AWARD NUMBER:
W81XWH-16-1-0463

TITLE:
Assessment of a Therapeutic Device for Treatment of Acute Lung Injury Using a Combat-Relevant Porcine Model

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REPORT DATE:
December 2020

TYPE OF REPORT:
Final

PREPARED FOR:
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
December 2020	Final	01Sep2016-31Aug2020

4. TITLE AND SUBTITLE

Assessment of a Therapeutic Device for Treatment of Acute Lung Injury Using a Combat-Relevant Porcine Model

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XHW-16-1-0463

5c. PROGRAM ELEMENT NUMBER

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Marvland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Purpose. Acute lung injury (ALI) occurs in >30% of combat casualties. Inflammation is a key component and therapeutic strategies to block it are expected to decrease morbidity/mortality. Investigations directed at ways to limit activation and accumulation of leukocytes at sites of inflammation may prove clinically effective. **Scope/Aims.** Modulating neutrophil & monocyte/macrophage activity, the effector cells of the innate immune system, is a novel approach to diminish inflammation-induced disease processes without interfering with other immunologic activity, thereby avoiding adverse side effects. Innovative BioTherapies developed a selective cytopheretic device (SCD) therapy (Rx), to address this unmet medical need. SCD_{Rx} has been shown to improve clinical outcomes of critically ill patients with multi-organ failure by mitigating the inflammatory cascade. **Specific Aim 1.** Optimize a two-hit porcine ALI model relevant to combat situations. **Specific Aim 2.** Assess efficacy of 24h SCD_{Rx} in the porcine model. **Progress to date.** Yr1: Animal approvals obtained. 17 studies conducted yielding study & analysis protocols (Aim 1 met). Yr2: Assessment of SCD_{Rx} efficacy in porcine model. 17 studies conducted and significant therapeutic benefit was observed (Aim 2 initiated). Yr3 and NCE: Continued assessment of SCD_{Rx} efficacy. 13 studies conducted. The project has been completed in its entirety.

15. SUBJECT TERMS
Acute lung injury, biomimetic device, inflammation, regenerative medicine, extracorporeal therapy.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
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1. **INTRODUCTION:**

Acute lung injury (ALI) progressing to acute respiratory distress syndrome (ARDS) affects nearly 200,000 Americans annually, develops in greater than 30% of combat casualties, and is associated with mortality rates of up to 50%. ALI and ARDS are currently treated solely based on supportive care as no pathophysiologic-based therapies for ARDS have been identified, leaving a large unmet medical need. Accordingly, the Department of Defense and Department of Veteran Affairs identified ALI to be a Topic Area of interest under the Peer Reviewed Medical Research Program (PRMRP). The awardee, Innovative BioTherapies, (IBT) is a start-up biotechnology company (founded 2003) based in Ann Arbor, MI, organized with the goal of developing bio-implantable/extracorporeal devices in the emerging field of regenerative medicine. IBT is actively advancing a platform technology, based on biomimetic membranes, that has improved clinical outcomes of critically ill patients with multiorgan dysfunction (MOD) by mitigating the inflammatory cascade. This technology has proven clinically effective to reduce biomarkers of inflammation, reduce organ dysfunction and decrease mortality rates in ICU patients with acute kidney injury (AKI) and multi-organ failure (MOF) receiving continuous renal replacement therapy (2-4). It has been effective in pre-clinical animal models in settings in which inflammation and MOD are present, including cardiopulmonary bypass and septic shock (5, 6), both of which are associated with ALI/ARDS. The project advanced under this contract seeks to assess the therapeutic impact of one of the biomimetic membrane-based devices, the selective cytopheretic device (SCD_{Rx}), in a preclinical, combat-relevant animal model of ALI. This proposal addresses several FY15 PRMRP sub-topic areas under the main topic area of ALI regarding preventative strategies and development of therapeutics for ALI. Activities under this 3-year proposal include development of a porcine model of ARDS relevant to combat trauma induced ALI (Year 1) followed by utilization of this animal model to evaluate SCD_{Rx} as a therapeutic intervention for ALI/ARDS (Years 2 and 3).

2. **KEYWORDS:**

- *Acute Lung Injury (ALI)*
- *Acute Respiratory Distress Syndrome (ARDS)*
- *Selective Cytopheric Device (SCD)*
- *Polytrauma*
- *Diffuse Alveolar Damage*

3. **ACCOMPLISHMENTS**:

- **Major Project Goals**

Specific Aim 1: Optimize a two-hit porcine ARDS model that is relevant to combat situation.	**Timeline months to complete task**	**Month of Proposal (0-36)**	**Anticipated Completion Date**	**Actual Completion Date**	**% Completed**
Major Task 1: Obtain approval for all animal work.	3.25	3.25	Dec 2016	Nov 1 2016	100%
Subtask 1: Complete and submit VA IACUC application. Obtain approval. NOTE: Will be submitted upon favorable grant review approximately 6 weeks before anticipated proposal start date.	1.5	-1.5.	Sept. 2016	Sept. 16, 2016	100%
Milestone Achieved: VA IACUC approval		0	Sept. 2016	Sept. 2016	100%
Subtask 2: Complete, submit ACURO application. Obtain approval.	3.25	3.25	Dec. 2016	Nov 1 2016	100%
Milestone Achieved: ACURO Approval		3.25	Dec. 2016	Nov 1 2016	100%
Major Task 2: Establish protocol for two-hit porcine ARDS model.	6.25	9.5	June 2017	July 2017	100%
Subtask 1: Perform blunt trauma with hemorrhage and fluid resuscitation under guidance of Dr. Alam.	0.5	3.75	Dec. 2016	Dec 2016	100%
Milestone Achieved: Staff are proficient in procedures involved with blunt trauma with hemorrhage and fluid resuscitation.		3.75	Dec. 2016	Dec 2016	100%
Subtask 2: Validate analysis protocols.	0.75	4.5	Jan. 2017	Jan. 2017	100%
Milestones Achieved: 1) All required antibodies and reagents are verified to be porcine specific. 2) LE flow panels are verified to be optimal for assessing LE phenotype and activation levels. 3) Staff are proficient in protocols for performing BAL and lung tissue processing.		4.5	Jan. 2017	*Jan. 2017*	*100%*
Subtask 3: Establish LPS dose to induce acceptable degree of ALI.	5	9.5	June 2016	July 2017	100%
Milestones Achieved: 1) LPS dose induces ALI, as defined by $Pa:FIO_2<300$, within 6 hours of LPS infusion start time. 2) 12 hour survival rate is ≥ 80%.		9.5	June 2016	July 2017	100%
Major Task 3: Verify reproducibility of two-hit porcine ARDS model up to 24 hr ARDS time course.	2.5	12	Aug. 2017	Feb 2018	100%
Subtask 1: Repeat study design determined in Aim1 /Major Task 2/Subtask 3 up to 24 hrs or until death, whichever occurs first.	1.5	11	Original July 2017 Adjusted Nov 2017	Feb 2018	100%
Milestones Achieved: 1) ALI, as defined by $Pa:FIO_2<300$, is achieved within 6 hours of LPS infusion start time in all pigs. 2) At least 80% of pigs survive 12 hours or longer.		11	Original July 2017 Adjusted Nov 2017	Feb 2018	100%

Subtask 2: Perform measurements and assays required to assess key endpoints/exploratory endpoints.	1	12	Original Aug 2017 Adjusted Dec 2017	Feb 2018	100%
Milestone Achieved: Experimental study design, with respect to analysis parameters and sample time points, will be finalized for Aim 2 study plan.		12	Original Aug 2017 Adjusted Dec 2017	Feb 2018	100%

	Timeline months to complete task	**Month of Proposal (0-36)**	**Anticipated Completion Date**	**Actual Completion Date**	**% Completed**
Specific Aim 2: Assess efficacy of 24 hour SCD_{Rx} in ARDS porcine model.					
Major Task 1: Perform 9 ARDS pig studies using final model optimized in Aim 1/Major Task 3/Subtask 1. (3 from each Cohort: Cohort defined in Methods on page 3 of SOW)	6	18	Feb. 2018	May 2018	See subtasks
Subtask 1: Perform 3 studies in each of the 3 cohorts.	5.5	17.5	Jan. 2018	May 2018	67% studies done in cohorts 1 and 2 only *(see below)*
Milestones Achieved: 1) ALI, as defined by Pa:FIO_2<300, is achieved within 6 hours of LPS infusion start time in cohort 1 and 3 pigs[1]. 2) At least 80% of pigs survive 12 hours or longer. 3) SCD therapy is successfully administered in Cohort 2 and 3. Please refer to footnote 1*		17.5	Jan. 2018	May 2018	83% studies not performed in Cohort 3 (milestone 3)
Subtask 2: Perform all measurements and assays required to assess key endpoints and exploratory endpoints.	6	18	Feb. 2018	May 2018	100%
Milestone Achieved: Assays results allow for comparison between cohorts.		18	Feb. 2018	May 2018	100%
Major Task 2: Perform 9 ARDS pig studies using final model optimized in Aim 1/Major Task 3/Subtask 1. (3 from each Cohort)	6	24	Aug. 2018	Aug 2018	See subtasks
Subtask 1: Perform 3 studies in each of the 3 cohorts.	5.5	23.5	July 2018	July 2018	67% studies done in cohorts 1 and 2 only (see below)
Milestones Achieved: 1) ALI, as defined by Pa:FIO_2<300, is achieved within 6 hours of LPS infusion start time in cohort 1 and 3 pigs[1]. 2) At least 80% of pigs survive 12 hours or longer. 3) SCD therapy is successfully administered in Cohort 2 and 3.*		23.5	July 2018	July 2018	83% studies not performed in Cohort 3 (milestone 3)
Subtask 2: Perform all measurements/assays required to assess key endpoints and exploratory endpoints.	6	24	Aug. 2018	Aug. 2018	100%
Milestone Achieved: Assays results allow for comparison between cohorts.		24	Aug. 2018	Aug 2018	100%

	Timeline months to complete task	Month of Proposal (0-36)	Anticipated Completion Date	Actual Completion Date	% Completed
Major Task 3: Perform 9 ARDS pig studies using final model optimized in Aim 1/Major Task 3/Subtask 1.	6	30	Feb. 2019	Aug 2020	100%
Subtask 1: Perform 3 studies in each of the 3 cohorts.	5.5	29.5	Jan. 2019	Aug 2020	100%
Milestones Achieved: 1) ALI, as defined by Pa:FIO_2<300, is achieved within 6 hours of LPS infusion start time in cohort 1 and 3 pigs[1]. 2) At least 80% of pigs survive 12 hours or longer. 3) SCD therapy is successfully administered in Cohorts 2 and 3.*		29.5	Jan. 2019	Aug 2020	100%
Subtask 2: Perform all measurements/assays required to assess key endpoints and exploratory endpoints.	6	30	Feb. 2019	Aug 2020	100%
Milestone Achieved: Assays results allow for comparison between cohorts.		30	Feb. 2019	Aug 2020	100%
Major Task 4: Perform 9 ARDS pig studies using final model optimized in Aim 1/Major Task 3/Subtask 1. (3 from each Cohort)	6	36	Aug. 2019	Aug 2020	100%
Subtask 1: Perform 3 studies in each of the 3 cohorts.	5.5	35.5	July 2019	Aug 2020	100%
Milestones Achieved: 1) ALI, as defined by Pa:FIO_2<300, is achieved within 6 hours of LPS infusion start time in cohort 1 and 3 pigs[1]. 2) At least 80% of pigs survive 12 hours or longer. 3) SCD therapy is successfully administered in Cohort 2 and 3.*		35.5	July 2019	Aug 2020	100%
Subtask 2: Perform all measurements/assays required to assess key endpoints and exploratory endpoints.	6*[2]	36	Aug. 2019	Aug 2020	100%
Milestone(s) Achieved: Assays results allow for comparison between cohorts.		36	Aug. 2019	Aug 2020	100%

*[1] *The possibility exists that cohort 2 (SCD at time of LPS infusion) may have altered ARDS onset or not develop ARDS, due to SCD impact.*

SUMMARY OF ACTIVITY

SPECIFIC AIM 1: OPTIMIZE A TWO-HIT PORCINE ARDS MODEL THAT IS RELEVANT TO COMBAT SITUATION

The overall goal of this Specific Aim was to define a clinical and combat-relevant animal model of endotoxin (lipopolysaccharide, LPS) induced ALI/ARDS after an episode of fluid resuscitated trauma. This sequence is intended to mimic the experience of a patient who is injured, resuscitated and then exposed to an inflammatory stimulus (such as infection) sometime during recovery that leads to ALI/ARDS. The optimized model is intended to be utilized to meet Specific Aim 2, evaluation of a novel therapeutic device, the SCD, for treatment of ALI/ARDS.

Specific Aim 1 was completed during Year 1 and Q1 of Year 2.

- **Major Task 1: Obtain approval for all animal work.**

Subtask 1: Complete and submit VA IACUC application. Obtain approval.
The completed Animal Component of Research Protocol (ACORP) was submitted to the VA AAHS Institutional Animal Care and Use Committee (IACUC) in June 2016 and was granted approval by the IACUC and AA VA subcommittee on Animals on September 16, 2016. (Milestone Achieved)

Subtask 2: Complete, submit ACURO application. Obtain approval.
Upon receipt of local approval, the competed Animal Use Appendix was submitted to the USAMRMC Animal Care and Use Review Office (ACURO) on September 20, 2016. The application was reviewed and granted approval on November 01, 2016. (Milestone Achieved)

- **Major Task 2: Establish protocol for two-hit porcine ARDS model**

Rationale: Inflammation is a key component in the pathophysiology of ARDS and recently published animal studies have been designed with 'two-hits' to better mimic co-morbidities and adequately reproduce the pathogenesis of ARDS [41]. Trauma (the first hit) evokes a pronounced inflammatory response that primes and activates cells of the innate immune system disrupting immunologic homeostasis. The second hit, often infectious in nature, occurs during an induced immunosuppressive state as initial inflammation subsides. With the second hit, a further inflammatory response is triggered whereby the previously primed immune system reacts beyond what is required for normal host defense, exacerbating tissue damage (including the lung) thus leading to ALI and a malignant inflammatory state.

Subtask 1: Perform blunt trauma with hemorrhage and fluid resuscitation under guidance of Dr. Alam.
Dr. Alam, Co-Investigator and practicing acute care surgeon at University of Michigan Hospital, provided experimental protocols and discussed techniques for hemorrhage and resuscitation of pigs based on his experience with trauma models. IBT veterinary surgeon, Dr. Johnston, and 2 IBT staff members (Drs. Chris Pino and Liandi Lou) visited Dr. Alam's laboratory on 3 separate occasions between September and November 2016 to learn the required techniques by observation of the procedures currently used for Dr. Alam's research.

Dr. Johnston conducted a follow up meeting with Dr. Alam in March 2017 where they reviewed data from the first 6 animal studies to ensure the trauma model was appropriate and reproducible.

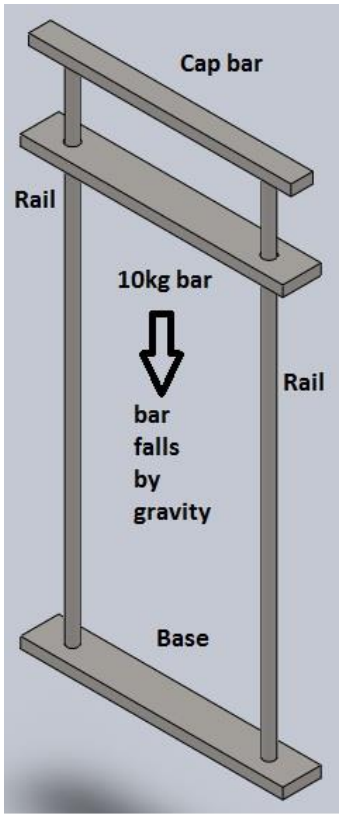


Figure 1. Design of an apparatus to produce soft tissue injury consisting of a 10kg bar that is raised by apparatus operators and dropped by gravity from a height of 1m to impact the fleshy region of a porcine thigh.

An initial step in model development was the manufacture of a customized apparatus designed to produce consistent soft tissue injury in a porcine model. In brief, the apparatus and method for injury is modeled after Stewart et al. where a 10kg steel bar is dropped from 1m to deliver 100j of energy to an area of 20cm^2, on the fleshy part of a porcine thigh (7). The design shown in Figure 1, details a dual rail system to guide the 10kg steel bar's freefall, to ensure reproducibility of soft tissue injury and safety of apparatus operators/staff involved.

Materials to produce this apparatus, especially the moving parts involved in trauma impact (left and right rails, and bearings embedded in the 10kg bar) were chosen to be stainless steel or non-corroding or tarnishing materials. The materials list ordered to build the apparatus are listed in Table 1. Total weight of the assembled apparatus was 36kg (79lb). Machining requirements included: 1) That the base must be drilled with through holes to affix left and right rails to the base via bolts. These holes must be countersunk so that the bolt head can be screwed in completely so that the bottom of base would be flat. 2) Left and right rails (1" diameter steel rod) must be drilled end on and tapped for bolts. 3) 10kg bar underside must be drilled & countersunk to allow easy travel along linear motion shafts (>1"diameter through holes) via bearings 3" from the left and right edges for an effective width between rails of 18" (1.5ft). 4) The Cap bar must be partially drilled, halfway through thickness, to allow for slip fit onto rails. This ensured proper spacing between rails and prevented binding of the 10kg bar during travel on the rails.

Material Name	**Description**	**Dimensions (L x W x H)**	**Weight (kg)**
10kg bar	Bar to fall by gravity, guided by rails	24" x 3.5" x 1"	10kg
Rails (x2)	1" diameter steel linear motion shaft	60"x1"x1"	5kg
Base	Base at least	24" x 3.5" x 1"	10kg
Cap Bar	Fixed distance to keep rail spacing	24" x 2" x 1"	6kg
Bearings (x2)	To ensure easy sliding of bar	1" diameter	0.1kg

Table 1. Materials used to build the Soft Tissue Injury Apparatus

As requested during the Ann Arbor VA IACUC review of the proposed animal protocol, preliminary calculations were undertaken to ensure that the proposed design of the apparatus would only produce soft tissue injury, and would not pose a substantial risk for porcine femur fracture. Bone fracture would be undesirable in

the experimental animals due to the pain and suffering that may be associated with this complication during the post trauma period. Calculations to demonstrate the safety of the proposed apparatus were based on the mechanical strength of bone data from the paper: "Design and Validation of a Compressive Tissue Stimulator with High-Throughput Capacity and Real-Time Modulus Measurement Capability" (8). In brief, in the studies reported, porcine cartilage and cancellous bone (aka trabecular, or spongy bone) from the head of a porcine femur was tested. In terms of bone breakage, cancellous bone of the femur would be more prone to breakage than cortical bone which is substantially stronger (normally orders of magnitude stronger). Table 2 reports the modulus of both porcine cartilage (3.4 MPa) and bone (123 MPa) (8), along with corresponding literature values. With its lower modulus, cartilage would be most susceptible to damage. However, damage to knee joint cartilage will be avoided by ensuring that the metal track system delivers the 10kg steel bar to the fleshy part of the thigh.

Modulus Values for Materials Measured by Mechanical Stimulator

Material tested	*Modulus (kPa) (measured values)*	*Modulus (kPa) (literature values)*
2% alginate	206 ± 65	163 ± 9[15]
3% alginate	317 ± 51	302 ± 4[15]
4% alginate	501 ± 87	476 ± 11[15]
Biological material tested	*Modulus (MPa) (measured values)*	*Modulus (MPa) (literature values)*
Porcine cartilage	3.4 ± 0.6	2.6 ± 0.1[17]
Collagenase digested cartilage	1.5 ± 0.2	NA
Porcine cancellous bone	123 ± 27	37–167[19]

Table 2. Mechanical strength of porcine bone from "Design and Validation of a Compressive Tissue Stimulator with High-Throughput Capacity and Real-Time Modulus Measurement Capability" (8)

With a modulus of 37-167 MPa reported in literature, cancellous bone is considerably stronger than cartilage, and less likely to sustain breakage. Ultimate break strength of a material normally strongly correlates with its modulus. However, bone density can also have a major impact on break susceptibility (1, 9, 10). Animals used for this porcine model are planned to be adolescents and should have minimal risk of osteoporosis with accompanying higher bone densities.

Calculation of Force produced by Soft Tissue Injury Apparatus for the proposed injury model:

Force = Mass x Acceleration

Force = 10kg (9.8 m/s^2) = 98N

Stress = Force/Area = 98N/20cm^2= 98N/(0.002m^2) = 49kPa

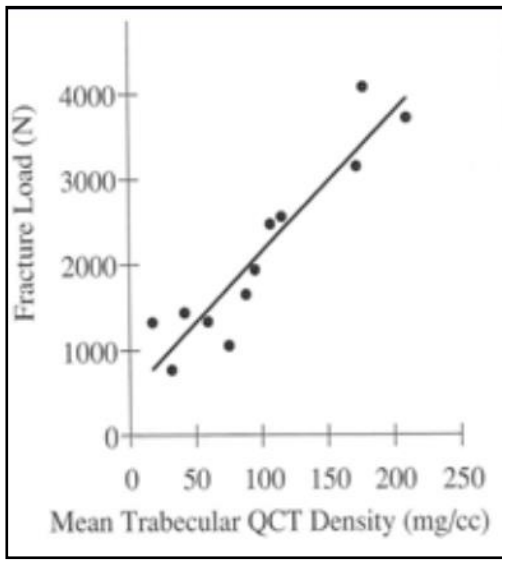


Figure 2. Risk of femur fractures as a function of Fracture Load in Newtons and bone density (1). Note that all fractures were sustained at loads >800N, which occurred at the lowest bone densities measured. This is an order of magnitude higher than the proposed injury model (98 N).

Typical fracture loads for porcine femurs reported in literature (>800N) are approximately an order of magnitude higher than the proposed injury model (98N), providing evidence that the apparatus has low risk of causing femur fracture (Figure 2).

Dr Pino, an engineer on staff at IBT working on the design and engineering aspects of the sort tissue injury apparatus, had all components machined to specifications by the University of Michigan LSA Scientific Machine Shop (Ann Arbor, MI). Dr. Pino transported and then assembled the apparatus and applied a medical grade lubricant (Nye Lubricants 760G, Fairhaven, MA) prior to testing the apparatus function on a porcine cadaver. The injury protocol was tested, with a drop at the 10kg bar from a height of 1m. Impact of the bar was recorded in a video, which demonstrated that significant soft tissue trauma could be rendered without causing porcine femur fracture (Figure 3). Dr. Pino was present and conducted soft tissue trauma with the animal study team using the apparatus during the porcine model development phase, to ensure that the injury protocol was safely, responsibly, and reproducibly followed.

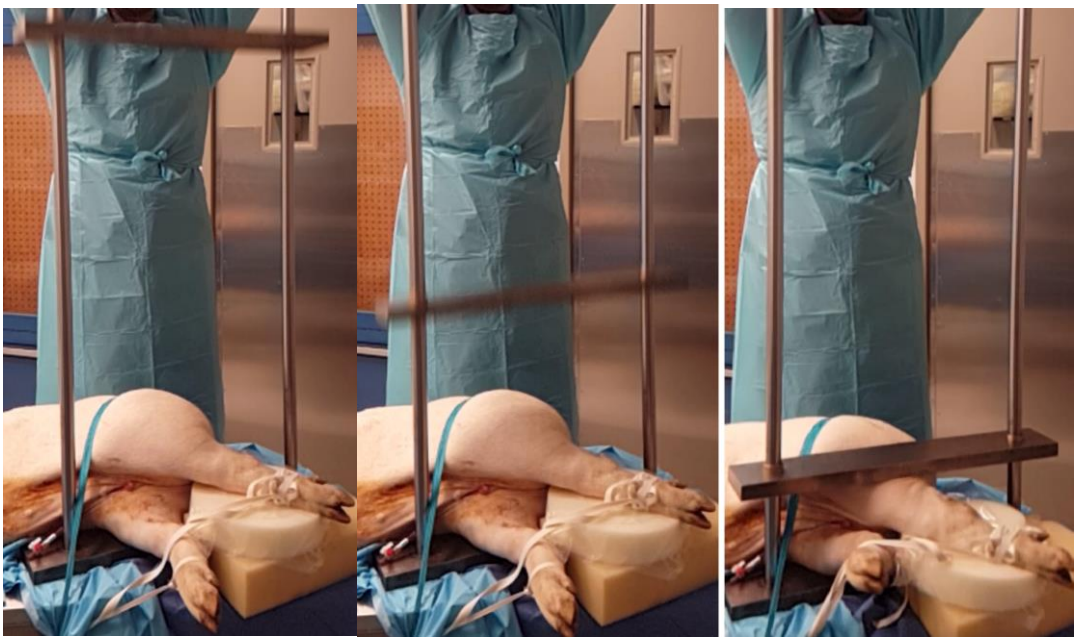


Figure 3. Images extracted from a video sequence of test run of the soft tissue injury apparatus used on a porcine cadaver, demonstrating the drop and impact of a 10kg bar.

All animal use activities were conducted at VAAAHS Research Facility under approved protocols. Animals were purchased from a single approved vendor (Oak Hill Genetics, Ewing IL) and received 1-2 weeks prior to use to permit assessment of heath and allow for acclimatization. The IBT animal research team under the direction of board certified veterinary surgeon, Dr. Johnston, developed an animal study plan based on utilization of familiar and previously successful procedures for the anesthesia and surgical instrumentation of the pigs based upon the team's experience in porcine models of ALI and sepsis. However, for this project several additional techniques had to be derived from reported models. Two pigs (n=2) were designated for training staff on the techniques for anesthesia, trauma and sampling procedures that would be required for the model. Both training animals were utilized in December 2016 to exercise techniques for "Hit 1", trauma with hemorrhagic shock and resuscitation. Briefly, pigs were sedated in the animal housing facility by intramuscular injection of Telazol (6 mg/kg) and xylazine hydrochloride (1-2 mg/kg) then transported to the animal operating theater where they were intubated and then maintained using inhaled isoflurane anesthesia in a mixture of air and oxygen and mechanical ventilation (GE Aespire 7100 with Cardiocap*5 monitor). Pigs were prepared for aseptic surgery then using a cut-down technique the blood vessels on one side of the neck were cannulated for monitoring and sample collection and the femoral vessels were cannulated to perform the hemorrhage and resuscitation procedures. Pigs were monitored using calibrated clinical patient monitoring systems consisting of

3 lead electrocardiogram, and pressure transducer systems for invasive measurement of central venous pressure, pulmonary artery pressures, arterial pressures (GE Solar 8000) and a thermodilution cardiac output monitor (Baxter Vigilance). Arterial blood gas values, serum electrolytes and lactate were measured at least hourly using an i-STAT (Abbott) handheld point of care analyzer. Once instrumented and stable, the anesthetized pig was moved to lateral recumbence and the custom-built traumatizer device described above was used to create a soft tissue injury to the pig's caudolateral thigh. Subsequent hemorrhage was achieved by controlled removal of 35% of estimated blood volume through the femoral arterial cannula using a Masterflex multichannel fluid pump. Shed blood was retained in sterile blood collection bags containing Citrate-Phosphate-Dextrose with Adenosine (CPD-A, Fenwall Inc)) anticoagulant and after a 60-minute period of hemorrhagic shock (maintained mean arterial pressure (MAP) of 35-40 mmHg), pigs were resuscitated by autologous transfusion using the shed blood plus additional crystalloid solution (0.9%NaCl or equivalent) to restore baseline hemodynamics.

Major Findings:
The anesthesia and cannulation procedures used, which were based upon prior models used in the IBT laboratory and Dr. Alam's model plus his recommendations, proved satisfactory to perform the necessary study interventions for Hit 1.

The custom-built traumatizer device effectively caused soft tissue trauma to the thigh as evidenced by skin indentation and discoloration immediately after impact and development of localized swelling without creation of open wound or bone fracture in either animal.

Hemorrhagic shock was successfully induced and maintained for 60 minutes in both pigs. A MAP of 35-40 mmHg could be maintained throughout this period by titrating the level of isoflurane anesthesia, without the need for fluid resuscitation. A measurable physiologic response due to the trauma and hemorrhage was observed in each animal evidenced by hypotension, a decline in cardiac output and progressive tachycardia (increased heart rate). Serum lactate, a biomarker of anaerobic respiration in hypoxic tissues, was found to be increased from a normal baseline value of <2.5 mmol/L to 4.07 mmol/L in Pig01 and 6.84 mmol/L in Pig02, consistent with a shock state. Hemorrhage in Pig01 was performed over 30 minutes by removal of blood in 3 increments (20%+10%+5% of blood volume per 10 minutes) as described in the model reference (Wilson *et. al*), however clotting was observed in the last bag making this blood unsuitable for reinfusion. Per protocol, this last bag of blood was collected at the slowest rate, 14 ml/min. Because of the clotting issue observed for Pig01, hemorrhage rate was increased to occur steadily over 20 minutes for Pig02. This hemorrhage rate utilized blood flow rates >50ml/min and no clotting was observed. Pig02 rapidly developed severe hypotension (MAP <30 mmHg) and drop in cardiac output to <1.0 L/min. These were treated with a small bolus of IV fluids according to Dr. Alam's hemorrhage protocol, and the pig survived to resuscitation. The response of Pig02 to the more aggressive hemorrhage was deemed potentially fatal. Based on these experiences, an intermediate hemorrhage protocol was developed for use on future pigs, targeting a 25-30 minute hemorrhage (to prevent severe cardiovascular collapse) using consistent flow rates >30 ml/min (to prevent clotting in the line).

Both pigs were successfully resuscitated to a stable cardiovascular status. Resuscitation was accomplished by rapid administration of 0.9% saline at 60 ml/kg (equivalent to the clinical "shock dose" of fluids administered to trauma patients) plus re-infusion of the shed blood (2/3 of the volume in Pig01 due to inability to use the last

blood bag which was clotted and 100% of the shed volume in Pig02). Vital signs and blood gas values (including lactate) were restored to baseline levels in both pigs with this protocol.

Research staff successfully performed all required procedures and gained experience in the sample collection and processing that would be required for the remainder of the project.

> *Milestone Achieved: Staff are proficient in procedures involved with blunt trauma with hemorrhage and fluid resuscitation.*

Subtask 2: Validate analysis protocols

1) Antibodies and reagents needed to be verified to be porcine specific.
Flow cytometric analysis has been historically performed at IBT using the BD Accuri C6 flow cytometer. At the time of submission of initial grant proposal, IBT's laboratory was limited in the number of parameters that could be simultaneously analyzed to six (forward and side scatter (FSC, SSC), and 4 color channels available on the Accuri C6). For this project, the cytometric data was slated to be obtained using the BD LSR II flow cytometer, available as core equipment at the VAMC. Prior to the initiation of this grant, IBT acquired the Attune flow cytometer from Thermo Fisher. As purchased, the Attune is equipped with three laser lines (488nm blue, 633nm red, 405nm violet laser), has automatic compensation capability, and can acquire up to 11 color channels. The Attune has the option to add an additional fourth 561nm yellow laser allowing for an additional 4 channels. After Pig07, all flow cytometry was performed on the Attune at IBT laboratories and data analyzed using FlowJo software. Regardless the flow cytometer employed, all data were analyzed using FlowJo software.

Single units of all antibodies were purchased, tested for reactivity, and titrations were performed on isolated lung cells, whole blood, whole blood processed with ammonium chloride lysis buffer to remove red blood cells, incubated whole blood, and lung cells processed for intracellular cytokine labeling. An example of titration data for anti-CD11R3 and anti-CD172a is shown in Figure 4. Neutrophils were gated by forward and side scatter profiles. The volume of blood [5 µL (0.5 µg) per 100 µL] was chosen for anti-CD11R3 to ensure saturation for CD11R3 which is reported as mean fluorescent intensity (MFI). For anti-CD172a, a volume of 0.5µl (0.5µg) per 100µL blood was chosen because it allowed for clear separation from negative events, enabling differentiation of monocytes and neutrophils from other cell populations.

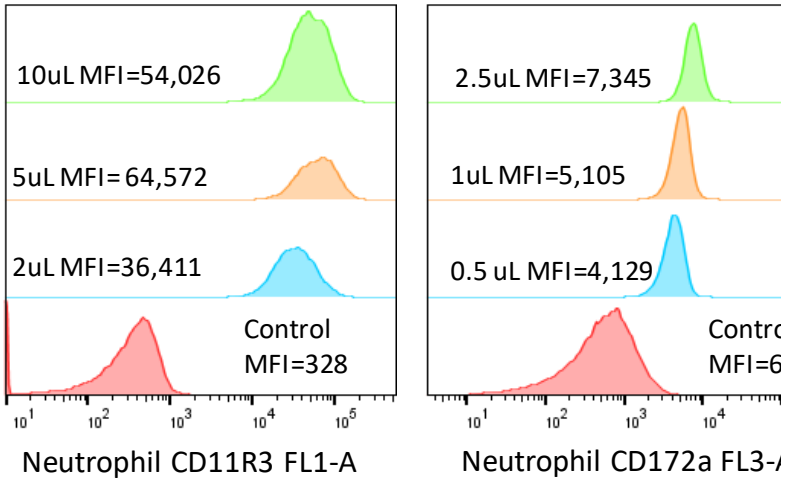


Figure 4. Data is shown for the titration of anti-CD11R3 and anti-CD172a antibodies on neutrophils gated by forward and side scatter profiles. 5µL (0.5µg) per 100µL blood was chosen for anti-CD11R3 to ensure saturation for CD11R3 which is reported as mean fluorescent intensity (MFI). 0.5µl (0.5µg) per 100µL blood was chosen for anti-CD172a because it allowed for clear separation from negative events, enabling differentiation of monocytes and neutrophils from other cell populations.

The titration results for all antibodies tested are shown in Table 3. Of the antibodies tested, anti-CD45 and anti-toll-like receptor 2 (CD282) did not show good reactivity in our system. Anti-CD45 was slated in the proposal for positive selection of leukocytes and exclusion of epithelial cells in single cell suspensions of lung tissue. However, the addition of 20µL of antibody resulted in only a small shift in MFI. When the complete panel was employed a much greater separation of cells of interest was afforded by CD172a and CD11R3 labeling. Anti-CD11antibodies have been used by others to identify monocytes, macrophages, neutrophils and eosinophils in single cell preparations from mouse and monkey lungs (11, 12). For pig, labeling with anti-CD172a antibody results in an even greater dynamic range of separation between cell types (Lung schematic, Figure 8). Work continued to determine if anti-CD45 conjugation was unsuccessful or interfered with the epitope recognition, but the addition of anti-CD45 was determined not be necessary to identify cells of interest in pig. Anti-CD282 was provided as a tissue culture supernatant from Dr. Xavier Dominguez (13) at no cost. For this antibody, further investigation would have required a great deal of effort to purify the antibody from the supernatant to perform the necessary conjugation for use in our multiplex systems. Although efforts continued to look for alternatives to this antibody, work proceeded with just toll-like receptor 4 (CD284), which worked well in our multiplex assays.

Table 3. Antibody Titration Record, 2 Hit Model (Trauma-LPS), Acute Lung Injury

Manufacturer	Target	Catalog Number	Lot Number	Conjugation for Initial Testing	Titration Result Amount Per Test	Confirmed Tar
BioRAD	CD11R3(2F4/11)	MCA2309F	1015	BL1 FITC	0.5ug/5uL	NE, EO, MO, subse
BioRAD	CD172a (BL1H7)/SWC3 FITC	MCA2312F	0515	BL1 FITC	0.05ug/0.5uL	MO, NE
BioRAD	CD163(2A 10/11) PE	MCA2311rpe	1607	BL2 PE	0.5ug/5uL	Subset MO
BioRAD	CD172a (BL1H7)/SWC3	MCA2312	1607	BL3 PERCP Cy5.5	0.05ug/0.5uL	MO, NE
BioRAD	CD203a SWC9 (PM18-7)	MCA1973GA	1602	BL4 PECy7	1.25ug/2.5uL	AMϕ
BioRAD	CD14(MIL-2)	MCA1218GA	0314	RL1 Alexa647	1ug/10uL	MO, Mϕ
BioRAD	CD14(tuk4)	MCA1568A647	0313	RL1 Alexa647	1ug/10uL	MO, Mϕ
ThermoFisher Scientific	CD284 (TLR4) HTA125	56-9917	E11982-1637	RL2 AlexaFluor700	1ug/5uL	Compensation Iss
BD	CD284 (TLR4) HTA125	743392	7072769	VL1- BV421	1ug/5uL	subset All
BioRAD	SLA DR Class II(2E9/13)	MCA2314GA	0615	RL3-APCCy7	0.5ug/5uL	MO, Mϕ
Dr. Javier Dominguez	CD282 (TLR2)	10/26/2016	10/26/2016	VL1-Alexa405 using zenon kit	75ul	Non reactive
BioRAD	SWC8, with anti MoIgM	MCA1219	1607	BL4 PECy7 , 2 step	5uL	NE, EO, subset L
BioRAD	CD45	MCA1222GA	0514	VL1-Dylight405	20uL	small shift all LE
R&D	TNFa (103302)	MAB6903	EXK0316031	VL1-Dylight405	0.5ug/5uL	Subset MO/Mϕ conf
R&D	IL-6 (77830)	MAB686	DAF0614081	VL1-Dylight405	0.5ug/5uL	Subset MO/Mϕ conf
R&D	IL-10 (262715)	MAB6932	UUT0411101	R3-APCCy7	0.5ug/5uL	Subset MO/Mϕ conf
R&D	IFN-g (154007)	MAB985	ISC0115021	R3-APCCy7	0.5ug/5uL	Subset MO/Mϕ conf

NE=Neutrophil, MO=Monocytes, EO=Eosinophils, Ly=Lymphocytes, A=Alveolar, Mϕ=Macrophage, LE=Leukocytes

The activation level of neutrophils is a primary factor to evaluate the dose of lipopolysaccharide essential to model development. Human neutrophils (14, 15) and monocytes (16, 17) mobilize intracellular stores of CD11b to the cell surface as they become (primed) activated, allowing a real-time measurement of systemic acute neutrophil (priming) and monocyte activation. For pilot studies, the clone 2F4/11, reactive to human CD11c, was selected from panel of human reactive CD11 antibodies. This antibody was found to be reactive to a 155kD alpha chain and CD18/β2 integrin. In pigs, anti-human CD11b specific antibodies had positive reactivity to the 165kD alpha chain expected for CD11b, however in pigs, these antibodies are reactive only to granulocytes. Of the antibodies reactive to human CD11c, only clone 2F4/11 strongly labeled granulocytes, monocytes and alveolar macrophages, the expected expression pattern comparable to human

CD11b. Because it is unclear whether the differences are due to species expression or differences in epitope recognition, the nomenclature CD11R3 was adapted (18). The clone was chosen for its strong reactivity to cells of interest and detectable upregulation upon stimulation.

For pilot studies, CD11R3, the porcine counterpart to human CD11b was used to track the activation state of neutrophils in response to experimental stimuli (See data in Model Comparison). Blood was labelled with a saturating amount of anti-human CD11R3-FITC (clone: 2F4/11), allowing for the quantitative analysis of expression to be reported as fluorescence intensity. Problems arose with this strategy due to differences in the flow cytometer instrument settings for the photomultiplier tubes (PMT) between the BD Accuri C6 (historical data), BD LSR II flow cytometer (VA core) and Thermo Fisher Attune (IBT). Additionally, compensation settings were altered depending on the antibody panels used. To allow for relative fluorescence activity to be compared between historic data evaluated on the BD Accuri C6, initial data from this project evaluated on the BD LSR II flow cytometer, and during protocol development when compensation and PMT parameters were altered on the Thermo Fisher Attune, cell sorting set-up beads for blue lasers (Life technologies, C16508) were added to each run. The control beads have consistent brightness, and therefore the changes in fluorescence due to compensation and instrument settings can be evaluated. The relationship between the power setting of the photomultiplier tube used for blue laser line 1 (also used for CD11R3-FITC) and the resulting mean fluorescence intensity (MFI) is exponential (Figure 5, left). For comparison of absolute numbers, experiments Pig01-Pig07, absolute data converted as a ratio of the closest values recorded for the blue laser beads. For experiments Pig08-Pig17 the PMT setting for blue laser 1 was kept consistent.

In all remaining work, the cell sorting set-up beads were used to verify consistency in flow cytometer function, but more importantly, to confirm that the animals have normal CD11R3 expression levels at baseline. Elevation in CD11R3 at baseline would indicate a previously existing condition that can influence experimental results. By using the normalization factor afforded by the compensation beads, the baseline CD11R3 MFI for the animals from our current supplier could be compared to baselines from historic data, and provided further assurance that animals are healthy upon arrival. As shown in Figure 5, right, similar animals used historically for an acid induced acute lung injury and sepsis models were not statistically different from those used for the current model. All future data will be obtained using the Attune flow cytometer at IBT and conversion of this data to compare between flow cytometers and settings will not be necessary. Data is reported as relative fluorescence intensity as compared to baseline.

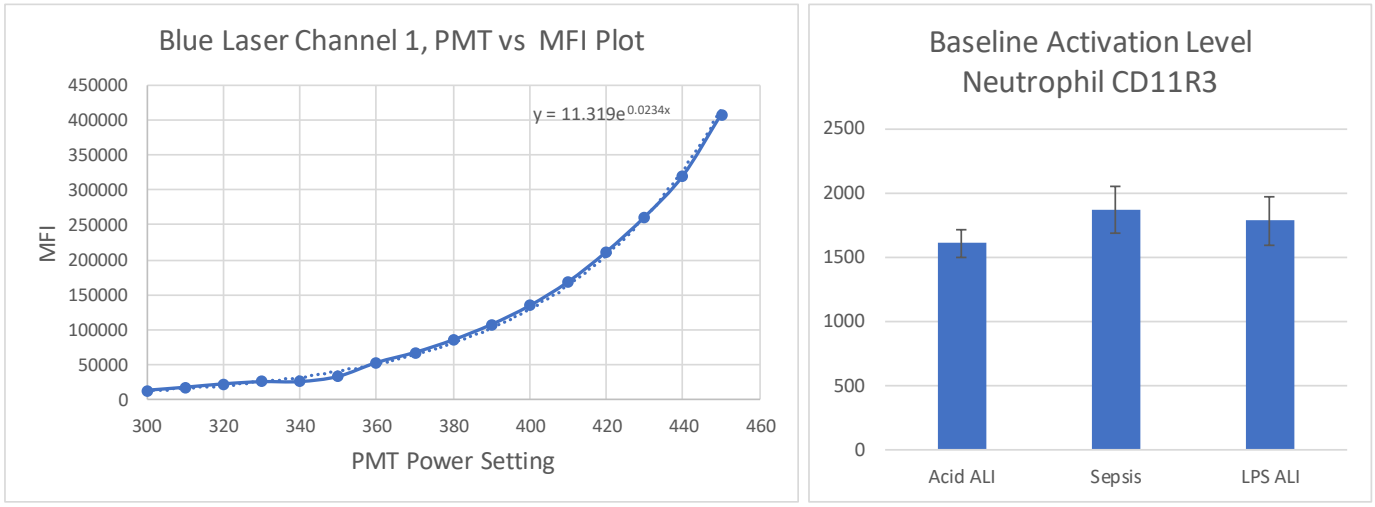


Figure 5. The exponential relationship between photomultiplier tube setting (PMT) and mean fluorescent intensity (MFI) is graphed in the left panel. A comparison of baseline levels from historic data of normal pigs (Used for acid induced Acute Lung Injury (ALI), Sepsis, and current model (LPS ALI) normalized using blue laser control beads is shown in right panel.

For multiplexed analysis, problems arose with using cell based single channel controls for calculating compensation. Antibody labels did not have clearly defined positive and negative populations, especially in the case of cells in bronchial alveolar lavage fluid (BALf), for which most cells are macrophages, and the positive cell populations for monocytes and neutrophils are rare. To overcome these issues the AbC total antibody compensation bead kit (Life Technologies) was used to make individual channel controls for compensation. For antibodies with confirmed reactivity, the specific lots tested were placed on hold with vendors except for CD11R3, which was purchased in a quantity that will suffice for completion of all Specific Aims. In all further work, additional controls were run to ensure that non-specific binding of antibody to FC receptors on myeloid cells is not occurring. FC blocking reagents were commercially available for human cells and mouse cells, but not for pigs. Pig blood was treated with ammonium chloride lysis buffer and rinsed to remove red blood cells and serum proteins followed by labelling with live-dead aqua (ThermoFisher L34966) to discriminate dead cells that represent another source of non-specific binding. Efforts continued to troubleshoot antibodies to CD45 and CD282 cells.

2) LE flow panels needed to be verified to be optimal for assessing LE phenotype and activation levels. Evaluation of neutrophil, monocyte and macrophage populations may provide insight to the transition from neutrophilic alveolitis to monocytic alveolitis (19). A Gating hierarchy was confirmed in systemic blood for the following: CD11R3, CD284 (toll-like receptor 4), and S(swine)LA DR II MFI in macrophages, neutrophils, monocytes and monocyte subsets (CD14+ CD163+, CD14+ CD163, CD14low CD163+). Anti-CD203 (SWC9), positively identifies alveolar macrophages (20, 21) and is included in the antibody panel used to analyze single cell suspensions of lung cells. Antibody to CD14 labels pig monocytes at variable intensity through maturation, macrophages and is also found on porcine neutrophils to a lesser degree. Antibody to CD163 recognizes a porcine monocyte maturation marker (22) and is highly expressed on a subset of monocytes and all macrophages. SLA DR Class II is differentially expressed on all cells of interest, but may be shed as cells become anergic (23). Antibody to CD284 recognizes toll-like receptor 4 which can be differentially expressed via a wide range of stressors (24, 25). Using the panels shown in Table 4,

macrophages, neutrophils, monocytes, and monocyte sub-populations were reliably identified. The identified populations could then be evaluated for expression of CD11R3, SLA DR II and CD284.

The typical expression profile for systemic blood is shown in Figure 6. Neutrophils and monocytes are identified by side-scatter area and CD172a expression. Monocyte subpopulations are further defined by CD14 and CD163 expression. For pig, two major subpopulations are present: CD14+CD163- and CD14+CD163+, with the CD163+ having slightly lower CD14 expression in normal blood.

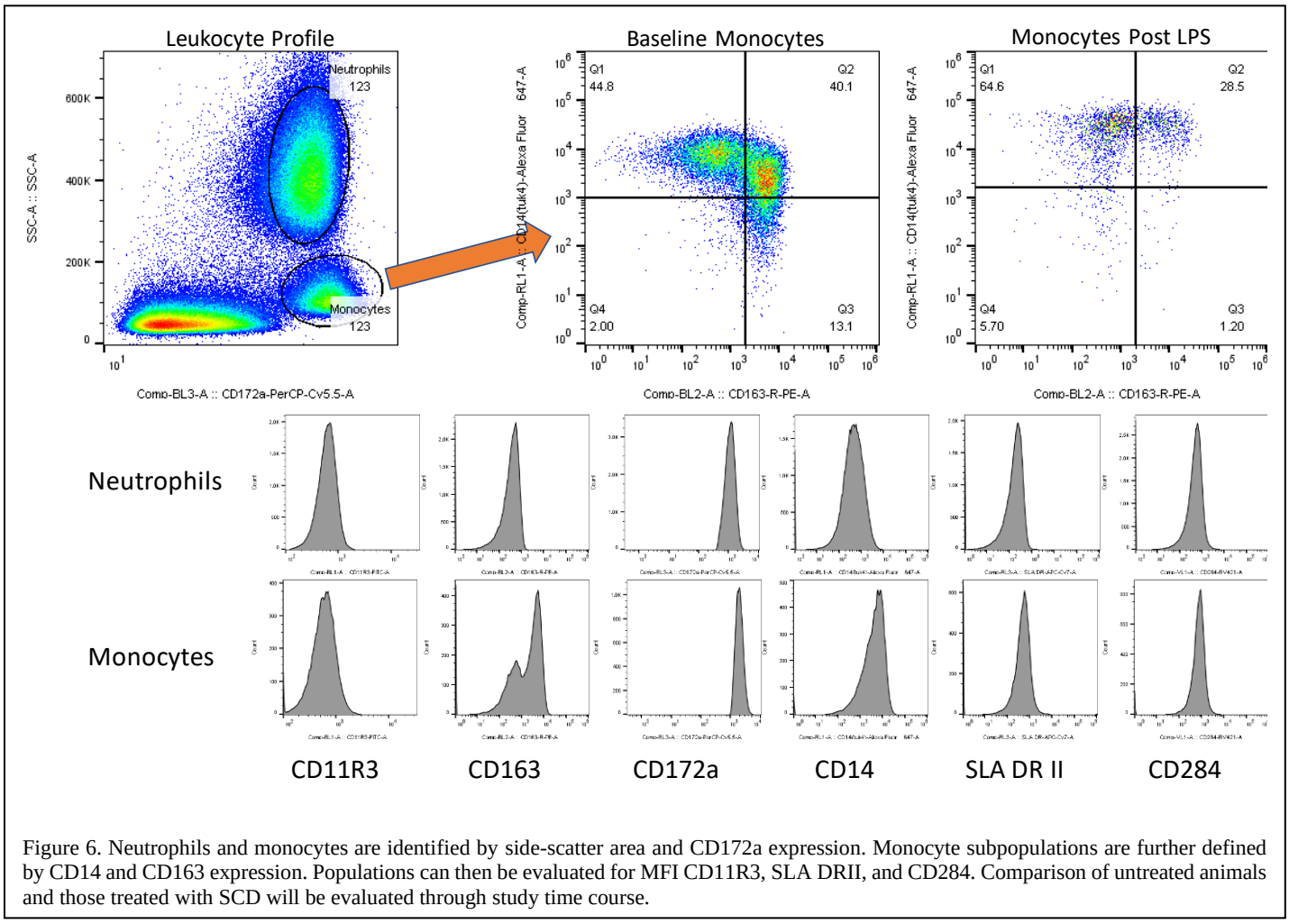


Figure 6. Neutrophils and monocytes are identified by side-scatter area and CD172a expression. Monocyte subpopulations are further defined by CD14 and CD163 expression. Populations can then be evaluated for MFI CD11R3, SLA DRII, and CD284. Comparison of untreated animals and those treated with SCD will be evaluated through study time course.

Blood from Pig08-Pig10 was evaluated through the study time course and data is shown in Figure 7. The monocyte populations showed a significant shift in CD163 expression (44±3.7% positive at baseline to 52±6.3% positive at Day 1 post trauma, $p<0.05$). With LPS infusion, there was a dramatic decrease in all leukocytes, but the remaining monocytes were only 16±4.9% CD163+ ($p<0.001$ compared to baseline) and continues to increase through the study time course. The relative fluorescence intensity (RFI) of CD14+CD163- monocytes, CD14+CD163+ monocytes and neutrophils for SLA DR II, CD11R3 and CD284 are shown in side panels normalized for baseline levels. SLA DR II, CD11R3 are elevated at 6 hours post LPS compared to baseline and decrease by 12 hours. CD284 expression remains relatively steady for neutrophils but continues to rise for monocytes. The ability to identify and analyze cells of interest was confirmed and protocols were validated for Specific Aim 2 studies comparing untreated and SCD treated animals using the final model.

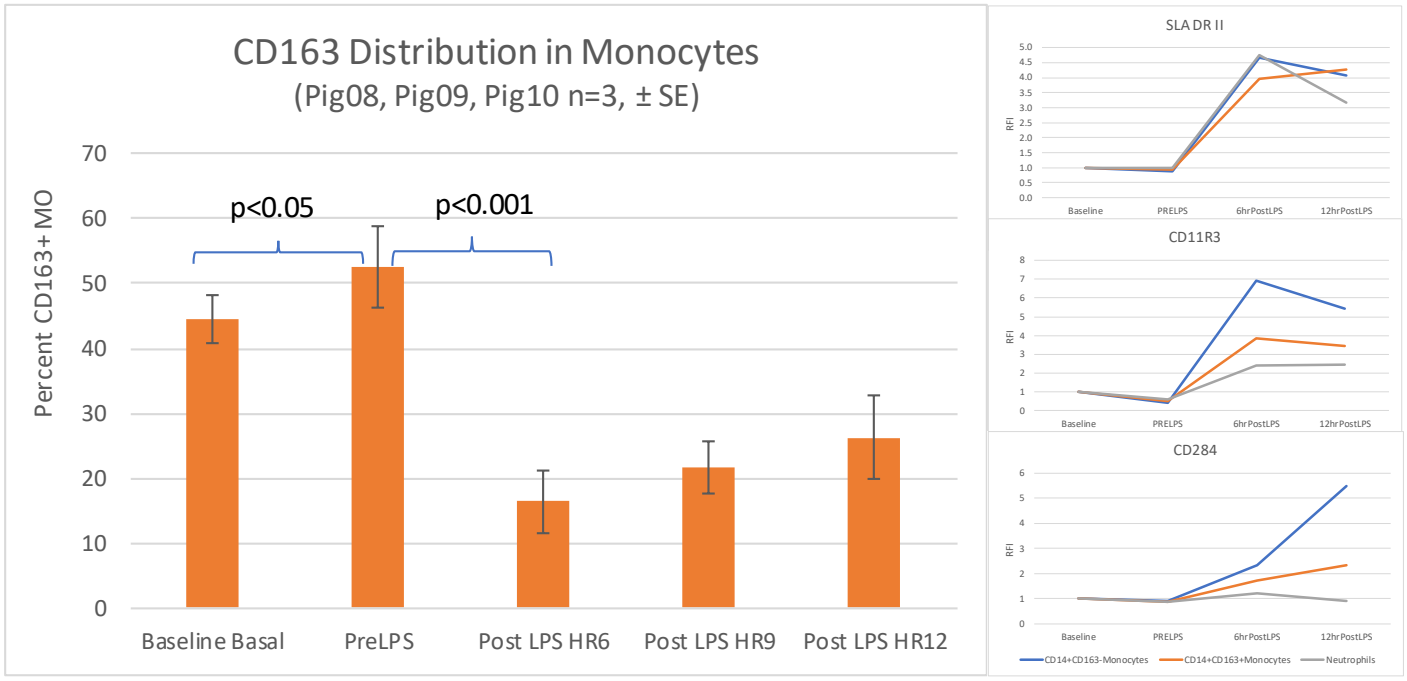


Figure 7. Samples were evaluated for three animals Pig08-10 to validate that protocols in preparation for Specific Aim 2 studies comparing untreated and SCD treated animals using the final model. Large Panel: The monocyte populations showed a significant shift from 44±3.7% CD163+ at baseline to 52±6.3% CD163+ Day 1 post trauma ($p<0.05$). With LPS infusion, there was a dramatic decrease in all leukocytes, but the remaining monocytes were only 16±4.9% CD163+ ($p<0.001$ compared to baseline). Side Panels: The Relative Fluorescent Intensity (RFI) of CD14+CD163- monocytes, CD14+CD163+ monocytes and neutrophils for SLA DR II, CD11R3 and CD284 are shown normalized to baseline levels. The ability to identify and analyze cells of interest was confirmed.

It has been hypothesized that alveolar macrophages repopulate from specific lung-resident ("interstitial") pools that enter the alveolar compartment only during extreme inflammatory events (11, 26). In the developed porcine model, where ALI is evidenced, most cells in the BALf are alveolar macrophages. Protocols for obtaining cells from mechanically dissociated lung and enzyme treated lung were developed because the interstitial cells may be more directly altered by the SCD placed in the systemic circulation as compared to the cells in the alveolar compartments. It was thought that analysis of these cells may show differences prior to those recovered in the BALf and may be more appropriate for the 3-day model time course (Model 2 and 3 described below).

Like blood, for analysis of single cell lung suspensions, populations of interest are gated away from lymphocytes, debris and epithelial cells by strong reactivity to CD172a. Alveolar macrophages are positive for CD203 and compared to monocytes and neutrophils, macrophages are more strongly positive for CD11R3, CD163 and SLA DR II. Examples of cytospins and scatter profiles of single cell lung suspensions from Pig07 sacrificed 12 hours post LPS infusion are shown in Figure 8. The same lung lobe was processed to obtain BALf, then mechanically disrupted, with copious rinsing to remove free cells, and finally the remaining tissue enzymatically digested with Liberase (Roche). Upon inspection by cytospin with differential staining and flow cytometry, the BALf contains almost exclusively alveolar macrophages, mechanically dissociated tissue has a greater number of cells identified as monocytes and neutrophils, and with enzyme treatment, an even greater percentage of neutrophils are present and few alveolar macrophages are present.

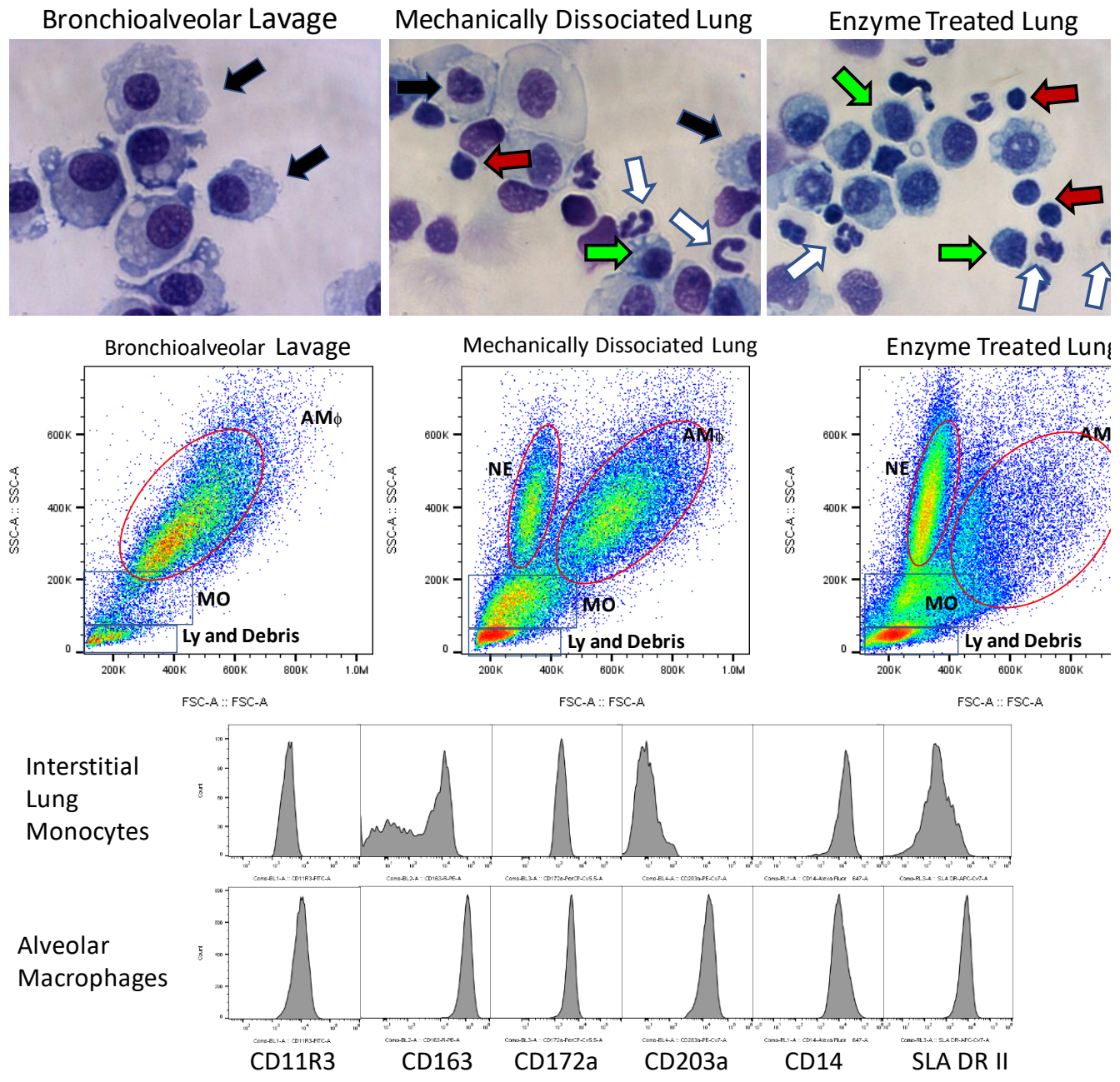


Figure 8. Cells can be identified in differentially stained cytospins from bronchioalveolar lavage fluid, mechanically dissociated lung and enzyme treated lung. In top panel, larger cells with plentiful vacuolized plasma are alveolar macrophages (AMϕ-black arrows); small segmented cells are neutrophils (NE-white arrows); small cells with a paucity of plasma are lymphocytes (Ly-red arrows); and monocytes (MO-green arrows). *Note: only a few of each cell type have been identified for clarity.*
In the bottom panels, alveolar macrophages, neutrophils and monocytes can also be identified by scatter profiles using flow cytometry. Populations of interest are gated away from lymphocytes, debris and epithelial cells by strong reactivity to CD172a. Monocyte subpopulations are further defined by CD14 and CD163 expression of CD203- cells. Populations can be evaluated for MFI CD11R3, SLA DRII, and CD284. Comparison of untreated animals and those treated with SCD was planned.

Cytokine expression under LPS stimulated conditions were evaluated for monocytes in whole blood, macrophages in BALf and dissociated lung cells (27). Intracellular cytokine labeling was accomplished using an Intrastain Kit (DAKO) on blood diluted 1:2 in RPMI, or lung cells in media alone supplemented with brefeldin A to inhibit Golgi secretion (28). Cells were labeled with surface markers, and then fixed and permeated and labeled with antibodies to cytokines. Monocytes and macrophages have a basal secretion of

cytokines and can have different responses to lipopolysaccharide (LPS) depending on their activation state. Secretion of TNFa, IL10, IL-6 and IFNg are slated for testing to further evaluate inflammatory state of these cells. Positive expression that increases with LPS treatment has been confirmed in blood monocytes, interstitial lung monocytes and alveolar macrophages for these cytokines. An example of data obtained for baseline blood cells and normal lung macrophages is shown in Figure 9 for TNFa. Brefeldin-A treated cells with and without LPS stimulation are shown compared to no brefeldin-A controls. Unstimulated monocytes can be split into categories according to their CD163 expression and TNFa production. CD163+ cells are negative for TNFa, while CD163- cells have both TNFa positive and negative populations. For comparison of SCD treated vs. untreated animals, gating for positive or negative cytokine parameters was determined using fluorescence minus one controls (29). Samples could then evaluated through both treated and untreated time courses before final gating parameters were established. Of note, during intracellular cytokine labelling, the permeation step alters the scatter profile making it more difficult to separate monocytes from neutrophils. Because of this, the addition of anti-SWC8 to the lung panel in the third violet laser channel (VL3) using BD anti-mouse IgM (clone R6-60.2) was planned.

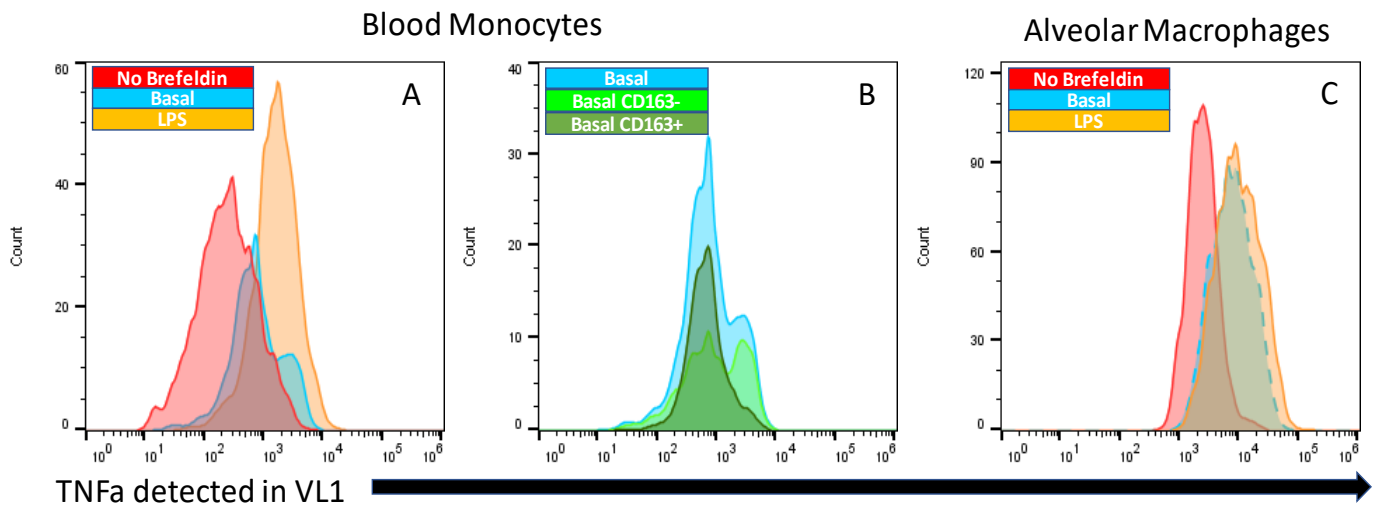


Figure 9. Example of intracellular TNFa detection in blood monocytes. Monocytes and macrophages have a basal secretion of TNFa that increases in response to lipopolysaccharide (LPS). No brefeldin (red), basal (blue) and LPS stimulated (orange) are shown for baseline pig blood (A) and alveolar macrophages (C). Unstimulated monocytes can be split into categories according to their CD163 expression and TNFa production (B). CD163+ cells are negative (dark green) while CD163- cells are both positive and negative for TNFa (light green).

Table 4. Multiplexed Antibody Panels

Systemic Blood- Monocyte Surface Characterization and MO And NE Activation

Analyze CD11R3 and CD284 in neutrophils, and CD11R3,CD284 and SLA DR II in all MO and MO subpopulations, (CD14+CD163-, CD14+CD163+, CD14low CD163+).

Vendor	Label	Titrated Amount	laser/fluor
ABDSerotec	CD11R3 (2F4/11)	0.5ug/5uL	BL1-FITC
ABDSerotec	CD163 (2A 10/11)	0.5ug/5uL	BL2-PE
ABDSerotec	CD172a (BL1H7)/SWC3	0.05ug/0.5uL	BL3 PERCP Cy5.5
ABDSerotec	CD14 (tuk4)	1ug/10uL	RL1-Alexa Fluor 647
ABDSerotec	SLA DR Class II (2E9/13)	0.5ug/5uL	RL3-APCCy7
MyBioSource	CD284 (TLR4) HTA125	1ug/5uL	VL1-BV421
ThermoFisher Scientific	LIVE/DEAD® Fixable Aqua Dead Cell Stain	1uL	VL2-405/aqua

Macrophage Surface Characterization and Activation
(Used for BAL, and Dissociated Lung, and Interstitial Lung (enzyme treated)

Analyze CD11R3, SLA DR II and CD284 in macrophages from dissociated lung tissue and BAL.

Vendor	Label	Titrated Amount	laser/fluor
ABDSerotec	CD11R3(2F4/11)	0.5ug/5uL	BL1-488/FITC
ABDSerotec	CD163(2A 10/11)	0.5ug/5uL	BL2-PE
ABDSerotec	CD172a (BL1H7)/SWC3	0.05ug/0.5uL	BL3 PERCP Cy5.5
ABDSerotec	CD203a SWC9 (PM18-7)	0.25ug/2.5uL	BL4-PECy7
ABDSerotec	CD14 (TUK4)	1ug/10uL	R1-Alexa Fluor 647
ABDSerotec	SLA DR Class II(2E9/13)**	0.5ug/5uL	RL3-APCCy7
MyBioSource	CD284 (TLR4) HTA125	1ug/5uL	VL1-BV421
ThermoFisher Scientific	LIVE/DEAD® Fixable Aqua Dead Cell Stain	1uL	VL2-405/aqua

Whole Blood ICC. Monocyte Surface Characterization and Intracellular Cytokines

Analyze Cytokines MFI in all MO and MO subpopulations, (CD14+CD163-, CD14+CD163+, CD14low CD163+).

Vendor	Label	Titrated Amount	laser/fluor
ABDSerotec	CD172a (BL1H7)/SWC3	0.05ug/0.5uL	BL1-FITC
ABDSerotec	CD163(2A 10/11)	0.5ug/5uL	BL2-PE
ABDSerotec	SWC8 (MIL2) (concentration not provided)	5uL	unconjugated
ThermoFisher Scientific	anti MO IgM PE-CY7(eB121-15F9)	1.25ug/2.5uL	BL4 PE-CY7
ABDSerotec	CD14 (MIL-2 or TUK4)	1ug/10uL	RL1-Alexa Fluor 647
R&D	IL-10 (262715) or IFN-g (154007)*	0.5ug/5uL	RL3-APCCy7
R&D	IL-6 (77830) or TNFa (103302)*	0.5ug/5uL	VL1-Dylight405
ThermoFisher Scientific	LIVE/DEAD® Fixable Aqua Dead Cell Stain	1uL	VL2-405/aqua

Lung Macrophage ICC. Surface Characterization and Intracellular Cytokines

Analyze Cytokines in macrophages from BAL and dissociated lung tissue.

Vendor	Label	Titrated Amount	laser/fluor
ABDSerotec	CD172a (BL1H7)/SWC3	0.05ug/0.5uL	BL1-FITC
ABDSerotec	CD163(2A 10/11)	0.5ug/5uL	BL2-PE
ABDSerotec	CD203a SWC9 (PM18-7)	0.25ug/2.5uL	BL4-PECy7
ABDSerotec	CD14 (MIL-2 or TUK4)	1ug/10uL	RL1-Alexa Fluor 647
R&D	IL-10 (262715) or IFN-g (154007)*	0.5ug/5uL	RL3-APCCy7
R&D	IL-6 (77830) or TNFa (103302)*	0.5ug/5uL	VL1-Dylight405

3) Staff needed to become proficient in protocols for performing BAL and lung tissue processing.
Following successful execution of the trauma and resuscitation procedures, pigs were humanely euthanized to train the post mortem evaluation and sample collection processes. En bloc excision of heart and lung best allowed for performing a BAL of the right middle lung lobe and access to appropriate anatomic sites for the remainder of the sampling. Recruitment maneuvers consisting of 3 rapid breaths to max pressure of 25-30mmHg followed by holding inflation pressure at 30mmHg for 30 seconds. to re-inflate atelectatic (collapsed) lung prior to removal from euthanized animals allowed for best preservation tissue architecture. For BAL, the right middle lobe was isolated at the bronchus closest to the trachea. Following a small incision, a sterile segment of polypropylene pressure tubing, cut at a diagonal and fenestrated near the tip with several punctures by an 18g needle (the "BAL cannula"), was inserted then gentle instillation and removal of three sequential 50ml aliquots of phosphate buffered saline (PBS) with 0.5mM ethylenediaminetetraacetic acid (EDTA) was performed. Recovered BALf was placed on ice, volume measured and then centrifuged at 350xg for 10 minutes. Supernatants were collected and stored in an ultra-low freezer for future assays. Any red blood cells, if present, were lysed using ammonium chloride buffer and remaining BALf cells were rinsed, suspended in PBS/EDTA and counted. A cytospin was prepared and differential staining performed to identify leukocyte subtypes.

The diaphragmatic lobe of the left lung was placed into a chopping apparatus (Ninja® blender) and ground to a paste. Representative samples were frozen and archived for protein analysis, and fresh tissue was transferred to IBT for evaluation of edema by determination of water content. Briefly, 1-2g samples were placed on to tared foil sheets and allowed to dry completely in a 70°C oven (as determined by no further decrease in weight). The percent water content can be expressed as the change between wet and dry weight, divided by the wet weight.

After extraction of BALf was completed, the left cranial lobe was rinsed free of blood and transferred to the IBT laboratories in organ preservation solution (UW Solution, Bridge to Life) for processing to obtain interstitial lung cells. The initial plan was to mechanically dissociate the lung in a Waring blender (30, 31), but this resulted in a substantial number of dead cells. Alternately, the tissue was manually minced into small aggregates after removal of major bronchi and washed with Dulbecco's Modified Eagle's medium (DMEM, Gibco) with antibiotics over a 212um sieve until fluid ran clear. Cells collected in this manner are referred to as dissociated lung cells. The tissue remaining on top of the sieve was subjected to enzyme digestion for 20 minutes at 37°C using 250Ku/mL DNase and 0.239wU/mL of Liberase in DMEM. Cells collected in this manner are referred to as enzyme treated lung cells. All cells were passed through a 32um sieve prior to further processing for antibody labelling or culture for intracellular cytokines.

A bronchus to the diaphragmatic lobe of the right lung was inflated with a 50/50 (v/v) optimum cutting temperature (OCT) compound (Tissue Tek)/PBS, via a cannula using a method similar to that described for BAL. Once inflated, the isolated lung lobe was placed into a pan on wet ice to allow it to become more firm, then appropriately sized sections were cut and placed into cryomolds with OCT. Filled cryomolds were allowed to freeze in the vapor phase on a surface precooled with liquid nitrogen. Prepared blocks were sectioned using a Lecia cryostat and labeled with antibody to CD11R3 (BioRAD) and visualized using anti mouse IgG Alexafluor 594 conjugate (Fisher Life Sciences). An example of antibody labeling from Pig02 is

shown in Figure 10. Images are slated to be used for image analysis using Image J software (NIH) to quantitate CD11R3 positive percent area normalized by DAPI positive percent area to provide a quantitative assessment of leukocyte recruitment into the lung in untreated animals compared to SCD treated.

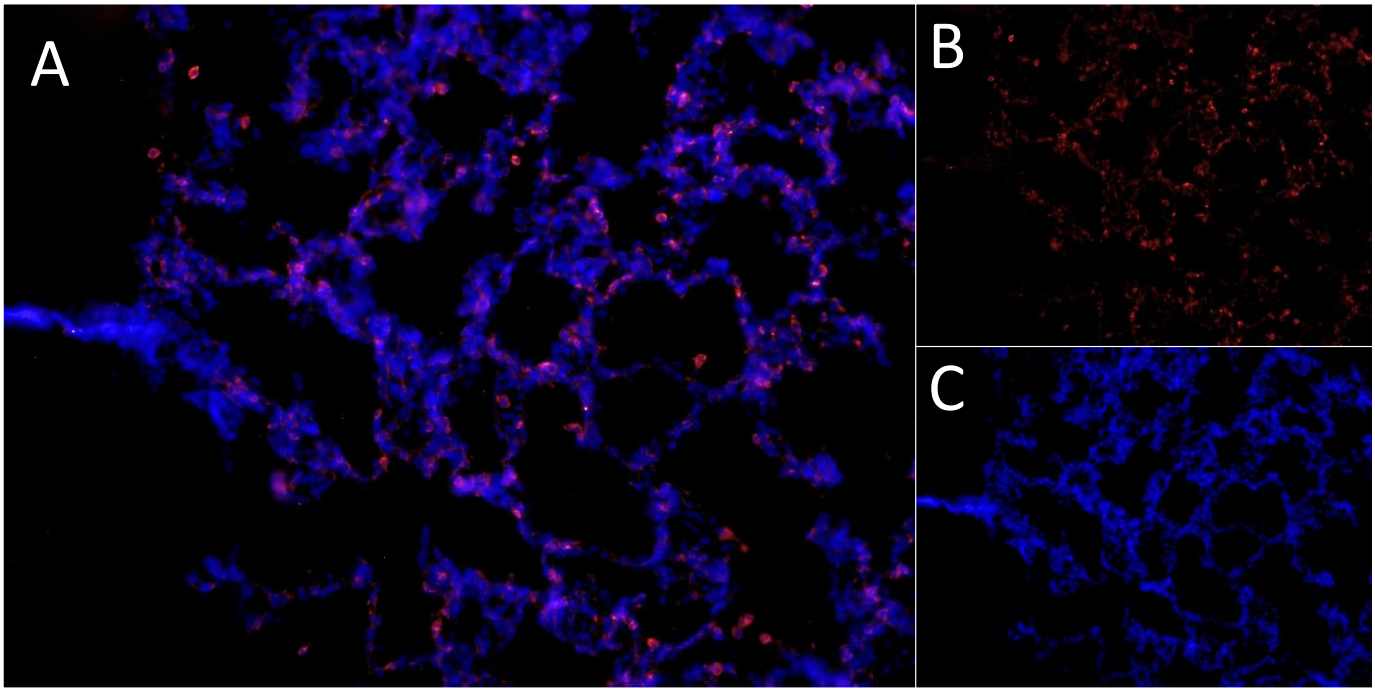


Figure 10. Example Pig02 cryosectioned lung labeled with antibody to CD11R3 detected with alexafluor 594 (Red) and counterstained with DAPI (Blue) to permit quantification of the amount of activated leukocytes within the lung. Overlaid images are shown in large panel A and individual channel images are shown for CD11R3 and DAPI in B and C respectively.

A separate bronchial segment of the right diaphragmatic lobe was used to prepare paraformaldehyde fixed samples for paraffin embedding and hematoxylin and eosin (H&E) staining. Like the lung lobes used for BAL and preparation of frozen sections, the lung was inflated with a solution of 4% paraformaldehyde freshly prepared from 16% ampules (Electron Microscopy Sciences). The bronchus was then ligated to retain fluid and the excised segment immersed in ample 4% paraformaldehyde solution. The section is fixed for 24 hours, then representative portions appropriately sized for paraffin embedding were transferred to fresh 4% paraformaldehyde for an additional 24 hours, and then transferred to 70% ethanol until processed by the Histology Core at the University of Michigan Dental school. Prepared H&E sections were evaluated for evidence of lung injury (see Figure 13 in Model Comparison).

Major Findings: The tool set was created to use flow cytometry to evaluate changes in blood- and lung-associated cell parameters when comparing untreated and SCD treated animals.

Milestones Achieved: 1) Required antibodies and reagents were verified to be porcine specific. 2) LE flow panels were verified to be optimal for assessing LE phenotype and activation levels. 3) Staff developed proficiency in protocols for performing BAL and lung tissue processing.

Subtask 3: Establish LPS dose to induce acceptable degree of ALI

Rationale: Infusion of LPS mimics many of the pathophysiologic changes associated with sepsis and has been shown to result in ALI in experimental animals (32). The development of stable lung injury from LPS is dependent on dosage, time, route of administration and species of model selected. Upon exposure to endotoxin, pigs develop clinical systemic inflammatory response syndrome (SIRS) that may progress to multi-organ dysfunction (MOD) consisting of cardiovascular instability, respiratory insufficiency and renal dysfunction (33). Previously reported LPS doses administered to pigs to induce experimental sepsis and organ injury range from 0.05 to 500 ug/kg with infusion rates and duration of endotoxin exposure varying from 30 minutes to several hours. Many experiments using LPS infusion in pigs are of short duration (<6hr) with rapid onset of organ dysfunction and high mortality due to progressive shock and organ failure. Studies extending beyond 6hr typically utilize uninjured animals and lower doses of LPS resulting in less hemodynamic instability and lower organ injury, however reported indices of pulmonary dysfunction often fall short of meeting the clinical definition of ALI/ARDS. The protocols described by Wilson et al. using previously traumatized and resuscitated pigs resulted in profound pulmonary hypertension and hypoxemia upon exposure to comparatively lower IV boluses of LPS (<150ug/kg) (34) supporting the premise that trauma predisposes to development of ALI/ARDS. While this published model appears suitable to investigate treatments for posttraumatic ARDS, even at the lowest reported bolus dosing of LPS (0.5ug/kg), rapid mortality was reported with >50% of pigs dying within 5hr of LPS exposure (35). Incrementally increasing the infusion rate of high dose LPS over 2.5hr in uninjured pigs has been shown to result in progressive acute lung injury and a sustained systemic inflammatory response while avoiding severe pulmonary hypertension and right heart dysfunction (36), which was theorized to contribute to the rapid lethality of the LPS infusion model in pigs. We planned to apply this incremental dosing regimen to traumatized pigs to develop a model using LPS to result in progressive ALI/ARDS with survival >12hr.

Empirically increasing dose and rate of LPS infusion was used to determine the optimal LPS dosing regimen with the goal of finding an LPS dosing protocol that reliably induces ALI within 6 hours while maintaining ≥80% survival rate at 12 hours. This temporal onset of ALI and survival time was important for the model to allow for sufficient duration of SCD therapy, which is based upon continuous cell processing activity during inflammation. Fifteen pigs (n=15) were utilized (Pig03-Pig17) for model development.

Eight studies (Pig03 through Pig10) were performed using the original animal model as described by Wilson *et al.* In brief, following the trauma, hemorrhage and resuscitation procedures on Day 0, pigs were recovered from anesthesia and returned to standard housing and care. On Day 3, pigs were re-anaesthetized and administered LPS to induce a systemic inflammatory response and ALI (Model 1). Three animals died prior to receiving LPS infusion; of these, Pig04 died during hemorrhagic shock and Pig05 and Pig06 died during the recovery from anesthesia. Respiratory complications were consistently encountered during recovery from anesthesia in this group of animals (Model 1). Pulmonary edema fluid was observed in both Pig05 and Pig06 at necropsy and hypoxemia secondary to pulmonary edema was suspected to be the cause of death as no other abnormalities were identified (Note: Pig06 was necropsied by UM Unit of Laboratory Animal Medicine veterinary pathologists). Fluid overload is a potential cause of flash pulmonary edema and while there was no other clinical evidence that the pigs were overloaded, the fluid resuscitation of the hemorrhagic shock was reduced to a 10-20 ml/kg bolus followed by slow infusion at 3mL/kg/hr for the next several animals in an attempt to avoid this fatal complication. Even with reduced fluid administration, complications were still encountered, including

prolonged time to extubation, respiratory distress upon extubation and hypoxemia. No additional pigs died during the anesthetic recovery period, however, intensive nursing care was often required for to 12-24 hours following trauma. These recovery complications prompted an official veterinary recommendation by the local attending veterinarian, Dr. Melissa Dyson, to try to refine the experimental protocol by combining the trauma procedure and the LPS administration to occur under a single anesthetic episode (see Appendix 1). Experiments using Pig11 through Pig13 were conducted under this recommendation. In brief, pigs were placed under general anesthesia and underwent the trauma, hemorrhage and resuscitation procedure as previously described. Pigs were then maintained under anesthesia in the operating theater for the remainder of the experimental period. Single dosage LPS infusion at varying doses, rates and durations was then investigated in this condensed model (Model 2). Pig11 received LPS the same day as the trauma, which was associated with immediate cardiovascular instability and death within 2 hours from the start of LPS infusion. For Pig12, LPS infusion was administered the day after trauma and found to successfully induce pulmonary dysfunction with survival to the study end point. This day-after-trauma LPS dosing protocol was repeated for Pig13, but this animal also experienced immediate cardiovascular compromise similar to that observed in Pig11. Using extensive resuscitative measures to prevent death, a 12-hour survival was achieved for Pig13. The degree of hemodynamic instability observed in 2 of the 3 pigs under the Model 2 LPS protocol would not be ideal for the intended model, prompting evaluation of a third dosing strategy. The veterinary recommendation to combine the trauma and LPS procedures under a single anesthetic episode was still followed in Pig studies 14-17. For these pigs, a priming dose of very low concentration LPS overnight post trauma was followed by a bolus of LPS the next day (Model 3). This strategy was based on recent publications describing LPS tolerance in pigs (37,38), a phenomenon which prevented cardiovascular collapse on exposure to subsequent high doses of LPS but exacerbated lung injury (37).

A table summarizing the strategy for each of the study animals is presented in Table 5. For clarity Model 1, 2 and 3 are designated using the colors black, red, and green respectively on graphs and figures. The pilot animals (Pig01 and Pig02) are included as no ALI controls where appropriate.

Models 1, 2 and 3 were compared to assess strengths and weakness of each, particularly the timing of onset of ALI (based on Pa:FiO2 $\leq$300) and the degree of lung injury created in each model (using BALf, lung edema, and histological changes). For graphs, data is expressed as the average ± standard error.

Table 5. Summary of Pig Studies FY01 used in Model Development

PIG ID#	Hemorrhage Notes	Resuscitation Notes	LPS treatment Day (from trauma)	LPS Strategy	Response to LPS	study END point	Signs of ALI	Model for DATA	
Pig01	35% in 30min	60ml/kg over 30min	N/A	N/A	-	-	N/A	**Control (no LPS)**	**Control**
Pig02	35% in 20min	60ml/kg over 30min	N/A	N/A	-	-	N/A	**Control (no LPS)**	
Pig03	35% in 30min	60ml/kg over 30min	3 days	0.5 ug/kg over 90 min then increased rate 1 ug/kg/hr total of 2.2mcg/kg over 3hrs	Minial hemodynamic respsone. Pa:FiO2 remained >400	12hr	NO	**use for data**	**Model 1**
Pig04	35% in 30min	_	N/A	N/A	N/A	-	-	**DIED**	
Pig05	35% in 30min	60ml/kg over 30min	N/A	N/A	N/A	-	-	**DIED**	
Pig06	35% in 30min	60ml/kg over 30min	N/A	N/A	N/A	-	-	**DIED**	
Pig07	35% in 30min	250ml maintenance fluids at 3 ml/kg/hr	3 days	0.5 ug/kg/hr then increased rate to effect Max rate = 33ug/kg/hr. Total of 30 ug/kg over2hrs	Immediate cardiovascular collapse but stabilized and then no increase in PA pressure. PaFiO2 remained >400	12hr	NO	**use for data**	
Pig08	35% in 30+min	500ml Maintenance 3ml/kg/hr	3 days	1ug/kg/hr then increased rate to effect Max rate = 8 ug/kg/hr. Total of 24ug/kg over 6hrs	PA pressures maintained in 40's. Fi:PaO2 ratio <300 at 4 hr	12hr	YES	**use for data**	
Pig09	35% in 30min	1000ml Maintenance 3ml/kg/hr	3 days	1ug/kg/hr then increased rate to effect Max rate = 15 ug/kg/hr Total of 42ug/kg over 6hrs	PA pressures maintained in 40's. Fi:PaO2 ratio <400 at 3 hr but never <300	12hr	YES	**use for data**	
Pig10	35% in 30+min	500mL (NaHCO3 added) Maintenance fluids 3ml/kg/hr	3 days	15ug/kg in 30min	Sharp rise in PA pressure and CV collapse but managed. Pa:FiO2 <300 in 30minutes	12hr	YES	**use for data**	
Pig11	35% in 30min	500mL (NaHCO3 added) Maintenance fluids 3ml/kg/hr	same day as trauma	15ug/kg in 30min	Sharp rise in PA pressure with progressive CV collapse leading to death. Pa:FiO2 <300 in 45 minutes	died at 2hr	-	**DIED**	**Model 2**
Pig12	35% in 30 min	500mL fluids (NaHCO3 added) Maintenance fluids 3ml/kg/hr	1 day	15ug/kg in 30 min repeated 3x for a total of 45ug/kg over 1.25 hours	Sharp rise in PA pressure but stable, Repeated LPS bolus because kept normalizing Pa:FiO2 <200 in 1 hour	12hr	YES	**use for DATA**	
Pig13	35% in 30min	1000mL (NaHCO3 added) Maintenance fluids 3ml/kg/hr	1 day	15 ug/kg in 30 min, then continued increasing infusion based on PA pressures, Total of 45ug/kg over 1.25 hrs	Sharp rise in PA pressures with progressive CV collapse. Able to prevent death with ER management Pa:FiO2 < 300 in 1 hour	22 hr	YES	**use for DATA**	
Pig14	35% in 30min	1000mL (NaHCO3 added) Maintenance fluids 3-5ml/kg/hr	low dose started Day 0 ARDS dose Day 1	Ultra low LPS dose at 0.063ug/kg/hr overnight starting 6hrs post trauma Day 1 = 4 ug/kg/kr x 6hr	Minimal respsones No change in Pa:FiO2	24hr	NO	**NO RESPONSE TO LPS**	**Model 3**
Pig15	45% in 30min	Aggressive resucitation plan (60ml/kg + addedNaHCO3) maintenance 10ml/kg/hr thereafter	low dose started Day 0 ARDS high dose day 1	Ultra low LPS dose at 0.063ug/kg/hr overnight starting 6hrs post trauma Day 1 = 15ug/kg/hr, increasing rate to get response. Max rate = 60ug/kg/hr maintained at 60 ug/kg/hr over 6hr	Mild hemodynamic respsones. Pa:FiO2 <300 in 1 hour	24hr	YES	**use for DATA**	
Pig 16	45% in 30min	Aggressive resucitation plan (60ml/kg + addedNaHCO3) maintenance 10ml/kg/hr thereafter	low dose started Day 0 ARDS high dose day 1	Ultra low LPS dose at 0.063ug/kg/hr overnight starting 6hrs post trauma Day 1 = 15 ug/kg/hr, increasing rate to get response. Max rate= 277 ug/kg/hr, maintained at 65 ug/kg/hr x 12hr	Mild hemodynmic respsonses initally but cardiovascualar instability with prolonged high LPS rates. Pa:FiO2 = 300 at 4 hour	24hr	YES	**use for DATA**	
Pig17	33% then pig arrested so stopped early	Aggressive resucitation plan (60ml/kg + addedNaHCO3) maintenance 10ml/kg/hr thereafter	low dose started Day 0 ARDS high dose day 1	Ultra low LPS dose at 0.063ug/kg/hr overnight starting 6hrs post trauma Day 1 = 15ug/kg/hr, increased rate to get response. Max rate =115ug/kg/hr maintained at 65ug/kg/hr x 6hr	Mild hemodynamic respsones. Pa:FiO2 <300 at 2 hr	died at 4hr	-	**use for DATA (no lung, BALf)**	

Assessment of Cardiovascular Parameters: Cardiovascular instability was an important factor in the optimization of the LPS dosing strategy. As anticipated, bolus administration of LPS caused acute pulmonary hypertension, which in some pigs resulted in right heart dysfunction and cardiovascular collapse. Hemodynamics were closely monitored to allow timely intervention as well as identification of patterns that may be associated with each LPS dose. A summary of key hemodynamic indices over time is depicted in Figure 11. Heart rate (HR-panel A), cardiac output (CO-panel B), mean arterial pressure (MAP-panel C) and mean pulmonary artery pressure (PAP-Panel D) followed a very consistent pattern in all pigs over the course of Hit 1 signaling the reproducibility of our trauma and resuscitation protocol. These parameters were also similar between groups immediately before beginning infusion of LPS (pre LPS), except for MAP which was higher for models 2 and 3, (maybe due to stability of being maintained under anesthesia) but all pigs were in the normal range at this study timepoint. Different hemodynamic patterns emerged upon administration of LPS using the 3 different dosing strategies. As could be expected, at the lowest doses/rates of LPS, which started at 0.05-1 µg/kg/hr and were administered to pigs in Model 1, the hemodynamic changes were gradual and were less dynamic than seen in the other models. For Model 1, all indices, including PAP stayed within the normal range over 12 hours. A 15-fold higher starting dose of LPS (15/µg/kg) was delivered as a bolus to pigs in Model 2. Similar to reported literature, this strategy caused rapid and sometimes profound alterations in cardiovascular parameters that are clearly evident within 1-2 hours of starting LPS. The most notable of these being PAP, which nearly doubled from 24 up to 40 mmHg within 1 hour. This increased PAP was associated with an increase in HR, consistent with a decrease in stroke volume as the right ventricle struggles to eject against this sudden pulmonary hypertension. The increased PAP persisted for several hours and resulted in cardiovascular instability in 2 of the 3 animals which is evident in the plotted averages for the HR, CO and MAP for Model 2 (Figure 11, red lines).

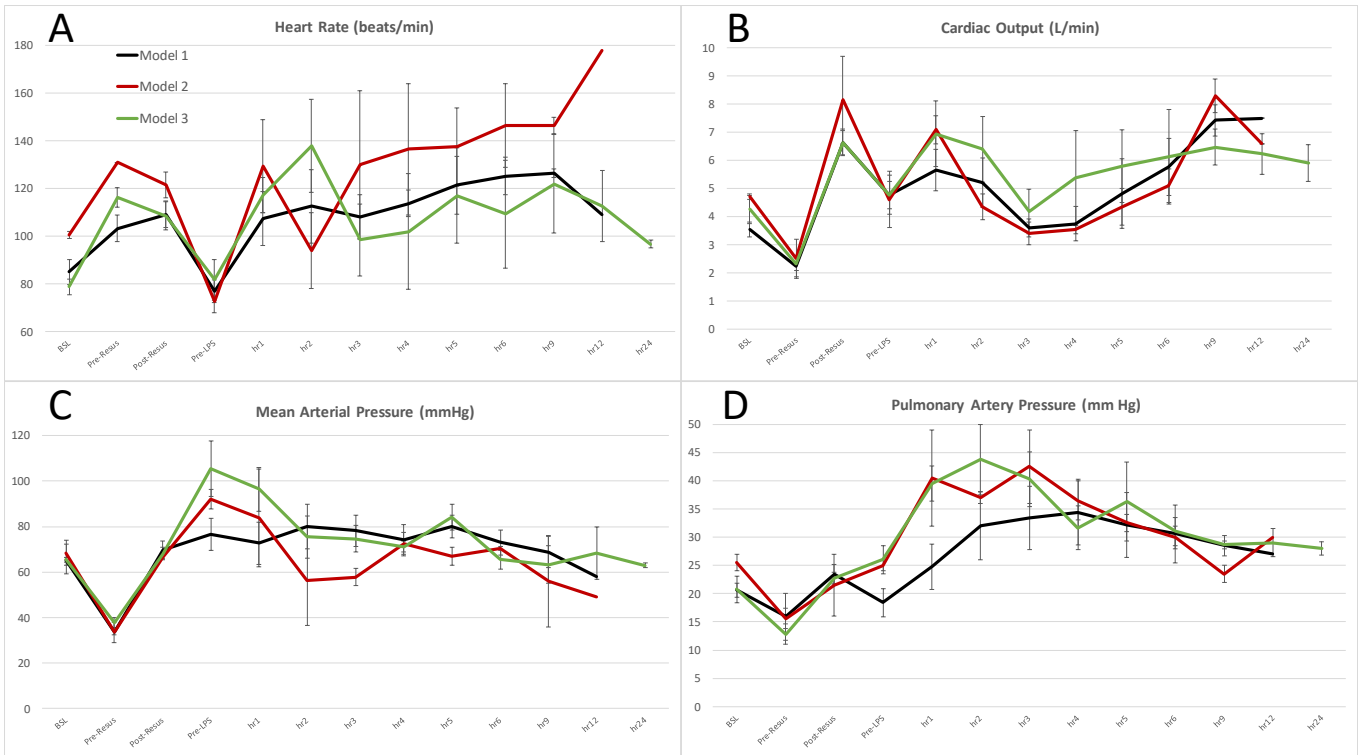


Figure 11. Comparison of hemodynamic Indices for ALI Models. Averages ± SE are shown for heart rate (A), cardiac output (B), mean arterial pressure (C), and pulmonary artery pressure (D).

Pigs in Model 3 received a priming dose of LPS (0.063 µg/kg/hr) overnight following trauma to induce LPS tolerance prior to receiving the "2nd Hit" with a high LPS bolus (starting at 4-15 µg/kg/hr). This strategy was intended to prevent/minimize the cardiovascular instability seen with Model 2 and this is, in fact, what was observed. Pig014 received LPS at 4µg/kg/hr and had no observable response to LPS. For subsequent animals a higher bolus of LPS was used (starting at 15 µg/kg/hr) and rate was titrated to PAP. Both PAP and HR did increase with exposure to high dose LPS but these increases were more gradual over the first hour (although this is not visible in the graph). More importantly, however, these changes were not associated with cardiovascular collapse in any of these pigs in Model 3. HR, MAP and CO were more stably maintained in these animals over 24 hours of study. The elevations in PAP were similar to that seen in Model 2 and this hemodynamic alteration is a desirable part of the ALI model, as it is reflective of pulmonary vascular dysfunction and contributes to precipitating the lung injury. Thus, inducing tolerance with very low dose LPS met the intended goal of inducing cardiovascular stability without compromising the ability to induce pulmonary dysfunction.

Assessment of lung injury: Per study protocol, pigs were mechanically ventilated while they were maintained under anesthesia. Ventilation was standardized to tidal volume (TV)=10ml/kg, positive end expiratory pressure (PEEP) = 5cmH20 and respiratory rate (RR) adjusted to maintain PaCO2 40-45mmHg. The fraction of inspired oxygen (FiO2) was adjusted to maintain a hemoglobin saturation >90% based on pulse oximetry. Arterial blood gas tensions were measured hourly. The ratio of the partial pressure arterial (Pa) oxygen to FiO2 is a widely used clinical indicator of significant pulmonary dysfunction in transfer of oxygen from the air to the blood. The Pa:FiO2 is used as part of the Berlin definition of ARDS (Pa:FiO2 <300mmHg in mechanically ventilated patients at a PEEP of 5 cmH2O) and correlates with severity and mortality for this disease. All pigs had a Pa:FiO2 > 400 at Baseline (BSL). The Pa:FiO2 was decreased after trauma (Pre-Resus) and resuscitation (Post-Resus), consistent with pulmonary dysfunction resulting from this modeled scenario. For Model 1 and Model 2, the average Pa:FiO2 had been restored to BSL values at the time when LPS administration was to commence (Pre LPS). In Model 3, using an ultra-low dose infusion of LPS (0.063ug/kg/hr) after trauma to induce LPS tolerance, the Pa:FiO2 remained below the BSL value. Pa:FiO2 continued to decrease with bolus administration of LPS in Model 3, falling below 300 by hour 2 and then remaining close to this critical value. LPS administration resulted in a rapid decrease in Pa:FiO2 to 200 in Model 2 which was followed by a recovery and then terminal decline that mirrored the hemodynamic instability that was also seen with this model. Decreases in Pa:FiO2 were not seen in Model 1.

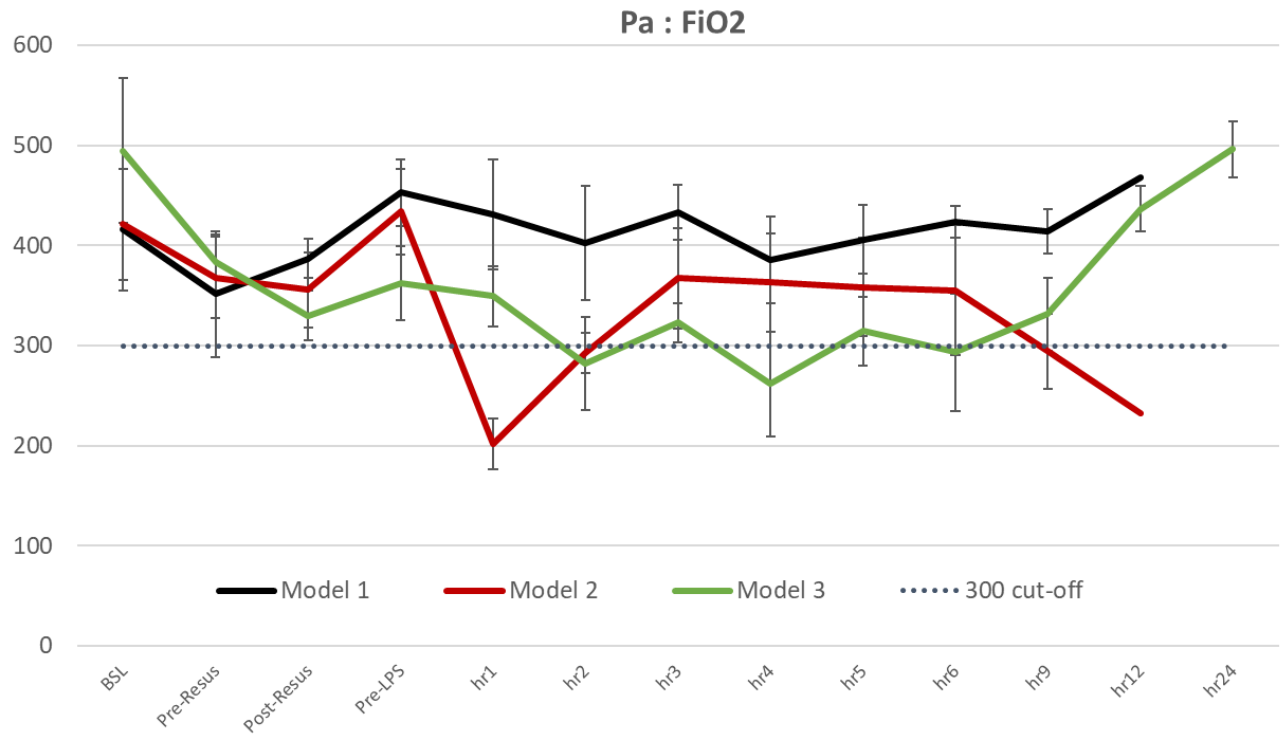


Figure 12. The ratio of partial pressure arterial (Pa) oxygen and fraction of inspired (Fi) oxygen (O2) is a comparison between the oxygen level in the blood and the oxygen concentration that is inhaled and used to determine the severity of lung dysfunction in transfer of oxygen to the blood. A PaO2/FiO2 ratio less than or equal to 300 defines a diagnosis of acute lung injury (ALI) in human clinical medicine. Model 1 did not reach the criteria for ALI. Model 2 resulted in a sharp decline in Pa:FiO2 which rebounded and was also associated with cardiovascular instability. Model 3 developed a gradual decrease of Pa:FiO2 with the ratio dropping below the 300 cut-off within 2 hours. *Note: the increase in PA:FiO2 after hour 9 in Model 3 was related to adjustments in the ventilation settings performed while developing treatment algorithms using the animals that were studied past the 12 hour timepoint (n=2). Settings were standardized in subsequent studies.*

Pulmonary compliance, a measurement of the ability of the lung to expand during each breath serves as an indicator tissue elasticity. Low compliance indicates a stiff lung in which extra work is required to exchange air and may result from infiltration of edema fluid and inflammatory cells or from fibrosis. Dynamic compliance (C_{dyn}) can be estimated using readings from the ventilator and is calculated as:

C_{dyn} = Tidal Volume / (Peak Inspiratory Pressure – Positive End Expiratory Pressure)

Ventilator settings in all pig studies were recorded every 15 minutes and used to calculate C_{dyn} hourly. Overall, a decrease in C_{dyn} was observed over time with each of the models but the calculated value was found to be strongly influenced by other ventilator settings that were not standardized, including respiratory rate and fresh gas flow. Operator preference and animal-to-animal variation in these requirements prevented meaningful interpretation of C_{dyn} during these model development studies. Predetermined key study timepoints were identified for obtaining measurements for C_{dyn} and a strict set of ventilator settings appropriate for measurement of C_{dyn} were identified. Ventilator settings were adjusted to these standardized parameters at each designated timepoint and after a few minutes of equilibration the measurements for C_{dyn} were recorded. Settings were then returned to original ventilation pattern for that animal allowing collection of C_{dyn} data with minimal compromise to the animal or alteration of the course of ALI, allowing for comparisons of this pulmonary parameter among treatment cohorts.

Lungs were harvested to permit grading of histologic injury, quantitation of LE infiltration and edema at the conclusion of each study. The pathological hallmark of ALI is diffuse alveolar damage (DAD) (39). In humans, DAD is characterized by neutrophil accumulation in the vascular, interstitial, and alveolar spaces (neutrophilic alveolitis); deposition of basement membranes as evidence that serum proteins have entered and precipitated in the airspaces (i.e., disruption of the alveolocapillary membrane); interstitial thickening; and formation of microthrombi. Representative samples from each right diaphragmatic lobe were fixed in 4% PFA, paraffin embedded and stained with hematoxylin and eosin (H&E). Morphometric evaluation of pathology can then be performed based on alveolar wall thickness, interstitial edema and infiltration of inflammatory cells. Histologic evidence of ALI was observed with each of the three models. The qualitative pathologic changes were mild for Model 1, with only slight venous congestion, septal thickening and few infiltrating inflammatory cells. More severe changes, including septal thickening and vascular congestion were prominent with Model 2 and Model 3 and both of these models demonstrated inflammatory cell infiltration into the interstitium and alveolar space (Figure 13). Quantitative analysis of pathology was not done during model development, but the sample processing proved adequate for this technique which was later used in assessment of SCD therapy in Specific Aim 2.

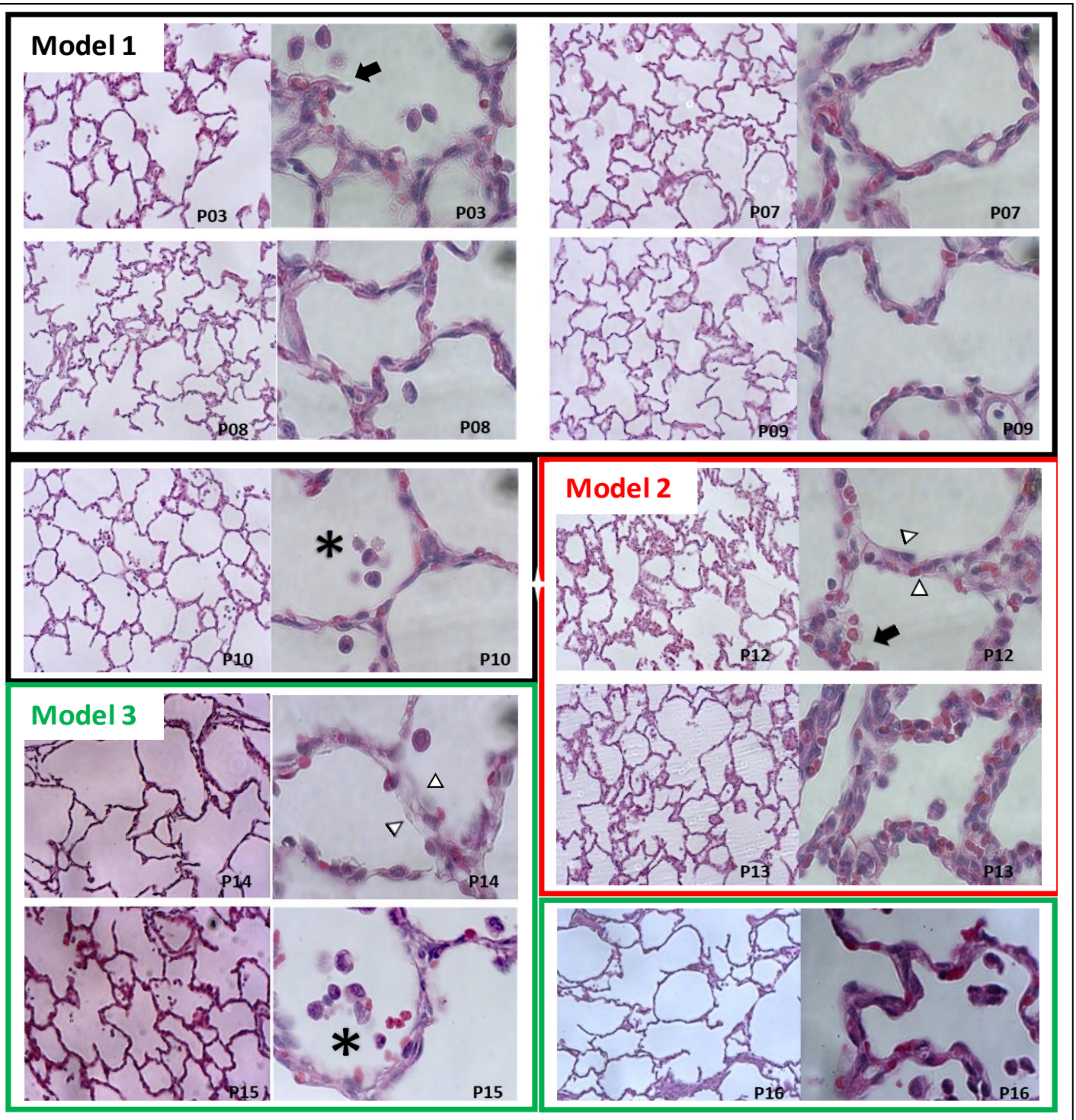


Figure 13. Representative hematoxylin and eosin (H&E) stained sections from paraformaldehyde fixed and paraffin embedded lung tissue generated from ALI model pigs. Models are designated by colored borders. Histologic evidence of diffuse alveolar damage, including vascular congestion (black arrow), septal thickening (white arrow heads), and barrier disruption with accumulation of inflammatory cells (*) in the alveolar spaces is observed in most specimens and appeared to be more severe in models 2 and 3.

BALf was obtained *post mortem* by the repeated instillation of saline supplemented with 0.2% EDTA into the right middle bronchus. In ARDS patients, the concentration of neutrophils in the BALF correlates with severity of ARDS and outcome (40, 41). Total cell counts and differentials, specifically for neutrophils relative to total counts, were determined from cytospins. Representative images are shown in Figure 14 . BALf obtained immediately after resuscitation of the practice pigs used in Subtask 1 (no ALI control) each contained <3% neutrophils, while a higher percentage of neutrophils was found in the BALf in each of the three ALI models. Direct comparisons of neutrophil % cannot be made since BALf was collected at 12 hours from pigs in Model 1 and 2 and at 24 hours in Model 3 but the high % neutrophils indicates that progressive neutrophilic alveolitis is a prominent feature of the lung injury with this model (Figure 15, left).

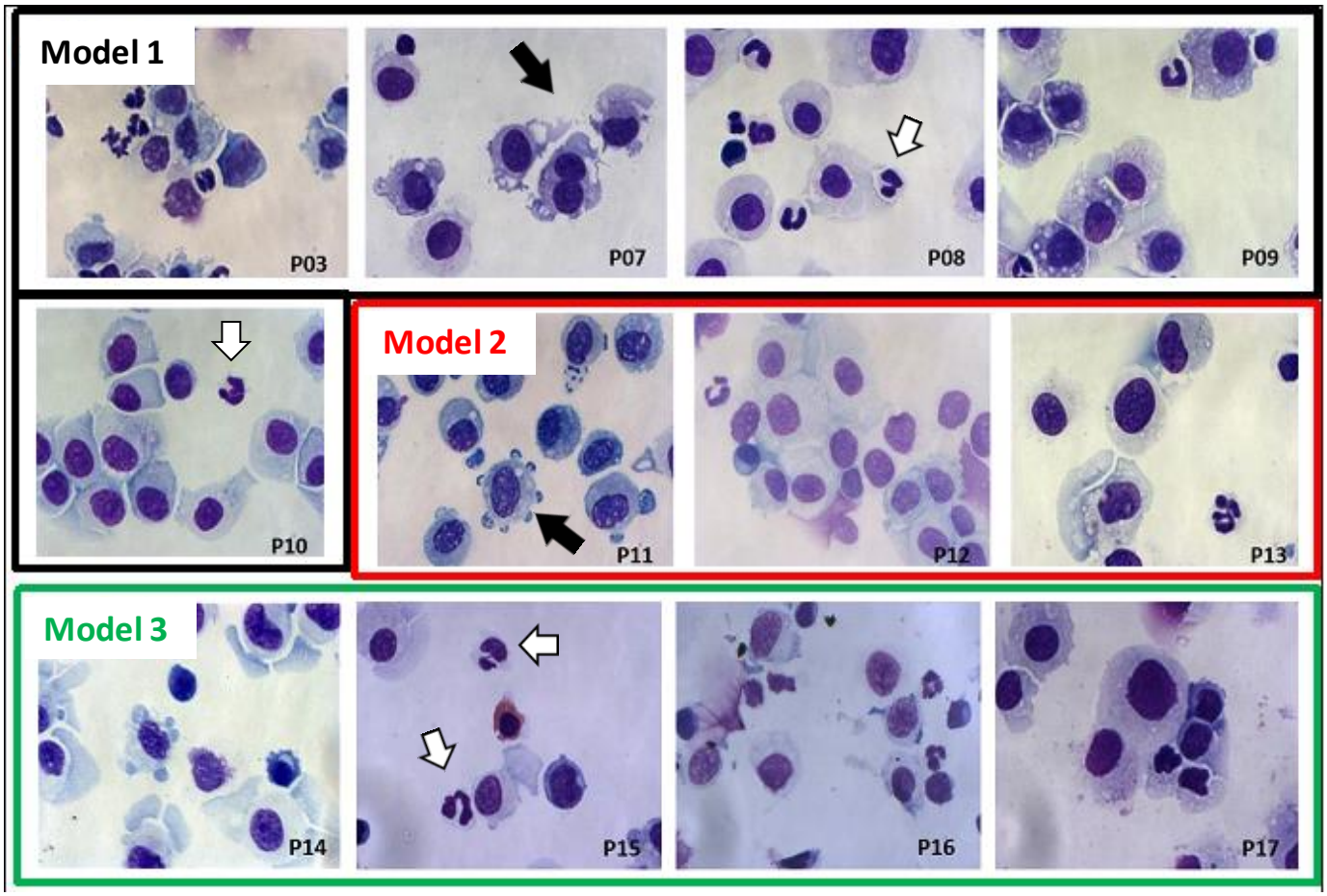


Figure 14. Cytospins from bronchioalveolar lavage fluid (BALf). The representative fields shown were obtained using an oil immersion 63x objective and phase contrast. Models are designated by colored borders. The predominant cell types identified in the BALf are the alveolar macrophage (black arrow) and neutrophil (white arrow). An increased percentage of neutrophils in the BALf is consistent with ALI as these inflammatory cells migrate into the alveolar spaces.

To further estimate the degree of tissue injury, pulmonary edema of excised lungs was quantified by water content using a wet:dry weight ratios (32). Water content was increased compared to the non ALI controls in each of the 3 ALI models (Figure 15, right). An increase in water content is expected with the development of pulmonary edema during ALI.

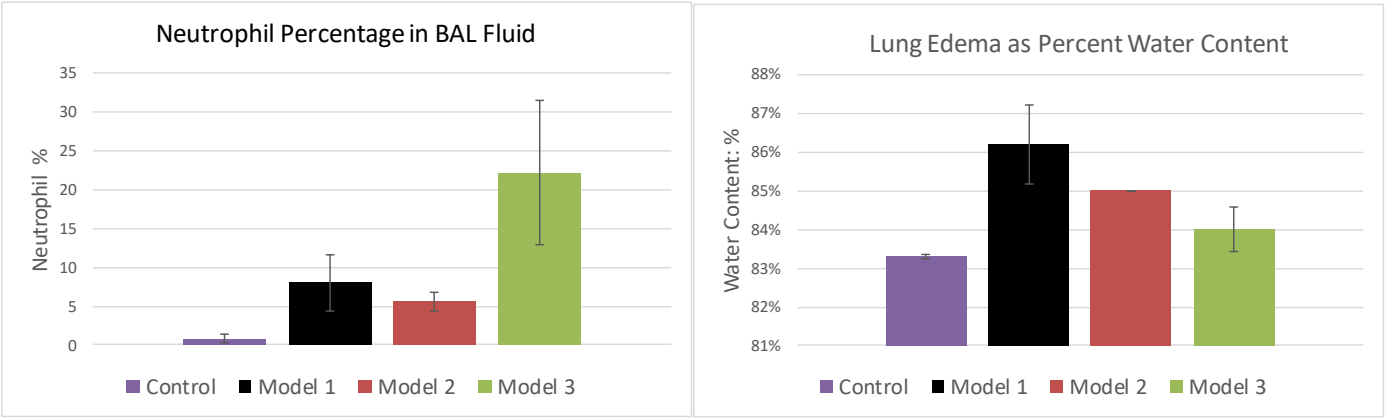


Figure 15. Acute lung injury (ALI) in models 1-3 was evidenced by the presence of neutrophils in bronchioalveolar lavage (BAL) fluid (Left) and edema (Right). Pilot animals terminated post resuscitation with no signs of acute lung injury (ALI) are included as no ALI controls for comparison.

Evaluation of Immunologic Parameters: Inflammation is a key component in the pathophysiology of ALI. With trauma and resuscitation (Hit 1) a pronounced systemic inflammatory response was expected that would prime and activate cells of the innate immune system disrupting immunologic homeostasis. With the high dose LPS injection, (Hit 2) a further inflammatory response was expected, whereby the previously primed leukocytes react beyond what is required for normal host defense, exacerbating tissue damage and leading to a malignant inflammatory state which causes multi-organ injury. Blood was collected and evaluated for complete blood counts using a HemaVet® analyzer and cell differentials were evaluated manually to accurately assess immature forms. As described in the development of leukocyte evaluation parameters, for model development, CD11R3 was chosen as a representative marker of cell activation to evaluate the dose of LPS essential to model development. Neutrophils (14, 15) and monocytes (16, 17) mobilize intracellular stores of CD11R3 to the cell surface as they become (primed) activated, allowing a real-time measurement of systemic acute neutrophil (priming) and monocyte activation.

Graphs of selected blood count parameters: white blood cells, neutrophils and the immature neutrophil subset are shown in Figure 16. For all models, Hit 1 caused a rapid increase in the circulating number of total neutrophils post resuscitation compared to baseline (BSL), even though overall white blood cell count remained stable. Administration of LPS as Hit 2 caused a profound decrease in all circulating white blood cells followed by a progressive influx of immature neutrophils. Interestingly, this leukocyte response to LPS was similar in all models and therefore not directly correlated to LPS dose. Of note, immature neutrophils were not detected at baseline, but were detected in all models post resuscitation, indicating an inflammatory response from Hit 1. This early neutrophil response was slightly more pronounced in pigs in Model 3, likely from the additional exposure to low dose LPS early post trauma. This modeled situation does closely mimic a clinical scenario in that open traumatic wounds would be exposed to LPS from environmental contamination. The influence of SCD therapy on the influx of immature neutrophils in the developed ALI model will be especially noteworthy. The homeostasis of neutrophil counts is maintained by a carefully detailed cytokine axis involving IL-17, IL-23 and G-CSF, as detailed in recent reviews (42-44). Pre-clinical animal models have suggested that SCD normalizes the apoptotic life span of NE down from the inflammatory promoted lifespan increase to greater than 24 hours, down towards a normal lifespan of 4-6 hours. This normalization is reflected in the decline in bone marrow release of immature NE and reduction of elevated NE counts during inflammation (5, 6, 45).

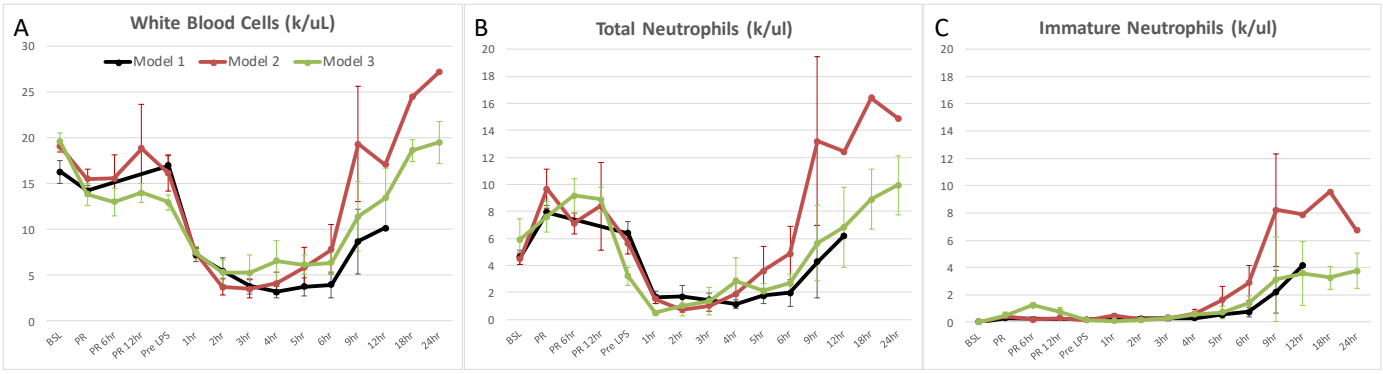


Figure 16. Leukocyte response in the "2 Hit' porcine model of ALI. Trauma with hemorrhagic shock and resuscitation (Hit 1) caused a rapid increase in the circulating number of total neutrophils (B) even though overall white blood cell count remained stable (A). Administration of LPS (Hit 2) then caused a profound decrease in all white blood cells (A) (including lymphocytes and monocytes which are not pictured) over the next serval hours followed by a progressive influx of neutrophils (B), particularly immature forms (C) consistent with an inflammatory response.

CD11R3 expression was measured as an indicator of acute activation on neutrophils. Acute activation was not detected post initial trauma and resuscitation (PR) in any model with 9 of the 10 animals used for model comparison exhibiting a relative decrease in CD11R3 expression. For Model 3, the priming dose of LPS (0.063µg/kg/hr) did result in a detectable change in CD11R3 expression that increased significantly from 12hr post resuscitation to the Hit 2, large LPS dose time point (designated "Pre LPS" on graph). For all models, a significant increase in CD11R3 was observed in response to large dose LPS infusion. This response was more pronounced with the higher doses of LPS used in Models 2 and 3. The rapid increase in CD11R3 was correlative to the rapid decrease in circulating neutrophils, the major contributor to the overall decrease in white blood cells. This finding is likely explained by margination and extravasation of activated cells into tissues. Total white cell counts rebounded between 4-6 hours for which immature neutrophils were a major contributor, suggesting recruitment of cells from the bone marrow. These cells could be visualized in a bi-modal histogram in the CD11R3 FL1 channel, with immature neutrophils having lower CD11R3 expression. CD11R3 expression in circulating neutrophils decreased to near baseline levels for Model 2 and 3 by 9 hours, but remained elevated for Model 1 (Figure 17).

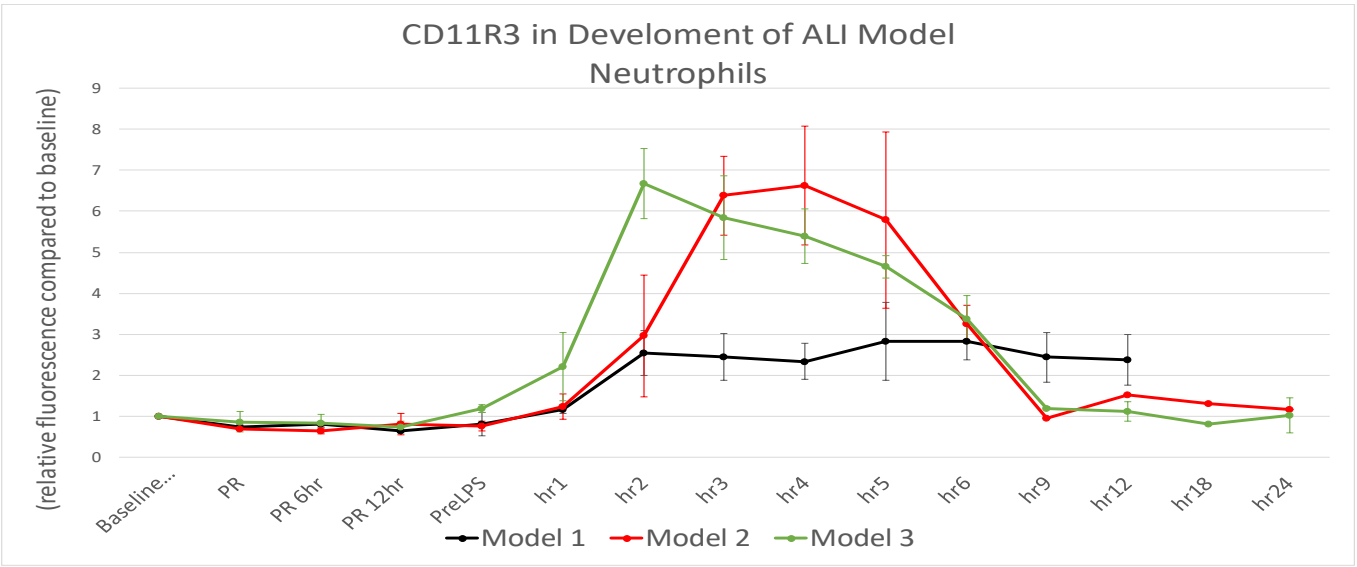


Figure 17. CD11R3 expression was measured as an indicator of acute activation on neutrophils. Acute activation was not detected post initial trauma and resuscitation (PR), but increase was detected in association with large dose lipopolysaccharide (LPS) infusion. This increase in neutrophil activation was more pronounced with the higher doses of LPS used in Model 2 and 3.

Major Findings:

● To avoid unmanageable respiratory compromise during the post trauma period, it was necessary to refine the model to maintain the animal under anesthesia for the duration of the experiment with trauma performed on Day 0 and LPS infusion for development of ALI performed on Day 1 (Models 2 and 3).

● Model 3 was found to avoid acute cardiovascular compromise from LPS administration, by using a low exposure priming dose (0.063 µg/kg/hr) for 12 hours followed by a higher LPS exposure (15+ µg/kg/hr) titrated to induce a pulmonary artery pressure of 45-50 mmHg for 6 hours.

● Model 3 consistently resulted in a Pa: Fi O2 ≤ 300 within 6 hours of the starting the high dose LPS.

● Pigs developed several key pathologic features of ALI. BALf contained an increased percentage of neutrophils and lung water content was increased at postmortem. Histologic abnormalities of diffuse alveolar damage were recognized, including septal thickening, interstitial accumulation of inflammatory cells and extravasation of neutrophils into the alveolar space.

● An inflammatory response to trauma and to LPS was evidenced by changes in leukocyte activation levels (CD11R3) and in leukocyte counts (notably neutrophils).

● In total, 10/12 pigs receiving LPS survived to 12 hours (83% survival), a necessary feature of the model to enable evaluation of sufficient duration of SCD therapy.

Milestones Achieved: 1) LPS dose induced ALI, as defined by Pa:FIO2<300, within 6 hours of LPS infusion start time. 2) 12 hour survival rate was ≥ 80%.

- **Major Task 3: Verify reproducibility of two-hit porcine ARDS model up to 24 hr ARDS time course.**

Subtask 1: Repeat study design determined in Aim1 /Major Task 2/Subtask 3 and monitor pigs for up to 24 hrs or until death, whichever occurs first.

As 12 hour survival was readily achievable with the LPS dosing strategies that were being utilized under Subtask 2, determining the feasibility of maintaining pigs beyond this timeframe was able to begin earlier than anticipated and started with Pig13. Using the optimal LPS dosing regimen (Model 3) determined in completion of Major task 2: subtask 3, a total of twelve pig studies (Pig13 – Pig24) were conducted over the remainder of FY01 and Q1 of FY02 under the goal of monitoring pigs for up to 24 hours from the start of LPS infusion or until death to finalize and validate a study protocol for use in Specific Aim 2.

The first few of these 24hr experiments, were used to optimize supportive care strategies and treatment algorithms were developed. As anticipated, LPS doses sufficient to induce ALI also resulted in injury and dysfunction of other organ systems. Algorithms were created for fluid administration, ventilation settings, use of vasopressors for cardiovascular support and for management of renal failure. The original study was to maintain committed preset ventilation settings in order to make direct comparisons of lung function between treatment cohorts. It became apparent that some adjustment in ventilatory settings during the experiment as pigs developed lung dysfunction was necessary for survival to 24 hours. A protocol to make predetermined adjustments based on measured parameters (PaO_2, $PaCO_2$, peak airway pressure) was developed and tested. Adjustment in ventilator settings more closely replicates the clinical scenario and use of a predetermined ventilation protocol should limit caregiver bias, thereby still allowing for comparisons of respiratory indices

between treatment cohorts. Adherence to the developed protocol prevented lethal aberrations in blood gas values that were seen in early studies when ventilator settings were kept static. A replacement fluid rate of 10 ml/kg/hr was found to maintain most animals but hypotension in Pig16 required treatment with vasopressors. Therefore, a protocol for administration of cardiovascular support using a fluid regimen and administration of phenylephrine and noradrenaline for animals with hypotension or low cardiac output was established to avoid early death from cardiovascular collapse. The treatment algorithm for fluids, medications and ventilator adjustments is presented in Table 6.

Table 6. Treatment Algorithm

	Parameter	Normal Range	Threshold values for intervention	Intervention
iStat values	**pH**	**7.35-7.50**	<7.25	1. Administer 50 ml of 8.4% bicarbonate IV over 5 minutes 2. if remains <7.3 then call immediately.
	PaO2	**>90-140 mmHg**	<90 mmHg	Increase FI O2 by 10 (i.e. from 31% to 41%) by increasing amount of delivered O2.
			>147 mmHg or >135 mmHg for 1hr	1. If PEEP >5 cmH2O, decrease PEEP by 2, lowest possible PEEP=5. 2. Decrease FI O2 5, lowest possible FI O2 =30%
	PaCO2	**35-45 mmHg**	<33 mmHg or <35 mmHg for 1h	Decrease TV by 10%.
			>50 mmHg or >47 mmHg for 1 hr	1. Increase RR by 2-3 breaths/min (may repeat) 2. Increase TV 10% up to a maximum of 15 mL/kg. 3. Call if CO2 remains high.
	Potassium (K+) *Must have urine output to give K+! *Monitor HR while giving K+!	**3.5-5.5 mEq/L** *too high can lead to arrythmias	<3.0 mEq/L	Add potassium chloride (KCl) to RF: 2 mEq/100 mL of RF - DO NOT BOLUS IV!
			6.1-6.5 mEq/L	Administer 50 mL IV bolus of Sodium Bicarb over 5 minutes. Recheck at next timepoint.
			6.6-7.5 mEq/L	Administer 50 mL sodium bicarb IV blous over 5 minutes and administer 20 mL 50% dextrose IV bolus over 5 minutes. Recheck in one hour.
			>7.5 mEq/L	Administer 10 mL calcium gluconate IV bolus over 5 minutes, 20 mL 50% dextrose IV bolus over 5 minutes, and 10 units of insulin IV over 1 minute. Recheck in one hour. Call if no respsone or arrythmias are noted!
	ionized calcium (iCa)	**0.95-1.45 mmol/L**	<0.95 mmol/L	1. Administer 1 x 10 mL vial of calcium chloride 10% IV bolus over 10 minutes. Recheck blood iCa level in 1 hour. 2. If still <0.95, add 1 mL CaCl2/100 mL RF to infusing RF.
	Bicarbonate (HCO3)	**22-30 mmol/L**	<20 mmol/L	Administer 25 mL of 8.4% sodium bicarbonate solution slow IV bolus over 10 minutes. Also, add 5 mL of 8.4% sodium bicarbonate solution /100 mL infusing RF. Recheck blood bicarbonate level in 1 hour.
	Glucose	**60-125 mg/dL**	<50 mg/dL	Give 20 mL 50% dextrose IV bolus over 5 minutes. Start IV infusion of 50% dextrose at 50 mL/hr or increase infusion by 20 mL/hr. Recheck in 1 hour.
			50-60 mg/dL	Give 10 ml 50% dextrose IV bolus over 5 minutes (if bag not running). Start IV infusion of 50% dextrose at 40 mL/hr or increase infusion by 15 mL/hr. Recheck in 1 hour.
			60-70 mg/dL	Start IV infusion of 50% dextrose at 20 mL/hr or increase by 10 mL/hr. Recheck in 1 hr.
			90-149 mg/dL	Decrease IV infusion of 50% dextrose by 10 mL/hr. Recheck in 1 hour.
			>150 mg/dL mmol/L or >130 mg/dL for 1 hr	Discontinue IV infusion of dextrose. Recheck in 1 hour.
			>180 mg/dL for 1 hr	Start IV infusion of insulin w/ infusion rate of 1 unit/hr, recheck in 30 min.
Ventilator values	**Pmax & MAP**	**<30 cmH2O**	>40 cmH2O & MAP <50 mmHg	1. Adjust I:E to 1:1. 2. Increase RR to 30 breaths/min and adjust TV to result in increased MV of 10%.
	Pmax	**≤10 cm H2O**	<20 cmH2O	Adjust RR back to 25 breaths/min and I:E to 1:2.
			>30 cmH2O	Increase PEEP to 10 cmH2O.
Patient monitoring values	**MAP**	**70-90 mmHg**	MAP <55 mmHg +/- CO <2.0 L/min	1. 5 mL/kg Hetastarch bolus 2. 5 mL/kg Hetastarch bolus (total possible fluid bolus 10 mL/kg/hr).
			<50 mmHg or <55 mmHg after max fluid bolus	Start norepinephrine IV at 0.03 ug/kg/min w/ initial bolus of 0.1mg; if MAP <50 mmHg after 5 min, double infusion rate.
			>100 mmHg	If norepinephrine infusion, decrease rate 10% every 5 min. Turn down RF rate by 25%.
	Core temperature	**38-39°C**	<38°C	Cover animal w/ blankets/blue pads and/or hot water blanket.
			38-39.9°C	Turn off active heating blankets, remove coverings
			39.5-39.9°C	Turn on fan or turn on cooling blanket.
			>40°C	Add ice to cooling blanket or ice packs around pig and turn on fan.
	***If interventions are numbered: try intervention #1 first, if no/not enough response, proceed to #2.**			
	Key	Value	description	Location of reading and/or where to adjust
		paO2	partial pressure of oxygen (blood)	iStat
		paCO2	partial pressure of carbon dioxide (blood)	iStat
		MAP	mean arterial pressure	Solar monitor
		Ppeak	maximum positive-end expired pressure	ventilator
		FI O2	fraction of inspired oxygen	anesthesia monitor
		PEEP	positive end expiratory pressure	ventilator
		TV	tidal volume	ventilator
		RR	respiration rate	ventilator
		MV	minute ventilation	ventilator (MV = TV x RR)
		I:E	inspiratory:expiratory ratio	ventilator
		RF	replacement fluid	Baxter IV fluid pump

A management strategy for acute renal failure was required as all pigs developed anuric renal failure and accumulated a significant amount of ascites fluid. Initially, placement of an abdominal drain was used to manage fluid accumulation, avoid compartment syndrome, and prevent accumulation of toxic metabolic byproducts. Despite this, several pigs died early from elevated serum potassium levels due to the insufficient renal function.

With a treatment algorithm established that enabled survival beyond 12 hours for most animals, the final methodology to be worked out was the exact dose of LPS to achieve consistent lung injury. Six studies were required to meet the goal, utilizing 4 more animals to complete Specific Aim 1 than anticipated, which pushed this task in Q1 of FY03. One pig died very early in the study and could not be used for data collection and several animals were needed to confirm the dose of LPS as marked variability in the response to LPS had been in observed. This inconstancy was attributed in part to animal to animal variability in response to LPS which is widely reported in the literature, especially in pigs (38, 46, 47), however, we also uncovered a discrepancy with the LPS lot #s. The LPS was purchased from Sigma-Aldrich (product # L4391 - Lipopolysaccharides from Escherichia coli O111:B4, γ-irradiated, BioXtra, suitable for cell culture, 1 mg vials) with a manufacturer guaranteed activity level of not less than 500,000 EU (endotoxin units)/mg. Upon review of the specifications provided with previously purchased lots, the listed activity levels ranged from >1.8 million units to >3.0 million units/mg. An extensive literature search was performed, yet only the manufacturer and serotype of LPS were described in animal studies utilizing LPS. Activity level of LPS was not reported in any of the reviewed literature. To avoid any potential interference from differing LPS activity levels in the assessment of SCD therapy in the ARDS model, LPS for the remainder of the project was purchased in bulk. The available supply was comprised of 2 different lots, so all acquired LPS was reconstituted, pooled, distributed into aliquots of either 100ug (for low dose exposures) or 1 mg (for high dose exposures) and frozen at -80°C. These LPS aliquots were then thawed as needed for each subsequent experiment. Using the pooled LPS, we were able to identify a standardized dose of 15µg/kg/hr x 3 hours, which evoked a consistent physiologic response without cardiovascular collapse and incited clinical features of ALI as seen during model development.

One of the pigs utilized during this period arrested unexpectedly during the hemorrhaging procedure and could not be resuscitated (ARDSp021). The other 5 studies progressed as expected with a decline in P_a:FiO_2 to <300 observed within 6 hours in all but one pig (ARDSp022). The P_a:FiO_2 did eventually drop to <300 in this animal by 12 hours. Fulminant ARDS was observed in two pigs evidenced by development of hypoxemia with severe pulmonary edema and pink foamy fluid filling the airway. Survival times for these first 5 animals were 7.25, 24, 4.5, 14, and 13 hours from the start of high dose LPS. Even with the use of the supportive care treatment algorithm developed early on, we eventually found that maintaining animals for greater than 15-18 hours was challenging. All pigs stopped making urine early in the study and despite the abdominal drain generalized edema formation plus acid-base disturbances with elevated serum potassium contributed to early demise <24h.

An anticipated potential complication in this model, it was evident that the dose of LPS sufficient to reliably induce ALI (15 µg/kg/hr x 3hrs) was inciting multi-organ dysfunction and further intervention would be necessary to support animals to achieve 24hr survival consistently. As renal dysfunction appeared to be an important contributor to the declining physical state of these pigs, it was concluded that renal replacement therapy would be necessary. Acute renal failure is a frequent sequalae to severe trauma, thus use of continuous renal replacement therapy (CRRT) is not uncommon in the trauma patient. SCD therapy is highly compatible

with CRRT as both can be delivered using the same extracorporeal blood circuit. A prescription for continuous venovenous hemofiltration (CVVH) was devised. The circuits designed for untreated and SCD treated cohorts are shown in Figure 18. Two additional pig studies were completed under Aim 1 using CVVH. The first animal survived 20h (ARDSp023). The next pig was given SCD therapy concurrently to ensure feasibility of the treatment in the model and this animal survived the entire 24-hour study period (ARDSp024).

Including these 2 studies, survival to 12 hours for this model was 8/10 (80%) of the animals that made it into the LPS period and therefore the Model 3 LPS dosing with use of CVVH was deemed suitable to advance to Specific Aim 2. Data from these 2 animals that received CVVH were included as part of the final study data set as described under Specific Aim 2.

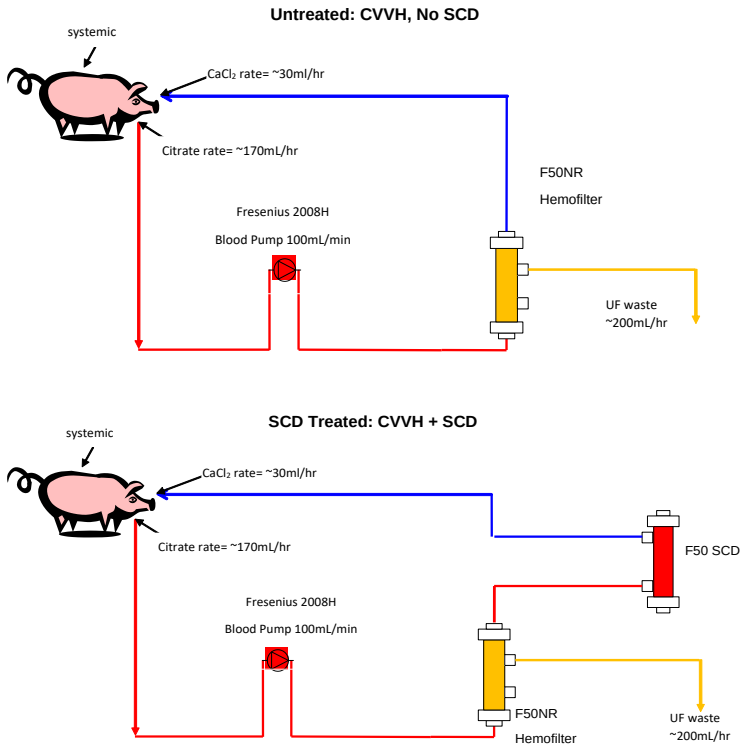


Figure 18. Schematic of extracorporeal circuits to be used for renal replacement therapy (CRRT) in the ALI studies, Untreated (top panel) vs. SCD treated (bottom panel). Continuous veno-venous hemofiltration (CVVH) is performed using a double lumen catheter in the right jugular vein, connected to a Fresenius hemodialysis tubing set. Citrate (ACD-A) is administered at the catheter outflow leaving animal, and calcium chloride ($CaCl_2$) at the venous return. Fresenius F50NR used as hemofilter. Blood flow rate (BFR) set at 100mL/min. Citrate (ACD-A) rate ~170mL/min, 2% Calcium chloride rate ~30mL/min via Baxter pumps. UF rate matched to citrate + $CaCl_2$ rate via Baxter pump. Replacement fluids are administered separate from circuit at an empiric rate of 10mL/kg/h.

<u>Major Findings:</u>

- *Using a pooled lot of LPS to ensure similar potency between studies, a dose of LPS of 15 µg/kg/hr x 3hrs was confirmed for induction of ALI.*
- *ALI was clinically evident in 8/10 pigs that received LPS infusion according to Model 3.*
- *8/10 (80%) pigs used under Model 3 survived to 12 hours.*
- *Supportive care interventions, including the use of CVVH, was determined necessary to reliably ensure survival of pigs beyond 12 hours in this Model. A final treatment algorithm was created to standardize care for animals used to meet Specific Aim 2.*

<u>Milestones Achieved</u>:

1) ALI, as defined by P_a:FIO_2<300, is achieved within 6 hours of LPS infusion start time in most pigs.
2) At least 80% of pigs survive 12 hours or longer.

Subtask 2: Perform measurements and assays required to assess key endpoints/exploratory endpoints.

Real-time measurements of respiratory parameters (P_aO_2, P_aCO_2, blood pH, airway resistance, pulmonary compliance) cardiovascular parameters (cardiac output, arterial pressure, pulmonary artery pressures, ventral venous pressure and systemic vascular resistance) and renal function (urine output) were obtained. As expected, LPS induced hemodynamic changes consistent with sepsis and aberrations in pulmonary parameters including elevation of pulmonary pressures, a decrease in P_aO_2 and declining pulmonary compliance were observed. Temporal variation in onset of clinical features to be used for determining the onset of ARDS were found from animal to animal, particularly the drop in P_a:FiO_2 to <300, which ranged from as early as 3 hours to as late as 12 hours. All but one of the untreated pigs did drop below this critical value. The single pig that received SCD_{Rx} during model development did not fall below 300.

Laboratory sampling with expedient analysis, including complete blood counts with differentials, flow cytometry for leukocyte activation, BALf cell counts and *postmortem* measurement of total lung water were performed on these animals to verify model characteristics. Lung tissue samples were collected from each of the pigs and prepared for histologic analysis. Pathologic evidence of ALI was observed in each of the animals. Pathologic changes ranged from mild to moderate and included septal thickening, vessel congestion, accumulation of proteinaceous material in the airway and infiltration of inflammatory cells in the tissue and airway. Of note, only mild lesions were observed in the pig that received SCD_{Rx}. For the rest of the animals, even in pigs where "real time" clinical measurements during the experiment were not as convincing of the ensuing disease process, significant histologic changes consistent with ALI were found. Taken together, these findings confirmed reliability of the model to induce ALI.

Evidence of a systemic inflammatory response to trauma as well as the LPS infusion were obtained in Model 3. This response entailed a post-trauma increase in white blood cell counts with neutrophilia as determined using manual complete blood counts. Using flow cytometry, a modest increase in expression of the CD11R3 integrin on inflammatory cells (neutrophils and monocytes) was observed post trauma and the priming dose of LPS. Immediately following high dose LPS exposure, CD11R3 expression increased dramatically while the circulating white cell counts severely decreased due to margination and extravasation of activated cells. A rebound in circulating leukocytes was observed over the 24-hour study time course with an influx of immature neutrophils indicative of recruitment from bone marrow and sustained inflammation.

Collated data for this subset is shown in Figure 19

Assay of serum, plasma, BALf and urine for exploratory markers was deemed unnecessary to confirm the model and was not performed based on the clinical and physical measures already obtained that corroborated the model's suitability to permit evaluation of SCD therapy for treatment in ALI/ARDS.

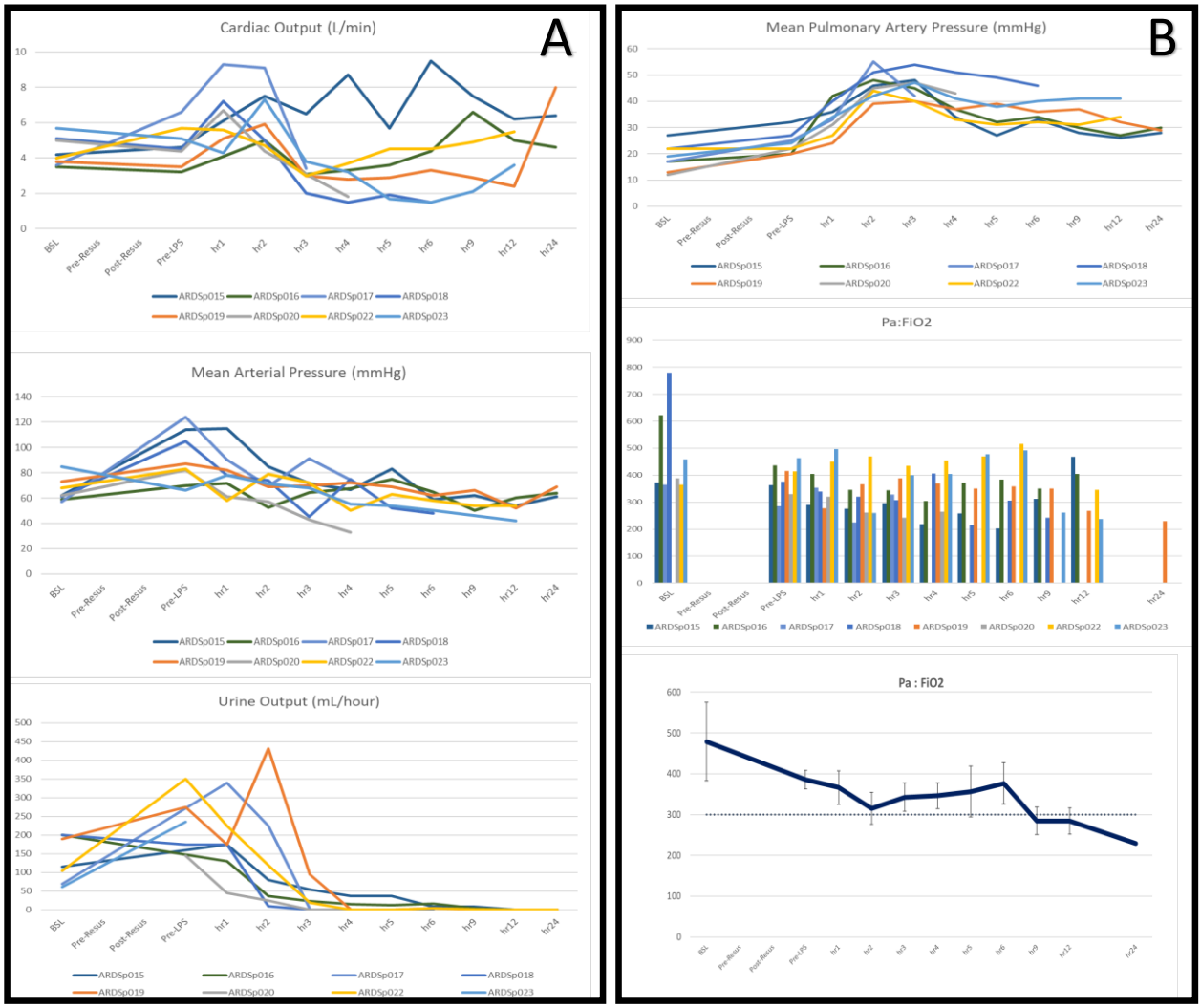


Figure 19. Key clinical values obtained from animals used to verify the final model. Panel A shows the cardiovascular instability encountered upon administration of high dose LPS with altered cardiac output, a progressive decrease in mean arterial pressure (hypotension) and the onset of anuric renal failure after 6 hours in all pigs. Yet, using the treatment algorithm pigs could be maintained for up to 24 hours. Panel B shows the pulmonary changes incurred with elevation in mean pulmonary artery pressure upon administration of LPS and the changes in Pa:FiO2 ratio over time in each animal (middle) and as an average for the group (bottom).

<u>Major Findings:</u>

- *Clinical measurements along with histologic changes confirmed that ALI is reliably induced in the pig model.*
- *P_a:FiO_2 declined to <300 in most pigs but timing was variable. A decrease this parameter, rather than an absolute value of <300 (which is part of the accepted human clinical definition of ARDS) was deemed suitable for indicating the onset of ARDS in this animal model.*
- *Other clinical measurements, such as the P_aO_2, pulmonary artery pressure, and peak airway pressure also provided indication of ALI, which was confirmed with histology.*
- *Laboratory assays for biomarkers of ALI and systemic inflammation corroborate the clinical evidence and provide additional parameters for which to compare treatment cohorts.*

<u>Milestones Achieved:</u> 1) Experimental study design, with respect to analysis parameters and sample time points, was finalized for Aim 2 study plan.

SPECIFIC AIM 2: ASSESS EFFICACY OF 24 HOUR SCD_{RX} IN ARDS PORCINE MODEL

ARDS pig studies using the final model optimized in Aim 1/Major Task 3/Subtask 1 were slated to occur under four identical allotments of 9 animal studies semi-annually over project Years 2 and 3. (Major Tasks 1-4 : Perform 9 ARDS pig studies using final model optimized in Aim 1/Major Task 3/Subtask 1. (3 from each cohort)

Original planned cohorts were:
1) untreated = supportive care alone.
2) supportive care + SCD_{Rx} at time of LPS infusion.
3) supportive care + SCD_{Rx} started at the time ARDS is verified.

Cohort 2 was intended to evaluate ability of SCD_{Rx} to mitigate or prevent ARDS in an at-risk patient and Cohort 3 was intended to evaluate ability of SCD_{Rx} to mitigate or reverse ARDS.
Based upon the human clinical definition of ARDS, a decrease in the P_a:FiO_2 <300 would serve as verification of ARDS onset in the pig model. However, a P_a:FiO_2 <300 did not occur within 6 hours reliably within every animal during model development. While the P_a:FiO_2 did eventually decrease to <300 within 12 hours in nearly all pigs, this inconsistency in the model complicated determination of the timing for initiation of SCD therapy as planned for Cohort 3. The variable onset of ALI meant that pigs would potentially receive vastly different durations of SCD therapy and there was concern that SCD treatment sessions of less than 12 hours might be insufficient to achieve a statistically significant treatment effect in this model. This concern stems from experience that modulation of the immune response through continuous processing of leukocytes during clinical use for sepsis associated AKI, SCD_{Rx} is delivered over several days. Furthermore, the completion of Specific Aim 1, with verification of the model, required 4 more animal studies than anticipated, decreasing the number of studies that could be performed during the rest of the Year 2 reporting period. For these reasons, it was decided to initially proceed with testing in Cohorts 1 and 2 only. Testing for Cohort 3 was postponed until Year 3 when a better understanding of the model had been obtained.

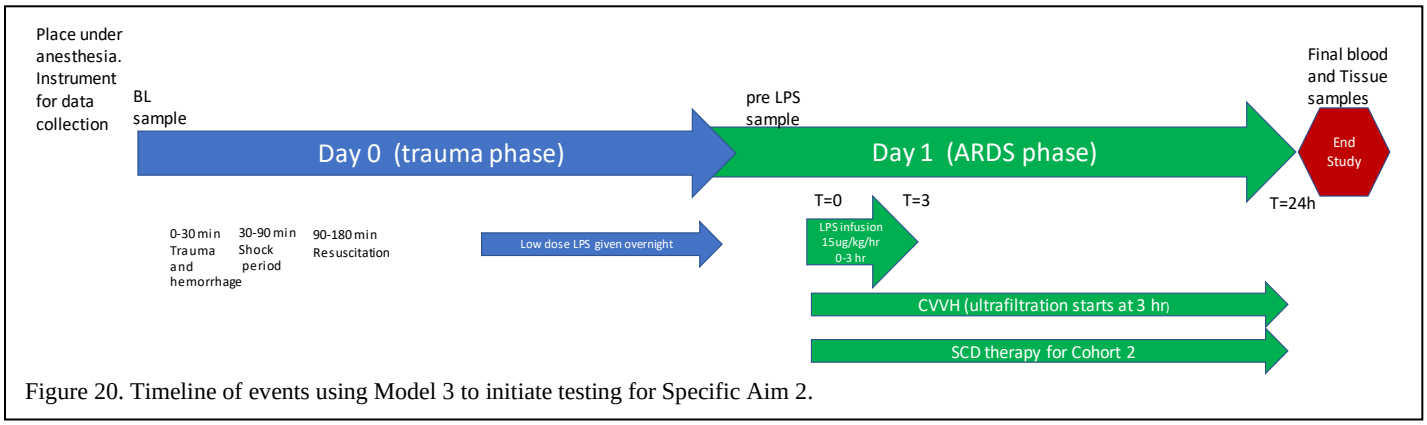


Figure 20. Timeline of events using Model 3 to initiate testing for Specific Aim 2.

Subtask 1: Perform studies in each of the cohorts

In Year 2, eleven (n=11) pig studies were conducted with 5 pigs allocated to Cohort 1 (untreated) and to 6 pigs to Cohort 2, in which SCD_{Rx} was initiated at the start of LPS infusion (Table 7). This total includes the 2 pigs that received CVVH during completion of Specific Aim 1. As dictated by the model, all these pigs were to receive CVVH as renal replacement therapy for fluid balance and avoidance of potentially toxic concentrations of metabolic byproducts, notably excessive serum potassium. The extracorporeal circulation was initiated at the start of LPS infusion but withdrawal of ultrafiltrate was delayed until infusion of LPS infusion was completed at hour 3 to avoid potentially filtering off LPS or inflammatory mediators during the inciting period. This allowed for features of renal failure to clinically manifest and also prevented a discrepancy in hemodynamic status between cohorts as extracorporeal circulation had to begin at time of LPS to deliver SCD_{Rx} in Cohort 2. A timeline of study events is depicted in Figure 20.

The study protocol was found to be suitable with 8/11 animals surviving to the 24-hour end point. One untreated animal died at 20 hours and two of the SCD treated pigs succumbed early (ARDSp026 at 9.5 hours, ARDSp034 at 12.5 hours) from complications associated with gastrointestinal compromise, abdominal compartment syndrome, and hemorrhagic diarrhea.

A decline in P_a:FiO_2 was observed in each of the untreated pigs in Cohort 1 with values $\leq$300 recorded at 3 hr (ARDSp028) and 11hr (ARDSp023) though, the lowest value recorded for ARDSp029 was 369 at 24 hr. The P_a:FiO_2 remained above 300 in the SCD treated animals, except immediately prior to death in the pig that succumbed at 9.5hr (ARDSp026).

The SCD treated pigs that survived the full 24 hours demonstrated markedly improved clinical status compared to the untreated animals. This was most notable in fluid and vasopressor requirements. These SCD treated pigs did not require any additional fluids beyond the maintenance rate of 10 mL/kg/hr while all the control pigs required extra fluid to be bolused intermittently as well as administration of vasopressor medications to maintain minimal hemodynamic target values of a mean arterial pressure of 60 mmHg and Cardiac output of 2.0 L/min as dictated in the treatment algorithm.

Table 7. Pigs used During YEAR 2 to Fulfill Specific Aim 2

PIG ID#	Cohort	LPS Strategy	Signs of ALI	Pa:FiO2	Study End	Comments
ARDSp024 CVVH	1	Ultra low LPS dose at 0.063ug/kg/hr overnight starting 6hrs post trauma Day 1 = 15ug/kg/hr x 3 hr	low Pa:Fi O2 High Pmax	dropped <300 at 12hr	20 hrs	Cardiovascualr collapse early partly due to circuit issues. Progressive MODS
ARDSp025 CVVH + SCD	2	Ultra low LPS dose at 0.063ug/kg/hr overnight starting 6hrs post trauma Day 1 = 15ug/kg/hr x 3 hr	↑ Pmax ↓ lung complience	consitantly greater than 300	24 hrs	VERY stable. No additional fluids or pressors required.
ARDSp026 CVVH + SCD	2	Ultra low LPS dose at 0.063ug/kg/hr overnight starting 6hrs post trauma Day 1 = 15ug/kg/hr x 3 hr VERY respsonsive to LPS (low +hi)	Increased Pmax	Pa:Fi 300-400 most of study. 200 near death.	9 hrs	Progressive cardiovascular collapse. Progressive acid-base disturbance. Hemorhhagic diarrhea. Abdominal compartment syndrome
ARDSp027 CVVH + SCD	2	Ultra low LPS dose at 0.063ug/kg/hr overnight starting 6hrs post trauma Day 1 = 15ug/kg/hr x 3 hr	minimal signs ALI	> 400 throughout	24 hrs	Pig VERY stable. No additional fluids or pressores required.
ARDSp028 CVVH	1	Ultra low LPS dose at 0.063ug/kg/hr overnight starting 6hrs post trauma Day 1 = 15ug/kg/hr x 3 hr	declining Pa:Fi O2 thoracic fluid intralobar edema	Pa:Fi O2 <300 at 3h with progressive decline	24 hrs	Initial hemodynamic decline but stabilized with fluids and pressors. Ascites fluid
ARDSp029 CVVH	1	Ultra low LPS dose at 0.063ug/kg/hr overnight starting 6hrs post trauma Day 1 = 15ug/kg/hr x 3 hr	Pa:Fi O2 declining visible edema	Pa:FI declining	24 hrs	Initial hemodynamic decline but stabilized with fluids and pressors
ARDSp030 CVVH + SCD	2	Ultra low LPS dose at 0.063ug/kg/hr overnight starting 6hrs post trauma Day 1 = 15ug/kg/hr x 3 hr	minimal signs of ALI	fell to mid 300s then improving	24 hrs	minimal added support
ARDSp031 CVVH + SCD	2	Ultra low LPS dose at 0.063ug/kg/hr overnight starting 6hrs post trauma Day 1 = 15ug/kg/hr x 3 hr	minimal signs of ALI	> 400 throughout	24 hrs	Required a bit of fluids and pressors Hemofilter clotted and was replaced
ARDSp032 CVVH	1	Ultra low LPS dose at 0.063ug/kg/hr overnight starting 6hrs post trauma Day 1 = 15ug/kg/hr x 3 hr	minimal signs of ALI but decline toward end of study	> 400 throughout	24 hrs	stable with support
ARDSp033 CVVH	1	Ultra low LPS dose at 0.063ug/kg/hr overnight starting 6hrs post trauma Day 1 = 15ug/kg/hr x 3 hr	Pa:Fi declining increased Pmax edematous lungs with foamy fluid	lowest was 332 (declining)	24 hrs	Stablized with support but declining >15 hrs
ARDSp034 CVVH + SCD	2	Ultra low LPS dose at 0.063ug/kg/hr overnight starting 8 hrs post trauma Day 1 = 15ug/kg/hr x 3 hr	minimal signs of ALI increased Pmax from Gi distention	> 400 throughout	12.75 hrs	Succumbed to GI distress and acidosis with progressive CV collapse. Abdominal compartment syndrome.

Data from these 11 animal experiments was collated and analyzed to determine impact of SCD_{Rx} in the ARDS model. Hemodynamic data averaged per cohort are presented in Figure 21. Improved hemodynamic stability was observed post LPS in the pigs that received SCD_{Rx} based upon significantly higher cardiac index and mean arterial pressure (MAP) with less fluid support. The difference in hemodynamics between treatment cohorts is reflected in the Vasopressor dependency index which is calculated hourly by adding up the dose of all administered vasoactive medications and dividing by the obtained MAP (48) for each pig. All five of the untreated pigs (100%) required administration of vasopressor medications to maintain minimal target hemodynamic values. This is contrasted by the need for vasopressor support in only 3 of the 6 SCD_{Rx} pigs (50%). The indices for Cohort 1 and 2 were similar during the first 9 hours post LPS, mainly reflecting pigs that developed gastrointestinal ischemia and cardiovascular collapse which contributed to early death for a few animals in each cohort, however the trends for each treatment cohort were clearly divergent after 12 hours, reaching statistical significance for the last hours of study.

Significant differences between treatment cohorts were observed in several clinically utilized pulmonary parameters (Figure 21). Both groups were maintained with an inspired oxygen content of approximately 0.25 as part of the standard care yet SCD treated pigs maintained significantly higher arterial oxygenation as demonstrated by the P_a:FiO_2 ratio. The P_aCO2 is presented to show that ventilation was not significantly different between groups as ventilator settings were standardized by the treatment algorithm. As anticipated, the P_a:FiO_2 declined over time in the untreated cohort. This value was more stable and returned toward baseline values with SCD_{Rx}. Due to the temporal differences in decline per animal the averaged P_a:FiO_2 values for each cohort never fell below 300. P_a:FiO_2 was higher in pigs treated with SCD_{Rx} after 9 hours, reaching statistical significance at several timepoints. CVVH during endotoxemia has been reported to increase arterial oxygenation (49), which may account for the higher P_a:FiO_2 values observed during these experiments compared to those during model development. These results do have implications regarding use of this single clinical parameter in defining our milestones and for defining the onset of ARDS, especially regarding initiation of SCD_{Rx} for Cohort 3. Perhaps, acceptance of a defined decline in P_a:FiO_2 as well as concordance with other aberrant pulmonary parameters, such as elevated PA pressure may be sufficient for defining onset of ALI within this model.

Decreased lung compliance due to pulmonary edema and atelectasis, a hallmark of human ARDS, is an important and easily assessed parameter in mechanically ventilated animals. Respiratory system compliance was assessed in vivo using automated measurement of respiratory mechanics from the ventilator readings. The respiratory compliance was calculated at designated timepoints using standardized ventilation settings of tidal volume =10 mL/kg, respiratory rate =14 breaths/minute, end expiratory pressure =0 and fresh gas flow = 2L/min. The peak inspiratory pressure was then recorded from the ventilator and compliance calculated using the formula:

Dynamic compliance (mL/cm H_2O) = tidal volume / (peak inspiratory pressure – end expiratory pressure)

As expected, compliance decreased in all pigs over time secondary to the injuries inflicted. The pigs that received SCD_{Rx} had a significantly higher lung compliance at 24 hours post LPS than the untreated animals.

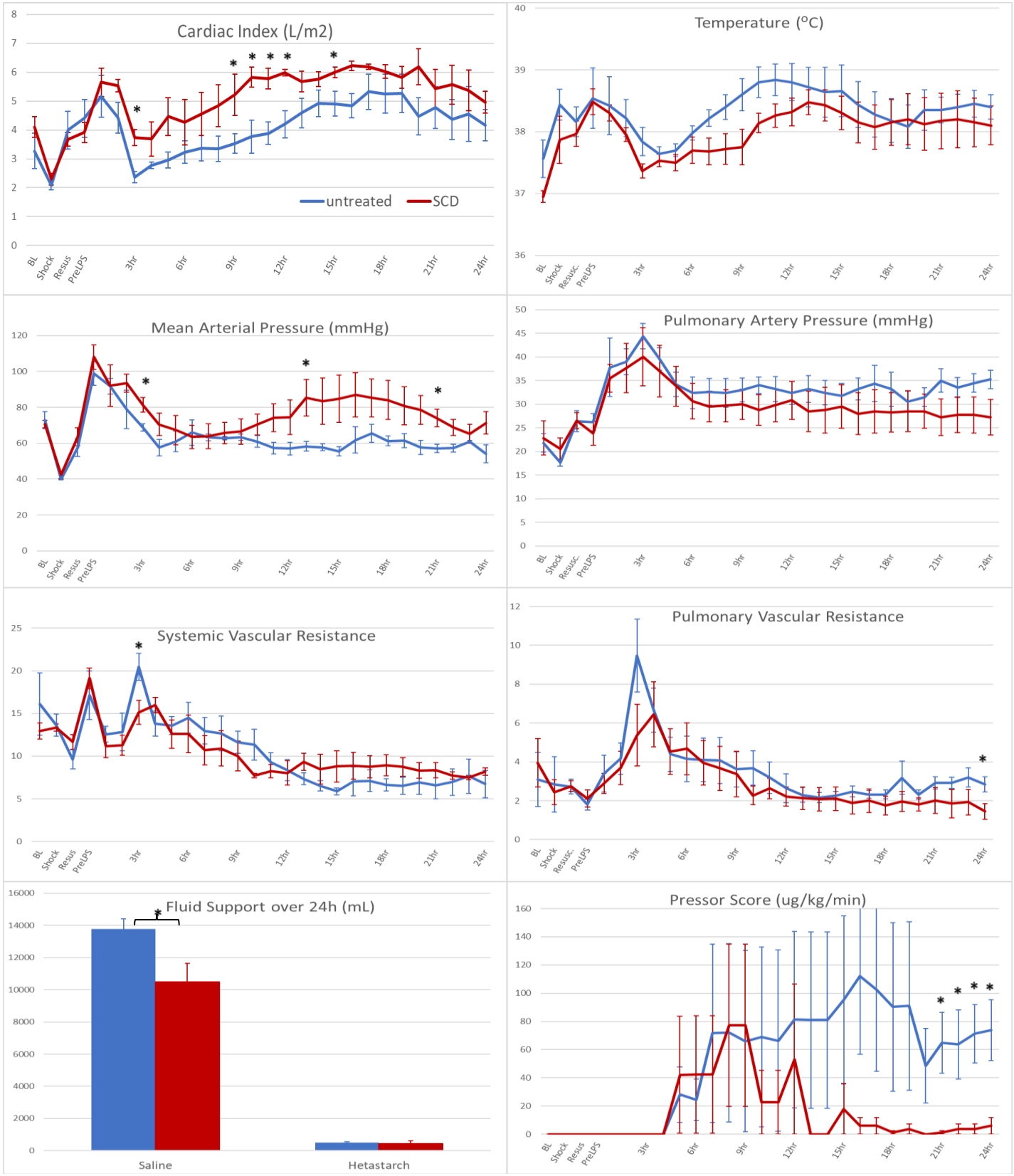


Figure 21. Hemodynamic data. Untreated (Cohort 1, Blue, n=5) and SCD (Cohort 2, Red, n=6), mean± SE, *significance p<0.05.

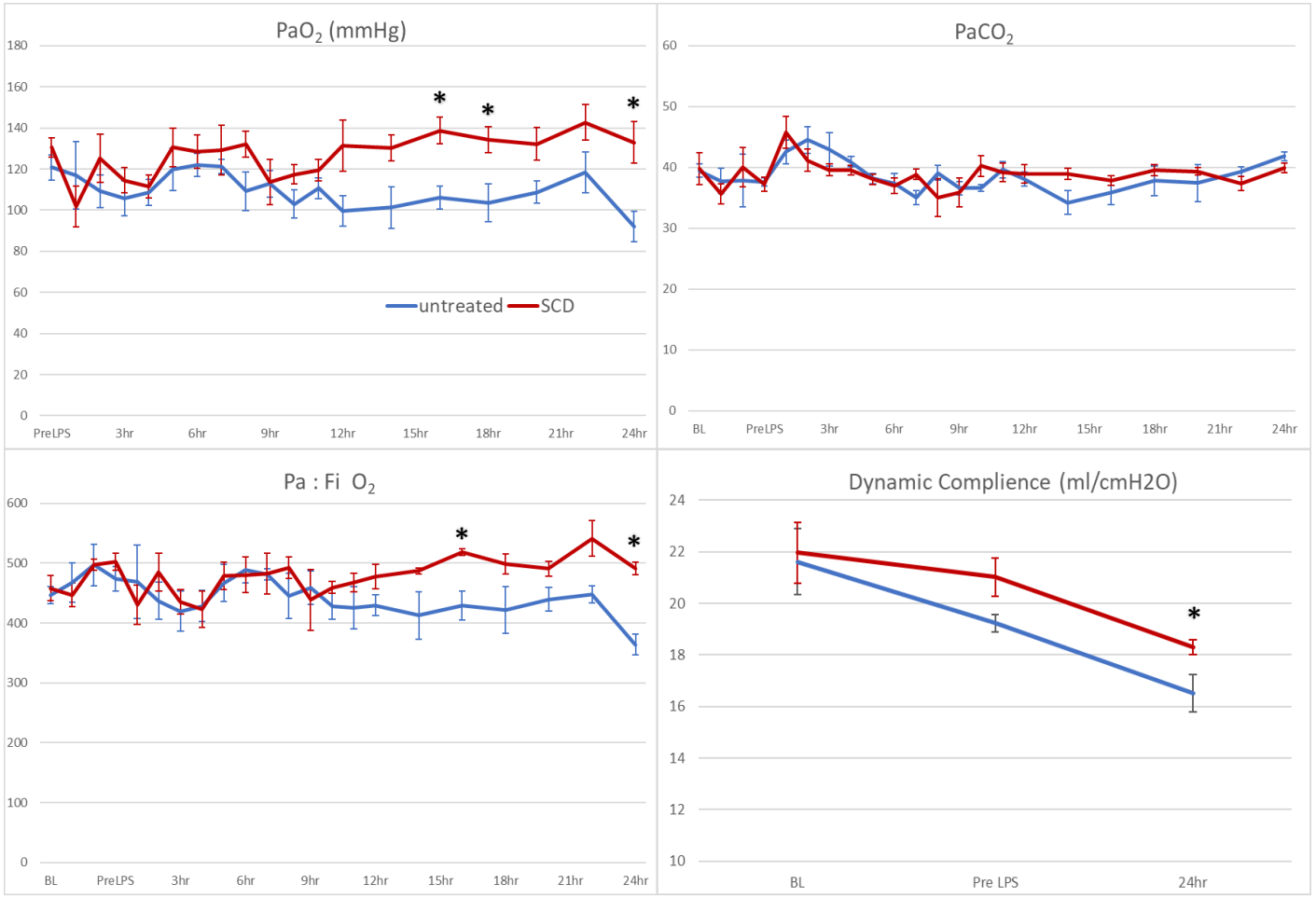


Figure 22. Clinically relevant pulmonary parameters. Untreated (Cohort 1, Blue, n=5) and SCD (Cohort 2, Red, n=6), mean± SE, *significance $p<0.05$.

Taken together, these findings suggested a positive clinical impact of SCD therapy in the setting of ALI/ARDS.

Subtask 2: Perform all measurements and assays required to assess key endpoints and exploratory endpoints.

Arterial blood was drawn into EDTA tubes and submitted to interrogation by a Hemavet® automated hematology analyzer to obtain complete blood counts to evaluate changes in leukocyte number. Differential cell counts were manually verified by microscopic evaluation of blood smears under oil immersion.

For all animals the overall white blood count remained stable through the initial insult period, with increased immature neutrophil counts and decreased monocyte counts observed post resuscitation. Upon initiation of high LPS infusion, a dramatic decrease in white blood count was observed. This decrease is observed for all leukocyte subsets. White blood cell counts rebounded at 6 hours, at which time there is an efflux of immature neutrophils from the bone marrow and marginated pools. White blood cell counts remain elevated through sacrifice. Significant differences in the absolute leukocyte numbers were not observed when comparing untreated animals assigned to cohort 1 to SCD_{Rx} animals assigned to cohort 2. However, trends emerged in that immature neutrophil release appeared to be attenuated in some SCD_{Rx} animals. This trend is of importance in that this phenomenon has been observed in several animal models used in testing SCD_{Rx}, including the porcine septic shock model where reaction to peritoneal instillation of bacteria results in a slower reaction to endotoxin. SCD_{Rx} attenuates the systemic inflammatory response in this model and improved survival (5). This attenuation of neutrophil recruitment, if present, may be harder to observe in the ALI model having sudden, high dose IV infusion of LPS. Monocyte numbers also trended lower for the SCD_{Rx} cohort in hours 6-18.

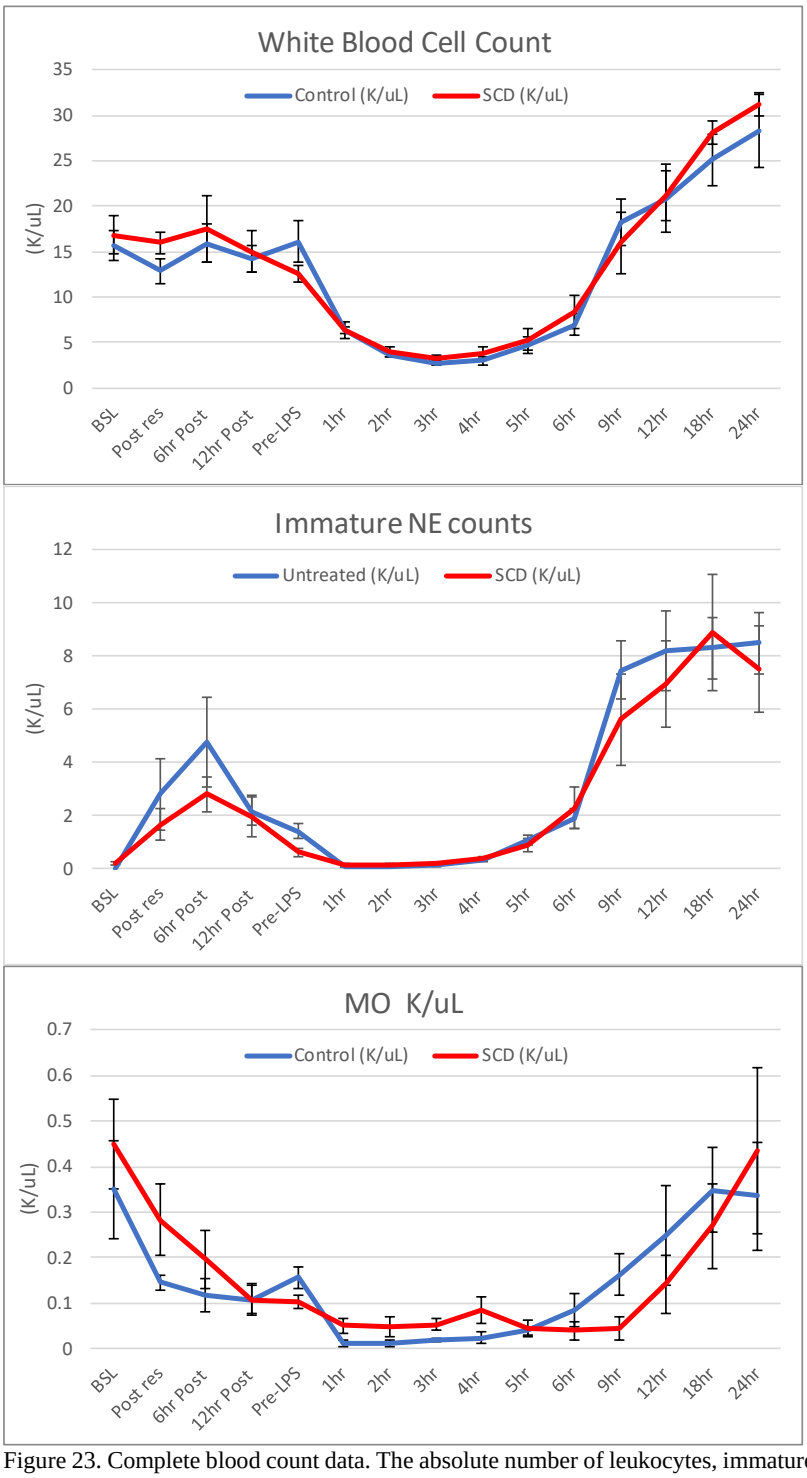


Figure 23. Complete blood count data. The absolute number of leukocytes, immature neutrophils, and monocytes are depicted in top, middle and bottom panels respectively. Untreated (Cohort 1, Blue, n=5) and SCD (Cohort 2, Red, n=6), mean± SE.

Plasma samples from arterial blood drawn into EDTA tubes and processed at baseline (immediately post arterial access, Day 0), pre-LPS (immediately post initiation of high dose LPS on Day 1), 2hr, 4hr, and 6hr, 12hr, 18hr and 24hr were analyzed by Luminex for porcine proteins (IFNα, IFNγ, IL-1β, IL-6, IL-8, IL-10, IL-12p40, and TNFα), using the Cytokine & Chemokine 9-Plex Porcine ProcartaPlex™ Panel (ThermoFisher, EPX090-60829-901). The assayed values and standard error for all assayed values are shown in Table 8: Systemic Plasma Pig Cytokine and Chemokine Concentrations as Assayed by Luminex. Comparison of baseline vs. Pre-LPS values shows a significant increase in the pro-inflammatory cytokine IL-6, reflective of the injury induced during the first phase of the two-hit model. IL-8 was also increased baseline to Pre-LPS but did not reach significance. Of interest, doing the same comparison, a significant decrease in TNFα was observed. TNFα is an acute phase cytokine; levels can begin to change immediately post insult. The higher levels at baseline can be a result of the initial response to anesthesia, or instrumentation required to obtain arterial blood or any combination of these stimulators. Pre-LPS blood sample is drawn from an animal that has been stable for several hours. For all animals, TNFα spiked at 2 hours while other analytes peaked from 4-6 hours following high-dose LPS infusion. Of interest, high TNFα values have been predictive of severity of gut dysfunction in experimentally induced swine dysentery (50). The three pigs that experienced intestinal embarrassment also had the highest spike in TNFα in response to high dose LPS.

Cytokine patterns are complex and often not predictive of outcomes (51), but systemic IL-6 concentrations and IL-6/IL-10 ratio have been found to have prognostic value in the overall outcome of sepsis and injury induced SIRS (52, 53). Plasma values fell within the assay detection range for all analytes except IFNγ. For plasma cytokines, significant differences were not yet demonstrated partly due to a high variability between individual animals. For example, IFNα data was driven by one animal, ARDSp033, that had very high values throughout the study (note the large SE). However, interesting trends were apparent, and it was believed that significance may achieved using the targeted study cohorts. Of note, the average concentrations for the proinflammatory cytokine IL-6 were lower in SCD treated animals, while the anti-inflammatory cytokines IL-10 and IL12p40 were increased. The average concentrations for untreated and SCD treated animals completed during Year 2 studies are given in Table 8: Systemic Plasma Pig Cytokine and Chemokine Concentrations as Assayed by Luminex are IL-6, IL-10 and IL12p40 are graphed in Figure 24.

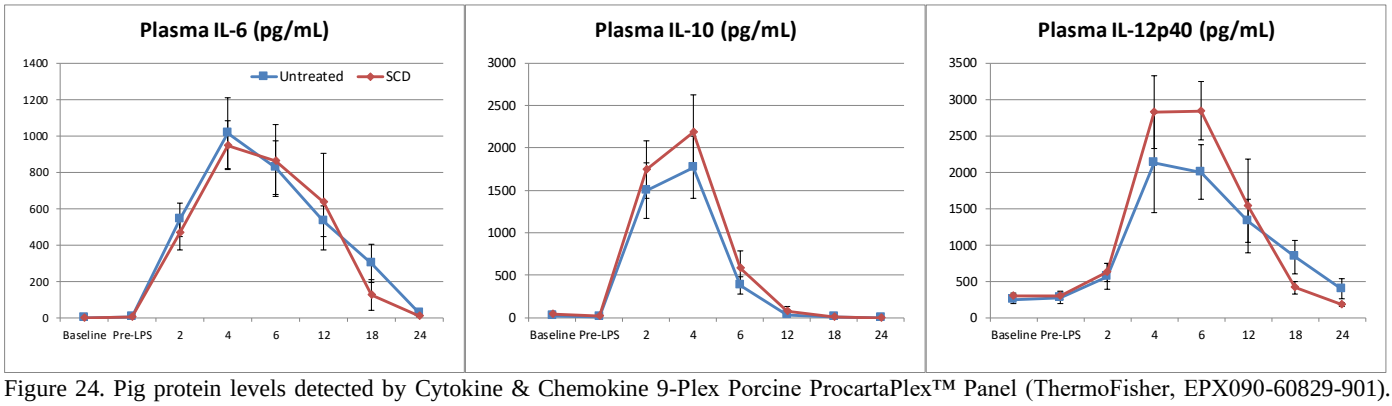


Figure 24. Pig protein levels detected by Cytokine & Chemokine 9-Plex Porcine ProcartaPlex™ Panel (ThermoFisher, EPX090-60829-901). Untreated (Cohort 1, Blue, n=5) and SCD (Cohort 2, Red, n=6), mean± SE.

Table 8: Systemic Plasma Pig Cytokine and Chemokine Concentrations as Assayed by Luminex

IL-1b (pg/mL)	Untreated	Baseline	Pre-LPS	2Hr	4Hr	6Hr	12Hr	18Hr	24Hr	SCD	Baseline	Pre-LPS	2Hr	4Hr	6Hr	12Hr	18Hr	24Hr
	Average	7.31	7.27	17.32	47.33	55.25	20.21	10.26	3.05	Average	12.72	7.55	16.98	63.91	93.23	23.42	10.07	3.50
	SE	1.45	1.43	2.75	7.50	18.73	5.88	2.66	1.76	SE	6.07	0.72	2.68	11.77	36.35	5.67	3.44	1.27
TTEST Untreated vs SCD		0.449	0.861	0.932	0.287	0.406	0.704	0.967	0.844	TTEST Baseline vs. Pre LPS			0.365	Assay Detection Range 3.74-15,300 pg/mL				
IL-4 (pg/mL)	Untreated	Baseline	Pre-LPS	2Hr	4Hr	6Hr	12Hr	18Hr	24Hr	SCD	Baseline	Pre-LPS	2Hr	4Hr	6Hr	12Hr	18Hr	24Hr
	Average	5.35	5.11	4.58	4.10	4.58	3.52	3.40	4.00	Average	5.40	4.96	4.08	4.40	3.68	3.48	4.00	4.60
	SE	0.65	0.86	0.36	0.67	0.46	0.48	0.60	0.00	SE	0.81	0.78	0.55	0.46	0.42	0.51	0.00	1.15
TTEST Untreated vs SCD		0.960	0.904	0.488	0.707	0.188	0.946	0.437	0.622	TTEST Baseline vs. Pre LPS			0.703	Assay Detection Range 44-5,900 pg/mL				
IL-6 (pg/mL)	Untreated	Baseline	Pre-LPS	2Hr	4Hr	6Hr	12Hr	18Hr	24Hr	SCD	Baseline	Pre-LPS	2Hr	4Hr	6Hr	12Hr	18Hr	24Hr
	Average	0.40	4.57	538.39	1011.46	825.80	530.11	296.97	24.38	Average	0.20	2.70	465.30	947.65	862.94	635.36	124.68	5.72
	SE	0.40	2.11	90.83	194.88	147.06	85.27	106.07	15.85	SE	0.20	0.66	94.84	131.83	198.27	265.81	82.29	2.96
TTEST Untreated vs SCD		0.648	0.383	0.596	0.786	0.888	0.716	0.283	0.291	TTEST Baseline vs. Pre LPS			0.003	Assay Detection Range 7.67-31,400 pg/mL				
IL-10 (pg/mL)	Untreated	Baseline	Pre-LPS	2Hr	4Hr	6Hr	12Hr	18Hr	24Hr	SCD	Baseline	Pre-LPS	2Hr	4Hr	6Hr	12Hr	18Hr	24Hr
	Average	12.39	8.02	1492.97	1767.70	379.92	25.49	2.82	0.00	Average	43.96	15.22	1739.56	2185.19	583.17	71.24	4.13	0.00
	SE	7.48	1.05	327.20	366.62	105.04	13.56	2.82	0.00	SE	25.95	3.68	339.57	434.94	200.21	62.21	4.13	0.00
TTEST Untreated vs SCD		0.312	0.119	0.618	0.493	0.420	0.493	0.796	NA	TTEST Baseline vs. Pre LPS			0.274	Assay Detection Range 3.76-15,400 pg/mL				
IL-12p40 (pg/mL)	Untreated	Baseline	Pre-LPS	2Hr	4Hr	6Hr	12Hr	18Hr	24Hr	SCD	Baseline	Pre-LPS	2Hr	4Hr	6Hr	12Hr	18Hr	24Hr
	Average	242.12	273.05	564.83	2131.00	1998.27	1330.06	831.28	393.29	Average	295.81	301.83	621.24	2827.22	2842.61	1534.90	410.29	176.56
	SE	49.47	79.38	173.68	683.26	371.40	290.50	232.67	134.32	SE	45.13	60.48	124.91	498.39	405.40	648.17	83.96	22.96
TTEST Untreated vs SCD		0.443	0.776	0.793	0.422	0.166	0.780	0.199	0.163	TTEST Baseline vs. Pre LPS			0.552	Assay Detection Range 45.14-184,900 pg/mL				
IL-8 (pg/mL)	Untreated	Baseline	Pre-LPS	2Hr	4Hr	6Hr	12Hr	18Hr	24Hr	SCD	Baseline	Pre-LPS	2Hr	4Hr	6Hr	12Hr	18Hr	24Hr
	Average	5.32	7.08	881.60	1386.63	351.43	11.66	3.07	0.00	Average	4.24	5.98	1237.66	1474.20	654.10	37.25	0.26	0.00
	SE	2.50	3.03	155.69	350.15	115.38	5.16	3.07	0.00	SE	2.49	3.86	338.08	341.26	424.28	33.75	0.26	0.00
TTEST Untreated vs SCD		0.767	0.832	0.396	0.863	0.544	0.475	0.475	#DIV/0!	TTEST Baseline vs. Pre LPS			0.219	Assay Detection Range 15.77-64,600 pg/mL				
IFNa (pg/mL)	Untreated	Baseline	Pre-LPS	2Hr	4Hr	6Hr	12Hr	18Hr	24Hr	SCD	Baseline	Pre-LPS	2Hr	4Hr	6Hr	12Hr	18Hr	24Hr
	Average	4.22	9.45	6.51	8.23	8.17	11.22	15.34	12.31	Average	0.25	0.50	0.85	1.06	0.76	0.45	0.35	0.22
	SE	3.98	9.12	6.03	7.50	7.56	10.29	14.23	11.13	SE	0.07	0.23	0.45	0.55	0.32	0.11	0.08	0.02
TTEST Untreated vs SCD		0.298	0.306	0.326	0.319	0.306	0.326	0.414	0.319	TTEST Baseline vs. Pre LPS			0.305	Assay Detection Range 0.81-3,300 pg/mL				
TNFa (pg/mL)	Untreated	Baseline	Pre-LPS	2Hr	4Hr	6Hr	12Hr	18Hr	24Hr	SCD	Baseline	Pre-LPS	2Hr	4Hr	6Hr	12Hr	18Hr	24Hr
	Average	5.16	3.03	2202.26	268.21	91.84	6.28	2.59	0.00	Average	10.94	0.89	1972.18	250.87	53.48	23.09	6.65	0.00
	SE	2.77	3.03	550.06	74.13	28.40	1.93	2.59	0.00	SE	4.94	0.89	387.21	109.05	16.69	20.60	6.65	0.00
TTEST Untreated vs SCD		0.361	0.480	0.734	0.903	0.255	0.440	0.551	#DIV/0!	TTEST Baseline vs. Pre LPS			0.039	Assay Detection Range 6.47-26,500 pg/m				

IFNg is not included in table because values fell below assay detection range. Untreated (Cohort 1, Blue, n=5) and SCD (Cohort 2, Red, n=6), mean± SE, significance $p<0.05$.
Cytokine & Chemokine 9-Plex Porcine ProcartaPlex™ Panel (ThermoFisher, EPX090-60829-901)

Cytometric Analysis of Leukocytes in Peripheral Blood and off SCD Membranes

All cytometric analysis for both blood and lung cells was performed on an Attune (ThermoFisher) flow cytometer, equipped with the following lasers: 488 nm blue, 405 nm violet laser, and 633 nm laser. Data were collected using Attune software (ThermoFisher) with automatic compensation. Samples were taken for single channel CD11R3 analysis on neutrophils gated by scatter profiles at Day 0 Baseline, Day 1 Pre-LPS and hourly through 6 hours. A full analysis panel to evaluate monocyte subsets was performed at Day 0 Baseline, Day 1 Pre LPS, 6hr, 12hr, 18hr, and 24hr, and then on cells eluted from SCD membranes for the SCD treated cohort. The antibody panels used to analyze cells from the lungs, blood and SCD membranes are shown in Table 9. Antibody Panels used for Blood and Lung. Evaluation of neutrophil, monocyte and macrophage populations may provide insight to the transition from neutrophilic alveolitis to monocytic alveolitis (19). A gating hierarchy was confirmed during FY01 work for lungs and systemic blood included: CD11R3, CD284 (toll-like receptor 4 (TLR4)), and S(swine)LA DR II MFI in macrophages, neutrophils, monocytes and monocyte subsets (CD14+ CD163+, CD14+ CD163, CD14low CD163+). Anti-CD203 (SWC9) is used to positively identify alveolar macrophages (20, 21) and is included in the antibody panel used to analyze single cell suspensions of lung cells. Antibody to CD14 labels pig monocytes at variable intensity through maturation and is also found on porcine neutrophils to a lesser degree. Antibody to CD163 is used as a porcine monocyte maturation marker (22) and is highly expressed on a subset of monocytes and all macrophages. SLA DR Class II is differentially expressed on all

Table 9. Antibody Panels used for Blood and Lung

Systemic Blood- Monocyte Surface Characterization and MO And NE Activation

Analyze CD11R3 and CD284 in neutrophils, and CD11R3,CD284 and SLA DR II in all MO and MO subpopulations, (CD14+CD163-, CD14+CD163+, CD14low CD163+).

Vendor	Label	Titrated Amount	laser/fluor
ABDSerotec	CD11R3 (2F4/11)	0.5ug/5uL	BL1-FITC
ABDSerotec	CD163 (2A 10/11)	0.5ug/5uL	BL2-PE
ABDSerotec	CD172a (BL1H7)/SWC3	0.05ug/0.5uL	BL3 PERCP Cy5.5
ABDSerotec	SWC8 (MIL2) (concentration not provided)	5uL	unconjugated
ThermoFisher Scientific	anti MO IgM PE-CY7(eB121-15F9)	1.25ug/2.5uL	BL4 PE-CY7
ABDSerotec	CD14 (tuk4)	1ug/10uL	RL1-Alexa Fluor 647
ABDSerotec	SLA DR Class II (2E9/13)	0.5ug/5uL	RL3-APCCy7
Novus	CD284 (TLR4) HTA125	0.8ug/1uL	VL1-BV421
ThermoFisher Scientific	LIVE/DEAD® Fixable Aqua Dead Cell Stai	1uL	VL2-405/aqua

Macrophage Surface Characterization and Activation

Analyze CD11R3, SLA DR II and CD284 in macrophages from dissociated lung tissue and BAL.

Vendor	Label	Titrated Amount	laser/fluor
ABDSerotec	CD11R3(2F4/11)	0.5ug/5uL	BL1-488/FITC
ABDSerotec	CD163(2A 10/11)	0.5ug/5uL	BL2-PE
ABDSerotec	CD172a (BL1H7)/SWC3	0.05ug/0.5uL	BL3 PERCP Cy5.5
ABDSerotec	CD203a SWC9 (PM18-7)	0.25ug/2.5uL	BL4-PECy7
ABDSerotec	CD14 (TUK4)	1ug/10uL	R1-Alexa Fluor 647
ABDSerotec	SLA DR Class II(2E9/13)**	0.5ug/5uL	RL3-APCCy7
Novus	CD284 (TLR4) HTA125	0.8ug/1uL	VL1-BV421
ThermoFisher Scientific	LIVE/DEAD® Fixable Aqua	1uL	VL2-405/aqua

Whole Blood ICC. Monocyte Surface Characterization and Intracellular Cytokines

Analyze Cytokines in all MO and MO subpopulations, (CD14+CD163-, CD14+CD163+, CD14low CD163+).

Vendor	Label	Titrated	laser/fluor
ABDSerotec	CD172a (BL1H7)/SWC3	0.05ug/0.5uL	BL1-FITC
ABDSerotec	CD163(2A 10/11)	0.5ug/5uL	BL2-PE
ABDSerotec	SWC8 (MIL2) (concentration not provided)	5uL	unconjugated
ThermoFisher Scientific	anti MO IgM PE-CY7(eB121-15F9)	2.5ug/1.25uL	BL4 PE-CY7
ABDSerotec	CD14 (MIL-2 or TUK4)	1ug/10uL	RL1-Alexa Fluor 647
R&D	IL-10 (262715) or IFN-g (154007)*	0.5ug/5uL	BL3 PERCP Cy5.5
R&D	IL-6 (77830) or TNFa (103302)*	0.5ug/5uL	VL1-Dylight405
ThermoFisher Scientific	LIVE/DEAD® Fixable Aqua	1uL	VL2-405/aqua

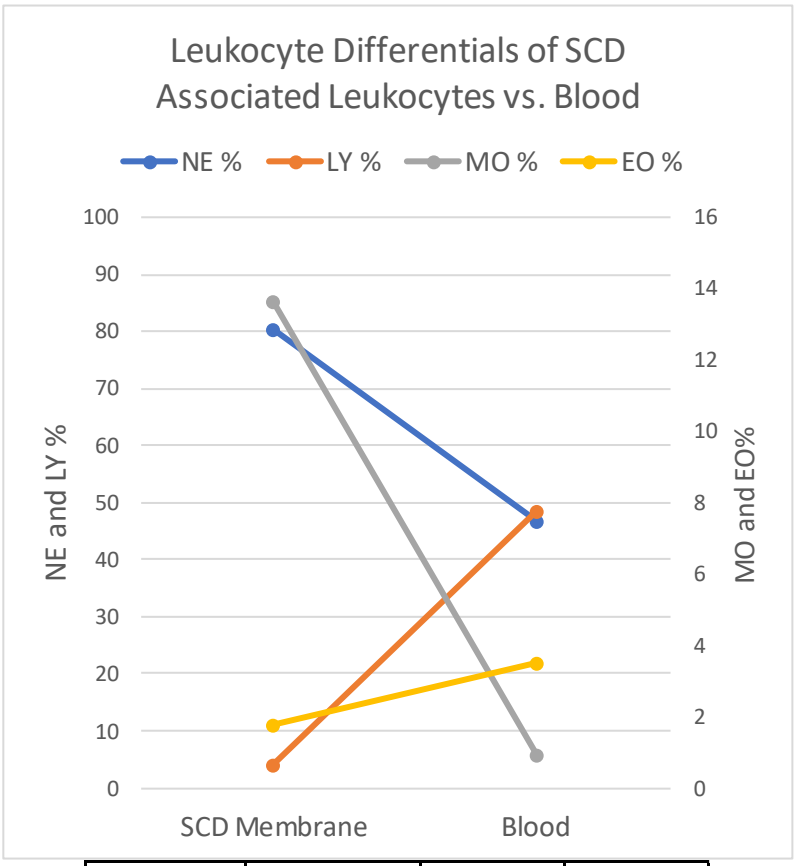
Lung Macrophage ICC. Surface Characterization and Intracellular Cytokines

Analyze Cytokines in macrophages from BAL and dissociated lung tissue.

Vendor	Label	Titrated	laser/fluor
ABDSerotec	CD172a (BL1H7)/SWC3	0.05ug/0.5uL	BL1-FITC
ABDSerotec	CD163(2A 10/11)	0.5ug/5uL	BL2-PE
ABDSerotec	CD203a SWC9 (PM18-7)	0.25ug/2.5uL	BL4-PECy7
ABDSerotec	CD14 (MIL-2 or TUK4)	1ug/10uL	RL1-Alexa Fluor 647
R&D	IL-10 (262715) or IFN-g (154007)*	0.5ug/5uL	BL3 PERCP Cy5.5
R&D	IL-6 (77830) or TNFa (103302)*	0.5ug/5uL	VL1-Dylight405
ThermoFisher Scientific	LIVE/DEAD® Fixable Aqua Dead	1uL	VL2-405/aqua

cells of interest but may be shed as cells become anergic (23). Antibody to CD284 recognizes toll-like receptor 4 which can be differentially expressed via a wide range of stressors (24, 25). Using the panels shown in Table 9. Antibody Panels used for Blood and Lung, macrophages, neutrophils, monocytes, and monocyte sub-populations were reliably identified. The identified populations were then evaluated for expression of CD11R3, SLA DR II and CD284.

In the SCD cohort, blood from the extracorporeal circuit was returned to the pigs prior to sacrifice. SCD membranes were then removed from the circuit, rinsed free of blood and treated with a solution containing EDTA to stabilize and release membrane associated leukocytes. The membrane associated leukocytes were compared to cells present in the circulation also drawn contemporaneously with sacrifice for all cytometric analysis parameters. On average, $1.56\pm0.27x10^9$ leukocytes were recovered from SCD membranes. A greater affinity of the SCD membrane to neutrophils and monocytes as compared to lymphocytes is shown in Figure 25. At sacrifice, neutrophils represented around 47% of circulating leukocytes and 80% of those recovered from the SCD. Monocytes represented only 1% of circulating leukocytes, but greater than 13% of cells recovered from the SCD. There was a compensatory decrease in lymphocytes and no significant change on eosinophil distribution.



Leukocyte Subset	SCD Membrane	Blood	t-Test
NE %	80.41	46.89	0.015
MO %	13.63	0.94	0.002
LY %	4.20	48.58	0.006
EO %	1.77	3.53	0.197

Figure 25. Evaluation of the distribution of SCD membrane associated leukocytes as compared to blood revealed an increase affinity for neutrophils and monocytes.

Neutrophil and Monocyte Activation

Human neutrophils (14, 15) and monocytes (16, 17) mobilize intracellular stores of CD11b to the cell surface as they become (primed) activated, allowing a real-time measurement of systemic acute neutrophil (priming) and monocyte activation. For this study, the clone 2F4/11, reactive to human CD11c, was selected from panel of human reactive CD11 antibodies. This antibody was found to be reactive to a 155kD alpha chain and CD18/β2 integrin. In pigs, anti-human CD11b specific antibodies had positive reactivity to the 165kD alpha chain expected for CD11b, however, in pigs these antibodies are reactive only to granulocytes. Of the antibodies reactive to human CD11c, only clone 2F4/11 strongly labeled granulocytes, monocytes and alveolar macrophages, the expected expression pattern comparable to human CD11b. Because it is unclear whether the differences are due to species expression or differences in epitope recognition, the nomenclature CD11R3 was adapted (18). The clone was chosen for its strong reactivity to cells of interest and detectable upregulation upon stimulation.

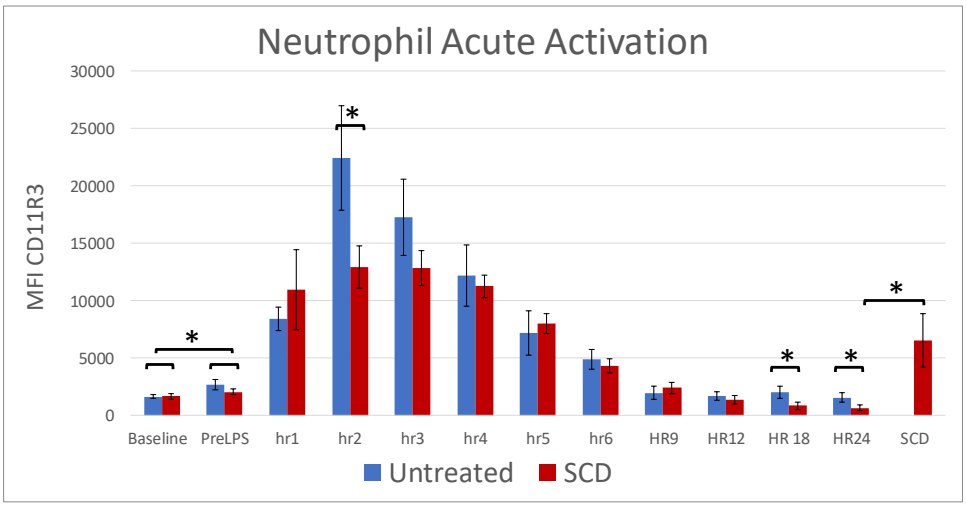


Figure 26. Neutrophil acute activation as detected by surface expression of CD11R3 in systemic blood (baseline-HR24) and cells eluted from SCD membrane at study termination (SCD). Untreated (Cohort 1, Blue, n=5) and SCD (Cohort 2, Red, n=6), mean± SE, significance p<0.05.

The first hit of the two-hit model was detectable by neutrophil expression of CD11R3 in that 10 of 11 animals (all except ARDSp031) had increased neutrophil CD11R3 expression from D0 baseline to D1 Pre-LPS, with the average MFI CD11R3±SE significantly increasing from 1,656±147 to 2,331±257, p=0.0108. With high dose LPS injection, CD11R3 expression increased dramatically concurrent with the decrease in systemic neutrophil numbers. Significantly lower CD11R3 expression levels by neutrophils in the SCD cohort compared to the untreated cohort were observed at 2, 18 and 24 hours. In the SCD cohort, blood was returned to the pigs prior to sacrifice. SCD membranes were rinsed free of blood and treated with a solution containing EDTA to stabilize and release membrane associated cells. The membrane associated cells were compared to cells present in the circulation also drawn contemporaneously with sacrifice. The CD11R3 expression by neutrophils eluted from SCD membranes at the study end (24 hours or death) was significantly higher than those in systemic blood, with average MFI CD11R3±SE being 1,739±502 vs. 6535±2348, p=0.0294. This data is shown graphically in Figure 26 (two far right red bars). This data supports the theory that the SCD sequesters activated leukocytes from the circulation as part of its therapeutic mechanism of action.

For monocytes, a significant increase in CD11R3 expression was not observed from D0 baseline to D1 Pre-LPS. CD11R3 was not measured hourly post high dose LPS injection for monocyte populations. For the SCD cohort, CD11R3 expression of monocytes eluted from SCD membranes at the study end (24 hours or death) was significantly higher than time matched monocytes in systemic blood (Figure 27). Monocyte average MFI CD11R3±SE for peripheral blood vs. those on the SCD was 996±146 vs. 3044±287, p=0.001.

In development of this model, the monocyte populations showed a significant increase from 44±3.7% CD163+ at baseline to 52±6.3% CD163+ Day 1 post trauma ($p<0.05$). For the current cohorts, a different trend emerged. Monocyte populations showed a significant decrease from 49±4% CD163+ at baseline to 32±4% CD163+ Day 1 post trauma ($p<0.01$). This difference may be due to the low dose LPS infusion on Day 0 that wasn't included for the initial animals. With high dose LPS infusion on Day 1, a dramatic decrease in all leukocyte absolute numbers was observed, but the monocytes remaining in circulation were only 20±4% CD163+ ($p<0.001$ compared to baseline) and continues to increase through the study time course. For the SCD treated cohort, the monocytes in the blood at time of death was compared to those eluted from the SCD. Significant differences were observed in CD14 and CD163 expression which is an indication of positive selection for a subset of monocytes. Using the MFI cut off value of 1000 to define % CD163+ resulted in no significant differences in % CD163+ systemically between untreated and SCD treated cohorts. However, the average MFI for CD163 in untreated controls is trending higher than in SCD treated animals.

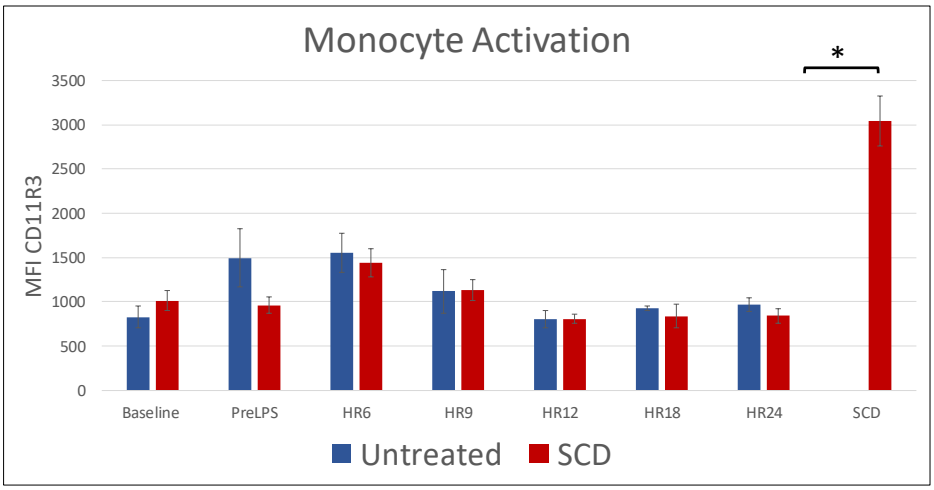


Figure 27. Monocyte activation as detected by surface expression of CD11R3 in systemic blood (baseline-Hr24) and cells eluted from SCD membrane at study termination (SCD). Untreated (Cohort 1, Blue, n=5) and SCD (Cohort 2, Red, n=6), mean± SE, significance $p<0.05$.

For pig, monocyte subsets have not been clearly defined by CD markers as compared to humans. Using available tools, a shift in monocyte phenotype has been detected in the final ARDS model. The shift is most evident by CD163 expression. At baseline, just under 50% of monocytes are CD163+. With LPS challenge, CD163+ cells leave the circulation and are replaced by CD14+ CD163- cells. In this model, the circulating population then returns to a majority of CD14+CD163+ with 24-hours of disease progression. In humans, circulating monocytes can be split into three basic phenotypes. The majority are described as classical CD14+ CD16-, non-classical/reparative CD14 low CD16+, and intermediate/proinflammatory CD14+ CD16+ phenotype. The proinflammatory monocytes in human can be readily identified by strong human (H) LADR expression. In the pig model, a significant shift to a lower swine (S) LADR expression level was observed in SCD treated animals at 18 hours, and trended lower in animals that survived through 24 hours (Figure 28). Although species similarity has not been confirmed for SLADR vs HLADR expression, these changes in expression panels within the circulating monocyte pool may indicate a reduction in proinflammatory monocytes with SCD_{Rx}. The collected cytometric data on peripheral blood and SCD eluted cells can be further analyzed to determine changes in CD11R3, TLR4, and SLADRII and CD14 expression for CD163 +/- subsets and neutrophils. This work was finalized once all experiments were completed to ensure consistency of gating.

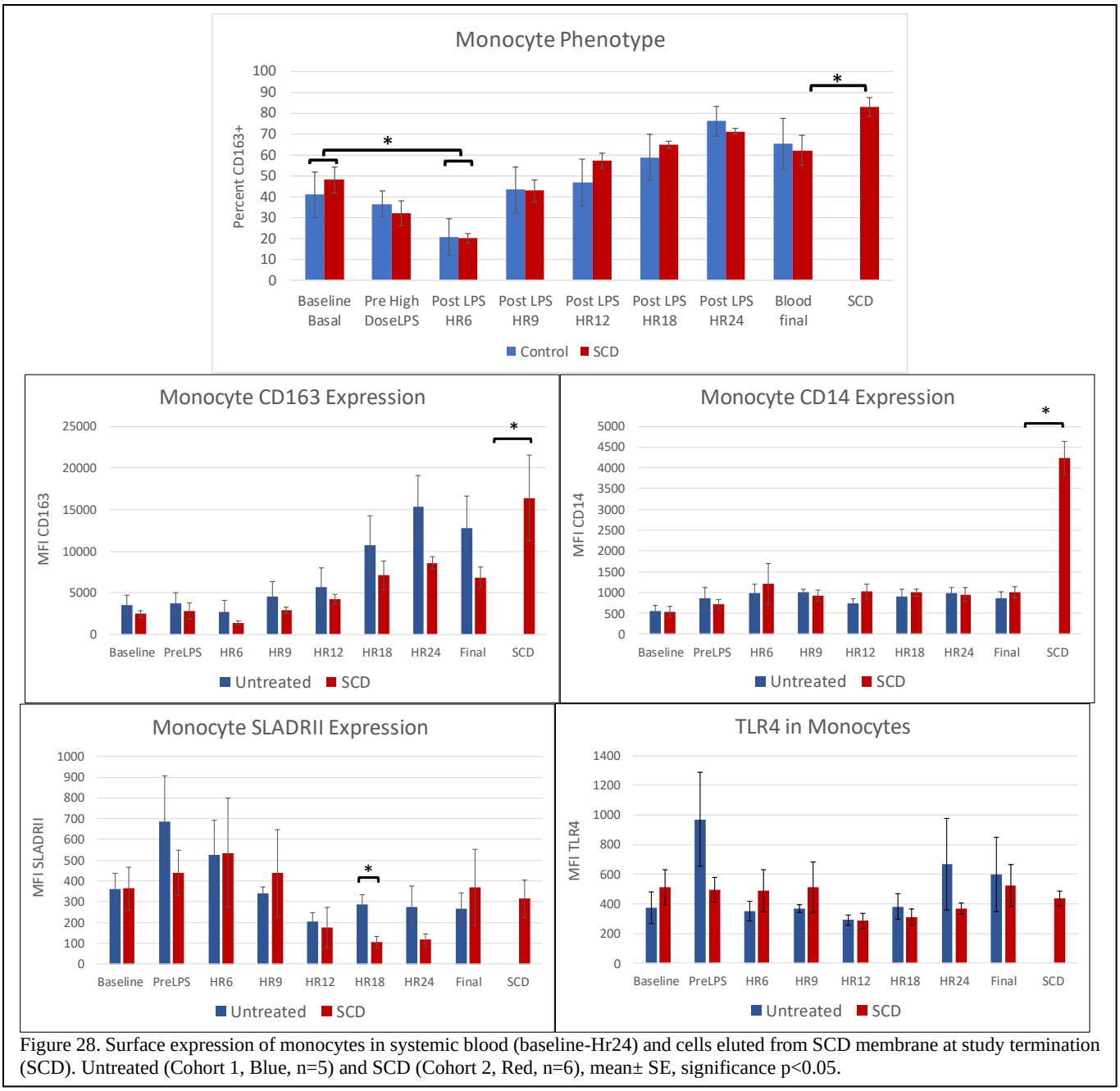


Figure 28. Surface expression of monocytes in systemic blood (baseline-Hr24) and cells eluted from SCD membrane at study termination (SCD). Untreated (Cohort 1, Blue, n=5) and SCD (Cohort 2, Red, n=6), mean± SE, significance p<0.05.

Evaluation of Lung Injury using Physical Parameters

Bronchoalveolar Lavage fluid (BALf) was obtained *post mortem* by the repeated instillation of saline supplemented with 0.2% EDTA into the right middle bronchus. Total cell counts and differentials, specifically for neutrophils relative to total counts, were determined from cytospins. BALf was centrifuged and supernatants assayed for total protein (BioRAD, Catalog#500-0116) to further determine the effects of neutrophil infiltration and extent of alveolar leak (32). BALf was assayed using the same Luminex panel as described for plasma (Cytokine & Chemokine 9-Plex Porcine ProcartaPlex™ Panel, ThermoFisher, EPX090-60829-901) and values normalized for total protein. Pulmonary edema of excised lungs was quantified by placing the entire left lobe into a Ninja food processor and processed until homogeneous. 1-2g samples were weighed (wet weight) and

dried until stable (dry weight) and then expressed as % water content [32]. The results for all animals conducted during FY02 are shown in Figure 29.

Table 10. BALf Pig Cytokine and Chemokine Concentrations as Assayed by Luminex Cytokine & Chemokine 9-Plex Porcine ProcartaPlex™ Panel (ThermoFisher, EPX090-60829-901)

	Animal	IFN-alpha	IFN-gamma	IL-1-beta	IL-4	IL-6	IL-8	IL-10	IL-12p40	TNF-alpha
Untreated	30	0.41	0.00	89.35	0.00	1013.41	8430.48	15.35	217.41	147.93
	34	1.09	0.00	46.53	25.56	166.33	1592.33	3.39	49.81	0.00
	35	1.71	0.00	133.03	68.70	111.43	1497.25	9.12	222.93	0.00
	38	1.19	0.00	87.20	0.00	18.43	5455.46	0.00	139.99	0.00
	39	20.61	0.09	45.19	4.43	76.89	1089.34	0.00	58.77	0.00
	Average	5.00	0.02	80.26	19.74	277.30	3612.97	5.57	137.78	29.59
	SE	3.91	0.02	16.25	13.12	185.59	1441.03	2.96	37.13	29.59

	Animal	IFN-alpha	IFN-gamma	IL-1-beta	IL-4	IL-6	IL-8	IL-10	IL-12p40	TNF-alpha
SCD	31	0.96	0.00	97.28	0.00	113.04	1344.25	0.00	29.18	0.00
	32	2.02	0.49	259.97	0.00	2555.91	16005.78	9.46	262.63	204.09
	33	1.72	0.00	138.12	0.00	65.87	1676.08	0.00	39.32	0.00
	36	1.11	0.48	42.96	0.00	26.38	1080.80	4.60	45.08	31.45
	37	1.09	0.00	69.66	0.00	41.15	996.70	0.00	28.54	0.00
	40	1.21	0.72	121.29	0.00	2067.10	14795.71	0.00	123.21	23.73
	Average	1.43	0.34	126.40	0.00	951.28	6911.01	2.81	99.76	51.85
	SE	0.17	0.13	31.03	0.00	478.65	2983.75	1.61	37.83	32.66
TTEST		0.328	0.104	0.297	0.130	0.361	0.521	0.341	0.377	0.768

In ARDS patients, the concentration of neutrophils in the BALf correlates with severity of ARDS and outcome (40, 41). Normally, BALf is almost devoid of these cells. For all study animals, neutrophils were found in the BALf, and total lung water content was higher than control animals, indicating that all had some degree of lung inflammation. The variables of edema, neutrophils and protein were not strongly correlative with each other and no statistical differences were observed in these parameters using raw data values. Even after normalizing Luminex data to protein levels, the detected cytokine and chemokine levels in the BALf were highly variable within cohorts. IFNα, IFNγ, IL-10 and TNFα had many values below the detection level of the assay. IL-1β, IL-6, IL-8 and IL-12p40 were within the detectable range for all animals, and levels were not significantly different between cohorts. Assayed pig protein levels are shown for all animals in Table 15. BALf Pig Cytokine and Chemokine Concentrations as Assayed by Luminex Cytokine & Chemokine 9-Plex Porcine ProcartaPlex™ Panel (ThermoFisher, EPX090-60829-901). Protein levels in BALf are expected to be higher with alveolar leak. Detected levels on pilot control animals that did not received LPS induced lung injury were 0.781 and 0.481 mg/mL. Only ARDSp024 and 33 had higher values, and therefore either the model doesn't consistently cause protein leakage or the efficiency of recovery of BALf may be inconsistent, which may contribute to the variability in this data.

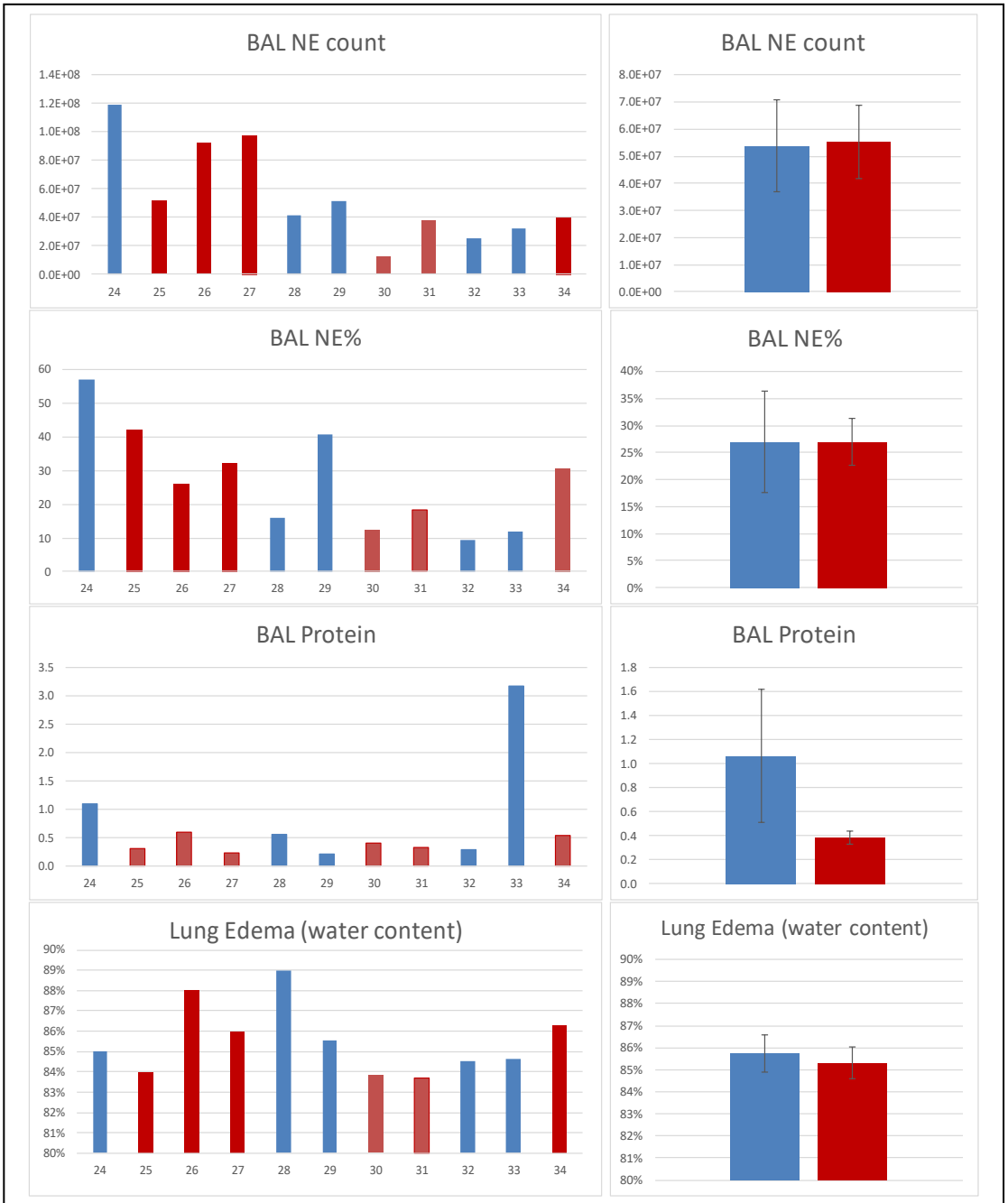


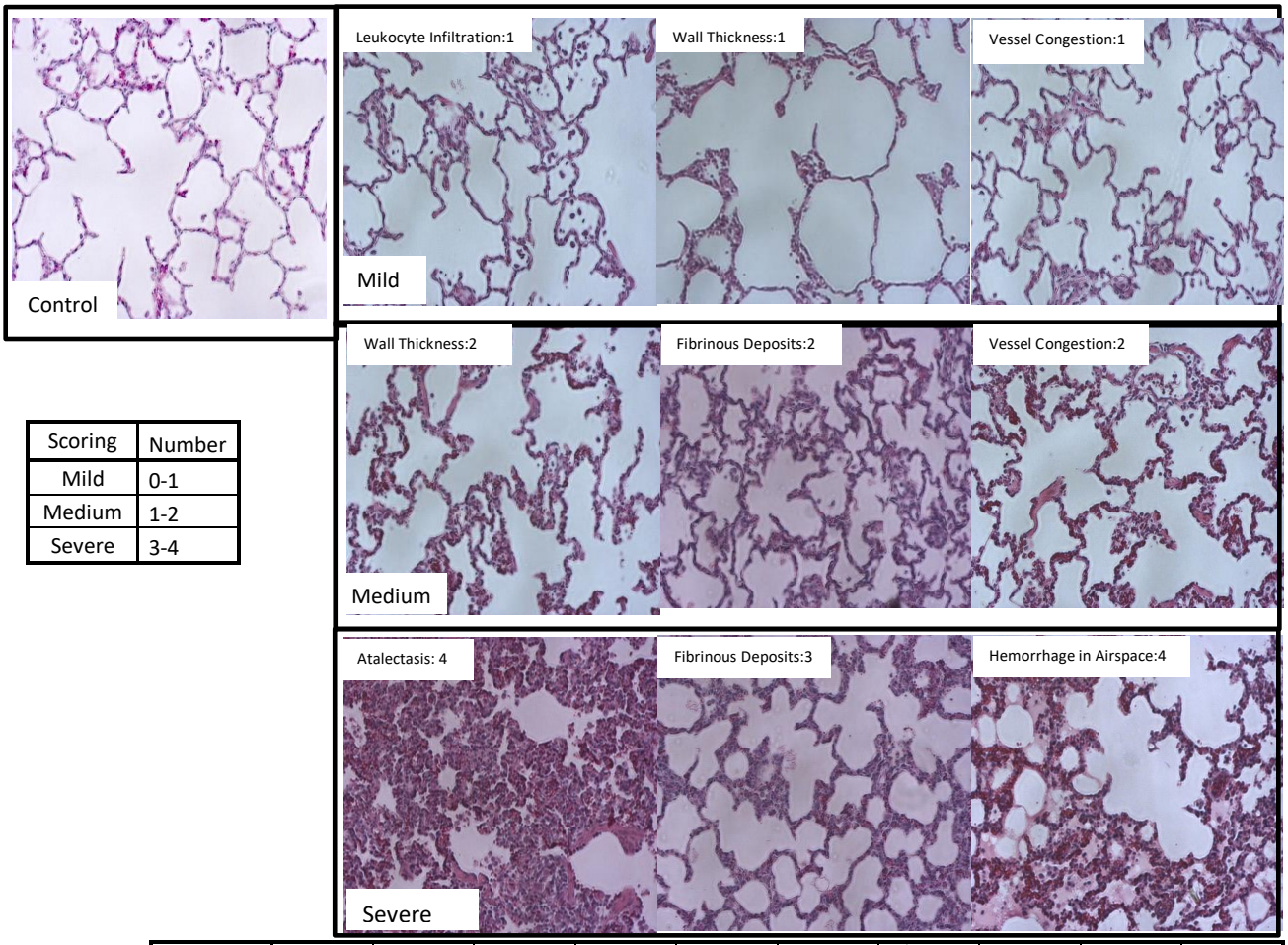
Figure 29. Evaluation of BALf and lung edema in Untreated (Cohort 1, Blue, n=5) and SCD (Cohort 2, Red, n=6). Individual studies on left, averages± SE on right. BAL NE count is the total number of NE recovered, %NE is % of total cells, protein is mg/mL, and edema is (wet weight-dry weight)/wet weight expressed as % water content.

Morphometric Evaluation of Lung

The pathological hallmark of ALI is diffuse alveolar damage (DAD) (39). In humans, DAD is characterized by: neutrophil accumulation in the vascular, interstitial, and alveolar spaces (neutrophilic alveolitis); deposition of hyaline membranes as evidence that serum proteins have entered and precipitated in the airspaces (i.e., disruption of the alveolocapillary membrane); interstitial thickening; and formation of microthrombi. Morphometric analysis of lung pathology in pigs at 24h was performed using reported methodology (54) based on alveolar wall thickness, interstitial congestion, airway hemorrhage and protein accumulation and leukocyte infiltration. Lung tissue from the right diaphragmatic lobe was fixed in 4% paraformaldehyde, serially rinsed and stored in ethyl alcohol prior to submission for sectioning, mounting and staining with hemoxylin and eosin. Photomicrographs were obtained from randomly selected areas of each prepared slide. Three high and three low magnification images from each animal were then randomly selected for evaluation. For this first set of animals, scoring for lung injury was performed independently by two lab personnel who were trained in the scoring system and blinded to treatment cohort. Individual scores were averaged to achieve a final score for each parameter for each animal. Results of this blinded scoring are shown in Figure 30.

Immunohistochemistry (IHC) was used to assess LE infiltration of lungs using CD11R3 (20, 55-57). A bronchus to the diaphragmatic lobe of the right lung was inflated with a 50/50 (v/v) optimum cutting temperature (OCT) compound (Tissue Tek)/PBS, via a cannula using a method similar to that described for BAL. Once inflated, the isolated lung lobe was placed into a pan on wet ice to allow it to become firm, then appropriately sized sections were cut and placed into cryomolds with OCT. Filled cryomolds were frozen in the vapor phase on a surface precooled with liquid nitrogen. Prepared blocks were sectioned using a Lecia cryostat and labeled with antibody to CD11R3 (BioRAD) and visualized using anti mouse IgG conjugated to Alexafluor 594 (Fisher Life Sciences). Images were analyzed using NIH Image J software to provide a semi-quantitative measurement of CD11R3+ leukocyte (monocytes and neutrophils) infiltration of lung tissue. Images were captured and processed using equivalent settings. The images were evaluated in three ways: 1) The total area of positive pixels for CD11R3 in the red channel normalized for the total area of positive pixels for DAPI in the blue channel (Area/Area), 2) the total Area of CD11R3+ cells normalized by total number of DAPI positive nuclei (Area/Count, and 3) the total number of CD11R3+ cells normalized by the total number of DAPI positive nuclei (Count/Count). Representative images are shown in Figure 51 for SCD_{Rx} animals that scored low (ARDSp026), and high (ARDSp30).

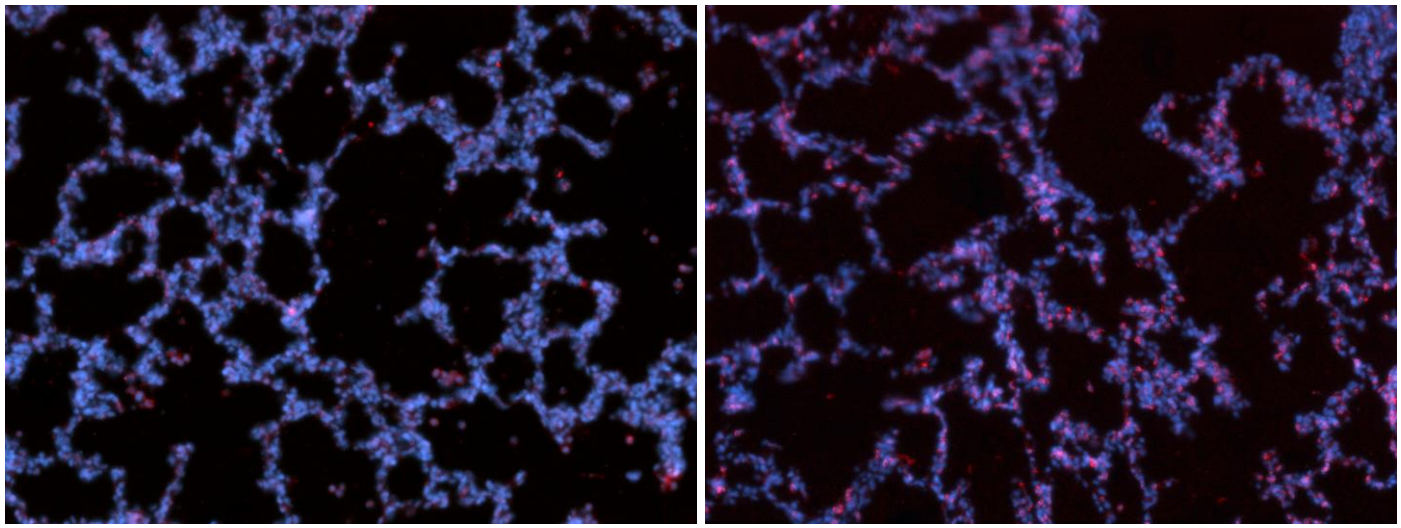
A reduced number of CD11R3+ cells in lung tissue was observed in SCD_{Rx} animals compared to the untreated cohorts. This difference did not quite reach statistical significance but is consistent with other parameters and the difference may become clearer as additional studies increase the numbers of animals per group. A reduction of LE infiltration into lung tissue has also been observed when using SCD_{Rx} in a porcine SSMOD model (5), in which survial was improved with SCD_{Rx}. Accordingly, a decrease in leukocyte extravasation and accumulation in lung tissue could lead to a reduction in lung injury, improved survival and possibly improved long-term lung function. The histologic scores for leukocyte infiltration match this trend and, in these studies, could be a contributing factor to the observed improvement in lung histopathology and function.



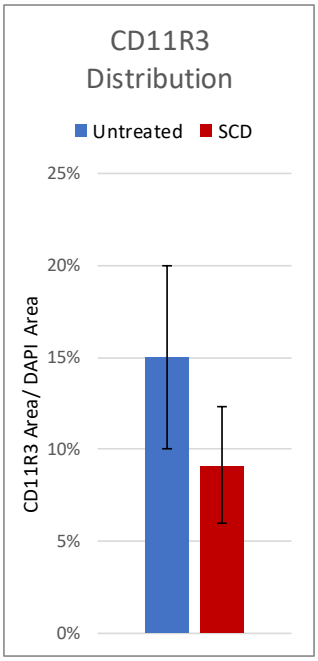
Scoring	Number
Mild	0-1
Medium	1-2
Severe	3-4

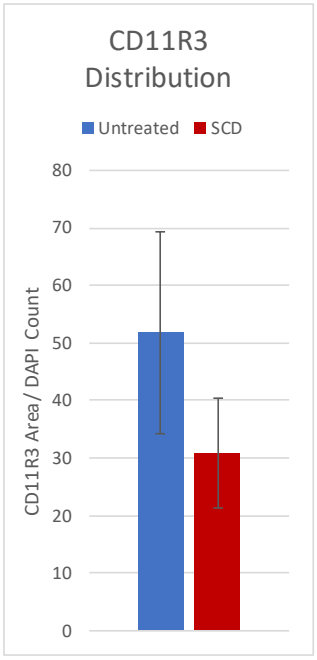
	Animal	Atalectasis	Fibrin Deposits	Hem. In Airway Low Resolution	Congestion	Wall Thickness	LE infiltration quadrant	Hem in Airway High Resolution	LE infiltration count	Total Score A	Total Score B
Untreated	ARDSp024	2.17	1.83	0.83	1.67	2.33	2.33	2.67	2.67	11.17	13.33
	ARDSp028	0.33	2.83	1.50	1.83	1.00	1.00	1.50	0.83	8.50	8.33
	ARDSp029	2.50	1.33	2.50	2.33	2.67	2.83	1.33	2.50	14.17	12.67
	ARDSp032	1.67	1.33	1.33	0.83	2.17	3.00	0.50	2.83	10.33	9.33
	ARDSp033	3.00	3.00	2.33	2.50	3.00	3.50	1.17	2.33	17.33	15.00
	Average	1.93	2.07	1.70	1.83	2.23	2.53	1.43	2.23	12.30	11.73
	SE	0.46	0.36	0.31	0.29	0.34	0.43	0.35	0.36	1.56	1.25
SCD	ARDSp025	0.33	1.50	0.83	0.83	1.00	1.33	1.00	1.00	5.83	5.67
	ARDSp026	0.33	0.50	1.33	1.00	1.17	1.33	0.83	1.83	5.67	5.67
	ARDSp027	0.83	1.00	1.67	1.17	1.50	1.67	1.50	1.33	7.83	7.33
	ARDSp030	1.00	1.83	0.83	1.00	2.33	2.33	0.17	2.00	9.33	8.33
	ARDSp031	1.17	0.83	1.33	1.17	1.67	2.33	0.83	1.83	8.50	7.50
	ARDSp034	2.17	1.33	1.17	1.50	2.17	2.50	1.33	2.83	10.83	11.33
	Average	0.97	1.17	1.19	1.11	1.64	1.92	0.94	1.81	8.00	7.64
	SE	0.28	0.20	0.13	0.09	0.22	0.22	0.19	0.26	0.82	0.86
TTEST	eq var.	0.047	0.023	0.073	0.016	0.081	0.104	0.116	0.173	0.015	0.011

Figure 30. Morphometric scoring of lung H&E Images. Scoring was performed by 2 blinded investigators on 3 high magnification and 3 low magnification images from each animal then all scores for each parameter were averaged. Total Score A was calculated using a reported scoring method and Total score B is an adapted method based upon scoring at high and low magnifications. Significance $p<0.05$ are highlighted green. Untreated (Cohort 1, n=5) and SCD (Cohort 2, n=6).



Study ID	Area/ Count	Area/ Area	Count/ Count
Untreated			
ARDSp024	111.89	0.30	0.33
ARDSp028	62.11	0.21	0.24
ARDSp029	33.94	0.07	0.22
ARDSp032	44.65	0.16	0.22
ARDSp033	6.43	0.02	0.05
Untreated	51.80	0.15	0.21
SE	17.53	0.05	0.05
SCD			
ARDSp025	62.66	0.19	0.24
ARDSp026	8.80	0.02	0.07
ARDSp027	24.61	0.06	0.21
ARDSp030	20.44	0.07	0.15
ARDSp031	37.43	0.11	0.23
ARDSp034	66.55	0.22	0.28
SCD	30.79	0.09	0.18
SE	9.58	0.03	0.03
TTEST	0.449	0.523	0.804





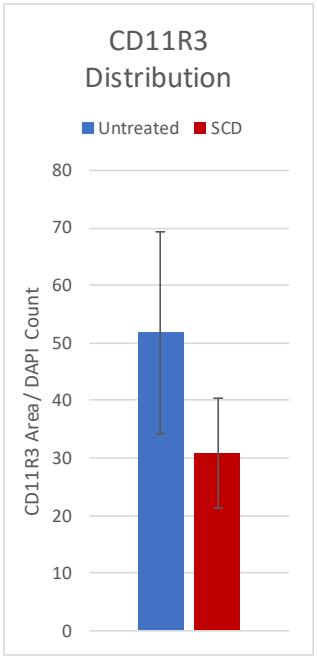


Figure 31. Top Panels, ARDSp026 and ARDSp030 Comparison of low to high scoring images. CD11R3 red, nuclei labeled with DAPI blue. Bottom panel includes individual study results and data analyzed in three ways. : 1) The total area of positive pixels for CD11R3 in the red channel normalized for the total area of positive pixels for DAPI in the blue channel (Area/Area), 2) the total Area of CD11R3+ cells normalized by total number of DAPI positive nuclei (Area/Count, and 3) the total number of CD11R3+ cells normalized by the total number of DAPI positive nuclei (Count/Count). Untreated (Cohort 1, Blue, n=5) and SCD (Cohort 2, Red, n=6), mean± SE, significance $p<0.05$.

Cytometric analysis of lung composition.

With CD11R3 analysis, it is not possible to differentiate monocytes, macrophages and neutrophils. As an alternative approach to accomplish that goal, cells were obtained from gentle enzymatic treatment of lung tissue post manually using dissecting scissors (30, 31). The same lung lobe was used for BAL and enzymatic treatment. Lungs were analyzed for the distribution of $CD172a^{+}$ pig myeloid derived cells as evaluated by flow cytometry and confirmed using manual cytospins. Enzyme dissociated lung cells were labeled with combinations of CD172a, CD14, CD163 (porcine monocyte maturation marker(22)), and SWC9 (positively

identifies alveolar macrophages (20, 21)) to determine overall cells distribution with the goal of quantitatively assessing shifts in cell density that may be attributable to SCD immune cell modulation. With the current cohorts, using this measurement parameter, significant differences in the distribution of cells in the lung was not observed between cell types. Results are shown in Table 11. Distribution of CD172a+ myeloid derived cells in lung tissue evaluated by cytometry. A larger number of CD172+ cells were recovered per gram of tissue from the treated cohorts. Cell numbers are normalized per gram tissue and therefore analysis may be affected by edema which can be normalized in future analysis. Of note, the percentage of interstitial monocytes is lower in the treated cohort, 20.6 vs. 27%, but this difference is not significant.

Table 11. Distribution of CD172a+ myeloid derived cells in lung tissue evaluated by cytometry

Study ID	Cells per Gram (wet weight)	Cells per Gram (dry weight)	CD203a+ CD163+ alveolar MP (%)	CD203- CD163+ Interstitial MP (%)	CD14+ MO (%)	SWC8+ NE (%)
Untreated						
ARDSp024	1.49E+07	9.91E+07	12.0	10.5	25.7	51.74
ARDSp028	1.69E+07	1.54E+08	7.6	49.8	1.9	40.69
ARDSp029	1.76E+07	1.22E+08	7.5	21.0	8.4	63.08
ARDSp032	7.02E+06	4.54E+07	3.7	29.0	4.6	62.62
ARDSp033	7.39E+06	4.81E+07	3.7	27.7	6.0	62.62
Untreated	**1.28E+07**	**9.36E+07**	**6.9**	**27.6**	**9.3**	**56.2**
SE	**2.31E+06**	**2.10E+07**	**1.5**	**6.4**	**4.2**	**4.4**
SCD Rx						
ARDSp025	9.92E+06	6.20E+07	14.0	18.8	14.7	52.46
ARDSp026	1.58E+07	1.32E+08	2.7	13.5	22.8	61.03
ARDSp027	1.03E+07	7.34E+07	4.9	19.4	2.3	73.4
ARDSp030	1.90E+07	1.18E+08	4.7	23.4	8.7	63.1
ARDSp031	3.54E+07	2.17E+08	6.7	37.0	2.7	53.5
ARDSp034	1.80E+07	1.31E+08	12.2	11.4	26.3	50.1
SCD	**1.81E+07**	**1.22E+08**	**7.6**	**20.6**	**12.9**	**58.9**
SE	**3.80E+06**	**2.25E+07**	**1.9**	**3.7**	**4.1**	**3.6**
TTEST (2-tailed) SCD vs. Untreated	0.287	0.385	0.808	0.351	0.559	0.631

Lung Cells Surface Marker and Intracellular Cytokine Cytometric Analysis

Samples from BALf and enzyme treated lung were incubated with antibodies to CD11R3 (pig homologue for human CD11b), SLADRII and TLR4 in combination with the monocyte/macrophage phenotype markers (Table 9) to investigate differential activation among interstitial and alveolar lung macrophage populations (58). Expression of toll-like receptors (TLR) by alveolar macrophages is upregulated by a variety of stressors, including ischemia-reperfusion and ventilator-induced lung injury, and in turn is required for ALI in animal models (24, 25). Evaluation of monocyte/macrophage populations may provide insight to the transition from neutrophilic alveolitis to monocytic alveolitis at 24-hours (19). In Humans, receptor profiles have been used to define the M1 vs M2 phenotypes which purportedly have different roles and may influence the course of disease (59-62). Recent analyses reveal that this concept of macrophage dichotomy is antiquated, and macrophages can be described as having a multidimensional complexity of phenotypes (62). Furthermore, parameters that can be used to describe this complexity are less clearly defined in pig. Further elucidation of pig monocyte/macrophage behavior requires a broad spectrum of tools. In addition to surface markers, secretory profiles were analyzed using both intracellular cytokines evaluated on individual cells using cytometry, and the secretory profile of isolated alveolar macrophages, interstitial monocyte/macrophages and blood monocytes were analyzed by Luminex.

For CD11R3, no changes were observed between treated and untreated cohorts. Within the alveolar compartment, the expression of TLR4 was significantly reduced for both alveolar macrophages, MFI

6966±1354 vs. 4022 ± 611 p=0.032, and neutrophils, 1007 ± 183 vs. 488 ± 192 p=0.043 for untreated and SCD cohorts respectively in Figure 32. This trend toward reduced TLR4 was also observed in interstitial macrophages and neutrophils but did not reach significance between cohorts analyzed at this point.

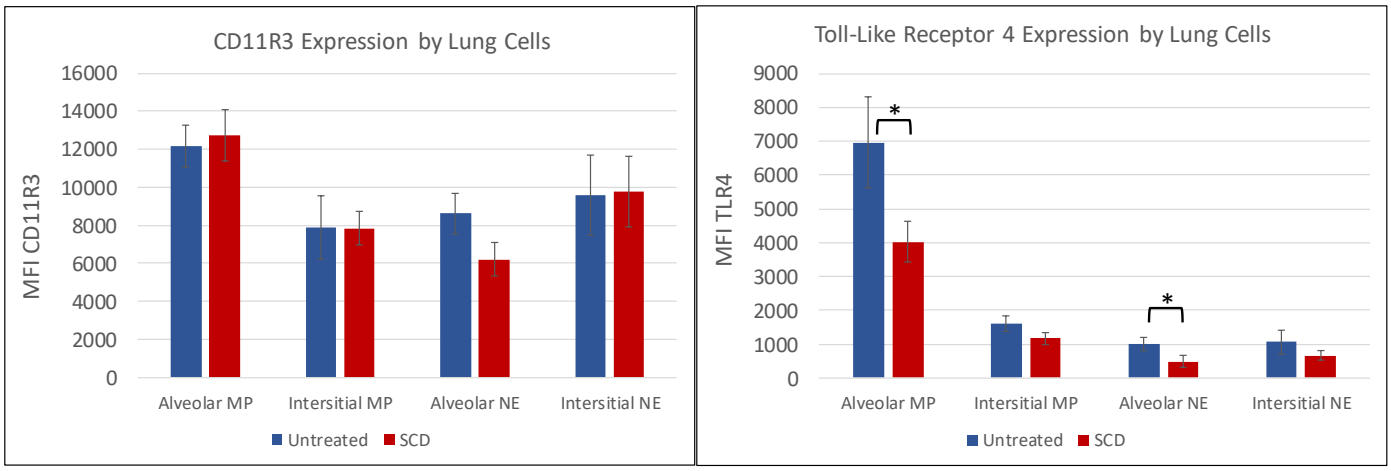
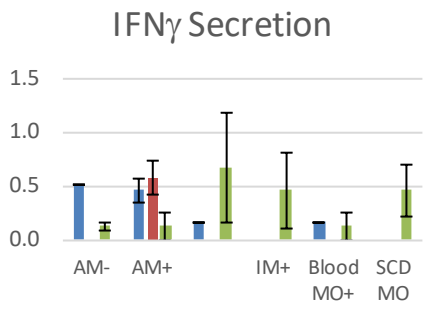


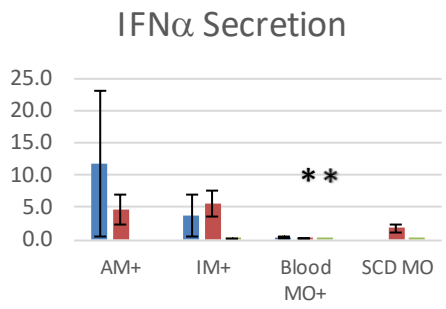
Figure 32. Surface expression of lung cells from the alveolar and interstitial compartments. Expression of TLR4 was significantly reduced for both macrophages, p=0.032, and neutrophils p=0.043 for untreated vs SCD cohorts. Untreated (Cohort 1, Blue, n=5) and SCD (Cohort 2, Red, n=6), mean± SE, significance p<0.05.

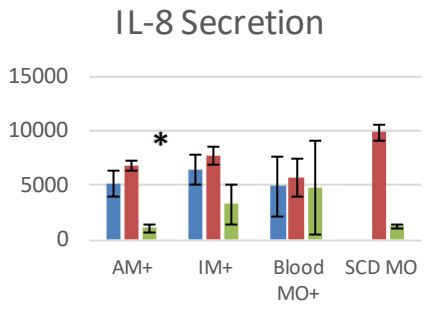
Lung Cells Intracellular Cytokines and Luminex

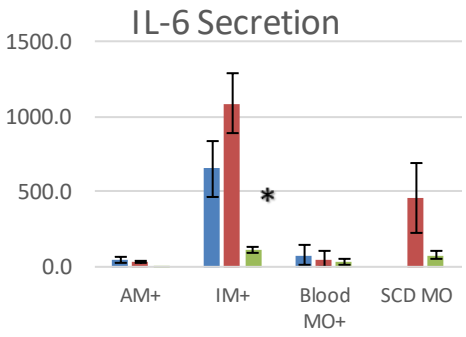
Alveolar Macrophages obtained from BALf, interstitial macrophages obtained from gentle enzymatic treatment of lung tissue were plated in RPMI +10% calf serum at 10^6 cells/2mL/tissue culture plate. Cytospins were performed and plating density adjusted for the number of cells of the macrophage and monocyte lineage. Monocytes and macrophages can be separated from other cell types by their ability to quickly stick to tissue culture plates. After 1-hour, non-adherent cells were removed leaving the desired number of macrophage and/or monocytes cells in each well. BAL cells were mostly alveolar macrophages, enzyme treated lungs were interstitial macrophages, blood derived cells were monocytes and SCD derived cells (from SCD membrane elution) were monocytes. Cells were then stimulated with 1μg/mL LPS. Basal and stimulated wells were collected, but only +LPS samples were assayed to date. Cytokines were detected for all porcine proteins (IFNα, IFNγ, IL-1β, IL-6, IL-8, IL-10, IL-12p40, and TNFα).

Interferons were not expected to be secreted by macrophage and monocytes in response to LPS but were included on the commercially available panel. Assay results were consistent with this prediction with results being around the detection levels. One animal, ARDSp029 had high levels of IFNα, which may be indicative of a viral infection, or impurities in the macrophage preparations. Statistical differences were not observed in IL-1β, IL-6, IL-8, IL-12p40, and TNFα. Of interest, the interstitial macrophages from SCD treated animals produced significantly more IL-10 than the untreated cohort. Calculation of the IL-6/IL-10 ratio revealed an even more significant difference in the secretory profiles. For both cohorts, interstitial macrophages were much more active for IL-6 and IL-10 than alveolar macrophages, and alveolar macrophages were more active for TNFα. Monocytes associated with the SCD membrane were much more active than contemporaneous blood monocytes. Secretory profiles of isolated monocytes and macrophages are shown in Figure 33.


IFNγ Secretion
1.5
1.0
0.5
0.0
AM-
AM+
IM+
Blood MO+
SCD MO


IFNα Secretion
25.0
20.0
15.0
10.0
5.0
0.0
* *
AM+
IM+
Blood MO+
SCD MO


IL-8 Secretion
15000
10000
5000
0
*
AM+
IM+
Blood MO+
SCD MO


IL-6 Secretion
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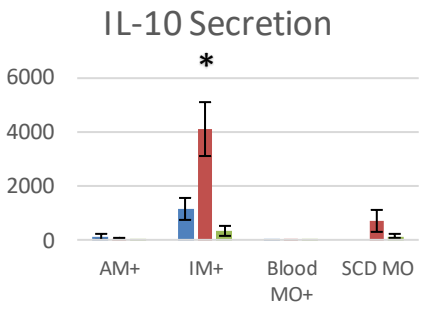
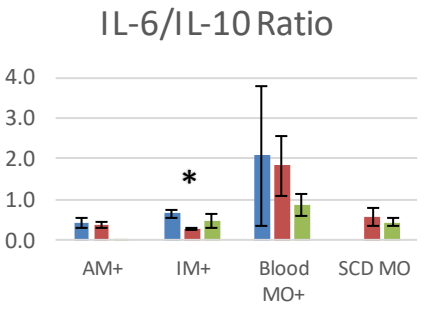
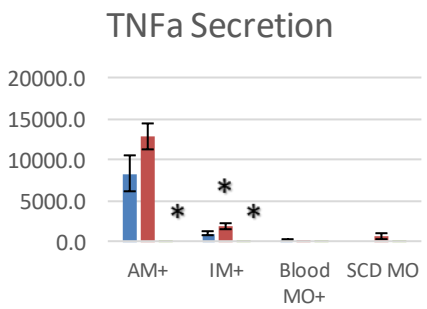
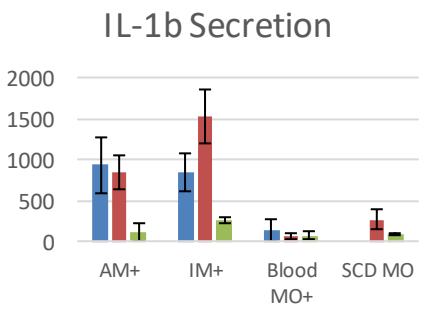
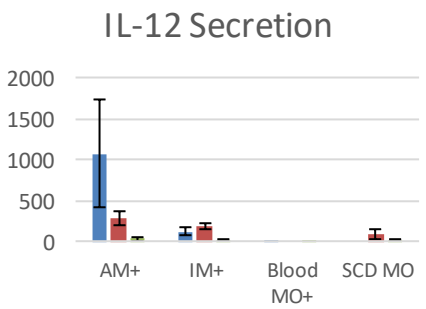
1000.0
500.0
0.0
*
AM+
IM+
Blood MO+
SCD MO


IL-10 Secretion
6000
4000
2000
0
*
AM+
IM+
Blood MO+
SCD MO


IL-6/IL-10 Ratio
4.0
3.0
2.0
1.0
0.0
*
AM+
IM+
Blood MO+
SCD MO


TNFa Secretion
20000.0
15000.0
10000.0
5000.0
0.0
* * *
AM+
IM+
Blood MO+
SCD MO


IL-1b Secretion
2000
1500
1000
500
0
AM+
IM+
Blood MO+
SCD MO


IL-12 Secretion
2000
1500
1000
500
0
AM+
IM+
Blood MO+
SCD MO

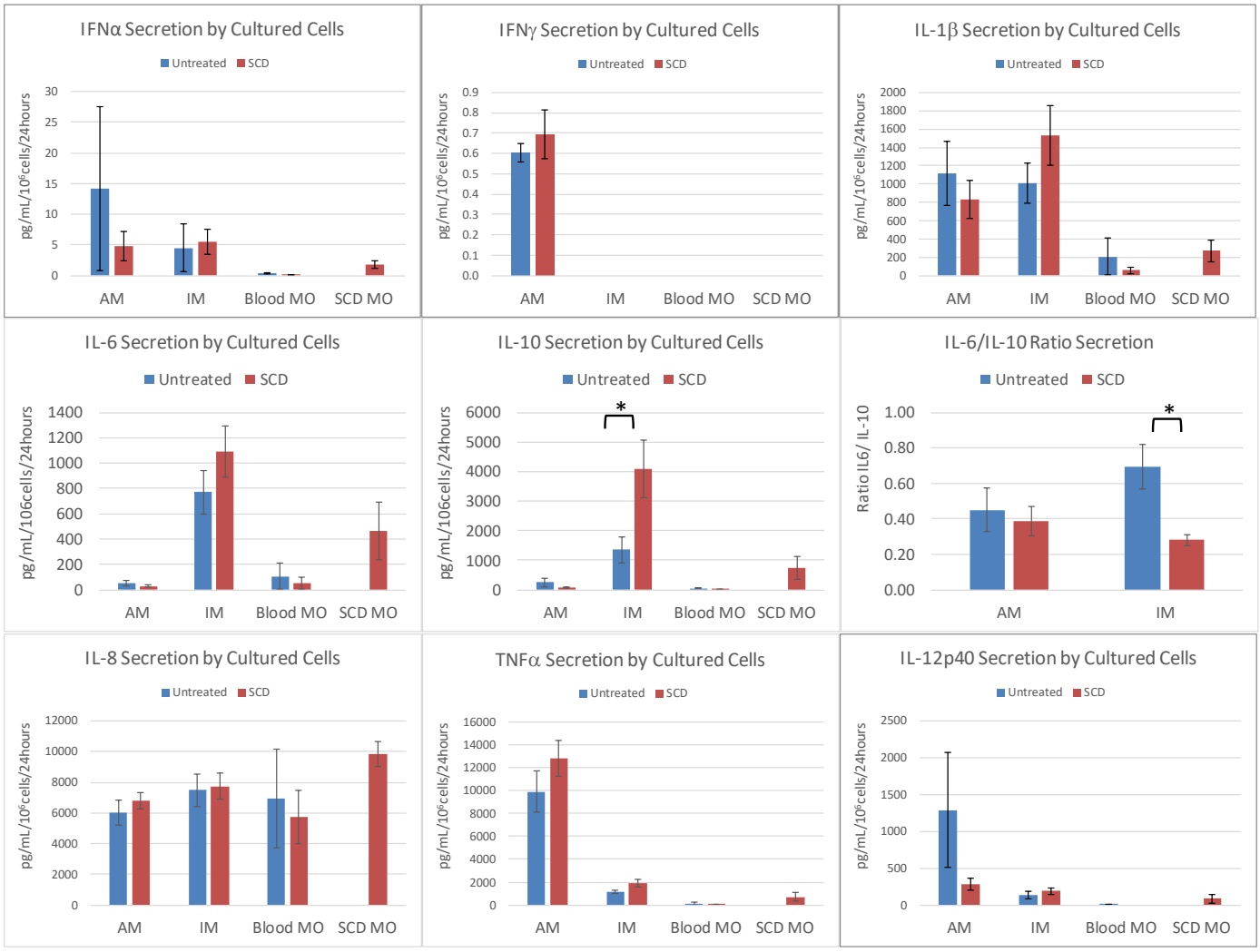


Figure 33. Secretory values of isolated cells of the monocyte and macrophage lineage collected at study termination. AM=alveolar macrophage, IM=interstitial macrophage, Blood MO= monocytes from systemic blood, SCD MO= monocytes from SCD membranes. Results are calculated as pg/mL/10^6 cells in 24 hours. A significant increase in IL-10 secretion was observed for interstitial macrophages of the SCD cohort (p=0.034). significant decrease in the IL6/IL-10 ratio was also observed (p=0.012). Untreated (Cohort 1, Blue, n=5) and SCD (Cohort 2, Red, n=6), mean± SE, significance $p<0.05$.

Monocyte/macrophage intracellular cytokine production was used to further assess the pro-vs. anti-inflammatory profiles. Cytokine expression under LPS stimulated conditions were evaluated in whole blood and dissociated lung cells (27). Intracellular cytokine labeling is accomplished using an Intrastain Kit (DAKO) on blood diluted 1:2 in media with brefeldin A to inhibit Golgi secretion [55]. Intracellular cytokine patterns are not directly correlative to secreted levels in isolated monocytes and macrophages in that the cell populations are not purified (remain mixed) and are stimulated for only 4 hours.

IL-6 secretion, a pro-inflammatory cytokine, trended lower in the SCD_{Rx} cohort compared to untreated for all cell populations. Meanwhile, the secretion of IL-10, an anti-inflammatory cytokine, by interstitial monocytes was significantly greater with SCD_{Rx} compared to the untreated cohorts. This observation is consistent with Luminex results on the isolated populations which also showed significantly higher IL-10 secretion by interstitial monocytes. Calculation of IL-6/IL-10 ratios enhances these differences, showing significance for both neutrophils and macrophages in the interstitial space. The relationship between IL-6 and IL-10 is shown graphically in Figure 34.

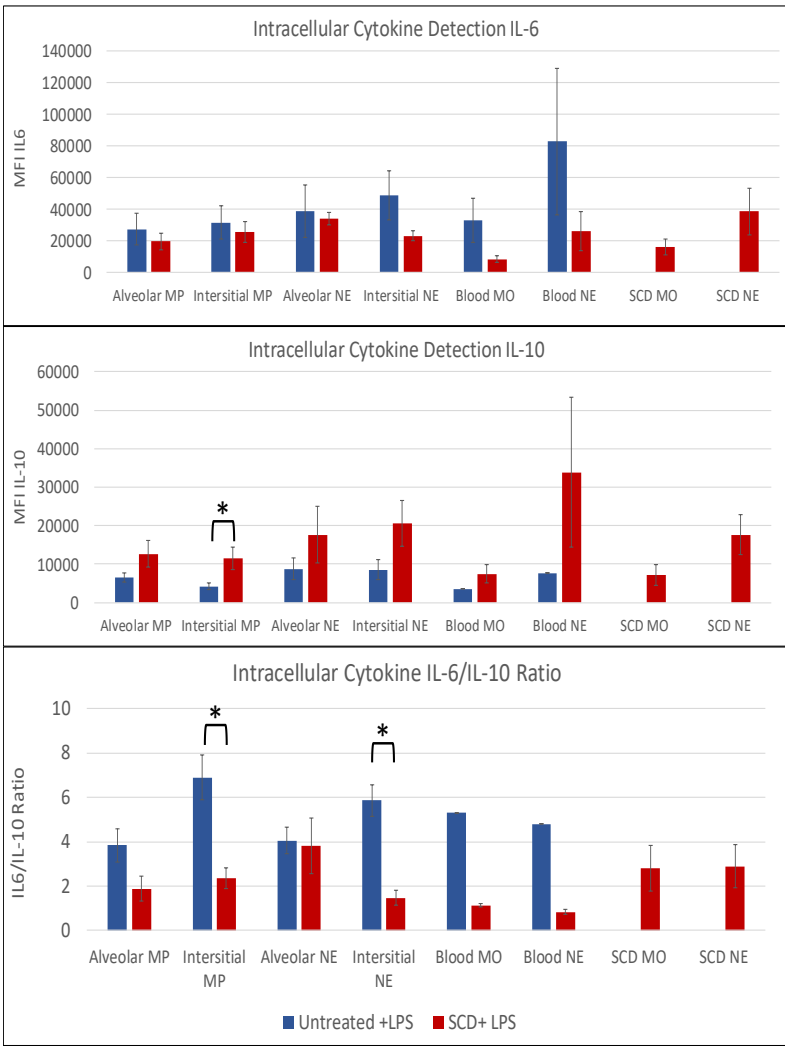


Figure 34. The relationship of IL-6 and IL-10 Intracellular cytokine levels in cells from lung, blood, and SCD membranes at study termination. Untreated (Cohort 1, Blue, n=5) and SCD (Cohort 2, Red, n=6), mean± SE, significance $p<0.05$.

For TNFα, the highest detection level was observed in cells eluted from the SCD membrane. As expected for IFNγ, detection was highest in neutrophil populations, but differences were not observed between cohorts (Figure 35).

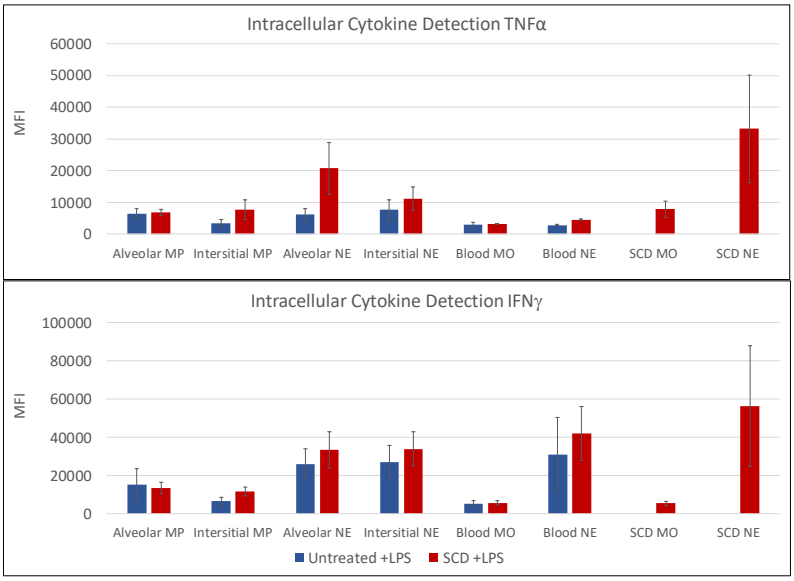


Figure 35. Intracellular detection of TNFα and IFNγ in cells from lung, blood, and SCD membranes at study termination. Untreated (Cohort 1, Blue, n=5) and SCD (Cohort 2, Red, n=6), mean± SE.

Analysis of animals from the untreated cohort 1 and SCD_{Rx} cohort 2 completed at this point were compelling in that even with a limited tool set, significant differences in the behavior of immune cells were observed. Should significant differences emerge, future work for peer reviewed publication will include correlation of secretory profiles in pig to surface markers and interpretation of these results in relation to the human immune system. The demonstration of anti-inflammatory immunomodulation by SCD_{Rx} during ALI/ARDS will support transition to clinical trials.

Major Findings:

- *Measurement cardiovascular and pulmonary parameters allowed for clinical assessment of animals.*
- *Complete blood counts with manual differentials were successful in the identification of immature NE and demonstration of a systemic inflammatory response.*
- *Cytokine analysis by Luminex resulted in appropriately scaled, interpretable results.*
- *The number of neutrophils in BAL and lung tissue did not correlate directly to lung function or pathologies but are indicative of lung inflammation.*
- *Morphometric scoring system was appropriate for evaluating tissues.*
- *Cytometric analysis of pig surface markers and intracellular cytokine levels detected changes in monocyte, macrophage and neutrophil behavior.*
- *The developed assay panel has revealed emerging trends towards differences in untreated (cohort 1) and SCD_{Rx} (Cohort 2) animals*

Milestone Achieved: Assays results allow for comparison between cohorts.

Activities: YEAR 3 (and the no cost extension period)

Work continued under Specific Aim 2: Assess efficacy of 24 hour SCD_{Rx} in ARDS porcine model

At the start of Year 3, the Year 2 results were critically reviewed. The results suggested that the developed pig ALI model was robust and suitable for finding signals for treatment effect with SCD_{Rx} in ARDS, however, the clinical time course achieved within the model was not of sufficient duration to allow for all 3 of the following conditions to be met which were needed to conduct studies in Cohort 3 :
1) waiting for clinically recognizable onset of ARDS *(which based on Pa:FiO2 definitions from humans could take up to 12 or more hours from the start of LPS infusion in the pig model)*
AND
2) then allowing sufficient duration of SCD treatment in the model *(believed to require >12 hours of continuous leukocyte processing activity to result in demonstrable benefit)*
AND
3) allowing for observation of a reversal of the disease process, *which may not be evident for hours to days, even if the device is highly effective.*

Extending the pig model beyond 24 hours post LPS infusion was not within the scope of the project due to the extensive resource costs and ethical cost of potentially prolonging animal suffering in a model that already required 48 hours of continuous nursing care of anesthetized pigs. However, it was determined that within the developed pig model, which uses a prolonged low dose LPS priming event followed by high dose LPS ARDS trigger, the SCD_{Rx} treated cohort already displayed a degree of reversibility of injury as treatment does not begin until greater than 12 hours after the start of LPS exposure. Thus, the intended milestones for the cohort were being met in current cohorts. For these reasons, testing in a cohort where SCD therapy is delayed until a clinical diagnosis of ARDS is met, as originally proposed, was removed from the study plan.

Year 2 results in the pig model clearly demonstrated that SCD_{Rx} potentially had significant beneficial treatment effects for patients at risk of or during early development of ARDS. Therefore, in order to properly promote SCD_{Rx} as a therapeutic option for ARDS, the therapy must be optimized for this indication. Increasing knowledge regarding the mechanism of action for the SCD has led to the understanding that this device, in part, alters the immune response to acute inflammatory insults by physical sequestration of subsets innate immune cells, primarily neutrophils. Data garnered through various pre-clinical work with this device supports the idea that the magnitude of physical binding of activated leukocytes during acute inflammation is proportional to the treatment effect. In theory, a device with a larger effective surface area for interaction with blood leukocytes will have greater therapeutic impact. This dose effect has been briefly explored (5), but much work remains to optimize dose of SCD_{Rx}, particularly for use in different indications. Since initiation of SCD treatment during ARDS was already being investigated in the current model, rather than initiate studies in the originally defined Cohort 3 efforts were redirected toward exploration of the dose effect of SCD_{Rx} based upon surface area to further assess the therapeutic benefit of SCD_{Rx} during ARDS.

The Year 2 animal studies were conducted using the clinically tested SCD-ARF, which has a lumen surface area of 1.0 m^2 (calculated ECS surface area of 1.4m^2). During Year 3 and the NCE period, studies were conducted using SCD with a larger lumen surface area of 1.8m^2 (calculated ECS surface area of 2.5m^2) which provides

considerably greater surface area for leukocyte interactions. For the conclusion of the project, Cohort 3 was redefined as animals treated with the larger 1.8 m^2 SCD devices. For these studies, SCD_{Rx} 1.8m^2 was initiated at the time of high dose LPS infusion as was done previously with the SCD 1.0m^2.

Subtask 1: Perform studies in each of the 3 cohorts

Five pig studies were completed in Y3Q1. Of these, a single animal was allocated to Cohort 1 (untreated) to provide a contemporaneous control and 4 pigs were allocated to the newly defined Cohort 3. Each of the studies proceeded as expected and no device related adverse events were observed. All 5 pigs survived to the 24 hour study endpoint. In Y3Q2-Q4, a major reduction in personnel resulting from a corporate restructuring of the awardee organization took place which limited work to *in vitro* assays and data analysis during this time. During resolution of the corporate issues, a No Cost extension was granted which allowed for completion of the work and an 8 additional animal studies to be conducted. Three of these pig studies did not yield data. ARDSp042 was euthanized during the overnight monitoring following the hemorrhage procedure due to refractory shock and ARDSp045 and ARDSp046 were excluded from the study immediately prior to high dose LPS infusion due to aberrant hemodynamic parameters suggestive of underlying illness or brain injury from the hemorrhagic shock which was not observed in prior animals. The remaining five studies generated usable data (3 in Cohort 1 and 2 in Cohort 3)

Data from the 10 successful Year 3 studies (6 pigs in Cohort 3 and 4 pigs in Cohort 1, see Table 12) were collated with all previous work under Specific Aim 2 and comparisons between cohorts were made to determine overall impact of SCD therapy during ALI and if significant improvement in treatment effects using SCD with a larger surface are of 1.8 m^2 were evident.

Hemodynamic data averaged per cohort are presented in Figure 36. Improved hemodynamic stability resulted lower fluid and pressor support requirements for SCD treated cohorts. A significantly higher cardiac index in the pigs that received 1.8 m^2 SCD_{Rx} was observed at several time points. The therapeutic impact resulting from the difference in hemodynamics between treatment cohorts is best reflected in the Vasopressor dependency index which was calculated hourly for each animal by tabulation of the doses of all administered vasoactive medications and dividing by the obtained mean arterial blood pressure (MAP) achieved at that time (48). All but one of the untreated pigs (89%) required administration of vasopressor medications to maintain minimum target hemodynamic values during the sepsis phase. In contrast, only fifty percent (50%) of the SCD_{Rx} pigs required any vasopressor support throughout the entire study period. The proportion of pigs requiring support was the same irrespective of the size of SCD used for treatment with 3 out of 6 animals in both the 1.0m^2 SCD and 1.8m^2 SCD cohorts requiring pressors. The pressor dependency index scores revealed that pigs treated with SCD required lower doses /less pressors as well as less fluid boluses to maintain target hemodynamic values (Figure 36).

Table 12. Pig studies conducted during FY03 and NCE to fulfill Specific Aim 2

PIG ID#	Cohort	LPS Strategy	Signs of ALI	Pa:FiO2	Study End	Comments
ARDSp035 CVVH +SCD 1.8m2	**3**	Ultra low LPS dose at 0.063ug/kg/hr overnight starting 6hrs post trauma Day 1 = 15ug/kg/hr x 3 hr	none	> 400 throughout	**24 hrs**	Stable made urine throughout
ARDSp036 CVVH + SCD 1.8m2	**3**	Ultra low LPS dose at 0.063ug/kg/hr overnight starting 6hrs post trauma Day 1 = 15ug/kg/hr x 3 hr	? Pmax ? lung complience	consitantly greater than 300	**24 hrs**	VERY stable. No additional fluids or pressores required.
ARDSp037 CVVH	**1**	Ultra low LPS dose at 0.063ug/kg/hr overnight starting 6hrs post trauma Day 1 = 15ug/kg/hr x 3 hr *VERY respsonsive to LPS (low +hi)*	Low compliance throughout study	Pa:Fi 300-400 most of study.	**24 hrs**	CV support required red belly fluid (no diarrhea) Made <5 ml urine per hour
ARDSp038 CVVH + SCD 1.8m2	**3**	Ultra low LPS dose at 0.063ug/kg/hr overnight starting 6hrs post trauma Day 1 = 15ug/kg/hr x 3 hr	minimal signs ALI	> 400 throughout	**24 hrs**	Required some fluids and pressors
ARDSp039 CVVH + SCD 1.8m2	**3**	Ultra low LPS dose at 0.063ug/kg/hr overnight starting 6hrs post trauma Day 1 = 15ug/kg/hr x 3 hr	increased Pmax	> 400 throughout	**24 hrs**	making A LOT of urine throughout
ARDSp040 CVVH + SCD 1.8m2	**3**	Ultra low LPS dose at 0.063ug/kg/hr overnight starting 6hrs post trauma Day 1 = 15ug/kg/hr x 3 hr	no signs of ALI	> 400 throughout	**24 hrs**	Initial hemodynamic decline but stabilized with fluids and pressors
ARDSp041 CVVH + SCD 1.8m2	**3**	Ultra low LPS dose at 0.063ug/kg/hr overnight starting 6hrs post trauma Day 1 = 15ug/kg/hr x 3 hr	flask pulmonary edema early on resolved	several drops to to mid 300s	**24 hrs**	Sensitve and crashed several times but did not require fluids or pressors
ARDSp042	**Euthanized early**	Ultra low LPS dose at 0.063ug/kg/hr overnight starting 6hrs post trauma Day 1 = 15ug/kg/hr x 3 hr	NA	NA	NA	Euthanized for refractory shock post trauma
ARDSp043 CVVH	**1**	Ultra low LPS dose at 0.063ug/kg/hr overnight starting 6hrs post trauma Day 1 = 15ug/kg/hr x 3 hr	minimal signs of ALI but decline toward end of study	fell to mid 300s but mostly >400	**24 hrs**	minimal added support
ARDSp044 CVVH	**1**	Ultra low LPS dose at 0.063ug/kg/hr overnight starting 6hrs post trauma Day 1 = 15ug/kg/hr x 3 hr	Pa:Fi declining increased Pmax edematous lungs with foamy fluid	declining to <300 prior to death	**3.75 hrs**	Died from progressive shock
ARDSp045	**EXCLUDED**	Ultra low LPS dose at 0.063ug/kg/hr overnight starting 8 hrs post trauma	NA	NA	NA	abberant hemodynamics prior to LPS
ARDSp046	**EXCLUDED**	Ultra low LPS dose at 0.063ug/kg/hr overnight starting 6hrs post trauma	NA	NA	NA	abberant hemodynamics prior to LPS
ARDSp047 CVVH	**1**	Ultra low LPS dose at 0.063ug/kg/hr overnight starting 8 hrs post trauma Day 1 = 15ug/kg/hr x 3 hr	? Pmax with thick phlegm	fell to mid 300s but mostly >400	**24 hrs**	Cardiac dysfunction with crash and pressor support. RBCs in abdmonial fluid

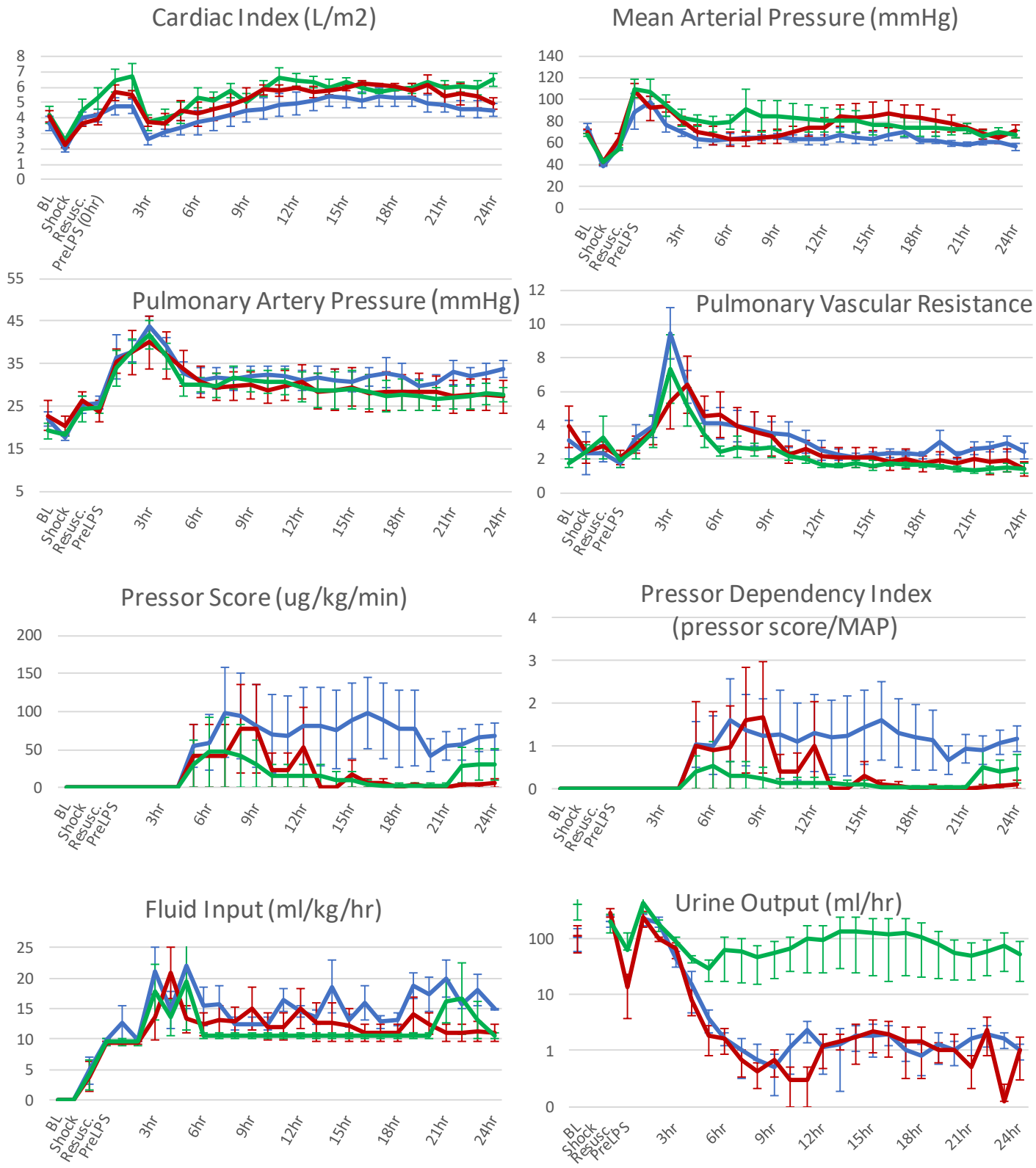


Figure 36. Clinically relevant hemodynamic data observed in ARDS pig model. Untreated (Cohort 1, Blue, n=6), SCD 1.0m2 (Cohort 2, Red, n=6), and SCD 1.8m2 (Cohort 3, Green, n=4) mean± SE

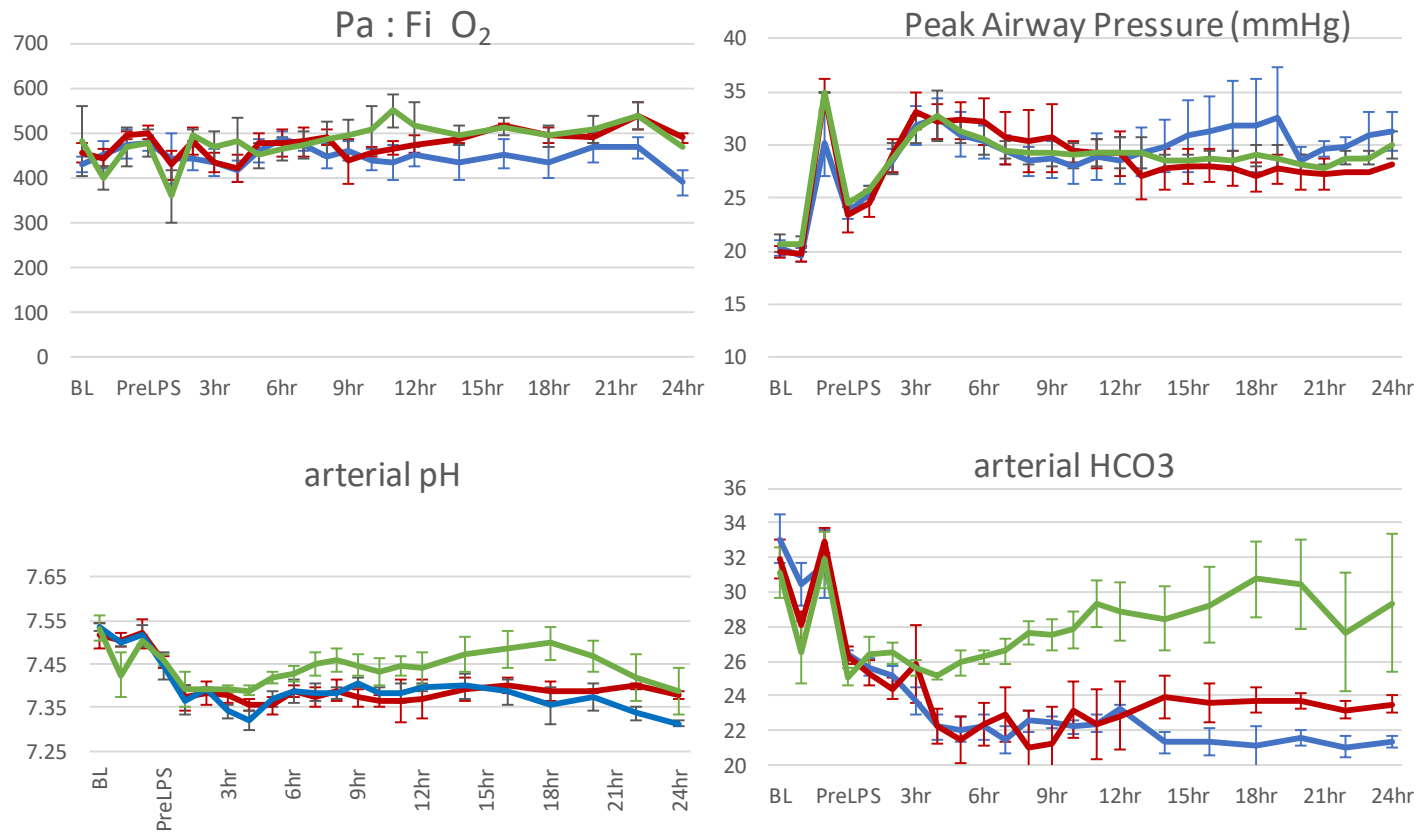


Figure 37. Clinically relevant pulmonary parameters observed in the ARDS pig model. Untreated (Cohort 1, Blue, n=5) SCD 1.0m2 (Cohort 2, Red, n=6) and SCD 1.8m2 (Cohort 3, Green, n=4), mean ± SE.

Pulmonary Parameters

The previously observed differences between the treatment cohorts in several clinically utilized pulmonary parameters (Figure 37) endured with use of SCD 1.8m^2, but were not significantly improved over what had been observed with SCD 1.0m^2. Lower pulmonary artery pressures through the study period were observed with SCD therapy, this difference being statistically significant at 24hr. Overall, arterial oxygen levels were not different between cohorts, possibly due to the individualized adjustment of ventilator settings to maintain reasonable ventilation. However, a trend for lower peak airway pressures and better maintained dynamic compliance of the pulmonary system was observed with SCD$_{Rx}$.

Close evaluation of all blood gas parameters measured using the i-STAT point of care analyzer (Abbot) did however reveal several important differences with the use of SCD 1.8m^2. The serum bicarbonate level was better maintained commensurate with higher pH of arterial blood post LPS administration. For this cohort, these values were more similar to preinjury values. This finding is likely reflective of the improved cardiovascular status of these animals and indicative of better preservation of renal function. Anuric renal failure was observed in all but 1 pig in both Cohorts 1 and 2 (with total urine outputs typically falling to <5 mL/hour) and was the reason continuous renal replacement therapy using continuous venovenous hemofiltration (CVVH) was added to the model. Four of the six pigs treated with SCD 1.8m^2 produced urine over the entire study. Pig035 maintained output >1 ml/kg/hr, Pig039-041 maintained near normal diuresis of 2-4 ml/kg/hr. This is especially noteworthy when considering that the pigs in this cohort had lower fluid inputs, receiving only the prescribed maintenance fluids for the majority of the study period.

Subtask 1 : Conduct studies in all cohorts

Major Findings:

- *SCD_{Rx} resulted in greater hemodynamic stability during the septic shock phase and improved pulmonary function over untreated pigs.*
- *A dose effect of SCD_{Rx} was observed with even greater hemodynamic stability with use of SCD 1.8m^2.*
- *Renal function was preserved with use of SCD 1.8m^2.*

Milestones Achieved:

- *The pig model continued to reliably demonstrate ALI (most visible in the in untreated cohort) based upon a decrease in Pa:FiO2 and negative changes in other pulmonary parameters.*
- *> 80% of pigs survived >12 hours allowing for sufficient duration of SCD therapy and for comparisons between cohorts.*
- *SCD_{Rx} was successfully administered in the re-defined cohort 3 (SCD1.8m^2).*

Subtask 2: Perform all measurements and assays required to assess key endpoints and exploratory endpoints.

Arterial blood drawn into EDTA tubes and submitted to interrogation by a Hemavet® automated hematology analyzer provided complete blood counts to evaluate changes in leukocyte number. Differential cell counts were manually verified by microscopic evaluation of blood smears under oil immersion.

The leukogram changes during the post trauma period remained representative of a low-grade inflammatory insult with increased immature neutrophil counts and decreased monocyte counts observed post resuscitation (data presented in sections under Year 1 and 2). Upon administration of high dose LPS infusion, a dramatic decrease in white blood count was observed. This decrease is observed for all leukocyte subsets. The disappearance of these cells from the circulation during the LPS infusion and the hours thereafter was attenuated by use of SCD_{Rx} and a trend for a greater effect with SCD 1.8m^2 was detected (Figure 38, top panel)
This attenuation was especially notable when evaluating neutrophils. In cohort 3, neutrophils begin to remerge into the systemic circulation sooner including an earlier emergence of immature neutrophil forms (a.k.a. “bar” or “band” neutrophils). It was also discovered that, with1.8m^2 $SCD_{Rx,}$ the neutrophil numbers did not rebound as high as seen in the untreated group (9.1 ± 1.1cells/µL versus 16 ± 1.9 cells/µL, respectively). A trend for leveling off or even resolving of the acute neutrophilia was seen with use of SCD 1.8m^2 as immature neutrophil counts for this cohort were statistically lower than the untreated cohort at 18 and 24 hours ($p<0.5$ for each time point, Figure 38, bottom panel).

Figure 38. Changes in leukocyte counts in relation to administration of high dose LPS from ARDS pig studies to date. LPS administration results in a profound decrease in circulating leukocyte counts which persists for many hours followed by a rebound and dramatic increase in leukocyte counts. SCD therapy attenuates this decrease with SCD 1.8m2 resulting in earlier resolution of leukopenia and reduced rebound leukophilia, especially in number of band neutrophils. Untreated (Cohort 1, Blue, n=5), SCD 1.0m2 (Cohort 2, Red, n=6), SCD 1.8m2 (Cohort 3, Green, n=6) mean± SE. * indicates significant differences between cohort 1 and 3 ($p<0.05$) at the end of the study period.

Note: For clarity, several early timepoints which also had significant differences between the SCD treated and untreated groups are not identified in these graphs.

Monocyte numbers for cohorts 2 and 3 trended lower that seen in the untreated animals during hours 6-18 (not shown). When coupled with the observed alteration in monocyte immunologic profiles described later in this section, this observation may be of clinical importance.

Arterial blood was drawn into EDTA tubes and processed to obtain plasma at regular intervals. Plasma from baseline (immediately post arterial access, Day 0), pre-LPS (immediately post initiation of high dose LPS on Day 1), 2hr, 4hr, and 6hr, 12hr, 18hr and 24hr was analyzed by Luminex for porcine proteins (IFNα, IFNγ, IL-1b, IL-6, IL-8, IL-10, IL-12p40, and TNFα), using the Cytokine & Chemokine 9-Plex Porcine ProcartaPlex™ Panel (ThermoFisher, EPX090-60829-901). The assayed values and standard error for all assayed values are shown in Table 13. IFN is not expected to be increased with this type of insult but it was included as part of the commercial assay and as expected, most concentrations observed were just at or below the detection level of the assay and no differences in interferon levels were observed between cohorts (although a pig in Cohort 1 had high baseline levels which remained elevated over the entire study). Because we had confirmed the inflammatory response to the trauma and resuscitation procedures in the early work, we did not analyze baseline samples for the last subset of pigs as this would have necessitated purchase of an additional assay kit in order to run all the samples. While the variability and inequity in sample number led to apparent differences at baseline, these differences disappeared following the consistent injury performed during the trauma phase and the PRE-LPS values were not statistically different between groups except for IL-1b. Moreover, the increase in the pro-inflammatory cytokines, such as IL-6, reflective of the injury induced during the first phase of the two-hit model is dwarfed by the massive increase seen following infusion of high dose LPS. TNFα spiked at 2 hours while other analytes peaked from 4-6 hours following high-dose LPS infusion.

Cytokine patterns are complex and despite countless clinical reports have not consistently been shown to be predictive of outcomes (51), but systemic IL-6 concentrations and IL-6/IL-10 ratio have been found to have prognostic value in the overall outcome of sepsis and injury induced SIRS (52, 53). In this pig model, trends in these cytokine levels were observed. Following LPS infusion, the average cytokine levels for untreated pigs and for pigs treated with SCD 1.0m^2 appeared quite similar, however, for animals treated with SCD 1.8m^2, the average concentrations for the proinflammatory cytokines IL-6 as well as TNFα were found to be lower. In fact, most cytokine levels, both pro and anti-inflammatory, were found to be lower in this cohort, although due to individual animal variability and relatively small cohort sizes we were unable to demonstrate statistically significance differences except in IL-12. This was an interesting finding in that the main physiological producers of IL-12 are phagocytes (monocytes/MΦ and neutrophils) and DCs in response to pathogens (bacteria, fungi, intracellular parasites and viruses) through Toll-like receptors (TLRs) and other receptors, to membrane-bound and soluble signals from activated T cells and NK cells, and to components of the inflammatory extracellular matrix. IL-12 is important in the differentiation of the immune response to trigger a more cell mediated attack on the invading pathogen (as opposed to antibody mediated). IL-12 belongs to a larger family of cytokines and is closely related to Il-23 and IL-27, which are involved in granulopoiesis (production and release of more leukocytes). Because of this interesting association and the corroborating evidence that the neutrophilic response to LPS was attenuated by treatment with SCD 1.8m^2, we assayed plasma for the presence of IL-23 (ElabScince E-EL-P1109) and Granulocyte Colony Stimulating Factor (G-CSF, ElabScince E-EL-P1320). Results are depicted in Figure 40. While G-CSF remained relatively low with no discernible difference between treatments, a very interesting pattern was observed for IL-23 that mirrored the serum levels of IL-12. Following LPS bolus, a rapid increase in IL-23 levels was observed in Cohorts 1 and 2

but this spike was attenuated in pigs treated with SCD 1.8m^2. These findings coupled with the rest of leukocyte analysis provide substantive insight to the mechanism of action of $SCD_{Rx,}$, demonstrating that through its binding and modulation of circulating leukocytes, which appears to be dose dependent, the systemic release of pro-inflammatory and granulopoitic cytokines in attenuated, which in turn reduces recruitment and activation of cellular effectors. Furthermore, based on lung histology, and the reduction in number of pigs that developed anuric renal failure, this immune modulation leads to a reduction in tissue injury.

One other notable finding was the pattern of IL-8 which had an early high spike and rapid disappearance of this cytokine for cohort 3. This was a slightly different pattern than was observed in the other cohorts. IL-8, also known as neutrophil chemotactic factor, has two primary functions. It induces chemotaxis in target cells, primarily neutrophils but also other granulocytes, causing them to migrate toward the site of infection. IL-8 also stimulates phagocytosis once they have arrived. As this cytokine is implicated in neutrophil activity, the finding suggests alteration of the acute immune response to LPS with use 1.8m^2 SCD_{Rx} which is supported by finding lower numbers of neutrophils in the lung tissues (see results from enzymatic digestion of lung tissue) and decreased migration of these cells into the airway based upon BAL. Neutrophils are believed to be central mediators of the pathogenesis of ALI, with activation of these cells responsible for much of the “bystander damage” from release of additional inflammatory mediators, proteases and reactive oxygen species. Thus, attenuation of the neutrophil response early in ALI using a 1.8m^2 SCD could conceivably lessen disease severity and progression.

The average concentrations for the serum cytokine levels have also been graphed for easier comparison in Figure 39.

Table 13: Systemic Plasma Pig Cytokine and Chemokine Concentrations as Assayed by Luminex

Cytokine		Baseline	Pre-LPS	2	4	6	12	18	24
IL-1b (pg/mL)	CNTL	6.29	4.30	10.71	50.75	52.11	13.39	8.67	1.75
	se	1.57	1.42	3.05	11.88	15.08	4.88	2.60	1.13
	SCD 1.0 m2	12.72	7.55	16.98	63.91	93.23	23.42	10.07	3.50
	se	6.07	0.72	2.68	11.77	36.35	5.67	3.44	1.27
	TTEST	0.329	0.103	0.173	0.465	0.272	0.216	0.755	0.350
	SCD 1.8 m2	33.45	15.34	23.85	35.72	23.13	5.39	10.07	3.65
	se	10.81	5.82	11.09	16.32	10.11	2.00	3.37	1.78
	TTEST	0.015	0.045	0.196	0.459	0.165	0.203	0.748	0.371
IL-4 (pg/mL)	CNTL	4.45	3.24	2.86	3.13	2.86	2.26	2.81	2.35
	se	1.04	1.12	0.92	0.85	0.94	0.73	0.76	0.84
	SCD 1.0 m2	5.40	4.96	4.08	4.40	3.68	3.48	4.00	4.60
	se	0.81	0.78	0.55	0.46	0.42	0.51	0.00	1.15
	TTEST	0.490	0.241	0.294	0.230	0.466	0.231	0.280	0.129
	SCD 1.8 m2	18.35	3.81	4.13	5.78	2.63	2.66	5.36	2.70
	se	6.16	2.59	1.36	1.90	1.39	1.22	2.51	0.78
	TTEST	0.025	0.827	0.427	0.181	0.887	0.761	0.317	0.761
IL-6 (pg/mL)	CNTL	22.12	23.54	548.66	869.33	716.08	474.90	268.41	35.11
	se	21.72	13.78	73.09	135.34	122.43	93.90	86.99	16.24
	SCD 1.0 m2	0.20	2.70	465.30	947.65	862.94	635.36	124.68	5.72
	se	0.20	0.66	94.84	131.83	198.27	265.81	82.29	2.96
	TTEST	0.337	0.244	0.493	0.699	0.520	0.513	0.313	0.216
	SCD 1.8 m2	49.34	34.46	441.86	523.08	467.41	229.79	55.25	58.29
	se	30.77	24.78	97.11	120.37	126.90	115.18	22.25	21.58
	TTEST	0.477	0.683	0.387	0.097	0.191	0.122	0.072	0.401
IL-10 (pg/mL)	CNTL	10.32	26.91	2618.91	3063.83	533.56	61.76	2.26	23.75
	se	6.45	21.60	627.83	872.47	131.04	44.49	2.26	23.75
	SCD 1.0 m2	43.96	15.22	1739.56	2185.19	583.17	71.24	4.13	0.00
	se	25.95	3.68	339.57	434.94	200.21	62.21	4.13	0.00
	TTEST	0.237	0.672	0.306	0.453	0.832	0.901	0.675	0.479
	SCD 1.8 m2	93.70	13.03	2200.80	1988.31	687.99	10.62	0.00	0.00
	se	93.70	9.18	806.49	1061.87	408.84	10.62	0.00	0.00
	TTEST	0.296	0.626	0.686	0.448	0.692	0.351	0.407	0.377

Cytokine		Baseline	Pre-LPS	2	4	6	12	18	24
IL-12 (pg/mL)	CNTL	225.45	231.81	609.02	2120.08	1857.25	1106.79	723.42	286.54
	se	43.70	48.80	115.17	486.73	327.56	230.02	210.04	95.58
	SCD 1.0 m2	295.81	301.83	621.24	2827.22	2842.61	1534.90	410.29	176.56
	se	45.13	60.48	124.91	498.39	405.40	648.17	83.96	22.96
	TTEST	0.289	0.383	0.945	0.346	0.080	0.476	0.317	0.421
	SCD 1.8 m2	399.42	217.89	341.48	700.51	584.24	289.45	276.54	197.08
	se	43.85	42.72	78.49	253.65	162.56	133.58	24.36	59.52
	TTEST	0.028	0.844	0.110	0.044	0.009	0.016	0.104	0.462
IL-8 (pg/mL)	CNTL	4.95	4.28	1805.29	2263.88	507.16	7.62	2.45	0.00
	se	2.08	1.97	999.80	1217.50	171.60	3.67	2.45	0.00
	SCD 1.0 m2	4.24	5.98	1237.66	1474.20	654.10	37.25	0.26	0.00
	se	2.49	3.86	338.08	341.26	424.28	33.75	0.26	0.00
	TTEST	0.831	0.673	0.662	0.615	0.729	0.285	0.528	NA
	SCD 1.8 m2	82.46	6.12	3165.64	1215.64	101.52	0.51	3.07	1.02
	se	22.12	2.50	1353.09	398.20	60.11	0.51	1.25	0.65
	TTEST	0.002	0.569	0.424	0.509	0.073	0.125	0.843	0.113
IFNa (pg/mL)	CNTL	3.53	5.92	4.20	5.72	5.85	8.02	12.29	8.24
	se	3.32	5.72	3.78	4.67	5.42	7.39	11.43	7.50
	SCD 1.0 m2	0.25	0.50	0.85	1.06	0.76	0.45	0.35	0.22
	se	0.07	0.23	0.45	0.55	0.32	0.11	0.08	0.02
	TTEST	0.346	0.433	0.463	0.411	0.406	0.413	0.463	0.417
	SCD 1.8 m2	0.34	0.15	0.76	1.26	0.13	0.12	0.17	0.11
	se	0.03	0.05	0.36	0.73	0.05	0.04	0.02	0.04
	TTEST	0.464	0.404	0.451	0.431	0.352	0.346	0.381	0.304
TNFa (pg/mL)	CNTL	4.30	28.36	2066.87	361.20	110.12	20.82	2.07	0.00
	se	2.42	26.26	452.88	121.70	49.86	16.40	2.07	0.00
	SCD 1.0 m2	10.94	0.89	1972.18	250.87	53.48	23.09	6.65	0.00
	se	4.94	0.89	387.21	109.05	16.69	20.60	6.65	0.00
	TTEST	0.255	0.388	0.885	0.538	0.363	0.933	0.444	NA
	SCD 1.8 m2	757.28	8.05	950.34	144.74	83.35	17.34	69.04	0.00
	se	694.24	7.77	741.91	123.08	67.80	11.29	69.04	0.00
	TTEST	0.207	0.529	0.195	0.251	0.750	0.874	0.306	NA

Table 13 continued. Cytokine & Chemokine 9-Plex Porcine ProcartaPlex™ Panel (ThermoFisher, EPX090-60829-901) Untreated (Cohort 1, Blue, n=9) SCD 1.0m2 (Cohort 2, Red, n=6), SCD 1.8m2 (Cohort 3, Green, n=6), mean± SE, Significant differences are highlighted as red text significance $p<0.05$. *IFNg is not included in table because values fell below assay detection range.*

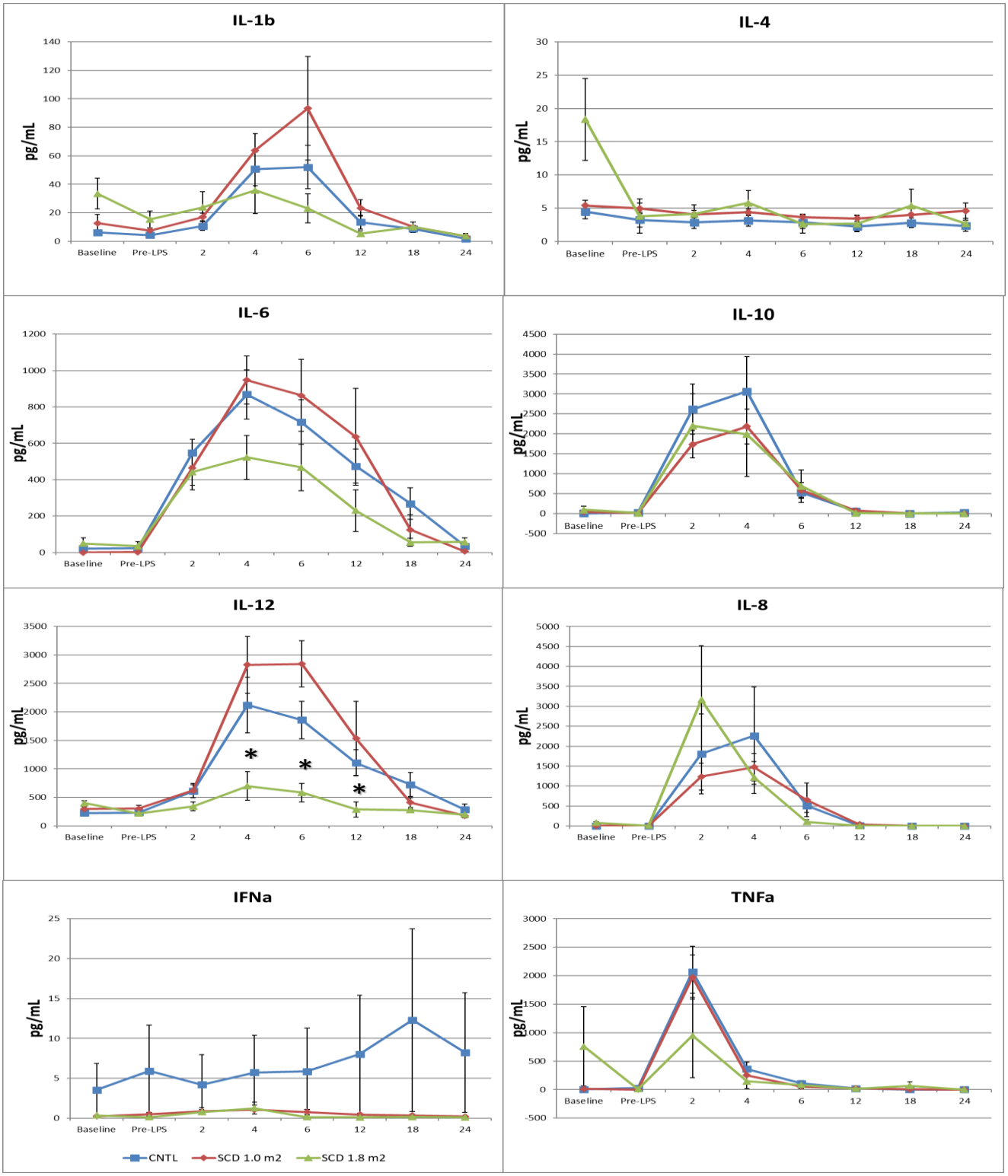


Figure 39. Graphic representation Pig protein levels detected by Cytokine & Chemokine 9-Plex Porcine ProcartaPlex™ Panel (ThermoFisher, EPX090-60829-901). Untreated (Cohort 1, Blue, n=9) SCD 1.0m2 (Cohort 2, Red, n=6), SCD 1.8m^2 (Cohort 3, Green, n=6), mean± SE. A trend for lower cytokine levels in Cohort 3 was observed, but this difference only reached statistical significance with IL-12. ($p<0.05$)

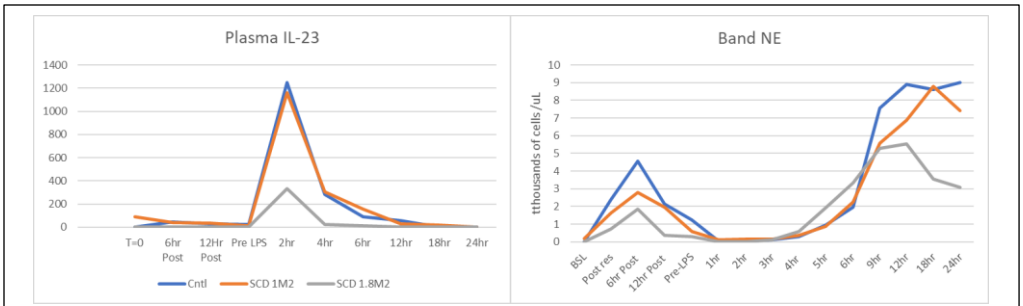


Figure 40. Plasma levels of the granulopoitic cytokine IL-23 were much lower with SCD1.8m^2 (p=0.01) which translated to lower recruitment of circulating band neutrophils.

Cytometric Analysis

All cytometric analysis for both blood and lung cells was performed on an Attune (ThermoFisher) flow cytometer, equipped with the following lasers: 488 nm blue, 405 nm violet laser, and 633 nm laser. Data were collected using Attune software (ThermoFisher) with automatic compensation. Samples were taken for single channel CD11R3 analysis on neutrophils gated by scatter profiles at Day 0 Baseline, Day 1 Pre-LPS and hourly through 6 hours. A full analysis panel to evaluate monocyte subsets was performed at Day 0 Baseline, Day 1 Pre LPS, 6hr, 12hr, 18hr, and 24hr, and then on cells eluted from SCD membranes for the SCD treated cohorts. The antibody panels used to analyze cells from the lungs, blood and SCD membranes are shown in Table 14. Evaluation of neutrophil, monocyte and macrophage populations may provide insight to the transition from neutrophilic alveolitis to monocytic alveolitis (19). A gating hierarchy was confirmed during FY01 work for lungs and systemic blood included: CD11R3, CD284 (toll-like receptor 4), and S(swine)LA DR

Table 14. Antibody Panels used for Blood and Lung

Systemic Blood- Monocyte Surface Characterization and MO And NE Activation

Analyze CD11R3 and CD284 in neutrophils, and CD11R3,CD284 and SLA DR II in all MO and MO subpopulations, (CD14+CD163-, CD14+CD163+, CD14low CD163+).

Vendor	Label	Titrated Amount	laser/fluor
ABDSerotec	CD11R3 (2F4/11)	0.5ug/5uL	BL1-FITC
ABDSerotec	CD163 (2A 10/11)	0.5ug/5uL	BL2-PE
ABDSerotec	CD172a (BL1H7)/SWC3	0.05ug/0.5uL	BL3 PERCP Cy5.5
ABDSerotec	SWC8 (MIL2) (concentration not provided)	5uL	unconjugated
ThermoFisher Scientific	anti MO IgM PE-CY7(eB121-15F9)	1.25ug/2.5uL	BL4 PE-CY7
ABDSerotec	CD14 (tuk4)	1ug/10uL	RL1-Alexa Fluor 647
ABDSerotec	SLA DR Class II (2E9/13)	0.5ug/5uL	RL3-APCCy7
Novus	CD284 (TLR4) HTA125	0.8ug/1uL	VL1-BV421
ThermoFisher Scientific	LIVE/DEAD® Fixable Aqua Dead Cell Stai	1uL	VL2-405/aqua

Macrophage Surface Characterization and Activation

Analyze CD11R3, SLA DR II and CD284 in macrophages from dissociated lung tissue and BAL.

Vendor	Label	Titrated Amount	laser/fluor
ABDSerotec	CD11R3(2F4/11)	0.5ug/5uL	BL1-488/FITC
ABDSerotec	CD163(2A 10/11)	0.5ug/5uL	BL2-PE
ABDSerotec	CD172a (BL1H7)/SWC3	0.05ug/0.5uL	BL3 PERCP Cy5.5
ABDSerotec	CD203a SWC9 (PM18-7)	0.25ug/2.5uL	BL4-PECy7
ABDSerotec	CD14 (TUK4)	1ug/10uL	R1-Alexa Fluor 647
ABDSerotec	SLA DR Class II(2E9/13)**	0.5ug/5uL	RL3-APCCy7
Novus	CD284 (TLR4) HTA125	0.8ug/1uL	VL1-BV421
ThermoFisher Scientific	LIVE/DEAD® Fixable Aqua	1uL	VL2-405/aqua

Whole Blood ICC. Monocyte Surface Characterization and Intracellular Cytokines

Analyze Cytokines in all MO and MO subpopulations, (CD14+CD163-, CD14+CD163+, CD14low CD163+).

Vendor	Label	Titrated	laser/fluor
ABDSerotec	CD172a (BL1H7)/SWC3	0.05ug/0.5uL	BL1-FITC
ABDSerotec	CD163(2A 10/11)	0.5ug/5uL	BL2-PE
ABDSerotec	SWC8 (MIL2) (concentration not provided)	5uL	unconjugated
ThermoFisher Scientific	anti MO IgM PE-CY7(eB121-15F9)	2.5ug/1.25uL	BL4 PE-CY7
ABDSerotec	CD14 (MIL-2 or TUK4)	1ug/10uL	RL1-Alexa Fluor 647
R&D	IL-10 (262715) or IFN-g (154007)*	0.5ug/5uL	BL3 PERCP Cy5.5
R&D	IL-6 (77830) or TNFa (103302)*	0.5ug/5uL	VL1-Dylight405
ThermoFisher Scientific	LIVE/DEAD® Fixable Aqua	1uL	VL2-405/aqua

Lung Macrophage ICC. Surface Characterization and Intracellular Cytokines

Analyze Cytokines in macrophages from BAL and dissociated lung tissue.

Vendor	Label	Titrated	laser/fluor
ABDSerotec	CD172a (BL1H7)/SWC3	0.05ug/0.5uL	BL1-FITC
ABDSerotec	CD163(2A 10/11)	0.5ug/5uL	BL2-PE
ABDSerotec	CD203a SWC9 (PM18-7)	0.25ug/2.5uL	BL4-PECy7
ABDSerotec	CD14 (MIL-2 or TUK4)	1ug/10uL	RL1-Alexa Fluor 647
R&D	IL-10 (262715) or IFN-g (154007)*	0.5ug/5uL	BL3 PERCP Cy5.5
R&D	IL-6 (77830) or TNFa (103302)*	0.5ug/5uL	VL1-Dylight405
ThermoFisher Scientific	LIVE/DEAD® Fixable Aqua Dead	1uL	VL2-405/aqua

II MFI in macrophages, neutrophils, monocytes and monocyte subsets (CD14+ CD163+, CD14+ CD163, CD14low CD163+). Anti-CD203 (SWC9) is used to positively identify alveolar macrophages (20, 21) and is included in the antibody panel used to analyze single cell suspensions of lung cells. Antibody to CD14 labels pig monocytes at variable intensity through maturation and is also found on porcine neutrophils to a lesser degree. Antibody to CD163 is used as a porcine monocyte maturation marker (22) and is highly expressed on a subset of monocytes and all macrophages. SLA DR Class II is differentially expressed on all cells of interest but may be shed as cells become anergic (23). Antibody to CD284 recognizes toll-like receptor 4 which can be differentially expressed via a wide range of stressors (24, 25). Using the panels shown in Table 14, macrophages, neutrophils, monocytes, and monocyte sub-populations were reliably identified. The identified populations were then evaluated for expression of CD11R3, SLA DR II and CD284 (TLR4).

Neutrophil and Monocyte Activation in Systemic Blood

Human neutrophils (14, 15) and monocytes (16, 17) mobilize intracellular stores of CD11b to the cell surface as they become (primed) activated, allowing a real-time measurement of systemic acute neutrophil (priming) and monocyte activation. For this study, the clone 2F4/11, reactive to human CD11c, was selected from panel of human reactive CD11 antibodies. This antibody was found to be reactive to a 155kD alpha chain and CD18/β2 integrin. In pigs, anti-human CD11b specific antibodies had positive reactivity to the 165kD alpha chain expected for CD11b, however, in pigs these antibodies are reactive only to granulocytes. Of the antibodies reactive to human CD11c, only clone 2F4/11 strongly labeled granulocytes, monocytes and alveolar macrophages, the expected expression pattern comparable to human CD11b. Because it is unclear whether the differences are due to species expression or differences in epitope recognition, the nomenclature CD11R3 was adapted (18). The clone was chosen for this project based upon its strong reactivity to cells of interest and detectable upregulation upon stimulation.

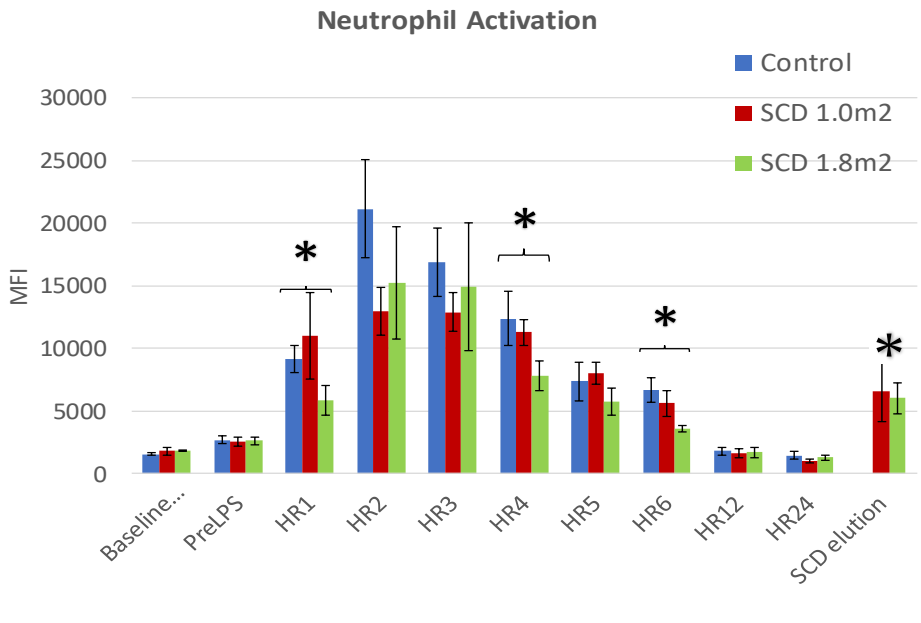


Figure 41. Neutrophil acute activation as detected by surface expression of CD11R3. Untreated (Cohort 1, Blue, n=9), SCD 1.0m2 (Cohort 2, Red, n=6), and SCD 1.8m2 (Cohort 3, Green, n=6), mean± SE, significance $p<0.05$.

As described during model development, the first hit of the two-hit model is detectable by increased neutrophil CD11R3 expression from D0 baseline to D1 Pre-LPS, with the average MFI CD11R3±SD significantly increasing from 1,691±512 to 2,618±727, p=0.0002 (average of all pigs studied). With high dose LPS injection, CD11R3 expression then increased dramatically concurrent with the decrease in systemic neutrophil numbers as these activated cells marginate and extravasate into tissues. Significantly lower CD11R3 expression levels on neutrophils in the SCD 1.8m^2 cohort compared to the untreated cohort were observed at 1,

4 and 6 hours after starting high dose LPS. This finding is compatible with the decreased severity of the hemodynamic response and attenuated disappearance of circulating leukocytes seen to high dose LPS in this cohort. As observed previously for the SCD 1.0m^2 treated cohort, CD11R3 expression by neutrophils eluted from SCD 1.8m^2 membranes at the study end (24 hours or death) was significantly higher than those in systemic blood at the time of device removal. This suggests that the most activated cells are being sequestered by the membrane. This data is shown graphically in Figure 41.

For monocytes, differences in CD11R3 expression on circulating cells were not observed between cohorts. However, in both the SCD treated cohorts, CD11R3 expression of monocytes eluted from SCD membranes at the study end (24 hours or death) was significantly higher than time matched monocytes in systemic blood (Figure 42). As seen with neutrophils, the SCD membrane appears to sequester the most activated cells, which is believed to result in the observed systemic effects of immune modulation.

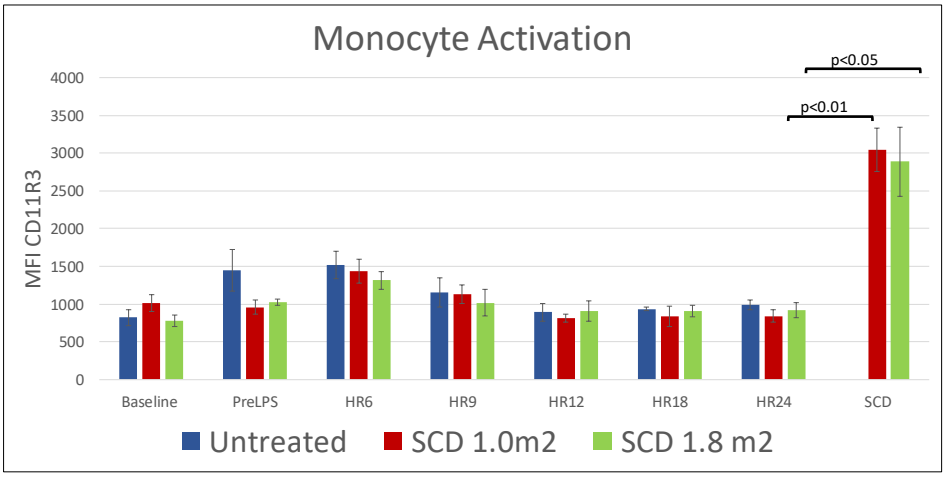


Figure 42. Monocyte activation as detected by surface expression of CD11R3. Untreated (Cohort 1, Blue, n=9) and SCD 1.0m2 (Cohort 2, Red, n=6), SCD 1.8m2 (Cohort 3, Green, n=6), mean± SE,

With high dose LPS infusion on Day 1, a dramatic decrease in all leukocyte absolute numbers is observed, but the monocytes remaining in circulation were only 20±4% CD163+ ($p<0.001$ compared to approximately 40% CD163+ at baseline). The percent of CD163+ cells then increases through the study time course. Using the MFI cut off value of 1000 to define %CD163+ resulted in no significant differences in circulating % CD163+ between untreated and SCD treated cohorts. It is however noteworthy that for both SCD treated cohorts, the percentage of CD163+ monocytes was significantly higher among cells eluted from the SCD membrane than was found among circulating cells at the time of removal of the device. This observation lends further support to the thesis that proinflammatory cells are selectively sequestered by the SCD and potentially result in immune modulation, even though differences in percentages of circulating cells were not readily apparent.

For pig, monocyte subsets have not been clearly defined by CD markers as compared to humans. Using available tools, a shift in monocyte phenotype has been detected in this ARDS model. The shift is most evident by CD163 expression. With LPS challenge, CD163+ cells leave the circulation and are replaced by CD14+ CD163- cells that then mature to CD14+CD163+. In humans, monocytes leave the bone marrow as CD14+ CD16-, and progress to CD14 low CD16+ cells. Depending on the maturation environment, some cells obtain the proinflammatory CD14+ CD16+ phenotype. These cells in human can be readily identified by strong human (H) LADR expression. In the pig model, (S)wine LADR expression increased with the trauma event, suggesting a pro-inflammatory state. A shift to a lower swine (S) LADR expression level was observed upon administration of LPS. Surprisingly, in SCD 1.8m^2 treated pigs, the (S) LADR expression level showed a trend for increasing toward the end of the study, although this was largely driven by one animal (p036), which had

much higher expression of all surface markers and approximately double (S) LADR expression than seen in any of the other pigs in any cohort. Overall, with or without data from pig036, any differences between cohorts were not significant and changes appeared to be reflective of the disease process. The collected cytometric data on peripheral blood and SCD eluted cells was further analyzed to assess changes in CD11R3, TLR4, and SLADRII and CD14 expression for CD163 +/- subsets and neutrophils but no other significant differences were observed.

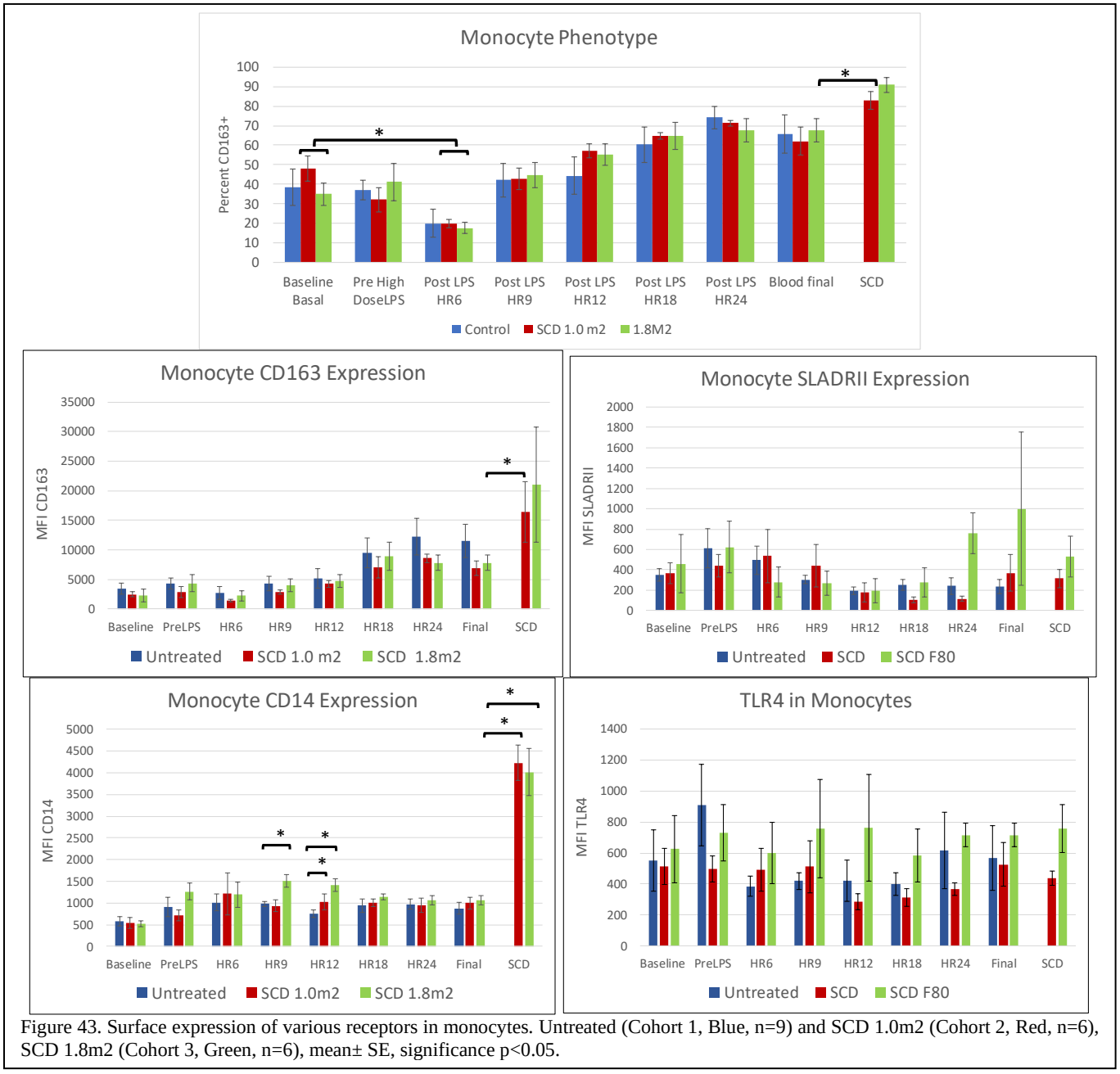
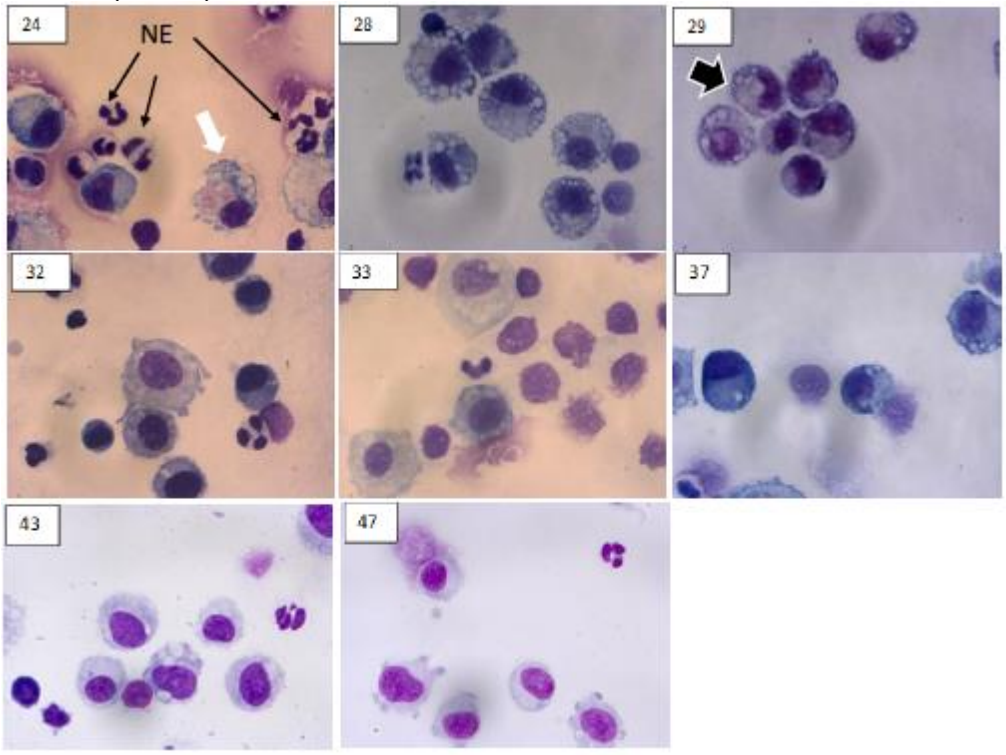


Figure 43. Surface expression of various receptors in monocytes. Untreated (Cohort 1, Blue, n=9) and SCD 1.0m2 (Cohort 2, Red, n=6), SCD 1.8m2 (Cohort 3, Green, n=6), mean± SE, significance p<0.05.

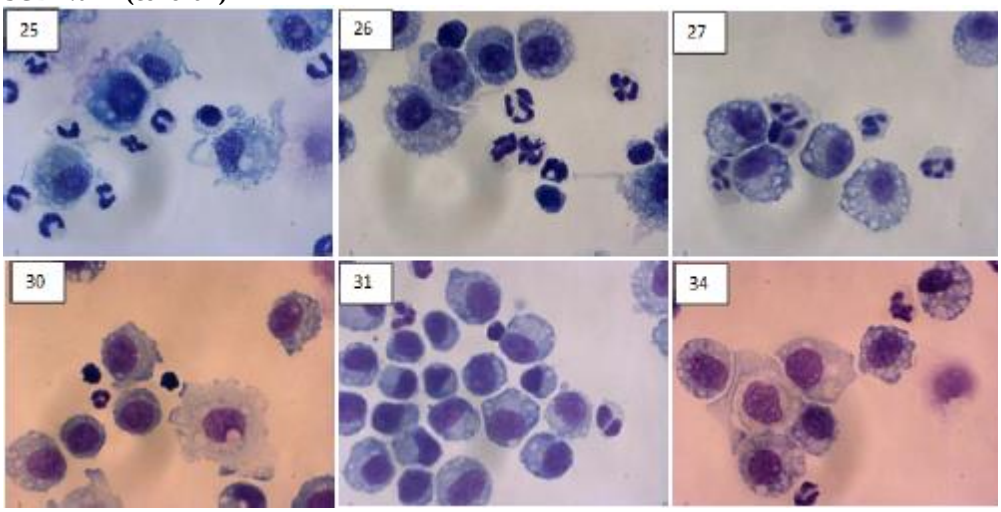
Evaluation of Lung Injury using Physical Parameters

Bronchoalveolar Lavage fluid (BALf) was obtained *post mortem* by the repeated instillation of saline supplemented with 0.2% EDTA into the right middle bronchus. Total cell counts and differentials, specifically for neutrophils relative to total counts, were determined from cytospins.

Untreated (cohort 1)



SCD 1.0m^2 (cohort 2)



SCD 1.8m^2 (cohort 3)

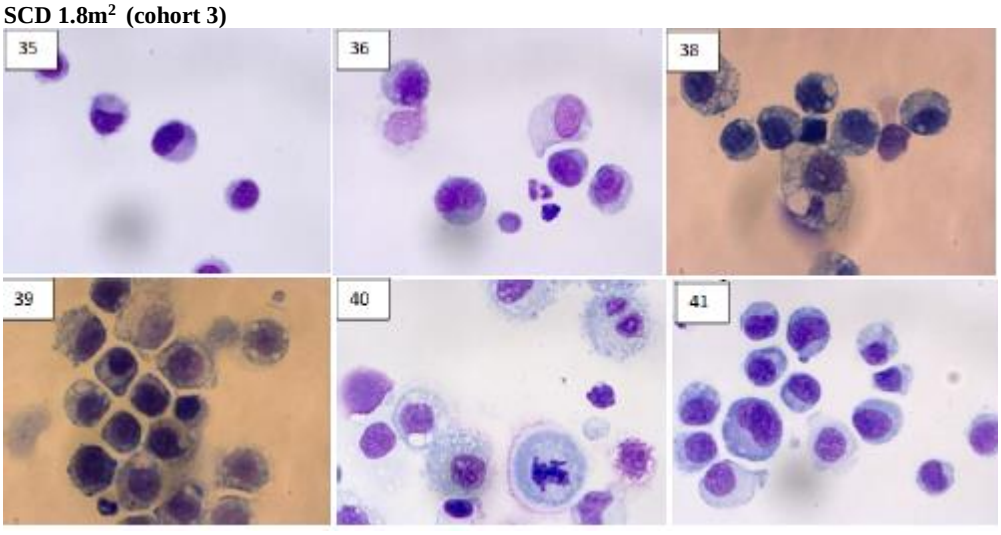


Figure 44. Representative microscopic images of BALf cytospins for individual studies.

BALf was centrifuged and supernatants assayed for total protein (BioRAD, Catalog#500-0116) to further determine the effects of neutrophil infiltration and extent of alveolar leak (32). BALf was assayed using the same Luminex panel as described for plasma (Cytokine & Chemokine 9-Plex Porcine ProcartaPlex™ Panel,ThermoFisher, EPX090-60829-901). Pulmonary edema of excised lungs was quantified by placing the entire left lobe into a Ninja food processor and processed until homogeneous. 1-2g samples weighed (wet weight) and dried until stable (dry weight) and then expressed as % water content (30, 31). The results for all animals used in data collection are shown in Figure 45 and Figure 46.

In ARDS patients, the concentration of neutrophils in the BALf correlates with severity of ARDS and outcome (40, 41). Normally, BALf is almost devoid of these cells. For all study animals, neutrophils were found in the BALf, and water content was higher than found for uninjured control animals, indicating that all study pigs had some degree of lung inflammation. This is especially apparent and noteworthy for protein, as BALf from uninjured lung should contain very little protein. Significant concentrations of protein were only found in untreated pigs. Overall, these disease markers trended much lower for pigs treated with SCD 1.8m^2.

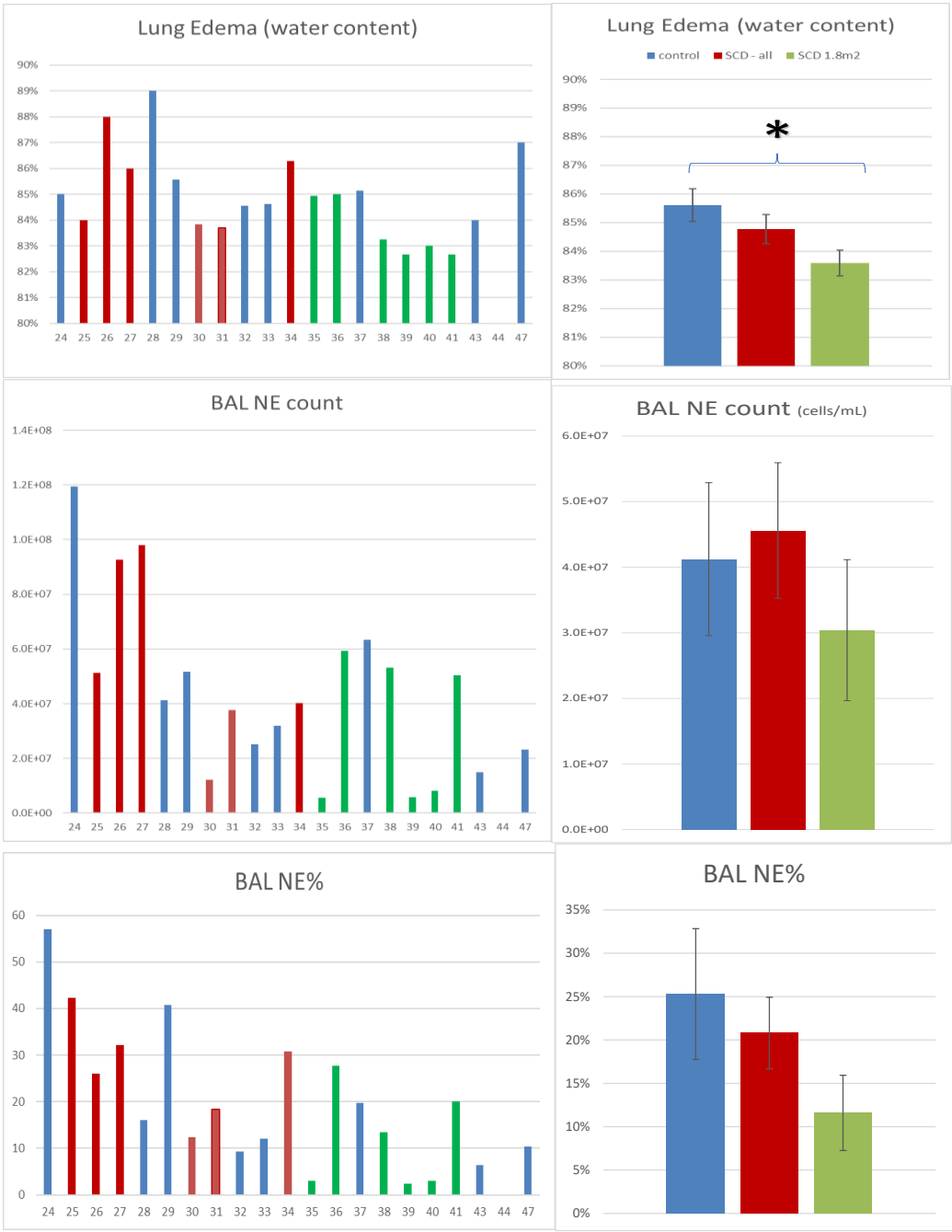


Figure 45. Evaluation of lung edema and presence of neutrophils (NE) in BALf from individual pigs on left and averages ± SE per treatment cohort on right. Edema is (wet weight-dry weight)/wet weight expressed as % water content and was found to be statistically lower when comparing the average all animals that received SCD 1.8m2 treatment to untreated. BAL NE count is the total number of NE recovered, %NE is % of total cells, Untreated (Cohort 1, Blue, n=8 samples) and SCD 1.0m2 (Cohort 2, Red, n=6), SCD 1.8m2 (Cohort 3, Green, n=6). A trend for lower amounts of NE in the SCD treated cohorts, especially for SCD 1.8m^2 was observed.

Figure 46. Levels of total protein and neutrophil -specific myeloperoxidase (MPO) in BALf from individual pigs on left and averages ± SE per treatment cohort on right. Only low amounts of protein were present in BALf of the SCD treated pigs. This difference was significant when comparing the average all animals that received SCD treatment to untreated (p=0.05). MPO activity was detected in several of the untreated pigs (Cohort 1, Blue, n=8) but only marginal MPO activity was observed in BALf from any of the SCD treated and SCD 1.0m2 (Cohort 2, Red, n=6), SCD 1.8m2 (Cohort 3, Green, n=6).

The detected cytokine and chemokine levels in the BALf were highly variable within cohorts. IFNα, IFNγ, IL-10, IL-4 and TNFα had many values below the detection level of the assay. IL-1b, IL-6, IL-8 and IL-12p40 were within the detectable range for most animals. BALf cytokine levels were not significantly different between cohorts, but average cytokine levels, for all but INFγ, were across the board lower for the SCD1.8m^2 treated cohort. Data is presented in Table 15.

Table 15. BALf Pig Cytokine and Chemokine Concentrations as Assayed by Luminex Cytokine & Chemokine 9-Plex Porcine ProcartaPlex™ Panel (ThermoFisher, EPX090-60829-901)

Untreated	IFN-alpha	IFN-gamma	IL1-beta	IL-10	IL-12p40	IL-4	IL-6	TNF-alpha	IL-8
p024 BALF	0.4504541	0.00	98.19576	16.86755	238.938	0.00	1113.735	162.5755	9265.094
p028 BALF	0.6003331	0.00	25.63703	1.86962	27.44501	14.0836	91.65048	0.00	877.373
p029 BALF	0.3505151	0.00	27.27148	1.86962	45.7	14.0836	22.84249	0.00	306.9356
p032 BALF	0.3505151	0.00	25.63578	0.00	41.15668	0.00	5.419462	0.00	1603.905
p033 BALF	65.476403	0.2902005	143.5736	0.00	186.7059	14.0836	244.2951	0.00	3460.829
p037 BALF	1.3618942	3.9031065	207.5385	393.1225	111.3073	1.827048	2074.617	772.1598	170.4085
p043 BALF	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	35.56211
p047 BALF	0.00	0.00	0.00	0.00	0.00	0.00	188.9625	41.56785	250.5729
av.	8.5737643	0.5241634	65.98151	51.71616	81.40663	5.509732	467.6903	122.0379	1996.335
SE	8.1303528	0.4840395	26.91179	48.81458	31.58297	2.519653	263.8623	94.98528	1113.762

SCD 1.0m2	IFN-alpha	IFN-gamma	IL1-beta	IL-10	IL-12p40	IL-4	IL-6	TNF-alpha	IL-8
p025 BALF	0.3004995	0.00	30.54638	0.00	9.161844	0.00	35.49388	0.00	422.0945
p026 BALF	1.1942961	0.2902005	153.9001	5.6	155.4795	0.00	1513.1	120.822	9475.422
p027 BALF	0.4004972	0.00	32.1811	0.00	9.161844	0.00	15.34873	0.00	390.5258
p030 BALF	0.4504792	0.1934729	17.44098	1.86962	18.30343	0.00	10.71042	12.76778	438.8046
p031 BALF	0.3504815	0.00	22.3595	0.00	9.161844	0.00	13.20779	0.00	319.941
p034 BALF	0.6502733	0.3869052	65.25658	0.00	66.28853	0.00	1112.099	12.76778	7960.092
av.	0.5577545	0.1450964	53.61411	1.24	44.59283	0.00	449.9932	24.39293	3167.813
SE	0.1365192	0.0695284	21.18936	0.922971	23.96091	0	277.6722	19.45414	1765.994
Ttest	0.41	0.52	0.74	0.39	0.40	**0.09**	0.96	0.40	0.57

SCD 1.8m2	IFN-alpha	IFN-gamma	IL1-beta	IL-10	IL-12p40	IL-4	IL-6	TNF-alpha	IL-8
p035 BALF	0.00	1.3464246	7.536757	74.55715	69.62419	1.317441	2.29461	69.471	15.3
p036 BALF	0.00	0.168526	9.960003	5.5	38.80747	0.425876	2.29642	31.66343	3.228295
p038 BALF	0.00	1.1783378	10.76816	0.00	59.21348	1.742252	3.439704	2.752276	67.56559
p039 BALF	0.07	4.7200921	14.04588	124.5128	90.46576	1.742252	4.589219	119.1649	7.256466
p040 BALF	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
p041 BALF	0.00	0.00	0.00	0.00	0.00	0.00	11.22523	0.00	6.237821
av.	0.01	1.2355634	7.0518	34.095	43.01848	0.871303	3.974197	37.17527	16.59803
SE	0.01	0.7382255	2.386609	21.69305	15.20997	0.338274	1.577673	19.78605	10.40548
Ttest	0.38	0.42	0.09	0.77	0.35	0.14	0.16	0.46	0.15

Levels of IL-6, an important pro-inflammatory cytokine, were low for each animal in the SCD 1.8m^2 cohort resulting in a much lower average value for this cohort (3.9±1.5 pg/mL), compared to untreated controls (467±263 pg/mL) as well as the SCD1.0m^2 cohort (449±277 pg/mL), each of which had animals with elevated values. Similarly, values for IL-8, which plays a role in attraction of neutrophils to areas of inflammation, was markedly lower in pigs treated with SCD 1.8m^2 (mean 16.5±10.4 pg/mL) compared to the other cohorts (untreated: 1996±113 pg/mL, SCD1.0m^2: 3167 ±1765 pg/mL). When considering cytokine levels along with the observed levels of protein and myeloperoxidase activity in the BALF, treatment with SCD1.8m^2 appears to result in immunomodulation detectable at the alveolar level. A protective effect of this immunomodulation is observed based upon the histologic injury scores described in the next section.

Morphometric Evaluation of Lung

The pathological hallmark of ALI is diffuse alveolar damage (DAD) (39). In humans, DAD is characterized by: neutrophil accumulation in the vascular, interstitial, and alveolar spaces (neutrophilic alveolitis); deposition of hyaline membranes as evidence that serum proteins have entered and precipitated in the airspaces (i.e., disruption of the alveolocapillary membrane); interstitial thickening; and formation of microthrombi. Morphometric analysis of lung pathology in pigs at 24h was performed using reported methodology (54) based on alveolar wall thickness, interstitial congestion, airway hemorrhage and protein accumulation and leukocyte infiltration. Lung tissue from the right diaphragmatic lobe was fixed in 4% paraformaldehyde, serially rinsed, and stored in ethyl alcohol prior to submission for sectioning, mounting and staining with hemoxylin and eosin (H&E). Photomicrographs were obtained from randomly selected areas of each prepared slide. Three high and three low magnification images from each animal were then randomly selected for evaluation. Scoring for lung

injury was performed independently by at least two lab personnel who were trained in the scoring system and blinded to pig identity and to treatment cohort. Individual scores were averaged to achieve a final score for each parameter for each animal. Results of this blinded scoring are shown in Figure 50.

Scores for each parameter were lower for SCD 1.0m^2 cohort and lower still for most categories in the SCD1.8m^2 cohort. Overall injury scores as assess by both the reported and the adapted method were significantly lower in both of SCD treated cohorts

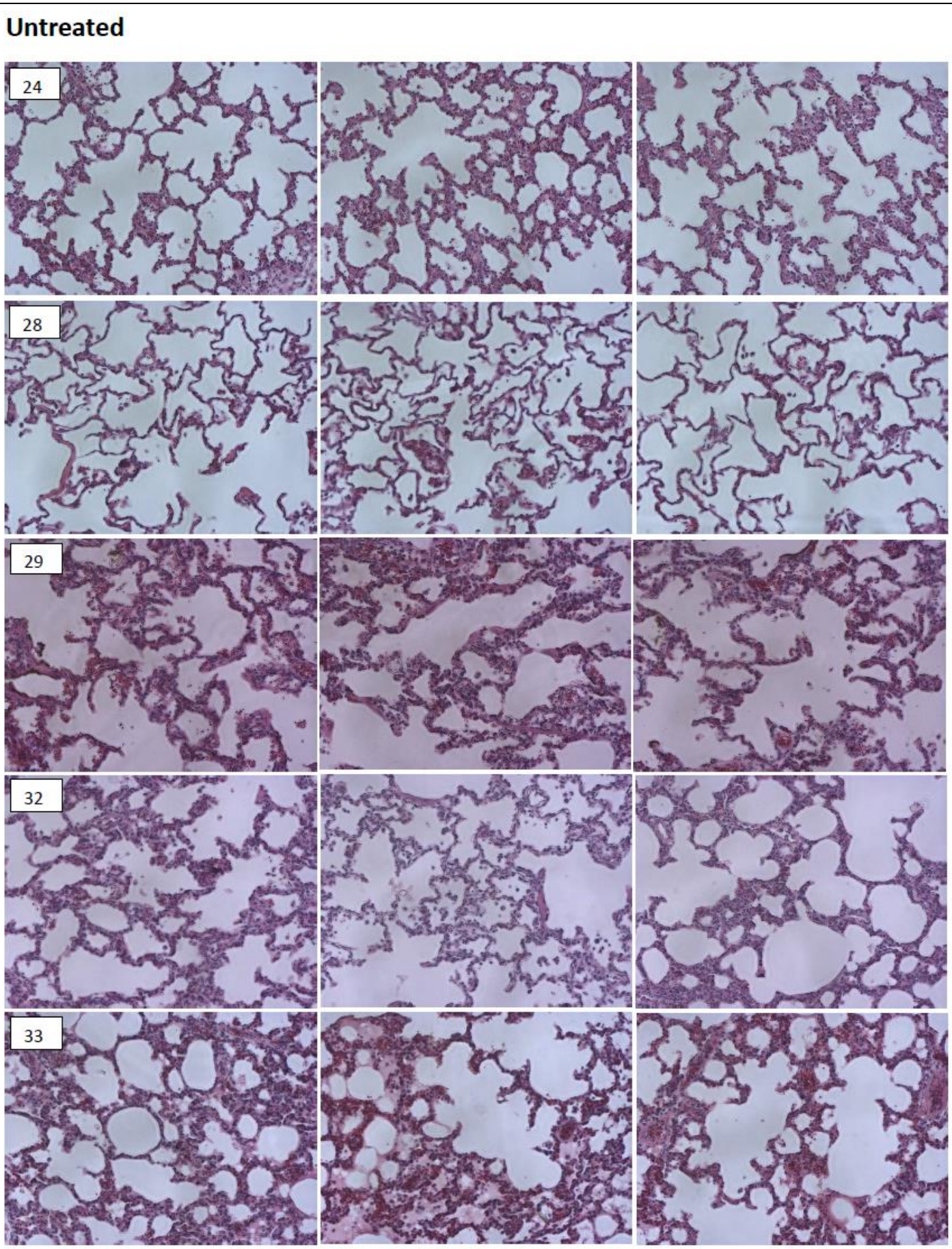


Figure 47. Representative microscopic images of H&E stained lung tissue from Untreated cohort.

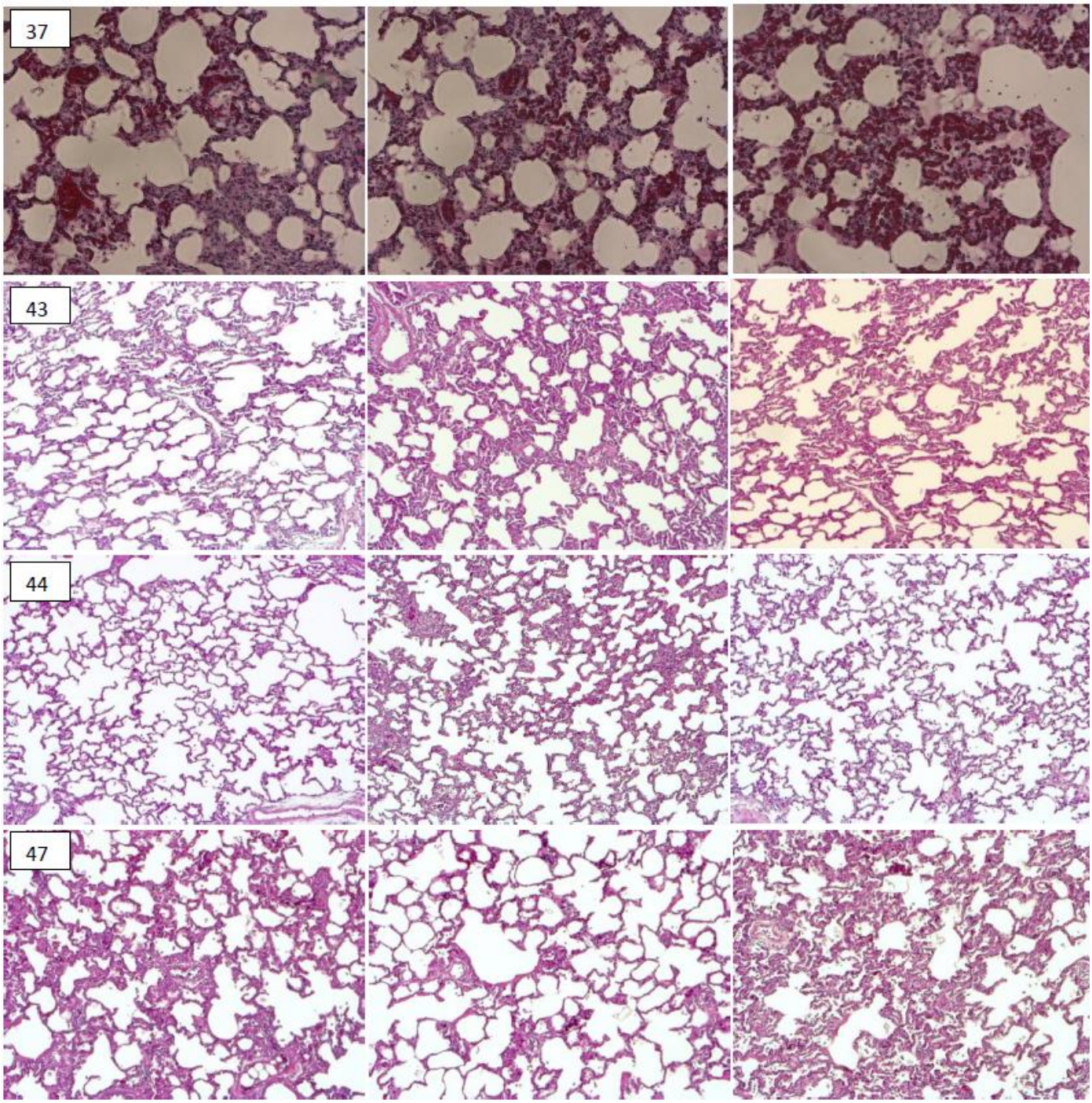


Figure 12 continued.

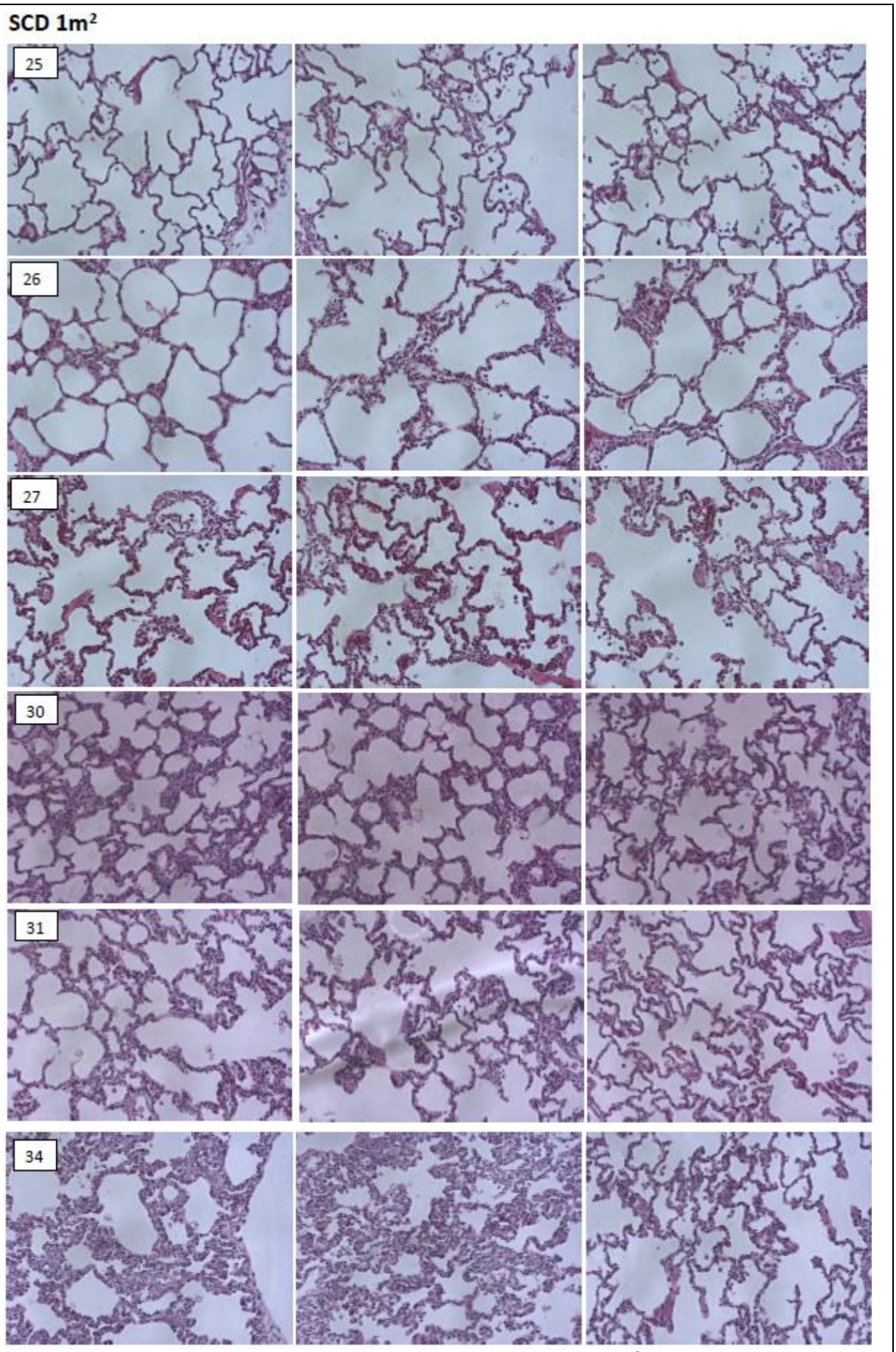


Figure 48. Representative microscopic images of H&E stained lung tissue from SCD1.0m^2 cohort.

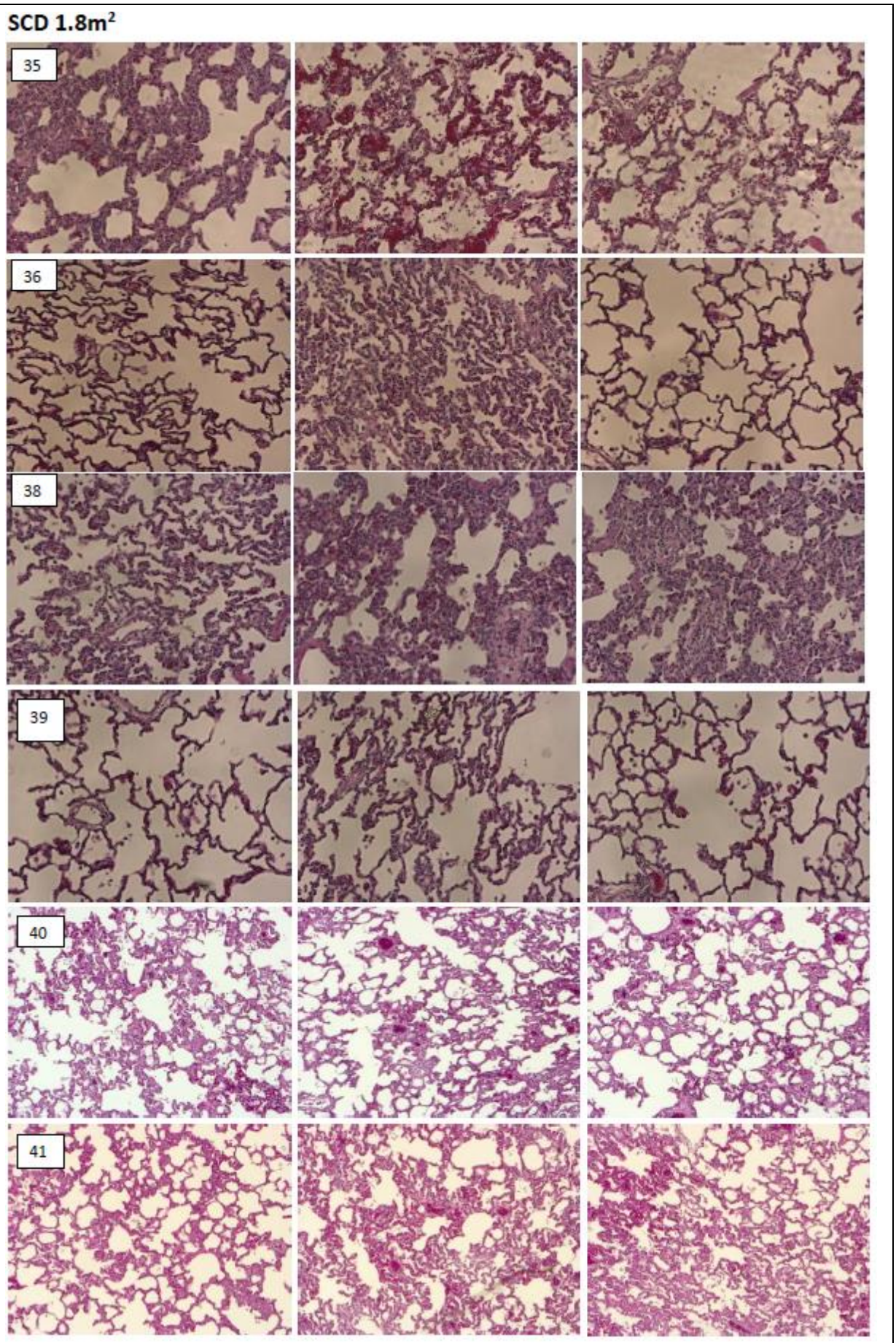
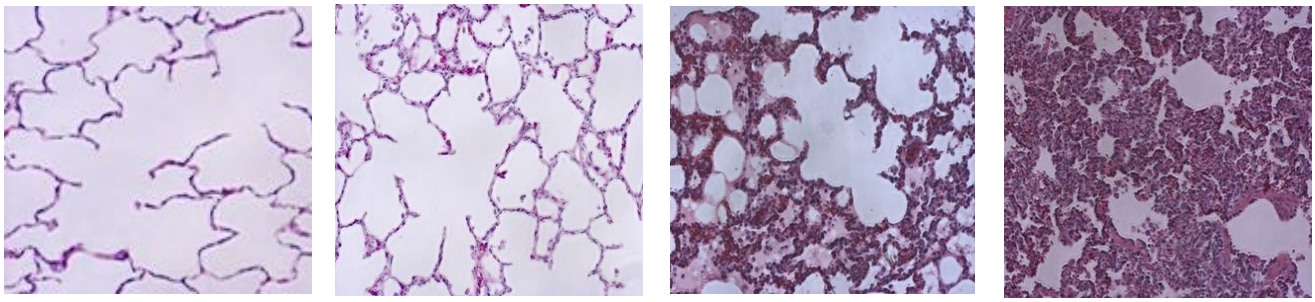


Figure 49. Representative microscopic images of H&E stained lung tissue from SCD1.8m^2 cohort.

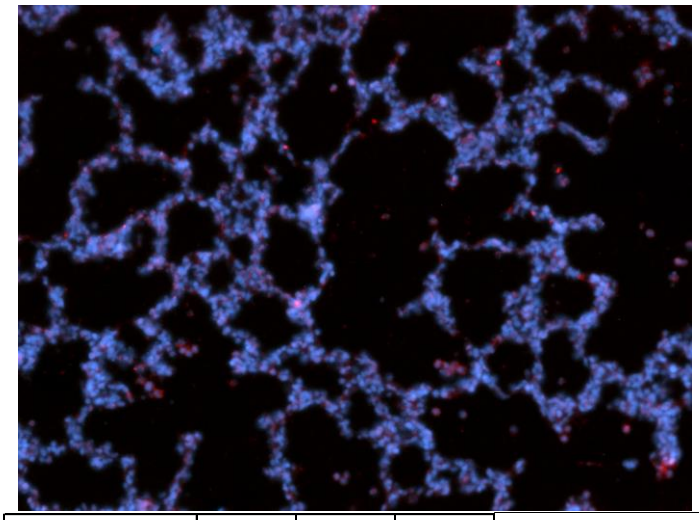


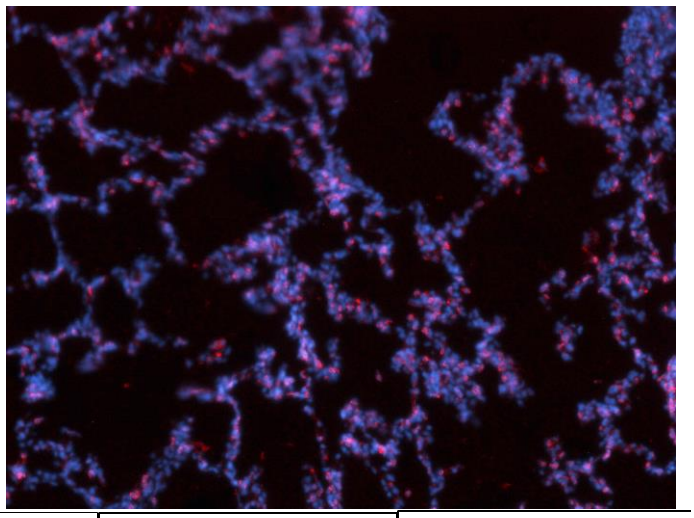
Normal lung | Mild scores 0-1 | Moderate scores 1-2 | Severe scores 3-4

	Animal ID	Atalectasis	Fibrin Deposits	Hem. In Airway Low Resolution	Congestion	Wall Thickness	LE infiltration quadrant	Hem in Airway High Resolution	LE infiltration count	Total Score A	Total Score B
Untreated	ARDSp024	2.17	1.83	0.83	1.67	2.33	2.33	2.67	2.67	11.17	13.33
	ARDSp028	0.33	2.83	1.50	1.83	1.00	1.00	1.50	0.83	8.50	8.33
	ARDSp029	2.50	1.33	2.50	2.33	2.67	2.83	1.33	2.50	14.17	12.67
	ARDSp032	1.67	1.33	1.33	0.83	2.17	3.00	0.50	2.83	10.33	9.33
	ARDSp033	3.00	3.00	2.33	2.50	3.00	3.50	1.17	2.33	17.33	15.00
	ARDSp037	1.33	1.17	0.17	2.83	2.17	2.83	0.17	2.33	10.50	10.00
	ARDSp043	1.83	0.50	0.67	1.83	2.25	2.08	0.50	2.13	9.17	9.04
	ARDSp047	2.08	1.08	1.33	2.17	1.50	2.17	1.00	2.00	10.33	9.83
	Average	1.86	1.64	1.33	2.00	2.14	2.47	1.10	2.20	11.44	10.94
	SE	0.28	0.31	0.28	0.22	0.22	0.27	0.28	0.22	1.03	0.85
SCD 1.0m2	ARDSp025	0.33	1.50	0.83	0.83	1.00	1.33	1.00	1.00	5.83	5.67
	ARDSp026	0.33	0.50	1.33	1.00	1.17	1.33	0.83	1.83	5.67	5.67
	ARDSp027	0.83	1.00	1.67	1.17	1.50	1.67	1.50	1.33	7.83	7.33
	ARDSp030	1.00	1.83	0.83	1.00	2.33	2.33	0.17	2.00	9.33	8.33
	ARDSp031	1.17	0.83	1.33	1.17	1.67	2.33	0.83	1.83	8.50	7.50
	ARDSp034	2.17	1.33	1.17	1.50	2.17	2.50	1.33	2.83	10.83	11.33
	Average	0.97	1.17	1.19	1.11	1.64	1.92	0.94	1.81	8.00	7.64
	SE	0.28	0.20	0.13	0.09	0.22	0.22	0.19	0.26	0.82	0.86
TTEST	eq var.	**0.024**	0.130	0.348	**0.003**	0.073	0.078	0.333	0.130	**0.015**	**0.010**
SCD 1.8m2	ARDSp035	1.00	0.83	1.17	1.83	2.00	2.33	0.83	3.00	9.16	9.49
	ARDSp036	0.50	0.00	0.00	0.83	1.17	1.67	0.17	2.00	4.17	4.67
	ARDSp038	1.50	0.17	0.17	1.50	1.67	2.33	0.67	3.17	7.34	8.68
	ARDSp039	0.00	0.17	0.17	0.50	1.00	0.83	0.50	1.83	2.67	4.00
	ARDSp040	1.58	1.33	0.50	2.42	1.75	2.08	0.17	1.42	9.67	8.67
	ARDSp041	2.33	1.42	0.83	2.75	2.00	2.42	1.25	2.00	11.75	11.75
	Average	1.15	0.65	0.47	1.64	1.60	1.94	0.60	2.24	7.46	7.88
	SE	0.34	0.26	0.18	0.36	0.17	0.25	0.17	0.28	1.41	1.21
TTEST	eq var.	0.066	**0.019**	**0.018**	0.189	**0.049**	0.095	0.088	0.463	**0.019**	**0.027**

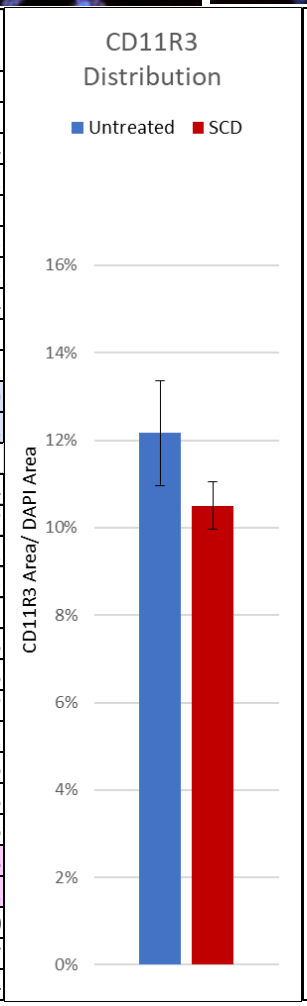
Figure 50. Morphometric scoring of lung H&E Images. Scoring was performed by 2 blinded investigators on 3 high magnification and 3 low magnification images from each animal then all scores for each parameter were averaged. Total Score A was calculated using a reported scoring method and Total score B is an adapted method based upon scoring at high and low magnifications. Significant differences ($p<0.05$) are highlighted by bold red font. Untreated (Cohort 1, n=8), SCD 1.0m2 (Cohort 2, n=6) and SCD 1.8m2 cohort 3, n=6)

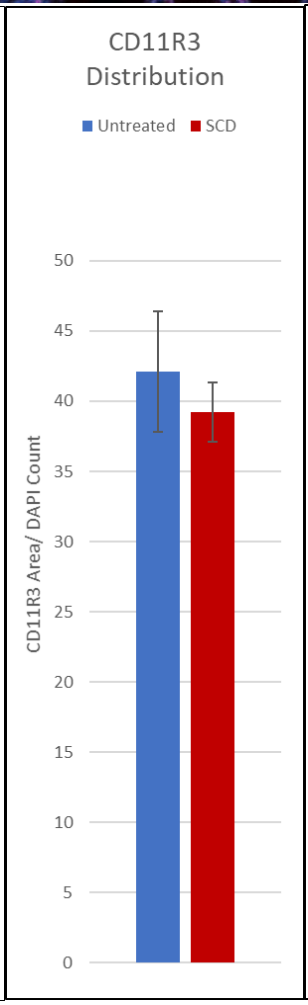
Immunohistochemistry (IHC) was used to assess leukocyte infiltration of lungs using CD11R3 (20, 55-57) a marker expressed specifically on activated leukocytes. A bronchus to the diaphragmatic lobe of the right lung was inflated with a 50/50 (v/v) optimum cutting temperature (OCT) compound (Tissue Tek)/PBS, via a cannula using a method similar to that described for BAL. Once inflated, the isolated lung lobe was placed into a pan on wet ice to allow it to become firm, then sections were cut and placed into cryomolds with OCT. Filled cryomolds were frozen in the vapor phase on a surface precooled with liquid nitrogen. Prepared blocks were sectioned using a Lecia cryostat and labeled with antibody to CD11R3 (BioRAD) and visualized using anti mouse IgG conjugated to Alexafluor 594 (Fisher Life Sciences). Images of processed tissue were captured and analyzed using equivalent settings across the entire project via NIH Image J software to provide a semi-quantitative measurement of CD11R3+ leukocyte (monocytes and neutrophils) infiltration of lung tissue. The images were then evaluated in three ways: 1) The total area of positive pixels for CD11R3 in the red channel normalized for the total area of positive pixels for DAPI in the blue channel (Area/Area), 2) the total Area of CD11R3+ cells normalized by total number of DAPI positive nuclei (Area/Count, and 3) the total number of CD11R3+ cells normalized by the total number of DAPI positive nuclei (Count/Count). Representative images are shown in Figure 51 for SCD_{Rx} animals that scored low (ARDSp026), and high (ARDSp30). During Year 2, a trend for a lower amount of CD11R3+ expression, by all 3 ways of quantitation was apparent in the SCD treated cohort. This trend was still apparent at the final analysis if you compare all SCD treated pigs to the untreated cohort. However, the marked variability between animals in each of the cohorts obscured any statistically significant differences.





Study ID	Area/ Count	Area/ Area	Count/ Count
Untreated			
ARDSp024	111.89	0.30	0.33
ARDSp028	62.11	0.21	0.24
ARDSp029	33.94	0.07	0.22
ARDSp032	44.65	0.16	0.22
ARDSp033	6.43	0.02	0.05
ARDSp037	45.50	0.10	0.25
ARDSp043	5.37	0.02	0.04
ARDSp044			
ARDSp047	27.05	0.10	0.15
Untreated	42.12	0.12	0.19
SE	4.28	0.01	0.01
SCD			
ARDSp025	62.66	0.19	0.24
ARDSp026	8.80	0.02	0.07
ARDSp027	24.61	0.06	0.21
ARDSp030	20.44	0.07	0.15
ARDSp031	37.43	0.11	0.23
ARDSp034	66.55	0.22	0.28
ARDSp035	30.12	0.07	0.18
ARDSp036	11.13	0.04	0.07
ARDSp038	74.20	0.16	0.31
ARDSp039	67.43	0.14	0.16
ARDSp040	58.59	0.14	0.18
ARDSp041	8.92	0.03	0.06
All SCD treated	39.24	0.11	0.18
SE	2.10	0.01	0.01
TTEST C vs all SCD	0.831	0.651	0.840
TTEST SCD1.0m2	0.748	0.849	0.837
TTEST C v SCD1.8m2	0.983	0.601	0.614





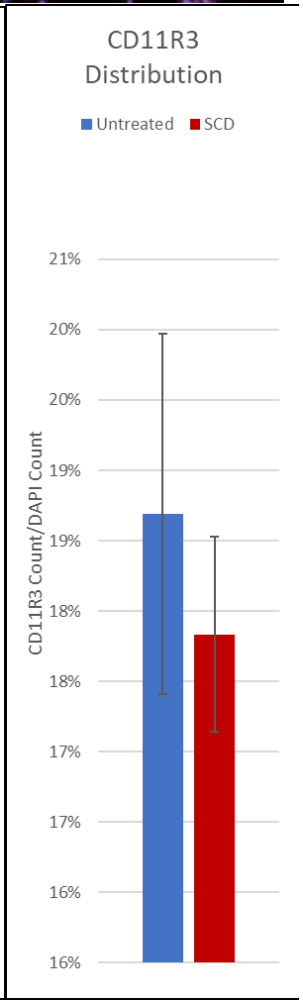


Figure 51. Representative images of lung tissue after immunohistochemical staining, Images from ARDSp026 (top left) and ARDSp030 (top right) provide comparison of low to high scoring images for presence of activated leukocytes, based on presence of CD11R3. CD11R3+ cells are red, and all cell nuclei are labeled with DAPI blue. A trend for lower CD11b+ cells in the SCD 1.0m2 treated cohort was observed and this trend continues if you compare all SCD treated animals to the untreated cohort. However, the marked variability between individual animals prevented detection statistically significant differences. Data from each animal is presented in the table below and the average for the cohorts is graphed for each method of comparison.

Cytometric analysis of lung composition.

Table 16. Distribution of CD172a+ myeloid derived cells in lung tissue evaluated by cytometry

	Enzyme Digested Lung Tissue Composition									
		From Cytospin					From flowcytometer with SWC8, %CD172+			
Control	Cells per gram	MP (%)	NE (%)	LY (%)	MO (%)	other (%)	CD203a+ CD163+ alveolarMP (%)	CD203- CD163+ Interstitial MP and MO (%)	MO	NE (%)
ARDSp024	1.49E+07	19.8	46.4	11.1	22.6	0.0	12.0	36.1	25.6	51.6
ARDSp028	1.69E+07	41.0	38.7	14.3	1.3	4.7	7.0	47.5	1.7	37.4
ARDSp029	1.76E+07	23.5	51.6	14.2	6.9	3.8	7.1	27.8	7.9	59.6
ARDSp032	7.02E+06	39.5	40.2	11.2	5.6	3.5	43.0	20.5	7.9	30.6
ARDSp033	7.39E+06	41.0	31.4	18.4	7.8	1.4	3.6	32.2	5.7	60.0
ARDSp037	5.50E+06	5.3	64.3	11.7	12.3	6.3	7.7	9.9	12.3	80.2
Control	**1.15E+07**	**28.4**	**45.4**	**13.5**	**9.4**	**3.3**	**13.4**	**29.0**	**10.2**	**53.2**
SE	**2.24E+06**	**6.0**	**4.7**	**1.1**	**3.0**	**0.9**	**6.0**	**5.3**	**3.4**	**7.3**
SCD 1.0m2										
ARDSp025	9.92E+06	24.58	45.85	16.94	10.96	0.00	12.9	30.8	13.5	48.2
ARDSp026	1.58E+07	13.4	49.3	17.6	19.0	0.0	2.7	36.3	22.8	61.1
ARDSp027	1.03E+07	27.7	54.0	11.3	2.7	4.3	4.6	20.4	2.2	68.9
ARDSp030	1.90E+07	33.8	40.3	13.2	10.5	2.2	4.5	30.5	8.3	59.9
ARDSp031	3.54E+07	38.6	47.2	11.4	2.4	0.3	6.2	37.0	2.5	49.9
ARDSp034	1.80E+07	23.5	28.1	21.2	26.2	0.0	11.0	33.9	23.6	45.0
SCD 1.0 m2	**1.81E+07**	**26.9**	**44.1**	**15.3**	**11.9**	**1.1**	**7.0**	**31.5**	**12.2**	**55.5**
SE	**3.80E+06**	**3.6**	**3.7**	**1.6**	**3.8**	**0.7**	**1.6**	**2.5**	**3.9**	**3.8**
TTEST	0.170	0.842	0.832	0.384	0.616	0.098	0.328	0.680	0.711	0.787
SCD 1.8m2										
ARDSp035	2.11E+07	13.67	29.00	55.67	0.00	2.00	13.0	24.6	0.0	58.0
ARDSp036	3.01E+07	35.7	33.7	22.0	6.7	0.00	15.0	29.9	7.1	51.8
ARDSp038	7.50E+06	31.8	26.1	34.1	8.0	0.0	18.4	27.8	9.3	23.6
ARDSp039	1.57E+07	40.9	26.2	24.3	5.6	3.0	33.3	21.8	6.7	44.9
SCD 1.8 m2	**1.86E+07**	**30.5**	**28.7**	**34.0**	**5.1**	**1.2**	**19.9**	**26.0**	**5.8**	**44.5**
SE	**4.74E+06**	**5.9**	**1.8**	**7.7**	**1.8**	**0.7**	**4.6**	**1.8**	**2.0**	**7.5**
TTEST	0.170	0.815	0.025	0.011	0.310	0.156	0.458	0.673	0.358	0.446

With CD11R3 analysis alone, it is not possible to differentiate monocytes, macrophages and neutrophils. Therefore it is not clear if differences in expression of this cell marker are to infiltration of specific cell types or related to activation of residence cells. As an alternative approach to accomplish that goal, cells were obtained from gentle enzymatic treatment of lung tissue post manually using dissecting scissors (30, 31). The same lung lobe was used for BAL and enzymatic treatment. Lungs were then analyzed for the distribution of $CD172a^+$ pig myeloid derived cells as evaluated by flow cytometry and confirmed using manual cytospins. Enzyme dissociated lung cells were labeled with combinations of CD172a, CD14, CD163 (porcine monocyte maturation marker(22)), and SWC9 (positively identifies alveolar macrophages (20, 21)) to determine overall cells distribution with the goal of quantitatively assessing shifts in cell density that may be attributable to immune cell modulation with use of SCD_{Rx}. A significant difference in the distribution of cells in the lung was observed between cohorts in that less neutrophils (and conversely a greater number of lymphocytes) were present when comparing the SCD $1.8m^2$ versus untreated control pigs. However, differences in expression of cell markers were not readily apparent therefore this analysis was not performed on the last few animal studies which were conducted during the NCE period. Results are shown in Table 16.

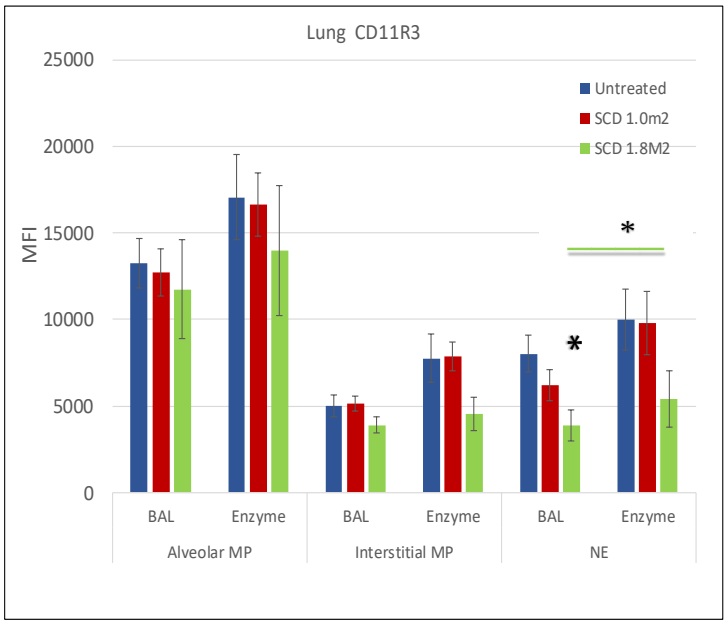
Interestingly, a larger number of CD172+ cells were recovered per gram of tissue from the treated cohorts. Cell numbers are normalized per gram tissue at time of harvest (wet weight) and therefore analysis may be affected by edema which can be normalized in future analysis for peer reviewed publication.

Lung Cells Surface Marker and Intracellular Cytokine Cytometric Analysis

Samples from BALf and enzyme treated lung were incubated with antibodies to CD11R3, SLA DRII and TLR4 in combination with the monocyte/macrophage phenotype markers (Table 14) to investigate differential activation among interstitial and alveolar lung macrophage populations (58). Expression of Toll-like receptors (TLR) by alveolar macrophages is upregulated by a variety of stressors, including ischemia-reperfusion and ventilator-induced lung injury, and in turn is required for induction of ALI in animal models (24, 25). Evaluation of monocyte/macrophage populations may provide insight to the transition from neutrophilic alveolitis to monocytic alveolitis at 24h (19) and importantly, if SCD_{Rx} can potentially alter this immunologic response. In Humans, receptor profiles define the M1 vs M2 monocyte/macrophage phenotypes (59-61), but these parameters are less clearly defined in pig. Further elucidation of pig monocyte/macrophage behavior requires a broad spectrum of tools. In addition to surface markers, secretory profiles were analyzed using both intracellular cytokines evaluated on individual cells using cytometry, and the secretory profile of isolated alveolar macrophages, interstitial monocyte/macrophages and blood monocytes were analyzed by Luminex.

For CD11R3, expression was found to be lower on neutrophils for pigs treated with SCD 1.8m2 (MFI 3863±9916 versus 8018±1044, $p<0.05$ for neutrophils specifically from the BAL and $p<0.001$ for all neutrophil groups combined).

Within the alveolar compartment, the expression of TLR4 was significantly reduced for pigs in the SCD 1.0m^2 treated cohort both alveolar macrophages, MFI 6966±1354 vs. 4022 ± 611 p=0.032, and neutrophils, 1007 ± 183 vs. 488 ± 192 p=0.043 for untreated and SCD 1.0 m^2 cohorts respectively. Significantly reduced TLR4 was also observed for interstitial macrophages and neutrophils. Results are presented in Figure 52. This finding was first observed during Year 2 and statistical differences were strengthened with the results from another untreated animal performed during Year 3. Surprisingly, this same trend was not observed for SCD 1.8m^2 cohort as mean values were very similar to the untreated cohort. However, this may be due in part to the animal to animal variation observed for these values with several but not all of the untreated pigs having high TLR expression. More animals are need per cohort to ascertain if significant difference in TLR4 for this cohort can also be observed.



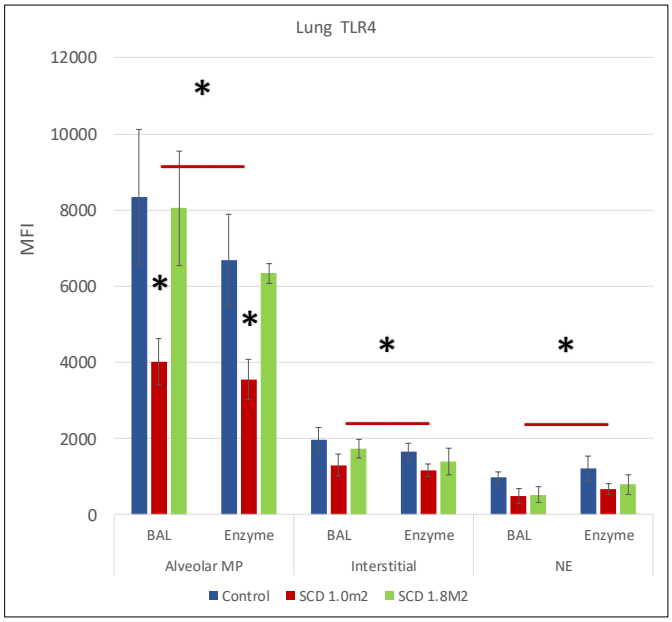


Figure 52. Surface expression of lung cells from the alveolar and interstitial compartments. Expression of CD11R3 was lower on neutrophils for pigs treated with SCD 1.8m2 compared to untreated. TLR4 was significantly lower for both macrophages, p=0.032, and neutrophils p=0.043 in SCD 1.0 m2 treated cohort but due to the small number of animals in the SCD 1.8 m2 cohort, differences were not apparent. BAL and enzyme cell data was examined individually and consolidated for each cell type, which is represented by the colored lines. Untreated (Cohort 1, Blue, n=6) and SCD 1.0m2 (Cohort 2, Red, n=6), SCD 1.8m2 (Cohort 3, Green, n=4), mean± SE, significance $p<0.05$ is indicated by the asterisks.

Lung Cells – Secreted and Intracellular Cytokines

Alveolar Macrophages obtained from BALf, interstitial macrophages obtained from gentle enzymatic treatment of lung tissue were plated in RPMI +10% calf serum at 10^6 cells/2mL/tissue culture plate. Cytospins were performed and plating density adjusted for the number of cells of the macrophage and monocyte lineage. Monocytes and macrophages can be separated from other cell types by their ability to quickly stick to tissue culture plates. After 1-hour, non-adherent cells were removed leaving the desired number of macrophage and/or monocytes cells in each well. BAL cells were mostly alveolar macrophages, enzyme treated lungs were interstitial macrophages, blood derived cells were monocytes and SCD derived cells were monocytes. Cells were then stimulated with 1ug/mL LPS. Basal and stimulated wells were collected, but only +LPS samples were assayed to date. Cytokines were detected for all porcine proteins (IFNα, IFNγ, IL-1b, IL-6, IL-8, IL-10, IL-12p40, and TNFα).

Interferons were not expected to be secreted by macrophage and monocytes in response to LPS but were included on the commercially available panel. Assay results were consistent with this prediction with results being just around the lower detection levels. Statistical differences were observed in IL-6, IL-8, IL-10, and TNFα but these differences were not consistent between the SCD treated cohorts. For all cohorts, interstitial macrophages were much more active for IL-6 and IL-10 than alveolar macrophages, and alveolar macrophages were more active for TNFα possibly detecting differences in how these cells function during an inflammatory insult and highlighting potential targets for SCD_{Rx}. Monocytes associated with the SCD membranes tended to more active than contemporaneous blood monocytes. Secretory profiles of isolated monocytes and macrophages are shown in Figure 53

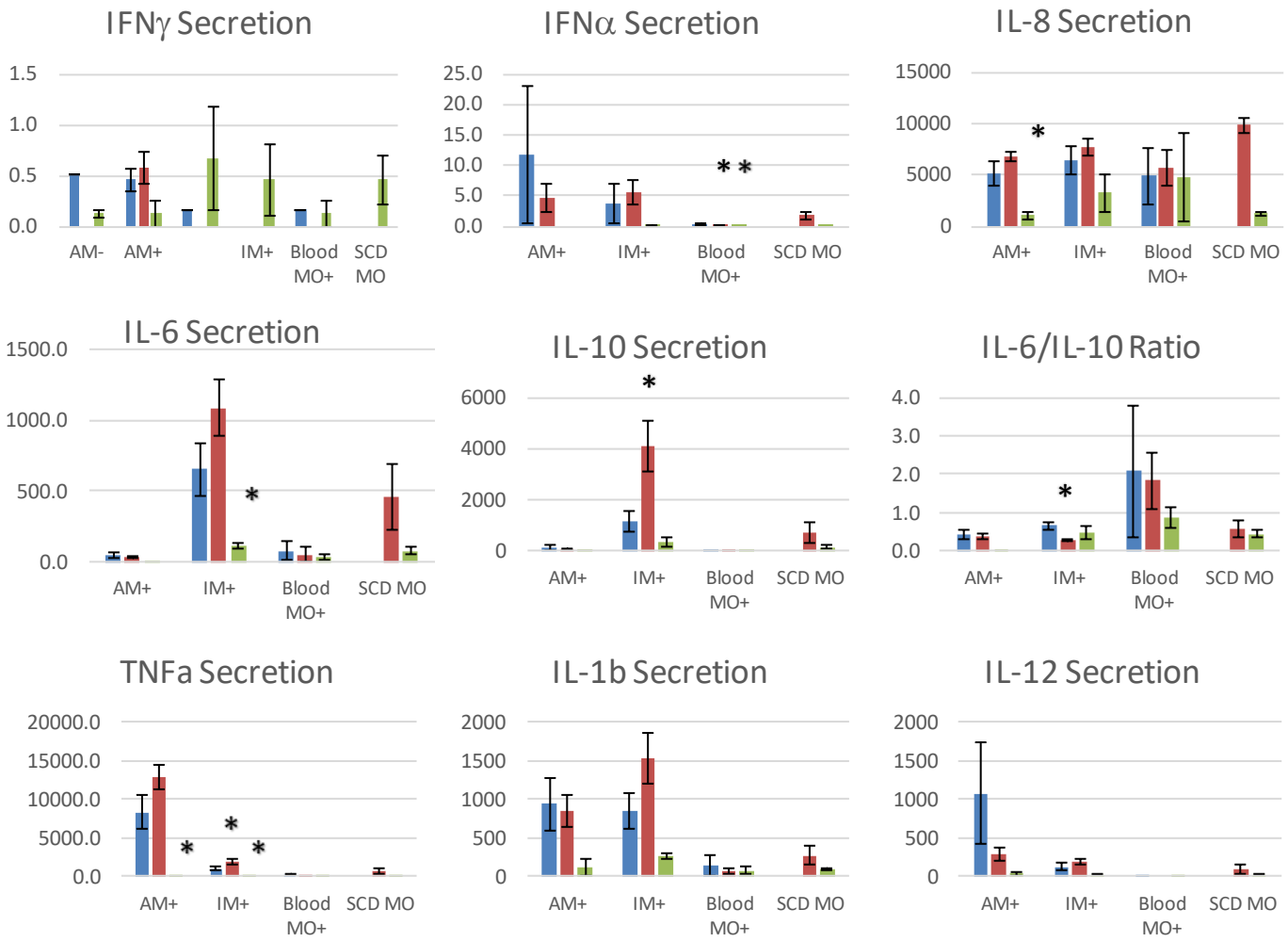


Figure 53. Secretory values of isolated cells of the monocyte and macrophage lineage. AM=alveolar macrophage, IM=interstitial macrophage, MO= monocyte. Results are calculated as pg/mL/10^6 cells in 24 hours. Other than IFNγ, a trend for lower cytokine secretion was observed from cells isolated from pigs in Cohort 3. Significantly lower IFNa secretion was observed for blood monocytes in both SCD treated cohorts. The secretions for other cytokines did not follow a consistent pattern between the cohorts so it is hard to draw conclusions about any observed differences. Untreated (Cohort 1, Blue, n=6), SCD 1.0m2 (Cohort 2, Red, n=6), and SCD 1.8m2 (Cohort 3, Green, n=4), mean± SE, significance $p<0.05$.

The intracellular production of cytokines by Monocyte/macrophages was used to further assess the pro-vs. anti-inflammatory profiles of these cells. Cytokine expression under LPS stimulated conditions was evaluated in whole blood and dissociated lung cells using flow cytometry (27). Intracellular cytokine labeling is accomplished using an Intrastain Kit (DAKO) on blood diluted 1:2 in media with brefeldin A to inhibit Golgi secretion (28). Intracellular cytokine patterns are not directly correlative to secreted levels in isolated monocytes and macrophages in that the cell populations are not purified (remain mixed) and are stimulated for only 4 hours. However, this analysis was done to provide insight into the phenotype of the cell based on which type of cytokines it is actively producing when stimulated.

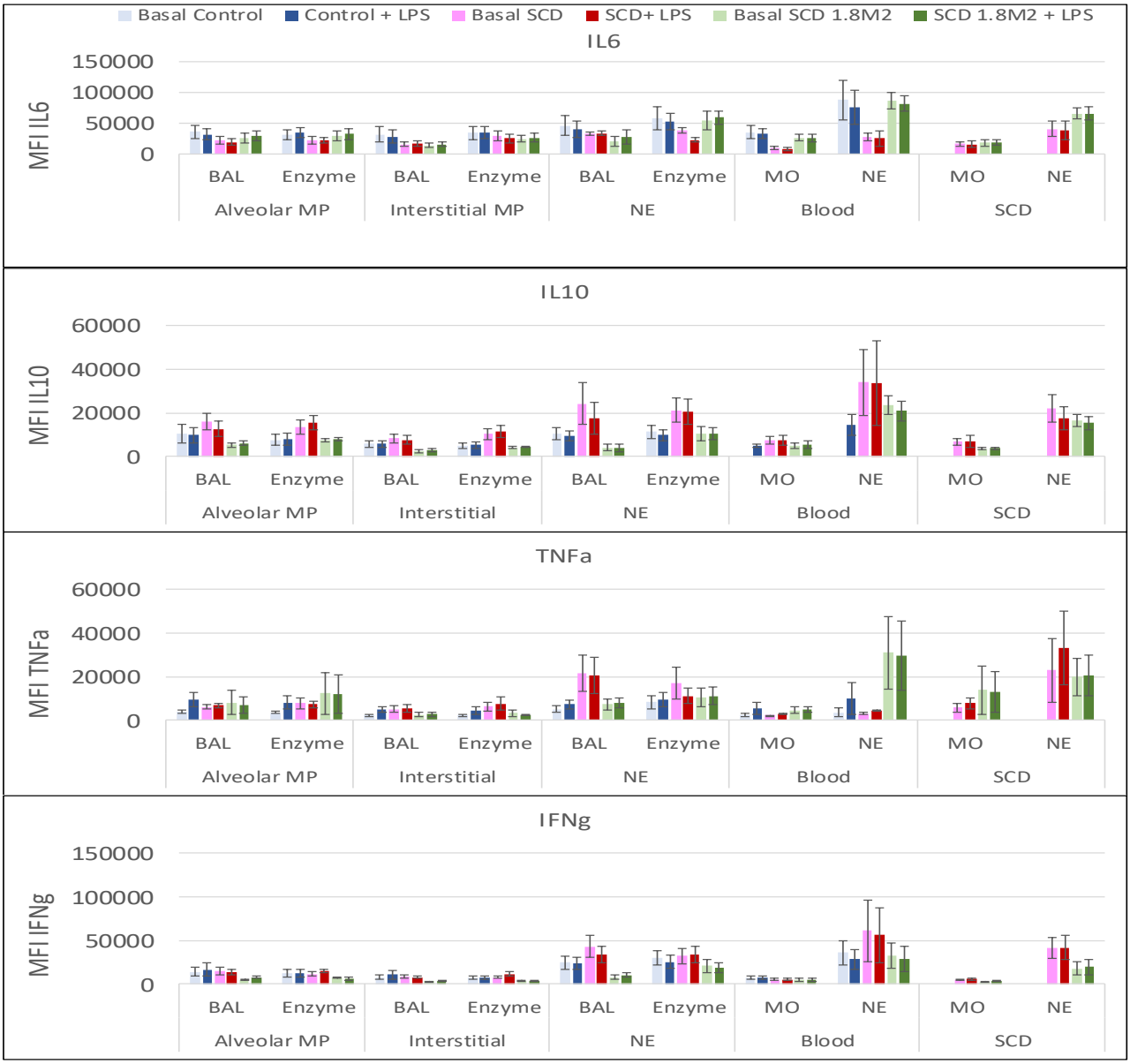


Figure 54. Intracellular cytokine levels in cells from lung and blood. While distinct patterns were not evident, IL-6 levels trended lower in both of the SCDRx cohorts compared to the untreated control cohort for all cell populations. Untreated (Cohort 1, Blue, n=6), SCD 1.0m2 (Cohort 2, Red, n=6), and SCD 1.8m2 (Cohort 3, Green, n=4), mean± SE, significance $p<0.05$).

IL-6 production trended lower in both of the SCD_{Rx} cohorts compared to untreated for all cell populations. (Figure 54). However, identifiable patterns or significant differences between the cohorts are not clearly evident for the remaining cytokines. Due to the labor-intensive nature of this analysis and lack of clear correlation, it was not performed on studies conducted during the no cost extension period when staffing was limited.

In summary, analysis of animals from the untreated cohort 1 and the SCD_{Rx} cohorts 2 and 3 completed are compelling in that even with a limited tool set, significant differences in the behavior of immune cells were observed. Future work in preparation for per reviewed publication will include correlation of secretory profiles in pig to surface markers and interpretation of these results in relation to the human immune system. The demonstration of anti-inflammatory immunomodulation particularly when combined with the improvements

seen in clinically applicable physical parameters and reduced histologic evidence of lung injury will help support transition of SCD_{Rx} to clinical trials.

Subtask 2 Major Findings:

- *SCD_{Rx} resulted in improved hemodynamic stability during the septic shock phase resulting in lower fluid and vasopressor support requirements. A dose effect was observed with even greater hemodynamic stability with use of SCD 1.8m2*
- *Improved pulmonary parameters were observed in SCD treated pigs over untreated pigs.*
- *Complete blood counts demonstrated a systemic inflammatory response to each insult in this 2"hit" model of ARDS.*
  SCD_{Rx} ameliorated the severe leukopenia and rebound leukophilia following administration of LPS. A dose effect was observed with use of SCD 1.8m^2 in that leukocyte counts did not drop as low and Neutrophil then counts rebounded and stabilized more quickly. Lower levels of the granolopoitinc cytokine IL-23 were found with use of SCD1.8m^2.
- *Cytokine analysis suggests immunomodulation of the inflammatory response during ARDS by SCD_{Rx}, particularly with use of SCD1.8$m,^2$ which resulted in lower circulating levels of pro-inflammatory cytokines TNF, IL-6 and IL-12.*
- *Cytometric analysis of pig surface markers and intracelluar cytokine levels detected changes in monocyte, macrophage and neutrophil behavior during the onset of ARDS and suggests that SCD_{Rx} may influence the phenotype of immune cells.*
- *Physical indicators of lung injury including lung edema, protein leak into the alveolar compartment and the number of neutrophils recovered in BALf were reduced with use of SCD_{Rx}. A dose effect was suggested in that all these parameters were lower in the pigs treated with SCD 1.8m^2.*
- *Reduced histopathologic evidence of lung injury based upon a morphometric scoring system was identified with use of SCD_{Rx}.*

Milestone Achieved: Results demonstrated immunomodulation during the course of ARDS with use of $SCD_{Rx,}$ which was associated with improved clinical status and reduced lung injury. These finding were more pronounced with use of SCD1.8m^2.

PROJECT CONCLUSIONS

During this project, a robust, combat relevant large animal model of acute lung injury (ALI) was successfully developed. The model proved reproducible, sustainable for >24h and demonstrated several key clinical and pathological hallmarks of ALI. This model is expected to be a publishable combat relevant large animal ARDS model that can be referenced to test various therapies intended to prevent or treat ARDS, specifically ARDS arising from severe trauma. Creation of the model allowed for evaluation of an innovative immunomodulatory device, called the Selective Cytopheretic Device (SCD), as a therapeutic intervention for ALI/ARDS, a disease associated with unacceptably high mortality and for which no specific pathophysiologic directed therapeutic options currently exist. In this model, we demonstrated that SCD therapy resulted in immunomodulation of the systemic pro-inflammatory state based upon immune cell activation and serum cytokine levels. This

immunomodulation was associated with improved clinical hemodynamic and pulmonary parameters and less severe lung injury. The greatest treatment benefit was realized with use of a larger 1.8 m2 device. The results of this project are compelling and provided substantive support for advancement of SCD therapy into human clinical trials for treatment of ALI associated with COVID-19.

- **Opportunities for Professional Development**
  This project was designed with collaboration between Innovative BioTherapies, Inc. and Dr Hassan Alam, then practicing acute care surgeon at the University of Michigan hospital and consultant for this project. Dr. Alam possesses over 20 years of trauma related research experience and has authored over 200 publications on the subject. He has participated in numerous projects focused on trauma, hemorrhagic shock and resuscitation, including several Department of Defense funded projects. Through this award, the research staff of Innovative BioTherapies received advanced training in porcine models of trauma and hemorrhagic shock. This opportunity included access to research protocols and discussions regarding pros and cons of various trauma models with Dr. Alam and visitation to the animal research Dr. Alam's animal research laboratory at the University of Michigan. Dr. Alam's research team has extensive experience with porcine models of trauma (namely traumatic brain injury) and hemorrhagic shock spanning over 15 years. IBT research staff visited Dr. Alam's lab on 3 separate occasions during this project period, in which they observed and learned techniques related to anesthesia and management, surgical instrumentation, traumatic brain injury, controlled hemorrhage with experimental shock, fluid resuscitation, anesthetic recovery and post- operative care of research swine. The newly learned techniques were directly applied to conducting the studies during year 1 of this project and were instrumental in development of a combat relevant porcine model of trauma associated Acute Lung injury (ALI).

  This project also extended a learning opportunity for the students enrolled in the post-doctoral clinical training program in Laboratory Animal Medicine at University of Michigan. This nationally recognized program which meets requirements for board certification in Laboratory Animal Medicine recently established an animal anesthesia surgery course for second year residents of the program. The course provides the residents with lectures, hands on laboratories and the opportunity to visit working labs to observes anesthesia and surgical procedures being utilized in actual research projects. Due to the depth and complexity of the research project funded by this award, ULAM approached IBT to host these residents over several experiments so that the residents may have the opportunity to spend a day observing work on our project and discussing the working with research staff. This project was selected for this learning opportunity because it incorporates advanced and extended anesthesia and monitoring, complex surgical techniques, models a disease process and includes simulation of intensive care. Three post-doctoral students (all veterinarians) each spent a full day at an animal study observing the procedures and discussing the techniques, protocols and model development with the IBT staff. ULAM residents will receive course credit for attending these sessions, which contributes to them achieving board certification in Laboratory Animal Surgery.
- **How were the results disseminated to communities of interest?**
  Manuscript preparation for publication of the model and results in peer reviewed journals is in progress.
- **What do you plan to do during the next reporting period to accomplish the goals?**
  Nothing to Report

4. **IMPACT:**

Translation to Clinical Trials.

The data developed with this DoD Award was used to move SCD_{Rx} into the clinical arena. The first evaluation of SCD therapy in ARDS patients was undertaken in 2020 under Emergency Use expanded access mechanism of the SCD in severely ill patients diagnosed with COVID-19 related ARDS at the University of Michigan after IRB approval.

Emergency use of the SCD to treat COVID-19 patients.

SCD treatment for COVID-19 associated ARDS has occurred under the emergency use expanded access mechanism. Four COVID-19 patients have been treated with SCD to date, all at the University of Michigan Hospital. The first 2 patients both had extremely poor prognoses, with hypoxemic respiratory failure on maximal therapy, receiving extracorporeal membrane oxygenation (ECMO). After being placed on SCD therapy, within 12h oxygenation improved for both patients with reduced oxygen requirements and all inflammatory markers were substantively reduced. In the first patient, a remarkable drop was observed in IL-6, decreasing from 231 pg/mL before therapy to 5.65 pg/mL at 30h after the start of SCD therapy (63). Both patients rapidly improved and were able to be weaned from ECMO, and subsequently released from the hospital alive. The compelling response to SCD therapy in these patients have been recently published (63). The third patient was chosen for treatment after he continued to deteriorate despite receiving steroids, IL-6 receptor blockade, antivirals, and convalescent plasma treatment. Patient was also receiving CRRT for acute kidney injury. Before SCD therapy, he had a pO2 of 59 on 100% O2 and PEEP 16. Within 6 hours of initiation of SCD treatment, oxygenation improved to pO2 of 117 on 90% O2 improving his pO2/FiO2 ratio from 59 to 130, preventing this patient from requiring ECMO. A fourth patient was treated due to persistent hypoxemic respiratory failure on ECMO after treatment with remdesivir, convalescent plasma, as well as azithromycin and ceftriaxone for superimposed Staphylococcus aureus pneumonia. In this patient, inflammatory markers remained persistently elevated during early treatment but had fallen to 22 pg/mL (from 177 pg/mL pre-treatment) for IL-6 and 35 pg/mL (from 134 pg/mL) for IL-10 by the end of SCR treatment on day 12.
No device related Serious Adverse Events (SAEs) were reported in these patients. Of note, SCD therapy was extended up to 17 days in these patients.

A prospective pilot IDE trial to evaluate use of SCD in COVD-19 patients

In addition to this expanded access treatment group, a prospective pilot IDE trial in COVID 19 patients has also been initiated: IDE G090189S34 (NCT04395911) has been approved by the FDA and by involved IRBs. The trial plans to enroll 35 patients, at up to 10 clinical sites, treatment arm only, ICU patients with COVID 19 with ARDS and/or AKI requiring CRRT. Daily 24-hour SCD treatment up to 10 days to assess safety and efficacy on 60 day mortality and renal recovery. Status: **ONGOING**

5. **CHANGES/PROBLEMS:**

- **Changes in approach and reasons for change**
  Nothing to report
- **Actual or anticipated problems or delays and actions or plans to resolve them**
  Nothing to report
- **Changes that had a significant impact on expenditures**
  Nothing to report

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
    - **Significant changes in use or care of human subjects**
      Nothing to report
    - **Significant changes in use or care of vertebrate animals.**
      Nothing to report
    - **Significant changes in use of biohazards and/or select agents**
      Nothing to Report

6. **PRODUCTS:**
   Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **Project Participants over the Course of the Award (Sept 2016- Aug 2020)**

Name	**Role**
Dr. H. David Humes	Principal Investigator
Dr. Jeffery Curtis	Co-Investigator
Dr. Kimberly A. Johnston	Co-Investigator
Deborah A. Buffington	Co-Investigator
Dr. Hassan Alam	Co-Investigator
Dr. Christopher Pino	Research Scientist/Biomedical Engineer
Angela Westover	Research Scientist
Dr. Liandi Lou	Research Associate
Linda Charles	Research Associate
Nicholas Greer	Research Associate
Rachel Baker	Research Assistant
Megan Davis	Research Assistant
Valerie Stolberg	Laboratory technician
Carson Hoke	Laboratory Assistant
Skylar Ketteler	Laboratory Assistant
Hailey Lindsey	Laboratory Assistant
Rex Underwood	Laboratory Assistant

- **Changes to active other support (PD/PI(s) or senior/key personnel)**

  Nothing to Report

- **What other organizations were involved as partners?**
  The only partner organizations are those listed as subcontractors in the award.

8. SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:**
  Not applicable

- **QUAD CHARTS:**
  Submitted with appendix material.

9. **APPENDICES:**
   Quad Chart

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Appendix I

Assessment of a Therapeutic Device for Treatment of Acute Lung Injury Using a Combat-Relevant Porcine Model

PR150432

W81XWH-16-1-0463

PI: H. D. Humes **Org:** Innovative BioTherapies, Inc. **Award Amount:** $2,696,788

Study/Product Aim(s)

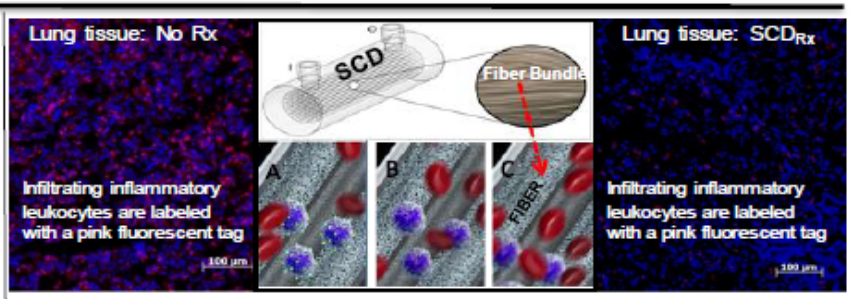
- **Aim 1.** Optimize a two-hit porcine acute respiratory distress syndrome (ARDS) model that is relevant to combat situation.
- **Aim 2.** Assess efficacy of 24 hour SCD_{Rx} in ARDS porcine model.

Selective cytopheretic device therapy (SCD_{Rx}) is an extracorporeal based therapy that has demonstrated efficacy in inhibiting leukocyte activation and organ injury in several acute and chronic disease indications for which inflammation is implicated.

Approach

A combat relevant porcine ARDS model (blunt trauma + hemorrhage/fluid resuscitation, followed by IV infusion of endotoxin) optimized in Aim 1, was used in the Aim 2 study series to determine impact of up to 24 hours SCD_{Rx} on survival time, respiratory function, pulmonary parenchymal damage, systemic inflammation and multi-organ dysfunction compared to standard supportive care alone.

Data generated will be used to advance SCD_{Rx} in clinical trials for ARDS.



Above center, depicts proposed SCD therapeutic action, is flanked by frozen lung sections from septic shock pigs, with no Rx (left panel) and after SCD_{Rx} (right panel). Lungs from untreated pigs have significant inflammatory leukocyte infiltration, while lungs from SCD treated pigs were afforded protection from this inflammatory insult. Top center panel shows an SCD, with blow up of device fiber bundle. Small Panels below illustrate modulation of circulating inflammatory leukocytes by SCD fibers.

Accomplishments: Significant therapeutic benefit was demonstrated with SCD_{Rx} in this porcine ALI model providing substantive evidence to advance the technology into an exploratory clinical trial for treatment in COVID-19 related ARDS (IDE G090189S34)

Timeline and Cost

	15				16				17				18				19				no cost extension
Activities	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	
Obtain approval for all animal work.																					
Establish protocol for two-hit porcine ARDS model																					
Assess efficacy of SCD_{Rx} in ARDS porcine model																					
Estimated budget	$41,711				$1,021,111				$812,234				$821,732								

Updated: December 2020

Goals/Milestones

CY15 Goal– Complete sub-contract facility administrative requirements.
☑ Execute VA Research Agreement ☑ VA IACUC approval

CY16 Goal 1– Obtain approval from DoD for animal work.
☑ DoD ACURO approval

CY16 Goal 2– Establish study protocols for 2-hit porcine ARDS model
☑ Blunt trauma with hemorrhage and fluid resuscitation
☑ Determine LPS dose ☑ Verify model reproducibility
☑ Validate analysis protocols

CY17 Goal – Assess efficacy of 24hr SCD_{Rx} in porcine ARDS model
☑ Complete 18 pig studies (17conducted) ☑ Analyze data from series

CY18 Goal – Assess efficacy of 24hr SCD_{Rx} in porcine ARDS model
☐ Complete 18 pig studies (5 conducted) ☑ Analyze data

CY19 Goal – ☑ Complete necessary remaining pig studies (8 conducted)
☑ Analyze and collate all data for final report

Comments/Challenges/Issues/Concerns: project completed

Budget Expenditure to Date

Projected Expenditure: $2,696,788 Actual Expenditure: $2,696,788