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The Endogenous Capacity to Produce Proinflammatory Mediators by the *Ex Vivo* Perfused Human Lung

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Abstract

Background: The *ex vivo* human perfused lung model has enabled optimizing donor lungs for transplantation and delineating mechanisms of lung injury. Perfusate and airspace biomarkers are a proxy of the lung response to experimental conditions. However, there is a lack of studies evaluating biomarker kinetics during perfusion and after exposure to stimuli. In this study we analyzed the *ex vivo* perfused lung response to three key perturbations: exposure to the perfusion circuit, exogenous fresh whole blood, and bacteria.

Results: 99 lungs rejected for transplantation underwent *ex vivo* perfusion. One hour after reaching experimental conditions, fresh whole blood was added to the perfusate (n=55). Two hours after reaching target temperature, *Streptococcus pneumoniae* was added to the perfusate (n=42) or to the airspaces (n=17). Perfusate and airspace samples were collected at baseline (once lungs were equilibrated for 1 hour, but before blood or bacteria were added) and 4 hours later. Interleukin (IL)-6, IL-8, Angiopoietin (Ang)-2, and soluble tumor necrosis factor receptor (sTNFR)-1 were quantified. Baseline perfusate and airspace biomarker levels varied significantly, and this was not related to pre-procurement P_aO_2:FiO_2 ratio, cold ischemia time, and baseline alveolar fluid clearance (AFC). After 4 hours of *ex vivo* perfusion, the lung demonstrated a sustained production of proinflammatory mediators. The change in biomarker levels was not influenced by baseline donor lung characteristics (cold ischemia time, baseline AFC) nor was it associated with measures of experimental epithelial (final AFC) or endothelial (percent weight gain) injury. In the presence of exogenous blood, the rise in biomarkers was attenuated. Lungs exposed to intravenous (IV) bacteria relative to control lungs demonstrated a significantly higher rise in perfusate IL-6.

Conclusions: The *ex vivo* perfused lung has a marked endogenous capacity to produce inflammatory mediators over the course of short-term perfusion that is not significantly influenced by donor lung characteristics or the presence of exogenous blood, and only minimally affected by the introduction of systemic bacteremia. The lack of association between biomarker change and donor lung cold ischemia time, final alveolar fluid clearance, and experimental percent weight gain suggest that the maintained ability of the human lung to produce biomarkers is not merely a marker of lung epithelial or endothelial injury, but may support the function of the lung as an immune cell reservoir.

Background

The *ex vivo* human perfused lung has been used for nearly 70 years to study mechanisms of lung function [1]. The model facilitates characterizing biological mechanisms that may preserve the lung for transplantation as well as response to clinically relevant pathological conditions, such as exposure to endotoxin or bacteria [1–3]. The experimental preparation has improved procurement of lungs for transplantation [4–9] and understanding mechanisms that contribute to primary graft dysfunction in lung transplant recipients [10]. It also offers insight into lung physiology in injury [11, 12] and as such, allows for the testing of new therapeutics (mesenchymal stem cells, microvesicles) [13–16].

The controlled conditions of the *ex vivo* perfused lung make it possible to collect samples from multiple compartments (perfusate, lung tissue, airspaces) and to study the response to experimental intervention. The quantification of biomarkers in these compartments is representative of injury [17–20]. However, whether biomarkers levels are associated with a negative outcome is uncertain in the *ex vivo* perfused lung model because the rise in biomarkers does not always correlate with validated measures of lung function [21]. Furthermore, removing biomarkers thought to induce injury, such as IL-8, with an adsorbent membrane, does not improve lung function during prolonged *ex vivo* lung perfusion (EVLP) [22].

The uncertainty in the field prompted us to perform a detailed study of several clinically relevant biomarkers that have been studied in clinical samples as well as in *ex vivo* and *in vitro* models of lung injury. The four biomarkers include three biomarkers associated with inflammation: Interleukin (IL)-6, IL-8, and soluble Tumor Necrosis Factor Receptor 1 (sTNFR1) [23–25], as well as one biomarker associated with endothelial activation: Angiopoietin-2 (Ang-2) [25, 26].

The goal of our study was to address four important poorly understood concepts. First, do donor lung characteristics (P_aO_2:FiO_2 ratio, cold ischemia time, baseline alveolar fluid clearance) influence baseline biomarker levels or biomarker kinetics in the perfusate and airspaces? Second, what is the impact of exogenous fresh whole blood on biomarker kinetics in the perfusate and in the airspaces during EVLP? Third, what is the impact of exposure to bacteria (intravenous [IV] or airspace infection with *Streptococcus pneumoniae*) on perfusate and airspace biomarker kinetics relative to uninfected control lungs? Fourth, do donor lung characteristics influence relationships between biomarker kinetics in the presence of exogenous blood or after exposure to bacteria?

Results

Donor lung characteristics

A total of 99 single human lungs were studied under the following six experimental conditions: (1) control lungs with exogenous blood (n = 22), (2) intravenous infection with *S. pneumoniae* with exogenous blood (n = 19), (3) airspace infection with *S. pneumoniae* with exogenous blood (n = 14), (4) control lungs without exogenous blood (n = 18), (5) intravenous infection with *S. pneumoniae* without exogenous blood (n = 23), (6) airspace infection with *S. pneumoniae* without blood (n = 3). The baseline donor characteristics among the six experimental conditions were well balanced (Table 1). There were no statistically significant differences in donor age, organ cold ischemia time, PaO_2:FiO_2 ratio, and baseline alveolar fluid clearance (AFC). Among the lungs perfused without exogenous blood, a greater proportion of control lungs relative to lungs intravenously exposed to bacteria received pre-procurement antibiotics (89% vs 52%, p = 0.01). This imbalance in pre-procurement antibiotics was greater in control lungs and therefore would not be expected to affect bacterial proliferation.

Table 1
Characteristics of donor lungs classified by experimental condition.

	With blood			Without Blood			
	Control lungs	IV bacteria	Airspace bacteria	Control lungs	IV bacteria	Airspace bacteria	P-value
Number of lungs	22	19	14	18	23	3	N/A
Donor age ^	44 (30, 55)	45 (32, 54)	45.5 (30, 55)	40 (38, 46)	53 (32, 64)	59 (55, 69)	0.4
Donor sex	15 (68%)	13 (68%)	9 (64%)	11 (61%)	16 (70%)	3 (100%)	0.9
Donor weight (kg) ^	88.5 (83.1, 104.3)	86.9 (72.7, 96)	83.9 (66.5, 112)	89.7 (75, 104)	83 (73.4, 96)	108 (85, 171)	0.5
Donor height (cm) #	172.5 (± 14.5)	174.4 (± 7)	172.1 (± 8.8)	173.7 (± 10.8)	169.9 (± 10.6)	179.3 (± 4)	0.5
Trauma	6 (27%)	5 (26%)	4 (29%)	5 (28%)	8 (35%)	2 (67%)	0.8
Cold ischemia Time (h) #	20 (± 13.5)	23.7 (± 13.3)	19.3 (± 14)	24.6 (± 10.6)	19.7 (± 10)	28 (± 8.5)	0.6
Pre-procurement antibiotics	13 (59%)	13 (68%)	13 (93%)	16 (89%)	12 (52%)	2 (67%)	0.05
CPR	14 (64%)	10 (53%)	10 (71%)	9 (50%)	12 (52%)	1 (33%)	0.7
NDD	19 (86%)	18 (95%)	13 (93%)	17 (94%)	20 (87%)	3 (100%)	0.9
PaO_2:FiO_2 ratio #	224 (± 74)	268 (± 103)	237 (± 136)	251 (± 111)	242 (± 100)	251 (± 104)	0.7
Normal baseline AFC	17 (77%)	15 (79%)	11 (79%)	16 (89%)	20 (87%)	2 (67%)	0.9
AFC at baseline ^	33.4 (9.5, 45.5)	27.3 (9.2, 40)	18.4 (15.4, 32.2)	28.8 (15, 33.3)	28 (18.2, 38.9)	20.6 (4.3, 49.1)	0.9

*Abbreviations: CPR: Cardio-Pulmonary Resuscitation; NDD: Neurological Declaration of Death; AFC: Alveolar Fluid Clearance

*Continuous data presented as means ± standard deviation (#) or medians with interquartile range (^)

*Group comparisons of continuous variables made using Kruskal-Wallis test

		With blood			Without Blood		
AFC at 5 h ^	14 (9.5, 36.2)	18.7 (9.5, 29.7)	2.7 (0, 16.9)	22.7 (14.8, 32)	15.2 (13.3, 32.4)	5 (0, 10)	0.02
Start lung weight (g) ^	402 (330, 532)	383 (351, 438)	372 (294, 396)	365 (297, 406)	360 (328, 414)	555 (373, 562)	0.2
End lung weight (g) ^	631 (564, 958)	784 (720, 1232)	767 (603, 891)	722 (630, 774)	779 (696, 987)	1328 (1312, 1344)	0.1
% weight gain ^	62 (44, 99)	115 (69, 186)	98 (83, 154)	99 (80, 128)	101 (82, 134)	138 (133, 142)	0.04
*Abbreviations: CPR: Cardio-Pulmonary Resuscitation; NDD: Neurological Declaration of Death; AFC: Alveolar Fluid Clearance							
*Continuous data presented as means ± standard deviation (#) or medians with interquartile range (^)							
*Group comparisons of continuous variables made using Kruskal-Wallis test							

Baseline biomarker variability and significant biomarker rise after 4 hours of EVLP

Because the first sample was collected before the addition of experimental conditions (exogenous fresh whole blood, intravenous or airspace infection with *S. pneumoniae*), we were able to study baseline biomarker levels in the perfusate (n = 97) and in the airspace (n = 88). Baseline (time 0 h) perfusate and airspace samples collected one hour after EVLP equilibration had a wide distribution in biomarker concentrations (Fig. 1).

Perfusate and airspace samples collected at baseline and after 4 hours of EVLP were used to study biomarker kinetics. The *ex vivo* perfused human lung demonstrated a maintained capacity to produce inflammatory cytokines at the 4 hour relative to the 0 hour time point in the perfusate and in the airspaces (Fig. 1) at a significance level of $p < 0.0001$. The substantial fold change in perfusate and airspace biomarkers is shown in **sFigure 1**.

A significant rise in the perfusate (**sTable 1**) and airspace (**sTable 2**) biomarkers was present in all six experimental conditions. This was especially true for IL-6 and IL-8 (**sFigure 2A-B** and **sFigure 3A-B**), with IL-6 levels increasing more than 200-fold (**sFigure 1**), up to a concentration of 1 µg/mL. The increase in perfusate and airspace sTNFR1 and Ang-2 was lower (**sFigure 2C-D** and **sFigure 3C-D**).

Donor characteristics and indicators of experimental lung injury are not associated with baseline biomarker levels and biomarker kinetics

Baseline biomarker levels were tested for their association with pre-procurement P_aO_2:FiO_2 ratio, cold ischemia time, and baseline AFC. Neither perfusate nor airspace baseline biomarker concentrations were associated with these donor lung characteristics (Fig. 2A). The only exception was for airspace IL-6 and IL-8 levels, whereby a higher baseline AFC was associated with a higher baseline biomarker concentration ($p < 0.0001$).

In spite of a substantial rise in perfusate and airspace IL-6 and IL-8, as well as to a lesser extent in sTNFR1 and Ang-2, there was no association between the change in any of the perfusate biomarker levels and indicators of lung injury at the end of the experiment (percent weight gain or final AFC) or baseline donor lung characteristics (Fig. 2B). The lack of association between change in biomarker levels and the final AFC and percent weight gain suggests that the presence of very high levels of proinflammatory mediators does not result in deterioration of epithelial or endothelial barrier function. There was weak but significant association between the baseline AFC and the increase in airspace IL-6 and IL-8 levels, suggesting that lungs with higher baseline AFC had a higher rise in airspace concentrations of these two inflammatory markers.

Exogenous blood attenuates perfusate IL-6 increase in lungs exposed to IV S. pneumoniae

The *ex vivo* human lung model has been shown to lack a response to the airspace administration of bacterial components (lipopolysaccharide, LPS) without the addition of exogenous blood [14]. Therefore, in many instances when the *ex vivo* perfused lung model is used for studying the response to injurious stimuli, exogenous fresh human whole blood has been added to the perfusate [1, 15]. However, the influence of the addition fresh whole blood to the *ex vivo* human perfused lung on pro-inflammatory biomarker levels in the perfusate and in the airspaces has not been studied in detail.

The differences between perfusate and airspace biomarker kinetics after 4 hours of *ex vivo* perfusion in the presence relative to the absence of fresh whole blood was compared in control lungs and in lungs exposed to IV *S. pneumoniae* (**sTable 3**). In control lungs, there were no statistically significant differences in the change in perfusate (Fig. 3A) or airspace (Fig. 3B) biomarkers after 4 hours of perfusion in lungs perfused with compared to without exogenous blood. In lungs exposed to IV *S. pneumoniae*, there was a significant difference in the increase in perfusate IL-6 (Fig. 3A) and airspace sTNFR1 (Fig. 3B) in lungs perfused with relative to without exogenous blood. In the absence of blood, perfusate IL-6 increase was 133,885 pg/ml higher relative to lungs perfused with blood ($p = 0.003$). In the presence of blood, airspace sTNFR1 increase was 1,793 pg/mL higher relative to lungs without blood ($p = 0.009$).

The effect of exogenous whole blood on biomarker kinetics in lungs exposed to airspace *S. pneumoniae* was not studied as there were only 3 lungs in this subgroup. Therefore, the previous observation that the addition of whole blood leads to a sharp increase in airspace biomarkers (IL-1β, TNF, and IL-8) when a bacterial component (LPS) is instilled into one of the lung lobes [14] could not be ascertained.

IV S. pneumoniae contributes to an increase in perfusate IL-6 in the absence of exogenous blood

We hypothesized that relative to control lungs, there would be a greater increase in lung endogenous biomarker production after *S. pneumonia* exposure. This was tested in (1) control lungs relative to lungs exposed to IV *S. pneumoniae* with exogenous blood, (2) control lungs relative to lungs exposed to IV *S. pneumoniae* without blood, and (3) control lungs relative to lungs exposed to airspace *S. pneumoniae* with exogenous blood (**sTable 4**).

Lungs exposed to IV *S. pneumoniae* perfused without exogenous blood relative to control lungs had a significantly greater increase in perfusate IL-6 (higher by 92,741 pg/mL, p = 0.04) and perfusate IL-8 (higher by 103,915 pg/mL, p = 0.05) (Fig. 4A). There were no differences in airspace biomarker levels (Fig. 4B). When exogenous blood was present in the model, lungs exposed to IV *S. pneumoniae* relative to control lungs had a greater increase in perfusate sTNFR1 (higher by 967 pg/mL, p = 0.04) (Fig. 4A), airspace sTNFR1 (higher by 1,492 pg/mL, p = 0.02), and airspace Ang-2 (higher by 823 pg/mL, p = 0.05) (Fig. 4B).

In lungs exposed to airspace *S. pneumoniae* relative to control lungs, there was no significant difference between perfusate or airspace biomarker kinetics (**sTable 4**).

Donor lung characteristics have minimal influence on biomarker kinetics

Donor lung characteristics may affect biomarker kinetics in lungs perfused with or without exogenous blood as well as in control lungs compared to lungs exposed to IV or airspace *S. pneumoniae*. To test this hypothesis, perfusate and airspace biomarker change was adjusted for cold ischemia time and baseline AFC in subgroups of lungs perfused with compared to without blood (**sTable 5**), and for cold ischemia time, baseline AFC, and pre-procurement antibiotics in control lungs compared to lungs exposed to IV *S. pneumoniae* (**sTable 6**) and airspace *S. pneumoniae* (**sTable 7**).

Overall the change in biomarker levels over the 4 hours of perfusion were not significantly different after adjustment for these potential confounders. Importantly, none of the significant biomarker kinetics discussed above were influenced by any of the adjustment variables.

Discussion

In the United States, lungs of approximately 75% eligible donors are ineligible for transplantation [27]. These lungs are an invaluable resource to study lung function and organ preservation to increase eligibility for transplantation. In this study, explanted human lungs rejected for transplantation underwent *ex vivo* lung perfusion and a detailed characterization of perfusate and airspace biomarker kinetics was performed under 6 experimental conditions.

During 4 hours of EVLP, we found that the lung has a remarkable capacity to produce proteins associated with immune (IL-6, IL-8, sTNFR1) and endothelial (Ang-2) responses. The baseline level and the change in

concentration of these mediators after 4 hours of *ex vivo* perfusion was unrelated to the duration of cold ischemia time and parameters associated with deranged lung epithelial (alveolar fluid clearance) or endothelial (percent weight gain) function [1]. The addition of fresh whole blood attenuated the increase in perfusate IL-6 in lungs exposed to intravenous bacteria. The addition of a lethal dose of Gram-positive bacteria (*S. pneumoniae*) did not significantly change perfusate or airspace biomarker kinetics, with the exception of perfusate IL-6 in the absence of exogenous blood.

A similar high rise in some biomarkers quantified in this study has been demonstrated in prior reports of EVLP [20, 28]. IL-6 and IL-8 are inflammatory cytokines traditionally synonymous with organ injury and poor outcome. In the EVLP model, several studies of cross-sectional cytokine levels suggest that donor lung levels of these biomarkers are inversely associated with graft function in the transplant recipient [17–20]. Interestingly, cytokines in the EVLP perfusate of successfully transplanted lungs also reach extremely high levels (as much as a 30–100 fold increase in IL-6 and IL-8) without evidence of primary graft dysfunction (PGD) [20, 21]. If IL-6 and IL-8 are always injurious, why do not more transplant recipients develop PGD?

High levels of IL-6, IL-8, sTNFR1, and Ang-2 in plasma samples of patients with the acute respiratory distress syndrome (ARDS) are well known for their association with poor outcomes [29, 30]. However, plasma biomarker levels are substantially lower than those reported in EVLP perfusate. This discrepancy between the extremely high levels of inflammatory cytokines in the EVLP perfusate and the lack of injury in every transplant recipient as well as the lack of association with experimental measurements of injury in our study (percent weight gain, final AFC) suggest that these cytokines have an additional biologic significance [31], especially in the EVLP model.

The source of biomarker production and the mechanism of accumulation are uncertain. The EVLP model lacks mechanisms associated with clearance, specifically the liver [32], the kidneys [33], and components of the vascular compartment, the absence of which may explain the striking difference between biomarker concentrations detectable in plasma relative to the perfusate and airspaces of the *ex vivo* perfused lung. In our experiments in which only 100 ml of fresh whole blood was added to 2L of EVLP perfusate (representative of a hematocrit of approximately 2%), there appeared to be a trend toward a dampening effect on the increase in perfusate IL-6 and IL-8 levels in control lungs as well as in lungs exposed to IV *S. pneumoniae.* This suggests that protective factors are present in blood that either reduce the production of these cytokines, that increase their enzymatic clearance, or that facilitate their sequestration [34]. Future investigation of whole blood components that may be responsible for the decrease in inflammatory biomarker levels may be relevant to transplantation as several studies support the notion that high IL-8 in perfusate is related to an increased incidence of PGD3 in the recipient [17–19].

A potential source of cytokines may be cell necrosis and apoptosis due to ischemia-reperfusion injury [35]. However, neither the baseline nor the change in biomarkers after 4 hours of EVLP was associated with cold ischemia time, suggesting that presumed cell necrosis and apoptosis cannot on their own explain the high abundance of cytokines in our model. In fact, prolonged hypothermia without reperfusion

did not increase pneumocyte apoptosis in a rat lung transplant model [36]. Also, there is evidence from studying tissue biomarker levels in the human EVLP model that inflammatory cytokine levels do not significantly differ after extended cold ischemia time [7].

Of note, not all biomarker levels increased to the same extend during EVLP. The small change in Ang-2 levels during the 4 hours of *ex vivo* perfusion relative to inflammatory biomarker levels is puzzling. This protein is produced by the endothelium and is stored in Weibel Palade bodies [26], where it has a long half-life (over 18 hours) and can be secreted within minutes of stimulation [37]. However, despite lung injury due to ischemia-reperfusion and addition of lethal doses of *S. pneumoniae*, the magnitude of Ang-2 change was minimal compared to the change in IL-6 and IL-8.

The observation that starting AFC is only significantly associated with baseline IL-6 and IL-8 levels in the airspace compartment is intriguing. It is plausible that when the lung epithelial function is intact and alveolar fluid is clearance is preserved [38], IL-6 and IL-8 may concentrate in the airspaces. It also implies that when interpreting levels of these cytokines in the airspace compartment of the EVLP model, it is important to measure and account for lung alveolar fluid clearance.

This study has some limitations. First, only two time points were studied during the course of perfusion, and as such, it is possible we missed important timepoint biomarker production trends, particularly in experimental conditions in the presence of bacteria. It is also possible that the perfusion was not long enough to appreciate differences between the experimental conditions included in this study. Secondly, we studied only four soluble proteins and to detect relevant differences between experimental conditions, other proteins may need to be studied. It is also plausible that other metrics, such as RNA expression, microvesicle or lipid production, could provide insight into biological differences between the experimental conditions. Third, our experiments were performed with a single bacterial pathogen, and as such, the data may not be extrapolated to infection with other pathogens.

Conclusions

Overall, the *ex vivo* perfused lung has a robust capacity to generate high levels of inflammatory proteins during 4 hours of perfusion. This was true in all experimental conditions, including control lungs, suggesting that future EVLP models assessing effect of injury or novel therapies should incorporate appropriate control lungs in the study design. The addition of blood does not increase biomarker levels while the addition of live bacteria results in a higher rise in only IL-6 in lungs perfused without exogenous blood. The substantial increase in biomarker levels between the two time points suggests that future studies of cross-sectional and longitudinal biomarker analysis should ensure strict adherence to pre-defined timing of sample collection. The remarkable lack of association between baseline proinflammatory biomarker levels as well as their increase over time and donor lung characteristics (cold ischemia time, starting AFC) and experimental outcomes (alveolar fluid clearance 5 hours after perfusion, and the percent weight gain) suggests that the maintained ability to produce biomarkers is not merely a

marker of lung epithelial or endothelial injury and may instead support the lung's role as an immune reservoir.

Materials And Methods

Ex vivo human perfused lung

Donor lungs rejected for transplantation were received from Donor Network West and the right or left lung was selected for EVLP based on gross appearance, as previously described [1]. Briefly, the main bronchus was intubated with an endotracheal tube and the pulmonary artery was cannulated. The lung was perfused with two liters of acellular DME-H21 media with 5% bovine serum albumin (BSA) and warmed to 37 °C. Subsequently, 8 cm H_2O of continuous positive airway pressure was applied using room air. Lung weight was obtained at the start (time 0 h, baseline) and at the end (time 6 h) of each experiment. One hour after experimental conditions were reached, 100 ml of exogenous fresh whole blood was added to the perfusate of some of the lungs.

Quantification of lung function: Alveolar Fluid Clearance

Alveolar Fluid Clearance (AFC) was determined once the *ex vivo* perfused lung was equilibrated on the circuit by introducing 100 ml of normal saline with 5% BSA into the distal airspaces. Samples for measurement of total protein by refractometry were collected at 5 and 35 minutes via a catheter inserted into the endobronchial tube. AFC was calculated using the formula: AFC (%/h) = $2(1-C_i/C_f)$, where C_i is the 5 minute sample protein concentration and C_f is the 35 minute sample protein concentration [3, 39]. The AFC for all experiments was calculated at the start of the experiment (time 0 h, baseline) and at 5 hours.

Infection with Streptococcus pneumoniae

S. pneumoniae serotype 19F (49619; ATCC, Manassas, VA) was grown in brain-heart broth (Becton-Dickinson, Sparks MD) and 10^{10} bacteria was resuspended in phosphate buffered saline for administration according to a weight-based adjustment of a severe pneumonia murine model [40, 41]. Two hours after reaching target temperature, bacteria were added intravenously into the perfusate (non-pulmonary sepsis model) or into the airspaces (pneumonia model).

Biomarker quantification

Perfusate and airspace samples were prospectively collected for biomarker quantification at two time points. The first sample was collected after the lungs were equilibrated on the circuit for 1 hour (immediately prior to the addition of exogenous whole blood and 1 hour prior to the introduction of *S. pneumoniae* in selected experiments; sample referred to as time 0 h). The second sample was collected 4 hours later (sample referred to as time 4 h). For lungs perfused with exogenous fresh whole blood, this corresponded to a 4-hour exposure to blood. For lungs exposed to *S. pneumoniae*, this corresponded to a 3-hour exposure to bacteria. All samples were cryopreserved at -80°C prior to protein quantification using the Simple Plex™ Ella multiplex microfluidic platform (Protein Simple, CA, USA). A 4-plex custom panel

was used to quantify Interleukin (IL)-6, IL-8, Angiopoietin (Ang)-2, and soluble tumor necrosis factor receptor (sTNFR)-1. Low abundance proteins (Ang-2) were tested at a 1:10 dilution and high-abundance proteins (IL-6, IL-8, sTNFR-1) were tested at a 1:1,000 dilution. The same dilutions were used for perfusate and airspace samples. Assays were performed according to manufacturer's protocol, as described previously [42]. Raw data were analyzed using the SimplePlex Explorer software.

Statistical Analysis

Relationships between continuous variables were performed using Pearson (normally distributed data) or Spearman (skewed data) correlation, with a Bonferroni correction for multiple testing. Comparison of baseline continuous variables among experimental groups were analyzed using the Kruskal Wallis test and dichotomous variables were compared using the chi2 test. Comparisons of biomarker kinetics among experimental conditions were made using generalized estimating equation models (GEE) using robust standard errors [43]. The analyses included: (1) impact of exogenous whole fresh blood on change in perfusate and airspace biomarker levels in lung subgroups stratified by exposure to bacteria (control/no bacteria, intravenous bacteria); (2) impact of addition of intravenous *S. pneumoniae* (non-pulmonary sepsis model) on change in perfusate and airspace biomarker levels in lung subgroups stratified by addition of exogenous whole fresh blood; (3) impact of addition of airspace *S. pneumoniae* (pneumonia model) on change in perfusate and airspace biomarker levels in lungs perfused with exogenous whole fresh blood. Too few lungs exposed to airspace *S. pneumoniae* were perfused without exogenous blood (n = 3) to test the impact of airspace bacteria on biomarker kinetics in this experimental subgroup. Interaction terms for cold ischemia time, baseline lung function (the surrogate of which was baseline AFC), lung response at end of experiment (AFC at 5 h, percent weight gain) were included individually in the above GEE models to test for the effect of these potential confounders on perfusate and airspace biomarker kinetics. In models assessing the impact of bacteria on biomarker kinetics, an interaction term for the administration of pre-procurement antibiotics was included. Statistical analyses and data presentation were performed using STATA v14.1 (StataCorp 2015).

List Of Abbreviations

IL-6 Interleukin-6

IL-8 Interleukin-8

Ang-2 Angiopoietin-2

sTNFR1 soluble Tumor Necrosis Factor Receptor-1

AFC Alveolar Fluid Clearance

IV Intravenous

EVLP *Ex vivo* lung perfusion

S. pneumoniae Streptococcus pneumoniae

LPS Lipopolysaccharide

Declarations

Ethics approval and consent to participate

Explicit approval for the use of donor lungs for research was sought from each donor's family by Donor Network West.

Consent for publication

Not applicable

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

AL designed the study, analyzed and interpreted the data, and prepared the manuscript; JTR designed the study, performed the experiments, and revised the manuscript; NN designed the study and performed the experiments; MAM designed the study, interpreted the data, and revised the final manuscript. All authors read and approved the final manuscript

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Figures

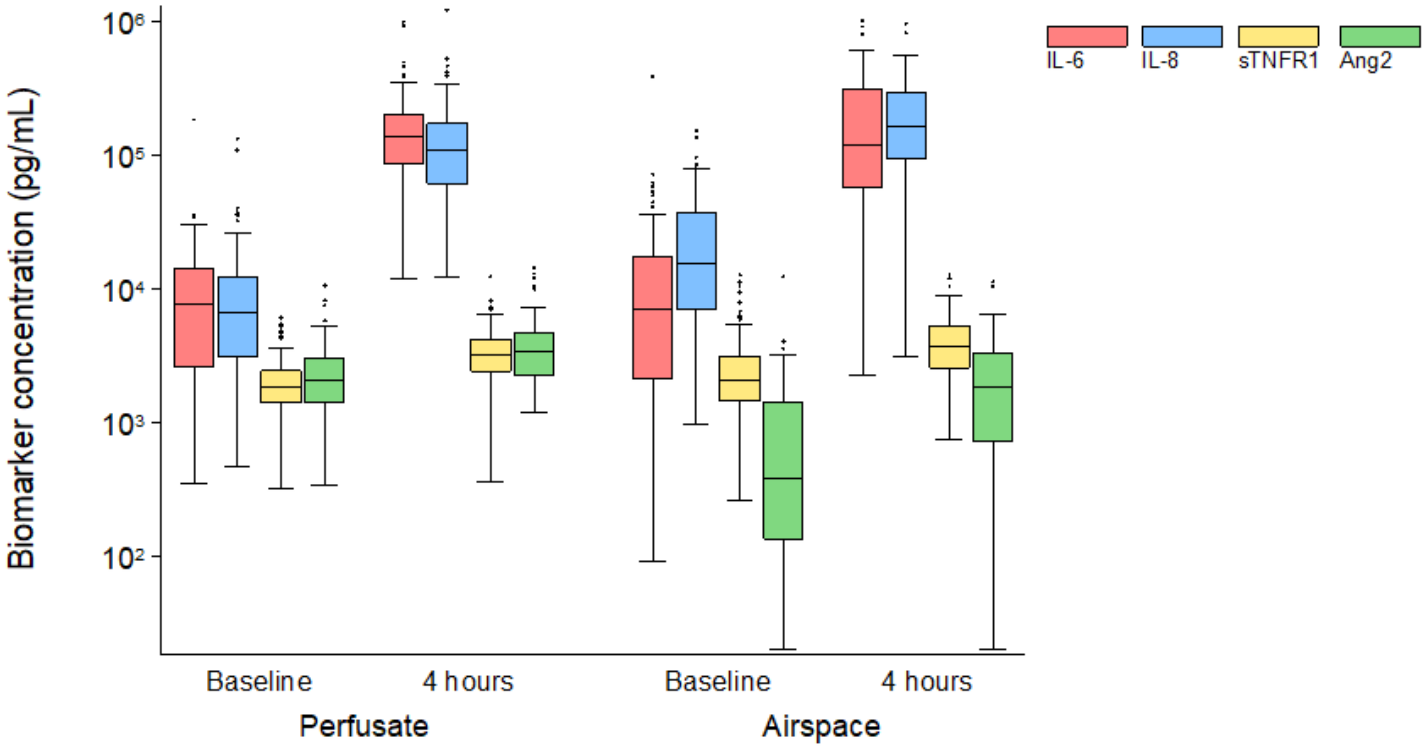


Figure 1

Perfusate and airspace biomarker concentrations at baseline and after 4 hours of ex vivo perfusion. Biomarker concentrations are presented on a Log10 scale.

Figure 2. (A)

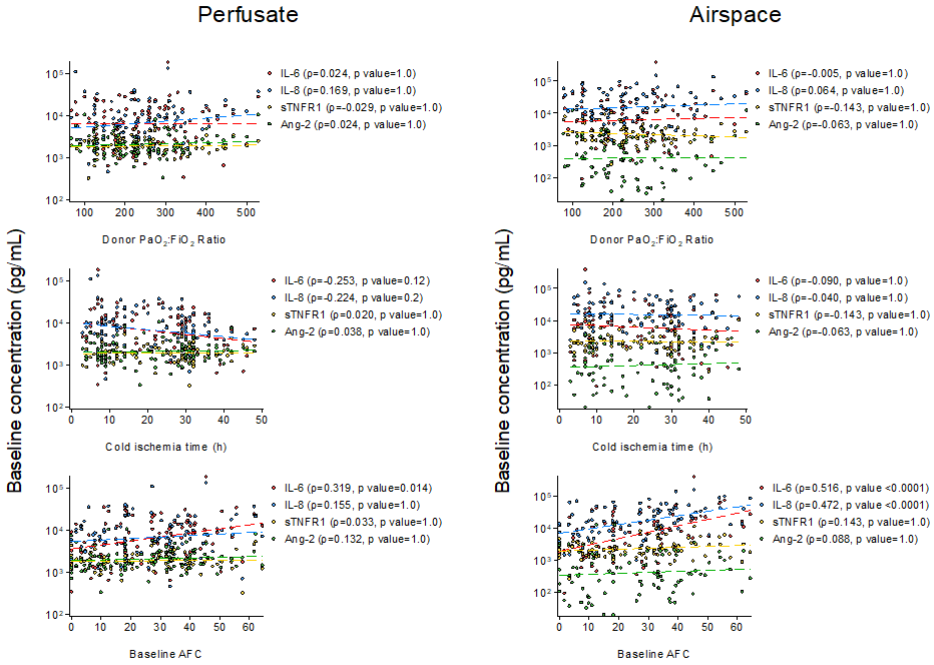


Figure 2. (B)

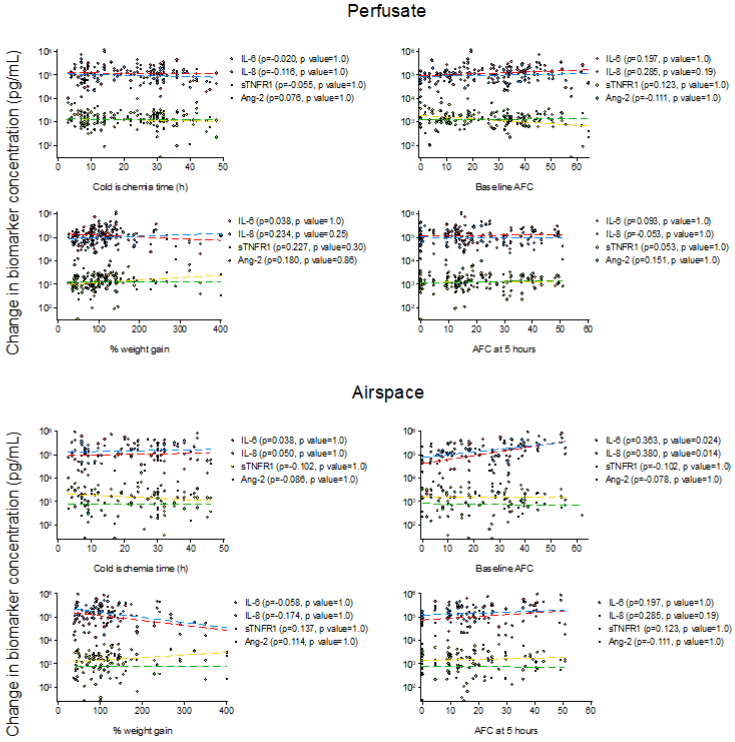


Figure 2

Perfusate and airspace biomarkers at baseline and change after perfusion relative to lung characteristics. Associations between (A) baseline perfusate (n=97) and airspace (n=88) biomarker levels and donor lung characteristics, and (B) change in perfusate (n=95) and airspace (n=65) biomarker concentrations after 4 hours of ex vivo lung perfusion and donor lung characteristics and indicators of lung epithelial and endothelial injury at the end of the experiment. P values are presented after a Bonferroni correction for multiple testing

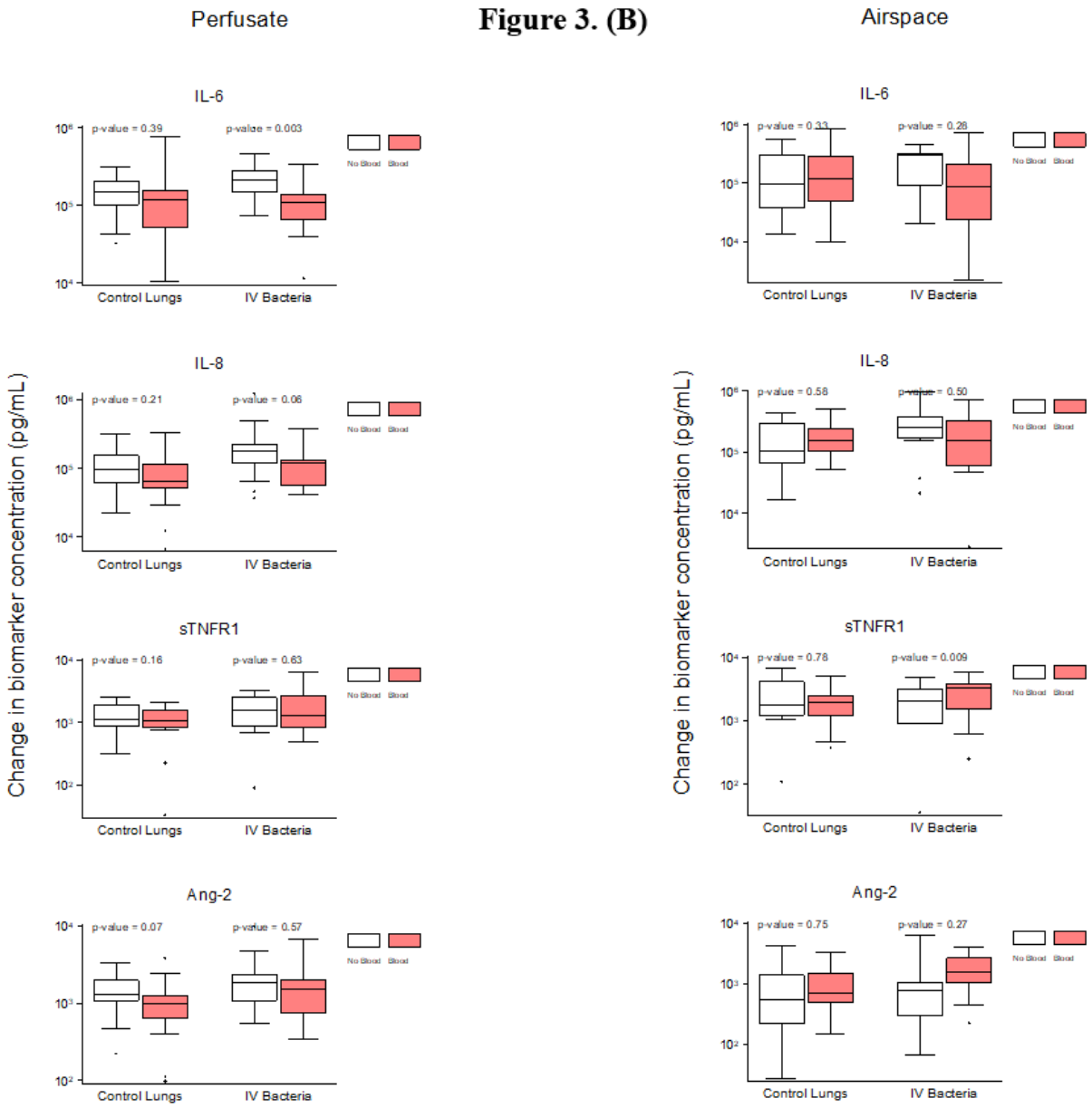


Figure 3

Impact of exogenous blood on biomarker change after 4 hours of ex vivo lung perfusion. (A) Comparison of change in perfusate biomarker levels after 4 hours of ex vivo perfusion in control lungs in the presence (n=21) or in the absence (n=17) of exogenous blood, and in IV S. pneumoniae exposed lungs in the presence (n=19) or in the absence (n=22) of exogenous blood. (B) Comparison of change in airspace biomarker levels after 4 hours of ex vivo perfusion in control lungs in the presence (n=16) or in the

absence (n=13) of exogenous blood, and in IV S. pneumoniae exposed lungs in the presence (n=13) or in the absence (n=14) of exogenous blood.

Figure 4. (A) Perfusate

Figure 4. (B) Airspace

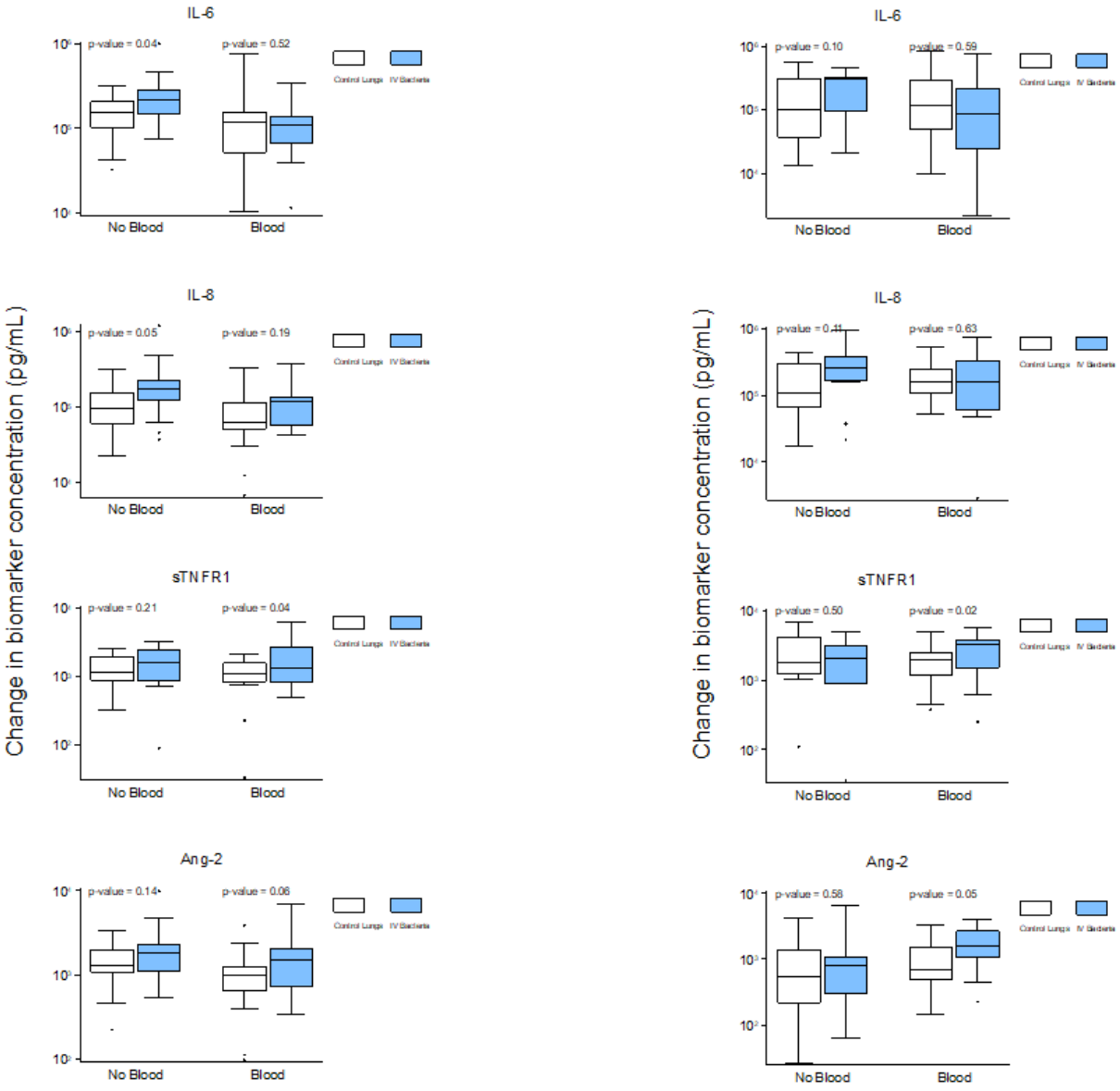


Figure 4

Impact of IV S. pneumoniae on biomarker change levels after ex vivo lung perfusion. (A) Comparison of change in perfusate biomarker levels after 4 hours of ex vivo perfusion in control lungs (n=17) relative to models with IV S. pneumoniae (n=22) without blood and in control lungs (n=21) relative to models with IV S. pneumoniae (n=19) with blood. (B) Comparison of change in airspace biomarker levels after 4 hours of ex vivo perfusion in control lungs (n=13) relative to models with IV S. pneumoniae (n=14) without blood and in control lungs (n=16) relative to IV S. pneumoniae (n=13) with blood.

Supplementary Files

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- PerfusedlungbiomarkersSupplementaryMaterialfinal.docx