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LUNG ISCHEMIA-REPERFUSION IS A STERILE INFLAMMATORY PROCESS INFLUENCED BY COMMENSAL MICROBIOTA IN MICE

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ABSTRACT—Lung ischemia-reperfusion (IR) complicates numerous clinical processes, such as cardiac arrest, transplantation, and major trauma. These conditions generate sterile inflammation, which can cause or worsen acute lung injury. We previously reported that lung and systemic inflammation in a mouse model of ventilated lung IR depends on Toll-like receptor 4 (TLR-4) signaling and the presence of alveolar macrophages. Here, we tested the hypothesis that the intestinal microbiome has a role in influencing the inflammatory response to lung IR. Lung IR was created in intubated mechanically ventilated mice via reversible left pulmonary artery occlusion followed by reperfusion. Inflammatory markers and histology were tracked during varying periods of reperfusion (from 1 to 24 h). Separate groups of mice were given intestinally localized antibiotics for 8 to 10 weeks and then were subjected to left lung IR and analysis of lungs and plasma for markers of inflammation. Alveolar macrophages from antibiotic-treated or control mice were tested *ex vivo* for inflammatory responses to bacterial TLR agonists, namely, lipopolysaccharide and Pam3Cys. We found that inflammation generated by left lung IR was rapid in onset and dissipated within 12 to 24 h. Treatment of mice with intestinally localized antibiotics was associated with a marked attenuation of circulating and lung inflammatory markers as well as reduced histologic evidence of infiltrating cells and edema in the lung after IR. Alveolar macrophages from antibiotic-treated mice produced less cytokines *ex vivo* when stimulated with TLR agonists as compared with those from control mice. Our data indicate that the inflammatory response induced by nonhypoxic lung IR is transient and is strongly influenced by intestinal microbiota. Furthermore, these data suggest that the intestinal microbiome could potentially be manipulated to attenuate the post-IR pulmonary inflammatory response.

KEYWORDS—Lung injury, alveolar macrophages, intestinal microbiome, microbiota, gut flora, IR injury, ischemia-reperfusion, ventilated lung IR

INTRODUCTION

Interruption in pulmonary blood flow can cause ischemia-reperfusion (IR) injury and lung dysfunction contributed to by induced inflammation (1–3). Lung IR injury in the context of hemorrhagic trauma, in-hospital cardiac arrest, and pulmonary emboli portends worse patient outcomes (reviewed in 4,5). Recent studies suggest that the intestinal microbial flora can modulate inflammatory responses of distant organs, including the lung (reviewed in 6–8). This raises the question of whether or not the intestinal microbial flora might have effects on inflammation induced by lung IR. Because critically ill patients often receive antibiotics that alter their intestinal flora, it is important to expand our understanding of the impact of these microbiome alterations on systemic and lung-specific immune responses. It is conceivable that manipulating the microbiome in a targeted fashion could have disease-specific benefits; however, widespread use of antibiotics to eliminate intestinal and other microbiome populations would likely cause greater harm than good. For example, medical and surgical patients who are identified as being at risk for pulmonary embolus (PE), such as those with cancer, hypercoagulable syndromes, or posttrauma, could benefit from such therapies that reduce their IR-driven lung inflammation should a PE occur. Conversely, the intestinal flora could be supplemented or augmented in patients at risk for nosocomial pneumonia to facilitate the lung's ability to clear microbial pathogens.

The innate immune system orchestrates sterile inflammation induced by tissue injury such as in trauma and other scenarios involving IR injury (9–12). In addition to serving as receptors for microbial products, the family of Toll-like receptors (TLRs) function as sensors of endogenous cellular and tissue damage (9, 13). For instance, TLR-4 recognizes lipopolysaccharide (LPS) as well as high-mobility group box 1 (HMGB1). As such, TLRs are centrally involved in initiating inflammatory responses to sterile and infectious injuries. Furthermore, the inflammasome, an intracellular immune-signaling complex

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that controls interleukin-1β (IL-1β) release, has been identified as a key intracellular regulator for both sterile and infectious inflammatory stimuli (reviewed in 14–16). At a cellular level, alveolar macrophages have been shown to influence local and remote inflammatory responses to trauma and IR in humans as well as in various animal models (reviewed in 17).

Studies in animals and humans have indicated that organ injury in IR is exacerbated by the extrinsic infiltration and activation of immune cells within the injured organ (1, 18). However, the spatiotemporal sequence of lung intrinsic and lung extrinsic events and the cellular and molecular pathways responsible for lung IR injury have not been fully delineated. Moreover, the lung immune response to IR may be unique because, as an organ, it experiences frequent alterations in segmental blood flow and ventilation, such as in the case of hypoxic pulmonary vasoconstriction.

We previously reported that TLR-4 and alveolar macrophages are key components of the early response to ventilated lung IR *in vivo*, and that IL-1β made by IR-challenged macrophages augmented endothelial inflammatory cytokine production *in vitro* (19). Commensal bacteria, also known as the host microbiome, provide an abundant source of diverse TLR ligands and other metabolic factors that can engage with the host immune system in various niches. In doing so, the microbiome may be able to modulate various inflammatory cells and processes, including possibly those activated or initiated after sterile IR in the lung.

In this study, we further characterized the kinetics of the inflammatory response to nonhypoxic lung IR and tested the hypothesis that a reduction of the intestinal microbial flora with intestinally localized antibiotics would affect this lung inflammatory response. We observed that lung IR inflammation was transient and that the reduction of the intestinal microbial flora markedly decreased lung IR-induced inflammation *in vivo* and reduced the responsiveness of alveolar macrophages to bacterial TLR agonists *ex vivo*. Our data suggest a novel role for the intestinal microbiome in regulating the magnitude of the lung inflammatory response after trauma through effects on alveolar macrophages.

MATERIALS AND METHODS

Animals

All animal studies were approved by the Institutional Animal Care and Use Committee at the University of California, San Francisco. Male mice (12–15 weeks old) were either purchased (The Jackson Laboratory, Bar Harbor, Me) or bred at the animal facility at University of California-San Francisco. Purchased mice were allowed to acclimatize to their new housing for at least 1 week before any experiments on them were conducted.

Investigators have reported strain variation in the inflammatory response to ventilated lung IR, and Dodd-o et al. (20) measured the magnitude of inflammatory changes observed in various laboratory strains of mice. They established the following hierarchy from highest IR-generated inflammation to lowest: SW/R > C3H > A/J > 129/J > BL6 > CBA/J > SJL/J > Balb/c. For our studies, we used C3H mice, which were shown to be strong responders.

Male mice were used primarily to reflect the fact that trauma disproportionately affects human males. Compared with our previous studies (19), we used larger groups (n = 10–15) to assess the effects of intestinal antibiotics on lung IR because we were uncertain of the magnitude of the treatment effect. All mice for a given experiment were either littermates or purchased/bred such that they were age matched. Because all mice used in these experiments were randomly chosen either to undergo the various surgeries (sham versus IR) or treatments (±antibiotic water *ad libitum*), there was no attempt made to blind the experimenters.

Antibiotic treatments

Mice were given oral antibiotics in their drinking water (120 units/mL polymyxin B and 0.6 mg/mL neomycin *ad libitum*; both Sigma-Aldrich, St. Louis, Mo) for 8 to 10 weeks. This duration of treatment was chosen to lower the bacterial burden in the intestinal tract and allow for a period of reequilibration to a new steady state with an absent/reduced commensal microbiome. Because we use 12- to 15-week-old adult mice for our experiments, we initiated antibiotic treatment immediately after weaning mice, at approximately 5 weeks of age. Antibiotics were added to the drinking water, and the antibiotic drinking water was then either used fresh or frozen for future use. Antibiotic drinking water was changed weekly. The continued microbicidal activity of the antibiotic drinking water was confirmed at the beginning of and at the end of each week when the water was changed. To do so, Luria-Bertani (LB) agar plates were coated with 1 mL of the antibiotic drinking water and then streaked with live *Escherichia coli* bacteria and incubated at 37°C for 48 h. Efficacy was confirmed based on lack of bacterial growth on the LB plates. We have not tested shorter durations of antibiotic treatment. Untreated control mice were given standard drinking water. There were no differences in weight gain or signs of pathogen overgrowth, such as can occur with *C. difficile* (no diarrhea), in mice that received antibiotic water as compared with control mice. Apart from the presence or absence of antibiotics in the drinking water, all mice were treated identically and were all housed in the same room in the animal facility.

Stool was obtained using sterile technique from within the intestine of antibiotic-treated or control mice, weighed and homogenized in sterile phosphate-buffered saline (PBS), and then equivalent amounts (adjusted for stool weight and PBS volume) were plated on LB agar plates and incubated overnight at 37°C under aerobic and anaerobic conditions. Antibiotic treatment did not result in the overgrowth of bacterial species under aerobic or anaerobic conditions.

Ventilated lung IR (unilateral left pulmonary artery occlusion) surgery

A murine model of unilateral left pulmonary artery occlusion was used, as we have described previously (19). Briefly, mice were anesthetized (using intraperitoneal tribromoethanol, Avertin; Sigma-Aldrich), orally intubated, given buprenorphine intraperitoneally (Harry Schein, Melville, NY), and placed on a rodent ventilator, using tidal volumes of 225 μL and a respiratory rate of 180 breaths/min (assuming an average mouse weight of 30 g). A left thoracotomy via the interspace between the second and third ribs was performed after which the left pulmonary artery was identified and an 8-0 prolene monofilament suture was passed between the left pulmonary artery and the left bronchus while visualizing under high-magnification microscopy. A slip-knot suture was tied, and the end of the suture was externalized through a narrow-bore (27-gauge) needle to the anterior chest wall. Before closure of the thorax, the left lung was inflated to occupy the left thoracic cavity. Local anesthetic (3–4 drops of 0.25% bupivacaine) was applied topically before skin closure. The total period of mechanical ventilation was approximately 20 to 25 min. After skin closure, mice were extubated. After 30 min of ischemia, the externalized suture was removed to release the ligature on the pulmonary artery and reestablish lung perfusion. At the end of the reperfusion period, mice were euthanized and the blood and lungs were collected.

Blood was collected in anesthetized mice via cardiac puncture using a heparinized syringe and centrifuged (14,000*g*, 5 min), and the plasma was separated, flash frozen in liquid nitrogen, and stored at −80°C. Lower portions of the left lungs were excised and placed in either Trizol (Life Technologies, Carlsbad, Calif) at −80°C for future RNA isolation or placed in histology cassettes in buffered formalin and, 24 h later, placed in 70% ethanol before processing. Lung sections were stained with hematoxylin and eosin (H&E) and analyzed histologically for neutrophil infiltration and edema formation. Levels of cytokines and chemokines were quantified in plasma.

Except where otherwise noted, the reperfusion time was 3 h. At this time point, we are able to detect both cellular recruitment to the lung and increased plasma levels of secreted cytokines and chemokines. Sham (control) mice underwent left thoracotomy and all other procedures at precisely the same time points as experimental mice, except that the left pulmonary artery was not isolated and a slip-knot was not tied or externalized.

All mice received equivalent durations of mechanical ventilation (20–25 min) and were left spontaneously breathing during their recovery from anesthesia and the remainder of the ischemia period and subsequent reperfusion or equivalent periods in the sham mice. This model is designed to minimize ventilator-induced lung injury by reducing both the duration of mechanical ventilation and the tidal volumes used to ventilate animals (7.5 mL/kg). This model also minimizes the potential confounding effects of atelectasis, which is believed to cause or exacerbate inflammation. This is accomplished by maintaining lung expansion with positive end-expiratory pressure, beginning immediately after the thoracotomy incision and during closure of the thoracotomy incision. Finally, the maintenance of oxygen delivery (room air, FiO_2 of 0.21) to the experimental

lung throughout the surgical, ischemia, and reperfusion periods should eliminate the potential contribution of lung tissue hypoxia to the inflammatory process. This latter phenomenon is uniquely possible in the lung using our surgical model because of the separate routes for the supply of nutrients (via blood) versus oxygen (via gas exchange) and the absence of a significant bronchial circulatory system in mice.

Although this lung IR procedure has high survival rates of 80% to 90% on average, some mice died from irreparable damage to the pulmonary artery or left bronchus during the slip-knot placement. Mice that did not survive the surgery or the reperfusion period procedure were excluded from the study.

Quantitative reverse transcription real-time polymerase chain reaction

TaqMan-specific inventoried gene primers for glyceraldehyde 3-phosphate dehydrogenase, β-actin, IL-6, IL-1β, tumor necrosis factor-α (TNF-α), chemokine (C-X-C motif) ligand 1 (CXCL-1), CXCL-2, and intercellular adhesion molecule 1 were used to measure the message levels of these genes in lung tissue (Life Technologies).

Lung tissue was homogenized (Tissue-Tearor; Biospec Products, Bartlesville, Okla) and total RNA was isolated using Trizol. We used the High-Capacity RNA-to-cDNA reverse transcription kit using 1 μg messenger RNA per reaction (Life Technologies). Quantitative real-time polymerase chain reaction was performed using the ABI Prism 7000 Sequence Detection System (Life Technologies). 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.

16S rRNA levels were measured from lung tissue cDNA using the following primers, which were generously provided by Susan Lynch, PhD (University of California, San Francisco): P891F (TGGAGCATGTGGTTTAATTCGA) and P1033R (TGCGGGACTTAACCCAACA) with UniProbe (CACGAGCTGACGA CARCCATGCA) as previously reported (21). Run method: UDG incubation at 50°C for 2 min, followed by polymerase chain reaction activation at 95°C for 10 min, and then followed by 40 cycles of 15 s at 95°C and 1 min at 60°C.

The average threshold count (Ct) value of two to three technical replicates was used in all calculations. The average Ct values of the internal controls (glyceraldehyde 3-phosphate dehydrogenase, β-actin) were used to calculate ΔCt values for the array samples. Data analysis was performed using the $2^{-\Delta\Delta Ct}$ method, and the data were corrected for statistical analysis using log transformation, mean centering, and autoscaling (22–24).

Sandwich enzyme-linked immunosorbant assay

Concentrations of IL-6, IL-1β, IL-33, chemokine (C-C motif) ligand 2 (CCL-2)/monocyte chemotactic protein 1 (MCP-1), CCL-3/macrophage inflammatory protein-1α (MIP-1α), CXCL-2/MIP-2α, and TNF-α in mouse plasma or cell culture supernatant were determined using the mouse Quantikine kit (R&D Systems, Minneapolis, Minn). The HMGB1 enzyme-linked immunosorbant assays (ELISAs) were performed per manufacturer's instructions (IBL International GmBH, Hamburg, Germany). All assays were performed according the manufacturer's supplied protocol. Standard curves were generated and used to determine the concentrations of individual cytokines or chemokines in the samples.

Microscopy and histology scoring of lung injury

Hematoxylin and eosin–stained paraffin-mounted lung sections were evaluated by bright field microscopy using an Olympus IX51 inverted microscope, and images were captured using a Retiga 2000R camera and the Qcapture Pro 7 software (Qimaging, British Columbia, Canada).

We assigned an investigator who was blinded to the group assignments to examine the lung H&E slides and determine the levels of lung injury with a semiquantitative scoring system. For each mouse, the investigator examined 20 fields at 200× total magnification, and scoring was performed as described elsewhere (25). Briefly, the first criterion was infiltration or aggregation of inflammatory cells in air space or vessel walls: 1 = only wall, 2 = few cells (1–5) in air space, 3 = intermediate, 4 = severe (air space congested). Second criterion was interstitial congestion and hyaline membrane formation: 1 = normal lung, 2 = moderate (>25% of lung section), 3 = intermediate (25%–50% of lung section), 4 = severe (>50% of lung section). Third criterion was hemorrhage: 0 = absent, 1 = present.

Ex vivo *stimulation of alveolar macrophages*

Alveolar macrophages were isolated via bronchoalveolar lavage (BAL) from antibiotic-treated or control mice that had not undergone surgery. Mice were euthanized under deep anesthesia (Avertin), and their tracheas were surgically exposed. Alveolar lavage was performed by injecting and withdrawing 10 mL of ice-cold PBS, 1 mL at a time into the trachea. Cells from two to four mice were pooled, counted, and pelleted at 800*g* × 5 min at 4°C. Cells were resuspended in Roswell Park Memorial Institute-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin and plated in a 48-well plate at 20,000 to 30,000 cells per well and were incubated for 21 h with LPS (1 μg/mL; Sigma-Aldrich) and Pam3Cys (2.5 μg/mL; EMC Microcollections, Tubingen, Germany). One hour before harvesting, the culture medium was collected and replaced with fresh medium containing nigericin (5 μM; Sigma-Aldrich). All media that were collected were centrifuged (800*g* × 5 min at 4°C) to remove debris and stored at −80°C until assayed by ELISA.

Statistical analysis

Data in the figures are expressed as mean ± SD. Data from *in vivo* studies comparing two conditions were analyzed using 2-tailed nonparametric Mann-Whitney *U* analyses. Data from *in vitro* studies comparing two conditions (*ex vivo* pooled alveolar macrophage stimulation studies) were analyzed using standard Student *t* test with equal SD to generate *P* values. For analyses of multiple groups (such as for the time course experiment in Fig. 1), simple 1-way analysis of variance (ANOVA) was used and Tukey correction for multiple comparisons was applied. GraphPad Prism was used for statistical analyses (GraphPad Software, La Jolla, Calif). For all *in vivo* experiments and *in vitro* analyses, values of $P < 0.05$ were considered significant. *P* values are represented as follows in the figures: *, <0.05; **, <0.01; ***, <0.001; ****, <0.0001. Experiments were repeated two or more times, as indicated in the figure legends. 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

Lung IR causes transient self-resolving inflammation and neutrophilic influx

We use a ventilated lung IR animal model to eliminate confounding influences of atelectasis or lung tissue hypoxia on

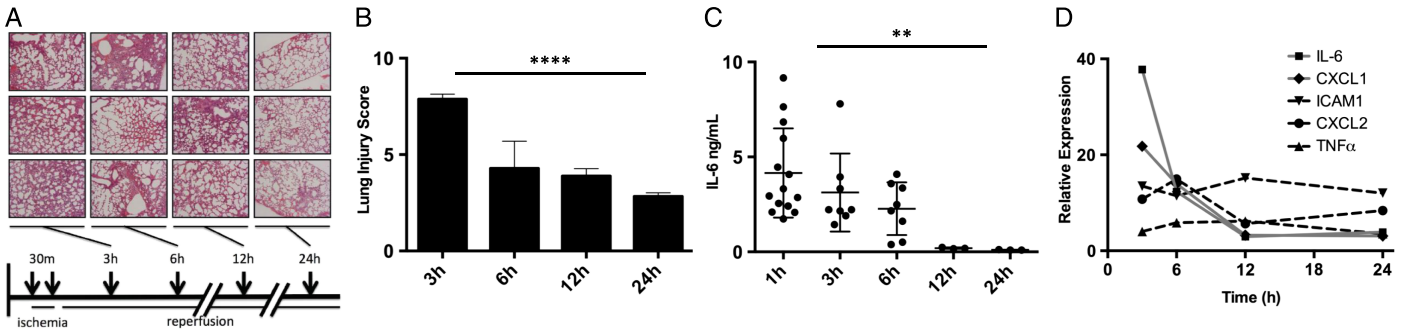

Fig. 1. **Early and transient inflammation after nonhypoxic lung IR**. A, H&E staining of left lower lung segments of C3H/HeOuJ mice that underwent ischemia (30 min) followed by reperfusion (3–24 h) (5× magnification). Each image is representative of one surgery, that is, three mice were examined at each reperfusion time point. The schematic of ischemia time and reperfusion times is included below the histology images. B, Semiquantitative lung injury scoring of the H&E-stained left lower lung segments. Normal lung score = 2; severely injured (maximum) score = 9. Statistics were performed via ordinary 1-way ANOVA for multiple comparisons. C, Plasma levels of IL-6 were measured at the noted reperfusion time points using single or multiplex ELISA. Statistics were performed via 1-way ANOVA (Kruskal-Wallis test) for multiple nonparametric comparisons. D, mRNA levels were measured at the noted reperfusion time points by quantitative real-time PCR. n = 3–13 depending on time point. Statistical analyses were performed via 1-way ANOVA (Kruskal-Wallis test) for multiple nonparametric comparisons.

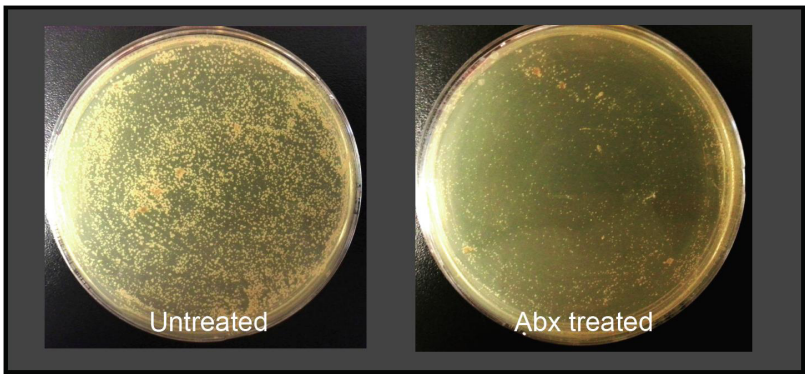

FIG. 2. **Oral antibiotic (Abx) treatment reduces gastrointestinal microbiota burden**. Stool from untreated control (left) and antibiotic-treated mice (right) was homogenized in sterile PBS and equivalent amounts (adjusted for stool weight and PBS volume) were plated on LB agar plates and incubated overnight at 37°C.

inflammatory responses. We investigated the time course of inflammation after lung IR and found that the neutrophilic influx to the IR-injured lung peaked at early time points (3–6 h) and abated within 12 to 24 h, as assessed by H&E staining (Fig. 1A) and lung injury score (Fig. 1B). We also measured the kinetics of induction of specific inflammatory cytokines and chemokines, which we previously established are induced by lung IR (19). We found that IL-6 was rapidly secreted (by 1–3 h after reperfusion). Similar to the lung histology, plasma levels of inflammatory mediators returned to baseline within 12 to 24 h (Fig. 1C for IL-6 secreted levels: 1-way ANOVA, $P < 0.01$; Tukey correction for multiple comparisons, $P = 0.05$, when comparing $t = 1$ h vs. $t = 12$ or 24 h). Similar expression patterns of IL-6 and CXCL-1 were observed at the mRNA level in the affected lungs but interestingly not for CXCL-2, TNF-α, or intercellular adhesion molecule 1 (Fig. 1D).

Antibiotic treatment depletes intestinal microbiota and attenuates lung IR inflammation by gross pathology

Using our ventilated lung IR model, we previously reported that TLR-4 and alveolar macrophages are required for lung IR inflammation (19). We tested the hypothesis that manipulating the intestinal microbiome would affect the inflammatory response to lung IR by altering alveolar macrophage function. We added antibiotics that are poorly absorbed from the gastrointestinal tract (neomycin and polymyxin B) to the drinking water of mice and performed bacterial cultures on the stool of mice to confirm that the inclusion of antibiotics in the drinking water qualitatively reduced the intestinal bacterial load (Fig. 2). We also performed anaerobic cultures to confirm that antibiotic-treated mice had reduced levels of strict anaerobic bacteria (data not shown). To address the possible contribution of the pulmonary microbiome in lung IR-generated inflammation, we measured the 16S rRNA levels in lung tissue from control and antibiotic-treated mice and observed no significant differences (Figure, Supplemental Digital Content 1, at http://links.lww.com/SHK/A308).

We assessed the degree of lung injury (at 3 h reperfusion), namely, neutrophil recruitment and lung edema, after ventilated IR and performed blinded semiquantitative lung injury scoring as well. The majority of antibiotic-treated mice (9 of 11) had severely attenuated IR-initiated inflammation by histology compared with control mice (9 of 12) (Fig. 3A). Lung scoring also demonstrated a significant decrease in the lung injury score in the control versus antibiotic-treated mice ($P < 0.01$; Fig. 3B).

Antibiotic treatment reduces the systemic circulating levels of inflammatory cytokines and chemokines after lung IR

Consistent with the effects of antibiotic treatment on lung injury by histology, plasma levels at 3 h of reperfusion of IL-6, CXCL-2/MIP-2α, and CCL-2/MCP-1 were also reduced in antibiotic-treated versus control mice ($P < 0.01$, $P < 0.05$, $P < 0.05$, respectively) (Fig. 4). Although CCL-3/MIP-1α and IL-1β levels trended lower in the antibiotic-treated cohort, these findings did not achieve statistical significance. There were no differences

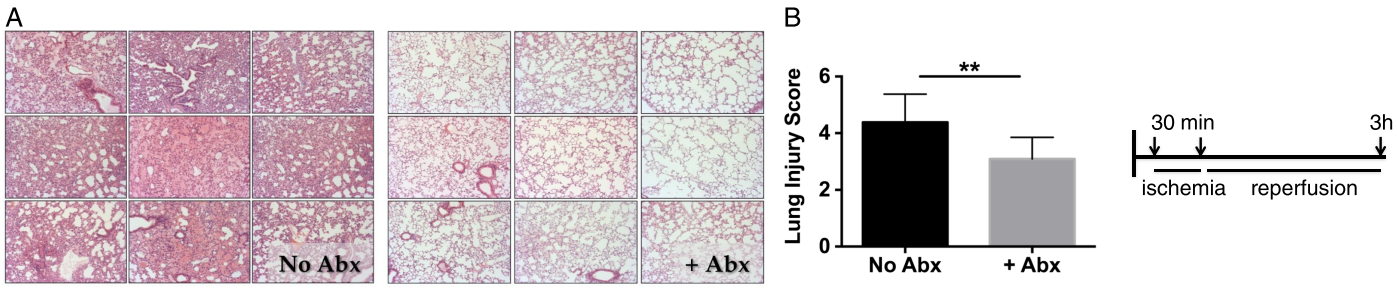

FIG. 3. **Antibiotic (Abx) treatment of mice results in reduced lung edema and alveolar disruption after nonhypoxic lung IR**. A, H&E staining of left lower lung segments (10× magnification) of mice that were given oral antibiotics (polymyxin B and neomycin *ad libitum* in drinking water) for 8 to 10 weeks and then were subjected to left lung ischemia reperfusion (30-min ischemia/3-h reperfusion). Images represent nine of 11 untreated control mice (left panel) and nine of 12 antibiotic-treated mice (right panel). Mice that did not survive the surgery (four control mice and three antibiotic-treated mice) were excluded from the study. B, Semiquantitative lung injury scoring of H&E-stained left lower lung segments from the control and antibiotic-treated mouse cohorts. Normal lung score = 2; severely injured (maximum) score = 9.

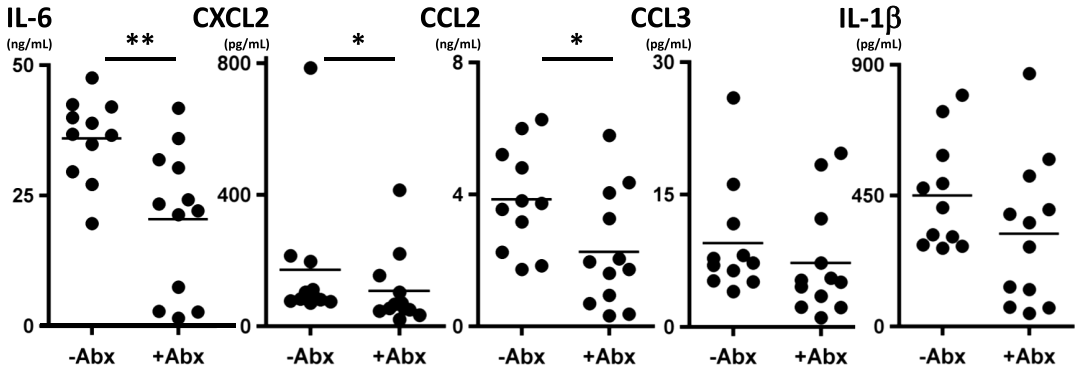

Fig. 4. **Antibiotic (Abx)-treated mice produce lower levels of inflammatory cytokine and chemokine after IR**. Plasma levels of IL-6, CXCL-2, CCL-2, CCL-3, and IL-1β were quantified in plasma collected from antibiotic-treated or control mice at 3 h after reperfusion by ELISA (data from all 11 untreated control and 12 antibiotic-treated mice are shown).

observable in other cytokines and DAMPs such as TNF-α, IL-33, or HMGB1 (data not shown).

Intestinal microbiota modulates alveolar macrophage activation by microbial TLR-2 and TLR-4 agonists

Intestinal microbiota has been shown by others to modulate lung immune responses (26). Because we had previously shown that alveolar macrophages were required for the initiation of lung IR inflammation (19), we hypothesized that intestinal commensal bacteria might modulate the ability of alveolar macrophages to regulate the lung IR inflammatory response. We compared inflammatory responses of BAL cells (predominantly alveolar macrophages) collected from mice that were and were not treated with antibiotics but that did not undergo surgery. These alveolar macrophages were treated *ex vivo* with LPS (TLR-4 agonist) or Pam3Cys (TLR-2 agonist). As compared with control mice, alveolar macrophages collected from antibiotic-treated mice were less responsive to the inflammatory effects of LPS *ex vivo*, as evidenced by lower levels of secreted IL-6, CXCL-2/MIP-2α, CCL-2/MCP-1, CCL-3/MIP-1α in culture supernatants compared with similarly stimulated alveolar macrophages from untreated control mice (Fig. 5A: LPS challenge – IL-6: $P < 0.05$; CXCL-2: $P < 0.01$; CCL-2: $P < 0.001$; CCL-3: $P < 0.05$; and Fig. 5B: Pam3Cys challenge – IL-6: $P < 0.0001$; CXCL-2: $P < 0.05$; CCL-2: $P < 0.001$; CCL-3: $P < 0.001$). Tumor necrosis factor-α levels trended lower in the antibiotic-treated cohort, but these findings did not achieve statistical significance.

Inflammasome pathways are stably muted in alveolar macrophages after exposure to antibiotics

Having previously shown that IL-1β is an important factor in IR for intercellular communication between macrophages and endothelial cells (19), we investigated whether inflammasome mediated release of active IL-1", was different in BAL macrophages collected from mice that were and were not treated with antibiotics but that did not undergo surgery. As compared with alveolar macrophages from control mice, alveolar macrophages from antibiotic-treated mice released significantly lower levels of IL-1β after stimulation with the inflammasome activator nigericin ($P < 0.01$; Fig. 6) even after 24 h in culture *ex vivo*.

DISCUSSION

Why relatively short interruptions in blood flow lead to inflammation remains an unresolved question of human physiology specifically whether or not this response serves an adaptive or maladaptive function. Frequently, in the clinical arena, IR is associated with significant detrimental effects on the host, with damage to the affected cells, tissues, and organs (lung IR reviewed in 1, 3). However, it is conceivable that IR responses may not necessarily be maladaptive. They could serve a beneficial role, but when things go awry, like in sepsis, damage ensues. By further understanding how this process of IR inflammation is regulated locally and systemically, we hope to gain insights into its overall role in health and disease. In this report, we identify a novel and

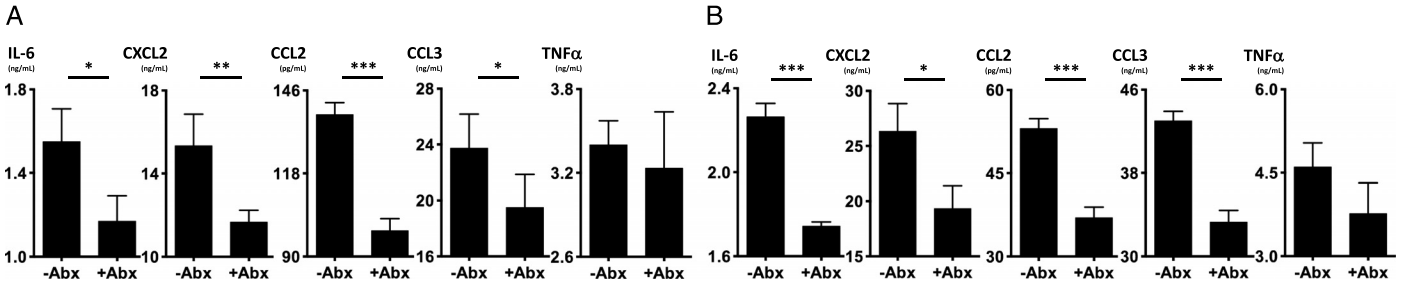

Fig. 5. **Alveolar macrophages from antibiotic-treated (+Abx) mice are less responsive to toll-like receptor (TLR) agonists *in vitro***. A, B, Alveolar macrophages were obtained from three each of control and antibiotic-treated mice that had not undergone surgery. The cells from the three mice were pooled, and equivalent numbers were seeded into three wells and treated overnight with LPS (1 μg/mL, A) or Pam3cys (2.5 μg/mL, B). Levels of IL-6, CXCL-2, CCL-2, CCL-3, and TNF-α were quantified in culture supernatants by ELISA. The experiment was repeated twice, and representative results are shown here.

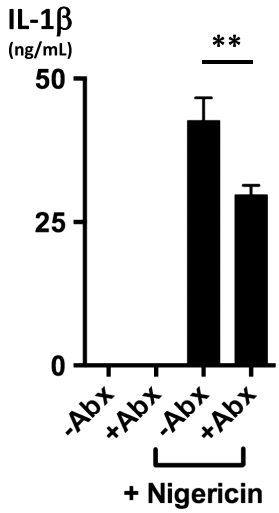

FIG. 6. **Inflammasome pathways are stably dampened in alveolar macrophages from antibiotic-treated (+Abx) mice**. After 24 h in culture *ex vivo*, alveolar macrophages from control and antibiotic-treated mice that had not undergone surgery (pooled from three mice each, just as in Fig. 5) were challenged with nigericin (5 μM) for 1 h to stimulate inflammasome-mediated IL-1β release. Levels of IL-1β were quantified in culture supernatants by ELISA, with the average of the three replicates represented on the graph. The experiment was repeated twice, and representative results are shown here.

heretofore unrecognized influence of the intestinal microbiome on distant organ injury, immune cell responses, and systemic inflammation induced by lung IR.

We first explored the timing of the inflammatory response and immune cell recruitment as well as its resolution after nonhypoxic lung IR in mice. We observed that short periods (30 min) of ischemia resulted in the early recruitment of neutrophils on reperfusion of the previously ischemic lung. However, this process resolved within 12 to 24 h with IL-6 and CXCL-1 levels also peaking early and then returning to baseline (as seen in Fig. 1). Although the rapid recruitment of neutrophils to the IR-injured tissue was expected, the fast resolution of this process with no apparent lung injury initially seemed to be inconsistent with clinical observations of sustained organ injury, dysfunction, and increased morbidity in patients. Although this may reflect differences in mouse and human biology, another possible explanation for this discrepancy is that, whereas neutrophils are attracted to lung tissue after nonhypoxic lung IR, the inflammatory response resolves in the absence of other insults, such as infection or lung tissue hypoxia, and does not result in significant lung damage and dysfunction. In fact, these neutrophils that are recruited do not generate reactive oxygen species (A. Prakash, unpublished data).

Investigators have shown that local IR can influence the composition of the intestinal microbiome (27). Conversely, we wanted to ask the question whether the microbiome could affect local and distant IR responses. We based this hypothesis on reports that the intestinal microbiome has immunomodulatory effects outside of the gastrointestinal tract and even in the lung (26, 28–30 and reviewed in 6–8). Thus, we sought to evaluate its role in influencing the inflammatory response to lung IR. We found that commensal microbiota indeed had a role in the initial induction of lung IR inflammation (as shown in Figs. 3 and 4). In addition, we found that, even before exposure to IR, the alveolar macrophages isolated from antibiotic-treated mice were less responsive to TLR-2 and TLR-4 agonists than those isolated from control mice (Fig. 5). These data suggest that alveolar macrophages may be a resident lung cell population whose ability to respond to lung IR is modulated by the presence, absence, or perhaps specific composition of the intestinal microbiome.

We hypothesize that circulating bacterial products or host signals derived from intestinal exposure to commensal flora may serve to prime lung immunity to respond to infectious and sterile injurants. Identifying such bacterial products and factors as well as host signals is of great interest and the focus of our ongoing experiments. These may include bacterial components, such as LPS, lipoproteins, or peptidoglycan, or bacterial metabolites that gain entry into the circulation and directly affect alveolar macrophages and other lung immune cells. In addition, or alternatively, the microbial flora might affect immune cells within or in proximity to the intestine that then migrate to the lung and directly or indirectly modulate the lung responses. These latter concepts are supported by a recent report that intestinal commensal flora control the trafficking of specific phagocytes to mesenteric lymph nodes (31). Thus, alterations in commensal flora could possibly indirectly or directly skew the alveolar macrophages from a proinflammatory to a proresolving phenotype (also described in the literature as "M1" and "M2," respectively).

Investigators have proposed a 2-step process for the activation of inflammatory cells via activation of the inflammasome, leading to IL-1β release (reviewed in 32, 33). We speculate that the commensal microbiota may provide this first "priming" signal, which signals alveolar macrophages to make pro-IL-1β. Alveolar macrophages would then be in a primed state to respond to second "triggering" signals, such as IR, resulting in inflammasome activation and active IL-1β release, further cytokine and chemokine release, and the rapid recruitment of effector cells, such as neutrophils. Our data showing that alveolar macrophages from antibiotic-treated mice produce significantly less IL-1β *ex vivo* after challenge with inflammasome stimulators (nigericin) than those from control mice (Fig. 6) is consistent with this 2-step model.

Another emerging microbiome population is the pulmonary microbiome (34). Although this bacterial population is largely thought to be present in the upper and not distal airways and alveoli during health, it is possible that the pulmonary microbiome also contributes to the lung IR inflammation. We chose to use oral antibiotics that are poorly absorbed from the intestinal tract to specifically affect the quantity and composition of intestinal bacteria. However, the aspiration or low-level systemic absorption of these antibiotics could also affect the composition of the commensal pulmonary microbiome. For example, aspirated polymyxin B could directly affect the lung IR responses by binding to and inactivating LPS. To address this issue, we measured bacterial load in antibiotic-treated and control mice by colony-forming unit counts and quantifying 16S rRNA levels. We found low to absent levels in blood and lung tissue, and these levels were not affected by our administration of oral antibiotics (data not shown and Figure, Supplemental Digital Content 1, at http://links.lww.com/SHK/A308).

Thus, our data support the hypothesis that these signals originate from the intestinal microbiome.

Overall, in the specific case of nonhypoxic lung IR, we speculate that inflammatory responses may serve a protective rather than a maladaptive role. In barrier organs that serve as a frontline against the entry of pathogens, such as the lung, a primed and rapid IR response with the recruitment of inflammatory cells may be advantageous. Because pneumonias frequently occur in the context of severe trauma, these cells could bolster antibacterial defenses and rapid clearance of invading pathogens. In contrast, in certain clinical scenarios, such as severe sterile IR injury, ventilator-induced lung injury, and acid aspiration, it may conversely prove advantageous to dampen inflammatory processes. Thus, manipulating the intestinal microbiome through decontamination approaches could prove useful in certain specific circumstances.

Other investigators have reported that intestinal commensal bacteria can influence bacterial killing by alveolar macrophages in a TLR-4–dependent manner (35). Without directly implicating the intestinal microbiome, another study demonstrated that intestinal IR leads to indirect lung injury (36). However, to our knowledge, ours is the first report demonstrating that manipulation of the intestinal microbiome leads to an altered inflammatory response to sterile injury induced by lung IR. We propose the means of communication between the intestine and the lung to be one or more of the following: 1) the direct translocation of intestinal bacteria; 2) direct translocation of bacterial products or metabolites; or 3) indirect reprogramming of local intestinal or migratory immune cells with direct or indirect signaling to the lung. Based on our results, we speculate that the variability of responses to lung IR injury in different individuals may result, at least in part, from the specific identity and diversity of the resident intestinal commensal bacteria. The fact that these bacterial populations are often unwittingly and indiscriminately targeted by antibiotic use and other nosocomial factors makes studying microbiome-influenced immune processes all the more relevant.

Although the strong advantage of our nonhypoxic lung IR model is its focus on the immune responses generated by "pure" lung IR, a limitation is the inability to model the pathophysiologic complexity of human trauma in mice. We are therefore currently interrogating how the immune responses to superimposed infections can be affected by the decontamination of the intestinal microbiome in the setting of lung IR in mice and in doing so we hope to better model complex trauma pathophysiology as experienced by patients. Other limitations include the possibility that mouse and human immunology may differ significantly in the responses to lung IR. Consequently, correlating human systemic immune responses to severe trauma to the presence or composition of intestinal microbiota may yield interesting information. These studies are ongoing at our institution. Further studies are also necessary to define the precise cellular and molecular mechanisms involved in the communication between the intestinal microbiome and lung and how they affect the resulting immune responses to IR.

We have demonstrated here that nonhypoxic lung ischemia reperfusion injury in mice is transient and is strongly influenced by intestinal commensal microbiota. By understanding how inflammation in the lung after IR is regulated, we may begin to devise strategies to manipulate this response in select groups of patients, for example, those at high risk for PE and the resulting sterile lung injury. Although we are not advocating the widespread initiation of antibiotics in trauma patients, our findings may be a first step toward understanding how to tailor therapies to mitigate lung and other organ injuries initiated by sterile injury through the manipulation of the intestinal microbiome.

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