Alveolar Macrophages and Toll-like Receptor 4 Mediate Ventilated Lung Ischemia Reperfusion Injury in Mice

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ABSTRACT

Background: Ischemia-reperfusion (I-R) injury is a sterile inflammatory process that is commonly associated with diverse clinical situations such as hemorrhage followed by resuscitation, transient embolic events, and organ transplantation. I-R injury can induce lung dysfunction whether the I-R occurs in the lung or in a remote organ. Recently, evidence has emerged that receptors and pathways of the innate immune system are involved in recognizing sterile inflammation and overlap considerably with those involved in the recognition of and response to pathogens.

Methods: The authors used a mouse surgical model of transient unilateral left pulmonary artery occlusion without bronchial involvement to create ventilated lung I-R injury. In addition, they mimicked nutritional I-R injury in vitro by transiently depriving cells of all nutrients.

Results: Compared with sham-operated mice, mice subjected to ventilated lung I-R injury had up-regulated lung expression of inflammatory mediator messenger RNA for interleukin-1β, interleukin-6, and chemokine (C-X-C motif) ligand-1 and -2, paralleled by histologic evidence of lung neutrophil recruitment and increased plasma concentrations of interleukin-1β, interleukin-6, and high-mobility group protein B1 proteins. This inflammatory response to I-R required toll-like receptor-4 (TLR4). In addition, the authors demonstrated in vitro cooperativity and cross-talk between human macrophages and endothelial cells, resulting in augmented inflammatory responses to I-R. Remarkably, the authors found that selective depletion of alveolar macrophages rendered mice resistant to ventilated lung I-R injury.

Conclusions: The data reveal that alveolar macrophages and the pattern recognition receptor toll-like receptor-4 are involved in the generation of the early inflammatory response to lung I-R injury.

TRAUMA, sepsis, organ transplantation, and thromboembolic events can result in periods of diminished blood flow, leading to ischemia. The restoration of blood flow (reperfusion) often is accompanied by injury to the affected organ(s), termed “ischemia reperfusion injury,” and ultimately can result in severe sterile inflammation.

The lung, as an organ, appears especially vulnerable to I-R injury, perhaps because of the extent of its vascular supply and continuous physiologic demand for oxygen uptake and gas exchange. However, lung vasculature possesses unique characteristics, such as exposure to highly variable oxygen tensions and adaptive hypoxic pulmonary vasoconstriction, which may make these vascular beds distinct in their response to I-R injury. Thus, understanding the key components and processes involved in lung I-R injury could significantly alter...
how the care for mechanically ventilated patients with acute lung injury is managed.

Toll-like receptors (TLRs) are a well-characterized group of immune receptors that recognize foreign molecules, such as those associated with pathogens.1 Interestingly, during the past decade, evidence has emerged that a subset of TLRs along with other receptors can sense cellular and tissue damage.2,3 That the body can reliably distinguish between sterile and infectious stimuli using a common set of sensors reveals yet another example of the elegant economy employed by the innate arm of the immune system.

In situations of extreme cellular damage, an overly exuberant inflammatory response can result in organ injury and dysfunction, followed by a period of immune hypoactivity that creates an environment in which infectious pathogens can thrive.4 I-R injury represents one such potentially maladaptive response of the innate immune system. Previous studies have implicated TLR4 as a key receptor involved in I-R injury in the lungs, brain, liver, and kidneys5–12 with TLR2 and TLR9 also potentially involved.13,14 The NALP3 (NLRP3) inflammasome, active caspase-1, and downstream cytokines such as interleukin (IL)-1β may also mediate the response to damage markers and nonprogrammed cell death.2,3,15,16 The final outcome of I-R injury involves the infiltration of neutrophils, whose activity causes additional damage to targeted tissues.9,10,12–14,17,18 The steps between the creation of the early post–I-R milieu and the later neutrophil infiltration are less well defined. Studies have suggested that resident monocytic cell populations may be involved in mediating the response to hepatic I-R injury.3,19

In the current study, we used a murine model of unilateral ventilated lung I-R injury in which left-sided lung blood flow, but not alveolar ventilation, was interrupted temporarily.17 This model is an improvement over hiliar clamping models, in which interruption of ventilation and perfusion does not distinguish I-R injury from atelectatic injury. Using this model, we found TLR4 to be required for the response to lung I-R injury. Through in vitro studies, we established that interactions between human endothelial cells and macrophages resulted in augmented inflammatory responses to simulated I-R conditions. This correlated with our observations that in vivo depletion of macrophages, specifically alveolar macrophages, protected mice from I-R–generated lung inflammation. Overall our data indicate a critical role for alveolar macrophages in the response to lung I-R injury, possibly through the sensing of released damage signatures by TLR4.

Materials and Methods

Animal Care

All studies were approved by the institutional animal care and use committee at University of California, San Francisco. All mice were purchased (The Jackson Laboratory, Bar Harbor, ME) or bred in the animal facility at University of California, San Francisco. Only male mice were used (8–16 weeks old, 20–35 g) for in vivo studies. CD11c-DTR mice were a gift from Audrey Gerard, Ph.D., and Max Krummel, Ph.D. (Department of Pathology, University of California, San Francisco; May 20, 2011), and Vicki Platz, Ph.D., and Zena Werb, Ph.D. (Department of Anatomy, University of California, San Francisco; October 31, 2011).

Reagents

Liposome-encapsulated clodronate was obtained from Nico Van Rooijen, Ph.D. (Department of Molecular Cell Biology, Free University Medical Center, Amsterdam, the Netherlands**). C12MDP (clodronate) was a gift of Roche Diagnostics GmbH (Mannheim, Germany). Diphtheria toxin (DTx) was purchased (Sigma-Aldrich, St. Louis, MO).

Cell Lines

All humans cells were incubated at 37°C under humidified 5% CO2. Human umbilical vein endothelial cells (HUVEC, passage 2–6) and human microvascular endothelial cells-lung (HMVEC-L, passage 2–6) were purchased (Lonza, Walkersville, MD). HUVEC were grown in EGM-2 (Lonza) and HMVEC-L in EGM-2-MV (Lonza). Endothelial growth medium was supplemented with 2% fetal calf serum. The monocytic cell line THP1 was grown and maintained in Roswell Park Memorial Institute 1640 (RPMI) medium supplemented with 10% fetal calf serum, L-glutamine, and antibiotics.20 THP1 monocytes were differentiated into THP1 macrophages by adding phorbol 12-myristate 13-acetate (100 nM) to the medium for 3 days, after which the medium was replaced with medium without phorbol 12-myristate 13-acetate and the cells allowed to rest for 5 additional days.20

In Vivo Mouse I-R Procedure

A murine model that temporarily interrupts pulmonary arterial blood flow while maintaining alveolar ventilation was used for this study, as reported previously.17 Mice were anesthetized with intraperitoneal tribromoethanol (Avertin®), intubated, and mechanically ventilated. Intraperitoneal buprenorphine was administered for analgesia. A left thoracotomy was performed and the left pulmonary artery isolated from the left bronchus. A slip knot (8–0 polypropylene suture) placed around the pulmonary artery was used to occlude pulmonary arterial blood flow for time periods ranging from 30 min to 2 h (ischemia time). One end of the suture used to make the slip knot was externalized to allow the knot to be released at the end of the ischemic period. The ventilated left lung was inflated to fill the left thoracic cavity using positive pressure, the thoracotomy closed, and the mouse extubated and allowed to recover from anesthesia. The period of reperfusion lasted 1–3 h, after which blood was collected and the lungs harvested and divided for RNA preparation and hematoxylin and eosin staining.

For the sham procedure, mice received a thoracotomy, but the left pulmonary artery was not isolated. The ventilated...
left lung was inflated to fill the left thoracic cavity using positive pressure, after which the thoracotomy was closed and the mouse exsanguinated. The mouse was sacrificed after a cumulative period of 30 min (analogous to the ischemic period) plus an additional 1–3 h (analogous to the reperfusion period).

**Plasma and Lung Collection and Processing**

Before the mice were sacrificed, blood was obtained when possible by cardiac puncture. Blood was collected in heparinized tubes and stored on ice before being centrifuged at 14,000g for 10 min at 4°C. The plasma upper layer was separated carefully and flash frozen in liquid N2 before being stored at −80°C. After the mice were sacrificed, lungs were removed and placed in Trizol® (Invitrogen/Life Technologies, Grand Island, NY) at 4°C and rapidly transferred to −80°C for future processing and analysis. As reported previously for this surgical model, the upper segment of the left lung was excluded because the thoracotomy and left pulmonary artery isolation procedures create direct damage to this part of the left lung, and this occurs both in sham and I-R surgeries but does not occur in the right lungs, specifically in the right lung sham surgery RNA sample used as the internal standard (relative quantification [RQ] = 1) for quantitative polymerase chain reaction (qPCR) analyses. In cases in which the lower half of the left lung was collected for both RNA preparation and histologic study, the lowest portion or lung base (furthest away from the surgical incision) was selected for histologic study and the other portion for RNA preparation.

**In Vitro Model of Nutritional I-R Injury**

Human umbilical vein endothelial cells and HMVEC-L were seeded on a 48-well plate (at 5–10,000 cells/well) and then grown either alone or with varying ratios of THP1 monocytes or freshly prepared peripheral blood mononuclear cells (PBMC) for 12–24 h in RPMI with 10% serum, at which time the HUVEC were at confluence (~20,000 cells/well). The THP1 monocytes initially were grown separately in suspension, and 1 day before being used for experiments, 20,000 cells were added to plated HUVEC in designated wells in a 48-well dish when HUVEC approached confluence. The THP1 macrophages, in contrast to the monocytes, attach to cell culture surfaces; so monocytes were first seeded in the densities noted (20,000 cells/well in a 48-well dish); after the 3 days of phorbol 12-myristate 13-acetate (PMA) treatment, these differentiated macrophages attached to the plate, and HUVEC were then seeded in these same wells at 2,500–5,000 cells/well; 2–3 days later, confluent HUVEC cultures were present in coculture with the initially seeded numbers of differentiated THP1 macrophages. For HMVEC-L/PBMC cocultures, HMVEC-L were seeded in a manner similar to that used for HUVEC and grown for 48–72 h, with media changed every 48 h. PBMCs were obtained from freshly separated carefully and flash frozen in liquid N2 before being stored at −80°C. After the mice were sacrificed, lungs were removed and placed in Trizol® (Invitrogen/Life Technologies, Grand Island, NY) at 4°C and rapidly transferred to −80°C for future processing and analysis. As reported previously for this surgical model, the upper segment of the left lung was excluded because the thoracotomy and left pulmonary artery isolation procedures create direct damage to this part of the left lung, and this occurs both in sham and I-R surgeries but does not occur in the right lungs, specifically in the right lung sham surgery RNA sample used as the internal standard (relative quantification [RQ] = 1) for quantitative polymerase chain reaction (qPCR) analyses. In cases in which the lower half of the left lung was collected for both RNA preparation and histologic study, the lowest portion or lung base (furthest away from the surgical incision) was selected for histologic study and the other portion for RNA preparation.

**Sandwich ELISAs**

Confluent monolayers of HUVEC was grown and treated with in vitro I-R conditions as described in the previous section. Supernatants were collected after centrifugation to remove cell debris, flash frozen in liquid N2, and stored in aliquots at −80°C. Concentrations of IL-6, IL-1β, and tumor necrosis factor-α (TNFα) in these supernatants were detected by ELISA (Human DuoSet or Quantikine kits, R&D Systems, Minneapolis, MN). IL-1β, IL-6, and high-mobility group protein B1 (HMGB1) concentrations were measured from mouse plasma samples by ELISA (mouse DuoSet or Quantikine kits, R&D Systems for IL-1β and IL-6; and HMGB1 ELISA kit from IBL International GmBH, Hamburg, Germany). All assays were performed according the manufacturer’s supplied protocol. Error bars depicted are the SD for the values from triplicate wells of each condition.

**Flow Cytometry**

Lungs and spleens were digested with prewarmed collagenase solution (Worthington Biochemical, Lakewood, NJ) and filtered through a 0.45-μM cell filter. The cells were then washed using Flow Cytometry Staining Buffer (R&D Systems) and incubated with 10 μg human immunoglobulin G (R&D Systems) in 0.2 ml Flow Cytometry Staining Buffer for 15 min at 4°C. After the cells were washed twice with Flow Cytometry Staining Buffer, they were incubated for 45 min at 4°C with primary antibodies, which included phycoerythrin, fluorescein isothiocyanate, and per CP-Cy 5.5-conjugated mouse anti-human CD11b, F4/80, and CD68 (all...
R&RD Systems; 1:10 dilution). All samples were washed two more times with Flow Cytometry Staining Buffer and then analyzed on a BD LSRII Flow Cytometer (BD Biosciences, San Jose, CA). Data analysis was done using FloJo software (Treestar, Ashland, OR).

**Real Time qPCR**

TaqMan®-specific gene primers—glyceraldehyde 3-phosphate dehydrogenase (GAPDH), intercellular adhesion molecule-1 (ICAM1), IL-6, IL-1β, TNFα, chemokine (C-X-C motif) ligand-1 (CXCL1), CXCL2, IL-17, interferon (IFN) β, IFNα/4, IFNα/7—and the manufacturer’s suggested assay reagents were purchased (Applied Biosystems, Foster City, CA). Lung tissue was homogenized using a Tissue-Tearor tissue homogenizer (Biospec Products, Bartlesville, OK) and messenger RNA (mRNA) isolated using Trizol® according to the manufacturer’s instructions. mRNA concentrations were determined with a NanoDrop® (Thermo Fisher Scientific, Waltham, MA), and mRNA to complementary DNA (cDNA) conversion was performed with the High Capacity RNA-to-cDNA Kit using 1–2 μg mRNA per reaction (Applied Biosystems). Fifty nanograms complementary DNA in 10 μl total reaction volume per well containing TaqMan® Fast Advanced Master Mix (Applied Biosystems) was used in all qPCR experiments, and qPCR was performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). Run method: Polymerase chain reaction activation at 95°C for 20 s was followed by 40 cycles of 1 s at 95°C and 20 s at 60°C. The average threshold count (Ct) value of 2–3 technical replicates was used in all calculations. The Ct value of the internal control mRNA, GAPDH was used to calculate ΔCt values for the array samples because this gene displayed the lowest SD among groups compared with other housekeeping genes tested. Initial data analysis was performed using the 2^−ΔΔCt method, and the data were corrected for statistical analysis using log transformation, mean centering, and autoscaling, as described by Willems et al.23 The method of calculation using the 2^−ΔΔCt method assumes an amplification efficiency of 100% between successive cycles. Relative mRNA data derived from the Ct values are expressed as mean ± SD. For all qPCR results, an internal standard (right lung upper lobe sham surgery) was included for comparison from experiment to experiment and set as RQ = 1.

**Liposome-encapsulated Clodronate Treatment**

Liposome-encapsulated clodronate was stored at 4°C and gently resuspended and allowed to reach room temperature before use. The liposome-encapsulated clodronate suspension (~7 mg/ml) was administered intraperitoneally to mice at 48 (70 mg/kg), 24 (35 mg/kg), and 6 h (35 mg/kg) before the experiment or specific procedure was conducted to deplete macrophage and dendritic cell populations.19 Left lung lower segments were collected for RNA preparation or histologic study (as described above).

**CD11c-DTR Mouse Experiments**

Diphtheria toxin (Sigma) was administered intraperitoneally (4 ng/g body weight) to CD11c-DTR mice once 24 h before the experiment or specific procedure was conducted. Left lung lower segments were collected for RNA preparation or histologic study.

**Microscopy**

Bright field and immunofluorescence microscopy of hematoxylin-and-eosin–stained (H&E) and unstained paraffin-mounted sections of lung was carried out using a Zeiss Axioscam microscope, and images were collected using Zeiss Axiosview software (Carl Zeiss Microscopy, Thornwood, NY).

**Statistical Analysis**

Data are expressed as mean ± SD. Data from in vivo studies comparing two conditions were analyzed using two-tailed nonparametric Mann–Whitney analyses. Data from in vitro studies comparing more than two conditions (time course and coculture conditions) were analyzed using two-way ANOVA with Bonferroni posttest to generate P values. GraphPad Prism was used for statistical analyses (GraphPad Software, La Jolla, CA). For all in vivo experiments, exact P values are reported, and for in vitro ANOVA analyses, P values < 0.05 were considered significant, and P values are represented as follows in the figures: * < 0.05; ** < 0.01; *** < 0.001. If any samples were excluded from analysis, the number of samples excluded and the reasons for exclusion are included in the figure legends for the corresponding figures.

**Results**

**Unilateral Pulmonary Artery Reversible Ligation Causes I-R Injury, Resulting in an Early Induction of Inflammatory Markers and Later Influx of Neutrophils**

We used a previously described but technically challenging surgical method of lung I-R injury that specifically and reversibly interrupts blood flow but not ventilation to the left lungs of mice.17 The period of ischemia (initially 120 min) was followed by reestablishment of blood flow for defined periods of reperfusion. First, we sought to confirm that this procedure resulted in I-R injury reflected by cellular infiltration in the affected lung tissue. We observed a marked increase in infiltrating leukocytes after the restoration of blood flow (3 h later), accompanied by obfuscation of the normal architecture specifically of the affected (left) lung (fig. 1A). Higher magnification (40×) identified these leukocytes to be polymorphonuclear neutrophils, which stereotypically are observed in I-R injury. The infiltration of neutrophils, along with histologic and gross visualization of blood accumulation in lungs after reperfusion along with previously published results, confirm the absence of the “no-reflow” phenomenon in this model of ventilated lung I-R injury.17

Even a shorter ischemic time of 30 min was able to generate neutrophil infiltrates in the I-R-affected (left) lungs.
Fig. 1. Unilateral pulmonary artery occlusion as a model of ventilated lung ischemia-reperfusion (I-R) injury in mice results in inflammatory marker up-regulation and neutrophil infiltration. Histologic findings from prolonged ventilated left lung ischemia (2 h) followed by reperfusion (3 h) in the left versus right lung in C57/BL6 wild-type mice (hematoxylin and eosin staining, original magnifications ×5 and ×40). Each image is representative of histologic images from four independent surgeries (A). Inflammatory cytokines and neutrophil-specific chemokines (mRNA) measured early (1 h) after reperfusion (ischemia time 30 min). Lower segments of left lungs were collected and relative mRNA levels measured by Q-PCR. All measurements were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) message levels measured by Q-PCR. All measurements were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) message levels and relative quantification (RQ) values obtained using the relative ΔΔCt method with an independent sample (right lung sham surgery) used to set as an internal reference RQ of 1. Values from sham surgery left lungs are higher than those from sham surgery right lungs (RQ = 1) because of the injury generated during the thoracotomy procedure that affects the left and not right lungs (see Materials and Methods). Each point represents RNA from left lung lower segments of individual mice that underwent either sham or I-R surgery. Mice that died before completion of surgery or collection of lungs or had inadvertent esophageal intubation were excluded from analysis (2 sham and 6 I-R mice) (B). Inflammatory cytokine and HMGB1 protein concentrations measured at 1 h after reperfusion (ischemia time 30 min). Plasma from blood collected from mice before collection of lungs was analyzed by enzyme-linked immunosorbent assay (ELISA) analysis for protein concentrations of IL-1β, IL-6, and HMGB1. Each point represents plasma from blood collected from individual mice that underwent either sham or I-R surgery. Mice that died before completion of surgery or collection of lungs, yielded insufficient blood volumes, from whom blood was not collected, or had inadvertent esophageal intubation were excluded from analysis (2 sham and 8 I-R mice) (C). Ct = threshold count; CXCL = chemokine (C-X-C motif) ligand; high-mobility group protein B1 = HMGB1; ICAM = intercellular adhesion molecule; IL = interleukin; mRNA = messenger RNA.
compared with contralateral (right) lungs and compared with left lungs from mice that received a sham operation (data not shown, see Supplemental Digital Content 1, fig. 1A, http://links.lww.com/ALN/A876, which compares gross neutrophilic infiltration by histologic findings between sham and I-R surgery left lungs after 30 min ischemic time). We chose this shorter ischemic period for all subsequent experiments. Of note, the contralateral right lungs that did not undergo I-R displayed detectable neutrophilic infiltrates consistent with the period of hyperperfusion experienced (fig. 1A and data not shown). Thus, we chose not to use contralateral right lungs as our comparison controls and instead used left lungs from mice that received a sham operation as comparison controls for all subsequent experiments.

We next analyzed the expression of specific markers of inflammation early in the reperfusion period. After 30 min of left pulmonary artery ligation followed by 1 h of reperfusion, lung tissue was assayed for concentrations of inflammatory cytokines, chemokines, and adhesion molecules. We chose the 1-h reperfusion time point because we wanted to observe early changes in gene expression that were generated by I-R injury before the secretion of, and response to, cytokines and chemokines and before the entry of infiltrating neutrophils into the lung. We observed an up-regulation of IL-6, IL-1β, CXCL1, and CXCL2 in the left lungs of mice after I-R relative to lungs from mice that underwent a sham procedure (fig. 1B, \( P = 0.0022, 0.0083, 0.0184, \) and 0.0035, respectively). ICAM1 and vascular cell adhesion molecule-1 were not induced at this early time point (fig. 1B, \( P = 0.5055 \) and data not shown). This is consistent with the reported delayed kinetics of ICAM1 induction in response to other stimulations, such as lipopolysaccharide (LPS), which is downstream of locally or systemically secreted cytokines, such as IFNγ, TNFα, or IL-1β (25,26 and reviewed in Long27). On the other hand, there is evidence of direct activation of ICAM1 in other circumstances.28 Other inflammatory, immune, and neutrophil-specific cytokines and effector molecules, such as TNFα, IFNα, IFNβ, IL-10, IL-17, and inducible nitric oxide synthase (iNOS) were not induced at this 1-h time point, whereas chemokine (C-C motif) ligand-2 (CCL2) was induced (data not shown). This up-regulation of the message level of inflammatory cytokines was confirmed by examining plasma samples from these mice, which showed increased IL-1β and IL-6, as well as the damage marker HMGB1 (fig. 1C, \( P = 0.0072, 0.0064, \) and 0.0045). In addition, we confirmed that these early inflammatory processes preceded perturbations in lung architecture with infiltration of neutrophils not seen by histology at 1 h after reperfusion (see also Supplemental Digital Content 1, fig. 1B, http://links.lww.com/ALN/A876, which compares histologic sections of lungs that received the sham surgery or the I-R surgery 1 h after reperfusion).

We also examined the traditional lung injury endpoints of edema fluid accumulation and found that at these early time points (1 and 3 h), significant fluid accumulation was not observed by high-resolution computed tomography scanning and wet-to-dry lung weight measurements (data not shown).

**TLR4 Is Required for the Full Inflammatory Response to Lung I-R Injury**

Since both TLR4 and TLR2 have been implicated in I-R injury in various organ systems, including TLR4 in the lung specifically,5–7,9–13,29–33 and TLR4 has been reported to be the receptor for HMGB1,34 we tested our model of surgically induced ventilated lung I-R injury on mice that were TLR4−/− or signaling defective and TLR2−/−.

We used C3H/HeJ mice that carry a point mutation in TLR4 rendering them unresponsive to LPS and analyzed their expression of specific markers of inflammation early in the reperfusion period after lung I-R surgery. We observed that 30 min of ischemia followed by 1 h of reperfusion in the left lung induced robust inflammatory markers in the wild-type control mice (C3H/HeOuJ) but not in the C3H/HeJ TLR4 mutant mice (fig. 2A). In fact, relative levels of cytokines and chemokines detected after I-R injury were lower in these TLR4 mutant mice (fig. 2A; \( P = 0.0541 \) [IL-6], 0.0093 [IL-1β], 0.0003 [CXCL1] and 0.0221 [CXCL2]). Moreover, this lack of induction of cytokines and chemokines was accompanied by an inability of the I-R–subjected TLR4–signaling–defective C3H/HeJ mice to recruit neutrophils to their left lungs after 3 h of reperfusion (fig. 2B). Of note, the C3H murine background has been reported to have more severe pathologic response to I-R injury than do other mouse strains,35 and this was reflected in our results, in which the C3H/HeOuJ control mice displayed greater lung neutrophil infiltrates with I-R than did the C57BL/6 mice (figs. 2B and see Supplemental Digital Content 1, fig. 1A, http://links.lww.com/ALN/A876, which compares neutrophil infiltration levels by histologic findings between C57BL/6 wild-type mice sham and I-R surgery left lungs).

The role of TLR4 in mediating early periods in the response to I-R injury was confirmed by performing the I-R surgery on the C57BL/6 TLR4−/− mouse. Compared with matched wild-type mice, C57BL/6 TLR4−/− mice (similar to the C3H/HeJ TLR4 mutant mice) did not experience neutrophil infiltration after I-R injury (see Supplemental Digital Content 1, fig. 2, http://links.lww.com/ALN/A876, which compares neutrophil infiltration levels by histologic findings between I-R left lungs of wild type and TLR4−/− mice). Thus, TLR4 plays an important and early role in lung I-R injury arguably as a sensor of cell damage signatures.

**Lung Inflammation Is Not Reduced in TLR2-deficient Mice after Lung I-R Injury**

We observed that compared with wild type (C57BL/6) mice, TLR2−/− mice have higher baseline levels of inflammatory markers (fig. 2C). However, TLR2−/− mice that underwent the I-R or sham surgeries did not express different levels of inflammatory markers compared with matched wild-type mice.
Fig. 2. Functional toll-like receptor (TLR) 4 is required for full pathologic response to ventilated lung ischemia-reperfusion (I-R) injury. Inflammatory cytokines and chemokine induction (mRNA) at 1 h after reperfusion measured in lung samples from C3H TLR4mut versus wild-type mice using Q-PCR. All measurements were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) message levels. Each point represents RNA from left lung lower segments of individual C3H wild-type or C3H TLR4mut mice that underwent I-R surgery. Mice that died before completion of surgery or collection of lungs or had inadvertent esophageal intubation and required multiple reintubation attempts were excluded from analysis (2 C3H wild-type and 1 C3H TLR4mut mouse) (A).

Hematoxylin and eosin histopathology of C3H wild-type and C3H TLR4 mut mice left lungs at 3 h after reperfusion after I-R surgery. Each image is representative of histologic images from three independent surgeries (B). Analysis of relative mRNA levels of inflammatory markers (as noted) for left lower lung segments from C57BL/6 wild-type versus C57BL/6 TLR2ko mice at 1 h after reperfusion after I-R surgery. Each point represents RNA from left lung lower segments of individual wild-type or TLR2ko mice that underwent I-R surgery. One wild-type mouse died before completion of surgery and was excluded from analysis (C).

Histologic analysis of C57/BL6 wild-type versus TLR2ko left lungs after 30 min ischemia and 3 h reperfusion. Each image is representative of histologic images from two independent surgeries (D). CXCL = chemokine (C-X-C motif) ligand; ICAM = intercellular adhesion molecule; IL = interleukin; mRNA = messenger RNA; WT = wild-type.
I-R or sham surgery mice (fig. 2C, P values: 0.5915 [IL-6], 0.5915 [IL-1β], 0.4828 [CXCL1], 0.8365 [CXCL2], and data not shown for sham operation). The degree of neutrophil infiltration in affected lung tissue in TLR2−/− mice that received the I-R procedure was similar to that seen for wild type C57BL/6 mice (fig. 2D) and unlike that seen for TLR4−/− mice on C57BL/6 background (see Supplemental Digital Content 1, fig. 2, http://links.lww.com/ALN/A876, which compares neutrophil infiltration levels by histologic findings between I-R left lungs of wild type and TLR4−/− mice). These data suggest that TLR2 does not play a major role in the genesis of lung I-R injury, in contrast to TLR4.

**Human Endothelial Cells In Vitro Nutritional I-R Injury Induces IL-6 but Not IL-1β**

To identify the cell types that were responsible for the inflammatory cytokine production described in the *in vivo* model of I-R injury, we simulated I-R nutritional deprivation conditions *in vitro* for human endothelial cells in culture. Nutrients (including serum, glucose, and growth factors) were omitted from cell culture media for a fixed time period (2 h); after replenishment with nutrient-rich or complete media, cytokine production was assessed by ELISA. Although these conditions do not recreate the blood-flow–dependent shear stress changes experienced by cells in *in vivo* I-R injury, *in vitro* nutritional I-R induced IL-6, but not TNFα or IL-1β protein, from human endothelial cells (fig. 3A). These findings are consistent with data from our lab showing that endothelial cells are generally incapable of making TNFα or IL-1β, even when exposed to bacterial pathogen-associated molecular patterns such as LPS and N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteiny1-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine (pam3cys), but which can strongly induce endothelial cell production of other cytokines, including IL-6 and IL-8.36

**Differentiated Macrophages and Not Monocytes Enhance the Ability of Endothelial Cells Specifically to Produce IL-6 in Response to In Vitro Nutritional I-R**

The lack of IL-1β production by endothelial cells under *in vitro* nutritional I-R conditions suggested that potentially another cell type was contributing to the I-R injury response in our mouse lung I-R model (fig. 1B). So, to extend our results to human cell types, we assessed the effects of coculturing human monocytes with human endothelial cells on the inflammatory response to *in vitro* nutritional I-R injury. As seen in figure 3B, the presence of THP1 monocytes in coculture with endothelial cells during *in vitro* nutritional I-R did not alter endothelial cells expression of TNFα, IL-1β, or IL-6 (fig. 3B).

On the other hand, when THP1 monocytes were differentiated into macrophages (THP1 macrophages) by treatment with PMA, the resulting macrophages produced IL-1β (but not TNFα) when exposed to *in vitro* nutritional I-R. In addition, although these macrophages did not produce IL-6 under these conditions, they enhanced the ability of endothelial cells to produce IL-6 (fig. 3C, P < 0.001 at the 20-h time point). The concentrations of IL-1β produced by THP1 macrophages were relatively modest. However, our data suggest that even very small amounts of IL-1β produced by 10-fold fewer THP1 macrophages compared with endothelial cells (undetectable by ELISA), was sufficient to amplify endothelial cell IL-6 production (data not shown), perhaps either via cell-cell contact between endothelial cells and macrophages or an alternative mechanism by which macrophage-secreted IL-1β sequestered at the surface of endothelial cells plays a role. In contrast, when cocultured under similar conditions with a lung epithelial cell line (A549), neither THP1 monocytes nor differentiated THP1 macrophages were capable of amplifying IL-6 production (data not shown).

To confirm that these findings applied to human lung endothelial cells and naturally occurring human macrophages, we repeated these experiments using HMVEC-L and PBMC and found that PBMCs also made IL-1β early in response to *in vitro* nutritional I-R, and their presence in coculture with HMVEC-L significantly enhanced production of IL-6 (fig. 3D, P < 0.01 for IL-1β at 1-h time point and P < 0.001 at 20 h).

Overall these data suggest that secreted factors, such as IL-1β, from macrophages in response to nutritional deprivation may act on endothelial cells and enhance their ability to produce IL-6 under *in vitro* nutritional I-R conditions.

**Macrophages Are Required for the Generation of a Full Inflammatory Response to I-R Injury In Vivo**

Resident phagocytes, namely macrophages, have been reported to be involved in the initial sensing of microbial or damage signatures (reviewed in Soehnlein and Lindbom37). Based on our *in vitro* data, we hypothesized that lung macrophages may act as sentinels or sensor cells that initiate and/or mediate early steps in the inflammatory process generated by I-R.

To test this hypothesis, we used liposome-encapsulated clodronate to deplete macrophages in C57BL/6 mice.38,39 We found that macrophage-depleted mice had reduced induction of inflammatory markers (IL-6, IL-1β, CXCL1, CXCL2: P = 0.0286, 0.0381, 0.1091, 0.0317, respectively) in lungs after the I-R procedure, compared with mice that had not undergone macrophage depletion (fig. 4A). Furthermore and in concordance with the qPCR data, there was markedly decreased neutrophil recruitment to the left lungs in the macrophage-depleted mice after the I-R surgery (fig. 4B). Flow cytometry analysis of spleens and lungs of mice treated with liposome-encapsulated clodronate confirmed the depletion of CD11b+, F4/80+, and CD68+ populations (see Supplemental Digital Content 1, fig. 3, http://links.lww.com/ALN/A876, which shows flow cytometry
Fig. 3. Generation of inflammatory cytokines from endothelial cells exposed to in vitro nutritional ischemia-reperfusion (I-R) injury conditions is amplified by the presence of macrophages. Interleukin (IL)-6 protein concentrations were measured by enzyme-linked immunosorbent assay (ELISA) analysis at indicated time points in human umbilical vein endothelial cells (HUVEC) after they were exposed to serum-free, nutrient-free, and glucose-free conditions for 2 h, followed by replenishment with rich complete media. Data are representative of three independent experiments (A). HUVEC were cocultured with THP1 human monocytes (THP-1 mo) as noted (HUVEC or THP-1 mo alone or HUVEC:THP-1 mo 1:1). Tumor necrosis factor-α (TNFα), IL-1β, and IL-6 protein concentrations were quantified from cell culture supernatants by ELISA. Data are representative of three independent experiments (B). HUVEC were cocultured with THP1 human differentiated macrophages (THPmph) as noted (HUVEC or THPmph alone or HUVEC:THPmph 1:1). TNFα, IL-1β, and IL-6 protein concentrations were assessed by ELISA from supernatants at times indicated. Data are representative of three independent experiments (C). Lung human microvascular endothelial cells (HMVEC) were cocultured with peripheral blood mononuclear cells (PBMC) as noted (HMVEC or PBMC alone or HMVEC:PBMC 1:1). IL-1β and IL-6 protein concentrations were assessed by ELISA from supernatants at times indicated. Data are representative of two independent experiments (D). THP1 = human monocytic leukemia cell line.
Analyses of spleen and lung cell populations with or without liposome-encapsulated clodronate administration). The liposome-encapsulated clodronate treatment (administered intraperitoneally 48, 24, and 6 h before surgery) depleted conventional macrophages (CD11b⁺/H11001⁺, F4/80⁺/H11001⁺) that were accessible to systemic blood circulation, as well as interstitial lung macrophages, including alveolar macrophages (CD68⁺/H11001⁺, CD11c⁺/H11001⁺), that were not accessible to systemic blood circulation but did not deplete other CD11b⁺ cells (such as granulocytes, neutrophils, NK cells, and so forth) (see Supplemental Digital Content 1, fig. 3, http://links.lww.com/ALN/A876, which shows flow cytometry analyses of spleen and lung cell populations with or without liposome-encapsulated clodronate administration). The depletion of alveolar macrophages and interstitial macrophages that were not in contact with blood flow was likely attributable to the depletion of precursors of these populations, which in turn resulted in a defect in repopulation of the lung macrophages and is consistent with other published data.⁴⁰

Alveolar Macrophages Are the Likely Sensor Cell for Lung I-R Injury

To further identify the subset of macrophage or phagocytic cells that may act as the sensor cell in the lung for sterile I-R injury, we depleted CD11c⁺ alveolar macrophages by treating CD11c-DTR transgenic mice⁴¹ with DTx. This treatment also targets conventional dendritic cells. The absence of alveolar macrophages was confirmed by fluorescent microscopy, which showed the absence of large ovoid green fluorescent protein-positive cells in the lungs of mice pretreated with DTx (see Supplemental Digital Content 1, fig. 4, http://links.lww.com/ALN/A876, which shows fluorescence images of unstained lung sections from mice that either did or did not have their CD11c⁺ cells depleted by pretreatment with intraperitoneal DTx). The depletion of alveolar macrophages caused markedly diminished neutrophil trafficking to the left lung after left-sided I-R surgery (fig. 5). Our liposome-encapsulated clodronate treatment of wild-type mice depleted most macrophage subsets (including alveolar macrophages), whereas DTx treatment of CD11c-DTR mice depletes CD11c⁺ cells (including alveolar macrophages). as interstitial lung macrophages, including alveolar macrophages (CD68⁺, CD11c⁺), that were not accessible to systemic blood circulation but did not deplete other CD11b⁺ cells (such as granulocytes, neutrophils, NK cells, and so forth) (see Supplemental Digital Content 1, fig. 3, http://links.lww.com/ALN/A876, which shows flow cytometry analyses of spleen and lung cell populations with or without liposome-encapsulated clodronate administration). The depletion of alveolar macrophages and interstitial macrophages that were not in contact with blood flow was likely attributable to the depletion of precursors of these populations, which in turn resulted in a defect in repopulation of the lung macrophages and is consistent with other published data.⁴⁰

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Since our results demonstrate that both treatments resulted in profound protection against lung I-R injury (by lack of neutrophil infiltration), we propose alveolar macrophages to be involved in sensing I-R damage signals and a key component of the innate immune response to lung I-R injury.

**Discussion**

In patients who experience severe trauma, undergo lung transplantation, have pulmonary emboli, or have severe sepsis, lung I-R injury can be severe enough to require mechanical ventilatory support, which in turn can contribute to overall morbidity and mortality (reviewed in den Hengst et al.45).

In the current study, we used a precise and sophisticated murine model to study isolated lung I-R injury. As opposed to the majority of published reports on lung I-R injury, in which hilar clamping is used,5,6 we focused on direct lung injury from I-R alone. The contribution of atelectasis, lung collapse, and mechanical ventilation to lung injury is well documented,43–46 and by allowing spontaneous ventilation throughout the majority of the I-R period in our model, we were able to minimize the deleterious effects of lung collapse and mechanical ventilation. In addition, we chose to study I-R injury in mice to take advantage of available gene knock-out and transgenic animals. Overall, we hope to investigate the molecular and cellular basis of this pathophysiological response. In our mouse I-R model, we first recapitulated neutrophil trafficking, a hallmark of clinical I-R injury, and this correlated well with the induction of inflammatory markers early in the reperfusion period. IL-6 and IL-1β were found to be specifically up-regulated both at the message and protein levels early in this process, whereas TNFα, type I IFN, IL-10, IL-17, and iNOS were not. We observed early up-regulation of specific chemokines that guide neutrophils to the site of injury (CXCL1 and CXCL2). This appeared to be an innate immune process because IL-17, an adaptive immune T-cell cytokine that results in neutrophil chemotaxis, was not induced by I-R in the lung. We were also able to detect a significant increase in HMGB1 concentrations in our I-R mice, and this damage marker may play a key role in ventilated lung I-R injury. Future work will attempt to identify the key damage markers released and their roles in mediating I-R injury. It is likely that reactive oxygen species generation plays an important role in this process because inhibition of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase has been shown to reduce I-R injury in the lung.47

Examining mice that were defective or deficient for TLR4 signaling revealed an important role for TLR4 in lung I-R injury. In contrast, TLR2−/− mice did not demonstrate similar levels of resistance to I-R injury. Our results are consistent with other published data that implicate TLR4 as a key receptor in lung I-R injury. However, the previous studies used hilar clamping and thus did not focus on I-R injury alone due to the fact that the atelectasis associated with occluding the left main bronchus along with the left pulmonary artery generates an additional source of inflammation that could also be TLR4-dependent.5,6 We focused specifically on ventilated I-R injury and showed that TLR4 signaling was involved early in lung I-R injury. We hypothesized that TLR4 directly or indirectly senses damage patterns released by endothelial cells in reperfused vasculature.

Human endothelial cells express TLR4 and TLR2. Specifically, they can respond to both TLR4 and TLR2 ligands to produce IL-6. As demonstrated by our in vitro data, simulated nutritional I-R injury in the absence of shear stress changes that accompany flow alterations generated endothelial cell IL-6, but not IL-1β production, soon after reperfusion (fig. 3). The lack of IL-1β production by endothelial cells led us to design coculture systems of endothelial cells with mononuclear cells to identify the cell type producing IL-1β. We hypothesized that IL-1β made by human macrophages acted on endothelial cells, amplifying their IL-6 production under nutritional I-R conditions (fig. 3, C and D). In studies examining endothelial cell response to LPS in the presence of PBMC monocytes, Ward et al. showed that IL-1β production fed back on endothelial cells to augment inflammatory cytokine production.48 We suggest that this paradigm of monocyte cell types serving as sensors or amplifiers of pathogen-mediated inflammatory responses may apply to conditions of sterile inflammation in the presence of damage markers both in humans and in mice.

A role for macrophages has been implicated in some I-R injury models of the heart, liver, and even lung (in the setting of lung transplantation).19,49–53 To demonstrate an in vivo role for macrophages in the lung I-R–mediated inflammatory process, we used liposome-encapsulated clodronate to deplete all macrophage cells in the mouse38 and observed a failure in neutrophil recruitment after lung I-R injury. Liposome-encapsulated clodronate treatment eliminates macrophage populations accessible to the bloodstream (splenic macrophages, for example) and can also eliminate resident tissue macrophage populations (such as alveolar macrophages and interstitial lung macrophages) by targeting circulating blood monocyte precursors.38,46,54–55

Alveolar macrophages are a major subset of macrophages that reside in the lung. Alveolar macrophages make up more than 80% of cells in the alveoli and are important for immune responses to inhaled particles and pathogens.56 In the current study, we show that alveolar macrophages play a key role in the response to lung I-R injury. Alveolar macrophage depletion with DTx in CD11c-DTR mice resulted in a near-complete absence of neutrophil trafficking to the lung after I-R injury (fig. 5). Our data suggest that these cells, which do not reside inside the pulmonary vasculature, may participate in the sensing of I-R and communicate with endothelial cells, leading to the production of inflammatory mediators. Although lung endothelial cells and alveolar macrophages may not physically be in contact with each other, their close proximity would allow them to communicate via secreted factors, such as IL-1β. Furthermore, alveolar macrophages may perform this I-R sensing role possibly...
through TLR4-mediated uptake of markers released by I-R–damaged endothelial cells or epithelial cells that then diffuse into the alveoli.

Although reports from others have suggested a role for monocytic cells in the process of neutrophil infiltration in a murine lung transplant model,19 our data using a more focused and precise lung I-R mouse model show that a specific subset, alveolar macrophages, likely act as the primary sensor cell type that responds to I-R injury. One group reported results contradictory to ours with worsening of lung I-R injury with alveolar macrophage depletion.57 However, their rat model involved ex vivo mechanical perfusion of isolated lungs and measurement of intrapulmonary neutrophil accumulation. In contrast, in our model we did not observe neutrophil trafficking at the early (1 h) reperfusion times (see Supplemental Digital Content 1, fig. 1B, http://links.lww.com/ALN/A876, which examines histologic lung sections 1 h after reperfusion).

Two other groups demonstrated a protective effect of alveolar macrophage-depletion in lung I-R injury. The first also used an ex vivo model to study the role of alveolar macrophages in lung I-R injury.58 Mice were exsanguinated and reperfused with a buffered solution to mimic mixed venous blood. The second used hilar clamping in a rat model of nonventilated I-R injury.59 We believe that our in vivo model better and more closely replicates clinical I-R injury scenarios. Furthermore, having validated this model in mice, we hope to further identify and dissect the key pathways important in lung I-R injury using available genetic knockouts and transgenic mice. Through in vivo cell depletion and reconstitution experiments, future experiments can examine the role of alveolar macrophages and specific signaling pathways within alveolar macrophages in this physiologically relevant model of ventilated lung I-R injury.

Two of the studies referred to previously also used liposome-encapsulated clodronate to deplete alveolar macrophages and examined lung vascular permeability.58,59 However, liposome-encapsulated clodronate may in fact affect lung vascular permeability independent of other treatments or procedures, perhaps making this alveolar-macrophage-depletion method unsuitable for examining changes in vascular permeability (data not shown).

Alveolar macrophage, by their numbers in the lung, their location close to the vasculature, and reported functionality in consuming dead cells and debris, are arguably ideally suited to perform the role of sterile damage or I-R sensor. However, some published reports have characterized alveolar macrophages as being immunosuppressive, rather than pro-inflammatory.54,57,60 Experiments are ongoing to directly address whether alveolar macrophages are necessary and sufficient for the generation of a full lung I-R inflammatory response. At this time we cannot formally rule out the possibility that another phagocytic CD11c+ population, such as a dendritic cell population, may contribute to initiating the response to lung I-R injury. It is also entirely possible that both dendritic cells and alveolar macrophages are immunomodulatory in distinct ways, depending on the type of sterile insult, the branch of immune system activated (adaptive vs. innate), and the concomitant presence of pathogen.

In summary, this study uses a murine model of lung injury that does not involve airway occlusion and specifically focuses on ventilated I-R by isolating the blood flow to a single lung. The data provide compelling evidence that alveolar macrophages serve the role of sensing and/or amplifying lung I-R injury and that TLR4 plays a critical part in this process. Thus, manipulating the activity and/or presence of TLR4 and alveolar macrophages potentially could permit control of the clinical response to lung I-R injury. In the future, one could envision this role of manipulating or modulating the immune system and its inflammatory responses falling to the anesthesiologist or perioperative physician in rapidly evolving clinical situations encountered in the operating theater and intensive care unit.

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References


