultured as a mixed monolayer at a ratio of 1:1. Exposure of mixed co-cultures to H/R (HR) induced a significant expression of IL-6, KC, MCP-1, MIP-2, TNF- α and RANTES. Pretreatment with TNF- α antibody (HR TNF) significantly attenuated the H/R-induced expression of cytokine/chemokines.

Summary

- The intercellular interactions between alveolar macrophages and type II epithelial cells modulate the activation of proinflammatory cytokines & chemokines in pulmonary IR injury.
- Alveolar macrophages activate type II epithelial cells via TNF- α after exposure to hypoxia/reoxygenation.
- Alveolar type II epithelial cells play an important role in acute IR injury via specific chemokines such as KC and MIP-2.