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

**PRINCIPAL INVESTIGATOR:**
H. David Humes, M.D.

**CONTRACTING ORGANIZATION:**
Innovative BioTherapies, Inc.

Ann Arbor, MI 48108-9649

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**6. AUTHOR(S)**
Dr. H. David Humes, Dr. Jeffrey Curtis, Dr. Kimberly Johnston, Angela Westover, Dr. Christopher Pino, Deborah Buffington

E-Mail: dhumes@innbio.com

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Innovative BioTherapies, Inc.
650 Avis Dr., Suite 300
Ann Arbor, MI 48108-9649

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**14. ABSTRACT**

**Purpose**. Acute lung injury (ALI) is recognized in >30% of combat casualties. Inflammation is a key component in ALI and therapeutic strategies to block inflammation are expected to decrease morbidity/mortality. Investigations directed at ways to limit activation and tissue accumulation of leukocytes at sites of inflammation may prove to be clinically effective. **Scope/Aims.** Specific approaches directed to target neutrophil and monocyte/macrophage activity, the effector cells of the innate immune system, may be a novel approach to diminish inflammation-induced disease processes without interfering with other immunologic activity, thereby avoiding adverse side effects. Innovative BioTherapies has developed an approach, known as selective cytopheretic device (SCD) therapy (Rx), in an effort to address this large unmet medical need. $SCD_{Rx}$ has been shown to improve clinical outcomes of critically ill patients with multi-organ failure (MOF) by mitigating the inflammatory cascade. **Specific Aim 1.** Optimize a two-hit porcine ALI model that is relevant to combat situation. **Specific Aim 2.** Assess efficacy of 24 hours of SCD therapy in an ALI porcine model. **Progress to date.** Yr1: Animal approvals obtained. 17 pig studies conducted. Finalized protocols to induce ALI in pigs were realized. Analysis protocols verified (Aim 1 met). Yr2: Assess efficacy of 24-hour SCDRx in ALI porcine model. 17 pig studies conducted. Significant therapeutic benefit was observed in clinically relevant hemodynamic and pulmonary parameters and immunomodulation suggested with assay parameters (Aim 2 initiated).

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

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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 (1-3). It has been effective in pre-clinical animal models in settings in which inflammation and MOD are present, including cardiopulmonary bypass and septic shock (4, 5), 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:FiO₂<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 24hr 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:FiO₂<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% |

| | Timeline months to complete task | Month of Proposal (0-36) | Anticipated Completion Date | Actual Completion Date | % Completed |
|---|---|---|---|---|---|
| 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% |
| | | | | | |
| **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. (3 from each Cohort)** | 6 | 30 | Feb. 2019 | | |
| Subtask 1: Perform 3 studies in each of the 3 cohorts. | 5.5 | 29.5 | Jan. 2019 | | |
| *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 | | |
| Subtask 2: Perform all measurements/assays required to assess key endpoints and exploratory endpoints. | 6 | 30 | Feb. 2019 | | |
| *Milestone Achieved: Assays results allow for comparison between cohorts.* | | 30 | Feb. 2019 | | |
| **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 | | |
| Subtask 1: Perform 3 studies in each of the 3 cohorts. | 5.5 | 35.5 | July 2019 | | |
| *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 | | |
| Subtask 2: Perform all measurements/assays required to assess key endpoints and exploratory endpoints. | 6[*2] | 36 | Aug. 2019 | | |
| *Milestone(s) Achieved: Assays results allow for comparison between cohorts.* | | 36 | Aug. 2019 | | |

*[*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.*

**Activities:**

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

The overall goal of this Specific Aim is 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 will then be utilized to meet Specific Aim 2, evaluation of a novel therapeutic device, the SCD, for treatment of ALI/ARDS.

- **Major Task 1 and Major Task 2 were completed during Year 1 of the project.**

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

  At the time of the last reporting, approximately 60% of this task had been completed as 3/5 studies needed to meet the milestones had been conducted.

  An amendment was submitted to the local IACUC on 08/02/17 requesting modifications to the animal use protocol required for Model 3, which had been deemed the most suitable model for the project (pilot studies for Model 3 were conducted under a temporary approved veterinary recommendation to ensure feasibility). The modifications entailed condensing the study to occur under a single anesthetic episode extending over 2 full days and use of low dose LPS to prime the animals for a more consistent response to the high dose LPS which induces ALI/ARDS. After obtaining necessary approvals from the local IACUC (approved 09/05/2017) and from ACURO (approved 11/02/2017), animal work was able to resume to verify reproducibility of Model 3 over the 24-hour time course post LPS (ARDS phase).

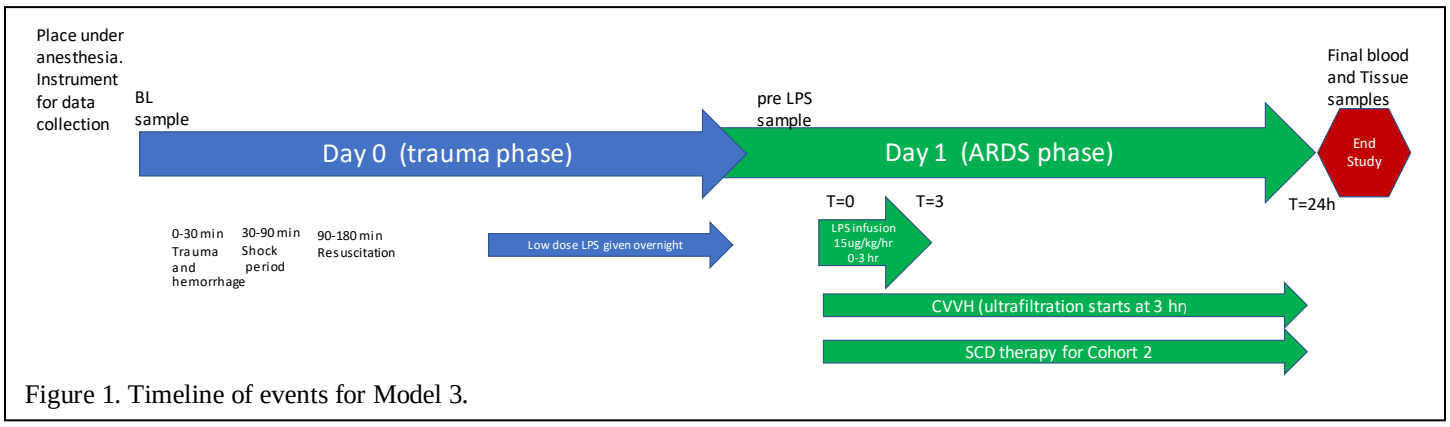



Figure 1. Timeline of events for Model 3.

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.

During this reporting period, additional pig studies were performed using the protocol developed as Model 3 to finalize and validate the study protocol. As determined during Major Task 2 during the last reporting period, the model involved running studies under a condensed format of a single anesthetic episode which included the trauma and hemorrhage procedure, priming the systemic responses with an infusion of ultra-low dose of LPS (0.063 µg/kg/hr) overnight post trauma to prevent sudden pulmonary vasoconstriction and cardiovascular collapse upon expose to a larger LPS dose, and administration of high dose LPS the following on day to induce a dependable systemic inflammatory response resulting in ALI. A timeline is shown in Figure 1. Animals were followed for 24 hours from the start of high dose LPS (ARDS phase). In order to complete this subtask, 6 more studies were performed, utilizing 4 more animals than anticipated. This is because 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. Marked variability in the response to LPS had been in observed in animals to date and this was also observed in the first 2 pigs during this reporting period. 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 (6-8), however, we also uncovered a relation to LPS lot #s. 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 previously purchased lots, the specifications provided with each lot had listed activity levels ranging from >1.8 million units to >3.0 million units/mg. An extensive literature search was performed, and only manufacturer and serotype of LPS were described in animal studies utilizing LPS. We have not found activity level of LPS to be reported in the literature relating to use of this compound in animal models. To avoid any potential complication from differing activity levels, the required amount of LPS for the remainder of the project was purchased in bulk. The available supply consisted of 2 different lots, so all acquired LPS was reconstituted, pooled, distributed into aliquots of either 100µg (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 animal experiment. During the 3 subsequent pig studies 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 mentioned, one of the six animals 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 their airway. Survival times for the 5 animals were 7.25, 24, 4.5, 14, and 13 hours from the start of high dose LPS. Table 1 lists the animals used to fulfill Specific Aim 1.

Even with the use of the basic supportive care treatment algorithm developed during Year 1, which included administration of fluid boluses and vasopressor medication for severe hypotension, we found that maintaining these animals for greater than 12 hours was challenging. All pigs stopped making urine within 3 hours and generalized edema formation plus acid-base disturbances with elevated serum potassium contributed to the early demise of these animals. As originally detailed within the grant proposal as a potential complication in creating 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. Due to the severity of the model, further intervention would be necessary to support animals to consistently achieve 24hr survival. Since renal dysfunction appeared to be an important contributor to the declining physical state of these pigs, a prescription for continuous venovenous hemofiltration (CVVH) was devised. Use of this renal replacement modality is not uncommon in the trauma patient and it is highly compatible with SCD therapy as both can be delivered using the same extracorporeal blood circuit. The circuits used for untreated and SCD treated cohorts are shown in Figure 2. Our lab is very familiar with administration of CVVH and it was easily implemented into the model. Two additional pig studies were completed with CVVH. The first animal survived 20h (ARDSp023). The next pig was also given SCD therapy concurrently to ensure feasibility of the treatment and this animal survived the entire 24-hour study period (ARDSp024).

Table 1. Pigs used to Fulfill Specific Aim 1.

| PIG ID# | LPS Strategy | LPS lot info. | Signs of ALI | Pa:Fi O2 <300 | Study End | Notes |
|---|---|---|---|---|---|---|
| **ARDSp015** | Ultra low LPS dose at 0.063ug/kg/hr overnight starting 6hrs post trauma<br>Day 1 = Higher dose/increased rates x 6hrs aiming for 15ug/kg/hr, increasing rate up to 60ug/kg/hr to get response | L4391 (067M4036V)<br>>1,800,000 EU/mg | ↑ PA pressures<br>↑ Pmax<br>low PaO2 | hour 1 | **24 hr** | showed tolerance to LPS which dampened reaction to High dose LPS |
| **ARDSp016** | Ultra low LPS dose at 0.063ug/kg/hr overnight starting 6hrs post trauma<br>Day 1 = Higher dose/increased rates x 12 hrs aiming for 15-30ug/kg/hr, had to increase rate up to 277ug/kg/hr to get response<br>then kep ~ 65 ug/kg/hr through hr 12 | L4391 (067M4036V)<br>>1,800,000 EU/mg | ↑ PA pressures<br>↑ Pmax<br>low PaO2 | hour 4 | **24 hr** | showed tolerance to LPS which dampened reaction to High dose LPS |
| **ARDSp017** | Ultra low LPS dose at 0.063ug/kg/hr overnight starting 6hrs post trauma<br>Day 1 = Higher dose x 6hrs aiming for 15-30ug/kg/hr, increasing rate up to 115ug/kg/hr to get response<br>then maintained at ~68ug/kg/hr | L4391 (067M4036V)<br>>1,800,000 EU/mg | - | hour 3 | 3.5 hr<br>Arrythmia | died from arrythmia when adjusting Swan Ganz catheter |
| **ARDSp018** | Ultra low LPS dose at 0.063ug/kg/hr overnight starting 6hrs post trauma<br>Day 1 = planned Hi dose x 6hrs aiming for 30-60ug/kg/hr,<br>start at 15 and raise to 30 x 3 hr then crashing | Low Dose: L4391 (067M4036V)<br>>1,800,000 EU/mg frozen<br>High Dose L3012 (026M4079V)<br>>3,000,000EU/mg<br>Made 11/26/2017 4°C | High PA press.<br>High Pmax<br>low PaO2<br>fulminant pulmonary edema coming out tube | hour 5 | 7.25 hr<br>CV collapse | Progressive cardiovascualr collapse even with pressors |
| **ARDSp019** | Ultra low LPS dose at 0.063ug/kg/hr overnight starting 6hrs post trauma<br>Day 1 = planned Hi dose x 3-6hrs<br>start at 1 doubled q 10 minutes kept at 15ug/kg/hr x 3 hr | Low Dose: L4391 (067M4036V)<br>>1,800,000 EU/mg frozen<br>High Dose L3012 (026M4079V)<br>>3,000,000EU/mg<br>Made 11/26/2017 4°C | ↑ PA pressures<br>High Pmax<br>low PaO2 | hour 12 | **24 hrs** | Required fluid and pressor support |
| **ARDSp020** | Ultra low LPS dose at 0.063ug/kg/hr overnight starting 6hrs post trauma<br>Day 1 = planned Hi dose x 3hrs<br>start at 1 doubled q 10 minutes kept at 30ug/kg/hr x 2 hr | Low Dose: L4391 (067M4036V)<br>>1,800,000 EU/mg frozen<br>High Dose 2018_0103 Conglomerate<br>~3,000,000EU/mg<br>Mixed 01032017 | High PA press.<br>High Pmax<br>Low PaO2 | hour 3 | **4.5 hr** | Rapid Cardiovascualr decline despite fluids and pressors.<br><br>Ascites fluid. |
| **ARDSp021** | not perfomed | | | | | died during hemorrhage |
| **ARDSp022** | Ultra low LPS dose at 0.063ug/kg/hr overnight starting 6hrs post trauma<br>Day 1 =15ug/kg/hr x 3 hr | Low Dose: L4391 (067M4036V)<br>>1,800,000 EU/mg frozen<br>High Dose 2018_0103 Conglomerate<br>~3,000,000EU/mg<br>Mixed 01032017 | ↑ PA pressures<br>↑ Pmax | lowest was 389 at hour 12 | **14 hr**<br>**cardiac arrest** | Died from Hi K+ |
| **ARDSp023** | Ultra low LPS dose at 0.063ug/kg/hr overnight starting 6hrs post trauma<br>Day 1 = 15ug/kg/hr x 3 hr | Low Dose: L4391 (067M4036V)<br>>1,800,000 EU/mg frozen<br>High Dose 2018_0103 Conglomerate<br>~3,000,000EU/mg<br>Mixed 01032017 | ↑ PA pressures<br>↑ Pmax<br>low PaO2 | hour 3 | 13 hr<br>cardiac arrest | Hemorhhagic diarrhea<br>Died from Hi K+ |
| **ARDSp024 w/CVVH** | Ultra low LPS dose at 0.063ug/kg/hr overnight starting 6hrs post trauma<br>Day 1 = 15ug/kg/hr x 3 hr | Low Dose: L4391 (067M4036V)<br>>1,800,000 EU/mg frozen<br>High Dose 2018_0103 Conglomerate<br>~3,000,000EU/mg<br>Mixed 01032017 | High Pmax | hour 14 | **20 hr** | Progressive MODS |
| **ARDSp025 w/CVVH + SCD** | Ultra low LPS dose at 0.063ug/kg/hr overnight starting 6hrs post trauma<br><br>Day 1 = 15ug/kg/hr x 3 hr | Low Dose: L4391 (067M4036V)<br>>1,800,000 EU/mg frozen<br><br>High Dose 2018_0103 Conglomerate<br>~3,000,000EU/mg<br>Mixed 01032017 | minimal signs of ALI | never dropped <300 | **24 hr** | Pig VERY stable. No additional fluids or pressors required. |

Including the 2 CVVH studies, survival to 12 hours for this model was 8/10 (80%) and therefore Model 3 with use of CVVH was deemed suitable to advance to Specific Aim 2. The 2 animals that received CVVH were also used for data collection as described under Specific Aim 2.

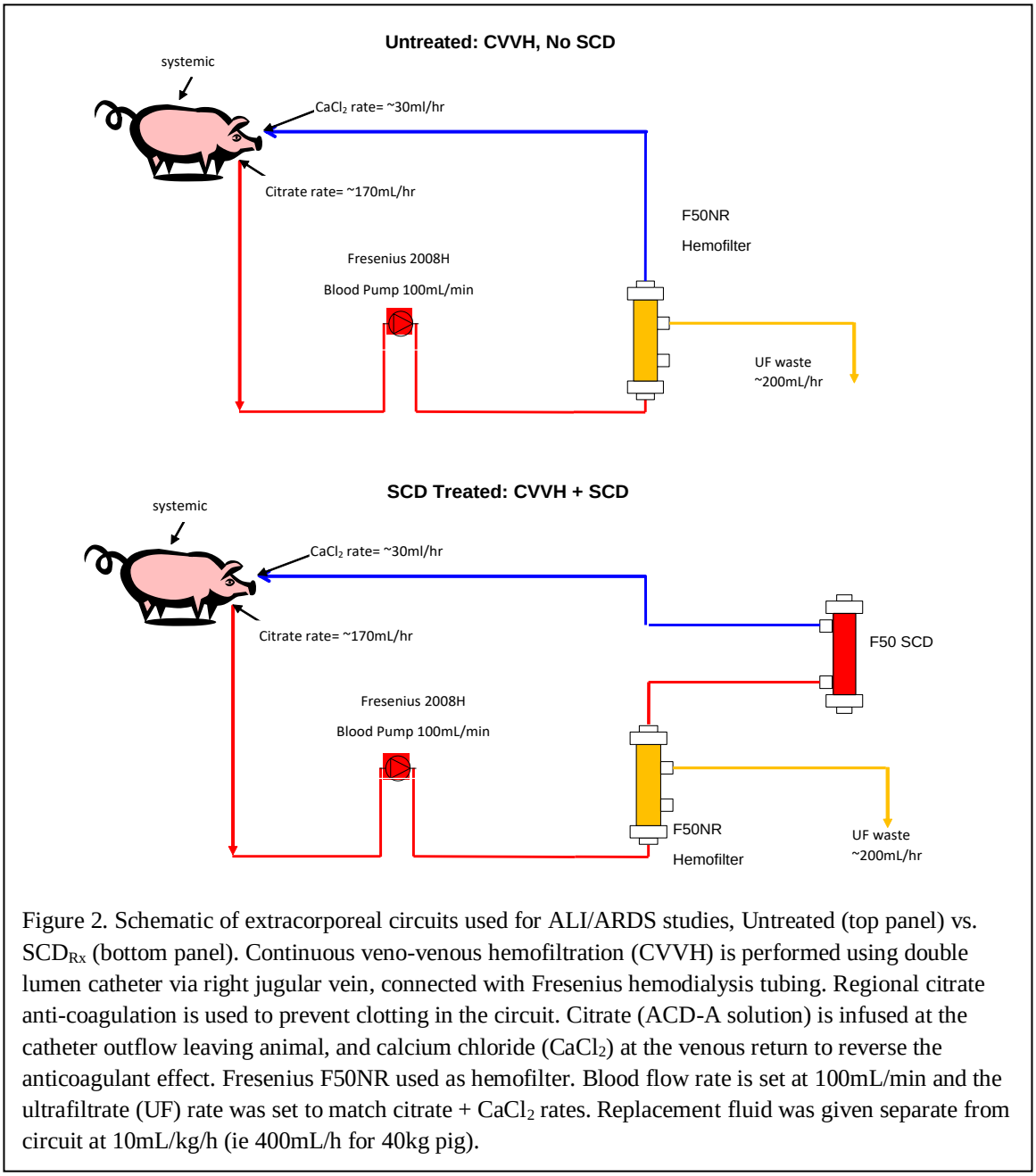



Figure 2. Schematic of extracorporeal circuits used for ALI/ARDS studies, Untreated (top panel) vs. $SCD_{Rx}$ (bottom panel). Continuous veno-venous hemofiltration (CVVH) is performed using double lumen catheter via right jugular vein, connected with Fresenius hemodialysis tubing. Regional citrate anti-coagulation is used to prevent clotting in the circuit. Citrate (ACD-A solution) is infused at the catheter outflow leaving animal, and calcium chloride ($CaCl_2$) at the venous return to reverse the anticoagulant effect. Fresenius F50NR used as hemofilter. Blood flow rate is set at 100mL/min and the ultrafiltrate (UF) rate was set to match citrate + $CaCl_2$ rates. Replacement fluid was given separate from circuit at 10mL/kg/h (ie 400mL/h for 40kg pig).

*Major Findings:*

●*In total, studies were performed on 10 pigs under Model 3 (3 from Year 1 + 5 for model validation this reporting period + 2 with CVVH as the final model).*

● *A dose of LPS of 15 µg/kg/hr x 3hrs was confirmed for induction of ALI using a pooled lot of LPS that will be consistent for the remainder of the project.*

● *ALI was clinically evident in 8/10 pigs that received LPS infusion according to Model 3.*

● *8/10 (80%) pigs used in Model 3 survived to 12 hours.*

● *Additional Supportive care interventions, namely adding the use of CVVH, was determined necessary to reliably ensure survival of pigs beyond 12 hours, which is favored for optimal investigation of the potential of SCD therapy for ALI/ARDS. All supporting interventions were further refined during this set of animals and a final treatment algorithm was created to standardize care for the study animals. Survival of the two pigs receiving CVVH was 20 and 24 hours.*

*Milestones Achieved:*

*1) ALI, as defined by $P_a$:$FiO_2$<300, is achieved within 6 hours of LPS infusion start time in almost all 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 during each animal study to date. As presented in the prior report, LPS induced hemodynamic changes consistent with sepsis and aberrations in pulmonary parameters such as elevation of pulmonary pressures, a decrease in $P_aO_2$ and declining pulmonary compliance were observed, suggesting ALI. Changes in clinical measurements to be used for determining the onset of ARDS were found to vary in timing from animal to animal, particularly the drop in $P_a$:$FiO_2$ to <300, which is a key measurement in the clinical definition of ARDS in man. The drop in Pa:FiO2 to <300 in the study animals ranged from as early as 3 hours to as late as 12 hours with all but one of the untreated pigs dropping below this critical value. (Figure 3). The pig that received $SCD_{Rx}$ did not fall below 300 and it is not presented in the figure.

Laboratory sampling that required 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 during each experiment. Lung tissue samples were also collected from each of the 10 pigs. Several samples were microscopically appraised to confirm pathologic evidence of ALI. Pathologic changes included septal thickening, vessel congestion, accumulation of proteinaceous material in the airway and infiltration of inflammatory cells in the tissue and airway. Taken together, the clinical parameters and the laboratory analysis findings confirmed reliability of the model to induce ALI.

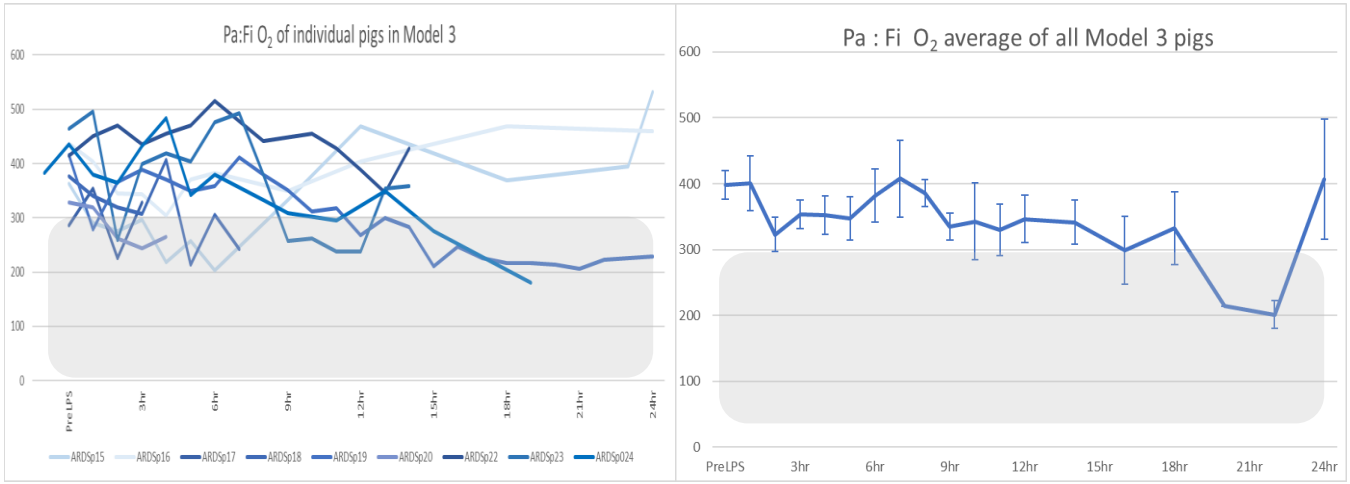



Figure 3. Pa:$FiO_2$ of Model 3 animals plotted individually in left panel with average plotted in right panel. A decline in this value was observed with all but one animal (ARDSp022) dropping below the critical value of a Pa:$FiO_2$<300 (shaded area) within 12 hours of the start of LPS. Animals that received lower doses of LPS as the Model was being refined (ARDSp015 and ARDSp016) showed recovery of this value to PreLPS levels. The average for the Model demonstrates the trend for declining Pa:FiO2 over the course of study.

Proof of a systemic inflammatory response to trauma as well as the LPS infusion were obtained and reported during the previous reporting period (Year 1). The data from the six untreated pig experiments performed during this period fell in line with the prior work. This evidence entailed a post-trauma increase in white blood cell counts with neutrophilia as determined using manual complete blood counts. A modest increase in expression of the CD11R3 integrin on inflammatory cells (neutrophils and monocytes) was also observed post trauma and the priming dose of LPS using flow cytometry. Immediately following high dose LPS exposure, the CD11R3 expression increased dramatically while the circulating white cell counts plummeted 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. As reported previously, this inflammatory response was not directly correlative to LPS dose, at least within the range used in our studies.

Collated data for this subset of animals used in finalizing the model is not shown. However, the two Model 3 animals that received CVVH as part of the final study plan are integrated in to the data sets collated for Specific Aim 2, Major Task 2, which are presented below under that heading.

Additional assays of serum, plasma, BALf and urine for exploratory markers were not necessary to confirm the model and were not performed at this point based on the substantial evidence already obtained to corroborate the model's suitability to permit evaluation of SCD therapy for treatment in ALI/ARDS.

*Major Findings:*

- *Clinical measurements along with histologic changes confirm that ALI is reliably induced in the pig model.*
- *$P_a$:$FiO_2$ declined to <300 in most pigs but the 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) may be more appropriate 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.*

● *Laboratory assays for biomarkers of ALI and systemic inflammation corroborate the clinical evidence and provide additional parameters for which to compare treatment cohorts.*

*Milestones Achieved: 1) Experimental study design, with respect to analysis parameters and sample time points, is finalized for Aim 2 study plan.*

**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 below)**

Subtask 1: Perform 3 studies in each of the 3 cohorts.

Cohorts are:
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.

*Note: Based upon the human clinical definition of ARDS, a $P_a:FiO_2$ <300 was intended to serve as verification of ARDS onset. The expectation of $P_a:FiO_2$ <300 to occur within 6 hours was not reliably achieved 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 complicates the timing for initiation of SCD therapy as planned for Cohort 3, as* $SCD_{Rx}$ *was to be deployed once a clinical diagnosis of ARDS was met. The variable onset of ALI means that pigs may receive different durations of SCD therapy, with <12 hours of therapy possibly being insufficient to achieve a statistically significant treatment effect. In addition, completion of Specific Aim 1 with verification of the current model required 4 more animal studies than anticipated, decreasing the number of studies that could be performed during the rest of the reporting period. For these reasons, it was decided to proceed with testing in Cohorts 1 and 2 only at this point. Testing for Cohort 3 is postponed until Year 3 when a better understanding of the model has been obtained.*

For this subtask, 3 pigs were allocated to Cohort 1 (untreated) and 3 pigs were allocated to Cohort 2, in which $SCD_{Rx}$ was initiated at the start of LPS infusion. As dictated by the model, all these pigs received CVVH. Extracorporeal circulation for CVVH (+/-$SCD_{Rx}$) was also initiated at the start of LPS infusion to enable direct comparison between cohorts. Withdrawal of ultrafiltrate, however, was delayed in both cohorts until infusion of LPS infusion was completed at hour 3 to avoid potentially filtering off LPS or inflammatory mediators during the inciting period. All pigs tolerated this protocol with 4/6 animals surviving to the 24-hour end point. One untreated animal died at 20 hours and an SCD treated pig succumbed at 9.5 hours from progressive cardiovascular compromise associated with gastrointestinal embarrassment and hemorrhagic diarrhea.

A decline in $P_a$:$FiO_2$ was observed in each of the untreated pigs in Cohort 1 with values ≤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 2 SCD treated pigs that survived the full 24 hours (ARDSp025 and ARDSp027) 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 3 of 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.

Collated data for this subset is not shown. All animals are integrated in to the data sets collated for Specific Aim 2, Major Task 2.

*Major Findings:*

- *As expected with the model, ALI was clinically observed in the untreated animals with a decline in $P_a$:$FiO_2$ observed in all animals in Cohort 1.*
- *$P_a$:$FiO_2$ remained >300 in the $SCD_{Rx}$ cohort over the study time course, with the exception of 1 animal that had a single low value immediately prior to death.*
- *83% of the pigs (5/6) survived greater than 12 hours. Four pigs survived the entire 24 hours.*
- *SCD therapy was administered to pigs in Cohort 2 as planned. $SCD_{Rx}$ was initiated the start of LPS infusion with continuous administration until the end of each experiment. No adverse events resulted from SCD therapy.*

*Milestones Achieved:*

*1) ALI, as defined by $P_a$:$FiO_2$ <300, was achieved following LPS infusion in cohort 1. (although this did not occur within 6 hours in all pigs)*
*2) At least 80% of pigs survive 12 hours or longer.*
*3) SCD therapy was successfully administered in Cohort 2*

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

Realtime measurements for clinical parameters of respiratory, cardiovascular and renal function were obtained and recorded as previously described for Specific Aim 1. Laboratory processes that necessitate 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. Serum, plasma and lung tissue samples were harvested and processed according to protocols determined in Specific Aim 1. In order to maximize efficient use of resources and to have a sufficient number of animals per cohort to perform statistical analysis, these samples were stored under appropriate conditions for batch analysis upon completion of Major task 2.

Details of the processing and analysis methods are described below in Major task 2, subtask 2 along with the results.

- **Major Task 2: Perform 9 ARDS pig studies using final model optimized in Aim 1/Major Task 3/Subtask 1. (3 from each Cohort)**

Subtask 1: Perform 3 studies in each of the 3 cohorts.

> *Note: As mentioned for Major task 1, the expectation of $P_a$:$FiO_2$<300 to occur within 6 hours was not reliably achieved within every untreated animal during model development which complicates timing for deployment of $SCD_{Rx}$ in Cohort 3. Therefore, testing was performed in Cohorts 1 and 2 only for this reporting period.*

Five animal experiments were performed. Two pigs were allocated to Cohort 1 (untreated) and 3 pigs were allocated to Cohort 2. Four of the pigs survived to 24 hours and one died at 12.5 hours with complications associated with gastrointestinal compromise and abdominal compartment syndrome (ARDSp034). Similar tends in the clinical parameters were observed in this group of experiments as were observed under Major task 1. The animals used to Fulfill Specific Aim 2 are listed in Table 2.

Table 2. Pigs used 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. |

With 11 animal experiments completed under the finalized model (6 pigs under Major Task 1 and 5 pigs under Major Task 2), available data was collated and analyzed. Hemodynamic data averaged per cohort are presented in Figure 4. 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 (9) for each pig. All five of the untreated pigs 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. The indices for Cohort 1 and 2 are 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. The trends for each treatment cohort are 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 5). SCD treated pigs maintained significantly higher arterial oxygenation as demonstrated by the $P_a$:$FiO_2$ ratio. Each group was maintained with an inspired oxygen content of approximately 0.25 as part of the standard care. 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 (10), 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.

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

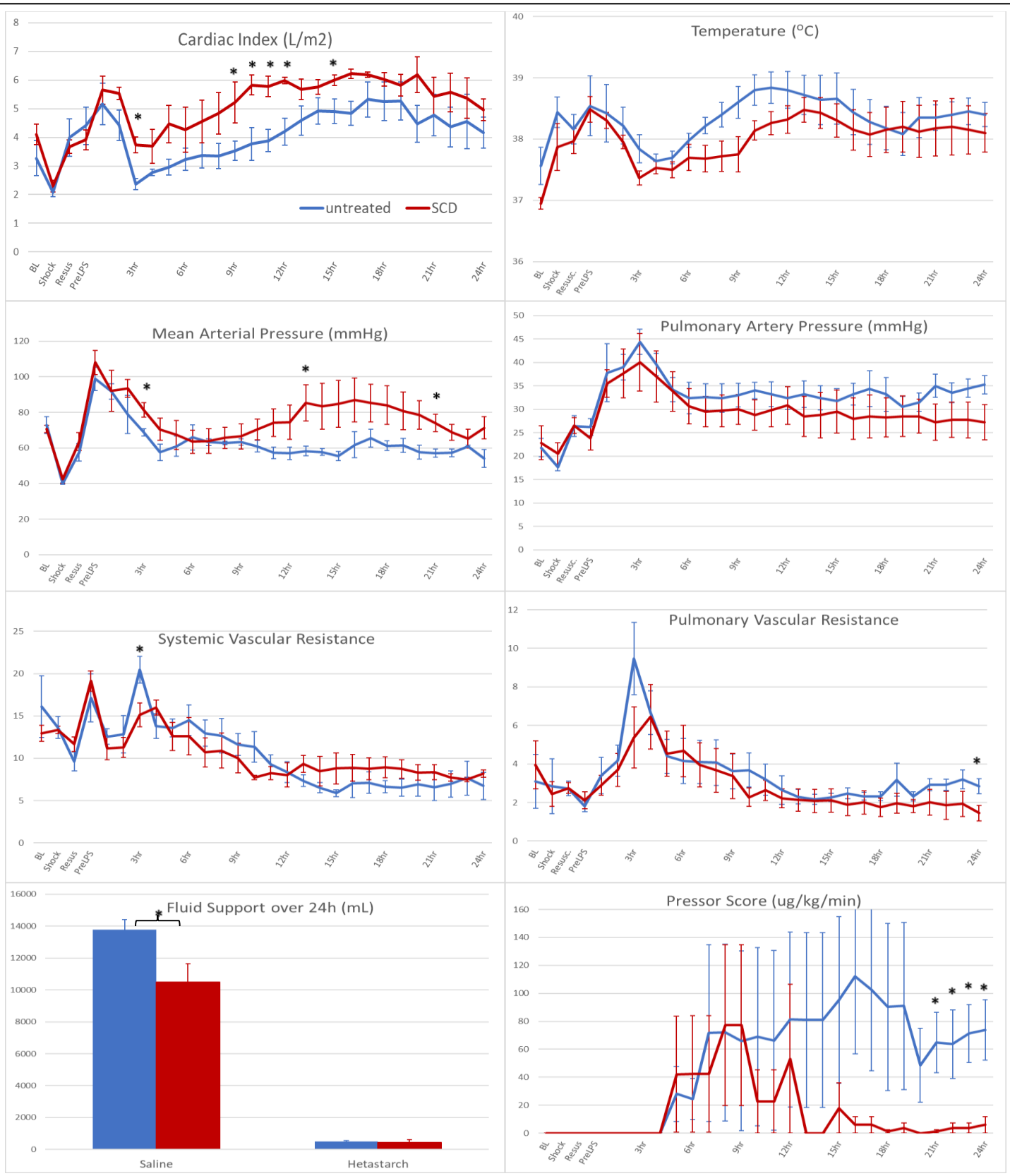



Figure 4.Hemodynamic data. Untreated (Cohort 1, Blue, n=5) and SCD (Cohort 2, Red, n=6), mean± SE, *significance p<0.05.

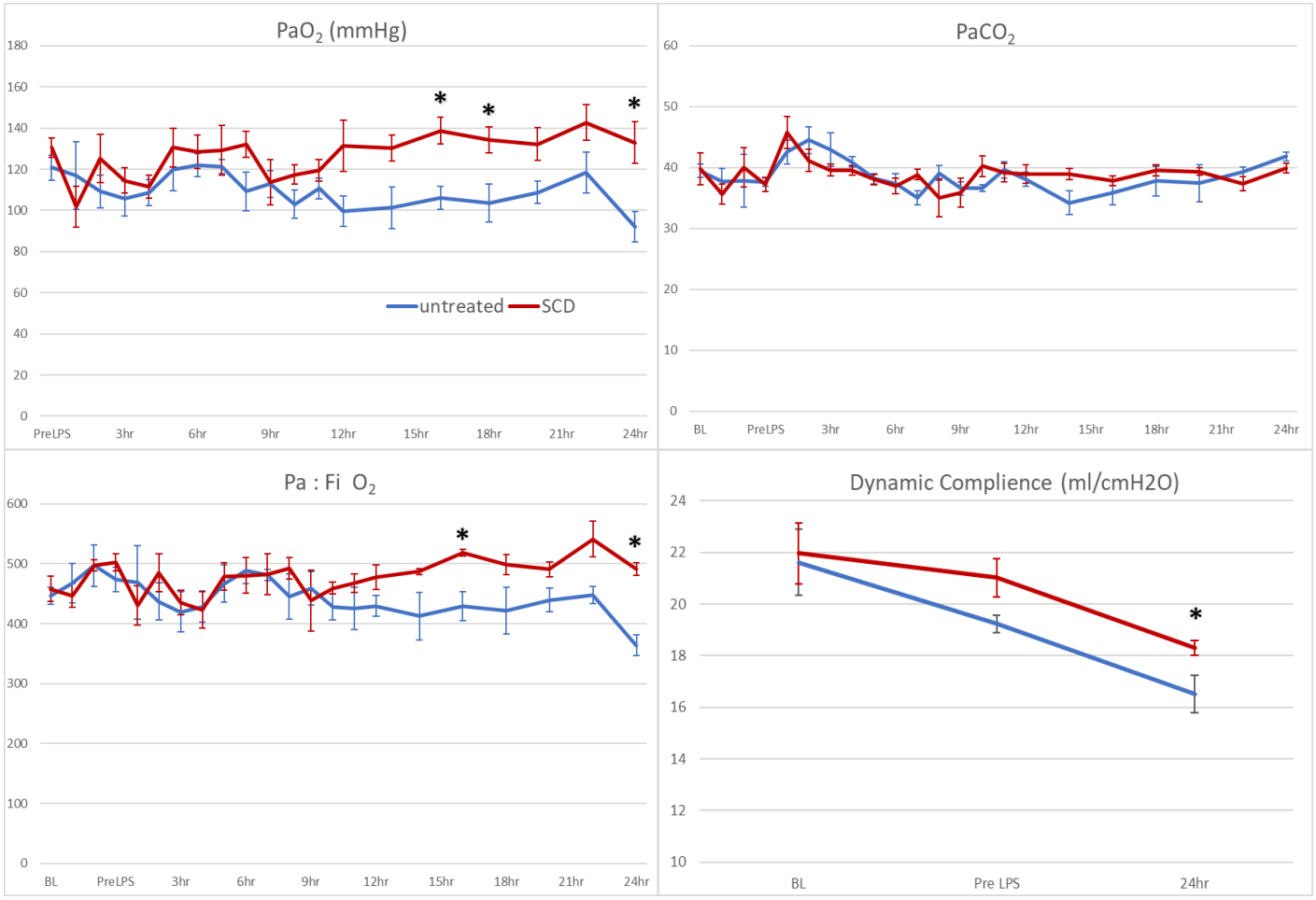



Figure 5. Clinically relevant pulmonary parameters. Untreated (Cohort 1, Blue, n=5) and SCD (Cohort 2, Red, n=6), mean± SE, *significance $p<0.05$.

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. To date, 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 may be emerging in that immature neutrophil release appears 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 (4). 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 are trending lower for the $SCD_{Rx}$ cohort in hours 6-18. When coupled with the

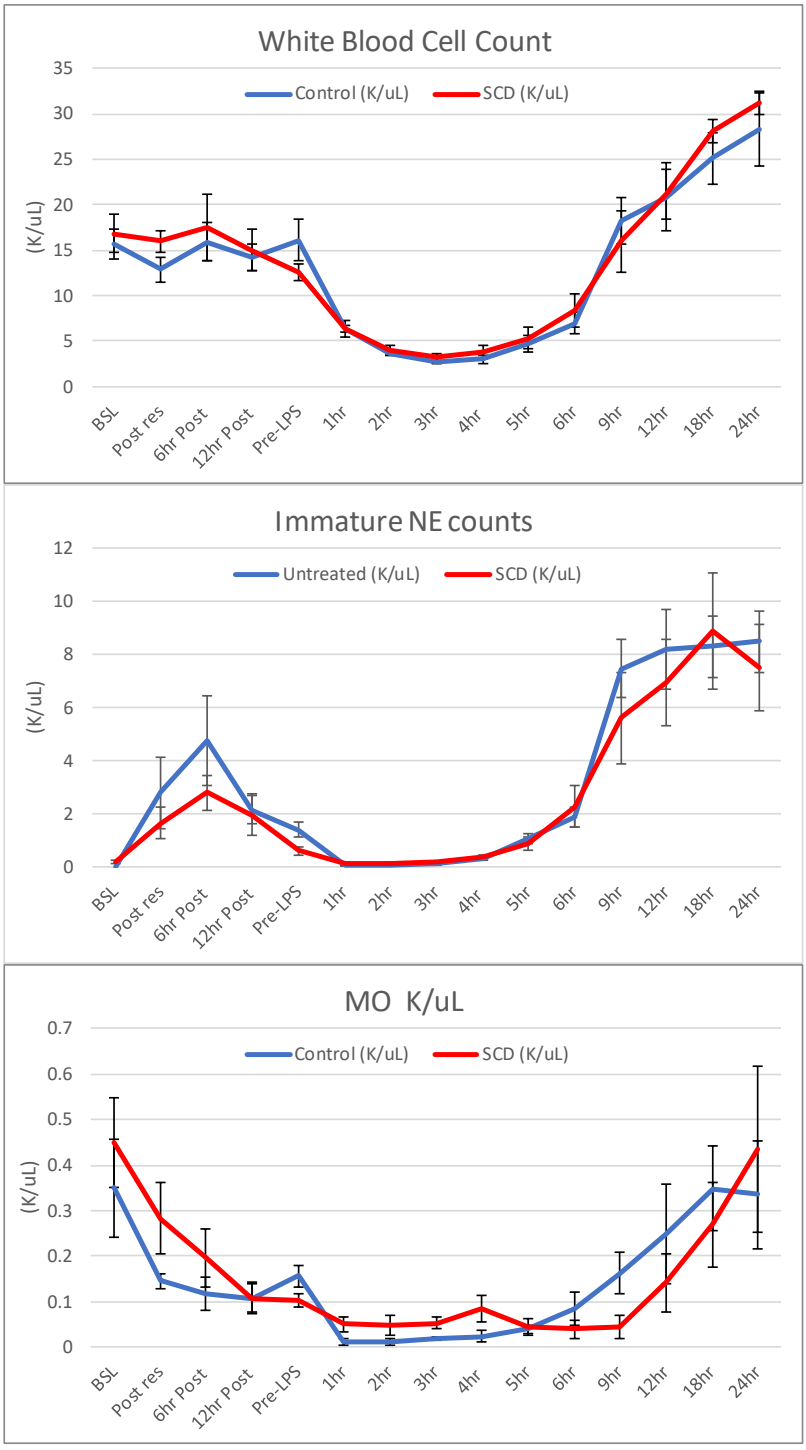



Figure 6. Complete blood count data. The absolute number of leukocytes, immature neutrophils, and monocytes are depicted in top, middle and bottom panels respectively. Untreated (Cohort 1, Blue, n=5) and SCD (Cohort 2, Red, n=6), mean± SE.

observed alteration in monocyte immunologic profiles described later in this section, this observation may be of 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-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 3. 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. The three pigs that experienced intestinal embarrassment also had the highest spike in TNFα in response to high dose LPS (11).

Cytokine patterns are complex and often not predictive of outcomes (12), 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 (13, 14). Plasma values fell within the assay detection range for all analytes except IFNγ. For plasma cytokines, significance has not yet been demonstrated partly due to a high variability between individual animals. For example, IFNα data is driven by one animal, ARDSp033, that had very high values throughout the study (note the large SE). Trends are emerging, and significance may yet be 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 given in Table 3 are IL-6, IL-10 and IL12p40 are graphed in Figure 7.

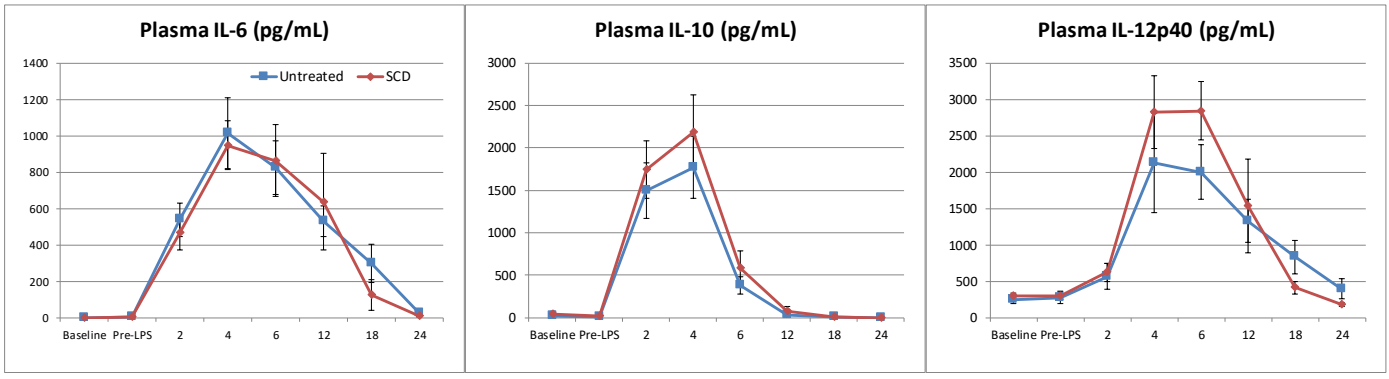



Figure 7. Pig protein levels detected by Cytokine & Chemokine 9-Plex Porcine ProcartaPlex™ Panel (ThermoFisher, EPX090-60829-901). Untreated (Cohort 1, Blue, n=5) and SCD (Cohort 2, Red, n=6), mean± SE.

Table 3: 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 4. Evaluation of neutrophil, monocyte and macrophage populations may provide insight to the transition from neutrophilic alveolitis to monocytic alveolitis (15). 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 (16, 17) 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 (18) 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

Table 4. 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+).

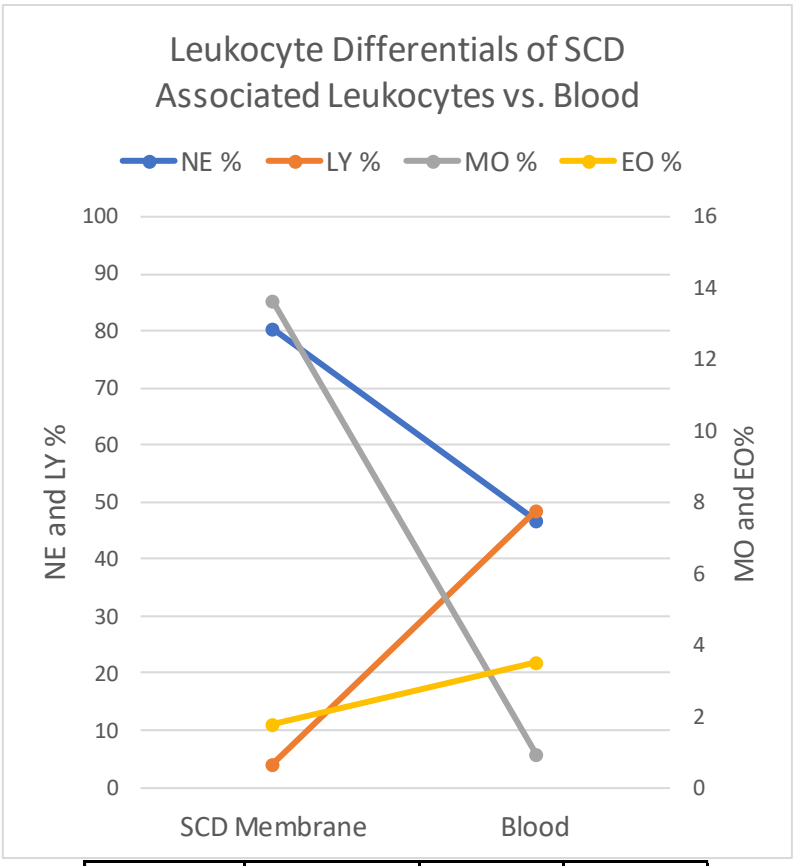
| Vendor | Label | Titrated | laser/fluor |
|---|---|---|---|
| ABDSerotec | CD172a (BL1H7)/SWC3 | 0.05ug/0.5uL | BL1-FITC |
| ABDSerotec | CD163(2A 10/11) | 0.5ug/5uL | BL2-PE |
| ABDSerotec | SWC8 (MIL2) (concentration not provided) | 5uL | unconjugated |
| ThermoFisher Scientific | anti MO IgM PE-CY7(eB121-15F9) | 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 |

become anergic (19). Antibody to CD284 recognizes toll-like receptor 4 which can be differentially expressed via a wide range of stressors (20, 21). Using the panels shown in Table 4, 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 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 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 8. 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 8. 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 (22, 23) and monocytes (24, 25) 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 (26). The clone was chosen for its strong reactivity to cells of interest and detectable upregulation upon stimulation.

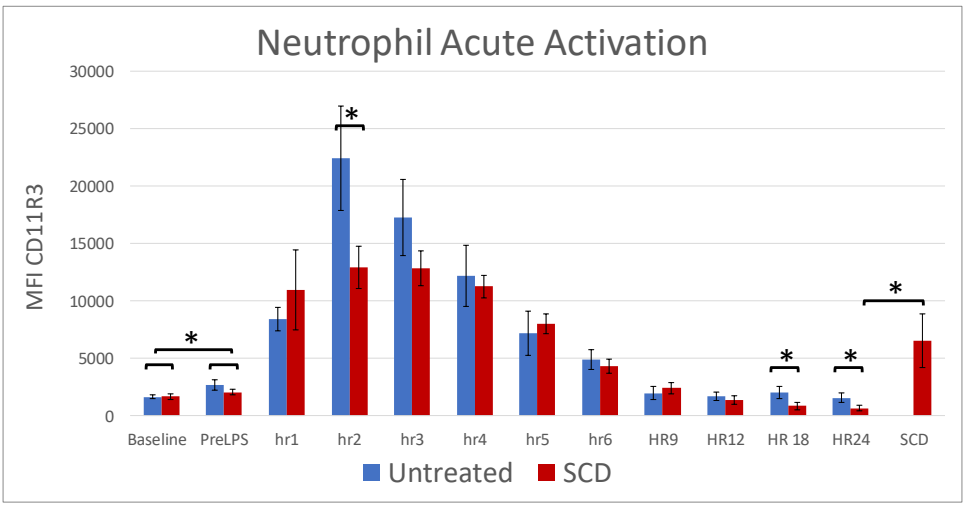



Figure 9. Neutrophil acute activation as detected by surface expression of CD11R3 in systemic blood (baseline-HR24) and cells eluted from SCD membrane at study termination (SCD). Untreated (Cohort 1, Blue, n=5) and SCD (Cohort 2, Red, n=6), mean± SE, significance $p<0.05$.

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 9 (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 10). 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 has 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 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.

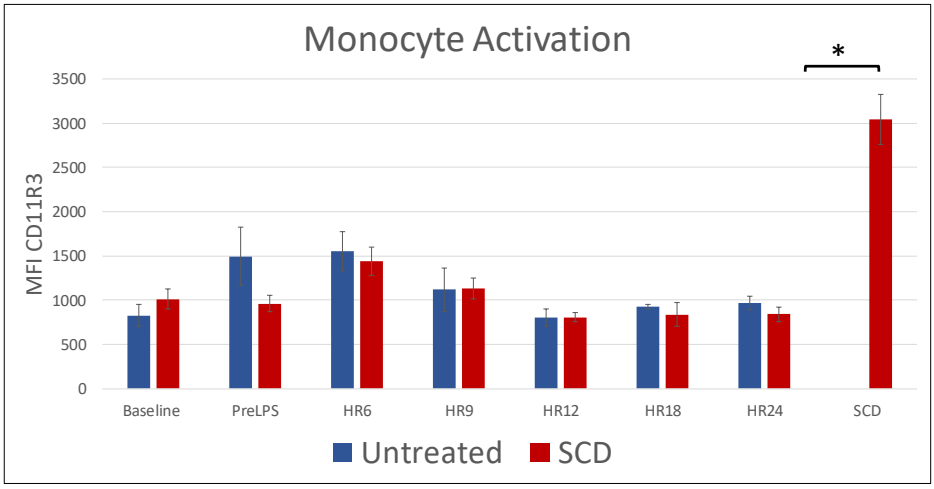



Figure 10. Monocyte activation as detected by surface expression of CD11R3 in systemic blood (baseline-Hr24) and cells eluted from SCD membrane at study termination (SCD). Untreated (Cohort 1, Blue, n=5) and SCD (Cohort 2, Red, n=6), mean± SE, significance $p<0.05$.

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 11). 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 will be completed once experiments are completed to ensure consistency of gating.

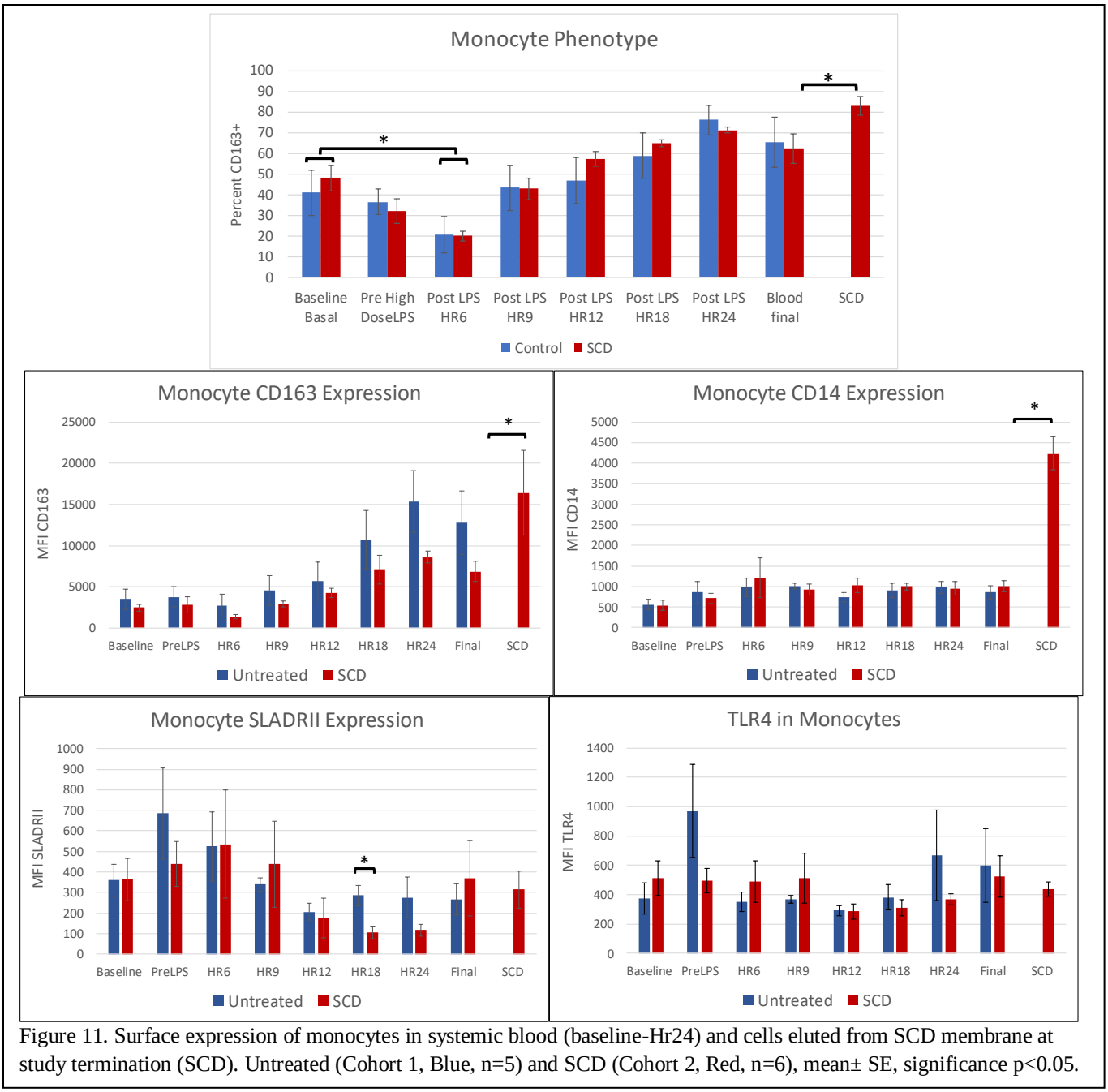



Figure 11. Surface expression of monocytes in systemic blood (baseline-Hr24) and cells eluted from SCD membrane at study termination (SCD). Untreated (Cohort 1, Blue, n=5) and SCD (Cohort 2, Red, n=6), mean± SE, significance $p<0.05$.

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 (27). 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 [94]. The results for all animals are shown in Figure 12.

Table 5. BALf Pig Cytokine and Chemokine Concentrations as Assayed by Luminex

| | 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 |

Cytokine & Chemokine 9-Plex Porcine ProcartaPlex™ Panel (ThermoFisher, EPX090-60829-901)

In ARDS patients, the concentration of neutrophils in the BALf correlates with severity of ARDS and outcome (28, 29). 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 have been 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 5. Protein levels in BALf are expected to be higher with alveolar leak. Detected levels on pilot control animals were 0.781 and 0.481 mg/mL. Only ARDSp024 and 33 had higher values, and therefore the efficiency of recovery of BALf may be inconsistent, which may contribute to the variability in this data.

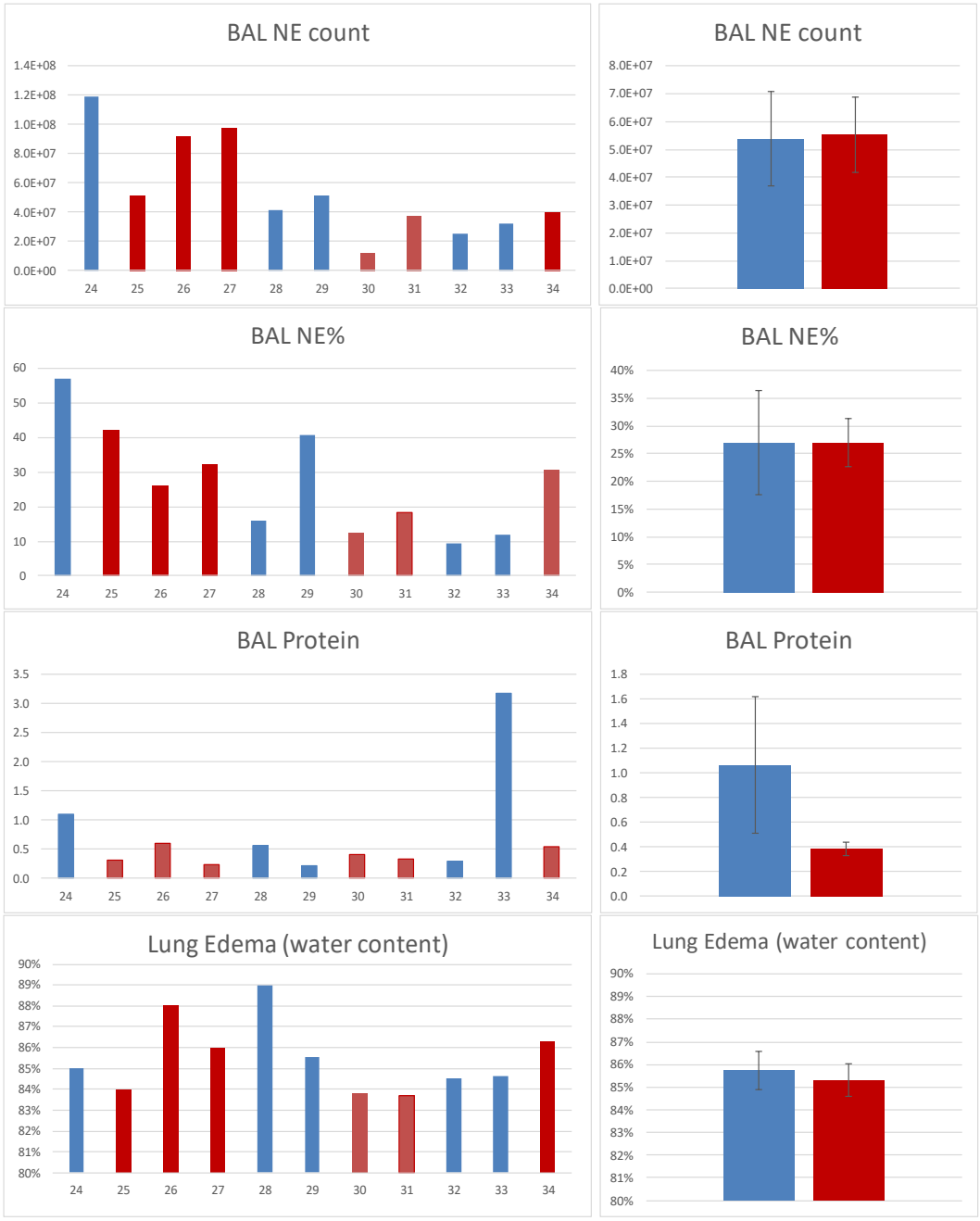
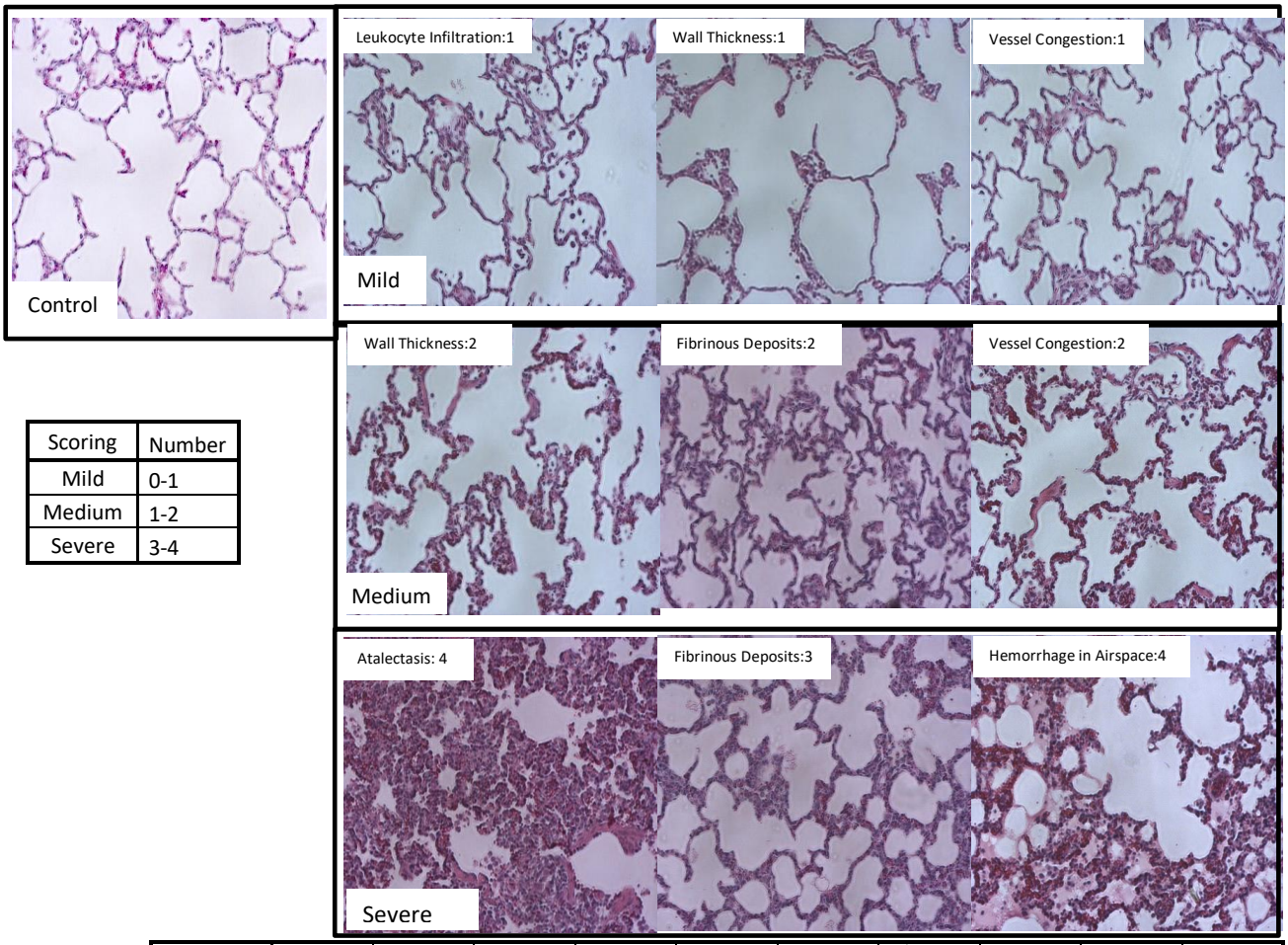



Figure 12. Evaluation of BALf and lung edema in Untreated (Cohort 1, Blue, n=5) and SCD (Cohort 2, Red, n=6). Individual studies on left, averages±SE on right. BAL NE count is the total number of NE recovered, %NE is % of total cells, protein is mg/mL, and edema is (wet weight-dry weight)/wet weight expressed as % water content.

Morphometric Evaluation of Lung

The pathological hallmark of ALI is diffuse alveolar damage (DAD) (30). 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 (31) 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. Lab personnel were used for this preliminary evaluation rather than submit slides to pulmonologists to conserve funds while we establish suitability of the scoring system for this project. Submission of slides from the complete set of animals to a qualified pulmonologist with guidelines for an appropriate scoring system will be done for the final report upon completion of all experiments. Individual scores were averaged to achieve a final score for each parameter for each animal. Results of this blinded scoring are shown in Figure 13.



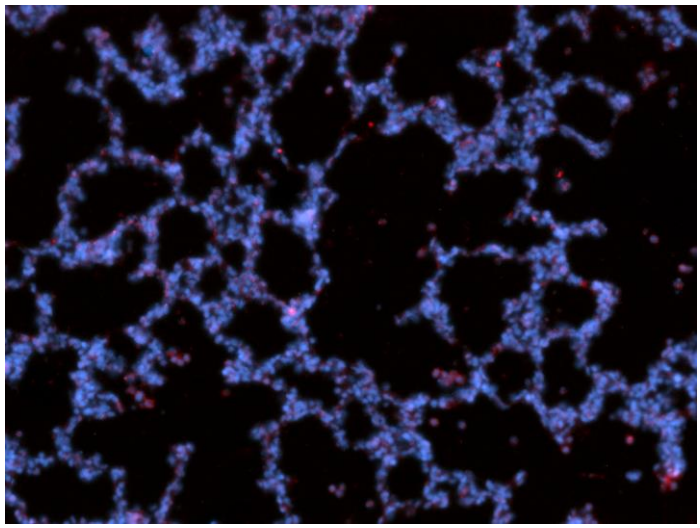

| Scoring | Number |
|---|---|
| Mild | 0-1 |
| Medium | 1-2 |
| Severe | 3-4 |

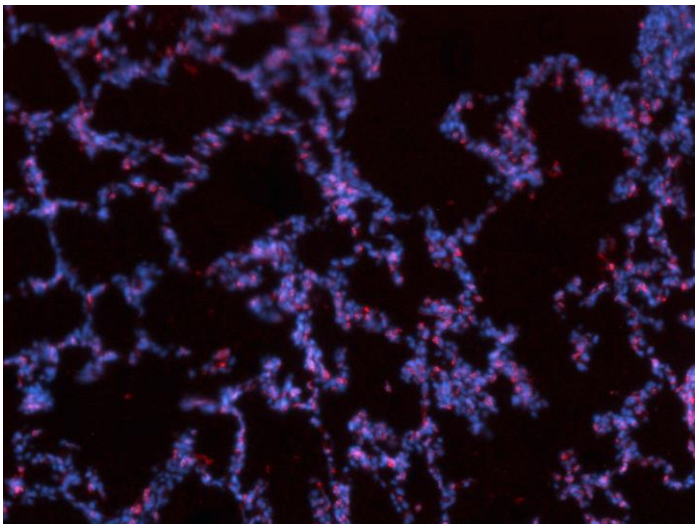
| | Animal | Atalectasis | Fibrin Deposits | Hem. In Airway Low Resolution | Congestion | Wall Thickness | LE infiltration quadrant | Hem in Airway High Resolution | LE infiltration count | Total Score A | Total Score B |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Untreated | ARDSp024 | 2.17 | 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 13. 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).

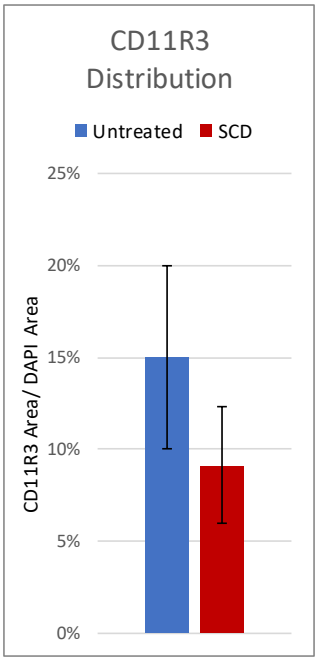
Immunohistochemistry (IHC) was used to assess LE infiltration of lungs using CD11R3 (16, 32-34). A bronchus to the diaphragmatic lobe of the right lung was inflated with a 50/50 (v/v) optimum cutting temperature (OCT) compound (Tissue Tek)/PBS, via a cannula using a method similar to that described for 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 14 for $SCD_{Rx}$ animals that scored low (ARDSp026), and high (ARDSp30).

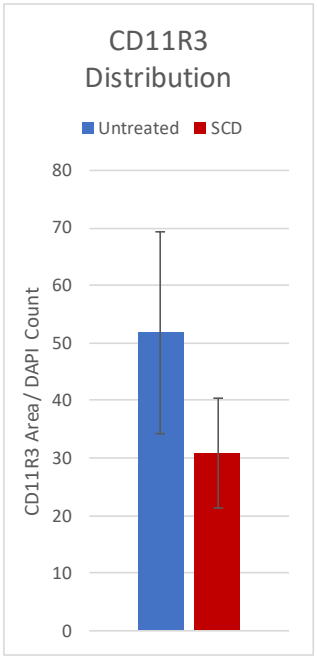
A reduced number of CD11R3+ cells in lung tissue was observed in $SCD_{Rx}$ animals compared to the untreated cohorts. This difference did not quite reach statistical significance but is consistent with other parameters and the difference may become clearer as additional studies increase the numbers 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 (4), 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.





| 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 |



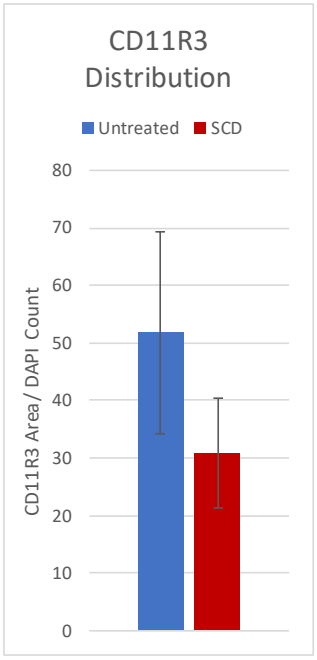




Figure 14. Top Panels, ARDSp026 and ARDSp030 Comparison of low to high scoring images. CD11R3 red, nuclei labeled with DAPI blue. Bottom panel includes individual study results and data analyzed in three ways. : 1) The total area of positive pixels for CD11R3 in the red channel normalized for the total 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 (35, 36). 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(18)), and SWC9 (positively identifies alveolar macrophages (16, 17)) 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 6. 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 6. 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 4) to investigate differential activation among interstitial and alveolar lung macrophage populations (37). 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 (20, 21). Evaluation of monocyte/macrophage populations may provide insight to the transition from neutrophilic alveolitis to monocytic alveolitis at 24-hours (15). In Humans, receptor profiles have been used to define the M1 vs M2 phenotypes (38-41). Recent analyses reveal that this concept of macrophage dichotomy is antiquated, and macrophages can be described as having a multidimensional complexity of phenotypes (41). 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 are 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

(Figure 15). This trend toward reduced TLR4 was also observed in interstitial macrophages and neutrophils but did not reach significance between cohorts analyzed to date.

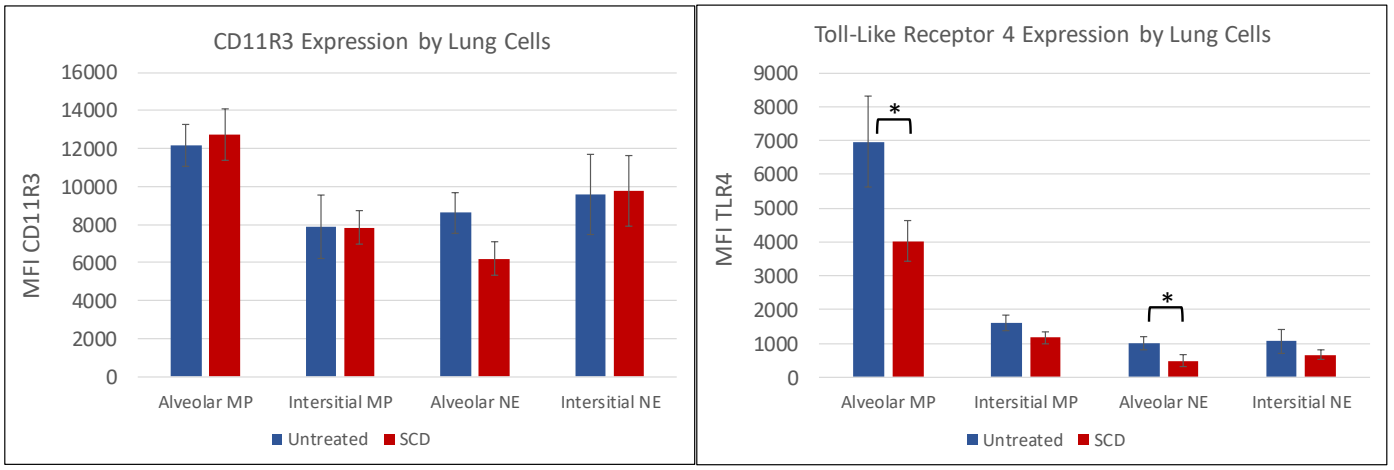



Figure 15. Surface expression of lung cells from the alveolar and interstitial compartments. Expression of TLR4 was significantly reduced for both macrophages, p=0.032, and neutrophils p=0.043 for untreated vs SCD cohorts. Untreated (Cohort 1, Blue, n=5) and SCD (Cohort 2, Red, n=6), mean± SE, significance $p<0.05$.

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 16.

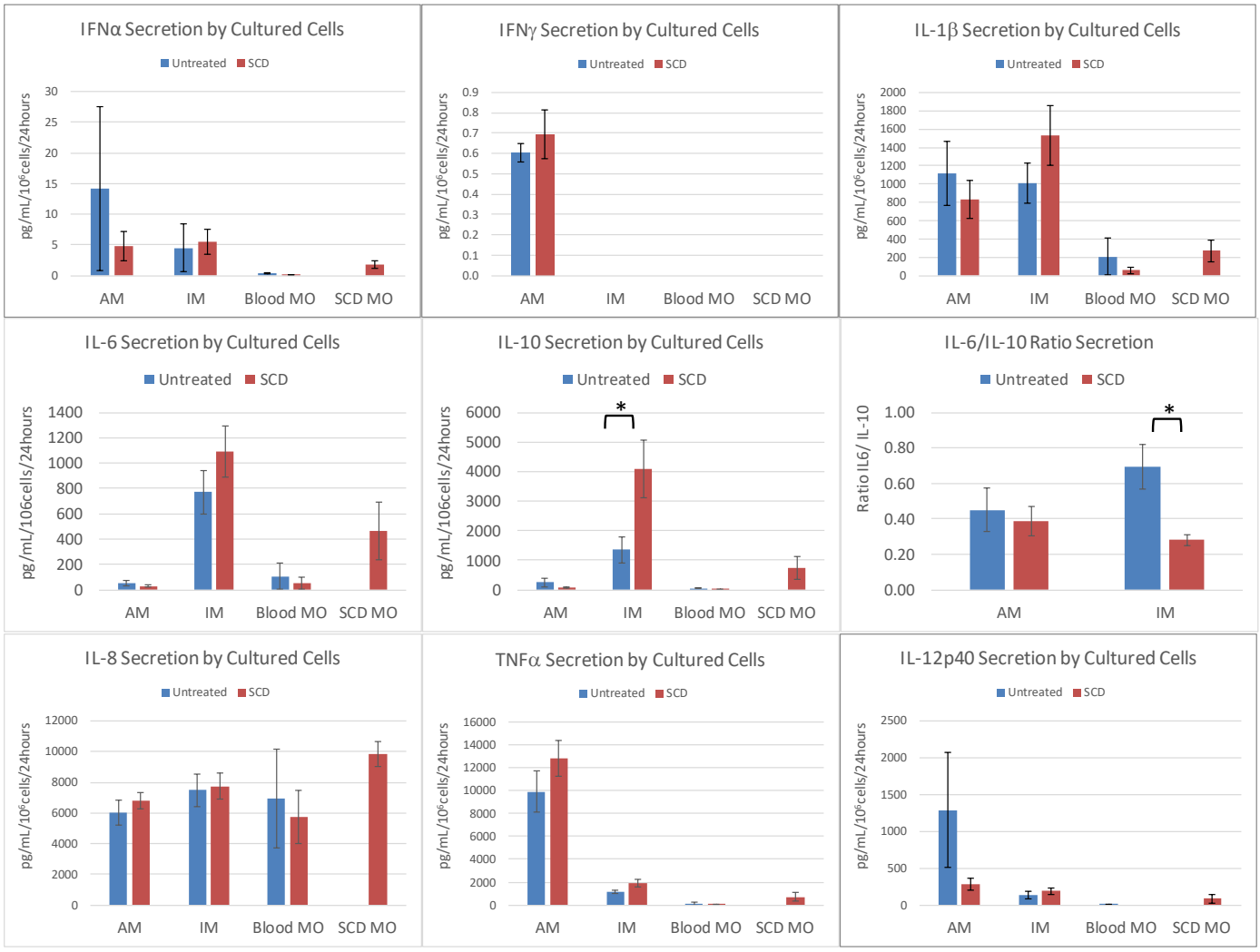



Figure 16. Secretory values of isolated cells of the monocyte and macrophage lineage collected at study termination. AM=alveolar macrophage, IM=interstitial macrophage, Blood MO= monocytes from systemic blood, SCD MO= monocytes from SCD membranes. Results are calculated as pg/mL/$10^6$ cells in 24 hours. 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 (42). 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 17.

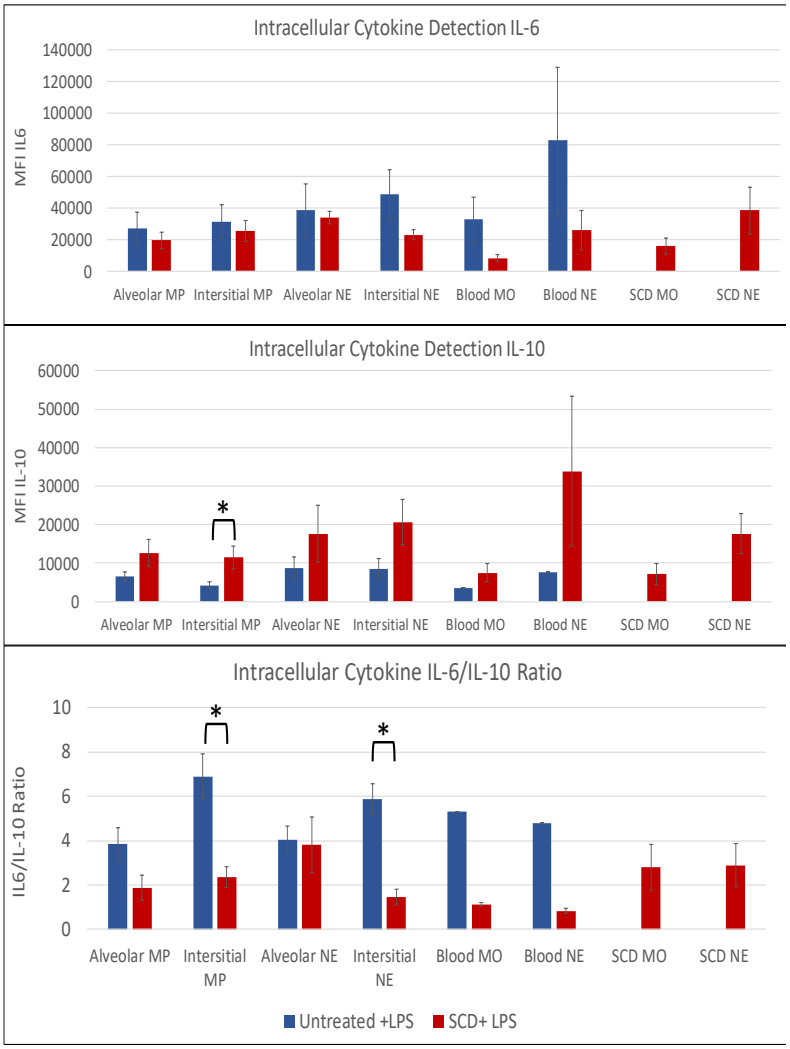



Figure 17. The relationship of IL-6 and IL-10 Intracellular cytokine levels in cells from lung, blood, and SCD membranes at study termination. Untreated (Cohort 1, Blue, n=5) and SCD (Cohort 2, Red, n=6), mean± SE, significance $p<0.05$.

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 18).

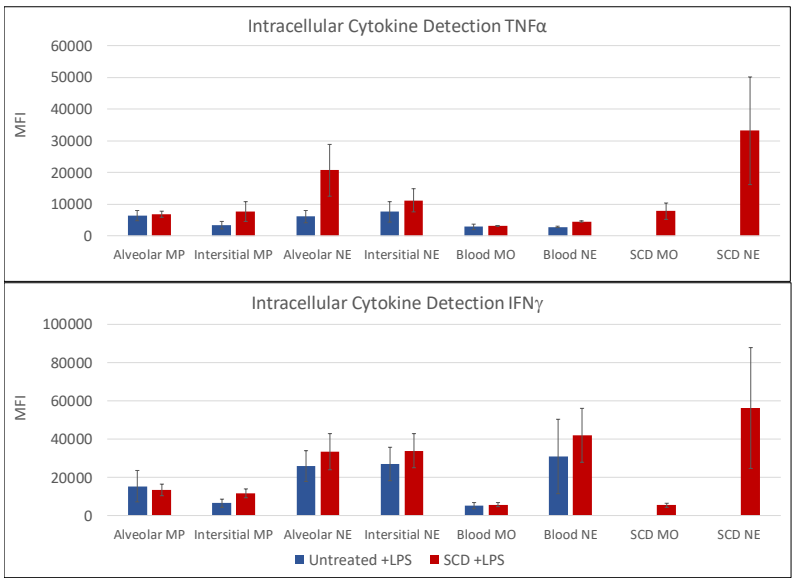



Figure 18. Intracellular detection of TNFα and IFNγ in cells from lung, blood, and SCD membranes at study termination. Untreated (Cohort 1, Blue, n=5) and SCD (Cohort 2, Red, n=6), mean± SE

Analysis of animals from the untreated cohort 1 and $SCD_{Rx}$ cohort 2 completed to date are compelling in that even with a limited tool set, significant differences in the behavior of immune cells were observed. Future work 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 allow 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.*

- **Opportunities for Professional Development**

  This project offered a learning opportunity for veterinarians 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 observe 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 observe the project and discuss the work with research staff. This project was selected for this learning opportunity because it incorporates advanced and extended anesthesia and monitoring, complex surgical techniques, includes a novel animal model of a disease process and simulation of intensive care. Two post-doctoral students 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 Medicine. The learning opportunity offered from this project will continue through the next reporting period for the next class of students.

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- **How were the results disseminated to communities of interest?**

*Nothing to Report*

- **What do you plan to do during the next reporting period to accomplish the goals?**

Summary of accomplishments thus far:

1) We have established a reproducible combat relevant large animal model of ALI/ARDS.

2) $SCD_{Rx}$ has demonstrated that it lessens or prevents multiple organ dysfunction in this model:

   - Cardiovascular parameters were significantly improved (cardiac index, blood pressure, pressor dependency scores)
   - Pulmonary parameters were significantly improved ($P_aO2$, $P_a$:$FiO_2$, pulmonary compliance)

3) $SCD_{Rx}$ was associated with immunomodulation.
   - Lower systemic pro-inflammatory cytokines IL-6 and TNFα. Increased anti-inflammatory cytokines IL-10 and IL-12p40.
   - Lower neutrophil activation as evaluated by expression of CD11R3
   - Lower TLR4 expression in alveolar macrophages and decreased IL-6/IL10 ratio in interstitial macrophages.
   - Lessened lung injury as evaluated by morphometric evaluation of lung damage: atelectasis, fibrin deposition and vascular congestion.

During the next reporting period work will continue to assess impact of $SCD_{Rx}$ in the now established pig model of ALI. As slated, this will encompass additional experiments which will be performed in each of the 3 designated treatment cohorts. These experiments will confirm and expand upon the findings to date.

Based upon literature review of ARDS models and a greater understanding of our chosen pig model achieved during this reporting period, we believe that with the priming events of trauma and exposure to low dose LPS, significant lung injury is incurred during the 3 hours of LPS infusion. Data obtained at 3 hours post LPS demonstrate a clinically significant increase in pulmonary artery pressure from baseline, a 50-60% increase in airway pressure, a greater than 10% decrease in arterial oxygenation from pre-LPS and a decrease in $P_a$:$FiO_2$, all indicative of diminished lung function. Based on these observations, we plan to deploy $SCD_{Rx}$ at 3 hours post-LPS for Cohort 3, to determine if the therapy can reverse the clinical picture and ameliorate the degree of lung injury. As this cohort will likely most closely represent the initial intended patient population, results from this cohort will be important in clinical translation of $SCD_{Rx}$ for treatment of ARDS. Morphometric analysis of histologic samples will be critical to assessment of therapeutic impact of $SCD_{Rx}$.

Upon competition of the final set of studies, data obtained from the 3 treatment cohorts over the entirety of the project will be collated and analyzed using the assay techniques developed over the first 2 years of the project.

*Assessment Parameters for efficacy of $SCD_{Rx}$ will include:*

Primary endpoints. Survival, pulmonary function, lung pathology.

Secondary endpoints. Leukocyte activation, release of immature neutrophils, MO/Mϕ phenotype, systemic cytokine profiles and other (as opposed to lung) end organ damage (heart, kidney, liver). Assessments will be conducted using the sampling, processing and analysis processes that were developed and tested in Year 1 under Specific Aim 1.

*Anticipated Findings:*

$SCD_{Rx}$ will demonstrate improved clinically relevant outcomes (with respect to Assessment Parameters) compared to supportive care alone in the combat applicable "2-hit" pig model of ALI.

With the anticipated promising data resulting from this project, discussions will be opened with project consultant, Dr. Theodore Standiford, to initiate pilot clinical trials for $SCD_{Rx}$ within the Clinical Trials Network for the Prevention and Early Treatment of Acute Lung Injury (PETAL). PETAL is a nationwide network of 12 Clinical Centers and a Coordinating Center funded by the National Heart, Lung and Blood Institute to test new treatments or approaches with the potential to improve clinical outcomes of patients with ARDS or at risk of developing ARDS. The University of Michigan is a key clinical site within the PETAL network. Dr. Sandiford's input will be invaluable in clinical translation of $SCD_{Rx}$. He is a Professor of Internal Medicine and Chief of the Division of Pulmonary and Critical Care Medicine at the University of Michigan and has served as Program Director of two large multi-investigator program project grants, including the University of Michigan Specialized Center of Research (SCOR) in Acute Lung Injury (2000-2002) and University of Michigan Specialized Center of Clinically-Oriented Research (SCCOR) in Acute Lung Injury (2003-2009). Dr. Standiford is also a permanent member of two NIH Study Sections, including Lung Biology and Pathology (LBPA) and the Lung Cell and Molecular Immunology (LCMI).

4. **IMPACT:**

*Nothing to Report*

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**

| | |
|---|---|
| Name: | Dr. H. David Humes |
| Project Role: | Principal Investigator |
| Researcher Identifier (e.g. ORCID ID): | 0000-0002-4309-1614 |
| Nearest person month worked: | 3 |
| Contribution to Project: | As PI for the project Dr. Humes is the central/global facilitator for the coordination of all studies in this project. Dr. Humes met weekly with the Co-Investigators (Co-I), and IBT team members to ascertain study progress, gave input on problems and to ensure comprehensive communications and provided input on data compilation and analysis. Dr. Humes reviewed the animal model set-up and analyzed the data generated from each study to date. Using his in-depth knowledge as well as his research and clinical experience, in collaboration with Co-I Deborah Buffington, Co-I and Veterinary Surgeon Kim Johnston, Co-I Dr. Alam, and Co-I Dr. Curtis, he made recommendations for adjustments and changes to the study design. |
| Funding Support: | |

| | |
|---|---|
| Name: | Deborah Buffington |
| Project Role: | Co-Investigator |
| Researcher Identifier (e.g. ORCID ID): | 0000-0002-1541-2003 |
| Nearest person month worked: | 4 |
| Contribution to Project: | Ms. Buffington met with the PI to ensure the project was moving forward per the proposed timeline. She provided her expertise to the coordination of the large animal studies with the contract facility and assisted in preparation of all reports and in optimization of the study plans. Ms. Buffington integrated adjunct funded projects so that there is no duplication of resource allocation and ensured all data is shared with IBT research scientists to most effectively and efficiently advance SCD therapy for treatment of acute lung injury (ALI). |

| | |
|---|---|
| | She gave advice as to experimental direction and was responsible for data review as well as coordination of data analysis. Ms. Buffington reviewed the report and ensured that budget/proposal guidelines were followed. |
| Funding Support: | |

| | |
|---|---|
| Name: | Dr. Kimberly Johnston |
| Project Role: | Co-Investigator |
| Researcher Identifier (e.g. ORCID ID): | 0000-0003-1899-7876 |
| Nearest person month worked: | 7 |
| Contribution to Project: | Dr. Johnston evaluated the overall health of the animals upon arrival at the contract facilities. She performed the required surgical procedures for the Trauma + Hemorrhage protocol, instrumentation of animals, initiation of ALI. Dr. Johnston provided oversight pertaining to animal management throughout each experiment. In addition, she completed all animal use protocols, reports, and amendments as required by the contract facility. Along with the Co-I's she took the lead troubleshooting animal health issues, thus ensuring all studies proceed according to the timeline presented in this proposal. Dr. Johnson participated in final data analysis and was responsible for report preparation at end of study. Dr. Johnston met weekly with Co-I's to discuss findings and troubleshoot potential problems. |
| Funding Support: | |

| | |
|---|---|
| Name: | Dr. Jeffrey Curtis |
| Project Role: | Co-Investigator |
| Researcher Identifier (e.g. ORCID ID): | 0000-0001-5191-4847 |
| Nearest person month worked: | 1 |

| | |
|---|---|
| Contribution to Project: | In Year 2, Dr. Curtis' provided insight into the analysis of circulating monocytes and alveolar macrophages with respect to phenotype. He reviewed pulmonary physiologic functional parameters and lung histology of the ALI pigs. |
| Funding Support: | |

| | |
|---|---|
| Name: | Dr. Hasan Alam |
| Project Role: | Co-Investigator |
| Researcher Identifier (e.g. ORCID ID): | 0000-0002-1024-5226 |
| Nearest person month worked: | 1 |
| Contribution to Project: | Dr. Alam provided guidance to IBT staff that was instrumental in assuring the injury from the blunt trauma with hemorrhage is relevant to that observed under military conditions. |
| Funding Support: | |

| | |
|---|---|
| Name: | Dr. Theodore Standiford |
| Project Role: | Consultant |
| Researcher Identifier (e.g. ORCID ID): | 0000-0002-5892-4470 |
| Nearest person month worked: | 0 |
| Contribution to Project: | Dr. Standiford will interpret results from data compiled on inflammatory biomarkers and will also grade the lung H&E slides with respect to degree of lung injury. Dr. Standiford's effort will be used in Year 3. |
| Funding Support: | |

| | |
|---|---|
| Name: | Angela Westover |
| Project Role: | Research Scientist |

| | |
|---|---|
| Researcher Identifier (e.g. ORCID ID): | 0000-0002-7556-9838 |
| Nearest person month worked: | 7 |
| Contribution to Project: | Ms. Westover conducted sample processing, data analysis, report preparation and assisted in the oversight and coordination of the sample processing and analysis. For year 2 of the proposed project, Ms. Westover completed the required protocols for processing of lung tissue samples for intracellular cytokines and phenotyping. On study days Ms. Westover assisted sample processing. Ms. Westover coordinated efforts to produce the Luminex cytokine data. She provided analysis and interpretation of all flow cytometry panels. She collated and interpreted all related data for presentation to the CO-I's and integrated these findings into the Year 2 report. She met weekly with the proposal Investigators to discuss findings and troubleshoot potential problems. |
| Funding Support: | |

| | |
|---|---|
| Name: | Christopher Pino |
| Project Role: | Research Scientist/Biomedical Engineer |
| Researcher Identifier (e.g. ORCID ID): | 0000-0003-4063-9215 |
| Nearest person month worked: | 8 |
| Contribution to Project: | Dr. Pino's effort was integral to the success of the trauma/hemorrhage step in the developed model of ALI. In Year 1, Dr. Pino researched and manufactured the traumatizer. He designed and manufactured the circuit used for blood removal and return to the animals. In Year 2, he prepared circuit materials, and provided for the calibration and maintenance of pumps and other required equipment prior to animal studies. Along with Dr. Lou, Dr. Pino, was responsible for performing the Trauma + Hemorrhage protocol and assisted with animal management. Dr. Pino attended weekly meetings with the research team to ensure studies are properly coordinated, discuss findings, and proposal objectives are being met. |
| Funding Support: | |

| | |
|---|---|
| Name: | Linda Charles |
| Project Role: | Research Associate |
| Researcher Identifier (e.g. ORCID ID): | n/a |
| Nearest person month worked: | 2 |
| Contribution to Project: | Along with Nick Greer, Ms. Charles provided the set-up and labeling of tubes for samples to be taken at indicated intervals. On study days Ms. Charles helped with sample processing and trained junior staff. She provided transportation of time sensitive samples to the applicant organization lab facilities for processing. She processed tissue samples for H&E, cryosectioning and neutrophil infiltration labeling. She performed and trained junior staff to process the BALf for supernatants and cells, as well as the cell counts and differentials. Ms. Charles attended weekly meetings with the research team to discuss findings, ensure studies are properly coordinated, and proposal objectives were being met. |
| Funding Support: | |

| | |
|---|---|
| Name: | Liandi Lou |
| Project Role: | Research Associate |
| Researcher Identifier (e.g. ORCID ID): | n/a |
| Nearest person month worked: | 7 |
| Contribution to Project: | In Year 2, Dr. Lou assisted Dr. Pino with preparation of circuit components and calibration and maintenance of pumps and required equipment prior to animal studies. Dr. Lou provided surgical and veterinary support to the animal during surgery. He was responsible for anesthesia management of animals during his shifts. Along with Dr. Pino, Dr. Lou was responsible for performing the Trauma + Hemorrhage protocol. He read and documented findings from systemic blood smears. He was responsible for compiling and collating pulmonary and hemodynamic data from the animal studies. Dr. Lou attended weekly meetings with the research team to discuss findings, ensure studies are properly coordinated, and proposal objectives were being met. |
| Funding Support: | |

| | |
|---|---|
| Name: | Nicholas Greer |
| Project Role: | Research Assistant |
| Researcher Identifier (e.g. ORCID ID): | n/a |
| Nearest person month worked: | 8 |
| Contribution to Project: | In Year 2, Mr. Greer maintained anesthesia, assisted in sample processing, data recording, running CBC on the Hemavet, and archiving samples for future batch testing. Along with Linda Charles, Mr. Greer provided the set-up and labeling of tubes for samples to be taken at indicated intervals. He maintained inventories at the animal facility to ensure that all necessary supplies were readily available. He has been responsible for processing the lung tissue for edema measurement (wet/dry weight) and transport of materials to and from the histology core at the University of Michigan. He acquired microscopic images from lung H&E and cells from BALf and performed differential counts on cytospins prepared from cells recovered in BALf. He performed chemical assays for protein concentration on BAL and plasma. |
| Funding Support: | |

| | |
|---|---|
| Name: | Valerie Stolberg |
| Project Role: | Laboratory Manager |
| Researcher Identifier (e.g. ORCID ID): | 0000-0001-6054-0592 |
| Nearest person month worked: | 2 |
| Contribution to Project: | Ms. Stolberg's responsibilities included sample processing during the ALI pig studies during the normal VA work day (between 8 am and 4 pm). |
| Funding Support: | |

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

**Dr. H. David Humes:**

| | | |
|---|---|---|
| BA170376 | Immunomodulatory Device for Treatment of Traumatic Brain Injury using a Combat Relevant Porcine Model | |
| | Pending 05/01/2018-04/30/2023 | 2.39 CM |
| 1R41HL134377 | Immunomodulatory Biomimetic Device to Treat Myocardial Stunning in ESRD Patients | |
| | Not Funded 07/01/2016-06/30/2017 | 0.49 CM |

**Deborah Buffington:**

| | | |
|---|---|---|
| BA170376 | Immunomodulatory Device for Treatment of Traumatic Brain Injury using a Combat Relevant Porcine Model | |
| | Pending 05/01/2018-04/30/2023 | 3.55 CM |
| 1R41HL134377 | Immunomodulatory Biomimetic Device to Treat Myocardial Stunning in ESRD Patients | |
| | Not Funded 07/01/2016-06/30/2017 | 0.83 CM |

**Dr. Kimberly Johnston:**

| | | |
|---|---|---|
| BA170376 | Immunomodulatory Device for Treatment of Traumatic Brain Injury using a Combat Relevant Porcine Model | |
| | Pending 05/01/2018-04/30/2023 | 4.72 CM |

**Dr. Hasan Alam:**

| | | |
|---|---|---|
| 2R01GM08412706A1 | Modulation of Acetylation in the Treatment of Lethal Injuries | |
| | Active 02/05/2016-01/31/2020 | 2.40 CM |
| BA150793 | Dose Optimization of Valproic Acid in a Swine Model of Traumatic Brain Injury, Hemorrhage, and Poly-Trauma, with the Initiation of a Clinical Trial | |
| | Active 09/01/2017-08/31/2022 | 1.20 CM |
| DM160428 | Testing of Novel Pro-Survival Strategies in the Setting of Prolonged Damage Control Resuscitation | |
| | Active 09/25/2017-09/24/2020 | 1.20 CM |
| N000140910378 | Phase I Trial of Valproic Acid in Healthy Volunteers / Trauma Patients | |
| | Closed 07/01/2016-06/30/2018 | 1.20 CM |

**Dr. Jeffrey Curtis:**

| | | |
|---|---|---|
| 1I10CX00911-01A2 | Modulation of Steroid Suppression by Alveolar Macrophage Efferocytosis | |
| | Active 10/01/2015-09/30/2019 | 2.40 CM |
| W81XWH-15-1-0705 | Beta-Blockers for the Prevention of Acute Exacerbations of COPD | |
| | Active 10/01/2015-09/30/2020 | 0.60 CM |
| R01AI120526 | Early Life Rhinovirus Infection and Childhood Asthma | |
| | Active 03/01/2016-02/28/2020 | 0.55 CM |

- **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:**
   References
   Quad Chart

   *Appendix material begins on the following page.*

**References**


1. Tumlin JA, Chawla L, Tolwani AJ, Mehta R, Dillon J, Finkel KW, DaSilva JR, Astor BC, Yevzlin AS, Humes HD. The effect of the selective cytopheretic device on acute kidney injury outcomes in the intensive care unit: a multicenter pilot study. Semin Dial. 2013;26(5):616-23. Epub 2012/10/31. PubMed PMID: 23106607.
2. Tumlin JA, Galphin CM, Tolwani AJ, Chan MR, Vijayan A, Finkel K, Szamosfalvi B, Dev D, DaSilva JR, Astor BC, Yevzlin AS, Humes HD, Group SCDI. A Multi-Center, Randomized, Controlled, Pivotal Study to Assess the Safety and Efficacy of a Selective Cytopheretic Device in Patients with Acute Kidney Injury. PLoS One. 2015;10(8):e0132482. PubMed PMID: 26244978; PMCID: PMC4526678.
3. Ding F, Yevzlin AS, Xu ZY, Zhou Y, Xie QH, Liu JF, Zheng Y, DaSilva JR, Humes HD. The effects of a novel therapeutic device on acute kidney injury outcomes in the intensive care unit: a pilot study. ASAIO J. 2011;57(5):426-32. Epub 2011/02/15. PubMed PMID: 21317636.
4. Ding F, Song JH, Jung JY, Lou L, Wang M, Charles L, Westover A, Smith PL, Pino CJ, Buffington DA, Humes HD. A biomimetic membrane device that modulates the excessive inflammatory response to sepsis. PLoS One. 2011;6(4):e18584. Epub 2011/05/03. PubMed PMID: 21533222; PMCID: 3077371.
5. Pino CJ, Lou L, Smith PL, Ding F, Pagani FD, Buffington DA, Humes HD. A selective cytopheretic inhibitory device for use during cardiopulmonary bypass surgery. Perfusion. 2012;27(4):311-9. Epub 2012/04/18. PubMed PMID: 22508804.
6. Castegren M, Skorup P, Lipcsey M, Larsson A, Sjolin J. Endotoxin tolerance variation over 24 h during porcine endotoxemia: association with changes in circulation and organ dysfunction. PLoS One. 2013;8(1):e53221. PubMed PMID: 23326400; PMCID: PMC3542331.
7. Nieman G, Brown D, Sarkar J, Kubiak B, Ziraldo C, Dutta-Moscato J, Vieau C, Barclay D, Gatto L, Maier K, Constantine G, Billiar TR, Zamora R, Mi Q, Chang S, Vodovotz Y. A two-compartment mathematical model of endotoxin-induced inflammatory and physiologic alterations in swine. Crit Care Med. 2012;40(4):1052-63. PubMed PMID: 22425816; PMCID: PMC3308118.
8. Weber TE, Schinckel AP, Spurlock ME. Evaluation of the Physiological Responses to Lipopolysaccharide in Different Genetic Populations of Pigs. Purdue University 2002.
9. Cruz DN, Antonelli M, Fumagalli R, Foltran F, Brienza N, Donati A, Malcangi V, Petrini F, Volta G, Bobbio Pallavicini FM, Rottoli F, Giunta F, Ronco C. Early use of polymyxin B hemoperfusion in abdominal septic shock: the EUPHAS randomized controlled trial. JAMA. 2009;301(23):2445-52. PubMed PMID: 19531784.
10. Ullrich R, Roeder G, Lorber C, Quezado ZM, Kneifel W, Gasser H, Schlag G, Redl H, Germann P. Continuous venovenous hemofiltration improves arterial oxygenation in endotoxin-induced lung injury in pigs. Anesthesiology. 2001;95(2):428-36. PubMed PMID: 11506117.
11. Kruse R, Essen-Gustavsson B, Fossum C, Jensen-Waern M. Blood concentrations of the cytokines IL-1beta, IL-6, IL-10, TNF-alpha and IFN-gamma during experimentally induced swine dysentery. Acta Vet Scand. 2008;50:32. PubMed PMID: 18700003; PMCID: PMC2527004.
12. Peng ZY, Wang HZ, Carter MJ, Dileo MV, Bishop JV, Zhou FH, Wen XY, Rimmele T, Singbartl K, Federspiel WJ, Clermont G, Kellum JA. Acute removal of common sepsis mediators does not explain the effects of extracorporeal blood purification in experimental sepsis. Kidney Int. 2012;81(4):363-9. Epub 2011/09/16. PubMed PMID: 21918497; PMCID: 3269547.
13. Jekarl DW, Lee SY, Lee J, Park YJ, Kim Y, Park JH, Wee JH, Choi SP. Procalcitonin as a diagnostic marker and IL-6 as a prognostic marker for sepsis. Diagn Microbiol Infect Dis. 2013;75(4):342-7. Epub 2013/02/09. PubMed PMID: 23391607.
14. Pierrakos C, Vincent JL. Sepsis biomarkers: a review. Crit Care. 2010;14(1):R15. Epub 2010/02/11. PubMed PMID: 20144219; PMCID: 2875530.
15. Tschernig T, Janardhan KS, Pabst R, Singh B. Lipopolysaccharide induced inflammation in the perivascular space in lungs. J Occup Med Toxicol. 2008;3:17. Epub 2008/08/01. PubMed PMID: 18667067; PMCID: 2518552.

16. Chamorro S, Revilla C, Alvarez B, Lopez-Fuertes L, Ezquerra A, Dominguez J. Phenotypic characterization of monocyte subpopulations in the pig. Immunobiology. 2000;202(1):82-93. Epub 2000/07/06. PubMed PMID: 10879692.
17. Piriou-Guzylack L, Salmon H. Membrane markers of the immune cells in swine: an update. Vet Res. 2008;39(6):54. Epub 2008/07/22. PubMed PMID: 18638439.
18. Ondrackova P, Nechvatalova K, Kucerova Z, Leva L, Dominguez J, Faldyna M. Porcine mononuclear phagocyte subpopulations in the lung, blood and bone marrow: dynamics during inflammation induced by Actinobacillus pleuropneumoniae. Vet Res. 2010;41(5):64. Epub 2010/06/04. PubMed PMID: 20519113; PMCID: 2898061.
19. Ward NS, Casserly B, Ayala A. The compensatory anti-inflammatory response syndrome (CARS) in critically ill patients. Clin Chest Med. 2008;29(4):617-25, viii. Epub 2008/10/29. PubMed PMID: 18954697; PMCID: 2786900.
20. Dai H, Pan L, Lin F, Ge W, Li W, He S. Mechanical ventilation modulates Toll-like receptors 2, 4, and 9 on alveolar macrophages in a ventilator-induced lung injury model. J Thorac Dis. 2015;7(4):616-24. Epub 2015/05/15. PubMed PMID: 25973227; PMCID: 4419314.
21. Merry HE, Phelan P, Doak MR, Zhao M, Hwang B, Mulligan MS. Role of toll-like receptor-4 in lung ischemia-reperfusion injury. Ann Thorac Surg. 2015;99(4):1193-9. Epub 2015/03/10. PubMed PMID: 25747278.
22. Hamblin A, Taylor M, Bernhagen J, Shakoor Z, Mayall S, Noble G, McCarthy D. A method of preparing blood leucocytes for flow cytometry which prevents upregulation of leucocyte integrins. J Immunol Methods. 1992;146(2):219-28. Epub 1992/02/05. PubMed PMID: 1347052.
23. Finn A, Rebuck N. Measurement of adhesion molecule expression on neutrophils and fixation. J Immunol Methods. 1994;171(2):267-70. Epub 1994/05/16. PubMed PMID: 7515088.
24. Lundahl J, Hallden G, Skold CM. Human blood monocytes, but not alveolar macrophages, reveal increased CD11b/CD18 expression and adhesion properties upon receptor-dependent activation. Eur Respir J. 1996;9(6):1188-94. Epub 1996/06/01. PubMed PMID: 8804936.
25. Fontes ML, Mathew JP, Rinder HM, Zelterman D, Smith BR, Rinder CS. Atrial fibrillation after cardiac surgery/cardiopulmonary bypass is associated with monocyte activation. Anesth Analg. 2005;101(1):17-23, table of contents. Epub 2005/06/25. PubMed PMID: 15976199.
26. Dominguez J, Alvarez B, Alonso F, Thacker E, Haverson K, McCullough K, Summerfield A, Ezquerra A. Workshop studies on monoclonal antibodies in the myeloid panel with CD11 specificity. Vet Immunol Immunopathol. 2001;80(1-2):111-9. PubMed PMID: 11445222.
27. Matute-Bello G, Downey G, Moore BB, Groshong SD, Matthay MA, Slutsky AS, Kuebler WM. An official American Thoracic Society workshop report: features and measurements of experimental acute lung injury in animals. Am J Respir Cell Mol Biol. 2011;44(5):725-38. Epub 2011/05/03. PubMed PMID: 21531958.
28. Parsons PE, Fowler AA, Hyers TM, Henson PM. Chemotactic activity in bronchoalveolar lavage fluid from patients with adult respiratory distress syndrome. Am Rev Respir Dis. 1985;132(3):490-3. Epub 1985/09/01. PubMed PMID: 4037522.
29. Steinberg KP, Milberg JA, Martin TR, Maunder RJ, Cockrill BA, Hudson LD. Evolution of bronchoalveolar cell populations in the adult respiratory distress syndrome. Am J Respir Crit Care Med. 1994;150(1):113-22. Epub 1994/07/01. PubMed PMID: 8025736.
30. Bachofen M, Weibel ER. Structural alterations of lung parenchyma in the adult respiratory distress syndrome. Clin Chest Med. 1982;3(1):35-56. Epub 1982/01/01. PubMed PMID: 7075161.
31. Kubiak BD, Albert SP, Gatto LA, Snyder KP, Maier KG, Vieau CJ, Roy S, Nieman GF. Peritoneal negative pressure therapy prevents multiple organ injury in a chronic porcine sepsis and ischemia/reperfusion model. Shock. 2010;34(5):525-34. PubMed PMID: 20823698.
32. Matsumoto H, Kumon Y, Watanabe H, Ohnishi T, Shudou M, Ii C, Takahashi H, Imai Y, Tanaka J. Antibodies to CD11b, CD68, and lectin label neutrophils rather than microglia in traumatic and ischemic brain lesions. J Neurosci Res. 2007;85(5):994-1009. Epub 2007/02/01. PubMed PMID: 17265469.

33. Gurney KJ, Estrada EY, Rosenberg GA. Blood-brain barrier disruption by stromelysin-1 facilitates neutrophil infiltration in neuroinflammation. Neurobiol Dis. 2006;23(1):87-96. Epub 2006/04/21. PubMed PMID: 16624562.
34. Moreno S, Alvarez B, Poderoso T, Revilla C, Ezquerra A, Alonso F, Dominguez J. Porcine monocyte subsets differ in the expression of CCR2 and in their responsiveness to CCL2. Vet Res. 2010;41(5):76. Epub 2010/07/31. PubMed PMID: 20670605; PMCID: 2941139.
35. Freeman CM, Curtis JL, Chensue SW. CC chemokine receptor 5 and CXC chemokine receptor 6 expression by lung CD8+ cells correlates with chronic obstructive pulmonary disease severity. Am J Pathol. 2007;171(3):767-76. Epub 2007/07/21. PubMed PMID: 17640964; PMCID: 1959492.
36. Freeman CM, Han MK, Martinez FJ, Murray S, Liu LX, Chensue SW, Polak TJ, Sonstein J, Todt JC, Ames TM, Arenberg DA, Meldrum CA, Getty C, McCloskey L, Curtis JL. Cytotoxic potential of lung CD8(+) T cells increases with chronic obstructive pulmonary disease severity and with in vitro stimulation by IL-18 or IL-15. J Immunol. 2010;184(11):6504-13. Epub 2010/04/30. PubMed PMID: 20427767; PMCID: 4098931.
37. Fairbairn L, Kapetanovic R, Beraldi D, Sester DP, Tuggle CK, Archibald AL, Hume DA. Comparative analysis of monocyte subsets in the pig. J Immunol. 2013;190(12):6389-96. Epub 2013/05/15. PubMed PMID: 23667115.
38. Urra X, Villamor N, Amaro S, Gomez-Choco M, Obach V, Oleaga L, Planas AM, Chamorro A. Monocyte subtypes predict clinical course and prognosis in human stroke. J Cereb Blood Flow Metab. 2009;29(5):994-1002. Epub 2009/03/19. PubMed PMID: 19293821.
39. Nahrendorf M, Pittet MJ, Swirski FK. Monocytes: protagonists of infarct inflammation and repair after myocardial infarction. Circulation. 2010;121(22):2437-45. Epub 2010/06/10. PubMed PMID: 20530020; PMCID: 2892474.
40. Nahrendorf M, Swirski FK, Aikawa E, Stangenberg L, Wurdinger T, Figueiredo JL, Libby P, Weissleder R, Pittet MJ. The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. J Exp Med. 2007;204(12):3037-47. Epub 2007/11/21. PubMed PMID: 18025128; PMCID: 2118517.
41. Ginhoux F, Schultze JL, Murray PJ, Ochando J, Biswas SK. New insights into the multidimensional concept of macrophage ontogeny, activation and function. Nat Immunol. 2016;17(1):34-40. PubMed PMID: 26681460.
42. Roberts RL, Hatori N, Drury JK, Stiehm ER. Purification and properties of porcine polymorphonuclear cells. J Immunol Methods. 1987;103(1):27-32. Epub 1987/10/23. PubMed PMID: 2821122.

# 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

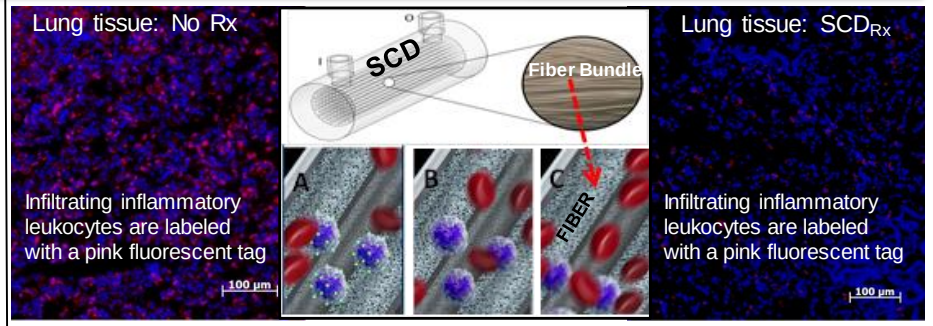
## Study/Product Aim(s)

- **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 plus hemorrhage/fluid resuscitation, followed by IV infusion of LPS endotoxin Day 3 post-trauma) optimized in Aim 1, will be 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.




Above center panel, depicting proposed SCD therapeutic action, is flanked by frozen lung sections from septic shock pigs, 1 with no Rx (left panel) and 1 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. Panels A, B and C illustrate capture of circulating inflammatory leukocytes on SCD fibers.

Accomplishments: Proceeded to testing $SCD_{Rx}$ in porcine ARDS model. Significant therapeutic benefit was observed in clinically relevant hemodynamic and pulmonary parameters and immunomodulation suggested with assay parameters.

## Timeline and Cost

| | CY | 15 | | | | 16 | | | | 17 | | | | 18 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Activities** | | 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 | | | | $1,045,631 | | | | $787,713 | | | | $821,733 | | | |

**Updated:** September 2018

## 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 animal studies ☐ Analyze data from study series

**Comments/Challenges/Issues/Concerns**

- Limited cohorts to keep Year 2 on pace with timeline.

**Budget Expenditure to Date**

Projected Expenditure: $1,875,055
Actual Expenditure: $1,875,055