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TITLE: Dried Plasma to Improve Outcomes in Polytrauma, Hemorrhage, and Trauma-Associated Sepsis (TAS): Novel Solutions for the Prolonged Field Care Environment

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14. ABSTRACT The goal of this project was to determine if plasma is an ideal fluid for resuscitation in the prolonged field care environment for trauma/hemorrhagic shock and trauma-associated sepsis (TAS). The use of a freeze dried(FD) plasma product was compared to fresh frozen plasma as a military-relevant alternative. We hypothesize that FD plasma- based resuscitation after TAS would be equivalent to FFP, superior to Lactated Ringers, and would reduce the endotheliopathy of sepsis (EOS), mitigate vascular and end organ injury, and decrease mortality, in clinically relevant mice and swine models of TAS. We first developed a novel model of prolonged hypotensive resuscitation in mice and found that plasma was superior to Hextend (then the fluid of choice on the battlefield) after hemorrhagic shock and prolonged hypotensive resuscitation. We next used this model to study sepsis. However, we were only able to demonstrate non-inferiority of plasma. In our mouse model of sepsis alone, we found FD-plasma to be noninferior to lactated Ringers with some mechanistic data suggesting an anti-inflammatory effect. In our swine model of combined hemorrhagic shock and sepsis we again demonstrated non-inferiority only of FD-plasma.					
15. SUBJECT TERMS Hemorrhagic shock, trauma, trauma-associated sepsis, sepsis, prolonged field care, endotheliopathy of trauma, hextend, fresh frozen plasma, freeze dried plasma					
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- 1. INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

The goal of the current project is to determine if plasma is an ideal fluid for resuscitation in the prolonged field care environment for trauma/hemorrhagic shock and trauma-associated sepsis (TAS). Additionally, use of a freeze dried(FD) plasma product compared to fresh frozen plasma will be tested. We hypothesize that FD plasma- based resuscitation after TAS will be equivalent to FFP, superior to Lactated Ringers, and will reduce the endotheliopathy of sepsis (EOS), mitigate vascular and end organ injury, and decrease mortality, in clinically relevant mice and swine models of TAS.

- 2. KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

Hemorrhagic shock, trauma-associated sepsis, sepsis, prolonged field care, endotheliopathy of trauma, fresh frozen plasma, freeze dried plasma, lactated Ringers
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- 3. ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

SOW

	Timeline (Months)	Site 1 (Kozar)	Site 2 (Pati)	Site 3 (Tiller)
Specific Aim 1: To determine the effects of spray- dried plasma compared to fresh frozen plasma and hextend on systemic, vascular, organ-specific pathophysiology and survival in a rodent model of hemorrhage shock and prolonged hypotensive resuscitation with trauma associated sepsis.				
Major Task 1: Obtain approval for mice experiments				
Subtask 1: Obtain local IACUC approval for mouse studies. (Estimated total number of animals: 245)	0-2	6/2017		
Subtask 2: Obtain ARUCO approval for mouse studies	0-4	9/2017		
Milestone: IACUC/ARUCO approvals	4			
Major Task 2: Preparation and testing of cecal slurry				
Subtask 1: Harvest cecal slurry (25 mice)	4-5	11/2017		
Subtask 2: Perform LD100 experiments (30 animals)	5-7	11/2017		
Milestone: Complete cecal slurry preparation and testing	7			

Major Task 3: Conduct short term study of HS and prolonged hypotensive resuscitation (PHR)				
Subtask 1: Perform short term hemorrhagic shock and PHR	7-12	8/2018		
Subtask 2: Analyze lung tissue for injury, inflammation, and permeability	10-15	12/2018		
Subtask 3: Analyze blood and BAL for cytokines	10-15		12/2018	
Milestone: Complete short term mouse surgeries and analysis for HS and PHR	15			
Major Task 4: Conduct short term sepsis studies in mice				
Subtask 1: Optimize cecal slurry model	13-15	3/2020		
Subtask 2: Perform short term experiments of sepsis	22-29	10/20		
Subtask 3: Perform analysis on from cecal slurry animals	25-30		12/2021	
Milestone: Complete short term mouse surgeries and analysis for sepsis	30			

Specific Aim 2: To determine the modulatory effects of SDP compared to hextend on hemodynamics, end-organ function, coagulopathy and survival in a swine model of TAS.				
Major Task 1: IACUC drafting and approval				
Subtask 1: Draft IACUC	21-22	1		11/2020
Subtask 2: Defend IACUC/obtain local and MRMC approval	23-25			3/ 2021
Milestone: IACUC approval	25			

Major Task 2: Model Refinement				
Subtask 1: Refine surgical parameters Optimize hemorrhage duration and time as well as the amount of bacteria infusion required to initiate sepsis.	26-27			6/2021
Major Task 3: Perform animal surgeries For all of Major Task 3, the two groups of animals will be TAS+ hextend and TAS+ FDP				
Subtask 1: Collect hemodynamic data SvO ₂ , CO, HR, SBP, DBP, MAP, CVP, respiration, TEE, StO ₂ , SpO ₂ (2 groups)	28-32			7/2021
Subtask 2: Collect arterial blood gas analysis data Arterial oxygenation, acidosis parameters, ion concentrations (n=6 per group; 2 groups)	28-32			9/2021

Subtask 3: Collect brain activity data Brain activity will be monitored by EEG and TCD (n=6 per group; 2 groups)	28-32			9/2021
Subtask 4: Collect blood chemistry data Metabolic blood chemistry panel including organ function markers (n=6 per group; 2 groups)	28-32			9/2021
Subtask 5: Complete blood counts Complete blood counts of WBC, LYM, MON, RBC, HGB, HCT, MCV, MCH, MCHC, RDW, PLT, MPV,	28-32			9/2021
Subtask 5: Inflammation Perform cytokine/chemokine analysis by luminex on serum samples (n=6 per group; 2 groups)	28-32			9/2021
Subtask 6: Coagulation PT, PTT INR by Stago (n=6 per group; 2 groups)	28-32			12/2021
Subtask 7: Endothelial integrity syndecan-1, hyaluronic acid, chondroitin sulfate, heparin sulfate by ELISAs in serum. (n=6 per group; 2 groups)	28-32			2/2022

Subtask 8: Lung leakage ELISA of albumin, cell counts in BAL fluid, PaO ₂ /FiO ₂ ratio. (n=6 per group; 2 groups)	28-32			12/2021
Subtask 9: Histology H&E staining of lung, liver and kidney for lesions (n=6 per group; 2 groups)	28-34			3/2022
Subtask 10: Survival Evaluate the percent survival and survival time (n=6 per group; 2 groups)	28-32			12/2021
Subtask 11: Data analysis	30-32			3/2022
Milestone: Completion of swine arm of TAS	32			3/2022
Major task 4: Manuscript write-up and presentations				
Subtask 1: Write up manuscripts and abstracts/posters	33-36			ongoing
Milestone: Results dissemination: manuscript and abstracts publications and presentations	36			ongoing

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Summary of activities 2017-2018

Aim 1. Major Task 1. Obtained IACUC and ACURO

Aim 1. Major Task 2. Harvested our first dose of cecal slurry and performed LD100 studies to determine the optimal dose of slurry to use.

CHALLENGES IN THIS YEAR: none

Summary of activities 2018-2019

Aim 1. Major Task 3: Conduct short term study of HS and prolonged hypotensive resuscitation (PHR)

We developed and published the first model of prolonged hypotensive resuscitation in a rodent model of hemorrhagic shock.

METHODS: Mice underwent laparotomy and hemorrhagic shock (MAP 35±5mmHg x 90 minutes) followed by PHR with either FFP or Hextend to maintain a MAP of 55-60mmHg for six hours. At the end of six hours, animals were euthanized and lung tissue harvested for measurement of histopathologic injury and inflammation, assessed by myeloperoxidase (MPO) immunofluorescence staining. Pulmonary syndecan-1 immunostaining was also assessed as an indicator of endothelial cell integrity. In a separate set of animals, permeability was assessed using Evans Blue dye.

RESULTS: All animals in the FFP, Hextend, and sham groups survived to the end of resuscitation. Resuscitation with FFP mitigated lung histopathologic injury compared to Hextend (4.38 ± 2.07 vs. 7.5 ± 0.93 , $p=0.002$) and was comparable to sham animals (4.0 ± 1.93 , $p=0.99$). FFP also lessened lung inflammation (2780 ± 1310 vs. 10466 ± 5180 RFU, $p=0.002$) and restored pulmonary syndecan-1 when compared to Hextend treated mice (5360 ± 2458 vs. 728 ± 454 RFU, $p=0.007$). Consistently, FFP mitigated lung hyperpermeability compared to Hextend (0.334 ± 0.056 vs. 0.651 ± 0.231 , $p=0.005$) but permeability was similar between FFP and sham animals (0.334 ± 0.056 vs. 0.335 ± 0.085 , $p=0.99$).

CONCLUSIONS: We have presented a novel model of prolonged hypotensive resuscitation of military relevance to the prolonged field care environment. In this model, FFP maintains its pulmonary protective effects compared to Hextend, which supports the need for further development and implementation of plasma-based resuscitation in the forward environment.

CHALLENGES IN THIS YEAR: There were several challenges faced. The first being we were sent female donor units of FFP. This was recognized due to unexpected mortality. Once recognized, male donor plasma was replaced by the company and used in subsequent experiments. Second, our first shipment of lyophilized plasma from France was sent to us reconstituted but got delayed in customs and had defrosted by the time it reached us. We then received the dried product, reconstituted it ourselves, but got unexpected results leading us to eventually conclude the high pH of this product was not tolerated by mice. We then had to find another company who would provide a freeze-dried product. This was all done in conjunction with the DOD, and we eventually were able to work with Teleflex.

Summary of activities 2019-2020

Major Task 3: Conduct short term study of HS and prolonged hypotensive resuscitation (PHR).

We were consistently having high mortality with our combined model of trauma/hemorrhagic shock and then sepsis. Mortality for FFP and lyophilized plasma (LP) was higher than Hextend. To attempt to explain this we tried using different donor units of FFP, pooling FFP, and buying new FFP to no avail. We concentrated these initial efforts on FFP as we have vast experience using FFP in our trauma/hemorrhagic shock model. After discussion with members of the DOD and revision of SOW, the decision was to proceed with a sepsis only (no trauma/ hemorrhagic shock) model to help differentiate the problems. There was concern that giving a second dose of human plasma to mice may be causing some type of transfusion reaction.

Major Task 4: Conduct short term sepsis studies in mice.

Subtask 1: We had to do additional experiments to make another batch of cecal slurry and then determine the optimal dose.

Subtask 2: Short term sepsis animal surgeries were started.

Specific Aim 2. Swine model

There were changes in personnel related to this aim. Dr Jacob Glaser left his position in the Navy and Dr AJ Burdette assumed a new position. Therefore Dr. Michael Tiller assumed Jacob Glaser's role and Dr. Leslie Neidert assumed Alexander Burdette's role. These changes were submitted and approved by the DOD.

CHALLENGES IN THIS YEAR: As detailed above and:

1. The LP and FFP groups after trauma-associated sepsis (hemorrhagic shock then sepsis) resulted in an unexplained mortality. To begin to understand these findings, we switched to a sepsis only model to first understand how plasma works in sepsis.
2. We did not see significant differences between Hextend and our plasma groups. The military also changed their guidelines and LR was recommended in place of Hextend. Additionally, LR is the standard of care for sepsis resuscitation. We therefore we revised our procedures to include LR rather than extend as a resuscitative fluid.
3. The COVID pandemic occurred and labs at the University of Maryland were closed for about 3.5 months. During that time we did need to write and submit an entirely new IACUC which was approved as was ACURO approval achieved.

Summary of activities 2020-2021

Specific Aim 1.

Major Task 2. Animal surgeries completed.

Major Task 3. Analysis began

Specific Aim 2.

Major Task 1: IACUC and MRMC approvals obtained

CHALLENGES IN THIS YEAR

COVID delays continued.

NAMRU originally sent 36 units of swine plasma to Teleflex for lyophilization. However, only 16 of them were viable by the time Teleflex received them. NAMRU has subsequently arranged for the remaining units of swine plasma to be shipped directly to Teleflex. All of the units were viable and all have been lyophilized.

Detailed activities of 2021 and 2022

Aim 1. Rodent model. Activities involved completing our nanostring analysis and manuscript preparation.

NanoString Gene Expression Analysis of Lung Tissue

Total RNA was isolated from fresh frozen lung lysates using the ALLPrep DNA/RNA mini kit (Qiagen, Germantown, MD) and quality control assessed with a DeNovix DS-11 Spectrophotometer (DeNovix, Wilmington, DE). Using nCounter technology from Nanostring, copy numbers were measured of the 750 genes in the mouse nCounter[®] Autoimmune Profiling Panel (NanoString, Seattle, WA). RNA was isolated from 5 randomly selected mice per group (Sham, LR, and LP), and the 4 mice per group with the highest RNA purity were processed for gene expression. Samples were normalized to positive controls and housekeeping genes using the Advanced Analysis geNorm algorithm. Differentially expressed genes between two groups (LR vs. Sham, LP vs. Sham, and LP vs. LR) were determined by Student's t-test (p -value < 0.05) using R software version 4.0.2 and were presented in a heat map using Cluster and TreeView Software. Volcano plots of all genes in the panel were generated using nCounter Advanced Analysis 2.0.134 (NanoString). Kyoto

Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were subsequently conducted on the differentially expressed genes using DAVID software. A pie distribution plot of the significant KEGG pathways (FDR adjusted p -value < 0.05) downregulated by LP vs. LR was generated using the Plotly package in R software. Pathways are presented in this plot with the name of the pathway and the number of genes in that pathway that were significantly downregulated.

Reverse Transcription Quantitative Real-Time PCR (RT-qPCR)

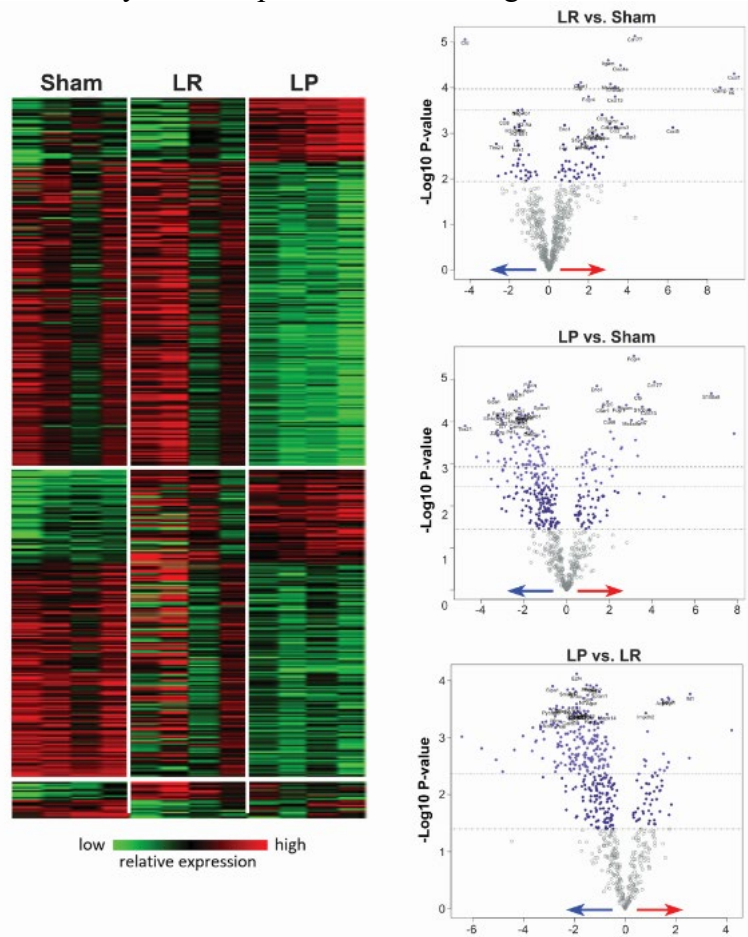
To verify select genes identified by NanoString, messenger RNA was reverse-transcribed (RT) using SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific) and quantitative PCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA). RT-qPCR was performed on the RNA isolated from the same 5 lung lysates per group as described above. The primer pair sequences were: ICAM: 5'-CAGTCTACAACCTTTTCAGCTC -3' and 5'- CACACTTCACAGTTACTTGG -3'; Ikbkg: 5'-GAGAAGAAGGAGTATTTGCAG-3' and 5'- CAAATGAAAGGAGTGGTGAG -3'; IL-6RA: 5'-AAAGTTCTACAGAAGCAACG -3' and 5'- TTGAGTCTCAGGATGATGAAG-3'; Jak3: 5'-ACAGGCTACGCTTTTATTTC-3' and 5'- AAACGTGTAAGTCAAGGATG-3'; Rela: 5'-GTATCCATAGCTTCCAGAAC-3' and 5'- CAAATGAAAGGAGTGGTGAG -3'; 5'-GAGAAGAAGGAGTATTTGCAG-3' and 5'- GAAAGGGGTTATTGTTGGTC-3'; E-selectin: 5'-CATGAAATGTCTTCCCAGTG-3' and 5'- ATCACATTTTCACAGCTGAAC-3'; STAT3: 5'-CGTCTGGAAAACCTGGATAAC-3' and 5'- TTAAGTTTCTGAACAGCTCC-3'; Traf1: 5'-CATCCTTTGATGGTACTTTCC-3' and 5'-TAGAAAGCTGGAGAGAAGAG-3'; VCAM1: 5'-ACTGATTATCCAAGTCTCTCC-3' and 5'-CCATCCACAGACTTTAATACC-3'. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as endogenous controls for the genes, respectively. Relative RNA amount was calculated using the $2^{-\Delta\Delta C_t}$ method.

Plasma Inflammatory Biomarker Analysis

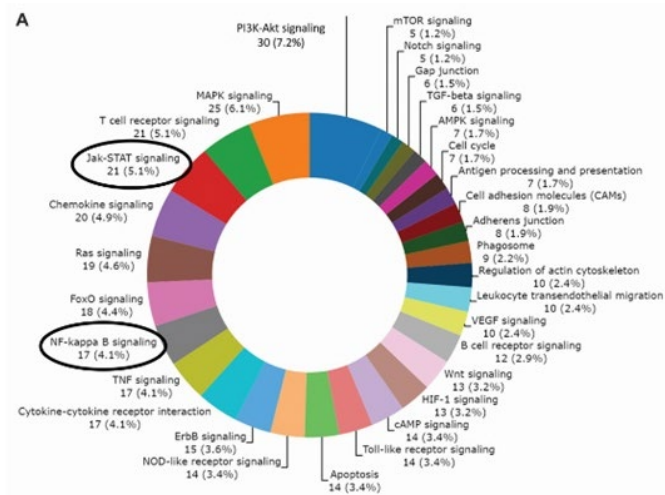
Using the same 5 mice per group as the pulmonary gene expression analysis, mouse plasma samples were analyzed for the following biomarkers using a custom multiplex Luminex® Discovery Assay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol: Angiopoietin-2 (Ang2), C-X-C Motif Chemokine Ligand 10 (CXCL10), CXCL12, Intercellular Adhesion Molecule-1 (ICAM-1), Interferon gamma (IFN- γ), Interleukin-1beta (IL-1 β), IL-2, IL-4, IL-6, IL-10, Matrix Metalloproteinase-8 (MMP8), MMP9, Receptor for Advanced Glycation Endproducts (RAGE), Syndecan-1, Tissue Inhibitor of Metalloprotease-4 (TIMP4), Tumor Necrosis Factor alpha (TNF α), TNF Receptor Superfamily Member 1a (TNFRSF1a), TNFRSF1b, TNF Superfamily Member 13b (TNFSF13b), and Vascular Endothelial Growth Factor (VEGF). In summary, plasma samples were thawed and centrifuged at 16,000 g for four minutes prior to use. Samples were then diluted 1:2 and 1:10 in appropriate diluent and pipetted onto a 96-well plate, mixed with magnetic beads coated with antibodies, and incubated for two hours at room temperature on a horizontal orbital microplate shaker at 800rpm. Three washes with wash buffer were performed, then the beads were incubated with the biotinylated antibody cocktail for 1 hour at room temperature on the orbital shaker at 800rpm. After another three washes, the beads were incubated with streptavidin-PE for 30 minutes on the shaker. The beads were washed a final three times then resuspended in wash buffer prior to being read on a MAGPIX System (Luminex Corp., Austin, TX). xPONENT 4.2 software (Luminex Corp.) was used for data acquisition.

Results

Inflammatory Gene Expression in the Lung



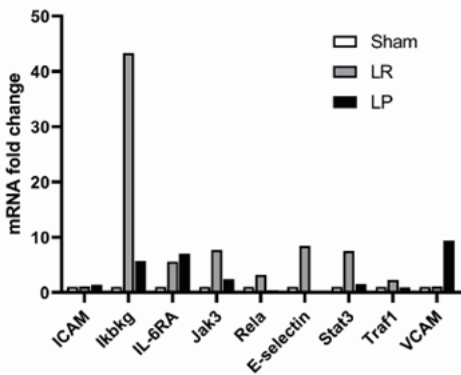
Inflammatory gene expression was analyzed using the nCounter® Autoimmune Profiling Panel (NanoString) and displayed in **Figure X**. A heat map and volcano plots were generated demonstrating that many more inflammatory genes were downregulated than upregulated on this panel among mice resuscitated with LP compared to mice resuscitated with LR. In total there were 187 genes that were significantly downregulated by LP compared to LR and 39 that were up-regulated.



Using KEGG pathway analysis, we identified multiple inflammatory signaling pathways which were significantly affected by resuscitation fluid. As shown in **Figure X** there were a number of KEGG signaling pathways with high numbers of genes that were downregulated among mice resuscitated with LP compared to LR, including JAK-STAT, chemokine, NF- κ B, TNF, cytokine-cytokine receptor interaction, and Toll-like receptor signaling.

To confirm these findings, RT-qPCR was performed, selecting genes involved in or known

to be affected by the JAK-STAT and NF- κ B signaling pathways (. In agreement with the NanoString pathway analysis, RT-qPCR demonstrated downregulation of genes involved in the NF- κ B (Ikbkg, Rela, and Traf1) and JAK-STAT pathways (Jak3, Stat3) in the LP group compared to LR. The expression of Il6ra (JAK-STAT



pathway) was significantly downregulated on the NanoString analysis but was found to be elevated in both the LR and LP groups using RT-qPCR. Finally, endothelial activation is known to induce the production of adhesion molecules E-selectin, Intercellular Adhesion Molecule-1 (ICAM-1), and Vascular Cell Adhesion Molecule-1 (VCAM-1), which are involved in leukocyte rolling and adhesion and regulated by the NF- κ B pathway.(35) We used RT-qPCR to analyze expression of these respective genes, which were not included on the NanoString panel. ICAM expression was similar across the three groups, E-selectin expression was increased among mice resuscitated with LR but not LP, and VCAM-1 expression was increased among mice resuscitated with LP but not LR (**Figure X**).

Plasma Biomarker Analysis

A custom multiplex Luminex® assay was used to evaluate levels of twenty biomarkers of inflammation and/or endothelial function in the plasma from N=5 mice per group. The results are displayed in Figure 5, demonstrating that there was significant variation in values particularly among the mice resuscitated with LR, whereas sham mice were tightly clustered with low expression of nearly all of these proteins, and mice resuscitated with LP fell in between depending on the biomarker. Elevated levels of the following biomarkers were found in a subset of LR mice but not in the Sham or LP groups: IFN- γ , IL1B, IL2, IL6, IL10, MMP9, Syndecan-1, TNF α , TNFRSF1a, TNFRSF1b, and VEGF.

Specific Aim 2. Major Task 2 and 3. Swine Model

Preoperative Preparations Day One

Fourteen male Yorkshire swine (*Sus scrofa domesticus*) weighing 40-60kg were randomized into two different groups (n=7/group). All animals were placed under general endotracheal anesthesia with isoflurane maintained at 1-3%, and Baseline Bronchiolavage (BAL) fluid was collected. Core body temperature was monitored via a rectal temperature probe and maintained between 36.0-38.0°C. End-tidal carbon dioxide (EtCO₂) was monitored continuously.

Under sterile conditions, lines were placed percutaneously using the Seldinger technique. The right femoral artery was catheterized for blood pressure monitoring, the right femoral vein for resuscitation, and the left femoral artery for hemorrhage and blood draws. Finally the jugular vein was catheterized with a 7Fr triple lumen catheter to be used for constant rate infusion (CRI) anesthesia. Bladder was monitored and emptied via decompressive cystocentesis as needed throughout the procedure. Following jugular cannulation, isoflurane anesthesia was switched over to CRI of ketamine, buprenorphine, propofol and dexmedetomidine over 15 minutes, followed by an additional 15 minutes were given to stabilize the animal.

Hemorrhagic Shock

Following baseline (BSLN) blood samples, hemorrhage was initiated via free-flow of arterial blood until the animal's mean arterial pressure (MAP) reached 30mmHg. Shed blood was collected in a blood donor bag containing anticoagulant citrate phosphate dextrose adenine solution at a 1:10 ratio and stored on a rocker for future resuscitation. After the target MAP was achieved the animals MAP was maintained between 30-35mmHg for up to 90 minutes through additional hemorrhage. Shock ended early if MAP dropped below 30mmHg for 10 minutes or below 20mmHg for 10 seconds. At the completion of shock blood samples were drawn and treatment began (T0).

Prolonged Field care, Hospital Period and Recovery

At the beginning of the four-hour prolonged field care phase (T0), animals in Group 1 were resuscitated with lactated ringers solution (LRS) followed by additional LRS to maintain systolic blood pressure (SBP) above 70mmHg. Prior to treatment the animals in Group 2 were given calcium gluconate (23% solution; Vedco, Saint Joseph, MO) then resuscitated using freeze dried plasma (FDP) followed by LRS to maintain SBP above 70mmHg.

Animals were further resuscitated with up to 1500mL of shed blood at the start of hospital period (T4) preceded by calcium gluconate to counteract the effects of the anticoagulant. LRS was given as needed to maintain a target MAP until recovery and observation (T7). Following collection of T7 blood samples, animals were recovered and moved to another room for observation. Post-operative health assessments were taken throughout observation to monitor animal pain until the sepsis treatment period (T48).

Preoperative Procedures Day Three

Prior to T48, animals were placed under general endotracheal anesthesia as described previously. The right femoral artery was catheterized for blood pressure monitoring, the right femoral vein access for resuscitation, and the jugular vein for constant rate infusion (CRI) anesthesia. Bladder was catheterized using a 14Fr foley catheter. Following jugular cannulation, isoflurane anesthesia was switched over to CRI as described previously.

Sepsis Induction

After collecting T48 samples, 27mL of live E. coli suspended in saline at a concentration of 6.00×10^7 /kg was administered over three hours at various rates. Once animals were considered septic through MAP falling below 60mmHg, resuscitation was provided with each group receiving the same treatment as described previously through a bolus of 14mL/kg. Maintenance fluids of LRS were provided to both groups to maintain a MAP greater than 65mmHg. At the end of sepsis (T60), final blood draws and BAL fluid were taken, and animals were humanely euthanized and taken to pathology for tissue collection. Lung, Liver, and Kidney tissues were later stained and scored by a board -certified pathologist blinded to the treatments.

Time Points and Analysis

Blood samples were collected in two ways; major and minor. Major draws occurred at BSLN, T0, T4, T7, T48 and T60 for all analyses. Minor blood draws occurred at T51, T54 and T57 for Arterial Blood Gas, Complete Blood Counts, and Complete Metabolic Panel. Arterial blood gas (ABG) parameters were assessed utilizing a GEM[®] Premier 4000 (Instrumentation Laboratory, Bedford, MA). Basic metabolic panels and liver associated enzymes were evaluated on Catalyst One Chemistry Analyzers (IDEXX Laboratories, Inc., Westbrook, ME).

A ProCartal Plex Pig Cyto/Chemokine kit (cat# EPX 090-60829-901, Invitrogen, Waltham, MA) was run with EDTA plasma. Serum was used to run the following ELISAs: Syndecan-1 (cat# E07S0301, BlueGene Biotech CO, Shanghai, China), Heparan sulfate (cat# MBS 265068, MyBioSource, Inc, San Diego, CA), Porcine IL-8/ CXCL8 (cat# P8000), Porcine IL-6 (cat# 6000B), Porcine IL-10 (cat# P1000), Porcine IL-1beta/ IL-1F2 (cat#PLB00B), and Porcine TNF alpha (cat#PTA00, R&D systems, Minneapolis, MN).

Results

Survival and Demographics

In each group, 6/7 animals survived to the Observation and Recovery Phase (T7) and 5/6 animals survived to the Sepsis Induction and Treatment Phase (T48). There were no significant differences in Time to Decompensation (LR: 60.0 ± 18.3 min vs. FDP: 69.9 ± 12.5 min) or Blood Loss (LR: $64.3 \pm 11.4\%$ vs. FDP: $63.0 \pm 7.8\%$) between the groups. Time to Sepsis was not significantly different between groups (LR: 370 ± 434 min vs. FDP: 200 ± 70 min). The LR group had 4/5 animals reach the end of the 12 hour Sepsis observation period, whereas FDP had 2/5 survive to T60, with no significant difference in Survival Time between the two groups (LR: 3581 ± 43 min vs. FDP: 3390 ± 258 min).

Physiologic Vitals- PFC

No significant differences were observed between the two groups in Heart Rate (HR), Mean Arterial Pressure (MAP), End Tidal CO₂ (EtCO₂), or SpO₂. Both groups had significantly lower MAP and significantly higher HR at T0 compared to Baseline (BSLN). MAP of LR animals remained lower out to T4 with Whole Blood resuscitation and then became significantly elevated from BSLN through T7, where the FDP group was able to bring their MAP within BSLN range 5 minutes after receiving resuscitation. HR returned to BSLN range within 5 minutes of resuscitation for the LR group, but remained significantly elevated in the FDP group through T7.

Hematologic Measures-PFC

The pH of both groups was significantly lower than BSLN at T0, but resolved by T4. There were no clinically significant changes in sodium, potassium, chloride, or calcium within or between groups from BSLN to T7. Lactate was significantly elevated following shock, and base excess and bicarbonate were significantly decreased after shock. But all three returned to BSLN ranges with resuscitation.

Red blood cells, hemoglobin, hematocrit, and platelets were not significantly different from BSLN at T0, but were all significantly decreased by T4 and remained lower at T7 despite values starting to trend back towards BSLN. No significant changes were seen in the overall White Blood Cell Count in either group. However, there was a significant increase in neutrophil count at T4 and T7 in the FDP group, and a significant decrease in lymphocytes at T4 and T7 in both groups.

Glucose and creatinine were significantly elevated at T0, but resolved by the end of PFC. Blood Urea Nitrogen (BUN) significantly increased and Total Protein, albumin, and globulin all significantly decreased from BSLN and remained through the end of hospital care. Alanine transferase, aspartate transaminase, amylase, and lipase did not demonstrate any clinically significant changes compared to BSLN.

Coagulation Measures-PFC

No clinically significant differences were observed within or between groups for ExTEM coagulation measures of clot time (CT), A10, clot formation time (CFT), maximum clot firmness (MCF), alpha angle, lysis index 30 (LI30), or max lysis (ML) or for FibTEM CT or LI60. Significant decreases from BSLN in FibTEM A10 and MCF measures occurred in the LR group at T4 in both groups, but only LR remained significantly reduced at T7.

No significant between group differences were observed in prothrombin (PT), international normalized ratio (INR), prothrombin time (PTT), antithrombin-III (ATIII), fibrinogen, D-Dimer, or von Willebrand Factor (vWF). PT and INR in both groups were significantly higher at T4 and T7. PTT and fibrinogen were significantly decreased in both groups at T0. The FDP group resolved by T7, but the LR group remained lower. ATIII significantly decreased in both groups at T0, but resolved by T7. vWF increased significantly at T0 in both groups, however LR resolved by the end of prolonged field care, whereas FDP did not recover by the end of hospital phase.

Physiologic Vitals- Sepsis

No significant differences were observed between the two groups during Sepsis in Heart Rate (HR), Mean Arterial Pressure (MAP), End Tidal CO₂ (EtCO₂), or SpO₂. In the FDP group, during sepsis introduction, MAP was significantly higher than T48 (start of bacterial infusion) at T49 and T50, but returned to starting ranges by T51 (end of bacterial infusion). HR in the LR group was significantly higher than BSLN at T54-56 and T58-59. No changes in EtCO₂ or SpO₂ occurred over time.

Hematologic Measures-Sepsis

In the LR group, pH was significantly decreased from T48 at T54-60, but no differences were seen in the FDP group. No significant differences were seen in the pO₂ or pCO₂ in either group. Both sodium and chloride were significantly decreased in the LR group starting at T54 and continuing through T60. Both groups saw significant increases in potassium from T57-60. No differences were seen in calcium, lactate, base excess, or bicarbonate over time.

Red blood cells, hemoglobin, and hematocrit were all significantly decreased from T48 in both groups from T51 until the end of the protocol, while platelets were significantly increased in both groups from T51-60. Total white blood cell count significantly decreased from T48 by the end of bacteria infusion in both groups. It remained suppressed through T54, where it started to recover at T57, no longer significant in the FDP group. Both groups returned to T48 levels by T60. Neutrophil count was nearly depleted at T51 and remained significantly decreased at T54. The decrease was no longer significant at T57 and T60, as the count started to return to T48 range. Lymphocyte count significantly decreased at T51-57 in both groups, but recovered by T60.

Glucose was significantly decreased in the LR group by T57 and the FDP group at T60. Both groups saw significant decreased in creatinine at T51, however this was resolved by T54 and no further significant differences were observed. BUN was significantly increased in the LR group at T54, with the FDP group showing a significant increase at T57. LR saw a significant decrease in Total Protein at T60, which was also significantly lower than that of FDP which saw no significant differences from T48. While neither group saw significant differences from their own T48 measure in albumin, the groups were significantly different from each other at T51 and T60. Alanine transferase, aspartate transaminase, amylase, and lipase did not demonstrate any clinically significant changes compared to T48.

Coagulation Measures- Sepsis

No significant differences in ExTEM clot time were observed in either group. ExTEM A10, alpha angle, and ML saw significant decreases from T48 at T60 in both LR and FDP groups, and LI30 was significantly increased by T60. FDP had a significant increase in CFT and a significant

decrease in MCF at T60. Both FibTEM A10 and MCF were significantly decreased in both groups at T60 compared to T48.

No significant changes occurred in PTT or D-Dimer during sepsis. PT and INR both saw significant time and treatment effects where the LR group was significantly higher at T60 from T48, as well as significantly higher than FDP at T60. ATIII in both groups were significantly lower than T48 at T60, whereas vWF was significantly higher in both groups at T60 compared to T48. Fibrinogen was in the LR group was significantly lower than T48 at T60.

Histopathology

Lung, liver, and kidney tissues were evaluated based on inflammation, stromal change, and vascular changes. Lungs showed signs of interstitial pneumonia and alveolar necrosis. There was evidence of neutrophil infiltration and necrosis of the liver, as well as glomerular congestion, thrombi, and tubule cellular casts present in the kidneys. No significant differences were seen in these findings between the groups.

Major Task 4 Manuscripts and presentations.

To date the following two manuscripts have been accepted/published:

Chipman AM, Wu F, Pati S, Burdette AJ, Glaser JJ, Kozar RA. Fresh frozen plasma attenuates lung injury in a novel model of prolonged hypotensive resuscitation. *J Trauma Acute Care Surg.* 2020 Aug;89(2S Suppl 2):S118-S125. doi: 10.1097/TA.0000000000002719. PMID: 32282752; PMCID: PMC7830779.

Barry M, Wu F, Pati S, Chipman A, Geng H, Kozar R. Lyophilized Plasma Resuscitation Downregulates Inflammatory Gene Expression in a Mouse Model of Sepsis. *J Trauma Acute Care Surg.* Accepted, not yet on PubMed

The following manuscript is in preparation for publication:

Neidert LE, Morgan CG, Castro C, Lonowski D, Romero MN, Cardin S, Kozar RA, Tiller MM. The Evaluation of Freeze Dried Plasma on Outcomes in a Porcine (*Sus scrofa domesticus*) Model of Hemorrhage and Trauma Associated Sepsis (TAS): Novel Solutions for the Prolonged Field Care Environment.

What opportunities for training and professional development has the project provided?

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to report

How were the results disseminated to communities of interest?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Results were disseminated via the above publications.

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

If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Future Directions:

1. Related to our novel model of prolonged hypotensive resuscitation following hemorrhagic shock: We did apply for subsequent DOD funding employing prolonged hypotensive resuscitation (DM210048). This grant was recommended for alternate funding only and we have not received additional notice at this time.
2. As we were unable to prove our original hypothesis, that freeze dried plasma would be equivalent to FFP but superior to Hextend (and later changed to LR) and reduce the endotheliopathy of sepsis, we do not propose future studies related to the use of plasma for resuscitation after sepsis. Because it was not inferior, and our molecular work suggested some anti-inflammatory effects, its use is not deleterious and in a prolonged battlefield scenario where a freeze-dried product is available.

The **lead candidate** (freeze dried plasma) would be classified as a therapeutic.

How does your candidate product aid the Warfighter, Veteran, Beneficiary, and/or General Population?

As above, for its use in sepsis, it was not superior to standard of care Lactated Ringers.

Describe the steps and relevant strategies required to move the candidate product to the next phase of development and/or commercialization.

We did not develop the product, it is being manufactured by Teleflex who is moving towards FDA approval, unrelated to its use in sepsis.

4. **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

1. *Development of a rodent model of prolonged hypotensive resuscitation after hemorrhagic shock.* This mouse model mimicked a potential battlefield scenario where immediate evacuation was not possible and we showed that plasma could still be effective even after hours of low blood pressure. This model can be used as the foundation for subsequent studies related to potential therapeutics after shock. It is a model applicable to the prolonged field care environment relevant to the military.
2. *Determined that the use of plasma was not superior to Lactated Ringers (an electrolyte solution) in improving blood pressure and improving function of endothelial cells after sepsis, a form of serious infection.* We also showed it was not worse but did demonstrate in the rodent model of sepsis alone some reduction in pro-inflammatory properties suggesting some benefit. Perhaps a better understanding of these properties may be beneficial

What was the impact on other disciplines?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to report

What was the impact on technology transfer?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report
No issues on intellectual property

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report

- 5. CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, “Nothing to Report,” if applicable:*

Nothing to report

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

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to report

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects or vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state “Nothing to Report.”*

- **Publications, conference papers, and presentations**
Report only the major publication(s) resulting from the work under this award.

Nothing to report.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume; year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Chipman AM, Wu F, Pati S, Burdette AJ, Glaser JJ, Kozar RA. Fresh frozen plasma attenuates lung injury in a novel model of prolonged hypotensive resuscitation. *J Trauma Acute Care Surg.* 2020 Aug;89(2S Suppl 2):S118-S125. doi: 10.1097/TA.0000000000002719. PMID: 32282752; PMCID: PMC7830779. *Federal funding was acknowledged*

Barry M, Wu F, Pati S, Chipman A, Geng H, Kozar R. Lyophilized Plasma Resuscitation Downregulates Inflammatory Gene Expression in a Mouse Model of Sepsis. *J Trauma Acute Care Surg.* Accepted for publication but not yet on PubMed *Federal funding was acknowledged*

Neidert LE, Morgan CG, Castro C, Lonowski D, Romero MN, Cardin S, Kozar RA, Tiller MM. The Evaluation of Freeze Dried Plasma on Outcomes in a Porcine (*Sus scrofa domesticus*) Model of Hemorrhage and Trauma Associated Sepsis (TAS): Novel Solutions for the Prolonged Field Care Environment. In preparation. *Federal Funding will be acknowledged*

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of*

publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).

Nothing to report

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to report

- **Website(s) or other Internet site(s)**

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report

- *Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.*

Nothing to report

- **Inventions, patent applications, and/or licenses**

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report

- **Other Products**

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *physical collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

Other: Related to the current project, we found that there was variability in effectiveness of different units of donor plasma.

This work was presented at the 49th Annual Meeting of the Western Trauma Association, March 03–08, 2019 in Snowmass, Colorado.

Chipman AM, Pati S, Potter D, Wu F, Lin M, Kozar RA. Is all plasma created equal? A pilot study of the effect of interdonor variability. J Trauma Acute Care Surg. 2020 Jan;88(1):121-127. doi: 10.1097/TA.0000000000002529. PMID: 31688783; PMCID: PMC7055504. Funding acknowledged.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate “no change”.

Example:

Name: Mary Smith

Project Role: Graduate Student

Researcher Identifier (e.g. ORCID ID): 1234567

Nearest person month worked: 5

Contribution to Project: Ms. Smith has performed work in the area of combined error-control and constrained coding.

Funding Support: The Ford Foundation (Complete only if the funding support is provided from other than this award.)

Rosemary Kozar

PI

Calendar months

2018 – 2.1

2019 – 2.4

2020 - 1.8

2021 – 1.0

Completed IACUC/ARUCO/HRPO and updates, assisted with planning, methods, analysis of data, trouble- shooting challenges, negotiations for plasma products, and completing all reports for DOD.

Feng Wu

Research Associate

Calendar months

2018 – 6.4

2019 – 6.0

2020 - 3.0

2021 – 2.0

Assisted with IACUC protocols, performed animal experiments, tissue processing and assays

Amanda Chipman

Surgical resident

Calendar months

2018 – 6.0

2019 – 6.0

2020 - 6.0

Assisting with animal experiments and tissue processing and assays

Shibani Pati

Co-Investigator

Calendar months

2018 – 0.78

2019 – 0.78

2020 - 0.78

2021 – 0.6

Assisted with experimental design and troubleshooting animal experiments, performing tissue processing and assays.

Christine Cavanaugh

Research Assistant

Calendar months

2018 – 4.8

Assisted with animal experiments and tissue processing

Daniel Potter

Research Associate

Calendar months

2018 – 0.9

2019 – 0.9

Performing assays on lung tissue, blood and bronchoalveolar fluid

CAPT Michael Tiller

Co-Investigator/ NAMRU-SA Site PI

Calendar Months

2020- 1

2021- 4

2022- 2

Completed IACUC/ACURO approval; planned and oversaw all experimental procedures; oversaw data analysis and clinical interpretations of data; assist in manuscript drafting

Leslie Neidert
NAMRU-SA Site Co-I

Calendar Months

2020- 2 2021- 4 2022- 3

Completed IACUC/ACURO approval; planned and oversaw all experimental procedures; oversaw data analysis and interpretations of data; oversaw manuscript drafting and assisted in DOD reports

Clifford Morgan
NAMRU-SA Site Co-I

Calendar Months

2020- 1 2021- 4 2022- 1

Assisted in IACUC/ACURO approval; planned and oversaw all experimental procedures; oversaw bacterial growth for sepsis induction; oversaw data analysis and interpretations of data; assist in manuscript drafting

Kassandra Ozuna
NAMRU-SA Program Manager

Calendar months

2020 – 1 2021- 2

Managed budget and acquisition of all animals and supplies; oversaw procurement of porcine FDP

Cecilia Castro
Research Associate

Calendar months

2021 – 4 2022- 1

Assisted with animal experiments and tissue processing; assisted with data analysis and manuscript drafting

Dominic Lonowski
Research Associate

Calendar months

2021 – 4 2022- 1

Assisted with animal experiments and tissue processing; assisted with data analysis and manuscript drafting

Melissa Romero
Research Associate

Calendar months

2021 – 4 2022- 1

Assisted with animal experiments and tissue processing; Managed bacterial growth for sepsis induction; assisted with data analysis and manuscript drafting

Valeria Lozano
Research Associate

Calendar months

2021 – 3

Assisted with animal experiments and tissue processing

Peter Hemond
Research Associate
Calendar months

2021 – 3

Assisted with animal experiments and tissue processing

Alejandra Lorenzen
Research Technician
Calendar months

2021 – 3

Assisted with animal experiments and tissue processing

Mary Salas
Lab Manager
Calendar months

2021 – 3

Managed acquisition of all and supplies and maintenance of lab equipment

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

New:

03/01/2022 – 01/31/2026

Kozar (Co-I)

25% effort

Trauma and Shock-Induced Microvascular Dysregulation and Coagulopathy Sponsor

What other organizations were involved as partners?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:

Organization Name:

Location of Organization: (if foreign location list country)

Partner’s contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner’s facilities for project activities);*
- *Collaboration (e.g., partner’s staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner’s staff use each other’s facilities, work at each other’s site); and*
- *Other.*

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ebrap.org/eBRAP/public/index.htm> for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on <https://www.usamraa.army.mil/Pages/Resources.aspx>) should be updated and submitted with attachments.*

9. **APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

Appendix: Original copies of the two accepted manuscripts



Published in final edited form as:

J Trauma Acute Care Surg. 2020 August ; 89(2) : S118–S125. doi:10.1097/TA.0000000000002719.

Fresh Frozen Plasma Attenuates Lung Injury in a Novel Model of Prolonged Hypotensive Resuscitation Plasma and Prolonged Hypotensive Resuscitation

Amanda M Chipman, MD¹, Feng Wu, PhD¹, Shibani Pati, MD, PhD², Alexander J Burdette, PhD³, Jacob J Glaser, MD³, Rosemary A Kozar, MD, PhD¹

¹Shock Trauma Center, University of Maryland School of Medicine, Baltimore, MD.

²Department of Lab Medicine, University of California San Francisco, CA.

³United States Naval Medical Research Unit-San Antonio, Fort Sam Houston, TX.

Abstract

BACKGROUND: Hemorrhagic shock (HS) remains a leading cause of early death among severely injured in both civilian and military settings. As future military operations will require strategies allowing prolonged field care (PFC) of the injured, we sought to develop an *in vivo* model of prolonged hypotensive resuscitation (PHR) and to evaluate the role of plasma-based resuscitation in this model. We hypothesized that resuscitation with fresh frozen plasma (FFP) would mitigate lung injury when compared with Hextend® in a rodent model of PHR.

METHODS: Mice underwent laparotomy and HS (MAP 35±5mmHg × 90 minutes) followed by PHR with either FFP or Hextend® to maintain a MAP of 55–60mmHg for six hours. Sham animals underwent cannulation only. At the end of six hours, animals were euthanized, and lung tissue harvested for measurement of histopathologic injury, inflammation and permeability using Hematoxylin & Eosin staining, myeloperoxidase (MPO) immunofluorescence staining and Evans Blue dye. Pulmonary syndecan-1 immunostaining was assessed as an indicator of endothelial cell integrity.

RESULTS: All animals in the FFP, Hextend®, and sham groups survived to the end of resuscitation. Resuscitation with FFP mitigated lung histopathologic injury compared to Hextend® (histologic injury score of 4.38±2.07 vs. 7.5±0.93, scale of 0–9, p=0.002) and was comparable to shams (histologic injury score of 4.0±1.93, scale of 0–9, p=0.99). FFP also reduced

achipman@som.umaryland.edu.

AUTHORSHIP

A.M.C. wrote the article and conducted analyses. F.W. accrued data and conducted analyses. S.P. and R.A.K. supervised writing the article and data interpretation. S.P., R.A.K., J.J.G. A.J.B. conceived the idea. All authors reviewed and edited the manuscript. The authors declare no conflicts of interest.

The authors have no conflicts of interest. The views expressed in this manuscript are those of the authors and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, nor the U.S. government. Some of the authors are military Service members and employees of the U.S. Government. This work was prepared as part of their official duties. Title 17, U.S.C., §105 provides that copyright protection under this title is not available for any work of the U.S. Government. Title 17, U.S.C., §101 defines a U.S. Government work as a work prepared by a military Service member or employee of the U.S. Government as part of that person's official duties.

This work was presented in part as a poster at the 42nd Conference on Shock, June 8–11, 2019, Coronado, California and at the Military Health System Research Symposium, August 19–22, 2019, Kissimmee, Florida.

lung inflammation (0.116 ± 0.044 vs. 0.308 ± 0.054 relative fluorescence of MPO, $p=0.002$) and restored pulmonary syndecan-1 (0.514 ± 0.061 vs. 0.059 ± 0.021 , relative syndecan-1 fluorescence, $p<0.001$) when compared to Hextend®. Consistently, FFP mitigated lung hyperpermeability compared to Hextend® (7.30 ± 1.34 vs. $14.91 \pm 5.55 \mu\text{gEB}/100\text{mg}$ lung tissue, $p=0.005$).

CONCLUSIONS: We have presented a novel model of PHR of military relevance to the PFC environment. In this model, FFP maintains its pulmonary protective effects using a PHR strategy compared to Hextend®, which supports the need for further development and implementation of plasma-based resuscitation in the forward environment.

Keywords

shock; hemorrhage; trauma; hypotensive resuscitation; hydroxyethyl starch; fresh frozen plasma

BACKGROUND

Prolonged field care (PFC) has recently emerged as an area of focus for the United States military due to the current reality of providing medical care to military forces in remote and austere locations(1). One of the major challenges in PFC is treating hemorrhagic shock, a leading cause of early death among the severely injured in both civilian and military settings(2,3). In fact, resuscitation in hemorrhage management remains a top priority of the Department of Defense research program(4). Current Tactical Combat Casualty Care (TCCC) guidelines for hemorrhagic shock call for damage control resuscitation strategies involving permissive hypotensive resuscitation as well as a focus on blood product based resuscitation(5). Permissive hypotension refers to managing patients in hemorrhagic shock by allowing for a lower, but still perfusing, blood pressure until active bleeding is controlled. However, while the principle of permissive hypotension has persisted as a tenet of trauma resuscitation for many years, it is unknown how to apply this principal beyond the “golden hour.” Given the anticipated need for up to 72 hours of prolonged field care, advanced resuscitative practices will need to involve sophisticated blood product utilization as a critical component of resuscitation(6). Currently, the degree to which hypotensive resuscitation can be utilized in a prolonged or delayed evacuation is unknown(7).

Equally important is the type of resuscitative fluid that is available in the PFC environment. While the military has used walking blood banks in the field hospital setting to allow for fresh whole blood (FWB) resuscitation of combat casualties, the PFC environment may not have this luxury, particularly with small combat operation teams that are in austere, far-forward environments. While it is recognized that whole blood, or blood components in a 1:1:1 ratio may be ideal, plasma based resuscitation may be more practical in the PFC environment, especially as researchers have been developing plasma formulations (e.g. lyophilized plasma) that can be transported more easily and stored for long periods of time(5,8). Freeze-dried plasma use has been well-documented clinically, and has been in clinical use since World War II(9). It is considered interchangeable with fresh frozen plasma (FFP) for trauma resuscitation, and is currently used by American military forces under a special, emergency use authorization from the Food and Drug Administration(10,11). Importantly, mortality benefits have been seen when FFP was given in the pre-hospital environment for patients in hemorrhagic shock(12). The benefit of plasma seems to extend

beyond its ability to correct trauma-induced coagulopathy and provide hemorrhage control and is thought to involve additional protective effects to a dysfunctional endothelium(13–15). Our previous investigations have demonstrated that resuscitation with plasma versus crystalloid provides benefits in both *in vitro* and *in vivo* models of hemorrhagic shock by reducing injury, inflammation and permeability(16,17).

As future areas of military operations will require strategies allowing PFC of the injured, we sought to develop a model of prolonged hypotensive resuscitation (PHR) and to evaluate the role of plasma-based resuscitation in this model. We hypothesized that PHR with FFP would mitigate lung injury when compared with Hextend® in our rodent model.

METHODS

Mouse model of hemorrhagic shock

The study protocol was reviewed and approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee in compliance with all applicable Federal regulations governing the protection of animals in research. The experiments reported herein were conducted in compliance with the Animal Welfare Act and per the principles set forth in the “Guide for Care and Use of Laboratory Animals,” Institute of Laboratory Animals Resources, National Research Council, National Academy Press, 2011. All animals were housed at constant room temperature with a 12:12-h light-dark cycle with access to food and water *ad libitum*. Male C57BL/6J mice were used at 8 to 10 weeks of age and weighing approximately 25 grams. Under isoflurane anesthesia, a midline laparotomy incision was made, the intestines inspected, and then the incision was closed. Mice then underwent femoral artery cannulation for blood withdrawal, resuscitation, and hemodynamic monitoring. Mean arterial blood pressure (MAP) was continuously recorded via the femoral arterial line. Mice then underwent hemorrhagic shock (MAP 35±5mmHg for 90 minutes) which was followed by PHR. During PHR, mice were resuscitated with either FFP or Hextend® to maintain a MAP of 55–60mmHg for six hours. Human, male FFP was obtained from Tennessee Blood Services (Memphis, TN). At the end of six hours, animals were euthanized and tissue was harvested for further analysis. Sham mice underwent femoral artery cannulation but no laparotomy or HS.

Lung histopathologic injury

The left lung was embedded in optimal cutting temperature compound (OCT) at the time of tissue harvest and stored at –80°C. Lung tissue was sectioned and stained with hematoxylin and eosin (H&E) and scored on a 3-point scale for alveolar thickness, capillary congestion, and cellularity as described by Hart et al. and as we have reported(16,18,19). The overall lung injury score was calculated by averaging the three parameters.

Lung inflammation

Lung inflammation was assessed by myeloperoxidase (MPO) immunostaining as an indicator of neutrophil influx. Lung tissue was sectioned and then incubated with MPO primary antibody (rabbit polyclonal antibody; Abcam, Cambridge, MA) followed by incubation with secondary antibody (goat anti-rabbit Alexa Fluor 488, Life Technologies,

Eugene, OR). Four random images were taken from each lung section with a fluorescent microscope (Nikon Eclipse E800) at 200X magnification. Immunofluorescence was quantified and normalized against total alveolar tissue present using Image J software. Results are reported as relative fluorescence units (RFU).

Pulmonary Permeability

In a separate set of animals, Evans blue (EB) dye extravasation was used as an indicator of vascular permeability, as we have previously described(20). Mice received a bolus injection of 3% Evans blue (4 ml/kg) one hour prior to sacrifice. At sacrifice, animals were perfused via the right ventricle with 10 ml PBS to remove the intravascular dye from vessels. Lungs were harvested and then incubated in formamide (1mL formamide per 100mg lung tissue) for 24 hours at 55°C to allow for dye extraction. After centrifugation, absorbance was measured in the supernatant at 620 nm using a plate reader. The amount of Evans blue dye extravasation is expressed as micrograms EB per 100mg lung tissue.

Pulmonary syndecan-1 immunostaining

To detect pulmonary syndecan-1, lungs were sectioned and then incubated with syndecan-1 primary antibody (mouse monoclonal antibody; Santa Cruz Biotechnology) followed by incubation with secondary antibody (goat anti-mouse Alexa Fluor 488; Life Technologies, Eugene, OR). Four random images were taken from each lung section with a fluorescent microscope at 200X and immunofluorescence was quantified and normalized against total alveolar tissue present using Image J software. Results are reported as RFU.

Cytokines and Chemokines

At the time of euthanasia, whole blood was obtained by cardiac puncture and the plasma fraction isolated by centrifugation. After euthanasia, the trachea of each mouse was cannulated and bronchoalveolar lavage (BAL) fluid was collected by three injections of 0.4mL of PBS into the lungs. The BAL fluid was then centrifuged and the collected supernatant was analyzed for cytokines and chemokines. The plasma and BAL levels of interleukin (IL)-6, tumor necrosis factor (TNF)- α , IL-10, Gro-alpha/ keratinocyte chemoattractant (KC), Regulated on Activation Normal T Expressed and Secreted (RANTES), interferon-inducible protein (IP)-10, macrophage inflammatory protein (MIP)-1 α , MIP-2, MIP-1 β , monocyte chemoattractant protein (MCP)-1, MCP-3, and Eotaxin were determined by mouse cytokine and chemokine array pro-inflammatory focused 26-plex using ProcartaPlex Multiplex Immunoassay kit (Lot number: 192621026) following the manufacturer's instructions. The plate was performed on a Luminex XMAP instrument.

Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA) with Bonferroni correction; p values < 0.05 were considered significant. Data are expressed as mean $\pm$ standard deviation (SD). For *in vivo* experiments, n=4–8/group. Sample size was based on our previous study using the same mouse model of hemorrhagic shock but with immediate resuscitation(20).

RESULTS

Resuscitation with FFP required less volume to maintain MAP

Mice resuscitated with FFP required less volume than mice resuscitated with Hextend® to maintain a MAP of 55–60mmHg during the six hour resuscitation period (0.211 ± 0.093 vs. 0.406 ± 0.135 mL, $p=0.003$). All animals in both the FFP and Hextend® groups survived to the study endpoint of six hours of hypotensive resuscitation. MAP was significantly higher for sham animals when compared to MAP for Hextend® and FFP animals at all experimental time points ($p<0.05$, two-way ANOVA) (Figure 1). MAP was similar for Hextend® and FFP animals at all time points, except for PHR hour 4 and PHR hour 5, where FFP animals had a significantly higher MAP than Hextend® animals (65.4 ± 10.1 vs. 55.1 ± 3.68 mmHg, $p=0.006$ and 64.3 ± 10.4 vs. 56.0 ± 4.3 mmHg, $p=0.037$, respectively).

Pulmonary injury and inflammation were reduced by FFP

Mice resuscitated with FFP demonstrated improved lung injury scores compared to mice resuscitated with Hextend®. FFP significantly mitigated lung histopathologic injury compared to Hextend® (histologic lung injury score 4.38 ± 2.07 vs. 7.5 ± 0.93 , on a 0–9 point scale, $p=0.002$), and was not significantly different when compared to sham animals (histologic lung injury score 4.38 ± 2.07 vs. 4.0 ± 1.93 , on a 0–9 point scale, $p=0.99$) (Figure 2). FFP also significantly reduced lung inflammation when compared to Hextend®, as measured by a quantified relative value of MPO staining using immunofluorescence (0.116 ± 0.044 vs. 0.308 ± 0.054 relative fluorescence of MPO, $p=0.002$) (Figure 3). Levels of MPO were similar between sham and FFP treated mice (0.082 ± 0.057 vs. 0.116 ± 0.044 relative fluorescence of MPO, $p=0.99$).

Fresh frozen plasma mitigated lung hyperpermeability

Resuscitation with FFP also significantly mitigated lung hyperpermeability compared to Hextend® (7.30 ± 1.34 vs. 14.91 ± 5.55 μ gEB/100mg lung tissue, $p=0.005$) (Figure 4). Pulmonary permeability was similar between the FFP treated mice and sham animals (7.30 ± 1.34 vs. 7.31 ± 2.05 μ gEB/100mg lung tissue, $p=0.99$).

Fresh frozen plasma restored pulmonary syndecan-1

Cell surface syndecan-1 was assessed as an indicator of endothelial injury. Mice resuscitated with FFP were found to have restored levels of pulmonary syndecan-1 as assessed by relative immunofluorescence staining when compared to Hextend® treated mice (0.514 ± 0.061 vs. 0.059 ± 0.021 , relative syndecan-1 fluorescence, $p<0.001$) (Figure 5).

Serum and BAL cytokine and chemokine profiles differed among FFP and Hextend® treated groups

Plasma levels of IL-6, IL-10, RANTES, IP-10, MCP-3, MCP-1 and Eotaxin were significantly higher in both Hextend® and FFP treated groups compared to shams (Figure 6A). FFP treated mice had significantly higher plasma levels of Gro- α /KC and MIP-1 β compared to shams. When compared to both sham and Hextend® treated animals, FFP treated animals had higher plasma levels of TNF- α and MIP-2. There was no significant

difference between plasma levels of IL-18, IL-22 and IL-1 β . BAL levels of IL-6, TNF- α , IL-10, RANTES, MIP-1 α , MCP-1, MCP-3, MIP-2, MIP-1 β , and Eotaxin were similar between sham, Hextend®, and FFP treated mice. Mice treated with FFP had significantly higher levels of Gro-alpha/KC when compared to shams and levels of IP-10 were significantly higher in sham mice than in those treated with either Hextend® or FFP (Figure 6B).

DISCUSSION

A number of studies have shown that the damage control strategy of hypotensive resuscitation for traumatic hemorrhagic shock reduces the need for blood transfusions, reduces the incidence of acute respiratory distress syndrome (ARDS) and multiple organ dysfunction, and may confer a survival benefit(21–24). As a result, this component of damage control resuscitation has been employed frequently in the military setting, where achieving definitive care post-injury within the “golden hour” may not be possible(1,25). However, the degree to which hypotensive resuscitation can be used in a PFC situation is unknown(7,26). We sought to develop a small animal model of hemorrhagic shock and PHR that could be used to study resuscitative strategies in the PFC environment that is frequently encountered during military operations.

Until recently, the Joint Trauma System had recommended the use of Hextend® (hydroxyethyl starch) as the preferred resuscitative fluid for patients in hemorrhagic shock in the absence of blood products, thus the current study employed the use of Hextend® rather than lactated Ringers as standard fluid(5,17). We have previously shown that plasma is superior to lactated Ringers in a mouse model of hemorrhagic shock with immediate resuscitation (13,16,27). We thus sought to evaluate the efficacy of FFP in a model of PHR after hemorrhagic shock. In the current study, we have shown that resuscitation with FFP mitigates lung injury, inflammation, and permeability when compared to resuscitation with Hextend® in a mouse model of PHR, similar to our prior findings with lactated Ringers and immediate resuscitation(17,20).

Since completion of this study, the Joint Trauma System no longer recommends the use of Hextend®. There have been conflicting reports around Hextend® and hetastarches in general(28–30). Much of the literature has supported the use of Hextend® and has shown that it is superior to normal saline and hypertonic saline and may be protective of the endothelial glycocalyx(31–34). While there is Level B evidence that Hextend® used in limited quantities does not result in coagulopathy, the FDA issued a safety communication on all hydroxy ethyl starches in 2013(35–38). This safety communication did not address the use of Hextend® in the prehospital environment for resuscitation after traumatic injury, however, concerns around potential adverse effects, specifically renal injury and coagulopathy, persist(37,39).

We have now shown that FFP caused less lung injury and inflammation, mitigated pulmonary permeability, and restored pulmonary syndecan-1 when compared to Hextend®. This finding is consistent with previous findings in the literature that have shown that FFP is protective when compared to hetastarches after traumatic injury(40,41), and further supports

the removal of Hextend® from prolonged field care guidelines as there is a potential additional detrimental effect on the lungs. Given that Hextend® has now been removed from the TCCC guidelines, we recognize that the next critical experiments would compare FFP versus a balanced salt solution such as Lactated Ringer's in our model of prolonged hypotensive resuscitation.

Animals resuscitated with FFP required less volume overall than animals resuscitated with Hextend®. Animals resuscitated with FFP required approximately 8.4mL/kg compared to animals resuscitated with Hextend® which required about 16.2mL/kg. These volumes are in line with standard volumes of resuscitative boluses given in the clinical environment. While some of the differences we observed in lung injury between the two groups may be partially explained by differences in the volume required for resuscitation, a number of factors that are inherent to FFP have been shown to be lung protective after hemorrhagic shock, such as fibrinogen, antithrombin III, and adiponectin(42–44). Additionally, even with more volume, animals resuscitated with Hextend® had significantly lower MAP at several time points, suggesting that resuscitation with lower volumes would have led to severe hypotension and possible death prior to the experimental endpoint.

After HS and PHR, plasma levels of IL-6, IL-10, RANTES, IP-10, MCP-3, MCP-1, and Eotaxin were significantly elevated in both Hextend® and FFP groups when compared to sham. IP-10 was also significantly elevated in the BAL of both Hextend® and FFP groups when compared to sham. With the exception of IL-10, which is typically thought of as an anti-inflammatory cytokine, all of these cytokines/chemokines are mediators of the inflammatory response. It is unsurprising that a number of inflammatory markers were increased in both the Hextend® and FFP groups, as hemorrhagic shock is a known inciting event for a large inflammatory cascade(32,45). Additionally, it has previously been shown that cytokine release correlates with the timing of fluid resuscitation(46). FFP treated mice also had significantly higher plasma levels of Gro-alpha/KC and MIP-1β compared to sham. Gro-alpha/KC was also higher in the BAL of mice treated with FFP than sham mice. Gro-alpha/KC is a chemoattractant of neutrophils and MIP-1β is crucial for the immune response towards infection and inflammation. FFP treated mice also had significantly higher plasma levels of TNF-α and MIP-2 when compared to both Hextend® and sham groups. MIP-2 is a chemotactic agent for leukocytes and hematopoietic cells and TNF-α is an inflammatory cytokine produced by macrophages and monocytes during acute inflammation. An immediate increase in serum TNF-α has been shown to be associated with survival in the setting of severe HS(47). Ultimately, a variety of complex interactions between pro- and anti-inflammatory cytokines and chemokines likely contribute to both injury and pulmonary protection in the setting of hemorrhagic shock and PHR.

Although we have developed a model of PHR that may allow for further study of HS and resuscitation in the PFC environment, only one time-point of PHR, six hours after hemorrhagic shock, was investigated. As animals were required to be under general anesthesia for PHR, we felt a longer period of time would be detrimental. Further studies of additional time points are needed. Additionally, we used human plasma in a murine model of hemorrhagic shock and PHR. The use of human plasma in mice allows us to study the clinical product used in humans, which we believe to be of important translational benefit,

although xeno-incompatibility is a possible confounder. However, we have previously shown a lack of species-specific differences in pulmonary indices following hemorrhagic shock(19). Although Hextend® and FFP were the resuscitative fluids selected for this study, we have shown that plasma-based resuscitation is effective and warrants further investigation. As dried plasma is considered functionally equivalent to other plasma products in trauma resuscitation, and may offer additional logistic advantages in the PFC environment, further study of lyophilized plasma in this model of HS and PHR is warranted(48–50).

In conclusion, we have developed a murine model of hemorrhagic shock and PHR, which will allow for further study of best practices for resuscitation after severe injury. This may be particularly useful in modeling the far forward and austere setting often encountered by the military. We have also demonstrated the protective effects of FFP compared to Hextend® in this model. This data provides proof-of-concept that plasma is effective even in PHR scenarios of trauma and hemorrhagic shock and supports further investigation and development of plasma formulations, such as lyophilized plasma, that can be utilized in the PFC environment.

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REFERENCES

1. Keenan S, Riesberg JC. Prolonged Field Care: Beyond the “Golden Hour.” *Wilderness Environ Med.* 2017 6;28(2):S135–9. [PubMed: 28601206]
2. Kauvar DS, Lefering R, Wade CE. Impact of Hemorrhage on Trauma Outcome: An Overview of Epidemiology, Clinical Presentations, and Therapeutic Considerations. *J Trauma Inj Infect Crit Care.* 2006 6;60(Supplement):S3–11.
3. Eastridge BJ, Mabry RL, Seguin P, Cantrell J, Tops T, Uribe P, Mallett O, Zubko T, Oetjen-Gerdes L, Rasmussen TE, et al. Death on the battlefield (2001–2011). *J Trauma Acute Care Surg.* 2012 12;73(6):S431–7. [PubMed: 23192066]
4. Martin MJ, Holcomb JB, Polk T, Hannon M, Eastridge B, Malik SZ, Blackman VS, Galante JM, Grabo D, Schreiber M, et al. The “Top 10” research and development priorities for battlefield surgical care. *J Trauma Acute Care Surg.* 2019 7;87:S14–21. [PubMed: 31246901]
5. Cap AP, Gurney J, Spinella PC, Strandenes G, Schreiber MA, Holcomb JB, Corley JB, Jenkins D, Eastridge BJ, Wier R, et al. Clinical Practice Guideline (JTS CPG) Damage Control Resuscitation (CPG ID: 18). 2019.
6. Butler FK, Holcomb JB, Shackelford S, Barababella S, Bailey JA, Baker JB, Cap AP, Conklin CC, Cunningham CW, Davis M, et al. Advanced Resuscitative Care in Tactical Combat Casualty Care: TCCC Guidelines Change 18–01:14 October 2018. *J Spec Oper Med.* 2018;18(4):37–55.
7. Eastridge BJ, Mabry RL, Blackburn LH, Butler FK. We don’t know what we don’t know: prehospital data in combat casualty care. *US Army Med Dep J.* 2011;11–4. [PubMed: 21607901]
8. Cap AP, Beckett AN, Benov A, Borgman MA, Bryant B, Chen J, Corley JB, Doughty H, Fisher AD, Glassberg E, et al. Clinical Practice Guideline (JTS CPG) Whole Blood Transfusion (CPG ID: 21). 2018.
9. Spinella PC, Cap AP. Whole blood. *Curr Opin Hematol.* 2016 11;23(6):536–42. [PubMed: 27607444]

10. Matijevic N, Wang Y-W, Cotton BA, Hartwell E, Barbeau JM, Wade CE, Holcomb JB. Better hemostatic profiles of never-frozen liquid plasma compared with thawed fresh frozen plasma. *J Trauma Acute Care Surg*. 2013 1;74(1):84–91. [PubMed: 23271081]
11. Pusateri AE, Given MB, Schreiber MA, Spinella PC, Pati S, Kozar RA, Khan A, Dacorta JA, Kupferer KR, Prat N, et al. Dried plasma: state of the science and recent developments. *Transfusion*. 2016 4;56:S128–39. [PubMed: 27100749]
12. Sperry JL, Guyette FX, Brown JB, Yazer MH, Triulzi DJ, Early-Young BJ, Adams PW, Daley BJ, Miller RS, Harbrecht BG, et al. Prehospital Plasma during Air Medical Transport in Trauma Patients at Risk for Hemorrhagic Shock. *N Engl J Med*. 2018 7 26;379(4):315–26. [PubMed: 30044935]
13. Kozar RA, Peng Z, Zhang R, Holcomb JB, Pati S, Park P, Ko TC, Paredes A. Plasma Restoration of Endothelial Glycocalyx in a Rodent Model of Hemorrhagic Shock. *Anesth Analg*. 2011 6;112(6):1289–95. [PubMed: 21346161]
14. Wu F, Chipman A, Pati S, Miyasawa B, Corash L, Kozar RA. Resuscitative Strategies to Modulate The Endotheliopathy of Trauma: From Cell to Patient. *Shock*. 2019 5 10;1.
15. Barelli S, Alberio L. The Role of Plasma Transfusion in Massive Bleeding: Protecting the Endothelial Glycocalyx? *Front Med*. 2018 4 18;5.
16. Pati S, Peng Z, Wataha K, Miyazawa B, Potter DR, Kozar RA. Lyophilized plasma attenuates vascular permeability, inflammation and lung injury in hemorrhagic shock. Raju R, editor. *PLoS One*. 2018 2 2;13(2).
17. Potter DR, Baimukanova G, Keating SM, Deng X, Chu JA, Gibb SL, Peng Z, Muench MO, Fomin ME, Spinella PC, et al. Fresh frozen plasma and spray-dried plasma mitigate pulmonary vascular permeability and inflammation in hemorrhagic shock. *J Trauma Acute Care Surg*. 2015 6;78:S7–17. [PubMed: 26002267]
18. Hart ML, Ceonzo KA, Shaffer LA, Takahashi K, Rother RP, Reenstra WR, Buras JA, Stahl GL. Gastrointestinal Ischemia-Reperfusion Injury Is Lectin Complement Pathway Dependent without Involving C1q. *J Immunol*. 2005 5 15;174(10):6373–80. [PubMed: 15879138]
19. Peng Z, Pati S, Fontaine MJ, Hall K, Herrera AV, Kozar RA. Lack of species-specific difference in pulmonary function when using mouse versus human plasma in a mouse model of hemorrhagic shock. *J Trauma Acute Care Surg*. 2016 11;81(5):S171–6. [PubMed: 27768665]
20. Wu F, Peng Z, Park PW, Kozar RA. Loss of Syndecan-1 Abrogates the Pulmonary Protective Phenotype Induced by Plasma After Hemorrhagic Shock. *Shock*. 2017 9;48(3):340–5. [PubMed: 28107214]
21. Owattanapanich N, Chittawatanarat K, Benyakorn T, Sirikun J. Risks and benefits of hypotensive resuscitation in patients with traumatic hemorrhagic shock: a meta-analysis. *Scand J Trauma Resusc Emerg Med*. 2018 12 17;26(1):107. [PubMed: 30558650]
22. Morrison CA, Carrick MM, Norman MA, Scott BG, Welsh FJ, Tsai P, Liscum KR, Wall MJ, Mattox KL. Hypotensive Resuscitation Strategy Reduces Transfusion Requirements and Severe Postoperative Coagulopathy in Trauma Patients With Hemorrhagic Shock: Preliminary Results of a Randomized Controlled Trial. *J Trauma Inj Infect Crit Care*. 2011 3;70(3):652–63.
23. Schreiber MA, Meier EN, Tisherman SA, Kerby JD, Newgard CD, Brasel K, Egan D, Witham W, Williams C, Daya M, et al. A controlled resuscitation strategy is feasible and safe in hypotensive trauma patients. *J Trauma Acute Care Surg*. 2015 4;78(4):687–97. [PubMed: 25807399]
24. Tran A, Yates J, Lau A, Lampron J, Matar M. Permissive hypotension versus conventional resuscitation strategies in adult trauma patients with hemorrhagic shock. *J Trauma Acute Care Surg*. 2018 5;84(5):802–8. [PubMed: 29370058]
25. Kotwal RS, Howard JT, Orman JA, Tarpey BW, Bailey JA, Champion HR, Mabry RL, Holcomb JB, Gross KR. The Effect of a Golden Hour Policy on the Morbidity and Mortality of Combat Casualties. *JAMA Surg*. 2016 1 1;151(1):15. [PubMed: 26422778]
26. Jenkins DH, Rappold JF, Badloe JF, Berséus O, Blackbourne CL, Brohi KH, Butler FK, Cap LAP, Cohen MJ, Davenport R, et al. Trauma Hemostasis and Oxygenation Research Position Paper on Remote Damage Control Resuscitation. *Shock*. 2014 5;41:3–12.
27. Pati S, Matijevic N, Doursout M-F, Ko T, Cao Y, Deng X, Kozar RA, Hartwell E, Conyers J, Holcomb JB. Protective Effects of Fresh Frozen Plasma on Vascular Endothelial Permeability,

- Coagulation, and Resuscitation After Hemorrhagic Shock Are Time Dependent and Diminish Between Days 0 and 5 After Thaw. *J Trauma Inj Infect Crit Care*. 2010 7;69(Supplement):S55–63.
28. Ogilvie MP, Pereira BMT, McKenney MG, McMahon PJ, Manning RJ, Namias N, Livingstone AS, Schulman CI, Proctor KG. First Report on Safety and Efficacy of Hetastarch Solution for Initial Fluid Resuscitation at a Level 1 Trauma Center. *J Am Coll Surg*. 2010 5;210(5):870–80. [PubMed: 20421069]
 29. Myburgh JA, Finfer S, Bellomo R, Billot L, Cass A, Gattas D, Glass P, Lipman J, Liu B, McArthur C, et al. Hydroxyethyl Starch or Saline for Fluid Resuscitation in Intensive Care. *N Engl J Med*. 2012 11 15;367(20):1901–11. [PubMed: 23075127]
 30. Weiskopf RB, James MFM. Update of use of hydroxyethyl starches in surgery and trauma. *J Trauma Acute Care Surg*. 2015 6;78:S54–9. [PubMed: 26002264]
 31. Jernigan PL, Hoehn RS, Cox D, Heyl J, Dorlac WC, Pritts TA. What If I Don't Have Blood? Hextend is Superior to 3% Saline in an Experimental Model of Far Forward Resuscitation After Hemorrhage. *Shock*. 2016 9;46:148–53. [PubMed: 27380529]
 32. Kane JM, Billiar TR. The Cytokine Response to Hemorrhagic Shock In: *J Anesth*. 1997 p. 42–51.
 33. Sheppard FR, Mitchell TA, Macko AR, Fryer DM, Schaub LJ, Ozuna KM, Glaser JJ. Whole blood and Hextend. *J Trauma Acute Care Surg*. 2018 7;85:S33–8. [PubMed: 29261592]
 34. Butler FK. Fluid Resuscitation in Tactical Combat Casualty Care: Yesterday and Today. *Wilderness Environ Med*. 2017 6;28(2):S74–81. [PubMed: 28601214]
 35. Deusch E, Thaler U, Kozek-Langenecker SA. The Effects of High Molecular Weight Hydroxyethyl Starch Solutions on Platelets. *Anesth Analg*. 2004 9;99(3):665–8. [PubMed: 15333389]
 36. Ogilvie MP, Ryan ML, Proctor KG. Hetastarch During Initial Resuscitation From Trauma. *J Trauma Inj Infect Crit Care*. 2011 5;70:S19–21.
 37. Ravi PR, Puri B. Fluid resuscitation in haemorrhagic shock in combat casualties. *Disaster Mil Med*. 2017 12 17;3(1):2. [PubMed: 28265454]
 38. James MFM, Michell WL, Joubert IA, Nicol AJ, Navsaria PH, Gillespie RS. Resuscitation with hydroxyethyl starch improves renal function and lactate clearance in penetrating trauma in a randomized controlled study: the FIRST trial (Fluids in Resuscitation of Severe Trauma). *Br J Anaesth*. 2011 11;107(5):693–702. [PubMed: 21857015]
 39. Hartog CS, Natanson C, Sun J, Klein HG, Reinhart K. Concerns over use of hydroxyethyl starch solutions. *BMJ*. 2014 11 10;349(nov10 1):g5981–g5981. [PubMed: 25385352]
 40. Torres LN, Sondeen JL, Ji L, Dubick MA, Filho IT. Evaluation of resuscitation fluids on endothelial glycocalyx, venular blood flow, and coagulation function after hemorrhagic shock in rats. *J Trauma Acute Care Surg*. 2013 10;75(5):759–66. [PubMed: 24158192]
 41. Jin G, DeMoya MA, Duggan M, Knightly T, Mejaddam AY, Hwabejire J, Lu J, Smith WM, Kasotakis G, Velmahos GC, et al. Traumatic Brain Injury and Hemorrhagic Shock. *Shock*. 2012 7;38(1):49–56. [PubMed: 22575994]
 42. Yu Q, Yang B, Davis JM, Ghosn J, Deng X, Doursout M-F, Dong J, Wang R, Holcomb JB, Wade CE, et al. Identification of Fibrinogen as a Key Anti-Apoptotic Factor in Human Fresh Frozen Plasma for Protecting Endothelial Cells In Vitro. *Shock*. 2019 6;1.
 43. Deng X, Cao Y, Huby MP, Duan C, Baer L, Peng Z, Kozar RA, Doursout M-F, Holcomb JB, Wade CE, et al. Adiponectin in Fresh Frozen Plasma Contributes to Restoration of Vascular Barrier Function After Hemorrhagic Shock. *Shock*. 2016 1;45(1):50–4. [PubMed: 26263440]
 44. Lopez E, Peng Z, Kozar RA, Cao Y, Ko TC, Wade CE, Cardenas JC. Antithrombin III Contributes to the Protective Effects of Fresh Frozen Plasma Following Hemorrhagic Shock by Preventing Syndecan-1 Shedding and Endothelial Barrier Disruption. *Shock*. 2020 2;53(2):156–63. [PubMed: 31389906]
 45. Lu S, Aguilar A, Subramani K, Poulouse N, Ayub A, Raju R. Alteration of cytokine profile following hemorrhagic shock. *Cytokine*. 2016 5;81:35–8. [PubMed: 26851979]
 46. Lee C-C, Chang I-J, Yen Z-S, Hsu C-Y, Chen S-Y, Su C-P, Chiang W-C, Chen S-C, Chen W-J. Delayed Fluid Resuscitation in Hemorrhagic Shock Induces Proinflammatory Cytokine Response. *Ann Emerg Med*. 2007 1;49(1):37–44. [PubMed: 17011072]
 47. Namas R, Ghuma A, Torres A, Polanco P, Gomez H, Barclay D, Gordon L, Zenker S, Kim HK, Hermus L, et al. An Adequately Robust Early TNF- α Response Is a Hallmark of Survival

Following Trauma/Hemorrhage. Ojcius DM, editor. PLoS One. 2009 12 22;4(12):e8406. [PubMed: 20027315]

48. Daniel Y, Habas S, Malan L, Escarment J, David J-S, Peyrefitte S. Tactical damage control resuscitation in austere military environments. J R Army Med Corps. 2016 12;162(6):419–27. [PubMed: 27531659]
49. Johnson VV, Swiatkowski SA. Scientific aspects of supplying blood to distant military theaters. Curr Opin Hematol. 2007 11;14(6):694–9. [PubMed: 17898577]
50. Hess JR, Lelkens CCM, Holcomb JB, Scalea TM. Advances in military, field, and austere transfusion medicine in the last decade. Transfus Apher Sci. 2013 12;49(3):380–6. [PubMed: 23856530]

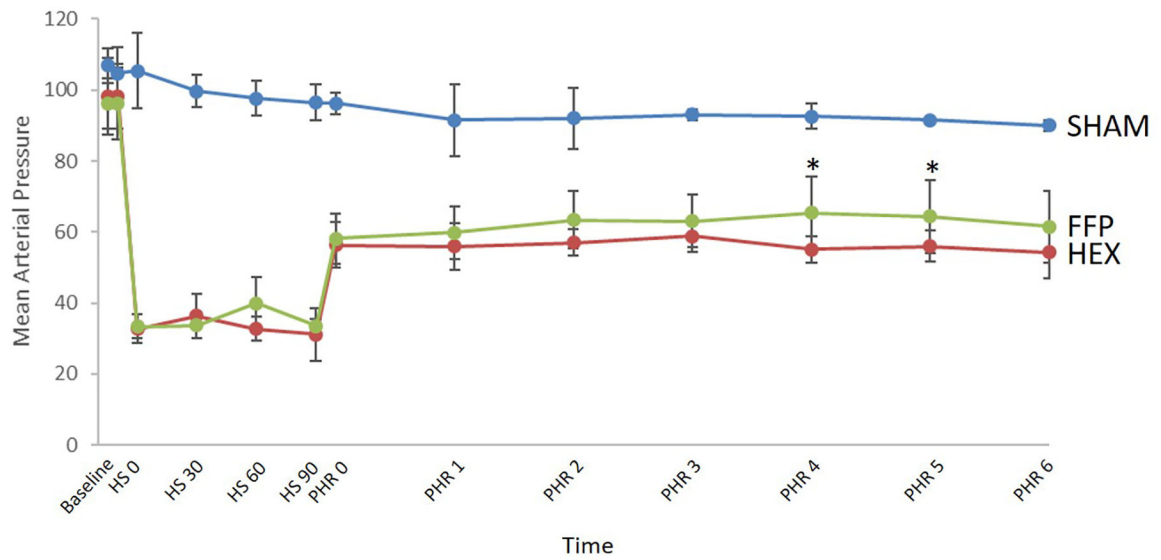


Figure 1: Mean Arterial Pressure Over Time

Mice underwent 90 minutes of hemorrhagic shock followed by prolonged hypotensive resuscitation with either FFP or Hextend® to maintain a mean arterial pressure (MAP) of 55–60mmHg for six hours and were compared to sham mice. Sham animals had a significantly higher MAP than FFP or Hextend® animals at all time-points. FFP and Hextend® animals had similar MAP for all time points except PHR hour 4 and PHR hour 5, which are denoted by an asterisk. Data is reported as mean $\pm$ SD with $n=6-8$ /group; two-way ANOVA. Abbreviations: Hex=Hextend®, FFP=fresh frozen plasma, HS=hemorrhagic shock, PHR=prolonged hypotensive resuscitation.

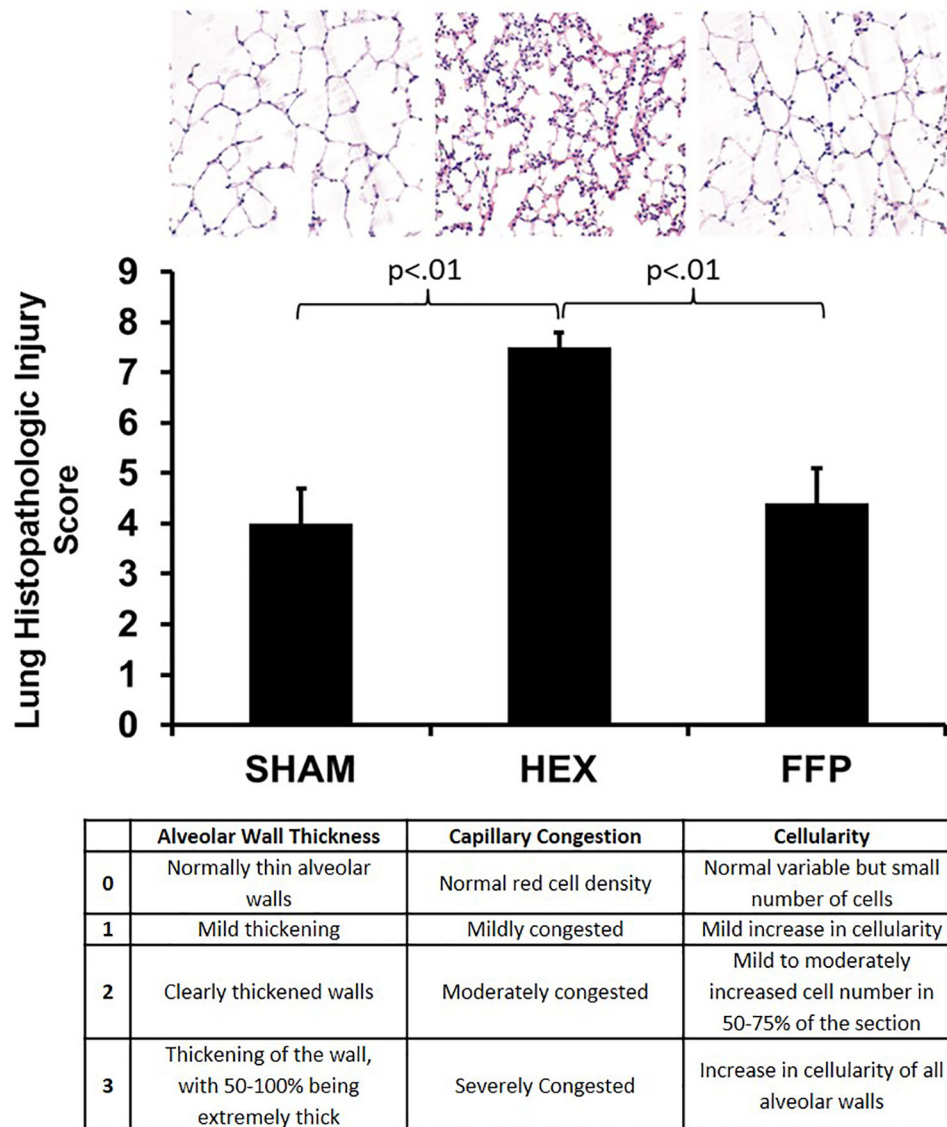


Figure 2: Lung histopathologic injury was reduced by FFP

Mice underwent 90 minutes of hemorrhagic shock followed by prolonged hypotensive resuscitation with either FFP or Hextend® to maintain a MAP of 55–60mmHg for six hours and were compared to sham mice. Shown are representative images and the corresponding lung injury scores. Table with scoring system for lung histopathologic injury modified from Hart et al., 2005(18). Data is reported as mean $\pm$ SD with $n=8$ /group and was analyzed by one-way ANOVA with Bonferroni *post hoc*. Abbreviations: Hex=Hextend, FFP=fresh frozen plasma.

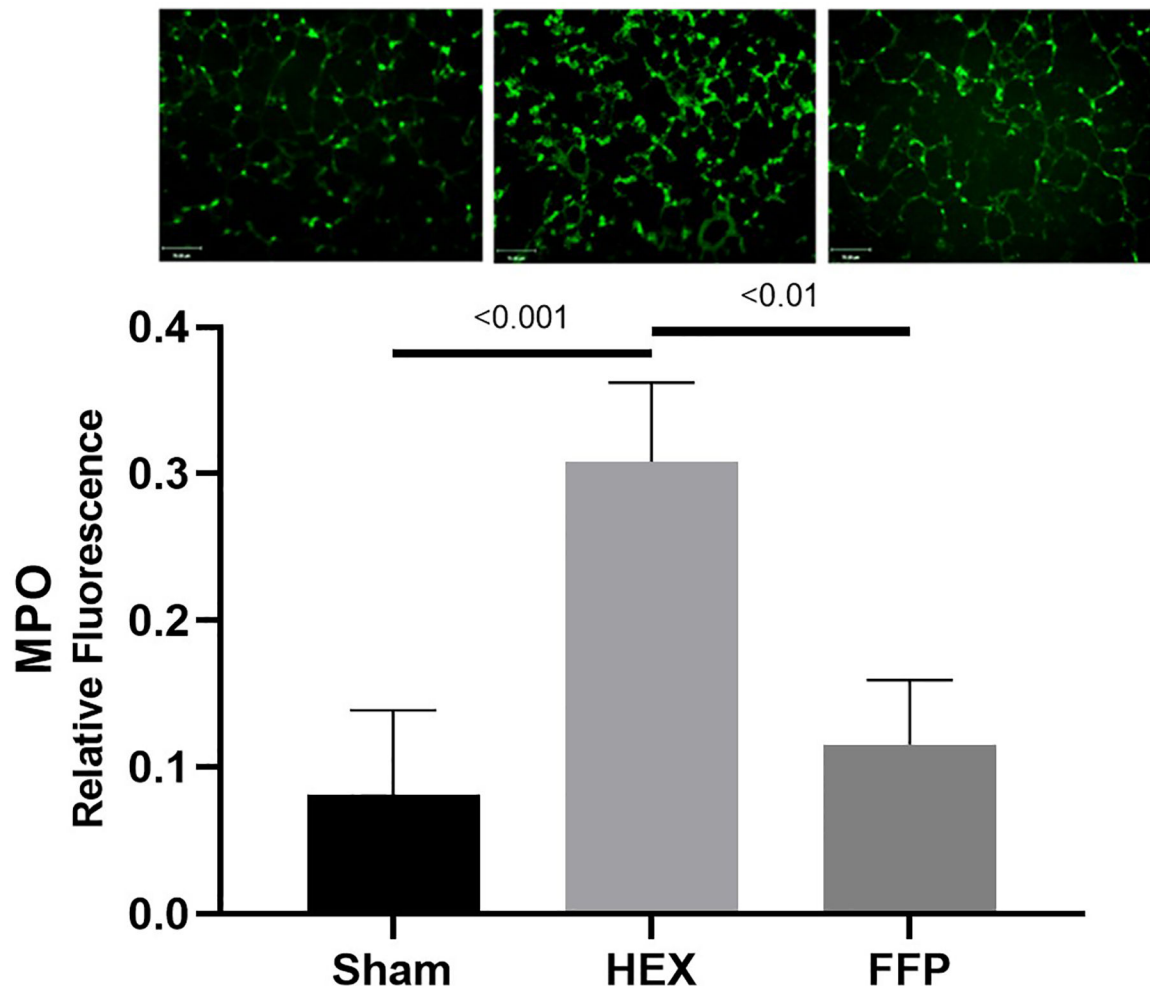


Figure 3: Pulmonary inflammation was reduced by FFP

Mice underwent 90 minutes of hemorrhagic shock followed by PHR with either FFP or Hextend® to maintain a MAP of 55–60mmHg for six hours and were compared to sham mice. Myeloperoxidase immunostaining was assessed as an indicator of neutrophil infiltration. Shown are representative images and the corresponding quantitation. Data is reported as mean ± SD with n=4/group and was analyzed by one-way ANOVA with Bonferroni *post hoc*. Abbreviations: Hex=Hextend®, FFP=fresh frozen plasma.

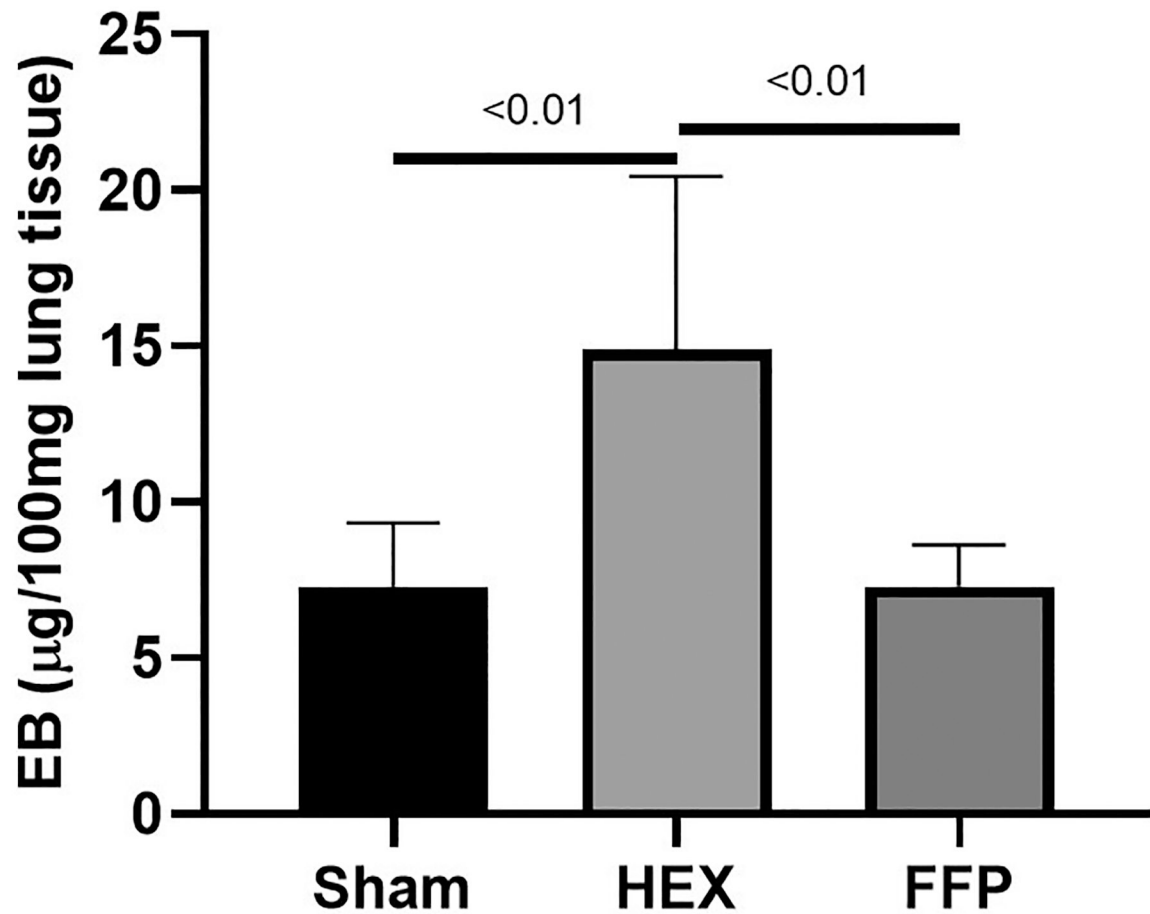


Figure 4: Pulmonary hyperpermeability was reduced by FFP

Mice underwent 90 minutes of hemorrhagic shock followed by PHR with either FFP or Hextend® to maintain a MAP of 55–60mmHg for six hours and were compared to sham mice. Pulmonary permeability was assessed with an Evans blue dye assay. Data is reported as mean±SD with n=6–8/group and was analyzed by one-way ANOVA with Bonferroni *post hoc*. Abbreviations: Hex=Hextend®, FFP=fresh frozen plasma, EB=Evans Blue.

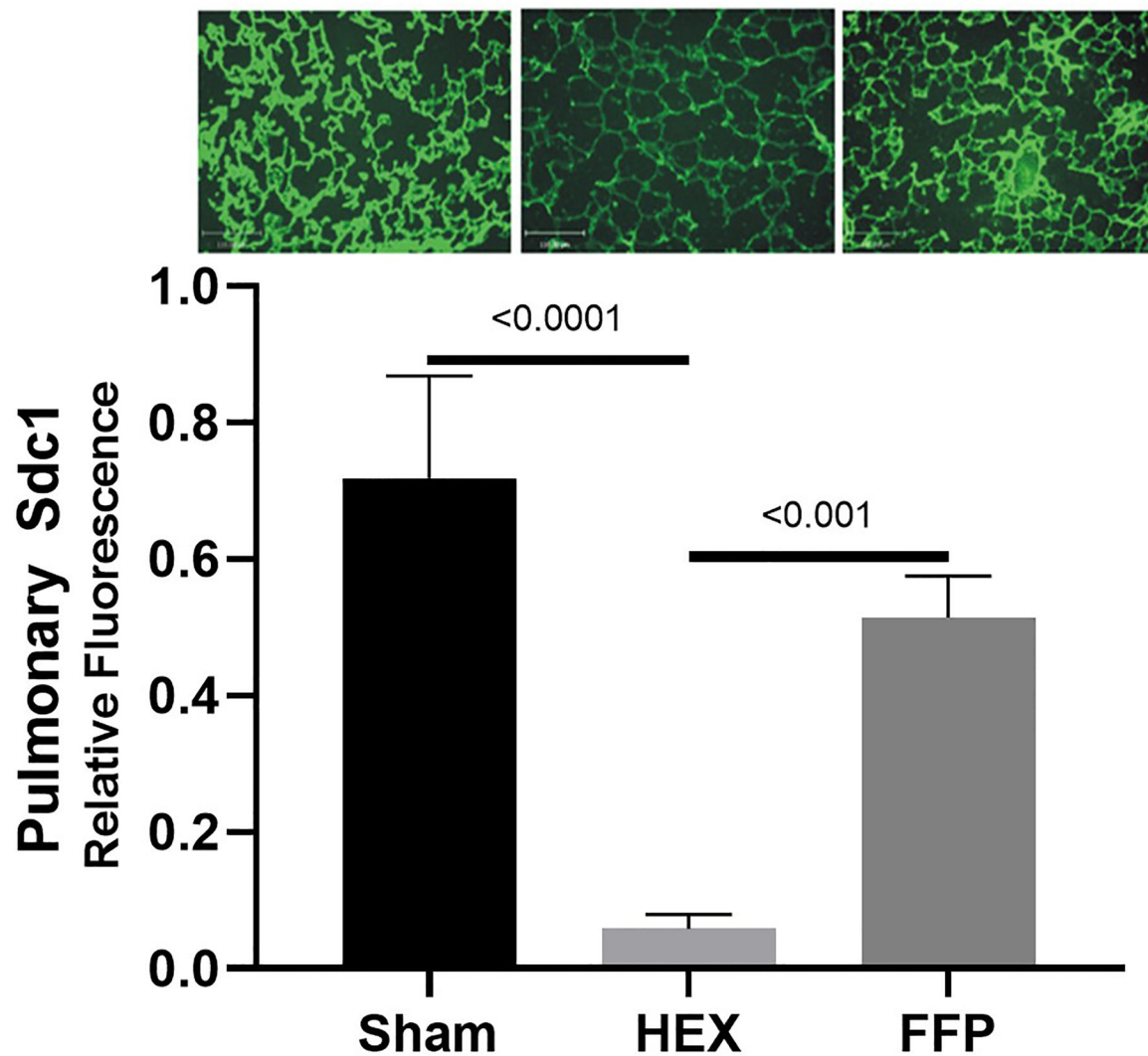


Figure 5: Pulmonary syndecan-1 immunostaining was increased by FFP

Mice underwent 90 minutes of hemorrhagic shock followed by PHR with either FFP or Hextend® to maintain a MAP of 55–60mmHg for six hours and were compared to sham mice. Pulmonary syndecan-1 immunostaining was assessed. Shown are representative images and the corresponding quantitation. Data is reported as mean±SD with n=4/group and was analyzed by one-way ANOVA with Bonferroni *post hoc*. Abbreviations: Hex=Hextend®, FFP=fresh frozen plasma.

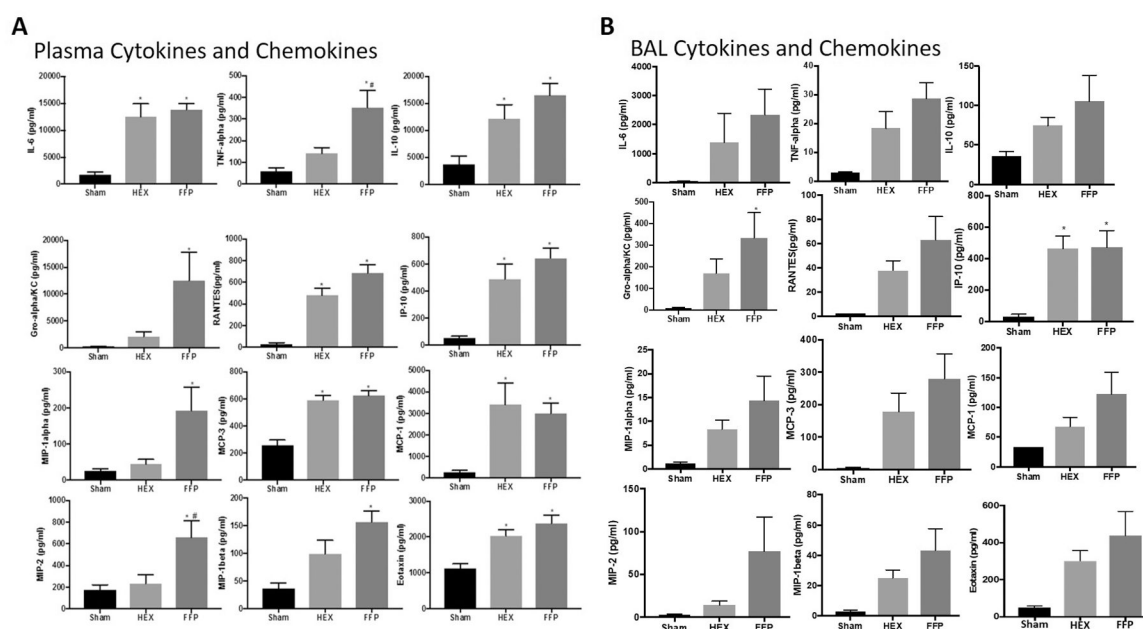


Figure 6: Cytokine and chemokine profiles differed among FFP and Hextend® treated groups
Mice underwent 90 minutes of hemorrhagic shock followed by PHR with either FFP or Hextend® to maintain a MAP of 55–60mmHg for six hours and were compared to sham mice. A. Plasma and B. Bronchoalveolar lavage was obtained from mice at time of sacrifice and assayed on a mouse cytokine and chemokine array pro-inflammatory Luminex Immunoassay. Selected cytokine and chemokine profiles are shown. *indicates $p < 0.05$ when compared to sham group, #indicates $p < 0.05$ when compared to Hextend® group. Abbreviations: IL=interleukin, TNF=tumor necrosis factor, KC=keratinocyte chemoattractant, IP=induced protein, MIP=macrophage inflammatory protein, MCP=monocyte chemoattractant protein.

Lyophilized Plasma Resuscitation Downregulates Inflammatory Gene Expression in a Mouse Model of Sepsis

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ABSTRACT

Background: Plasma resuscitation may improve outcomes by targeting endotheliopathy induced by severe sepsis or septic shock. Given the logistical constraints of using fresh frozen plasma (FFP) in military settings or areas with prolonged pre-hospital care, dried products such as lyophilized plasma (LP) have been developed. We hypothesized that resuscitation with LP would decrease lung injury, inflammation, and mortality in a mouse sepsis model.

Methods: Adult male C57BL/6J mice received an intraperitoneal injection of cecal slurry. Twenty-two hours later the mice were anesthetized, the femoral artery was cannulated, and the mice were randomized to receive resuscitation with LP (10 mL/kg) or lactated Ringer's (LR, 30 mL/kg) over one hour. At 48-hours post cecal slurry injection, bronchoalveolar lavage (BAL) fluid was collected, the lungs were harvested, and plasma was obtained. Mortality and BAL total protein concentration (as an indicator of permeability) were compared between groups. The lungs were analyzed for histopathology and inflammatory gene expression using NanoString, and the plasma was analyzed for biomarkers of inflammation and endothelial function.

Results: There was no significant difference in short-term mortality between LR and LP mice, 38% versus 47%, respectively ($p=0.62$). BAL protein levels were similar among mice resuscitated with LR or LP, and there was a lack of significant histopathologic lung injury in all groups. However, LP resuscitation resulted in downregulation of pulmonary inflammatory genes, including signaling pathways such as JAK-STAT and NF- κ B, and a circulating inflammatory biomarker profile similar to sham animals.

Conclusion: Resuscitation with LP did not improve mortality or reduce permeability or injury in this model compared to LR. However, LP downregulated pulmonary inflammatory gene signaling and may also reduce circulating biomarkers of inflammation. Future studies should evaluate LP resuscitation in combination with antibiotics and other therapeutics to determine whether the anti-inflammatory effects of LP may improve outcomes in sepsis.

Level of Evidence: N/A (Basic Science)

Study Type: Original Research (Basic Science)

Keywords: sepsis, septic shock, lyophilized plasma, resuscitation

BACKGROUND

Sepsis accounts for approximately 20% of all global deaths and is a leading cause of death in intensive care units.(1, 2) Sepsis is also a serious complication following major trauma and is associated with prolonged hospital care and high mortality.(3) In addition to prompt administration of antibiotics, rapid fluid administration and vasopressors to maintain blood pressure are key elements in the management of patients with sepsis or septic shock.(4) However, there is no clear consensus on the optimal resuscitative fluid.(5)

Crystalloid solutions are widely available, inexpensive, and currently recommended by the Surviving Sepsis Campaign as first-line therapy for fluid administration in septic patients given the lack of benefit demonstrated with other fluids.(4) Balanced crystalloids such as lactated Ringer's (LR) or PlasmaLyte are preferred due to concerns regarding hyperchloremic metabolic acidosis and acute renal injury with administration of large volumes of normal saline.(6-9) Colloid solutions such as albumin or hydroxyethyl starch (HES) have not been shown to be superior to crystalloids and are also more expensive(10)—HES, in fact, may be associated with worse outcomes and is no longer utilized.(9, 11) Consistent with these findings, the Joint Trauma System Clinical Practice Guideline for Sepsis Management in Prolonged Field Care currently recommends initial resuscitation with IV/IO LR or PlasmaLyte.(12)

Plasma-based resuscitation could potentially play a therapeutic role in septic shock by mitigating shock-induced endotheliopathy, which is characterized by glycocalyx shedding, vascular permeability, inflammation, coagulopathy, and ultimately organ failure.(13-15) Fresh frozen plasma (FFP) resuscitation has been demonstrated in animal models to decrease the

endotheliopathy associated with trauma and hemorrhagic shock.(14, 16-19) Given that sepsis-induced endotheliopathy is characterized by similar findings, FFP could be an ideal resuscitative fluid in these patients. Recently, resuscitation with FFP was shown in a rat model of sepsis to increase 48-hour survival, improve pulmonary function, and decrease markers of inflammation, endothelial injury, and catecholamines compared to normal saline.(20) Currently there is one active clinical trial we are aware of investigating the use of plasma transfusion in septic patients, but the study outcomes are unknown (NCT04580563).

The logistical challenges of FFP, however, complicate its use in the battlefield or other remote settings requiring prolonged pre-hospital care. As a frozen product, FFP requires cold chain management often over long distances to reach military settings, and once thawed must be refrigerated and used within 5 days, thus limiting its availability. Recognizing this gap, dried plasma technologies such as lyophilized plasma (LP) have been developed to provide plasma-based resuscitation in these settings because of their improved storage profile and ability to be reconstituted rapidly.(21) The composition of FFP and LP have been demonstrated to be highly similar; the lyophilization process results in only a small reduction in some coagulation factors and does not significantly change thromboelastographic parameters or protein composition.(22-24) LP has been shown to be safe and effective in treating bleeding patients.(25, 26) Moreover, in a mouse model of hemorrhagic shock, LP was shown to reduce inflammation and vascular permeability similar to FFP, suggesting that LP can recapitulate the effects of FFP on shock-induced endotheliopathy.(27)

The purpose of this study was to evaluate LP as an initial resuscitative fluid in a mouse model of sepsis using intraperitoneal administration of a cecal slurry. We hypothesized that LP would decrease lung injury, inflammation, and mortality compared to lactated Ringer's (LR) resuscitation.

METHODS

Animals

All experimental procedures were approved by the Institutional Animal Care and Use Committee. The experiments were conducted in compliance with the ARRIVE guidelines for animal models (SDC ARRIVE Guidelines Checklist, <http://links.lww.com/TA/C512>) and the National Institutes of Health (NIH) guidelines on the use of laboratory animals. Adult male C57BL/6J mice (9–12 weeks old) were used for all studies.

Cecal Slurry

The cecal slurry was prepared according to Starr et al.(28) Cecal contents were collected from 25 mice of the same strain, age, and sex as the mice used in the sepsis model. Under isoflurane anesthesia, mice were euthanized, the cecum was dissected from each mouse, and the cecal contents from all 25 mice were pooled together, weighed, and mixed in 10% glycerol in phosphate buffered saline (PBS) with the ratio of 1 mL buffer to 100 mg of stool. The cecal slurry was serially strained using meshes of progressively smaller pore sizes: 860, 380, 190, and finally 74 microns. This produced a single batch of cecal slurry that was used for all animals in this experiment to ensure consistent injury across animals. The cecal slurry was aliquoted and frozen at -80°C and thawed prior to use.

Lyophilized Plasma

Research grade lyophilized plasma (LP) derived from human plasma was reconstituted with sterile water to its original volume and aliquoted by Teleflex Inc. (Wayne, PA). Samples were shipped on dry ice and maintained at -80°C until time of use. Approximately 15 minutes prior to administration the aliquot of LP was placed in a 37°C water bath and thawed.

Sepsis Protocol

Mice received an intraperitoneal injection of cecal slurry at a weight-based dose of 10 μ L/g of body weight. Sham animals received saline in equal volume. At 22 hours post cecal slurry, sepsis scores were calculated using the modified murine sepsis score (MSS) from Mai et al. which includes evaluation of appearance, level of consciousness, activity level, response to stimulus, eye opening, and respiration quality.(29) The mice were then placed under isoflurane anesthesia for a one-hour period of resuscitation. The femoral artery was cannulated for continuous hemodynamic monitoring and resuscitation. Mean arterial blood pressure (MAP) was recorded at baseline then every 5 minutes during resuscitation. Mice were resuscitated with 10 mL/kg body weight of LP or 30 mL/kg body weight of lactated Ringer's (LR) over 15 min. These bolus volumes are consistent with a recent study of plasma resuscitation in sepsis by Chang et al.(20) as well as the Surviving Sepsis Campaign's recommendation of at least 30 mL/kg of IV crystalloid fluid during initial resuscitation.(4) If the MAP dropped below 60 mmHg, an additional 0.1 mL of fluid was given in 15 min increments to maintain a MAP greater than 60 mmHg. Sham mice underwent anesthesia but no resuscitation. The femoral catheter was removed at 60 min post initial resuscitation, and mice were allowed to recover from anesthesia. Twenty-four hours later, animals were placed under isoflurane anesthesia and euthanized by cardiac puncture. Blood was collected in citrate-coated

tubes and centrifuged at 2000 g for 10 minutes for isolation of the plasma fraction. The right lung was ligated, resected, and frozen on dry ice and stored at -80°C for RNA and protein extraction. The trachea was then cannulated and bronchoalveolar lavage (BAL) was performed on the left lung by injecting and aspirating 0.4 mL PBS three times through the catheter and pooling the retained BALs. The left lung was then filled with 0.4 mL of 50% OCT in PBS and saved in OCT. The left lung was snap frozen and maintained at -80°C until it was sectioned for histopathologic analysis. The schematic model for this protocol is depicted in Figure 1.

Lung Histology

Lungs were sectioned on a Leica CM 1950 Cryostat (Wetzlar, Germany) at 10 µm thickness. Tissue sections were then stained with Hematoxylin & Eosin and imaged with a Revolve microscope (Echo Inc., San Diego, CA). Representative images from a minimum of three lung sections per mouse were evaluated by a blinded researcher using a semi-quantitative scoring system to evaluate inflammatory cell infiltration as previously described.(30)

NanoString Gene Expression Analysis of Lung Tissue

Total RNA was isolated from fresh frozen lung lysates using the ALLPrep DNA/RNA mini kit (Qiagen, Germantown, MD) and quality control assessed with a DeNovix DS-11 Spectrophotometer (DeNovix, Wilmington, DE). Using nCounter technology from Nanostring, copy numbers were measured of the 750 genes in the mouse nCounter® Autoimmune Profiling Panel (NanoString, Seattle, WA). RNA was isolated from 5 randomly selected mice per group (Sham, LR, and LP), and the 4 samples per group with the highest RNA purity were processed for gene expression. Samples were normalized to positive controls and housekeeping genes using the

Advanced Analysis geNorm algorithm.(31) Differentially expressed genes between two groups (LR vs. Sham, LP vs. Sham, and LP vs. LR) were determined by Student's t-test (p -value < 0.05) using R software version 4.0.2 and were presented in a heat map using Cluster and TreeView Software.(32) Volcano plots of all genes in the panel were generated using nCounter Advanced Analysis 2.0.134 (NanoString). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway(33) enrichment analyses were subsequently conducted on the differentially expressed genes using DAVID software.(34) A pie distribution plot of the significant KEGG pathways (FDR adjusted p -value < 0.05) downregulated by LP vs. LR was generated using the Plotly package in R software. Pathways are presented in this plot with the name of the pathway and the number of genes in that pathway that were significantly downregulated.

Reverse Transcription Quantitative Real-Time PCR (RT-qPCR)

To verify select genes identified by NanoString, messenger RNA was reverse-transcribed (RT) using SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific) and quantitative PCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA). RT-qPCR was performed on the RNA isolated from the same 5 lung lysates per group as described above. The primer pair sequences were: ICAM: 5'-CAGTCTACAACCTTTTCAGCTC -3' and 5'-CACACTTCACAGTTACTTGG -3'; Ikbkg: 5'-GAGAAGAAGGAGTATTTGCAG-3' and 5'-CAAATGAAAGGAGTGGTGAG -3'; IL-6RA: 5'-AAAGTTCTACAGAAGCAACG -3' and 5'-TTGAGTCTCAGGATGATGAAG-3'; Jak3: 5'-ACAGGCTACGCTTTTATTTTC-3' and 5'-AAACGTGTAAGTCAAGGATG-3'; Rela: 5'-GTATCCATAGCTTCCAGAAC-3' and 5'-CAAATGAAAGGAGTGGTGAG -3';5'-GAGAAGAAGGAGTATTTGCAG-3' and 5'-GAAAGGGGTTATTGTTGGTC-3'; E-selectin:

5'-CATGAAATGTCTTCCCAGTG-3' and 5'-ATCACATTTTCACAGCTGAAC-3'; STAT3: 5'-CGTCTGGAAACTGGATAAC-3' and 5'-TTAAGTTTCTGAACAGCTCC-3'; Traf1: 5'-CATCCTTTGATGGTACTTTCC-3' and 5'-TAGAAAGCTGGAGAGAAGAG-3'; VCAM1: 5'-ACTGATTATCCAAGTCTCTCC-3' and 5'-CCATCCACAGACTTTAATACC-3'. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the endogenous control. Relative RNA amount was calculated using the $2^{-\Delta\Delta C_t}$ method.

Plasma Inflammatory Biomarker Analysis

Using the same 5 mice per group as the pulmonary gene expression analysis, mouse plasma samples were analyzed for the following biomarkers using a custom multiplex Luminex® Discovery Assay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol: Angiopoietin-2 (Ang2), C-X-C Motif Chemokine Ligand 10 (CXCL10), CXCL12, Intercellular Adhesion Molecule-1 (ICAM-1), Interferon gamma (IFN- γ), Interleukin-1beta (IL-1 β), IL-2, IL-4, IL-6, IL-10, Matrix Metalloproteinase-8 (MMP8), MMP9, Receptor for Advanced Glycation Endproducts (RAGE), Syndecan-1, Tissue Inhibitor of Metalloprotease-4 (TIMP4), Tumor Necrosis Factor alpha (TNF α), TNF Receptor Superfamily Member 1a (TNFRSF1a), TNFRSF1b, TNF Superfamily Member 13b (TNFSF13b), and Vascular Endothelial Growth Factor (VEGF). In summary, plasma samples were thawed and centrifuged at 16,000 g for 4 min prior to use. Samples were then diluted 1:2 and 1:10 in appropriate diluent and pipetted onto a 96-well plate, mixed with magnetic beads coated with antibodies, and incubated for two hours at room temperature on a horizontal orbital microplate shaker at 800rpm. Three washes with wash buffer were performed, then the beads were incubated with the biotinylated antibody cocktail for 1 hour at room temperature on the orbital shaker at 800rpm. After another three washes, the beads were

incubated with streptavidin-PE for 30 minutes on the shaker. The beads were washed a final three times then resuspended in wash buffer prior to being read on a MAGPIX System (Luminex Corp., Austin, TX). xPONENT 4.2 software (Luminex Corp.) was used for data acquisition.

Statistical Analysis

Based on our previous studies with the primary endpoints used in this study (i.e. BAL and lung histopathology), approximately 8 surviving mice per group were required for significance analysis. No surviving animals were excluded from analysis. Mortality was compared using the Pearson Chi-square test. Sepsis scores and MAPs were compared between LR and LP groups using Student's t-test. BAL total protein levels were compared among sham, LR, and LP groups using one-way ANOVA with post-hoc Tukey's tests. $P < 0.05$ was considered significant. Differential expression of pulmonary inflammatory genes and signaling pathways were analyzed as described above. Data in this manuscript are presented as mean $\pm$ SD.

RESULTS

Mortality of Cecal Slurry-Induced Sepsis

We chose to use a cecal slurry to induce sepsis in mice as it has been shown to be reproducible.(28) To test the efficacy of stored cecal slurry stock for inducing sepsis, mice were injected with varying doses of slurry from 5 μ L/g body weight to 20 μ L/g body weight then resuscitated with LR and monitored up to 48 hours: 5 μ L/g was non-lethal, 10 μ L/g increased mortality to 38%, and at 20 μ L/g no animals survived. Therefore 10 μ L/g was chosen for subsequent study.

Sepsis Physiologic Outcomes and Mortality

N=7 sham, 13 LR, and 17 LP mice were used for this experiment. Sepsis scores were calculated using the modified Murine Sepsis Score (MSS)(29) prior to resuscitation, demonstrating sepsis progression in the mice that had received cecal slurry administration that was similar between the mice that were randomized to receive LR versus LP resuscitation (Table 1). Intraperitoneal administration of cecal slurry caused notable hypotension by 22 hours post-injection compared to sham animals. MAPs at one hour post resuscitation were lower in the sepsis groups compared to shams and were similar between LR and LP mice. There was substantial mortality with this model with 38% mortality in the LR group and 47% in the LP group, however these two groups did not differ from each other ($p=0.62$). In the LR group, two mortalities occurred 1 hour after resuscitation, and the remaining three died overnight (<18 hours after resuscitation). Similarly, in the LP group, one mortality occurred 1 hour after resuscitation, one occurred 3 hours after, and the remaining six occurred overnight. The total protein concentration was measured from BAL fluid collected from each mouse as a marker of protein permeability across the alveolar epithelium. Total protein levels between the sham, LR, and LP groups were not significantly different from each other ($p=0.11$) (Table 1).

Lung Histopathology

Representative images of the lung after H&E stain are shown in Figure 2. At this time point we found that the lungs from each group appeared similar. Notably, there was a paucity of inflammatory infiltrates or alveolar wall thickening, indicating a lack of significant lung injury in this model among mice that survived at 48 hours.

Inflammatory Gene Expression in the Lung

Inflammatory gene expression was analyzed using the nCounter[®] Autoimmune Profiling Panel (NanoString) and displayed in Figure 3. A heat map and volcano plots were generated demonstrating that many more inflammatory genes were downregulated than upregulated on this panel among mice resuscitated with LP compared to mice resuscitated with LR. In total there were 187 genes that were significantly downregulated by LP compared to LR and 39 that were upregulated (Supplemental Figure 1, <http://links.lww.com/TA/C513>). A complete list of these genes, their fold change, and *p*-values are displayed in Supplemental Tables 1 and 2, <http://links.lww.com/TA/C514>.

Using KEGG pathway analysis, we identified multiple inflammatory signaling pathways which were significantly affected by resuscitation fluid. As shown in Figure 4a, there were a number of KEGG signaling pathways with high numbers of genes that were downregulated among mice resuscitated with LP compared to LR, including JAK-STAT, chemokine, NF- κ B, TNF, cytokine-cytokine receptor interaction, and Toll-like receptor signaling.

To confirm these findings, RT-qPCR was performed, selecting genes involved in or known to be affected by the JAK-STAT and NF- κ B signaling pathways (Figure 4b). In agreement with the NanoString pathway analysis, RT-qPCR demonstrated downregulation of genes involved in the NF- κ B (*Ikbkg*, *Rela*, and *Traf1*) and JAK-STAT pathways (*Jak3*, *Stat3*) in the LP group compared to LR. The expression of *Il6ra* (JAK-STAT pathway) was significantly downregulated by LP in the NanoString analysis but was found to be elevated in both the LR and LP groups using RT-qPCR. Finally, endothelial activation is known to induce the production of adhesion molecules E-

selectin, Intercellular Adhesion Molecule-1 (ICAM-1), and Vascular Cell Adhesion Molecule-1 (VCAM-1), which are involved in leukocyte rolling and adhesion and regulated by the NF- κ B pathway.(35) We used RT-qPCR to analyze expression of these respective genes, which were not included on the NanoString panel. ICAM expression was similar across the three groups, E-selectin expression was increased among mice resuscitated with LR but not LP, and VCAM-1 expression was increased among mice resuscitated with LP but not LR (Figure 4b).

Plasma Biomarker Analysis

A custom multiplex Luminex® assay was used to evaluate levels of twenty biomarkers of inflammation and/or endothelial function in the plasma from N=5 mice per group. The results are displayed in Figure 5, demonstrating that there was significant variation in values particularly among the mice resuscitated with LR, whereas sham mice were tightly clustered with low expression of nearly all of these proteins, and mice resuscitated with LP fell in between depending on the biomarker. Elevated levels of the following biomarkers were found in a subset of LR mice but not in the Sham or LP groups: IFN- γ , IL1B, IL2, IL6, IL10, MMP9, Syndecan-1, TNF α , TNFRSF1a, TNFRSF1b, and VEGF.

DISCUSSION

Though we hypothesized that LP resuscitation would decrease lung injury and mortality compared to LR resuscitation in a mouse model of sepsis, we found no such differences in these endpoints. However, at the molecular level, we identified significant alterations in inflammatory gene expression profiles in the lung, supporting an anti-inflammatory effect of LP. This was demonstrated by downregulation of numerous genes in an inflammatory gene panel as well as

important signaling pathways such as NF- κ B, JAK-STAT, chemokine and cytokine receptor interactions, TNF, and Toll-like receptor signaling, all of which have been shown to play important roles in mediating overwhelming inflammation, organ injury, and death from sepsis.(36-41)

We also used RT-qPCR to confirm the NanoString panel results on a small selection of genes involved in the NF- κ B and JAK-STAT pathways, with similar findings. In addition, with RT-qPCR we evaluated the expression of several endothelial surface markers involved in leukocyte adhesion that are regulated by NF- κ B. These included E-selectin, which was downregulated by LP compared to LR resuscitation, and VCAM-1, which surprisingly was higher in the LP group, potentially related to other transcriptional factors not evaluated in this study. Finally, plasma analysis of inflammatory biomarkers demonstrated that many of these circulating proteins were highly variable in LR mice, a finding that was not seen to such an extent among the LP mice. However, these biomarkers were analyzed in only a small number of mice per group, and the differences were not statistically significant.

The immune system response to sepsis is complex and includes activation of both pro- and anti-inflammatory pathways early and simultaneously in sepsis. The net effect is generally thought to include an initial hyperinflammatory phase lasting several days, followed by a prolonged immunosuppressive phase.(42, 43) Hyperinflammation can lead to major complications including multi-organ dysfunction syndrome (MODS) and disseminated intravascular coagulation (DIC). Theoretically, mitigating the hyperinflammatory phase of sepsis could be beneficial early in sepsis, though notably a number of anti-inflammatory agents effective in animal models have failed to improve outcomes in clinical trials.(44, 45) In this study we found that LP modulates the

hyperinflammatory phase of sepsis, but it is unknown whether the use of LP as a volume expander and inhibitor of hyperinflammation would attenuate organ failure and mortality in patients with sepsis. Indeed, in this model there was no associated improvement in mortality with LP resuscitation. It is possible that the anti-inflammatory effects of LP were not sufficient to overcome the high mortality rates in the septic mice, particularly in the absence of antibiotics. Investigating plasma or LP resuscitation in the presence of antibiotics may improve the ability to detect more broad-spanning therapeutic effects, though survivors did not demonstrate improvement in other biologic endpoints that were examined. As the use of LP increases in clinical trials and in settings where injured patients develop sepsis, we will be able to further elucidate the potential impact of these anti-inflammatory effects compared to crystalloid resuscitation.

Currently there are no proven therapies to target the endotheliopathy that develops in patients with sepsis or septic shock. The ability of LP to modulate the inflammatory cascade in sepsis as shown in this study suggests that plasma could potentially play a therapeutic role in sepsis as it does in hemorrhagic shock. This has important implications for current and future combat, as complications such as sepsis not only amplify tissue and organ damage after severe polytraumatic injury but also increase mortality.(46, 47) An autopsy review of US Casualties during Operation Iraqi Freedom and Enduring Freedom found that sepsis was the fourth leading cause of death among potentially survivable injuries, while hemorrhage remained the leading cause.(48) Future conflicts will rely on missions in resource-limited, austere environments where the injured fighter may have to wait days before evacuation. The military advantage of using a dried plasma product such as LP, which is stored at room temperature and rapidly reconstituted, cannot be overstated.

There are several limitations of this study. First, we did not include fresh frozen plasma (FFP) as a resuscitation group. Chang et al. demonstrated in a similar sepsis model in rats using cecal ligation and puncture that FFP resuscitation improved survival and pulmonary function and decreased inflammatory markers.(20) In our preliminary studies, there were no differences between LP, human FFP, or fresh mouse plasma in terms of mortality or sepsis scores (unpublished data), so for this study LP was the only plasma product administered. Moreover, FFP versus LP have been extensively studied in models of hemorrhagic shock both *in vitro* and *in vivo* with no discernable difference.(21, 27, 49-51) Second, the LP used in this study was reconstituted and frozen in small aliquots, then thawed before administration. This was done to avoid reconstituting a new unit of LP for each mouse throughout the study period, which would have wasted large volumes of product, and it is possible this additional freeze-thaw cycle could have a small impact on its efficacy. Of course, the logistical advantage of LP in patient settings is that it is stored at room temperature and reconstituted at the time it is needed. Third, the LP used in this study was derived from human plasma but administered in mice, raising the possibility of cross-species interactions. However, previous work with human plasma in mouse models of hemorrhage has not shown differences in terms of pulmonary function and inflammation compared to mouse plasma.(49) Fourth, assays were only able to be performed on tissue from surviving mice, which could have introduced survivor bias or masked differences between treatment groups. Finally, we evaluated all endpoints at 48 hours after cecal slurry injection, modeling our study design to that of Chang et al.(20), and we did not find evidence of overt histologic lung injury at this time point. Other time points may better elucidate changes in histopathology or levels of circulating inflammatory proteins. We were also unable to replicate the survival benefit of plasma found by

Chang et al., but perhaps differences in rodent species and minor differences in resuscitation could explain this discrepancy.

In conclusion, LP resuscitation did not improve physiologic endpoints in a mouse model of sepsis, however LP does appear to cause downregulation of inflammatory genes and multiple pro-inflammatory signaling pathways. Future studies should evaluate LP resuscitation in combination with other therapies to determine whether the anti-inflammatory effects of LP may improve outcomes in sepsis.

Authorship Contributions: All authors participated in manuscript preparation. **SP** and **RK**: planning, funding acquisition, supervision, data interpretation. **MB**: planning, performed the experiments, statistical analysis, data interpretation, figure preparation. **FW**: planning, performed the experiments, statistical analysis. **HG**: gene expression statistical analysis, figure preparation. **AC**: planning, performed the experiments.

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Conflict of Interest Statement: The authors have no conflicts of interest.

References

1. Rudd KE, Johnson SC, Agesa KM, Shackelford KA, Tsoi D, Kievlan DR, et al. Global, regional, and national sepsis incidence and mortality, 1990-2017: analysis for the Global Burden of Disease Study. *Lancet*. 2020;395(10219):200-11.
2. Mayr FB, Yende S, Angus DC. Epidemiology of severe sepsis. *Virulence*. 2014;5(1):4-11.
3. Eriksson J, Lindström AC, Hellgren E, Friman O, Larsson E, Eriksson M, et al. Postinjury Sepsis-Associations With Risk Factors, Impact on Clinical Course, and Mortality: A Retrospective Observational Study. *Crit Care Explor*. 2021;3(8):e0495.
4. Evans L, Rhodes A, Alhazzani W, Antonelli M, Coopersmith CM, French C, et al. Surviving Sepsis Campaign: International Guidelines for Management of Sepsis and Septic Shock 2021. *Crit Care Med*. 2021;49(11):e1063-e143.
5. Chang R, Holcomb JB. Choice of Fluid Therapy in the Initial Management of Sepsis, Severe Sepsis, and Septic Shock. *Shock*. 2016;46(1):17-26.
6. Williams EL, Hildebrand KL, McCormick SA, Bedel MJ. The effect of intravenous lactated Ringer's solution versus 0.9% sodium chloride solution on serum osmolality in human volunteers. *Anesth Analg*. 1999;88(5):999-1003.
7. Kellum JA, Song M, Almasri E. Hyperchloremic acidosis increases circulating inflammatory molecules in experimental sepsis. *Chest*. 2006;130(4):962-7.
8. Chowdhury AH, Cox EF, Francis ST, Lobo DN. A randomized, controlled, double-blind crossover study on the effects of 2-L infusions of 0.9% saline and plasma-lyte® 148 on renal blood flow velocity and renal cortical tissue perfusion in healthy volunteers. *Ann Surg*. 2012;256(1):18-24.

9. Rochwerg B, Alhazzani W, Sindi A, Heels-Ansdell D, Thabane L, Fox-Robichaud A, et al. Fluid resuscitation in sepsis: a systematic review and network meta-analysis. *Ann Intern Med.* 2014;161(5):347-55.
10. Lewis SR, Pritchard MW, Evans DJ, Butler AR, Alderson P, Smith AF, et al. Colloids versus crystalloids for fluid resuscitation in critically ill people. *Cochrane Database Syst Rev.* 2018;8:CD000567.
11. Haase N, Perner A, Hennings LI, Siegemund M, Lauridsen B, Wetterslev M, et al. Hydroxyethyl starch 130/0.38-0.45 versus crystalloid or albumin in patients with sepsis: systematic review with meta-analysis and trial sequential analysis. *BMJ.* 2013;346:f839.
12. Joint Trauma System Clinical Practice Guideline. Sepsis Management in Prolonged Field Care. 2020.
13. Johansson PI, Stensballe J, Ostrowski SR. Shock induced endotheliopathy (SHINE) in acute critical illness - a unifying pathophysiologic mechanism. *Crit Care.* 2017;21(1):25.
14. Holcomb JB, Pati S. Optimal trauma resuscitation with plasma as the primary resuscitative fluid: the surgeon's perspective. *Hematology Am Soc Hematol Educ Program.* 2013;2013:656-9.
15. Gurney JM, Kozar RA, Cancio LC. Plasma for burn shock resuscitation: is it time to go back to the future? *Transfusion.* 2019;59(S2):1578-86.
16. Milford EM, Reade MC. Resuscitation Fluid Choices to Preserve the Endothelial Glycocalyx. *Crit Care.* 2019;23(1):77.
17. Kozar RA, Pati S. Syndecan-1 restitution by plasma after hemorrhagic shock. *J Trauma Acute Care Surg.* 2015;78(6 Suppl 1):S83-6.

18. Kozar RA, Peng Z, Zhang R, Holcomb JB, Pati S, Park P, et al. Plasma restoration of endothelial glycocalyx in a rodent model of hemorrhagic shock. *Anesth Analg*. 2011;112(6):1289-95.
19. Peng Z, Pati S, Potter D, Brown R, Holcomb JB, Grill R, et al. Fresh frozen plasma lessens pulmonary endothelial inflammation and hyperpermeability after hemorrhagic shock and is associated with loss of syndecan 1. *Shock*. 2013;40(3):195-202.
20. Chang R, Holcomb JB, Johansson PI, Pati S, Schreiber MA, Wade CE. Plasma Resuscitation Improved Survival in a Cecal Ligation and Puncture Rat Model of Sepsis. *Shock*. 2018;49(1):53-61.
21. Pusateri AE, Given MB, Schreiber MA, Spinella PC, Pati S, Kozar RA, et al. Dried plasma: state of the science and recent developments. *Transfusion*. 2016;56 Suppl 2:S128-39.
22. Steil L, Thiele T, Hammer E, Bux J, Kalus M, Völker U, et al. Proteomic characterization of freeze-dried human plasma: providing treatment of bleeding disorders without the need for a cold chain. *Transfusion*. 2008;48(11):2356-63.
23. Bux J, Dickhörner D, Scheel E. Quality of freeze-dried (lyophilized) quarantined single-donor plasma. *Transfusion*. 2013;53(12):3203-9.
24. Martinaud C, Civadier C, Ausset S, Verret C, Deshayes AV, Sailliol A. In vitro hemostatic properties of French lyophilized plasma. *Anesthesiology*. 2012;117(2):339-46.
25. Sailliol A, Martinaud C, Cap AP, Civadier C, Clavier B, Deshayes AV, et al. The evolving role of lyophilized plasma in remote damage control resuscitation in the French Armed Forces Health Service. *Transfusion*. 2013;53 Suppl 1:65S-71S.
26. Nguyen C, Bordes J, Cungi PJ, Esnault P, Cardinale M, Mathais Q, et al. Use of French lyophilized plasma transfusion in severe trauma patients is associated with an early plasma

- transfusion and early transfusion ratio improvement. *J Trauma Acute Care Surg.* 2018;84(5):780-5.
27. Pati S, Peng Z, Wataha K, Miyazawa B, Potter DR, Kozar RA. Lyophilized plasma attenuates vascular permeability, inflammation and lung injury in hemorrhagic shock. *PLoS One.* 2018;13(2):e0192363.
28. Starr ME, Steele AM, Saito M, Hacker BJ, Evers BM, Saito H. A new cecal slurry preparation protocol with improved long-term reproducibility for animal models of sepsis. *PLoS One.* 2014;9(12):e115705.
29. Mai SHC, Sharma N, Kwong AC, Dwivedi DJ, Khan M, Grin PM, et al. Body temperature and mouse scoring systems as surrogate markers of death in cecal ligation and puncture sepsis. *Intensive Care Med Exp.* 2018;6(1):20.
30. Barry M, Trivedi A, Pathipati P, Miyazawa BY, Vivona LR, Togarrati PP, et al. Mesenchymal Stem Cell Extracellular Vesicles Mitigate Vascular Permeability and Injury in the Small Intestine and Lung in a Mouse Model of Hemorrhagic Shock and Trauma. *J Trauma Acute Care Surg.* 2021.
31. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 2002;3(7):RESEARCH0034.
32. Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci U S A.* 1998;95(25):14863-8.
33. Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Res.* 2000;28(1):27-30.

34. Dennis G, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, et al. DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol.* 2003;4(5):P3.
35. Shapiro NI, Schuetz P, Yano K, Sorasaki M, Parikh SM, Jones AE, et al. The association of endothelial cell signaling, severity of illness, and organ dysfunction in sepsis. *Crit Care.* 2010;14(5):R182.
36. Cai B, Cai JP, Luo YL, Chen C, Zhang S. The Specific Roles of JAK/STAT Signaling Pathway in Sepsis. *Inflammation.* 2015;38(4):1599-608.
37. Bosmann M, Ward PA. The inflammatory response in sepsis. *Trends Immunol.* 2013;34(3):129-36.
38. Liu SF, Malik AB. NF-kappa B activation as a pathological mechanism of septic shock and inflammation. *Am J Physiol Lung Cell Mol Physiol.* 2006;290(4):L622-L45.
39. Tsujimoto H, Ono S, Efron PA, Scumpia PO, Moldawer LL, Mochizuki H. Role of Toll-like receptors in the development of sepsis. *Shock.* 2008;29(3):315-21.
40. Schulte W, Bernhagen J, Bucala R. Cytokines in sepsis: potent immunoregulators and potential therapeutic targets--an updated view. *Mediators Inflamm.* 2013;2013:165974.
41. Peters K, Unger RE, Brunner J, Kirkpatrick CJ. Molecular basis of endothelial dysfunction in sepsis. *Cardiovasc Res.* 2003;60(1):49-57.
42. Hotchkiss RS, Monneret G, Payen D. Sepsis-induced immunosuppression: from cellular dysfunctions to immunotherapy. *Nat Rev Immunol.* 2013;13(12):862-74.
43. Chen J, Wei H. Immune Intervention in Sepsis. *Front Pharmacol.* 2021;12:718089.
44. Nedeva C, Menassa J, Puthalakath H. Sepsis: Inflammation Is a Necessary Evil. *Front Cell Dev Biol.* 2019;7:108.

45. Okeke EB, Uzonna JE. In Search of a Cure for Sepsis: Taming the Monster in Critical Care Medicine. *J Innate Immun.* 2016;8(2):156-70.
46. Murray CK. Infectious disease complications of combat-related injuries. *Crit Care Med.* 2008;36(7 Suppl):S358-64.
47. Murray CK. Epidemiology of infections associated with combat-related injuries in Iraq and Afghanistan. *J Trauma.* 2008;64(3 Suppl):S232-8.
48. Kelly JF, Ritenour AE, McLaughlin DF, Bagg KA, Apodaca AN, Mallak CT, et al. Injury severity and causes of death from Operation Iraqi Freedom and Operation Enduring Freedom: 2003-2004 versus 2006. *J Trauma.* 2008;64(2 Suppl):S21-6; discussion S6-7.
49. Peng Z, Pati S, Fontaine MJ, Hall K, Herrera AV, Kozar RA. Lack of species-specific difference in pulmonary function when using mouse versus human plasma in a mouse model of hemorrhagic shock. *J Trauma Acute Care Surg.* 2016;81(5 Suppl 2 Proceedings of the 2015 Military Health System Research Symposium):S171-S6.
50. Diebel ME, Diebel LN, Liberati DM. Protective effects of plasma products on the endothelial-glycocalyx barrier following trauma-hemorrhagic shock: Is sphingosine-1 phosphate responsible? *J Trauma Acute Care Surg.* 2019;87(5):1061-9.
51. Spoerke N, Zink K, Cho SD, Differding J, Muller P, Karahan A, et al. Lyophilized plasma for resuscitation in a swine model of severe injury. *Arch Surg.* 2009;144(9):829-34.

Figure 1. Schematic Model of Mouse Sepsis Protocol. Cecal slurry (10 μ L/g body weight) was administered via intraperitoneal (IP) injection. At t=22hrs post injection, the mice were anesthetized and the femoral artery was cannulated for hemodynamic monitoring and resuscitation with LR (30 mL/kg body weight) or LP (10 mL/kg). Blood pressures were monitored for 1 hour post-resuscitation. At t=48 hours post injection, the mice were re-anesthetized, blood was collected via cardiac puncture, bronchoalveolar lavage was performed, and the lungs were harvested. Sham animals instead received saline via IP injection and similarly underwent femoral artery cannulation at t=22 hours however did not receive any resuscitation products. IP = intraperitoneal; LR = lactated Ringer's; LP = lyophilized plasma.

Figure 2. Lung Histopathology. Sections of lung were stained with H&E for all mice that survived to the 48-hour time point (N= 7 Sham, 8 LR, 9 LP). At the 48-hour time point, the lungs from all three groups appeared similar; there was no evidence of significant lung injury in terms of inflammatory infiltrates or alveolar wall thickening.

Figure 3. Inflammatory Gene Expression in the Lung. Total RNA was isolated from lungs of N=4 mice per group (sham, LR, LP) and analyzed using the 750 gene Autoimmune Profiling Panel from NanoString. (A) A heat map of genes that were differentially expressed between any two groups ($p<0.05$) was generated to display the gene expression profile for each mouse, with red indicating higher relative expression and green indicating lower relative expression. This demonstrates overall relative downregulation of genes on this panel in the lungs of mice resuscitated with LP compared to those resuscitated with LR. (B) The volcano plots display differential expression of all genes on the panel between each set of two groups: LR vs. sham, LP

vs. sham, and LP vs. LR. Genes to the right of zero on the x-axis are up-regulated, and genes to the left of zero are downregulated. Values higher on the y-axis are more statistically significant. Most notably the LP vs. LR plot demonstrates that a large number of inflammatory genes in the lung were downregulated by LP resuscitation compared to LR.

Figure 4. Inflammatory Gene Expression Pathway Analysis. Pathway analysis was conducted to determine which signaling pathways were affected by LP vs. LR resuscitation. (A) The donut chart displays KEGG pathways that were significantly downregulated (FDR adjusted p -value < 0.05) in the lungs of mice resuscitated with LP compared to those resuscitated with LR. There were multiple pathways involved in inflammatory signaling that were downregulated by LP resuscitation, including JAK-STAT, chemokine, NF- κ B, TNF, and cytokine-cytokine receptor signaling. (B) To confirm these findings, we performed RT-qPCR on N=5 mice per group, selecting genes involved in Jak-STAT and NF- κ B signaling pathways. The mRNA fold change in the LR or LP groups relative to sham is displayed here for each gene.

Figure 5. Plasma Biomarker Analysis. Twenty plasma biomarkers of inflammation and endothelial function were evaluated using a custom Luminex® assay for N=5 mice per group. All measurements are expressed in pg/mL. Differences were not significant between groups, however there was increased variability in multiple biomarkers among LR mice not seen with the LP or sham groups.

SUPPLEMENTAL DIGITAL CONTENT

Supplemental Figure 1. Differential Gene Expression. Venn diagrams demonstrating the number of genes on the NanoString panel that were significantly upregulated or downregulated for each comparison. In total there were 39 genes upregulated by LP compared to LR (16 unique to LP vs. LR, and 23 that were also upregulated by LR vs. Sham and/or LP vs. Sham). There were 187 genes downregulated by LP compared to LR (38 unique to LP vs. LR, and 149 that were also downregulated by LR vs. Sham and/or LP vs. Sham). The list of these genes and their relative expression are displayed in Supplemental Tables 1 and 2, <http://links.lww.com/TA/C514>.

Supplemental Table 1. List of genes upregulated by LP versus LR and/or Sham. This table displays the 39 genes that were significantly upregulated ($p < 0.05$) by LP compared to LR and/or sham mice. The table is organized into three sections corresponding to the Venn diagram in Supplemental Figure 1, <http://links.lww.com/TA/C513>, starting with the 3 genes upregulated across all three comparisons (LP vs. LR, LR vs. Sham, and LP vs. Sham), then the 20 genes upregulated by LP vs. both LR and Sham, and finally the 16 genes upregulated by LP compared to LR only. LP=lyophilized plasma; LR=lactated Ringer's.

Supplemental Table 2. List of genes downregulated by LP versus LR and/or Sham. This table displays the 187 genes that were significantly downregulated ($p < 0.05$) by LP compared to LR and/or sham mice. The table is organized into three sections corresponding to the Venn diagram in Supplemental Figure 1, <http://links.lww.com/TA/C513>, starting with the 4 genes downregulated across all three comparisons (LP vs. LR, LR vs. Sham, and LP vs. Sham), then the 145 genes downregulated by LP vs. both LR and sham, and finally the 38 genes downregulated by LP compared to LR only. LP=lyophilized plasma; LR=lactated Ringer's.

Figure 1

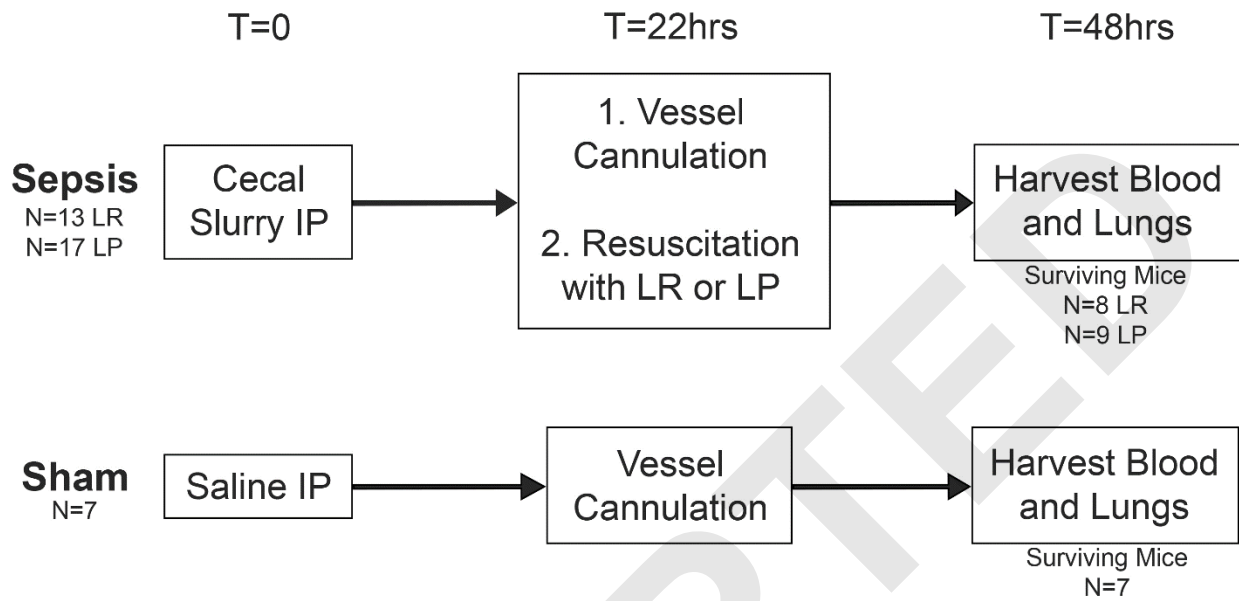


Figure 2

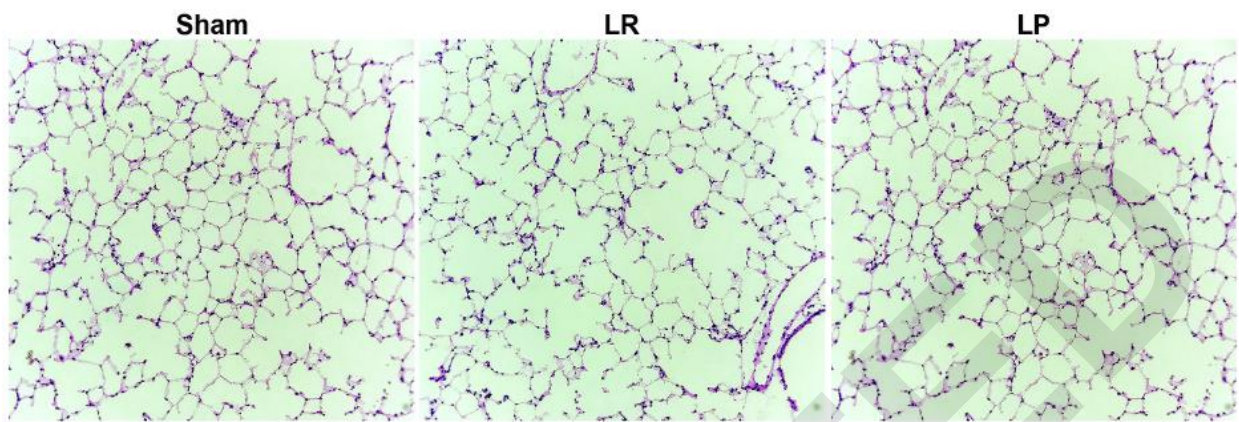


Figure 3

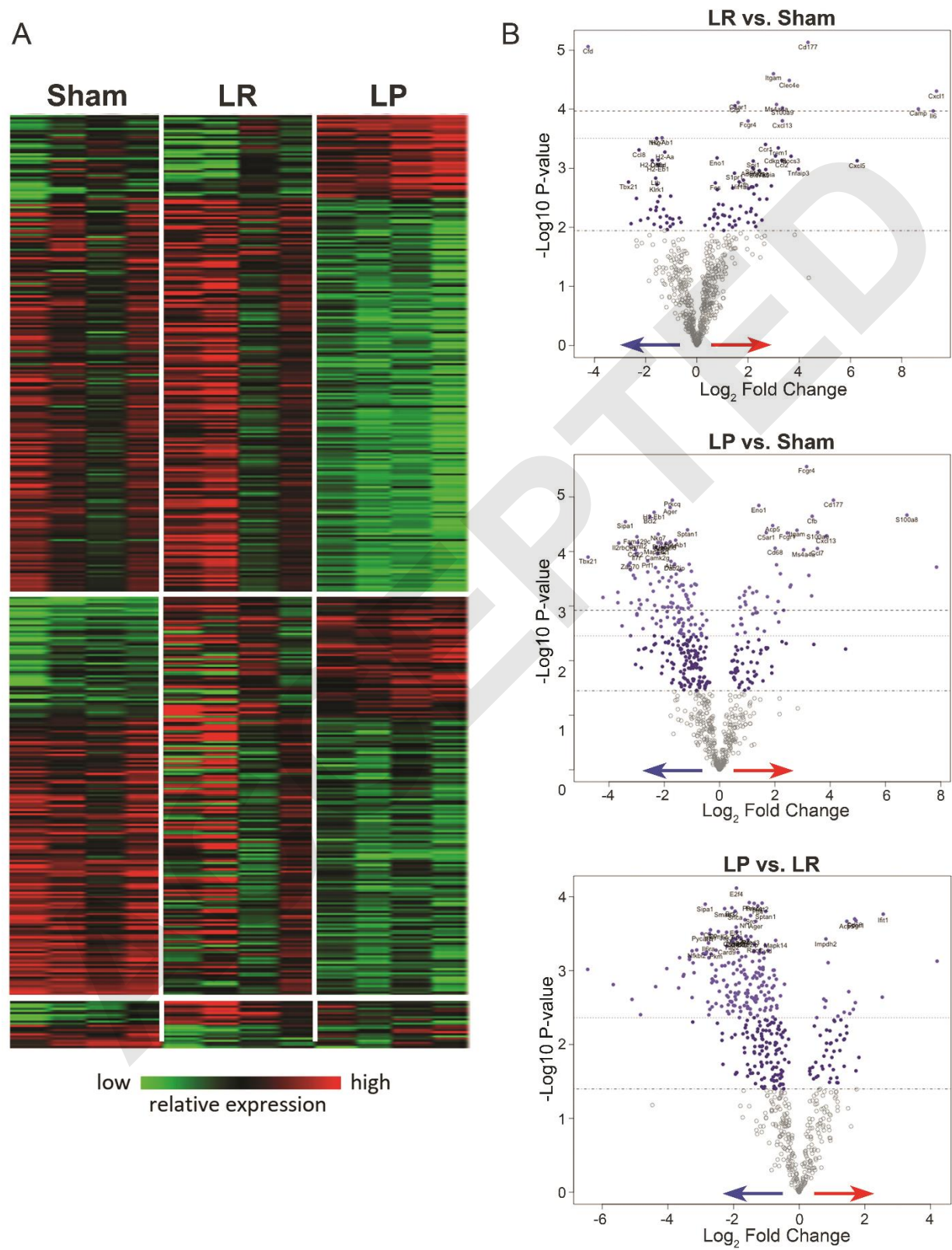


Figure 4

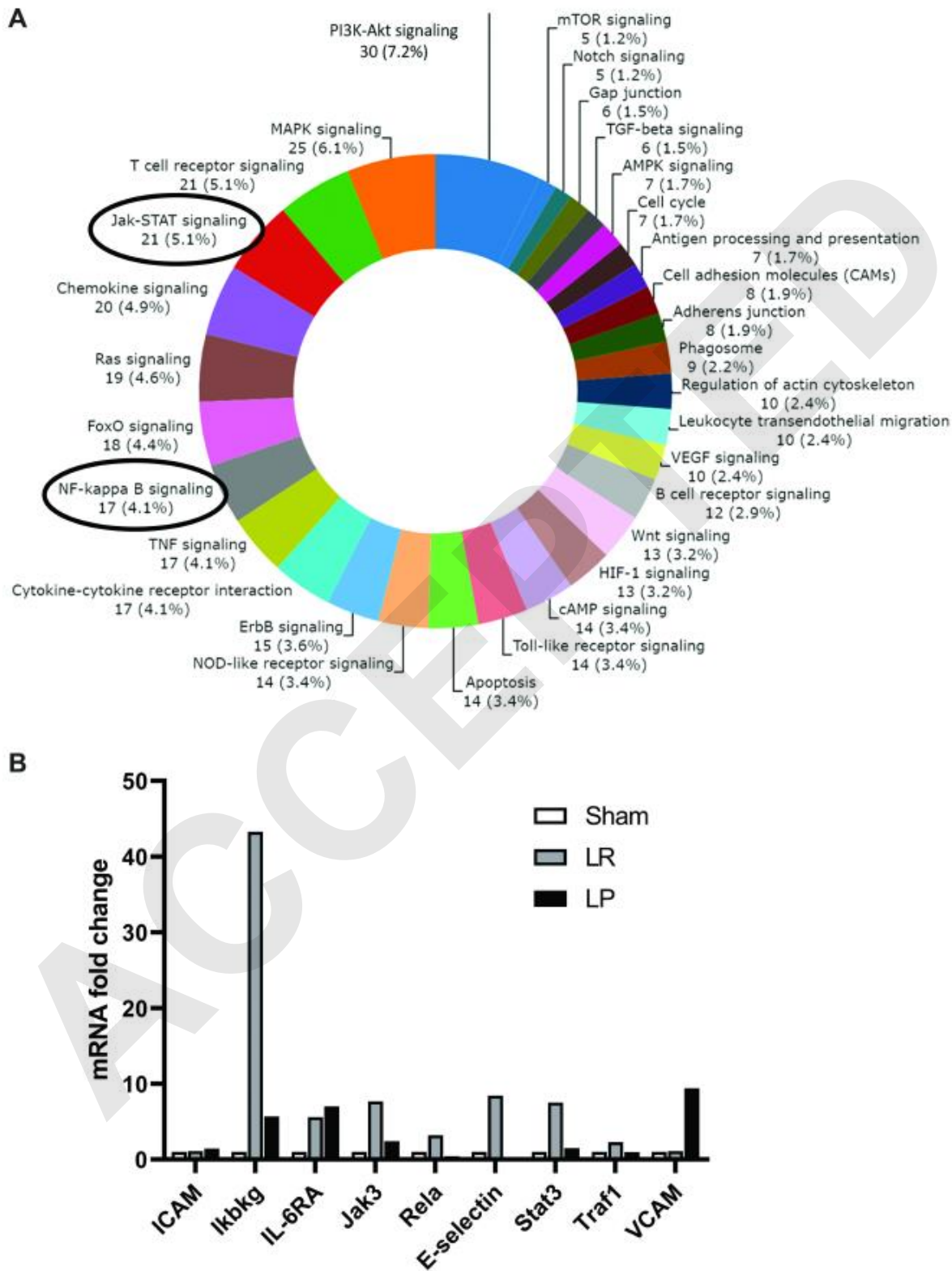


Figure 5

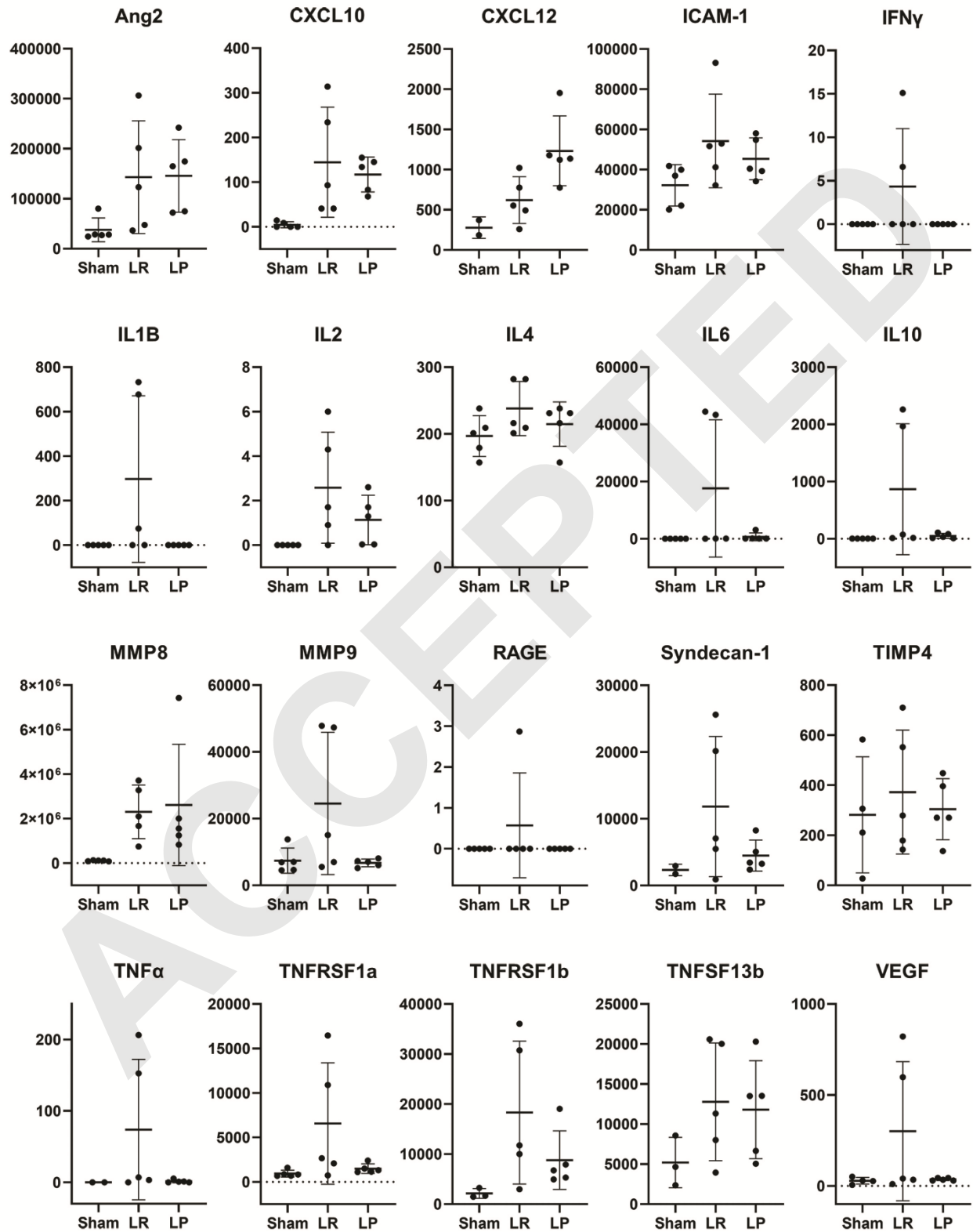
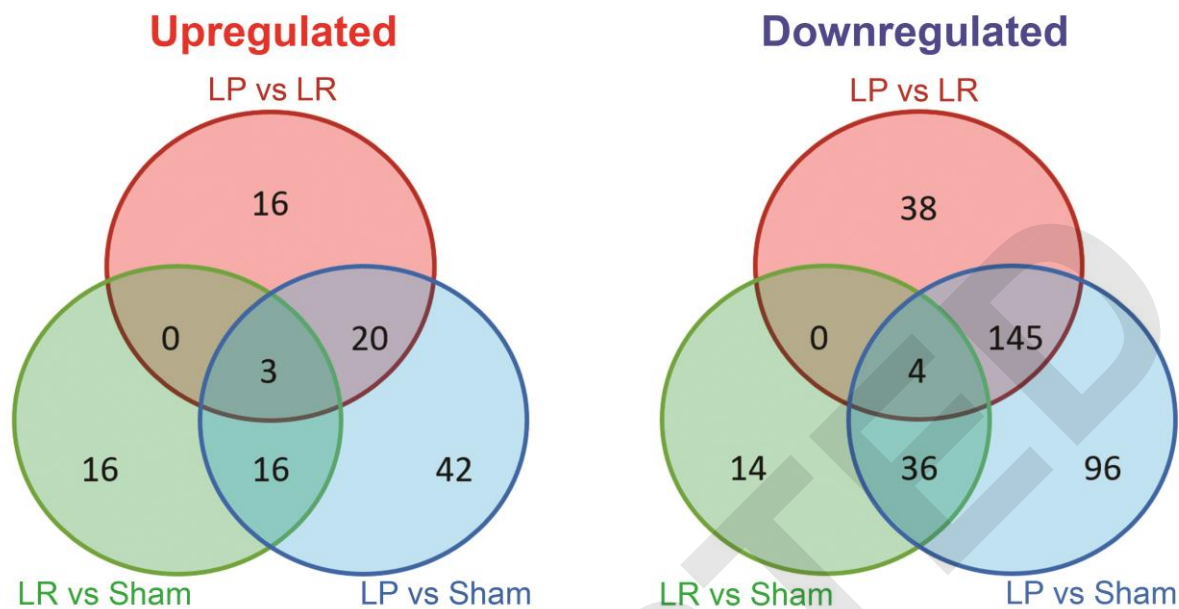


Table 1. Sepsis Physiologic Outcomes and Mortality

	Sham (N=7)	LR (N=13)	LP (N=17)
Sepsis Score Pre-Resuscitation	0±0	0.99±0.27	0.94±0.20
MAP Pre-Resuscitation (mmHg)	104±12	61±8	67±3
MAP Post-Resuscitation (mmHg)	N/A	54±12	57±14
Mortality	0% (0/7)	38% (5/13)	47% (8/17)
BAL Protein (mg/mL)	80.3±17.2	129.7±51.8	123.4±56.4

Table 1. Sepsis Physiologic Outcomes and Mortality. Mice injected with cecal slurry were hypotensive compared to sham animals 22-hours post injection. MAPs, murine sepsis scores, mortality, and BAL total protein levels were similar between mice resuscitated with LR or LP. Values are displayed as mean±SD. MAP = mean arterial pressure; BAL = bronchoalveolar lavage; LR = lactated Ringer's; LP = lyophilized plasma.

Supplemental Figure 1



Supplemental Table 1. List of genes upregulated by LP versus LR

Gene	Log₂ Fold Change LP vs. LR	P-value	Log₂ Fold Change LP vs. Sham	P-value	Log₂ Fold Change LR vs. Sham	P-value
Cfb	1.57	0.032	3.10	<0.001	1.53	0.037
Fcgr4	1.03	0.030	2.70	<0.001	1.67	0.004
Eno1	0.53	0.047	1.23	<0.001	0.70	0.020
S100a8	4.26	0.003	6.98	0.001		
Ifit1	2.02	0.020	0.83	0.023		
Cd68	1.67	0.008	1.86	0.005		
Lilra5	1.64	0.028	1.59	0.009		
Fcgr1	1.62	0.003	2.14	0.002		
Cd34	1.57	0.036	2.04	0.002		
Cd52	1.53	0.031	1.33	0.001		
Ifitm1	1.41	0.021	1.59	0.015		
Acp5	1.33	0.002	1.67	0.001		
Jun	1.20	0.021	1.56	0.019		
Fpr1	1.17	0.027	1.79	<0.001		
Herc6	1.07	0.030	0.82	0.003		
Il18	0.99	0.026	0.85	0.040		
Prdx2	0.92	0.018	0.47	0.008		
Tlr4	0.91	0.032	1.19	0.007		
Impdh2	0.75	0.005	0.72	0.005		
Rhoa	0.74	0.016	0.67	0.022		
Cybb	0.73	0.040	1.56	0.048		
Ly96	0.68	0.007	0.88	0.013		
Ifi30	0.62	0.007	0.85	0.020		
Bst2	1.54	0.040				
Ifi44	1.38	0.020				
Il33	1.31	0.043				
Siglecf	1.23	0.028				
Slamf6	1.15	0.036				
Nudt1	1.13	0.015				
Hist1h2be	1.08	0.050				
Irf7	1.00	0.008				
Hmgb1	0.95	0.034				
C2	0.92	0.030				
Sod1	0.85	0.038				
Oas1a	0.81	0.029				

Maged1	0.78	0.009
Mtr	0.52	0.044
Dtx3l	0.45	0.029
Casp1	0.29	0.019

ACCEPTED

Supplemental Table 2. List of genes downregulated by LP versus LR

Gene	Log₂ Fold Change LP vs. LR	P-value	Log₂ Fold Change LP vs. Sham	P-value	Log₂ Fold Change LR vs. Sham	P-value
Tbx21	-1.45	0.018	-3.32	0.002	-1.87	0.021
Padi2	-1.15	0.026	-2.57	0.002	-1.42	0.023
Btla	-1.12	0.033	-2.09	0.005	-0.97	0.024
Eomes	-1.02	0.013	-1.69	0.002	-0.67	0.024
Nlrp3	-2.74	0.040	-1.38	0.030		
Arhgef1	-2.60	0.014	-3.08	0.003		
Sipa1	-2.58	0.003	-3.23	0.001		
Il2rb	-2.50	0.009	-3.42	0.004		
Csf2ra	-2.42	0.025	-2.00	0.013		
Dynll2	-2.40	0.003	-2.95	0.002		
Il6ra	-2.37	0.031	-2.08	0.007		
Tyk2	-2.37	0.005	-3.10	0.005		
Pkm	-2.32	0.014	-1.42	0.029		
Il7r	-2.31	0.018	-2.81	0.002		
Pycard	-2.26	0.006	-1.51	0.030		
Crcp	-2.24	0.022	-2.35	0.002		
Zap70	-2.17	0.006	-2.69	0.001		
Mbp	-2.05	0.011	-1.60	0.018		
Cd28	-2.05	0.041	-2.23	0.004		
Smad3	-2.02	0.022	-1.53	<0.001		
Jak3	-1.97	0.022	-1.65	0.007		
Bcl2	-1.96	0.001	-2.47	0.001		
Creb3	-1.94	0.034	-1.83	0.013		
Stat3	-1.93	0.049	-0.90	0.021		
Camk2g	-1.87	0.005	-2.28	0.002		
Tab2	-1.86	0.023	-1.52	0.003		
Rela	-1.84	0.021	-1.46	0.009		
Tnfsf13b	-1.83	0.023	-1.92	0.004		
Tmem176a	-1.80	0.004	-1.65	0.024		
Cdk8	-1.77	0.032	-1.45	0.002		
E2f4	-1.76	0.003	-1.78	0.001		
Mapk7	-1.76	0.014	-1.83	0.008		
Apc	-1.75	0.020	-1.64	0.001		
Atp6v0a1	-1.75	0.016	-1.54	0.012		
Rnaseh2a	-1.72	0.018	-1.91	0.011		

Nod1	-1.71	0.002	-1.75	0.007
Traf1	-1.71	0.027	-2.25	0.001
Nprl3	-1.71	0.008	-1.92	0.015
Cd86	-1.69	0.011	-1.40	0.010
Snca	-1.68	0.001	-1.90	0.003
Prf1	-1.68	0.001	-2.30	0.001
Dnmt3a	-1.68	0.017	-1.92	0.014
Grk6	-1.68	0.017	-1.29	0.013
Map3k7	-1.67	0.029	-1.25	0.003
Cd96	-1.64	0.005	-2.12	0.001
Ltbp1	-1.64	0.025	-1.78	0.004
Atp6v1c2	-1.63	0.039	-2.53	0.006
Il17rc	-1.61	0.007	-1.95	0.009
Grb2	-1.61	0.021	-1.04	0.010
Cd6	-1.56	0.050	-2.17	0.019
Tnfsf13	-1.56	0.032	-2.14	0.018
Atm	-1.56	0.003	-1.81	0.001
Ptger4	-1.55	0.026	-1.20	0.015
Nf1	-1.54	0.006	-1.09	0.006
Mtor	-1.52	0.016	-1.53	0.005
Dab2ip	-1.51	0.003	-1.68	0.003
Rassf5	-1.48	0.025	-0.86	0.031
Pten	-1.48	0.004	-1.21	0.002
Elk1	-1.46	0.007	-1.68	0.001
H2-T23	-1.46	0.021	-2.26	0.008
Ikbkg	-1.45	0.038	-1.67	0.008
Src	-1.44	0.004	-1.27	0.005
Atg4b	-1.43	0.033	-1.30	0.007
Lat	-1.41	0.038	-2.05	0.015
Sppl2b	-1.39	0.021	-1.35	0.016
Plekhhb2	-1.38	0.025	-1.52	0.006
Ube2l6	-1.37	0.003	-1.88	0.015
Rac1	-1.37	0.005	-0.96	0.020
Ap2a1	-1.36	0.019	-1.16	0.027
Map4k2	-1.35	0.044	-2.30	0.001
Trp53	-1.35	0.005	-1.47	0.017
Cd74	-1.35	0.036	-2.25	0.002
Gata3	-1.34	0.048	-2.37	0.001
Tnfrsf14	-1.34	0.043	-1.40	0.006
Ager	-1.33	0.003	-1.91	<0.001

Pard3	-1.33	0.010	-1.19	0.006
Fam129c	-1.32	0.005	-1.66	0.002
Ddx24	-1.31	0.014	-1.06	0.005
Kmt2a	-1.27	0.010	-1.66	0.002
Vamp2	-1.27	0.024	-1.37	0.002
Rnf126	-1.25	0.032	-1.06	0.006
Prkcq	-1.24	0.001	-1.65	<0.001
Jag1	-1.23	0.031	-1.18	0.021
Creb1	-1.21	0.006	-1.47	0.003
Gsdmd	-1.21	0.037	-1.39	0.026
Sirt1	-1.21	0.024	-1.44	0.006
Neo1	-1.21	0.013	-1.65	0.001
Flt4	-1.20	0.006	-0.57	0.019
Stat5a	-1.19	0.020	-1.18	0.009
Hsp90b1	-1.17	0.016	-1.32	0.024
Tapbp	-1.16	0.011	-0.77	0.021
Hras	-1.13	0.017	-1.20	0.004
Ptk2	-1.13	0.008	-1.06	0.012
Nptn	-1.12	0.015	-1.26	0.005
Crebbp	-1.12	0.023	-0.59	0.010
Casp8	-1.11	0.005	-0.88	0.023
Akt2	-1.10	0.003	-0.72	0.013
Ptpn11	-1.09	0.038	-0.74	0.002
Itpr3	-1.07	0.044	-0.79	0.007
Ptpn18	-1.06	0.046	-1.21	0.034
Trim26	-1.04	0.036	-1.26	0.004
Nfatc1	-1.04	0.037	-1.20	0.014
Gsk3b	-1.03	0.015	-0.76	0.009
Mcl1	-1.02	0.044	-0.70	0.021
Tatdn1	-1.02	0.009	-1.02	0.021
Sptan1	-1.01	0.002	-1.25	0.001
Camk2d	-1.01	0.038	-0.62	0.015
Plcb2	-1.00	0.043	-1.80	0.001
Otud7b	-1.00	0.037	-0.63	0.001
Psmb10	-1.00	0.004	-1.09	0.014
Gmpr	-1.00	0.046	-1.08	0.007
Eif4a2	-1.00	0.027	-1.47	0.004
Ptk2b	-0.99	0.025	-0.85	0.026
Capn1	-0.97	0.038	-1.02	0.009
Otulin	-0.97	0.024	-0.92	0.005

Hist2h3c2	-0.96	0.028	-1.58	0.032
Ap1b1	-0.94	0.044	-0.81	0.026
H2-M3	-0.94	0.038	-0.79	0.021
Sigirr	-0.91	0.010	-1.33	0.005
Tgfbr1	-0.90	0.017	-0.59	0.038
Acin1	-0.90	0.009	-0.92	0.005
S1pr5	-0.89	0.004	-1.33	0.010
Fasl	-0.89	0.023	-0.53	0.008
Irf3	-0.86	0.024	-1.13	0.001
Ets1	-0.84	0.043	-0.63	0.006
Plcg1	-0.79	0.007	-0.96	0.005
Il10rb	-0.79	0.043	-0.53	0.021
Trim8	-0.78	0.015	-0.56	0.028
App	-0.76	0.024	-0.87	0.012
Serping1	-0.76	0.002	-0.62	0.028
Tns1	-0.75	0.016	-1.02	0.007
Ep300	-0.75	0.011	-0.46	0.040
Irak1	-0.74	0.039	-0.38	0.030
Chuk	-0.74	0.036	-0.51	0.024
Dvl2	-0.73	0.019	-1.34	0.004
Adam17	-0.73	0.031	-0.37	0.028
Mapk14	-0.72	0.005	-0.46	0.031
Pdpn	-0.70	0.015	-0.52	0.033
Bdnf	-0.68	0.035	-1.08	0.003
Nfe2l1	-0.67	0.012	-0.66	0.016
Ccr7	-0.63	0.049	-0.81	0.030
Stat5b	-0.60	0.032	-0.80	0.003
Xrcc6	-0.57	0.037	-0.73	0.005
Xiap	-0.55	0.031	-0.34	0.022
Ube2l3	-0.54	0.048	-0.95	0.008
Adar	-0.53	0.040	-0.75	0.004
Nfatc2	-0.47	0.023	-1.17	0.011
Akt3	-0.44	0.005	-0.56	0.001
Hsp90ab1	-0.42	0.015	-0.56	0.023
Tnfrsf9	-1.77	0.050		
Pstpip1	-1.76	0.040		
Ripk3	-1.69	0.046		
Bmper	-1.66	0.032		
Card9	-1.65	0.007		
Gzmm	-1.51	0.001		

Itga5	-1.40	0.041
Tnfrsf1a	-1.38	0.030
Hdc	-1.24	0.012
Il4	-1.23	0.003
Itgb2	-1.20	0.040
Kif22	-1.16	0.050
Hist1h3b	-1.14	0.012
Kir3dl1	-1.13	0.010
Alas2	-1.11	0.008
Il17f	-1.05	0.041
Grn	-1.02	0.012
Ceacam3	-1.01	0.034
Cyld	-1.00	0.008
Ifngr1	-0.88	0.034
Atg101	-0.82	0.043
Tcirg1	-0.80	0.044
Ctla4	-0.76	0.033
Il2	-0.74	0.020
Cd79a	-0.69	0.018
Ap2a2	-0.69	0.037
C1ra	-0.68	0.008
Il2rg	-0.62	0.040
Braf	-0.62	0.029
Yy1	-0.56	0.013
Ras2	-0.50	0.017
Alox5	-0.47	0.042
Mapk11	-0.47	0.008
Dnmt1	-0.45	0.037
Cflar	-0.44	0.018
Cd164	-0.39	0.045
Stat6	-0.32	0.029
Atf2	-0.30	0.045

The ARRIVE Essential 10: Compliance Questionnaire

Use this questionnaire to evaluate how well a manuscript complies with the ARRIVE Essential 10. It can be applied to any manuscript describing comparative experiments in living animals, by assessors such as journal staff, editors, or peer reviewers.

Item	Question(s)	Answers
1 Study Design	Are all experimental and control groups clearly identified?	☒ Yes, for at least one experiment ☐ No
	Is the experimental unit (e.g. an animal, litter or cage of animals) clearly identified?	☒ Yes, for at least one experiment ☐ No
2 Sample Size	Is the exact number of experimental units in each group at the start of the study provided (e.g. in the format 'n=')?	☒ Yes, for at least one experiment ☐ No
	Is the method by which the sample size was chosen explained?	☒ Yes, for at least one experiment ☐ No
3 Inclusion & Exclusion Criteria	Are the criteria used for including and excluding animals, experimental units, or data points provided?	☒ Yes, for at least one experiment ☐ No
	Are any exclusions of animals, experimental units, or data points reported, or is there a statement indicating that there were no exclusions?	☒ Yes, for at least one analysis ☐ No
4 Randomisation	Is the method by which experimental units were allocated to control and treatment groups described?	☒ Yes, for at least one experiment ☐ No
5 Blinding	Is it clear whether researchers were aware of, or blinded to, the group allocation at any stage of the experiment or data analysis?	☒ Yes, for at least one experiment ☐ No
6 Outcome Measures	For all experimental outcomes presented, are details provided of exactly what parameter was measured?	☒ Yes, for at least one experiment ☐ No
7 Statistical Methods	Is the statistical approach used to analyse each outcome detailed?	☒ Yes, for at least one analysis ☐ No
	Is there a description of any methods used to assess whether data met statistical assumptions?	☐ Yes, for at least one analysis ☐ No ☒ Not applicable
8 Experimental Animals	Are all species of animal used specified?	☒ Yes, for at least one experiment ☐ No
	Is the sex of the animals specified?	☒ Yes, for at least one experiment ☐ No ☐ Not applicable to species
	Is at least one of age, weight or developmental stage of the animals specified?	☒ Yes, for at least one experiment ☐ No
9 Experimental Procedures	Are both the timing and frequency with which procedures took place specified?	☒ Yes, for at least one experiment ☐ No
	Are details of acclimatisation periods to experimental locations provided?	☒ Yes, for at least one experiment ☐ No
10 Results	Are descriptive statistics for each experimental group provided, with a measure of variability (e.g. mean and SD, or median and range)?	☒ Yes, for at least one experiment ☐ No ☐ Not applicable to the type of data collected
	Is the effect size and confidence interval provided?	☐ Yes, for at least one experiment ☐ No ☒ Not applicable to the type of analysis used

Notes on questionnaire design

The ARRIVE guidelines are a useful resource for authors preparing manuscripts describing animal research, and also provide a framework to evaluate the transparency of those manuscripts. To assess reporting quality, numerous studies have in the past sought to operationalise reporting guidelines (including ARRIVE). Typically, this involves scoring a manuscript's degree of compliance with guideline items in a binary fashion (e.g. an item is either not reported or reported) [1-3], a graded fashion (e.g. not, partially, or completely reported) [4,5], or a combination of the two [6].

This questionnaire has been designed to be as concise and user-friendly as possible. The number of questions used to assess a manuscript's compliance has been kept to a minimum, and in most cases each question is designed to be answered in a binary fashion. Compliance with some Essential 10 sub-items is inherently impossible to judge in this way, instead requiring a subjective judgement on the level of detail provided. For this reason, not all sub-items are represented by a question in this questionnaire.

To facilitate binary answers, it has been necessary to identify the minimum information in a manuscript sufficient to comply with each question. The strengths of this approach include the relatively short length of the questionnaire (and the correspondingly low time burden of using it), and the avoidance of ambiguity that would arise from a graded answering system, in which an intermediate score (e.g. 'partially/insufficiently reported') could denote a number of distinct deficiencies in compliance with an item (e.g. either only part of the item was complied with, or only the reporting of some experiments in the manuscript complied with the item.)

Limitations of this approach centre on the necessity to identify the minimum information sufficient to comply with each question. In some cases, this has resulted in questions that require a guideline sub-item's criteria to have been fulfilled in the reporting of only one experiment in a manuscript. As a result, not all experiments in a manuscript may be described in a way that fulfils that criterion, despite the manuscript being considered to comply with the guidelines overall.

References

1. Hair *et al* (2020). *Res Integ Peer Rev*. doi: [10.1186/s41073-019-0069-3](https://doi.org/10.1186/s41073-019-0069-3)
2. Tihanyi *et al* (2019). *J Surg Res*. doi: [10.1016/j.jss.2018.10.038](https://doi.org/10.1016/j.jss.2018.10.038)
3. Zhao *et al* (2020). *BMC Vet Res*. doi: [10.1186/s12917-020-02664-1](https://doi.org/10.1186/s12917-020-02664-1)
4. Han *et al* (2017). *Plos One*. doi: [10.1371/journal.pone.0183591](https://doi.org/10.1371/journal.pone.0183591)
5. Chatzimanouil *et al* (2019). *J Am Soc Nephrol*. doi: [10.1681/ASN.2018050515](https://doi.org/10.1681/ASN.2018050515)
6. Leung *et al* (2018). *Plos One*. doi: [10.1371/journal.pone.0197882](https://doi.org/10.1371/journal.pone.0197882)

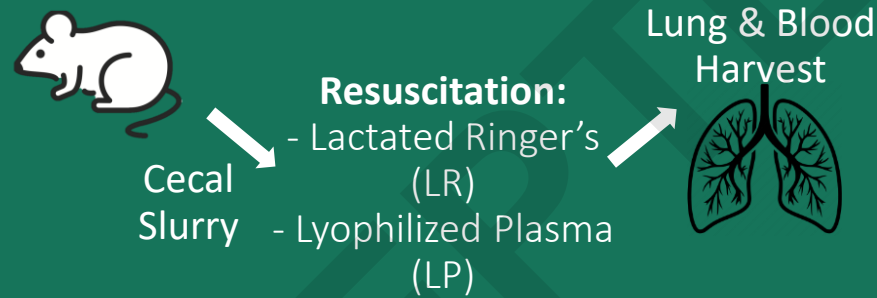
Lyophilized Plasma Resuscitation Downregulates Inflammatory Gene Expression in a Mouse Model of Sepsis

Lyophilized Plasma (LP) overcomes the logistical burdens of Fresh Frozen Plasma in military and remote environments.

LP is safe and effective in trauma patients with hemorrhage and may mitigate endotheliopathy of trauma.

The therapeutic potential of LP as an initial resuscitation fluid in sepsis is unknown.

Mouse Sepsis Model Intraperitoneal Cecal Slurry Injection



Endpoints:

- Mean Arterial Pressure
- Bronchoalveolar Lavage
- Lung Histology
- Lung Inflammatory Gene Expression (NanoString & RT-qPCR) and Signaling Pathways
- Plasma Inflammatory Biomarkers
- Mortality

LP resuscitation downregulated numerous inflammatory genes and pro-inflammatory signaling pathways in the lung: NF- κ B, JAK-STAT, chemokine/cytokine receptor interactions, TNF, and Toll-like receptor signaling.

However, LP resuscitation did *not* improve physiologic endpoints or mortality compared to LR.

Barry et al. *Journal of Trauma and Acute Care Surgery*. Month
Year [doi]

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