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

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14. ABSTRACT

Purpose. Acute lung injury (ALI) occurs in >30% of combat casualties. Inflammation is a key component and therapeutic strategies to block it are expected to decrease morbidity/mortality. Investigations directed at ways to limit activation and accumulation of leukocytes at sites of inflammation may prove clinically effective. **Scope/Aims.** Modulating neutrophil & monocyte/macrophage activity, the effector cells of the innate immune system, is a novel approach to diminish inflammation-induced disease processes without interfering with other immunologic activity, thereby avoiding adverse side effects. Innovative BioTherapies developed a selective cytopheretic device (SCD) therapy (Rx), to address this unmet medical need. SCD_{Rx} has been shown to improve clinical outcomes of critically ill patients with multi-organ failure by mitigating the inflammatory cascade. **Specific Aim 1.** Optimize a two-hit porcine ALI model relevant to combat situations. **Specific Aim 2.** Assess efficacy of 24h SCD_{Rx} in the porcine model. **Progress to date.** Yr1: Animal approvals obtained. 17 studies conducted yielding study & analysis protocols (Aim 1 met). Yr2: Began assessment of SCD_{Rx} efficacy in porcine model. 17 studies conducted and significant therapeutic benefit was observed (Aim 2 initiated). Yr3: Continued assessment of SCD_{Rx} efficacy (18 studies planned). 5 studies were conducted using a larger device and even greater benefit was observed. A corporate restructuring resulted in loss of personnel and interrupted the work. A 1 year no cost extension has been requested to complete the project.

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 $Pa:FIO_2<300$, within 6 hours of LPS infusion start time. 2) 12 hour survival rate is ≥ 80%.		9.5	June 2016	July 2017	100%
Major Task 3: Verify reproducibility of two-hit porcine ARDS model up to 24 hr ARDS time course.	2.5	12	Aug. 2017	Feb 2018	100%
Subtask 1: Repeat study design determined in Aim1 /Major Task 2/Subtask 3 up to 24 hrs or until death, whichever occurs first.	1.5	11	Original July 2017 Adjusted Nov 2017	Feb 2018	100%
Milestones Achieved: 1) ALI, as defined by $Pa:FIO_2<300$, is achieved within 6 hours of LPS infusion start time in all pigs. 2) At least 80% of pigs survive 12 hours or longer.		11	Original July 2017 Adjusted Nov 2017	Feb 2018	100%

Subtask 2: Perform measurements and assays required to assess key endpoints/exploratory endpoints.	1	12	Original Aug 2017 Adjusted Dec 2017	Feb 2018	100%
Milestone Achieved: Experimental study design, with respect to analysis parameters and sample time points, will be finalized for Aim 2 study plan.		12	Original Aug 2017 Adjusted Dec 2017	Feb 2018	100%
	Timeline months to complete task	**Month of Proposal (0-36)**	**Anticipated Completion Date**	**Actual Completion Date**	**% Completed**
Specific Aim 2: Assess efficacy of 24 hour SCD_{Rx} in ARDS porcine model.					
Major Task 1: Perform 9 ARDS pig studies using final model optimized in Aim 1/Major Task 3/Subtask 1. (3 from each Cohort: Cohort defined in Methods on page 3 of SOW)	6	18	Feb. 2018	May 2018	See subtasks
Subtask 1: Perform 3 studies in each of the 3 cohorts.	5.5	17.5	Jan. 2018	May 2018	67% studies done in cohorts 1 and 2 only *(see below)*
Milestones Achieved: 1) ALI, as defined by Pa:FIO_2<300, is achieved within 6 hours of LPS infusion start time in cohort 1 and 3 pigs[1]. 2) At least 80% of pigs survive 12 hours or longer. 3) SCD therapy is successfully administered in Cohort 2 and 3. Please refer to footnote 1*		17.5	Jan. 2018	May 2018	83% studies not performed in Cohort 3 (milestone 3)
Subtask 2: Perform all measurements and assays required to assess key endpoints and exploratory endpoints.	6	18	Feb. 2018	May 2018	100%
Milestone Achieved: Assays results allow for comparison between cohorts.		18	Feb. 2018	May 2018	100%
Major Task 2: Perform 9 ARDS pig studies using final model optimized in Aim 1/Major Task 3/Subtask 1. (3 from each Cohort)	6	24	Aug. 2018	Aug 2018	See subtasks
Subtask 1: Perform 3 studies in each of the 3 cohorts.	5.5	23.5	July 2018	July 2018	67% studies done in cohorts 1 and 2 only (see below)
Milestones Achieved: 1) ALI, as defined by Pa:FIO_2<300, is achieved within 6 hours of LPS infusion start time in cohort 1 and 3 pigs[1]. 2) At least 80% of pigs survive 12 hours or longer. 3) SCD therapy is successfully administered in Cohort 2 and 3.*		23.5	July 2018	July 2018	83% studies not performed in Cohort 3 (milestone 3)
Subtask 2: Perform all measurements/assays required to assess key endpoints and exploratory endpoints.	6	24	Aug. 2018	Aug. 2018	100%
Milestone Achieved: Assays results allow for comparison between cohorts.		24	Aug. 2018	Aug 2018	100%

	Timeline months to complete task	Month of Proposal (0-36)	Anticipated Completion Date	Actual Completion Date	% Completed
Major Task 3: Perform 9 ARDS pig studies using final model optimized in Aim 1/Major Task 3/Subtask 1.	6	30	Feb. 2019		**55%**
Subtask 1: Perform 3 studies in each of the 3 cohorts.	5.5	29.5	Jan. 2019		(5 studies done)
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		(done on the 5 completed studies)
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		**0%**
Subtask 1: Perform 3 studies in each of the 3 cohorts.	5.5	35.5	July 2019		0%
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		0%
Subtask 2: Perform all measurements/assays required to assess key endpoints and exploratory endpoints.	6[*2]	36	Aug. 2019		0%
Milestone(s) Achieved: Assays results allow for comparison between cohorts.		36	Aug. 2019		0%

*[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 2: ASSESS EFFICACY OF 24 HOUR SCD_{RX} IN ARDS PORCINE MODEL

- **Major Task 3: Perform a total of 9 ARDS pig studies using final model optimized in Aim 1/Major Task 3/Subtask 1**

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

Originally Proposed cohorts were to be:
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.

During Year 2, a total of 10 studies were performed under Specific Aim 2: major tasks 1 and 2. Animals were assigned to cohorts 1 and 2 but were not assigned to cohort 3. The reasons for not performing studies in cohort 3 was discussed in the annual report for year 2 but will be summarized here to provide rationale for the direction of study activities as pursued during Year 3. Briefly, based upon the human clinical definition of ARDS, a $P_a:FiO_2$ <300 was intended to serve as verification of ARDS onset. The originally proposed study plan had an expectation that a $P_a:FiO_2$ <300 was going to occur within 6 hours, however in the actual developed model, this was not reliably achieved within every animal. While the $P_a:FiO_2$ did decrease and was indicative of lung injury, the timing was highly variable and the value did not actually consistently reach <300 once we started utilizing continuous venovenous hemofiltration (CVVH) to support the pigs. Therefore, in the actually developed model, the timing for initiation of SCD therapy as planned for Cohort 3 was not clear. The later onset and variable presentation of clinical ARDS meant that if we followed the original study plan, pigs may receive vastly different durations of SCD therapy. This would likely make the outcomes difficult to compare. Moreover, <12 hours of SCD_{Rx}, as might be the case if does not occur if ARDS is not diagnosed *until 12 hours post LPS in this 24 hour model, could prove to be an insufficient duration to achieve a statistically significant treatment effect. For these reasons, it was decided in Year 2 to only proceed with testing in Cohorts 1 and 2 and reevaluate the model before initiating studies in Cohort 3 during Year 3.*

At the start of Year 3, Year 2 results were critically reviewed. The results suggested that the developed pig ALI model is robust, however the clinical time course achieved within the model is not sufficiently long to allow for 1) waiting for clinically recognizable onset of ARDS (which was based on human definitions and which can take up to 12 or more hours from the start of LPS infusion) and then 2) for sufficient duration of SCD treatment (requires >12 hours of therapy) and then 3) still allow for observation of a reversal of the disease process which may not be evident for hours to days, even if the device is highly effective. Extending the model is not within the scope of the project due to the extensive resource costs and ethical cost of potentially prolonging animal suffering. However, it was determined that within the current pig model, which uses a prolonged low dose LPS priming event followed by high dose LPS ARDS trigger, the SCD_{Rx} treated cohort already displays a degree of reversibility of injury as treatment does not begin until greater than 12 hours after the start of LPS exposure. Thus, the intended goals of the subtask and the milestones are still being met. For these reasons, testing in a cohort where SCD therapy is delayed until a clinical diagnosis of ARDS is met, as originally proposed, has been removed from the study plan.

Year 2 results clearly demonstrated that SCD_{Rx} potentially has significant beneficial treatment effects for patients at risk of or during early development of ARDS as observed in the pig model, therefore this avenue of testing merits further investigation. If SCD_{Rx} is to become a therapeutic option for ARDS, therapy must be optimized for this indication. Increasing knowledge regarding the mechanism of action for the SCD has led to the understanding that this device alters the immune response to acute inflammatory insults by sequestration of innate immune cells, primarily neutrophils. Recent data supports the idea that the amount of binding of activated leukocytes during acute inflammation is proportional to the treatment effect. Therefore, in theory, a device with a larger effective surface area for interaction with blood leukocytes will have greater therapeutic impact. This dose effect has been briefly explored (4), but much work remains to optimize dose of SCD_{Rx}, particularly for use in different indications. Since the effect of delayed treatment was already being investigated based upon the current model, exploration of the dose effect of SCD_{Rx} based upon surface area was undertaken to further assess the therapeutic benefit of SCD_{Rx} during ARDS.

During Year 2, studies were performed using the clinically utilized SCD-ARF, which has a lumen surface area of 1.0 m^2 (calculated ECS surface area of 1.4m^2). For Year 3, studies were initiated using SCD with a larger lumen surface area of 1.8m^2 (calculated ECS surface area of 2.5m^2) which provided considerably greater surface area for leukocyte interactions. SCD_{Rx} using a 1.8m^2 SCD in studies for year 3 was initiated at the time of LPS infusion as was done previously. Animals treated with the larger 1.8 m^2 devices were designated as Cohort 3.

Five pig studies were completed in Y3Q1. Of these, a single animal was allocated to Cohort 1 (untreated) to provide a contemporaneous control and 4 pigs were allocated to the newly defined Cohort 3, in which SCD_{Rx} 1.8m^2 was initiated at the start of LPS infusion. Each of the studies proceeded as expected and no device related adverse events were observed. All 5 pigs survived to the 24 hour study endpoint. Data were collated and then compared to the previous cohorts to determine if noteworthy treatments effects using SCD with a larger surface are of 1.8 m^2 were evident.

Hemodynamic data averaged per cohort are presented in Figure 1. Even greater improvements in hemodynamic stability were observed post LPS in the pigs that received 1.8 m^2 SCD_{Rx} based upon significantly higher cardiac index and mean arterial pressure (MAP). These improved hemodynamics resulted lower fluid and pressor support requirements. The therapeutic impact resulting from the difference in hemodynamics between treatment cohorts is best reflected in the Vasopressor dependency index which is calculated hourly for each animal by tabulation of the doses of all administered vasoactive medications and dividing by the obtained mean arterial blood pressure (MAP) achieved at that time (6). All of the untreated pigs to date (100%) have required administration of vasopressor medications to maintain minimum target hemodynamic values during the sepsis phase. In contrast, only fifty percent (50%) of the SCD_{Rx} pigs required any vasopressor support throughout the entire study period. The proportion of pigs requiring support was the same irrespective of the size of SCD used for treatment (3/6 for 1.0m2 SCD and 2/4 for 1.8m2 SCD), yet the pressor dependency index scores revealed that pigs treated with SCD 1.8m2 required lower doses /less pressors (as well as less fluid boluses) to maintain target hemodynamic values (Figure 1).

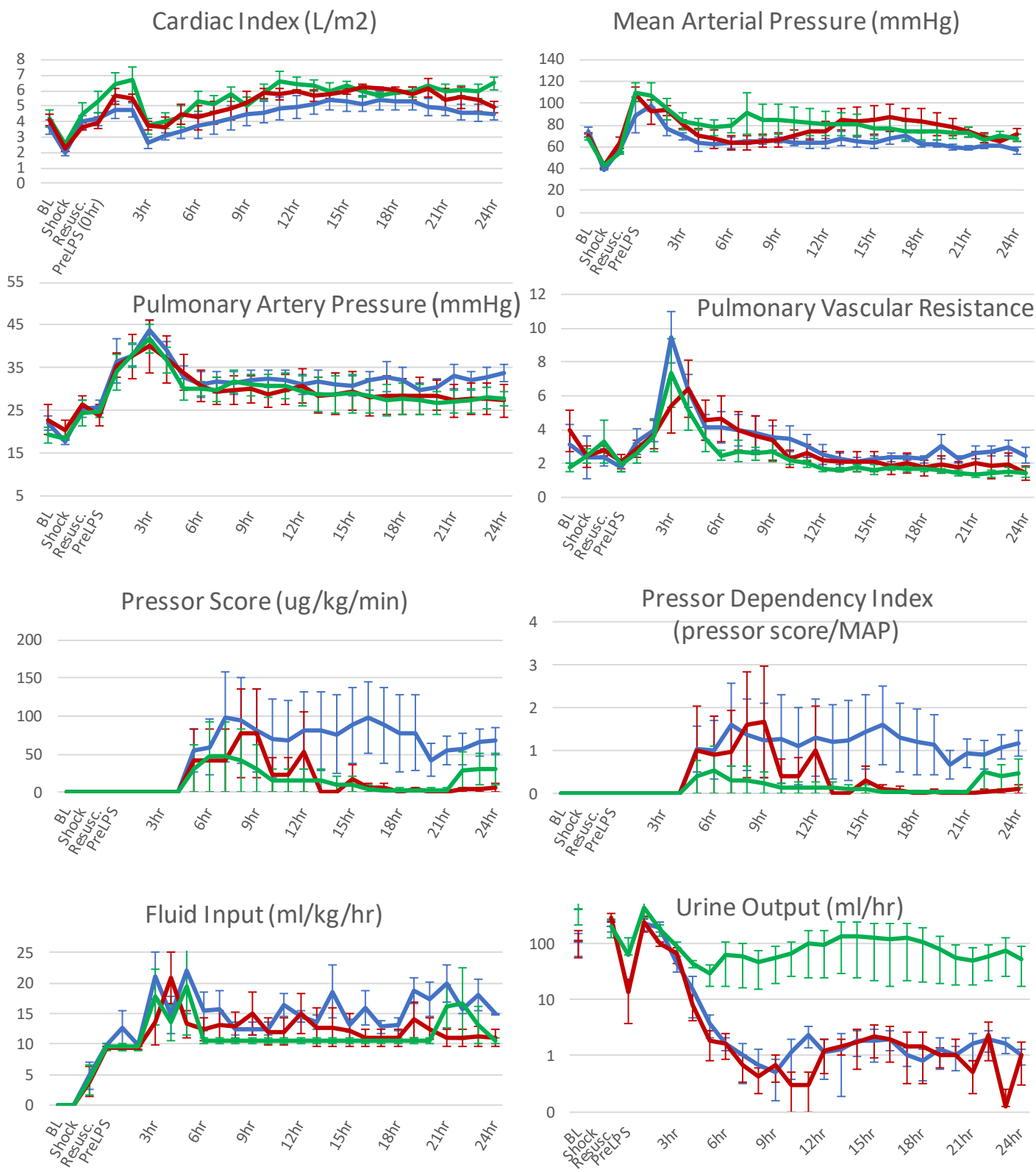


Figure 1. Clinically relevant hemodynamic data observed in ARDS pig model. Untreated (Cohort 1, Blue, n=6), SCD 1.0m2 (Cohort 2, Red, n=6), and SCD 1.8m2 (Cohort 3, Green, n=4) mean± SE

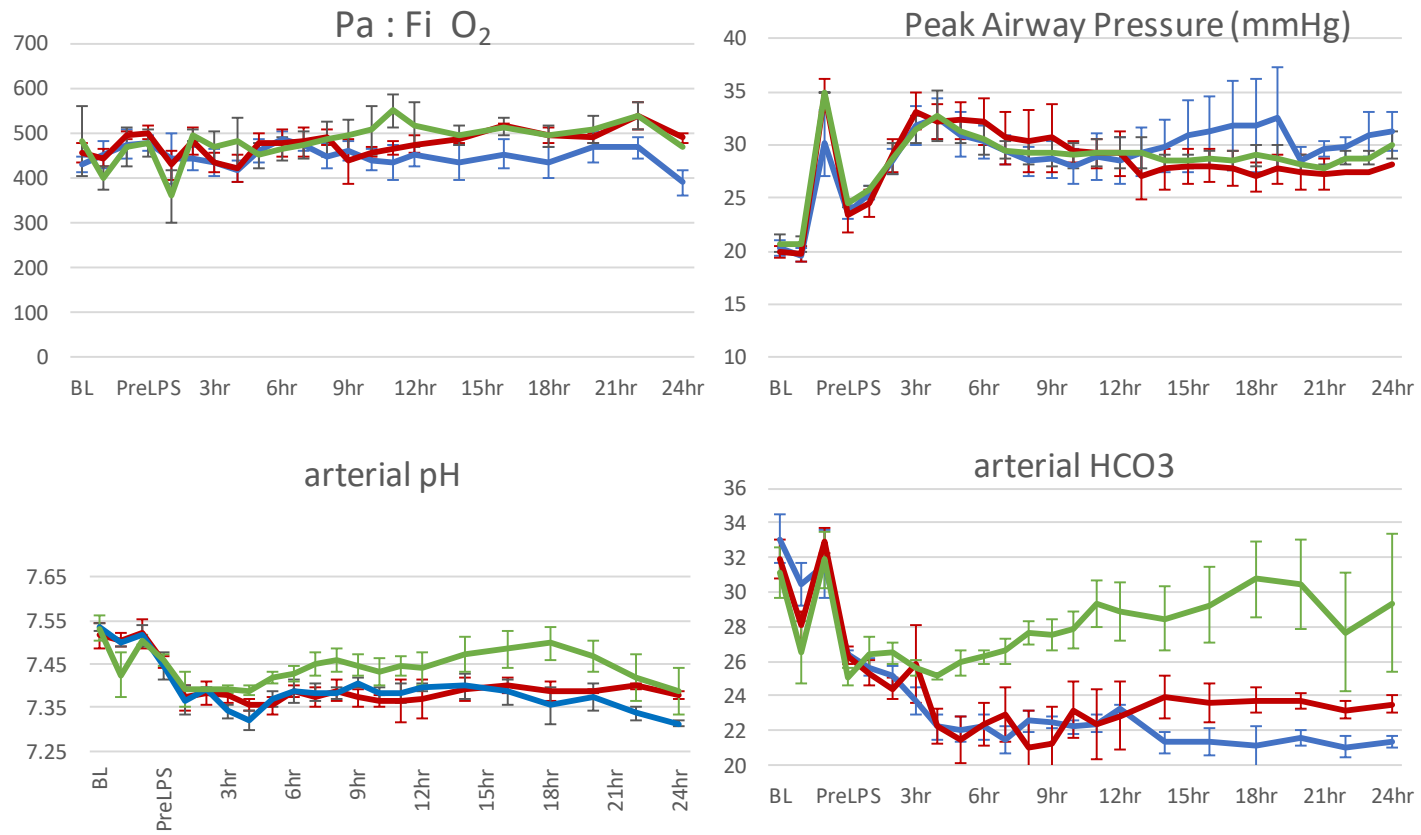


Figure 2. Clinically relevant pulmonary parameters observed in the ARDS pig model. Untreated (Cohort 1, Blue, n=5) SCD 1.0m2 (Cohort 2, Red, n=6) and SCD 1.8m2 (Cohort 3, Green, n=4), mean ± SE.

The previously observed differences between the treatment cohorts in several clinically utilized pulmonary parameters (Figure 2) endured with use of SCD 1.8m^2, but were not significantly improved over what had been observed with used of SCD 1.0m^2. Overall, SCD treated pigs maintain higher arterial oxygenation as demonstrated by the Pa:FiO2 ratio along with a trend for lower peak airway pressures and better maintained dynamic compliance of the pulmonary system. Close evaluation of all blood gas parameters measured using the i-STAT point of care analyzer (Abbot) did however reveal several important differences with the use of SCD 1.8m^2. The serum bicarbonate level was better maintained commensurate with higher pH of arterial blood post LPS. For this cohort, these values were more similar to preinjury values. This finding is likely reflective of the improved cardiovascular status of these animals and may also potentially be indicative of better preservation of renal function. Anuric renal failure has been observed in all pigs in both Cohorts 1 and 2 (with total urine outputs typically <5 mL/hour) and was the reason continuous renal replacement therapy using continuous venovenous hemofiltration (CVVH) was added to the model. During the Year 3 studies using SCD 1.8m2, 2 of the 4 pigs treated with this larger device continued to produced urine over the entire study. Pig035 maintained output >1 ml/kg/hr while Pig039 retained near normal outputs of 2-4 ml/kg/hr. This is especially noteworthy when considering that the pigs in this cohort had lower fluid inputs, receiving only the prescribed maintenance fluids for the majority of the study period.

A corporate restructuring took place Dec 20th, 2018, whereby a significant decrease in the number of available research personnel prevented conduction of additional animal studies, thus only 5 of the projected 18 studies were completed. (see details under Section 5. Changes/Problems) During the remainder of Year 3, analysis of the samples collected during the 5 conducted studies was performed and the resulting data was analyzed and collated (subtask 2, see below).

Major Findings:

- *Measurement cardiovascular and pulmonary parameters allows for clinical assessment of animals. SCD_{Rx} results in greater hemodynamic stability during the septic shock phase and improved pulmonary function over untreated pigs.*
- *A dose effect of SCD_{Rx} was observed with even greater hemodynamic stability with use of SCD 1.8m^2.*
- *Renal function was better preserved with use of SCD 1.8m^2.*

Milestones Achieved:

- *Using the redefined model to continue testing, ALI is being induced (most visible in the in untreated cohort) based upon a decrease in Pa:FiO2 and changes in other pulmonary parameters.*
- *> 80% of pigs survived >12 hours which allows for sufficient duration of SCD therapy and allows for comparisons between cohorts.*
- *SCD_{Rx} was successfully administered in Cohort 2(SCD 1.0m^2) and the re-defined cohort 3 (SCD1.8m^2).*

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

As done in all previous studies, 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.

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

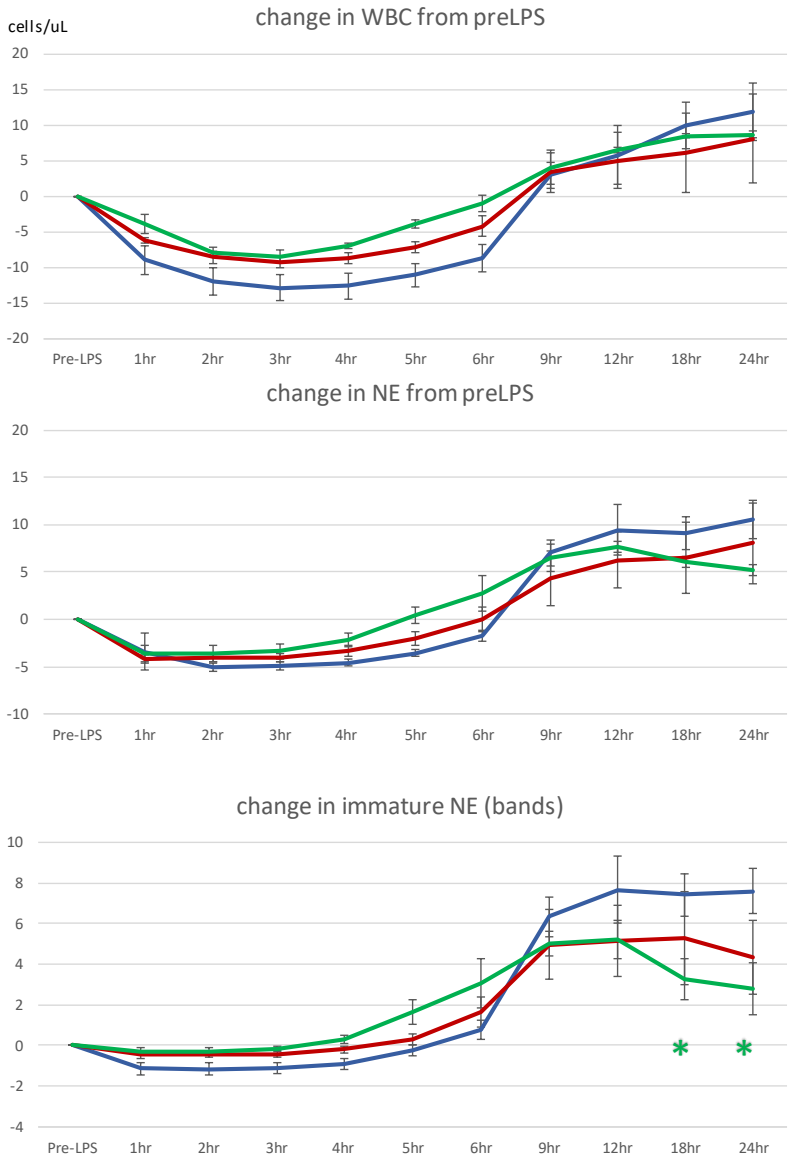


Figure 3. Changes in leukocyte counts in relation to administration of high dose LPS from ARDS pig studies to date. LPS administration results in a profound decrease in circulating leukocyte counts which persists for many hours followed by a rebound and dramatic increase in leukocyte counts. SCD therapy attenuates this decrease with SCD 1.8m2 resulting in earlier resolution of leukopenia and reduced rebound leukophilia, especially in number of band neutrophils. Untreated (Cohort 1, Blue, n=5), SCD 1.0m2 (Cohort 2, Red, n=6), SCD 1.8m2 (Cohort 3, Green, n=6) mean± SE. * indicates significant differences between cohort 1 and 3 ($p<0.05$) at the end of the study period.
Note: For clarity, several early timepoints which also had significant differences between the SCD treated and untreated groups are not identified in these graphs.

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

Arterial blood was drawn into EDTA tubes and processed to obtain plasma at regular intervals. Plasma from baseline (immediately post arterial access, Day 0), pre-LPS (immediately post initiation of high dose LPS on Day 1), 2hr, 4hr, and 6hr, 12hr, 18hr and 24hr was analyzed by Luminex for porcine proteins (IFNα, IFNγ, IL-1b, IL-6, IL-8, IL-10, IL-12p40, and TNFα), using the Cytokine & Chemokine 9-Plex Porcine ProcartaPlex™ Panel (ThermoFisher, EPX090-60829-901). The assayed values and standard error for all assayed values are shown in Table 1. IFN is not expected to be increased with this type of insult but it was included in the commercial assay and as expected, most concentrations observed were just at the detection level of the assay. One control animal had high levels of IFN which suggests a viral infection and makes the mean data appear as if there is a therapeutic influence of SCD_{Rx} on this parameter. More importantly, 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. TNFα spiked at 2 hours while other analytes peaked from 4-6 hours following high-dose LPS infusion.

Cytokine patterns are complex and despite countless clinical reports have not consistently been shown to be predictive of outcomes (7), 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 (8, 9). In this pig model, trends are emerging, and significance may yet be achieved upon completion of the targeted study cohorts. Of note, the average concentrations for the proinflammatory cytokine IL-6 were found to be lower in SCD treated animals and lower still in the SCD $1.8m^2$ treated cohort 3. In fact, most cytokine levels, both pro and anti-inflammatory, were found to be lower in this cohort. One notable exception to this being, IL-8 which had an early high spike and rapid disappearance of this cytokine for cohort 3. This was a slightly different pattern than was observed in the other cohorts. IL-8, also known as neutrophil chemotactic factor, has two primary functions. It induces chemotaxis in target cells, primarily neutrophils but also other granulocytes, causing them to migrate toward the site of infection. IL-8 also stimulates phagocytosis once they have arrived. As this cytokine is implicated in neutrophil activity, the finding suggests alteration of the acute immune response to LPS by SCD_{Rx}. Yet with only a handful of pigs currently in this cohort, these findings, while compelling, do not yet reach statistical significance and additional studies are needs to determine if these trends continue. Furthermore, caution is warranted in overinterpretation of these preliminary results as samples from these few animals were analyzed separately from the Year 2 study set and as a whole the cytokine concentrations appeared to trend much lower. Differences in assay kits could account for this variability, however, the single untreated animal in this series had higher levels of most cytokines than seen in SCD $1.8m^2$ treated animals assayed in the same time period and cytokine levels for this animal were in line with other animals in the untreated cohort. Analysis will be repeated and verified upon completion of all studies.

The average concentrations for the serum cytokine levels to date have also been graphed for easier comparison in Figure 4.

Table 1: Systemic Plasma Pig Cytokine and Chemokine Concentrations as Assayed by Luminex

Cytokine		Baseline	Pre-LPS	2	4	6	12	18	24
IL-1b (pg/mL)	CNTL	6.29	6.45	15.45	51.94	63.40	17.86	8.67	2.44
	se	1.57	1.43	2.92	7.67	17.33	5.35	2.60	1.50
	SCD 1.0 m2	12.72	7.55	16.98	63.91	93.23	23.42	10.07	3.50
	se	6.07	0.72	2.68	11.77	36.35	5.67	3.44	1.27
	TTEST	0.329	0.509	0.708	0.414	0.476	0.494	0.755	0.619
	SCD 1.8 m2	33.45	13.89	17.56	25.77	34.24	8.08	10.07	5.47
	se	10.81	5.20	6.81	7.12	11.48	1.65	3.37	2.13
	TTEST	0.015	0.136	0.755	0.046	0.250	0.189	0.748	0.270
IL-4 (pg/mL)	CNTL	4.45	4.33	3.82	4.18	3.82	3.01	2.81	3.29
	se	1.04	1.05	0.82	0.55	0.85	0.65	0.76	0.72
	SCD 1.0 m2	5.40	4.96	4.08	4.40	3.68	3.48	4.00	4.60
	se	0.81	0.78	0.55	0.46	0.42	0.51	0.00	1.15
	TTEST	0.490	0.638	0.792	0.756	0.892	0.596	0.280	0.345
	SCD 1.8 m2	18.35	5.71	5.45	7.93	3.95	3.99	5.36	1.99
	se	6.16	3.62	1.58	2.00	1.76	1.39	2.51	1.02
	TTEST	0.025	0.671	0.341	0.061	0.941	0.490	0.317	0.318
IL-6 (pg/mL)	CNTL	22.12	25.59	513.14	940.72	767.39	495.39	268.41	42.97
	se	21.72	21.10	78.34	174.14	133.53	77.80	86.99	22.28
	SCD 1.0 m2	0.20	2.70	465.30	947.65	862.94	635.36	124.68	5.72
	se	0.20	0.66	94.84	131.83	198.27	265.81	82.29	2.96
	TTEST	0.337	0.304	0.706	0.975	0.698	0.596	0.313	0.186
	SCD 1.8 m2	49.34	47.75	347.19	412.89	386.31	125.18	55.25	24.77
	se	30.77	36.85	53.56	77.21	149.89	63.98	22.25	5.51
	TTEST	0.477	0.588	0.158	0.048	0.100	0.010	0.072	0.502
IL-10 (pg/mL)	CNTL	10.32	6.69	1531.83	1761.86	404.47	21.24	2.26	0.00
	se	6.45	1.59	269.97	299.40	89.21	11.86	2.26	0.00
	SCD 1.0 m2	43.96	15.22	1739.56	2185.19	583.17	71.24	4.13	0.00
	se	25.95	3.68	339.57	434.94	200.21	62.21	4.13	0.00
	TTEST	0.237	0.059	0.642	0.441	0.434	0.408	0.675	
	SCD 1.8 m2	93.70	13.85	1075.05	1056.79	355.13	0.00	0.00	0.00
	se	93.70	13.85	361.70	545.62	349.61	0.00	0.00	0.00
	TTEST	0.296	0.538	0.332	0.252	0.873	0.190	0.407	

Table 1 continued.

Cytokine		Baseline	Pre-LPS	2	4	6	12	18	24
IL-12 (pg/mL)	CNTL	225.45	253.04	514.22	1957.00	1884.23	1210.72	723.42	382.25
	se	43.70	67.83	150.57	584.39	323.98	265.52	210.04	104.63
	SCD 1.0 m2	295.81	301.83	621.24	2827.22	2842.61	1534.90	410.29	176.56
	se	45.13	60.48	124.91	498.39	405.40	648.17	83.96	22.96
	TTEST	0.289	0.603	0.596	0.284	0.095	0.632	0.317	0.131
	SCD 1.8 m2	399.42	227.53	280.35	479.11	466.22	197.88	276.54	188.03
	se	43.85	40.07	36.30	91.56	132.70	94.77	24.36	42.25
	TTEST	0.028	0.785	0.254	0.079	0.010	0.018	0.104	0.162
IL-8 (pg/mL)	CNTL	4.95	6.41	2357.48	3122.34	534.21	9.72	2.45	0.00
	se	2.08	2.56	1481.35	1759.09	205.63	4.64	2.45	0.00
	SCD 1.0 m2	4.24	5.98	1237.66	1474.20	654.10	37.25	0.26	0.00
	se	2.49	3.86	338.08	341.26	424.28	33.75	0.26	0.00
	TTEST	0.831	0.926	0.478	0.379	0.804	0.396	0.528	
	SCD 1.8 m2	82.46	9.18	4489.18	1572.16	75.00	0.77	3.07	2.04
	se	22.12	2.50	1678.61	490.44	54.83	0.77	1.25	1.02
	TTEST	0.002	0.482	0.377	0.507	0.174	0.163	0.843	0.034
IFNa (pg/mL)	CNTL	3.53	7.89	5.46	6.88	6.83	9.36	12.29	9.88
	se	3.32	7.61	5.03	6.27	6.31	8.60	11.43	8.96
	SCD 1.0 m2	0.25	0.50	0.85	1.06	0.76	0.45	0.35	0.22
	se	0.07	0.23	0.45	0.55	0.32	0.11	0.08	0.02
	TTEST	0.346	0.355	0.383	0.377	0.359	0.373	0.463	0.373
	SCD 1.8 m2	0.34	0.22	0.25	0.20	0.19	0.17	0.17	0.17
	se	0.03	0.03	0.03	0.02	0.02	0.02	0.02	0.03
	TTEST	0.464	0.443	0.432	0.419	0.425	0.418	0.381	0.371
TNFa (pg/mL)	CNTL	4.30	2.53	1842.58	223.50	76.53	5.23	2.07	0.00
	se	2.42	2.53	575.40	75.24	27.78	1.89	2.07	0.00
	SCD 1.0 m2	10.94	0.89	1972.18	250.87	53.48	23.09	6.65	0.00
	se	4.94	0.89	387.21	109.05	16.69	20.60	6.65	0.00
	TTEST	0.255	0.554	0.856	0.841	0.493	0.365	0.444	
	SCD 1.8 m2	757.28	12.07	0.00	0.00	1.03	0.00	69.04	0.00
	se	694.24	11.61	0.00	0.00	1.03	0.00	69.04	0.00
	TTEST	0.207	0.354	0.034	0.045	0.061	0.058	0.306	

Cytokine & Chemokine 9-Plex Porcine ProcartaPlex™ Panel (ThermoFisher, EPX090-60829-901) IFNg is not included in table because values fell below assay detection range. Untreated (Cohort 1, Blue, n=6) SCD 1.0m2 (Cohort 2, Red, n=6), SCD 1.8m2 (Cohort 3, Green, n=4), mean± SE, Significant differences are highlighted as red text significance $p<0.05$.

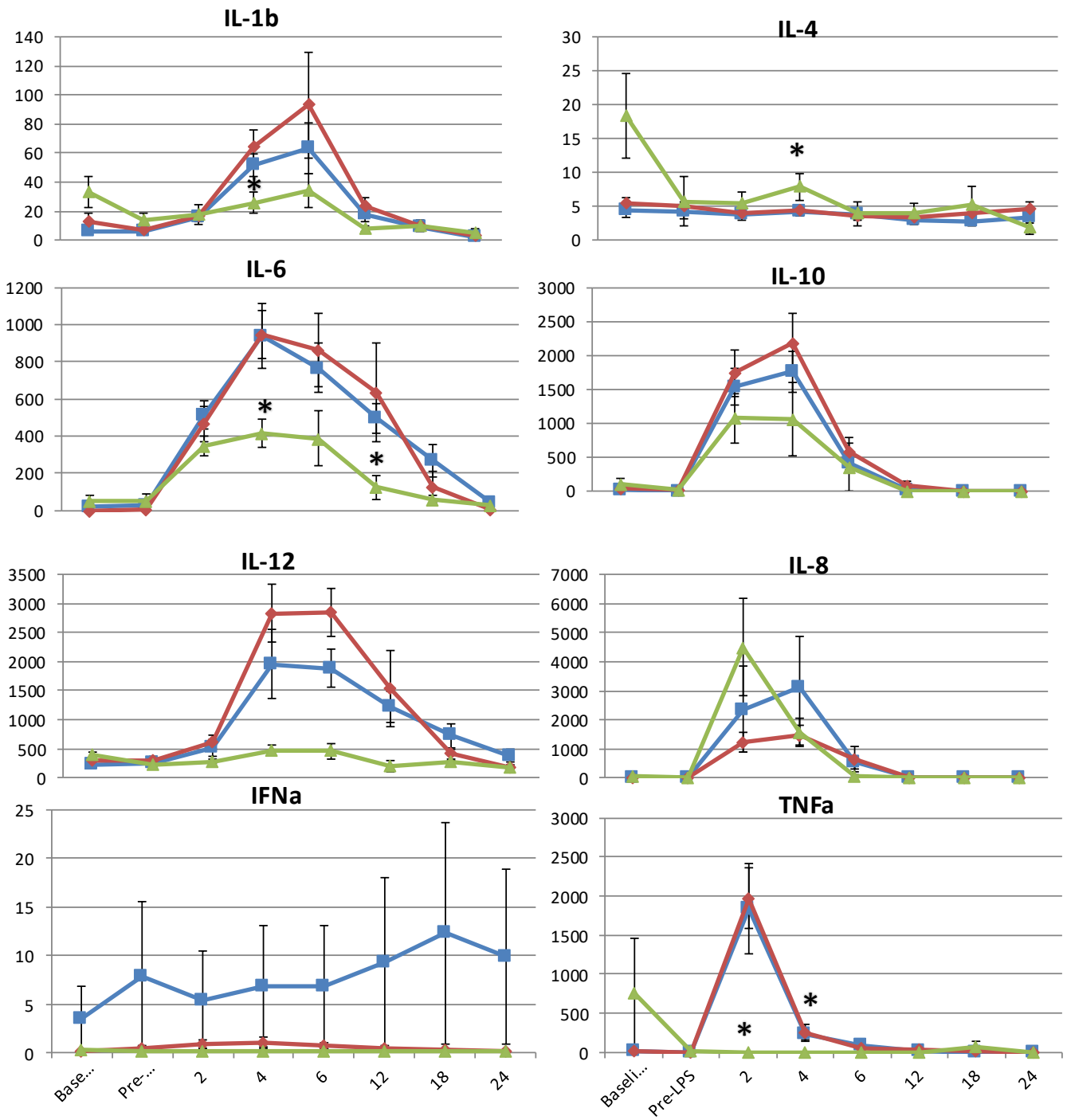


Figure 4. Graphic representation Pig protein levels detected by Cytokine & Chemokine 9-Plex Porcine ProcartaPlex™ Panel (ThermoFisher, EPX090-60829-901). Untreated (Cohort 1, Blue, n=6) SCD 1.0m2 (Cohort 2, Red, n=6), SCD 1.8m2 (Cohort 3, Green, n=4), mean± SE, significance $p<0.05$.

Cytometric Analysis

All cytometric analysis for both blood and lung cells was performed on an Attune (ThermoFisher) flow cytometer, equipped with the following lasers: 488 nm blue, 405 nm violet laser, and 633 nm laser. Data were collected using Attune software (ThermoFisher) with automatic compensation. Samples were taken for single channel CD11R3 analysis on neutrophils gated by scatter profiles at Day 0 Baseline, Day 1 Pre-LPS and hourly through 6 hours. A full analysis panel to evaluate monocyte subsets was performed at Day 0 Baseline, Day 1 Pre LPS, 6hr, 12hr, 18hr, and 24hr, and then on cells eluted from SCD membranes for the SCD treated cohorts. The antibody panels used to analyze cells from the lungs, blood and SCD membranes are shown in Table 2. Evaluation of neutrophil, monocyte and macrophage populations may provide insight to the transition from neutrophilic alveolitis to monocytic alveolitis (10). A gating hierarchy was confirmed during FY01 work for lungs and systemic blood included: CD11R3, CD284 (toll-like receptor 4), and S(swine)LA DR 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 (11, 12) 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 (13) and is highly expressed on a subset of monocytes and all macrophages. SLA DR Class II is differentially expressed on all cells of interest but may be shed as cells become anergic (14). Antibody to CD284 recognizes toll-like receptor 4 which can be differentially expressed

Table 2. 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

via a wide range of stressors (15, 16). Using the panels shown in Table 2, macrophages, neutrophils, monocytes, and monocyte sub-populations were reliably identified. The identified populations were then evaluated for expression of CD11R3, SLA DR II and CD284 (TLR4).

Neutrophil and Monocyte Activation in Systemic Blood

Human neutrophils (17, 18) and monocytes (19, 20) 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 (21). The clone was chosen for this project based upon its strong reactivity to cells of interest and detectable upregulation upon stimulation.

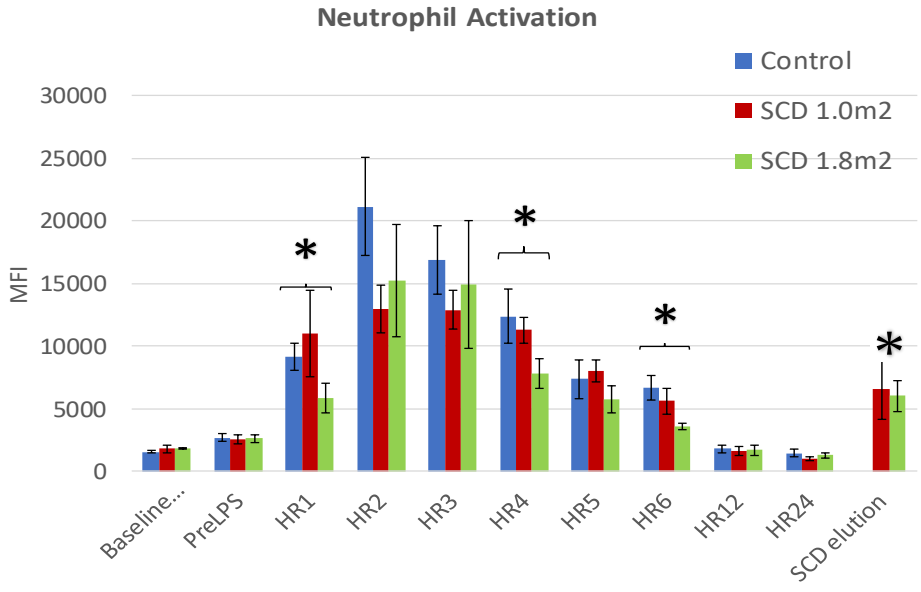


Figure 5. Neutrophil acute activation as detected by surface expression of CD11R3. Untreated (Cohort 1, Blue, n=6), SCD 1.0m2 (Cohort 2, Red, n=6), and SCD 1.8m2 (Cohort 3, Green, n=4), mean± SE, significance p<0.05.

As described during model development, the first hit of the two-hit model is detectable by increased neutrophil CD11R3 expression from D0 baseline to D1 Pre-LPS, with the average MFI CD11R3±SD significantly increasing from 1,691±512 to 2,618±727, p=0.0002 (average of all pigs to date). With high dose LPS injection, CD11R3 expression then increased dramatically concurrent with the decrease in systemic neutrophil numbers as these activated cells marginate and extravasate into tissues. Significantly lower CD11R3 expression levels on neutrophils in the SCD 1.8m^2 cohort compared to the untreated cohort were observed at 1, 4 and 6 hours after starting high dose LPS. This finding is compatible with the decreased severity of the hemodynamic response and disappearance of circulating leukocytes seen to high dose LPS in this cohort. As observed previously for the SCD 1.0m^2 treated cohort, CD11R3 expression by neutrophils eluted from SCD 1.8m^2 membranes at the study end (24 hours or death) was significantly higher than those in systemic blood at the time of device removal. This suggests that the most activated cells are being sequestered by the membrane. This data is shown graphically in Figure 5.

For monocytes, differences in CD11R3 expression on circulating were not observed between cohorts. For both the SCD treated cohorts, CD11R3 expression of monocytes eluted from SCD membranes at the study end (24 hours or death) was significantly higher than time matched monocytes in systemic blood (Figure 6).

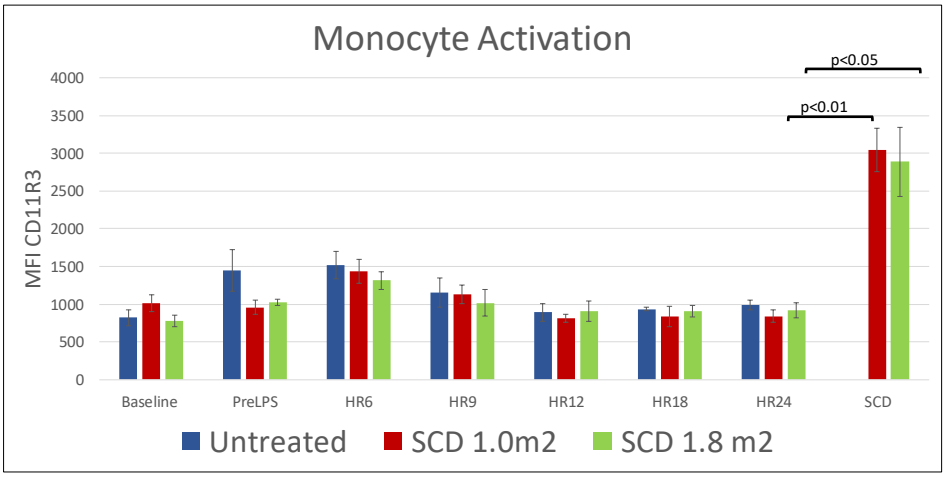


Figure 6. Monocyte activation as detected by surface expression of CD11R3. Untreated (Cohort 1, Blue, n=6) and SCD 1.0m2 (Cohort 2, Red, n=6), SCD 1.8m2 (Cohort 3, Green, n=4), mean± SE,

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

For pig, monocyte subsets have not been clearly defined by CD markers as compared to humans. Using available tools, a shift in monocyte phenotype has been detected in this ARDS model. The shift is most evident by CD163 expression. With LPS challenge, CD163+ cells leave the circulation and are replaced by CD14+ CD163- cells that then mature to CD14+CD163+. In humans, monocytes leave the bone marrow as CD14+ CD16-, and progress to CD14 low CD16+ cells. Depending on the maturation environment, some cells obtain the proinflammatory CD14+ CD16+ phenotype. These cells in human can be readily identified by strong human (H) LADR expression. In the pig model, (S)wine LADR expression increased with the trauma event, suggesting a pro-inflammatory state. A shift to a lower swine (S) LADR expression level was observed upon administration of LPS. Surprisingly, in SCD 1.8m^2 treated pigs, the (S) LADR expression level showed a trend for increasing toward the end of the study, although this was largely driven by one animal (p036), which had much higher expression of all surface markers and approximately double (S) LADR expression than seen in any of the other pigs. With only four animals in Cohort 3, it is too soon to tell if the current observations have real significance. 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 all experiments are completed to ensure consistency of gating.

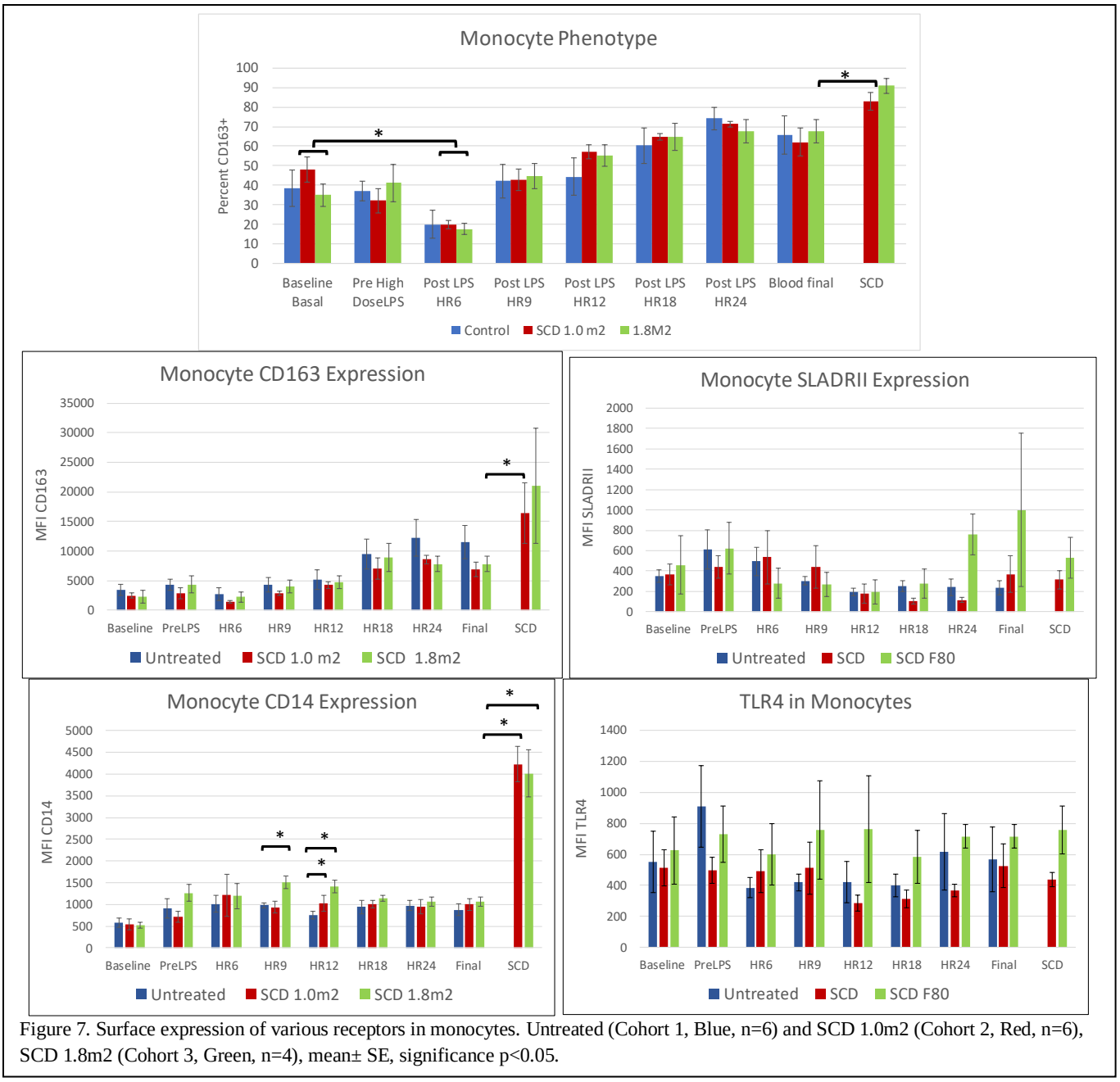


Figure 7. Surface expression of various receptors in monocytes. Untreated (Cohort 1, Blue, n=6) and SCD 1.0m2 (Cohort 2, Red, n=6), SCD 1.8m2 (Cohort 3, Green, n=4), mean± SE, significance $p<0.05$.

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 (22). BALf was assayed using the same Luminex panel as described for plasma (Cytokine & Chemokine 9-Plex Porcine ProcartaPlex™ Panel,ThermoFisher, EPX090-60829-901). Pulmonary edema of excised lungs was quantified by placing the entire left lobe into a Ninja food processor and processed until homogeneous. 1-2g samples weighed (wet weight) and dried until stable (dry weight) and then expressed as % water content (23, 24). The results for all animals are shown in Figure 8.

Figure 8. Evaluation of BALf and lung edema in 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. Untreated (Cohort 1, Blue, n=6) and SCD 1.0m2 (Cohort 2, Red, n=6), SCD 1.8m2 (Cohort 3, Green, n=4). Trends are emerging for lower values of these injury indices in the SCD treated cohorts, especially for SCD 1.8m^2.

In ARDS patients, the concentration of neutrophils in the BALf correlates with severity of ARDS and outcome (25, 26). Normally, BALf is almost devoid of these cells. For all study animals, neutrophils were found in the

BALf, and water content was higher than found for uninjured control animals, indicating that all study pigs had some degree of lung inflammation. The variables of edema, neutrophils and protein were not strongly correlative with each other suggesting that the pattern of lung injury is variable, at least over the first 24 hours. No statistical differences have yet been observed in these parameters using raw data values, although trends are emerging in that the highest observed values do occur in the untreated cohort. This is especially apparent and noteworthy for protein, as BALf from uninjured lung should contain very little protein. Significant concentrations of protein have only been found in untreated pigs thus far. Values for each of the BAL parameters are trending much lower for pigs treated with SCD 1.8m^2.

The detected cytokine and chemokine levels in the BALf were highly variable within cohorts. IFNα, IFNγ, IL-10, IL-4 and TNFα had many values below the detection level of the assay. IL-1b, IL-6, IL-8 and IL-12p40 were within the detectable range for all animals, and levels were not significantly different between cohorts. Data is presented in Table 3. For IL-4, the SCD 1.0m^2 cohort was statistically lower than untreated controls. This was related to the fact that IL-4 was undetectable in BALf from all animals in this cohort. Low levels of IL-4 were measured in BALF samples from pigs in the SCD 1.8m^2 cohort, much lower than seen in most control pigs (1.3±0.3 v 7.3±3.0 pg/mL). IL-4 is purported to play a role in chronic inflammation and wound repair and may be associated with alternative activation of tissue macrophages into different phenotypes. More data is needed to determine if this will emerge as a significant finding.

Table 3. BALf Pig Cytokine and Chemokine Concentrations as Assayed by Luminex

Untreated	IFN-alpha	IFN-gamma	IL1-beta	IL-10	IL-12p40	IL-4	IL-6	TNF-alpha	IL-8
p024 BALF	0.4504541	0.00	98.19576	16.86755	238.938	0.00	1113.735	162.5755	9265.094
p028 BALF	0.6003331	0.00	25.63703	1.86962	27.44501	14.0836	91.65048	0.00	877.373
p029 BALF	0.3505151	0.00	27.27148	1.86962	45.7	14.0836	22.84249	0.00	306.9356
p032 BALF	0.3505151	0.00	25.63578	0.00	41.15668	0.00	5.419462	0.00	1603.905
p033 BALF	65.476403	0.2902005	143.5736	0.00	186.7059	14.0836	244.2951	0.00	3460.829
p037 BALF	1.3618942	3.9031065	207.5385	393.1225	111.3073	1.827048	2074.617	772.1598	170.4085
av.	11.431686	0.6988845	87.97535	68.95488	108.5422	7.346309	592.0933	155.7892	2614.091
SE	10.810061	0.6425942	31.06349	64.88661	35.67772	3.025294	342.0344	126.1005	1417.557
SCD 1.0m2	IFN-alpha	IFN-gamma	IL1-beta	IL-10	IL-12p40	IL-4	IL-6	TNF-alpha	IL-8
p025 BALF	0.3004995	0.00	30.54638	0.00	9.161844	0.00	35.49388	0.00	422.0945
p026 BALF	1.1942961	0.2902005	153.9001	5.6	155.4795	0.00	1513.1	120.822	9475.422
p027 BALF	0.4004972	0.00	32.1811	0.00	9.161844	0.00	15.34873	0.00	390.5258
p030 BALF	0.4504792	0.1934729	17.44098	1.86962	18.30343	0.00	10.71042	12.76778	438.8046
p031 BALF	0.3504815	0.00	22.3595	0.00	9.161844	0.00	13.20779	0.00	319.941
p034 BALF	0.6502733	0.3869052	65.25658	0.00	66.28853	0.00	1112.099	12.76778	7960.092
av.	0.5577545	0.1450964	53.61411	1.24	44.59283	0.00	449.9932	24.39293	3167.813
SE	0.1365192	0.0695284	21.18936	0.922971	23.96091	0	277.6722	19.45414	1765.994
Ttest	0.34	0.41	0.38	0.32	0.17	**0.04**	0.75	0.33	0.81
SCD 1.8m2	IFN-alpha	IFN-gamma	IL1-beta	IL-10	IL-12p40	IL-4	IL-6	TNF-alpha	IL-8
p035 BALF	0.00	1.3464246	7.536757	74.55715	69.62419	1.317441	2.29461	69.471	15.3
p036 BALF	0.00	0.168526	9.960003	5.5	38.80747	0.425876	2.29642	31.66343	3.228295
p038 BALF	0.00	1.1783378	10.76816	0.00	59.21348	1.742252	3.439704	2.752276	67.56559
p039 BALF	0.07	4.7200921	14.04588	124.5128	90.46576	1.742252	4.589219	119.1649	7.256466
av.	0.02	1.8533452	10.5777	51.14249	64.52772	1.306955	3.154988	55.7629	23.33759
SE	0.02	0.9903475	1.344539	29.76329	10.75689	0.310293	0.548898	25.16386	14.95467
Ttest	0.42	0.33	0.08	0.84	0.36	0.15	0.21	0.55	0.18

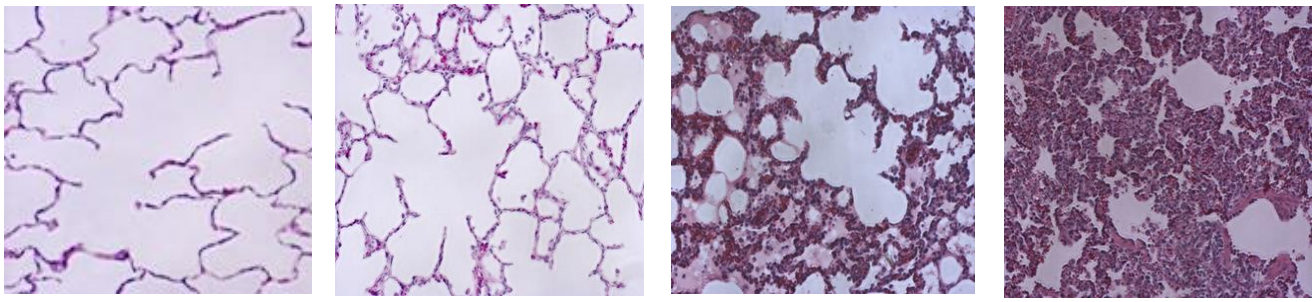
Cytokine & Chemokine 9-Plex Porcine ProcartaPlex™ Panel (ThermoFisher, EPX090-60829-901)

Morphometric Evaluation of Lung

The pathological hallmark of ALI is diffuse alveolar damage (DAD) (27). 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 (28) 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. Scoring for lung injury was performed independently by at least two lab personnel who were trained in the scoring system and blinded to pig identity and to treatment cohort. Lab personnel were used for this preliminary evaluation rather than submit slides to pulmonologists to conserve funds while we establish treatment cohorts for final analysis. Submission of photomicrographs 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 9.

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



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

	Animal ID	Atalectasis	Fibrin Deposits	Hem. In Airway Low Resolution	Congestion	Wall Thickness	LE infiltration quadrant	Hem in Airway High Resolution	LE infiltration count	Total Score A	Total Score B
Untreated	ARDSp024	2.17	1.83	0.83	1.67	2.33	2.33	2.67	2.67	11.17	13.33
	ARDSp028	0.33	2.83	1.50	1.83	1.00	1.00	1.50	0.83	8.50	8.33
	ARDSp029	2.50	1.33	2.50	2.33	2.67	2.83	1.33	2.50	14.17	12.67
	ARDSp032	1.67	1.33	1.33	0.83	2.17	3.00	0.50	2.83	10.33	9.33
	ARDSp033	3.00	3.00	2.33	2.50	3.00	3.50	1.17	2.33	17.33	15.00
	ARDSp037	1.33	1.17	0.17	2.83	2.17	2.83	0.17	2.33	10.50	10.00
	Average	1.83	1.92	1.45	2.00	2.22	2.58	1.22	2.25	12.00	11.44
	SE	0.39	0.33	0.36	0.29	0.28	0.35	0.36	0.29	1.31	1.06
SCD 1.0m2	ARDSp025	0.33	1.50	0.83	0.83	1.00	1.33	1.00	1.00	5.83	5.67
	ARDSp026	0.33	0.50	1.33	1.00	1.17	1.33	0.83	1.83	5.67	5.67
	ARDSp027	0.83	1.00	1.67	1.17	1.50	1.67	1.50	1.33	7.83	7.33
	ARDSp030	1.00	1.83	0.83	1.00	2.33	2.33	0.17	2.00	9.33	8.33
	ARDSp031	1.17	0.83	1.33	1.17	1.67	2.33	0.83	1.83	8.50	7.50
	ARDSp034	2.17	1.33	1.17	1.50	2.17	2.50	1.33	2.83	10.83	11.33
	Average	0.97	1.17	1.19	1.11	1.64	1.92	0.94	1.81	8.00	7.64
	SE	0.28	0.20	0.13	0.09	0.22	0.22	0.19	0.26	0.82	0.86
TTEST	eq var.	**0.050**	**0.040**	0.265	**0.008**	0.064	0.069	0.253	0.141	**0.013**	**0.010**
SCD 1.8m2	ARDSp035	1.00	0.83	1.17	1.83	2.00	2.33	0.83	3.00	9.16	9.49
	ARDSp036	0.50	0.00	0.00	0.83	1.17	1.67	0.17	2.00	4.17	4.67
	ARDSp038	1.50	0.17	0.17	1.50	1.67	2.33	0.67	3.17	7.34	8.68
	ARDSp039	0.00	0.17	0.17	0.50	1.00	0.83	0.50	1.83	2.67	4.00
	Average	0.75	0.29	0.38	1.17	1.46	1.79	0.54	2.50	5.84	6.71
	SE	0.32	0.18	0.27	0.30	0.23	0.36	0.14	0.34	1.48	1.39
TTEST	eq var.	**0.041**	**0.003**	**0.032**	**0.046**	**0.044**	0.084	0.088	0.299	**0.008**	**0.013**

Figure 9. Morphometric scoring of lung H&E Images. Scoring was performed by 2 blinded investigators on 3 high magnification and 3 low magnification images from each animal then all scores for each parameter were averaged. Total Score A was calculated using a reported scoring method and Total score B is an adapted method based upon scoring at high and low magnifications. Significant differences ($p<0.05$) are highlighted by bold red font. Untreated (Cohort 1, n=5), SCD 1.0m2 (Cohort 2, n=6) and SCD 1.8m2 cohort 3, n=4)

Immunohistochemistry (IHC) was used to assess LE infiltration of lungs using CD11R3 (11, 29-31) a marker expressed specifically on activated leukocytes. A bronchus to the diaphragmatic lobe of the right lung was inflated with a 50/50 (v/v) optimum cutting temperature (OCT) compound (Tissue Tek)/PBS, via a cannula using a method similar to that described for BAL. Once inflated, the isolated lung lobe was placed into a pan on

wet ice to allow it to become firm, then sections were cut and placed into cryomolds with OCT. Filled cryomolds were frozen in the vapor phase on a surface precooled with liquid nitrogen. Prepared blocks were sectioned using a Lecia cryostat and labeled with antibody to CD11R3 (BioRAD) and visualized using anti mouse IgG conjugated to Alexafluor 594 (Fisher Life Sciences). Tissues from pig studies completed during Year 3 have been processed at the time of this report. Images for pigs performed during Year 3 still need to be analyzed using NIH Image J software to provide a semi-quantitative measurement of CD11R3+ leukocyte (monocytes and neutrophils) infiltration of lung tissue. These images will be captured and processed using equivalent settings. The images are then evaluated in three ways: 1) The total area of positive pixels for CD11R3 in the red channel normalized for the total area of positive pixels for DAPI in the blue channel (Area/Area), 2) the total Area of CD11R3+ cells normalized by total number of DAPI positive nuclei (Area/Count, and 3) the total number of CD11R3+ cells normalized by the total number of DAPI positive nuclei (Count/Count). Representative images are shown in Figure 10 for SCD_{Rx} animals that scored low (ARDSp026), and high (ARDSp30). During Year 2, a trend for a lower amount of CD11R3+ expression, by all 3 ways of quantitation was apparent in the SCD treated cohort (data presented in FY02 report). Data is still being analyzed from tissues processed during Year 3.

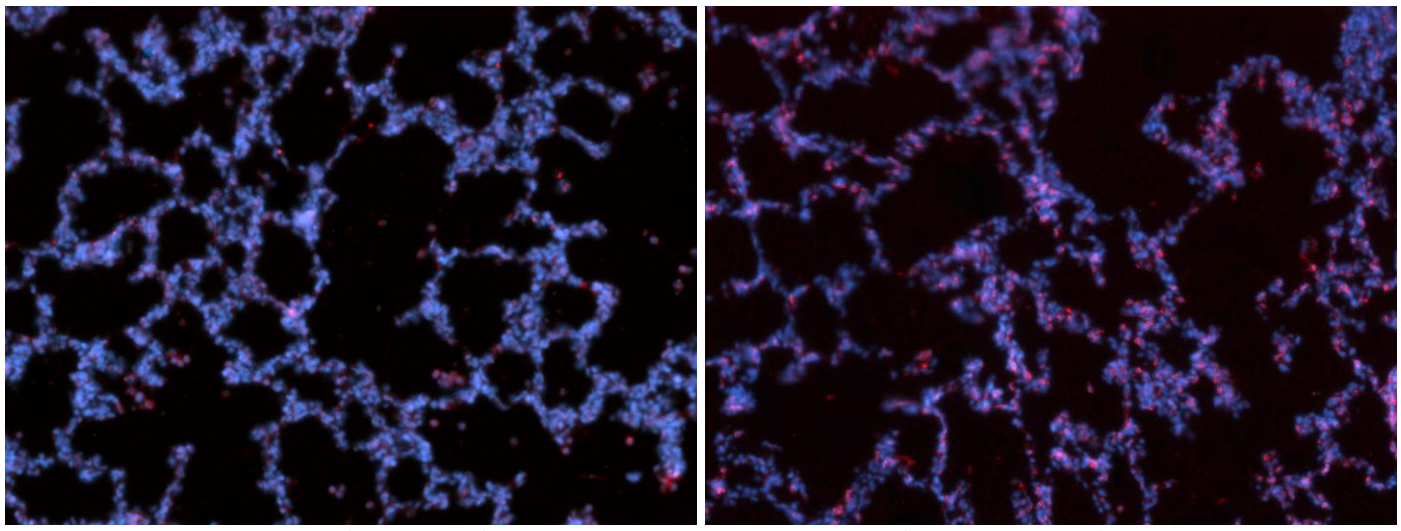


Figure 10. Representative images of lung tissue after immunohistochemical staining, Images from ARDSp026 (left) and ARDSp030 (right) provide comparison of low to high scoring images for presence of activated leukocytes, based on presence of CD11R3. CD11R3+ cells are red, and all cell nuclei are labeled with DAPI blue. During Year 2, a trend for lower CD11b+ cells in the SCD 1.0m2 treated cohort was observed. Imaging and analysis from cohort 3, pigs treated with SCD 1.8m^2, have not yet been completed.

Cytometric analysis of lung composition.

With CD11R3 analysis alone, it is not possible to differentiate monocytes, macrophages and neutrophils. Therefore it is not clear if differences in expression of this cell marker are to infiltration of specific cell types or related to activation of residence cells. As an alternative approach to accomplish that goal, cells were obtained from gentle enzymatic treatment of lung tissue post manually using dissecting scissors (23, 24). The same lung lobe was used for BAL and enzymatic treatment. Lungs were then analyzed for the distribution of $CD172a^+$ pig myeloid derived cells as evaluated by flow cytometry and confirmed using manual cytospins. Enzyme

Table 4. Distribution of CD172a+ myeloid derived cells in lung tissue evaluated by cytometry

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

dissociated lung cells were labeled with combinations of CD172a, CD14, CD163 (porcine monocyte maturation marker(13)), and SWC9 (positively identifies alveolar macrophages (11, 12)) to determine overall cells distribution with the goal of quantitatively assessing shifts in cell density that may be attributable to immune cell modulation with use of SCD_{Rx}. A significant difference in the distribution of cells in the lung was observed between cohorts in that less neutrophils (and conversely a greater number of lymphocytes) were present when comparing the SCD 1.8m^2 versus untreated control pigs. Results are shown in Table 4.

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

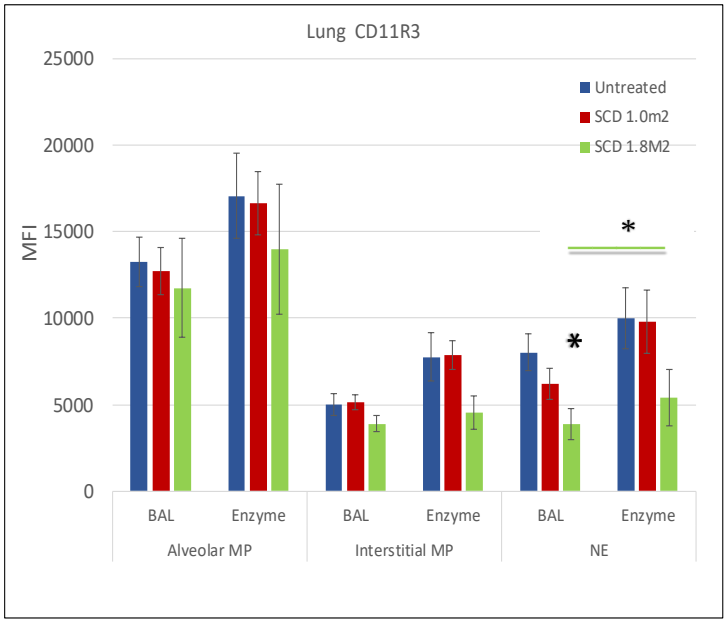
Lung Cells Surface Marker and Intracellular Cytokine Cytometric Analysis

Samples from BALf and enzyme treated lung were incubated with antibodies to CD11R3, SLA DRII and TLR4 in combination with the monocyte/macrophage phenotype markers (Table 2) to investigate differential activation among interstitial and alveolar lung macrophage populations (32). Expression of Toll-like receptors (TLR) by alveolar macrophages is upregulated by a variety of stressors, including ischemia-reperfusion and

ventilator-induced lung injury, and in turn is required for induction of ALI in animal models (15, 16). Evaluation of monocyte/macrophage populations may provide insight to the transition from neutrophilic alveolitis to monocytic alveolitis at 24h (10) and importantly, if SCD_{Rx} can potentially alter this immunologic response. In Humans, receptor profiles define the M1 vs M2 monocyte/macrophage phenotypes (33-35), but these parameters are less clearly defined in pig. Further elucidation of pig monocyte/macrophage behavior requires a broad spectrum of tools. In addition to surface markers, secretory profiles were analyzed using both intracellular cytokines evaluated on individual cells using cytometry, and the secretory profile of isolated alveolar macrophages, interstitial monocyte/macrophages and blood monocytes were analyzed by Luminex.

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

Within the alveolar compartment, the expression of TLR4 was significantly reduced for pigs in the SCD 1.0m^2 treated cohort both alveolar macrophages, MFI 6966±1354 vs. 4022 ± 611 p=0.032, and neutrophils, 1007 ± 183 vs. 488 ± 192 p=0.043 for untreated and SCD 1.0 m^2 cohorts respectively. Significantly reduced TLR4 was also observed for interstitial macrophages and neutrophils. Results are presented in Figure 11. This finding was first observed during Year 2 and statistical differences were strengthened with the results from another untreated animal performed during Year 3. Surprisingly, this same trend was not observed for SCD 1.8m^2 cohort as mean values were very similar to the untreated cohort. However, this may be due in part to the animal to animal variation observed for these values with several but not all of the untreated pigs having high TLR expression. More animals are need per cohort to ascertain if significant difference in TLR4 for this cohort can also be observed.



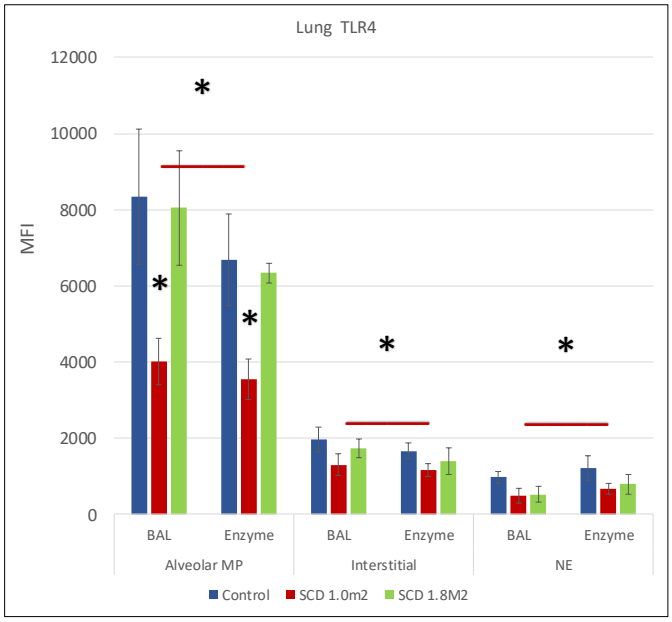


Figure 11. Surface expression of lung cells from the alveolar and interstitial compartments. Expression of CD11R3 was lower on neutrophils for pigs treated with SCD 1.8m2 compared to untreated. TLR4 was significantly lower for both macrophages, p=0.032, and neutrophils p=0.043 in SCD 1.0 m2 treated cohort but due to the small number of animals in the SCD 1.8 m2 cohort, differences were not apparent. BAL and enzyme cell data was examined individually and consolidated for each cell type, which is represented by the colored lines. Untreated (Cohort 1, Blue, n=6) and SCD 1.0m2 (Cohort 2, Red, n=6), SCD 1.8m2 (Cohort 3, Green, n=4), mean± SE, significance $p<0.05$ is indicated by the asterisks.

Lung Cells – Secreted and Intracellular Cytokines

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

Interferons were not expected to be secreted by macrophage and monocytes in response to LPS but were included on the commercially available panel. Assay results were consistent with this prediction with results being just around the lower detection levels. Statistical differences were observed in IL-6, IL-8, IL-10, and TNFα but these differences were not consistent between the SCD treated cohorts. For all cohorts, interstitial macrophages were much more active for IL-6 and IL-10 than alveolar macrophages, and alveolar macrophages were more active for TNFα possibly detecting differences in how these cells function during an inflammatory insult and highlighting potential targets for SCD_{Rx}. Monocytes associated with the SCD membranes tended to more active than contemporaneous blood monocytes. Secretory profiles of isolated monocytes and macrophages are shown in Figure 12

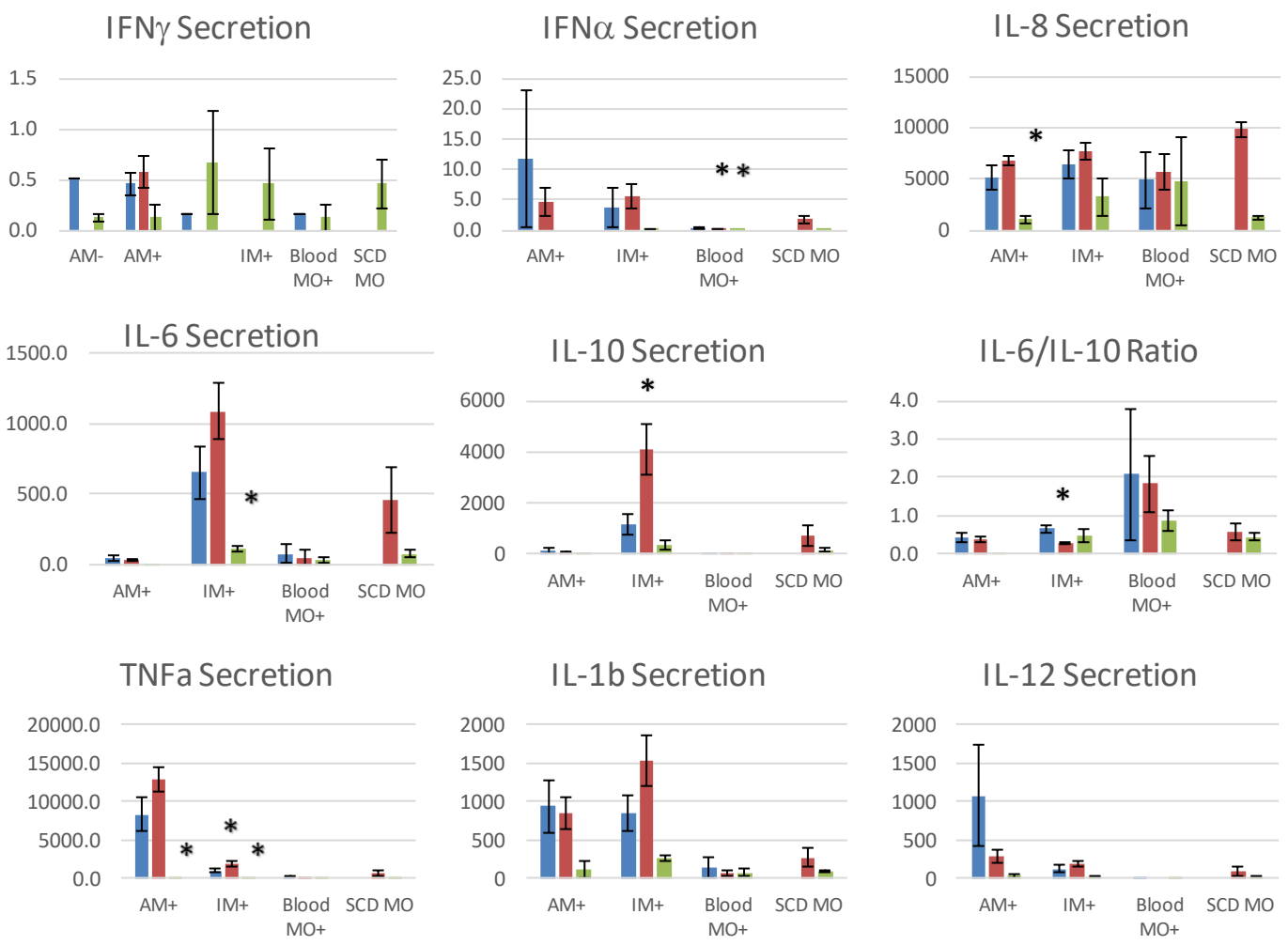


Figure 12. Secretory values of isolated cells of the monocyte and macrophage lineage. AM=alveolar macrophage, IM=interstitial macrophage, MO= monocyte. Results are calculated as pg/mL/10^6 cells in 24 hours. Other than IFNγ, a trend for lower cytokine secretion was observed from cells isolated from pigs in Cohort 3. Significantly lower IFNa secretion was observed for blood monocytes in both SCD treated cohorts. The secretions for other cytokines did not follow a consistent pattern between the cohorts so it is hard to draw conclusions about any observed differences. Untreated (Cohort 1, Blue, n=6), SCD 1.0m2 (Cohort 2, Red, n=6), and SCD 1.8m2 (Cohort 3, Green, n=4), mean± SE, significance $p<0.05$.

The intracellular production of cytokines by Monocyte/macrophages was used to further assess the pro-vs. anti-inflammatory profiles of these cells. Cytokine expression under LPS stimulated conditions was evaluated in whole blood and dissociated lung cells using flow cytometry (36). Intracellular cytokine labeling is accomplished using an Intrastain Kit (DAKO) on blood diluted 1:2 in media with brefeldin A to inhibit Golgi secretion (37). Intracellular cytokine patterns are not directly correlative to secreted levels in isolated monocytes and macrophages in that the cell populations are not purified (remain mixed) and are stimulated for only 4 hours. However, this analysis may provide insight into the phenotype of the cell based on which type of cytokines it is actively producing when stimulated.

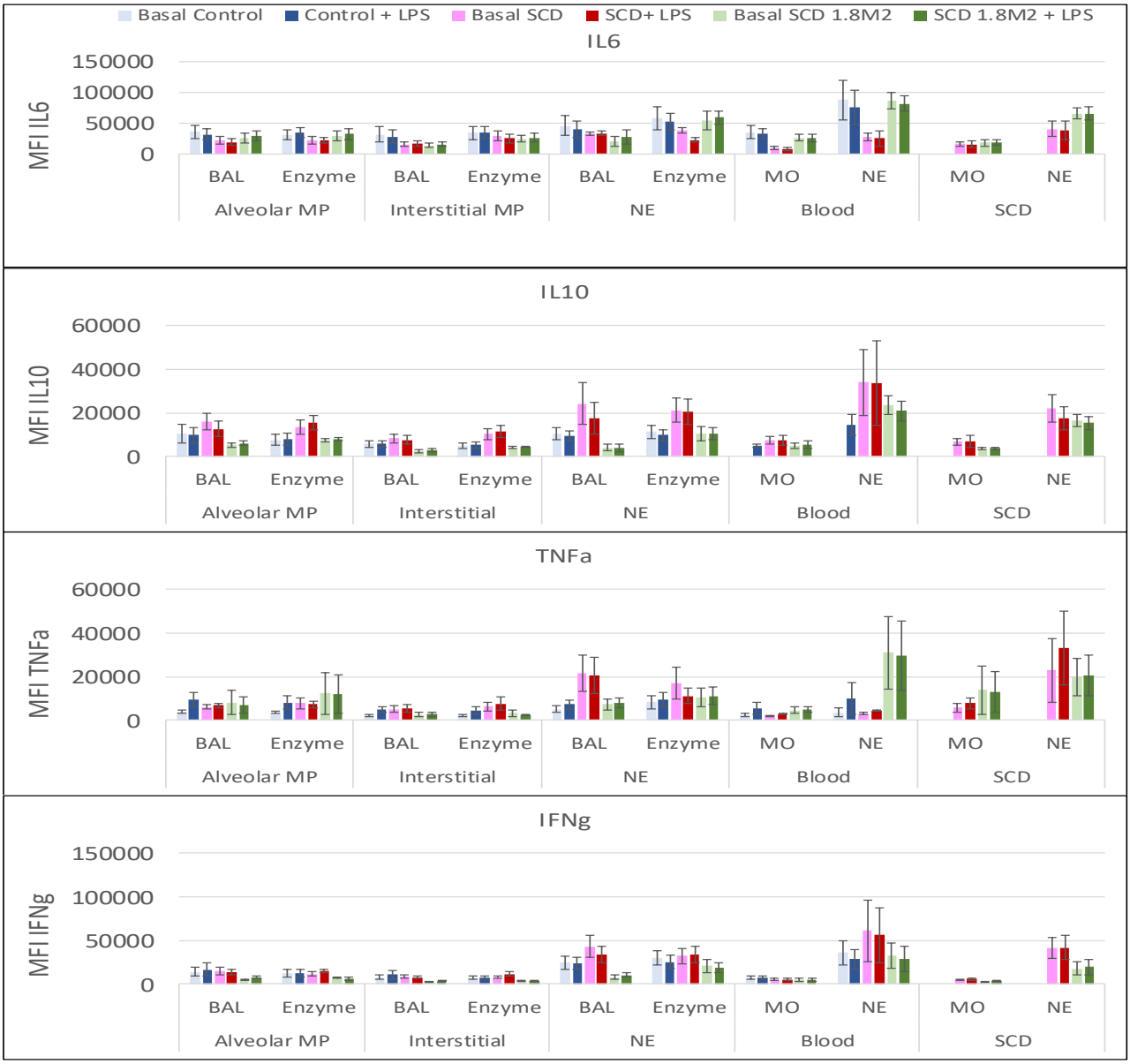


Figure 13. Intracellular cytokine levels in cells from lung and blood. While distinct patterns were not evident, IL-6 levels trended lower in both of the SCDRx cohorts compared to the untreated control cohort for all cell populations. Untreated (Cohort 1, Blue, n=6), SCD 1.0m2 (Cohort 2, Red, n=6), and SCD 1.8m2 (Cohort 3, Green, n=4), mean± SE, significance $p<0.05$).

IL-6 production trended lower in both of the SCD_{Rx} cohorts compared to untreated for all cell populations. (Figure 13). However, identifiable patterns or significant differences between the cohorts are not clearly evident for the remaining cytokines.

In summary, analysis of animals from the untreated cohort 1 and the SCD_{Rx} cohorts 2 and 3 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 particularly when combined with the improvements seen in clinically applicable physical parameters and reduced histologic evidence of lung injury will support transition of SCD_{Rx} to clinical trials.

Major Findings:

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

Milestone Achieved: Assay results allow comparison between cohorts and are able to demonstrate immunomodulation during the course of ARDS with use of SCD_{Rx}.

- **Opportunities for Professional Development**
  Nothing to Report
- **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?**

A one year no cost extension has been requested to continue the originally proposed work which was delayed by a corporate restructuring involving the awardee, Innovative BioTherapies and its parent company, SeaStar medical (formerly CytoPherx Inc.). During the next year, the remaining pig studies will be completed and then all acquired data from all the treatment cohorts will be collated and analyzed using the assay techniques developed over the first 2 years of the project in order to fully assess impact of SCD_{Rx} in the established pig model of ALI.

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 and have proven suitable Years 2 and 3.

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. Furthermore, a dose effect with treatment using the larger SCD $1.8m^2$ will be revealed allowing for optimization of SCD_{Rx} for ARDS.

With the 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**

  As discussed at the beginning of this this report, the model that resulted from Year 1 efforts does not allow for the assessment of SCD_{Rx} in cohort 3 as it was originally proposed, which was to delay SCD_{Rx} until a diagnosis of ARDS was clinically apparent. The current porcine model is not of sufficient duration to enable assessment of SCD_{Rx} in this manner. Instead, based upon improved understanding of SCD_{Rx}, Cohort 3 was redefined to encompass testing the dose effect of SCD_{Rx}. This is being accomplished by testing of a SCD with a larger effective surface area (lumen SA of $1.8m^2$ versus the $1.0m_2$ original SA) in the porcine model.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

  The awardee, Innovative BioTherapies (IBT), existed as a wholly owned subsidiary of CytoPherx Inc. which has since undergone a merger with Immunocept Inc and a name change to SeaStar Medical. A corporate restructuring of SeaStar Medical and its subsidiaries took place in December. 2018, whereby all operations of IBT *were significantly downsized. This restructuring included termination of all IBT employees, including the PI for this award, Dr. David Humes as well as Co-investigators Dr. Kimberly Johnston and Deborah Buffington.*

  Dr. Johnston as well as a few other project participants (Angela Westover, Christopher Pino, and Liandi Lou) were rehired as employees of SeaStar Medical. Dr Humes has yet been to unable to negotiate a suitable contract but has remained acting PI for the project. The reduction in personnel prevented conduction of the outstanding animal studies slated for FY03 of the project. The remaining active personnel, under guidance of Co-Investigators, Dr. Johnston and Dr. Jeffry Curtis, were able to analyze samples from the studies that had been conducted prior to the December 2018 restructuring. Data from these studies were then collated and compared to existing findings and are presented in this report. Results are promising for use of SCD_{Rx} as a novel therapy for ALI, but the studies are incomplete.

  As of August 2019, SeaStar Medical has re-entered contract negotiations with Dr. Humes and as both parties hope to finish the work on the project, a request for a 1 year no cost extension has been submitted. In order to resume work, contracts will have to be renegotiated between the awardee and project performance site, which was the Veterans Affairs Ann Arbor Heath System. The animal use protocol at the VAAAHS has also expired and will need to be renewed before animal studies can be resumed. Replacement staff will need to be hired to cover overnight shift work and some sample processing duties which are required during the 48 hour pig studies.

- **Changes that had a significant impact on expenditures**

  Due to the afore mentioned corporate restructuring, only 5 of the proposed 18 animal studies were conducted during Year 3. This reduction in effort has left approximately $448,000 in requested funds which have not been drawn down or used. A 1 year no cost extension has been requested to enable completion of the project utilizing the remaining funds.

- **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:	1
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 and analyzed the data generated from each animal study to date. Using his in-depth knowledge as well as his research and clinical experience, in collaboration with Co-I's, he made recommendations for adjustments and changes to the study design. Due to corporate restructuring, Dr. Humes employment with Innovative Therapies was terminated in Dec 2018. However, he continued to provide project guidance to remaining investigators while contract negotiations are underway.
Funding Support:	

Name:	Deborah Buffington
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0002-1541-2003
Nearest person month worked:	1
Contribution to Project:	Ms. Buffington met regularly 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).
Funding Support:	

Name:	Dr. Kimberly Johnston
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0003-1899-7876
Nearest person month worked:	4
Contribution to Project:	Dr. Johnston's primary role was to evaluate the overall health of the animals upon arrival at the contract facilities and perform the required surgical procedures for the Trauma + Hemorrhage protocol, instrumentation of animals, initiation of ALI/ARDS with LPS infusion. Dr. Johnston provided oversight pertaining to animal management throughout each experiment and report/data preparation at end of study. In addition, she generated and maintained all animal use protocols, reports, and amendments as required by the contract facility. She assumed many of the responsibilities of PI following Dr Humes termination and with input from the Co-I's input and with available resources attempted to continue the project as close as possible to the timeline presented in this proposal. Dr. Johnston met intermittently with Co-I's to discuss findings and troubleshoot potential problems. Dr. Johnson was responsible for overseeing data collation and analysis and was responsible for integration of results into the annual report.
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 3, Dr. Curtis' maintained his role as site PI for the animal studies. He consulted with Dr Johnston regularly about the status of the project. Dr Curtis also met with Ms. Westover as cytometric data was generated. Dr Curtis provided insight into the analysis of circulating monocytes and alveolar macrophages with respect to phenotype.
Funding Support:	

Name:	Dr. Hasan Alam
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0002-1024-5226
Nearest person month worked:	0
Contribution to Project:	Dr. Alam provided guidance to IBT staff that was instrumental in model development in assuring the injury from the blunt trauma with hemorrhage is relevant to that observed under military conditions. No effort was required for Year 3.
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 has reviewed and 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. He will relate the findings from the animal studies to the human clinical situation and guide future direction of the SCD

	technology into a pilot clival trial. Dr Standiford's effort will be used in completion of the project.
Funding Support:	

Name:	Angela Westover
Project Role:	Research Scientist
Researcher Identifier (e.g. ORCID ID):	0000-0002-7556-9838
Nearest person month worked:	3
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 as the laboratory manager at the primary site. For Year 3 of this project, Ms. Westover completed the required protocols for processing of lung tissue samples for intracellular cytokines and phenotyping. On study days Ms. Westover assisted with sample processing and was responsible for cell isolation and cell culture activities. Ms. Westover coordinated efforts to produce the Luminex cytokine data and was responsible for resulting data analysis and collation. She conducted then provided analysis and interpretation of all flow cytometry panels. She collated and interpreted all related data for presentation to the CO-I's and helped integrate these findings into the Year 3 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:	3

Contribution to Project:	During year 3 Dr. Pino prepared circuit materials and provided for the calibration and maintenance of pumps and other required equipment prior to animal studies. He also maintained the traumatizer apparatus. Along with Dr. Lou, Dr. Pino, was responsible for performing the Trauma + Hemorrhage protocol on animals and assisted with animal management during live animal studies. Dr. Pino attended weekly meetings with the research team to ensure studies are properly coordinated, discuss findings, and proposal objectives are being met. Dr Pino was responsible for image analysis from immunohistochemistry and he contributed to report generation.
Funding Support:	

Name:	Liandi Lou
Project Role:	Research Associate
Researcher Identifier (e.g. ORCID ID):	n/a
Nearest person month worked:	4.5
Contribution to Project:	In Year 3, 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 animals 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. Post study, he read and documented findings from systemic blood smears and he was responsible for compiling and collating pulmonary and hemodynamic data from the animal studies. Dr. Lou assisted in generalized data entry, data collation and report generation.
Funding Support:	none

Name:	Nicholas Greer
Project Role:	Research Assistant

Researcher Identifier (e.g. ORCID ID):	n/a
Nearest person month worked:	2.5
Contribution to Project:	In Year 3, 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. During live animal studies, Mr. Greer maintained anesthesia, assisted in sample collection and processing, and data recording during overnight shifts. Post study, Mr. Greer ran automated CBC on the Hemavet, and archived other samples for future batch testing. 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. Mr. Greer also performed immunohistochemical staining of lung tissues and then image analysis to determine leukocyte infiltration for comparisons between cohorts.
Funding Support:	none

Name:	Valerie Stolberg
Project Role:	Laboratory Manager at contract facility
Researcher Identifier (e.g. ORCID ID):	0000-0001-6054-0592
Nearest person month worked:	1
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). Ms. Stolberg also ran batched samples from Year 3 pig studies on the Luminex to obtain cytokine concentrations.
Funding Support:	

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

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:**
   Quad Chart

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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

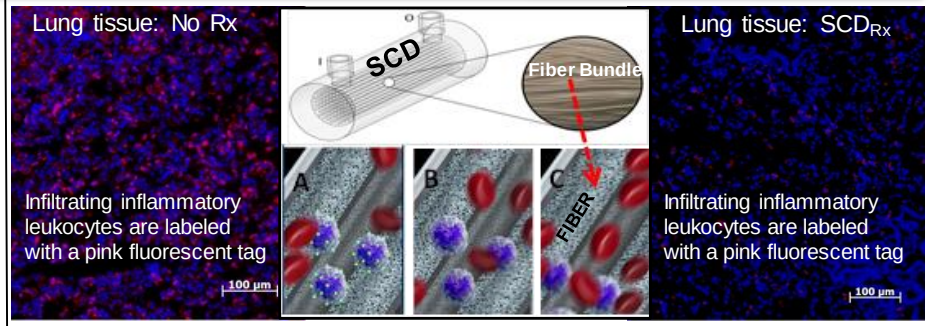
Study/Product Aim(s)

- **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 endotoxin) 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 modulation of circulating inflammatory leukocytes by SCD fibers.

Accomplishments: Significant therapeutic benefit demonstrated with SCD_{Rx} in this porcine ARDS model has been observed. provides substantive evidence to advance the technology into an exploratory clinical trial within the ARDS network.

Timeline and Cost

no cost extension

	15				16				17				18				19			
Activities	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
Obtain approval for all animal work.																				
Establish protocol for two-hit porcine ARDS model																				
Assess efficacy of SCD_{Rx} in ARDS porcine model																				
Estimated budget	$41,711				$1,021,111				$812,234				$821,732				$0			

Updated: August 2019

Goals/Milestones

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

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

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

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

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

Comments/Challenges/Issues/Concerns

- Corporate restructuring limited the # of animal studies performed.
- A no cost extension has been requested to complete the project

Budget Expenditure to Date

Projected Expenditure: $2,696,788 Actual Expenditure: $ 2,248570