Title

Alveolar macrophages and Toll-like receptor 4 mediate ventilated lung ischemia reperfusion injury in mice.

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Lung Ischemia Reperfusion (IR) is a Sterile Inflammatory Process Influenced by Commensal Microbiota in Mice

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Running Head: Commensals modulate lung IR inflammation

ABSTRACT

Background: 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 augment acute lung injury. We previously reported that lung and systemic inflammation in a mouse model of ventilated lung IR depends on Toll-like receptor (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.

Methods: Lung IR was created in intubated mechanically ventilated mice via reversible left pulmonary artery occlusion followed by reperfusion. Inflammatory markers and histology were tracked over varying periods of reperfusion (from 1h to 24h). Separate groups of mice were given intestinally-localized antibiotics for 8-10 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 LPS and Pam3Cys.

Results: Inflammation generated by left lung IR was rapid in onset and dissipated within 12-24h. Treatment of mice with intestinally localized antibiotics was associated with a marked attenuation of circulating and lung inflammatory markers, and histologic evidence of infiltrating cells and edema in the lung following IR. Alveolar macrophages from antibiotic-treated mice produced less cytokines *ex vivo* when stimulated with TLR agonists as compared to those from control mice.

Conclusions: Our data indicate that the inflammatory response induced by lung IR is transient and is strongly influenced by intestinal microbiota. 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. Since 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 that are identified as being at risk for pulmonary embolus (PE), such as those with cancer, hypercoagulable syndromes, or post trauma, 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, TLR4 recognizes LPS

as well as HMGB1. As such TLRs are centrally involved in initiating inflammatory responses to sterile and infectious injury. Furthermore, the inflammasome, an intracellular immune signaling complex that controls 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 since 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 TLR4 and alveolar macrophages are key components of the early response to ventilated lung IR and that IL-1β made by IR challenged macrophages augmented endothelial inflammatory cytokine production *in vitro*[20]. Commensal bacteria, aka, the host microbiome provides 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 following sterile IR in the lung.

In this study, we further characterized the kinetics of the inflammatory response to non-hypoxic lung IR and tested the hypothesis that 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 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 suggests a novel role for the intestinal microbiome in regulating the magnitude of the lung inflammatory response following 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 and colleagues measured the magnitude of inflammatory changes observed in various laboratory strains of mice[21]. 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 to our previous studies[20], we used larger groups (n=10-15) to assess the effects of intestinal antibiotics on lung IR, since 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. Since all mice used in these experiments were randomly chosen either to undergo the various surgeries (sham vs. 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 8-10 weeks. This duration of treatment was chosen in order to lower the bacterial burden in the intestinal tract and allow for a period of re-equilibration to a new steady-state with an absent/reduced commensal microbiome. Because we use 12-15 week old adult mice for our experiments, we initiated antibiotic treatment immediately after weaning mice, at around 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. Mice received fresh antibiotic drinking water once a week. 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, LB agar plates were coated with 1mL of the antibiotic drinking water and then streaked with live *E.coli* bacteria and incubated at 37ºC for 48h. 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 to 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 (PSB), and then equivalent amounts (adjusted for stool weight and PBS volume) were plated on LB agar plates and incubated overnight at 37ºC under aerobic and anaerobic conditions. Antibiotic treatment did not result in the overgrowth of bacterial species under aerobic or aneaerobic conditions.

Ventilated Lung Ischemia Reperfusion (Unilateral left pulmonary artery occlusion) Surgery

A murine model of unilateral left pulmonary artery occlusion was used, as we have described previously [20]. Briefly, mice were anesthetized (using IP tribromoethanol (Avertin®); Sigma-Aldrich), orally intubated, given buprenorphine (IP; Harry Schein, Melville, NY), and placed on a rodent ventilator, using tidal volumes of 225μL, and a respiratory rate of 180 breaths/min (assuming an average mouse weight of 30g). A left thoracotomy via the interspace between the 2nd and 3rd 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

microscope magnification. A slip-knot suture was tied and the end of the suture externalized through a narrow bore (27g) needle to the anterior chest wall. Prior to 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 prior to skin closure. The total period of mechanical ventilation was approximately 20-25min. After skin closure and recovery from anesthesia, mice were extubated. After 30min 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, centrifuged (14,000g, 5min) and the plasma 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, CA) at -80°C for future RNA isolation or placed in histology cassettes in buffered formalin and 24h later placed in 70% ethanol before processing. Lung sections were stained with hemotoxylin and eosin, and analyzed histologically for neutrophil infiltration and edema formation. Levels of cytokines and chemokines were quantified in plasmas.

Except where otherwise noted, the reperfusion time was 3h. At this time point, we are able to see 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 the left pulmonary artery was not isolated and a slip-knot was not tied or externalized.

All mice received equivalent durations of mechanical ventilation (20-25min), 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 (7.5cc/kg). This model 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 (PEEP), beginning immediately after the thoracotomy incision throughout the entire period of surgery through 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 due to the separate routes for the supply of nutrients (via blood) *vs.* oxygen (via gas exchange) and the absence of a significant bronchial circulatory system.

While this lung IR procedure has high initial survival rates of 80-90% on average, some mice die 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, beta actin, interleukin (IL) 6, IL-1β, tumor necrosis factor (TNF) α, chemokine (C-X-C motif) ligand (CXCL) 1, CXCL2, and ICAM1 were used to measure the message levels of these genes in lung tissue (Life Technologies, Carlsbad, CA).

Lung tissue was homogenized (Tissue-Tearor - Biospec Products, Bartlesville, OK) and total RNA 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 20s was followed by 40 cycles of 1s at 95°C and 20s 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 (CACGAGCTGACGACARCCATGCA) as previously reported [22]. Run method: UDG incubation at 50°C for 2min, followed by polymerase chain reaction activation at 95°C for 10min and then followed by 40 cycles of 15s at 95°C and 1min at 60°C.

The average threshold count (Ct) value of 2–3 technical replicates was used in all calculations. The average Ct values of the internal controls (glyceraldehyde 3-phosphate dehydrogenase, beta actin) was 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 [23–25].

Sandwich enzyme-linked immunosorbant assay (ELISA)

Concentrations of IL-6, IL-1β, IL-33, chemokine (C-C motif) ligand (CCL) 2/monocyte chemotactic protein (MCP) 1, CCL3/macrophage inflammatory protein (MIP) 1α, CXCL2/MIP2α, and TNFα in mouse plasma or cell culture supernatant were determined using the mouse Quantikine kit (R&D Systems, Minneapolis, MN). HMGB1 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 sample.

Microscopy and Histology Scoring of Lung Injury

Hematoxylin and Eosin (H&E) stained paraffin-mounted lung sections were evaluated by bright field microscopy using an Olympus IX51 inverted microscope and images 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 semi-quantitative scoring system. For each mouse, the investigator examined 20 fields at 200x total magnification and scoring was performed as described elsewhere[26]. Briefly, the first criterion was infiltration or aggregation of inflammatory cells in airspace 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 2-4 mice were pooled, counted and pelleted at 800g x 5min at 4°C. Cells were re-suspended in Roswell Park Memorial Institute-1640 (RPMI-1640) medium supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin and plated in a 48-well plate at 20,000-30,000 cells per well and were incubated for 21h with LPS (Sigma-Aldrich; 1 μg/ml) and Pam3Cys (EMC Microcollections, Tubingen, Germany; 2.5 μg/ml). One hour before harvesting, the culture medium was collected and replaced with fresh medium containing Nigericin (Sigma-Aldrich; 5μM). All media that was collected was centrifuged (800g x 5min 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

analyses. Data from *in vitro* studies comparing two conditions (*ex vivo* pooled alveolar macrophage stimulation studies) were analyzed using standard Student's t-test with equal SD to generate *P* values. For analyses of multiple groups (such as for the time course experiment in Figure 1), simple 1-way ANOVA was used and Tukey's correction for multiple comparisons applied. 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* analyses, p values < 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 2 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 Ischemia Reperfusion (IR) causes a transient self-resolving inflammation and neutrophilic influx

We utilize a ventilated lung IR animal model in order to eliminate confounding influences of atelectasis or lung tissue hypoxia on the inflammatory responses. We investigated the time course of inflammation following lung IR and found that the neutrophilic influx to the IR-injured lung peaked at early time points (3-6h) and abated within 12-24h, as assessed by H&E staining (**Figure 1A**) and lung injury score (**Figure 1B**). We also measured the kinetics of induction of specific inflammatory cytokines and chemokines, which we previously established are induced by lung IR[20]. We found that IL-6 was rapidly secreted (by 1-3h post reperfusion). Similar to the lung histology,

plasma levels of inflammatory mediators returned to baseline within 12-24h (**Figure 1C** for IL-6 secreted levels**:** 1-way ANOVA $p<0.01$; Tukey's correction for multiple comparisons $p=0.05$ when comparing t=1h *vs* t=12h or 24h**).** Similar expression patterns of IL-6 and CXCL1 were observed at the messenger RNA level in the affected lungs, but interestingly not for CXCL2, TNFα or ICAM1 (**Figure 1D**).

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

Using our ventilated lung IR model, we previously reported that TLR4 and alveolar macrophages are required for lung IR inflammation[20]. We tested the hypothesis that manipulating the intestinal microbiome would alter the inflammatory response to lung IR in an alveolar macrophage dependent fashion. 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 (**Figure 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 S1**).

We assessed the degree of lung injury (at 3h reperfusion), namely neutrophil recruitment and lung edema, following ventilated IR by histology and performed blinded semi-quantitative 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) (**Figure 3A).** Lung scoring also demonstrated a significant decrease in the lung injury score in the control vs. antibiotic treated mice ($p<0.01$, **Figure 3B**).

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

Consistent with the effects of antibiotic treatment on lung histology, plasma levels at 3h of reperfusion of IL-6, CXCL2/MIP2α, and CCL2/MCP1 were also reduced in antibiotic-treated *vs*. control mice ($p<0.01$, $p<0.05$, $p<0.05$, respectively) (**Figure 4**). While CCL3/MIP1α and IL-1β levels trended lower in the antibiotic-treated cohort, these findings did not achieve statistical significance. There were no differences observable in other cytokines and DAMPs such as TNFα, IL-33, or HMGB1 (data not shown).

Intestinal microbiota modulate alveolar macrophages activation by microbial TLR2 and TLR4 agonists

Intestinal microbiota have been shown by others to modulate lung immune responses[27]. Since we had previously shown that alveolar macrophages were required for the initiation of lung IR inflammation[20], we hypothesized that intestinal commensal bacteria might modulate the ability of alveolar macrophage to regulate the lung IR inflammatory response. We compared inflammatory responses of BAL (bronchoalveolar lavage) 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 (TLR4 agonist) or Pam3Cys (TLR2 agonist). As compared with control mice, equivalent numbers of 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, CXCL2/MIP2α, CCL2/MCP1, CCL3/MIP1α in culture supernatants compared to similarly stimulated alveolar macrophages from untreated control mice (**Figure 5A**: LPS challenge – IL6: $p<0.05$; CXCL2: $p<0.01$; CCL2: $p<0.001$; CCL3: $p<0.05$; and **Figure 5B:** Pam3Cys challenge – IL6: $p<0.0001$; CXCL2: $p<0.05$; CCL2: $p<0.001$; CCL3: $p<0.001$). TNFα 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 showed that IL-1β is an important factor for intercellular communication between macrophages and endothelial cells[20], we investigated whether inflammasome pathways, which regulate the release of active IL-1β, were functioning differently 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 following stimulation with the inflammasome activator, nigericin ($p<0.01$; **Figure 6**) even after 24h 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, lung IR is associated with significant detrimental effects on the host with damage to the affected cells, tissues, and organs (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 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 following non-hypoxic lung IR in mice. We observed that short periods (30min) of ischemia resulted in the early recruitment of neutrophils upon reperfusion of the previously ischemic lung. However, this process resolved within 12-24h with IL-6 and CXCL1 levels also peaking early and then returning to baseline (as seen in Figure 1). While the rapid recruitment of neutrophils to the IR injured tissue was expected, the fast resolution of this process with no apparent lung injury initially appeared to be inconsistent with clinical observations of sustained organ injury, dysfunction and increased morbidity in patients. While this may reflect differences in mouse and human biology, another possible explanation for this discrepancy is that while neutrophils are attracted to lung tissue following non-hypoxic 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[28]. Conversely, we wanted to ask the question whether the microbiome could affect 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 ([27,29–31] 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 generating the initial induction of lung IR inflammation (as shown in Figures 3 and 4). Additionally, we found that even before exposure to IR, the alveolar macrophages isolated from antibiotic treated mice were less responsive to TLR2 and TLR4 agonists than those isolated from control mice (Figure 5). These data suggest that alveolar macrophages may be the resident lung cell type 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 macrophage and other lung immune cells. Additionally, or alternatively, the microbial flora might

affect immune cells within or in proximity to the intestine that then either 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[32]. Thus alterations in commensal flora could possibly indirectly or directly skew the alveolar macrophages from a pro-inflammatory to a pro-resolving phenotype (also described in the literature as "M1" and "M2", respectively).

Investigators have proposed a two-step process for the activation of inflammatory cells via activation of the inflammasome leading to IL-1β release (reviewed in [33,34]). We speculate that the commensal microbiota may provide this first "priming" signal, which signals alveolar macrophages to induce pro-IL-1β levels. 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. Supporting this concept is 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 (Figure 6).

Another emerging microbiome population is the pulmonary microbiome[35]. Although this bacterial population is largely thought to be present in the upper airways and not in the 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 CFU counts and quantifying 16S rRNA. 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 S1). Thus, the data support the hypothesis these signals originate from the intestinal microbiome.

Overall, in the specific case of lung IR, we speculate that inflammatory responses may serve a protective rather than 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. Since 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 TLR4-dependent manner[36]. Without directly implicating the intestinal microbiome, another study demonstrated that intestinal IR leads to indirect lung injury[37]. 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 that the means of communication between the intestine and the lung could 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 important.

While the strong advantage of our non-hypoxic lung IR model is the 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 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. 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 ventilated lung ischemia reperfusion injury in mice is transient and is strongly influenced by intestinal commensal microbiota. By understanding how inflammation in the lung following 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 PEs and the resulting sterile lung injury. While we are not advocating for the widespread initiation of antibiotics in trauma patients, our intriguing findings may be a first step towards understanding how to tailor therapies to mitigate lung and other organ injury initiated by sterile injury through the manipulation of the intestinal microbiome.

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Figure Legends

Figure 1. Early and transient inflammation following ventilated lung ischemia reperfusion.

(A) H&E staining of left lower lung segments of C3H/HeOuJ mice that underwent ischemia (30min) followed by reperfusion (3h to 24h) (5X magnification). Each image is representative of one surgery: i.e. 3 mice were examined at each reperfusion time point. The schematic of ischemia time and reperfusion times is included below the histology images.

(B) Semi-quantitative lung injury scoring of the H&E stained left lower lung segments. Normal lung score = 2 and severely injured (maximum) score = 9. Statistics were performed via ordinary one-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 one-way analysis of variance (Kruskal-Wallis test) for multiple non-parametric comparisons.

(D) mRNA levels were measured at the noted reperfusion time points by quantitative real-time PCR. n=3-13 depending on time point. Statistics were performed via one-way analysis of variance (Kruskal-Wallis test) for multiple non-parametric comparisons.

Figure 2. Oral Antibiotic (Abx) treatment reduces gastrointestinal microbiota burden.

Stool from untreated control (left) and antibiotic-treated mice (right) was homogenized in sterile phosphate buffered saline (PSB) and equivalent amounts (adjusted for stool weight and PBS volume) were plated on Luria-Bertani (LB) agar plates and incubated overnight at 37C.

Figure 3. Antibiotic (Abx) treatment of mice results in reduced lung edema and alveolar disruption following ventilated lung ischemia reperfusion.

(A) H&E staining of left lower lung segments (10x magnification) of mice that were given oral antibiotics (polymyxin B and neomycin *ad libitum* in drinking water) for 8-10 weeks, and then were subjected to left lung ischemia reperfusion (30min ischemia/3h reperfusion). Images represent 9 of 11 untreated control mice (left panel) and 9 of 12 antibiotic-treated mice (right panel). Mice that did not survive the surgery (4 control mice and 3 antibiotic-treated mice were excluded from the study).

(B) Semi-quantitative lung injury scoring of H&E stained left lower lung segments from the control and antibiotic treated mouse cohorts. Normal lung score = 2 and severely injured (maximum) score = 9.

Figure 4. Antibiotic treated mice produce decreased levels of inflammatory cytokine and chemokine following IR.

Plasma levels of IL-6, CXCL2, CCL2, CCL3, and IL-1β were quantified in plasmas collected from antibiotic-treated or control mice at 3h following reperfusion by ELISA (data from all 11 untreated control and 12 antibiotic-treated mice plasma samples shown).

Figure 5. Alveolar macrophages from antibiotic-treated (+Abx) mice are less responsive to toll-like receptor (TLR) agonists *in vitro*.

(A) and (B) Alveolar macrophages were obtained 3 each of control and antibiotic-treated mice that had not undergone surgery. The cells from the 3 mice were pooled and equivalent numbers were seeded into 3 wells and treated overnight with LPS (1μg/mL, A) or Pam3cys (2.5μg/mL, B). Levels of IL-6, CXCL2, CCL2, CCL3, and TNFα were quantified in culture supernatants by ELISA. The experiment was repeated twice and representative results are shown here.

Figure 6. Inflammasome pathways are stably dampened in alveolar macrophages from antibiotic-treated (+Abx) mice.

After 24h in culture *ex vivo*, alveolar macrophages from control and antibiotic-treated mice that had not undergone surgery (pooled from 3 mice each, just as in Figure 5) were challenged with nigericin (5μM) for 1h 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 are represented on the graph. The experiment was repeated twice and representative results are shown here.

Figure 1

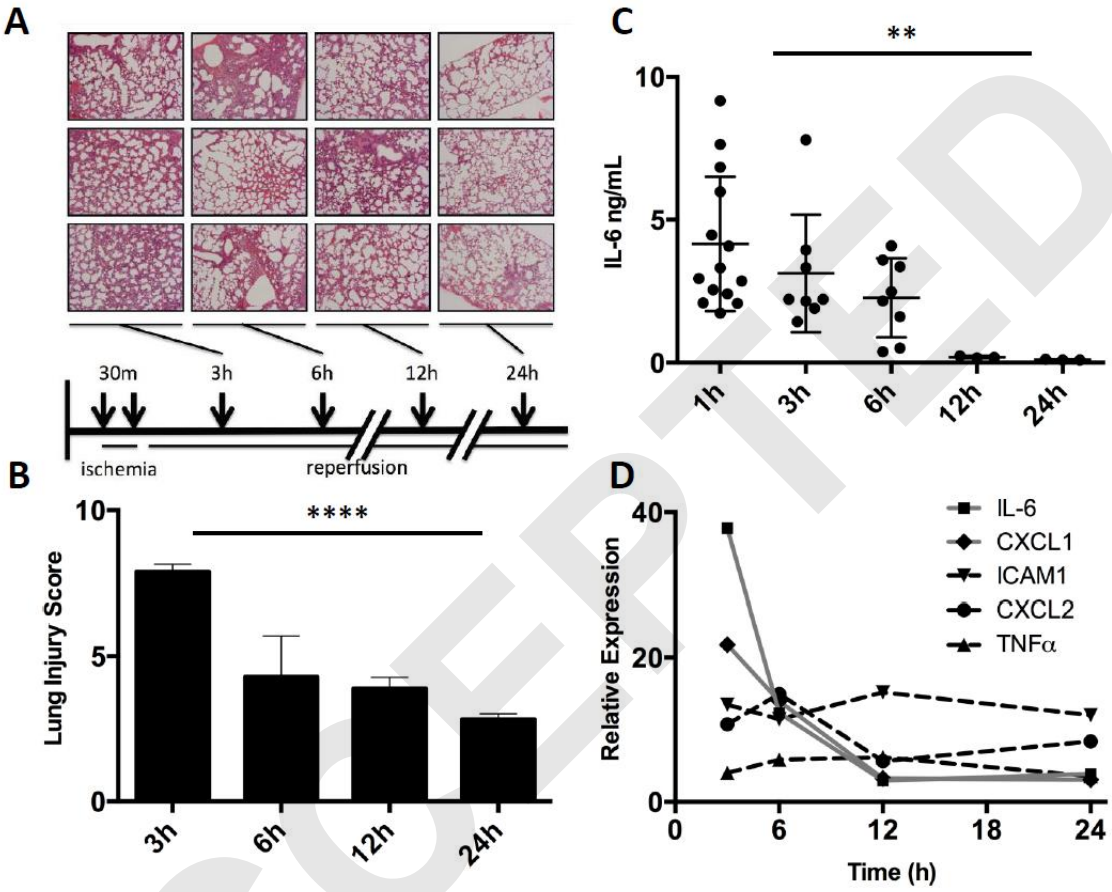
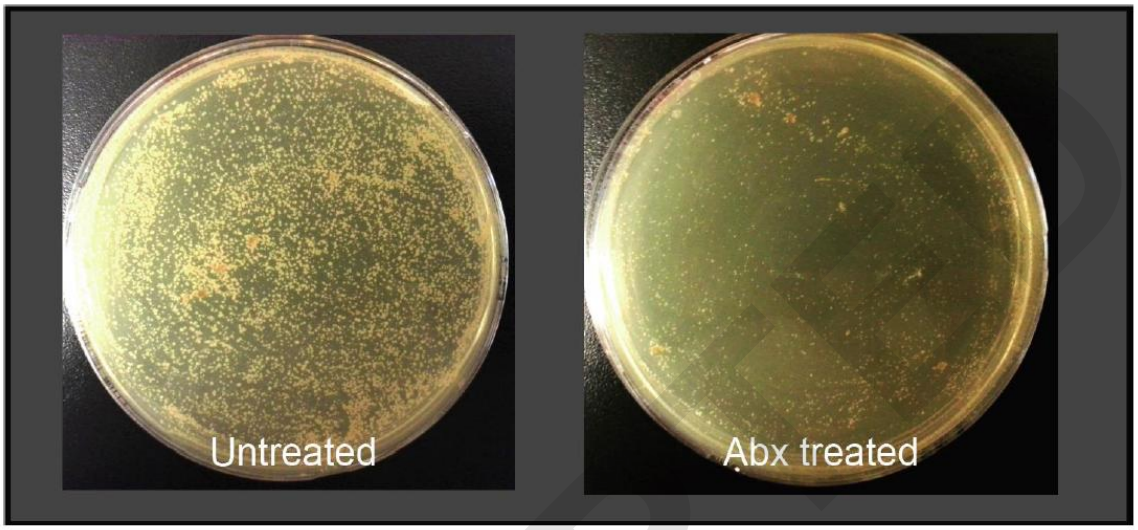


Figure 2



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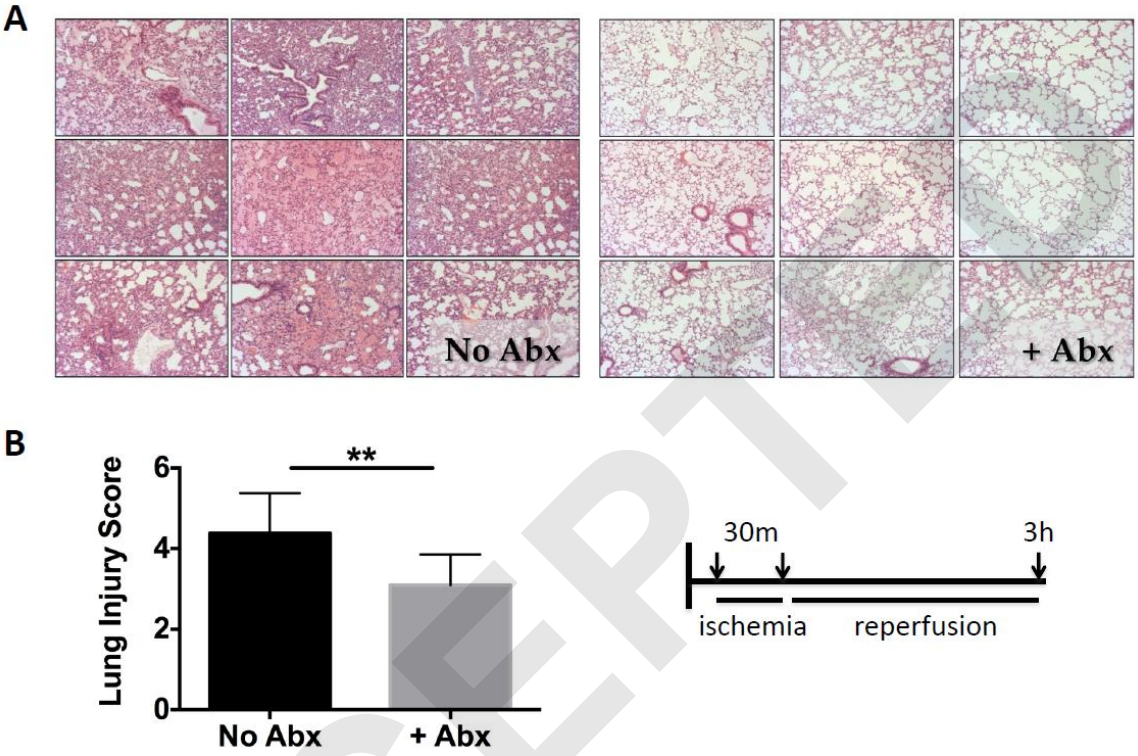


Figure 4

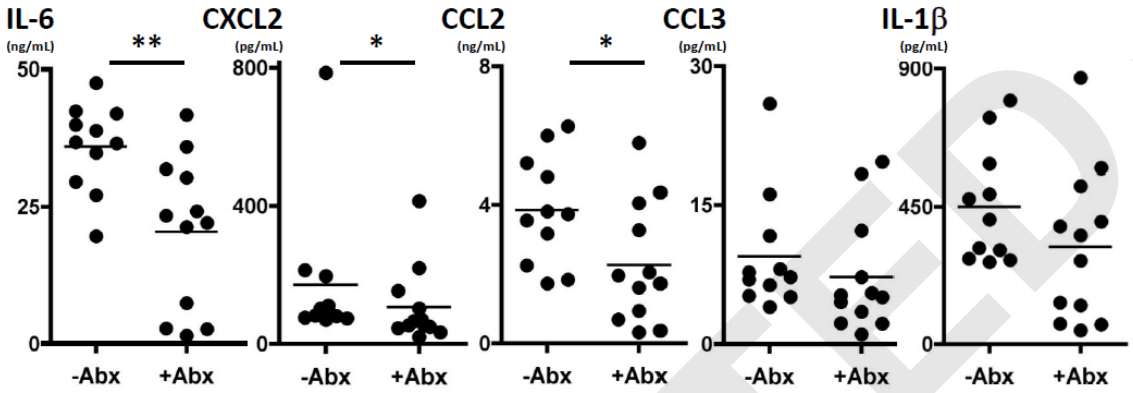


Figure 5

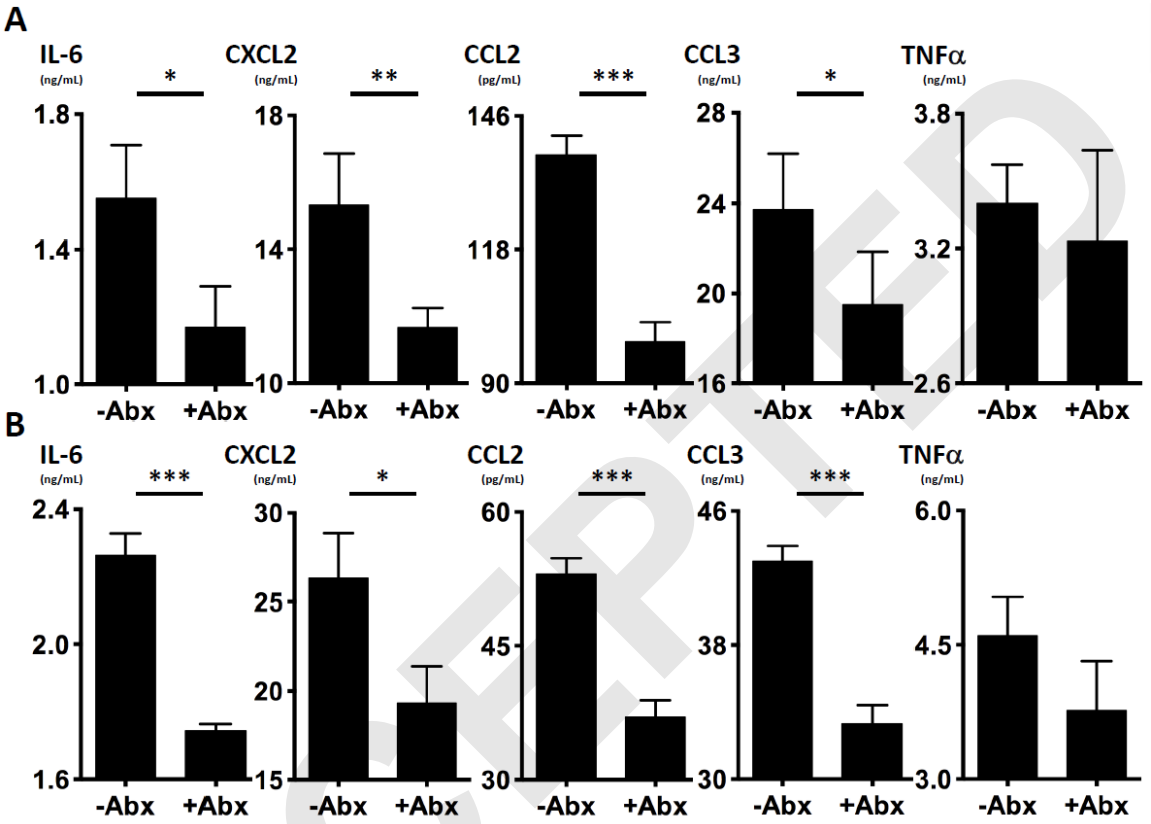
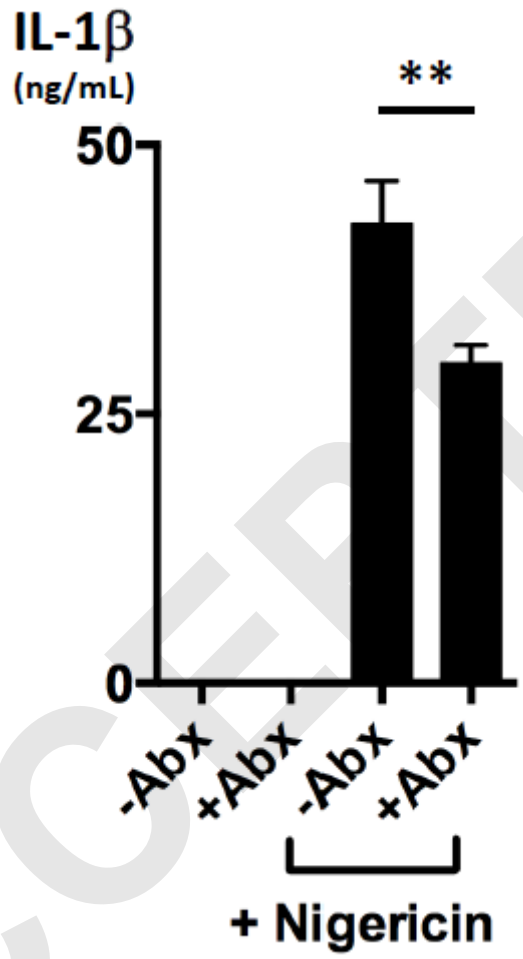
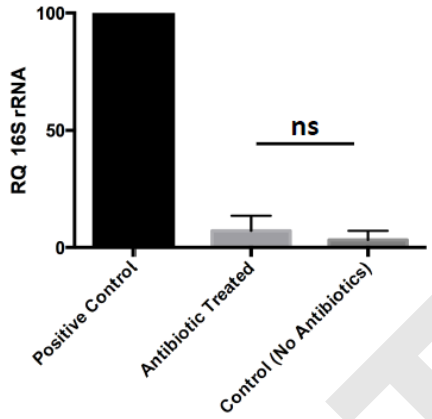


Figure 6



Supplemental Figure S1



Distal lung tissue is not colonized by commensal flora.
Quantification of 16S rRNA levels using qPCR in left lung tissue obtained from control mice and antibiotic treated mice that underwent IR surgery. There are low background levels of 16S rRNA signal in both groups. In comparison, left lung tissue from mice that received sham surgery and were infected with E. Coli (IP for 2h) was used as a positive control (and set to an RQ of 100).