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1. INTRODUCTION

Lung infection is extremely common after injury. Injuries like combat wounds are linked to infections distal from the injury site due in part to release of specific molecules called "Damage Associated Molecular Patterns" (DAMPs). Cellular injury renders the host immunologically susceptible to infection. After major injury, DAMP release alters immune responses creating a systemic environment that is permissive of infection. This program collectively studies the production of multiple DAMPs in clinical trauma patient populations that have been aligned with highly specific laboratory models. This approach allows mechanistic studies by which production of DAMPs, released as a result of tissue injury, predisposes the host to pneumonia. Importantly, we are developing novel therapeutic treatment strategies and deliverables that will reduce the incidence of acute lung injury and promote recovery from trauma.

2. KEYWORDS

DAMP – Danger Associated Molecular Pattern (DAMP), Carbon Monoxide (CO), Heme, Gasotransmitter, Neutrophils, Formyl peptides, Mitochondria, Innate Immunity, ATP/ADP/AMP, CD39, Reactive Oxygen Species (ROS), Trauma Patients, Acute Lung Injury, Pneumonia, Oligonucleotides, CyTOF, Computational Modeling, Signal Transduction, Therapeutics.

3. ACCOMPLISHMENTS

What were the major goals of the project?

Major goals: clarify how injury, by releasing "danger-associated molecular patterns" (DAMPs), modulates innate immunity, diminishing host response to infection. We are studying the effects of DAMPs released by injury, translating basic discoveries so as to improve the outcomes of injured warfighters. Mitochondria (MT) are immunologically related to bacteria. Thus, MT harbor powerful DAMPs including heme, formyl peptides, DNA, nucleotides and reactive oxygen species. When these are released by tissue trauma they hamper innate immune surveillance of pathogens at barrier sites like the lung. Our goal is to identify means to restore normal host responses to bacterial challenge after trauma.

Minor goals: We have developed animal models (trauma and burn) ± infection that we are working toward mimicking what is observed in the patients that have been recruited. In addition to the functional readouts in vivo in mice, we are now able to compare murine leukocytes (neutrophils, macrophages, T-cells) in blood and bronchoalveolar lavage with similar cells collected from injured patients. These methodologies are a large focus for this year, enabling us to ensure that all readouts are validated and reproducible using ever larger and better-developed clinical databases.

PROJECT 1 (HAUSER): Mitochondrial f-peptides and DNA released by injury suppress neutrophil function

Major activities, objectives, and results.

- As part of our studies of how mitochondrial DAMPs evoke mononuclear cell chemotactic responses during sepsis we inhibited cytoskeletal re-arrangement in PBMC treated with LPS using Latrunculin A. This was it critically necessary to demonstrate that release of biologically active agonists is an active response to danger signals rather than due to cell death. The elaboration of chemokines and cytokines into conditioned medium was evaluated using a Luminex bead panel. Three conditions were explored: 1. Human PBMC treated with 0.1% DMSO (vehicle) for 2 h followed by 4 h incubation with 1 μg/mL LPS, 2. Human PBMC treated with Lantrunculin A (vehicle) for 2 h followed by 4 h incubation with 1 μM LPS, 3. PBMC treated with 100 ng/mL LPS for 24 h. Disruption of actin filaments could reduce mitochondrial release from PBMC and decrease chemokine or cytokine release by PBMC (05/07/2019). The results demonstrate that release of biologically active materials in response to DAMPs is indeed active. **(Fig 1)**
- One of the major aims at this point in the program is to generate a colony of FPR-1 knockout mice for preclinical studies of the role of the receptor in infections. We have begun to do that although the process has been slow. To evaluate the genotyping of FPR1-KO animals we extract DNA from mouse-tail samples. This is followed by qPCR with specific primers that detect wild type and FPR1-KO genetic material by application of PCR products to DNA gels (10, 11, 12/07/2019, 22/08/2019, 10, 11/09/2019). These show successful breeding of the FPR-1 knockout mice **(Fig 2)**. We are now slowly beginning to enlarge our $FPR1^{-/-}$ colony and hope to be able to begin experiments within a few months.
- In preparing for experiments evaluating the effects of mitochondrial DAMPs on bacterial killing by immune cells we have noted inconsistencies in the growth of different bacterial species. To create more stable data going forward with anti-infective studies we decided that we needed to know the exact growth rates of the different clinically relevant isolates we plan to use **(Fig 3)**. Hourly growth of each bacterial strain was determined and the appropriate OD600 values were determined to obtain consistent number of bacteria for use in our experimental models **(Fig 4)**. *E. coli*, *P. aeruginosa*, and *S. aureus* were used to establish growth rate (16,17, 19, 23, 24, 25, 26, 30/07/2019).
- To apply our studies of the effects of danger signals on innate immune cells we have started working with clinical samples from patients with 1) documented bacteremia, 2) "severe sepsis" (SIRS with a suspected infective plus organ dysfunction), 3) septic shock (infection with SBP<90), and 4) ER patients with no clinically determined pathology. Initial studies are in progress using 12 samples from each group. Plasma samples were spun at 12,000 g for 15 min at 4°C. Cell-free samples were then divided into four groups to be used for 1) qPCR for mitochondrial DNA and bacterial 16s DNA, 2) cytokine and chemokine assay by Luminex platform (in association with Program 5, 3) Plasma will be used for stimulation of normal PMN. 4) Residual pellets will be assayed for exosomes. Similarly, 11 samples from trauma patients known to have high concentrations of ND6 (ELISA) were chosen from the HALO plasma repository and treated similarly for the same purposes (12, 25/09/2019).
  These studies should help us to determine the effects of sterile and infective Danger environments on innate immune and neutrophil function. It should also help us to understand the circulating immune environment in sepsis and sterile SIRS. This will be of particular value in terms of creating diagnostic tests that will justify early antibiotic treatment of sepsis and early discontinuation of antibiotic treatment in patients with SIRS of sterile origin.
- In addition to the studies noted above, during this quarter a large amount of Project 1 effort was devoted to supporting program wide studies. These studies were aimed at defining the phenotypic variations in peripheral mononuclear cells as well as alveolar innate immune cells that occurred in clinical trauma. Considerable effort was invested in assays of mitochondrial DAMPs both peripherally and in the bronchoalveolar lavage fluids of trauma patients and volunteer controls. In addition, technical work was done supporting BAL studies by back-calculating BAL fluid dilution using microtiter assays of urea nitrogen (BUN). All of these efforts will be reflected in data reported by other projects in the program, especially Project 5.

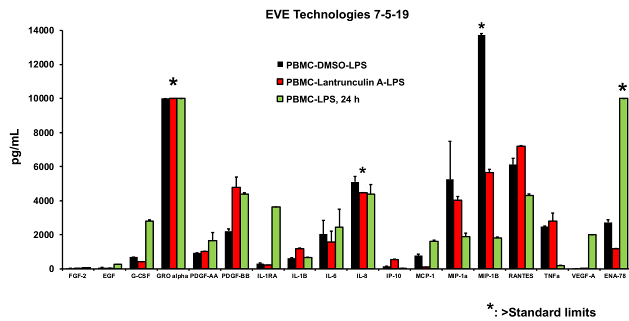
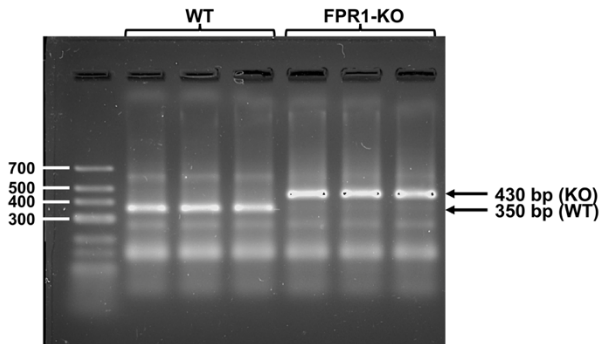


Figure 1: Lantruculin A did not have any effects on the production of chemokine/cytokine except MIP-1B. ENA-78 production was significantly higher when PBMC were stimulated with 100 ng/mL LPS for 24 h. Production of GROα and IL-8 was very high. Our previous results also suggested no change in mtDNA release by treatment with Lantrunculin A. We could not detect any inhibitory effects by Lantrunculin A.



Due to prolonged exposure to SYBR sage DNA gel staining non-specific bands became visible

Figure 2: FPR310 (Forward), AFPR660 (Reverse), and SNeo2 (reverse) primers were used to distinguish FPR1-KO and WT animals. As shown below, we can clearly distinguish between these groups and confirm that our colony is indeed phenotypically FPR1$^{-/-}$.

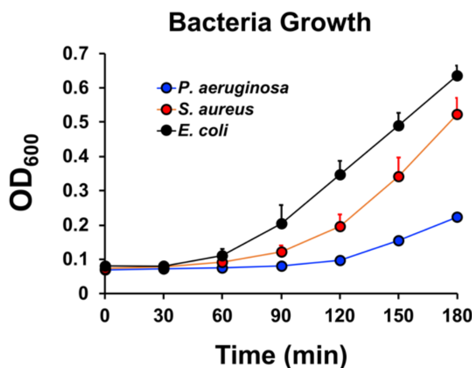


Figure 3: *P. aeruginosa*, *S. aureus*, *E. coli* were three bacteria that we use for both in vitro and in vivo experiments where their number is very crucial for experimental protocols. We only know the real bacterial number next day (overnight growth in agar plates) after we used them for experiments. It is important to know approximate number at the time of experiments. First, as shown below, we examined growth of bacteria after x1/100 dilution of overnight grown bacteria at time "0". Growth curves are different by type of bacteria. *P. aeruginosa* is the slowest growing bacteria whose growth curve becomes an exponential phase after 2h incubation.

OD=0.1…8E+07 CFU/mL

	1.665	NA	1.449	1.613	**2 mL PBS**
E. coli	x1/160	x1/120	x1/100	x1/120	
diluted-OD	0.112	0.112	0.103	0.109	**0.109**
CFU/mL	83000000	72000000	65000000	Not reliable	**73333333.3**
	1.227	1.1	1.138		**2 mL PBS**
P. aeruginosa	x1/40	x1/30	x1/30		
diluted-OD	0.099	0.1	0.102		**0.1**
CFU/mL	73000000	87500000	73000000		**77833333**
	1.453	1.597	1.39		**2 mL PBS**
S. aureus	x1/90	x1/105	x1/65		
diluted-OD	0.097	0.101	0.099		**0.099**
CFU/mL	82166667	95700000	82166667		**86677778**

Figure 4: We spun down bacteria after 3h incubation and then re-suspended them in PBS. Reflecting the growth curves, OD600 values were ***E. coli>S. aureus>P. aeruginosa.*** However, OD600=0.1 gave us $8x10^7$ CFU/mL for all three types of bacteria from three different experiments for each bacterium. We will use this data for the future studies to have consistent bacterial numbers.

Summary: During this period, we described and confirmed the immune signals produced by PBMC after exposure to mitochondrial (mt) formyl peptides (mtFP) and showed they are very different from signals elicited by bacteria. These findings may be used in future tests to discriminate Sepsis from SIRS. We studied but could not confirm a role for active MT release by mononuclear cells in PMN chemotaxis after trauma. We conducted studies of how mtDNA released from injury sites affects neutrophil migration towards inflammatory chemoattractants. Our previous studies suggested mtDNA activate PMN to release neutrophil extracellular traps

(NETs) and kill bacteria. NETs however, can also harm tissue if released when there are no bacteria present. We know mtFPs are released after injury and are strong chemo-attractants. So we sought to assess whether PMN pre-exposure to mtDNA could regulate PMN chemotaxis. We also studied and unified PMN isolation methods across the project as a whole so as to study priming and activation in a uniform and rigorous fashion.
In further studies we investigated the roles of the FP receptors FPR-1 and FPR-2 in nosocomial pneumonia after trauma. We showed FPR-1 was specifically responsible for suppression of chemokine and leukotriene receptors. This decreases PMN responses to bacteria and the defects found parallel findings in human trauma patients. The risk of pneumonia was reduced markedly in mouse models by FPR-1 blockade. The important manuscript produced from these data has been accepted by the high-impact journal "*Critical Care Medicine*". Of great importance, this manuscript demonstrates that FPR blockade increases PMN responses to bacteria rather than suppressing them, as feared. Last, we continue to assay trauma plasmas for mtDNA and mt-formyl peptides. These generally reflect the severity of injury. Ongoing studies will assess their relationship to posttraumatic infection. Also, we have assayed clinical plasmas looking for bacterial 16s-DNA. This will confirm infections where the clinical definitions are not clear.

PROJECT 2 (OTTERBEIN): Heme Metabolism and The Innate Response to Trauma and Infection

Major activities, objectives, and results.

- Much work was performed on the mouse model of trauma ± infection seeking to demonstrate that heme was is a key danger molecule released during trauma that leads to decreased bacterial clearance in the lung. These studies included two separate heme oxygenase null mice and hemopexin null mice.
- We spent a large amount of effort understanding the lung airway milieu; the infiltrating cell phenotypes and lavage fluid analysis and how this might explain, in part, increased susceptibility to infection
- Compared killing capabilities of neutrophils purified from bone marrow and whole blood in terms of their killing capabilities and have characterized new methodologies.
- Began to characterize a novel approache to utilizing Carbon Monoxide (CO) as a therapeutic against inflammation using serum from humans and mice that had been exposed to CO.

What was accomplished under these goals?

- BAL fluid analyses from trauma+infected mice showed increased lung injury as reflected in protein accumulation, which correlated with greater amounts of myeloperoxidase (**Figures 1A-1B**). PMN counts were the same, but TLR2 expression was significantly less. TLR2 is the receptor for *S. aureus* phagocytosis (**Figures 1C-D**). Analysis of blood levels of PMN showed increased numbers of Ly6G+ PMN with corresponding increases in HO-1 expression in this population.

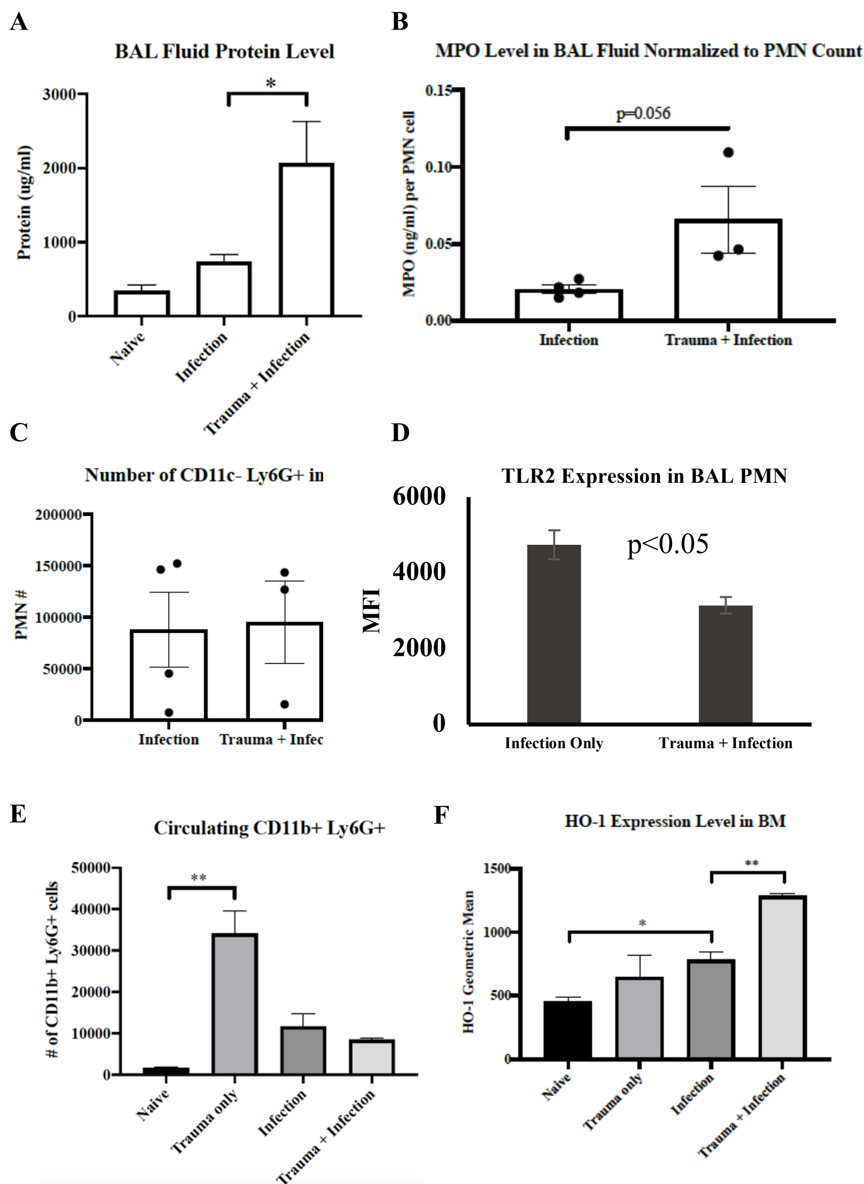
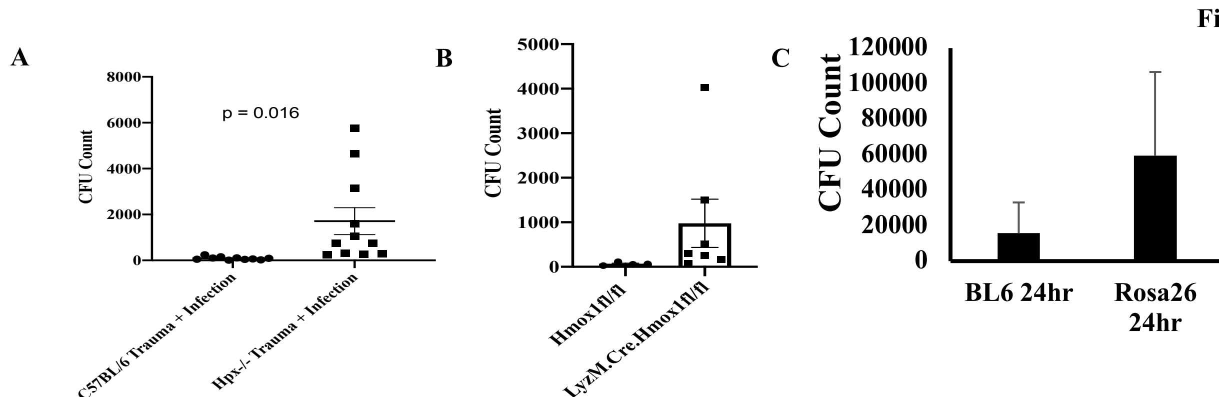


Figure 1. A-B. BAL fluid protein and myeloperoxidase (MPO) from mice subjected to a liver crush injury followed 4h later by an intratracheal administration of *S. aureus*. A lavage was performed 24h later and total protein and MPO was performed by ELISA. **C-D**. Cells from these same mice were profiled for a neutrophil phenotype and TLR2 expression. **E-F.** FACs data showing expression of PMN in the blood and corresponding HO-1 expression. Data represent mean±SD of 3-4 mice/group.

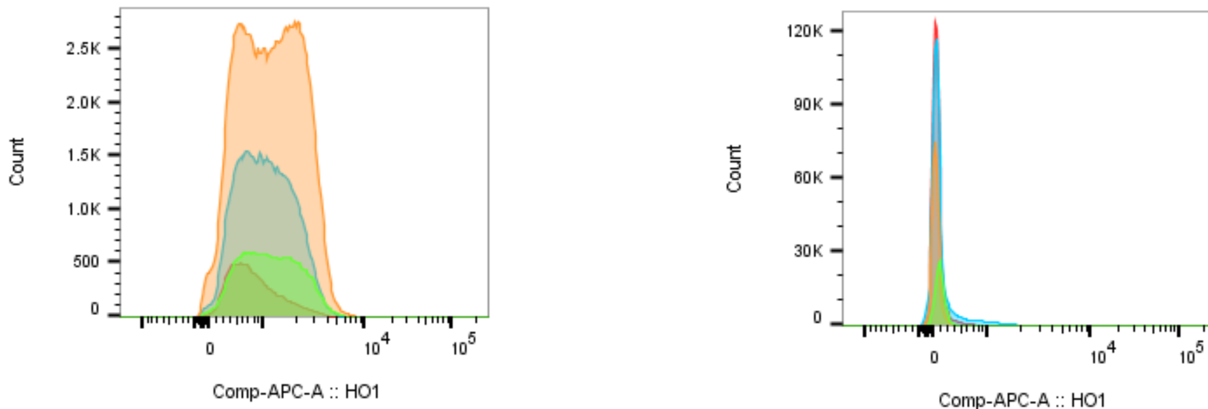
- Knowing that serum heme was elevated after trauma, and that administration of heme in the absence of trauma recapitulated trauma itself, we next tested the crush+infection in hemopexin (Hpx) deficient mice and observed that these animals showed even further sensitivity to infection after trauma due in part to the excess amounts of circulating heme (**Figure 2A**). Similarly, mice lacking HO-1 globally (*Rosa 26*) or specifically in myeloid cells (*LyzM-Cre-Hmox*) were also much more sensitive to bacterial challenge in the absence of trauma (**Figures 2B-C**).

Figure 2



- Given that PMN infiltration into the airways after infection was no different between Rosa26 mice and controls, yet the bacterial counts were much greater, we measured HO-1 expression in the PMN and, as expected see no HO-1 in the infiltrating PMN from the Rosa mice (**Figure 3**).

Figure 3



- As CO is the presumed mechanism by which HO-1 functions to facilitate bacterial clearance, and is also anti-inflammatory, we asked if the serum from mice exposed to CO only would impart anti-inflammatory effects on macrophages and PMN in vitro. Figure 3 shows that macrophages treated with CO-exposed serum had a remarkable ability to block LPS-induced increases in IL-6 expression as measured by ELISA. Similar effects were observed on PMN bacterial killing (data not presented) Ongoing work will begin to study potential mechanisms of action.

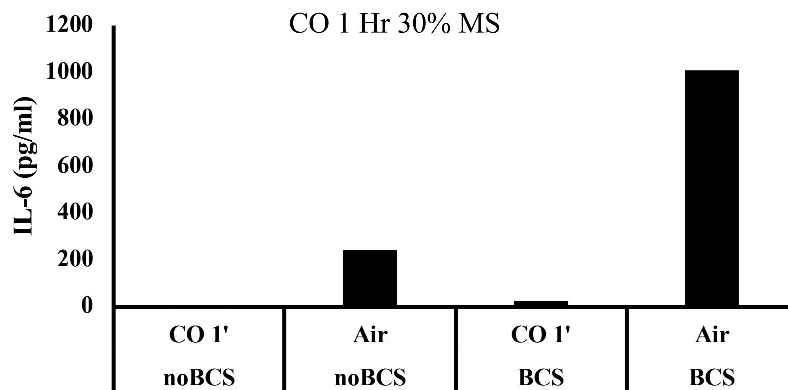


Figure 4. RAW 264 macrophages were treated with LPS (1 ug/ml) for 5h in the presence of 30% mouse serum (MS) from a normal or CO exposed mouse (1h at 250 parts per million). Cells were cultured with or without bovine calf serum (10%) per standard culturing conditions for RAW cells. IL-6 levels were determined by ELISA. Results represent one of 3 independent experiments.

Summary: we continue to characterize the animal model to understand more of the mechanism by which trauma conditions mice to be more susceptible to bacterial infection. We are focused on heme given the large literature base that free heme is pro-oxidant and causes tissue injury. We have brought in genetically modified animals that take time to breed and validate genotype, but having these genetic tools to ask the biological and physiologic question is critical data. We appreciate the need for translation of our work and have begun to test the hypothesis that CO exposure may provide a unique avenue by which to utilize the serum as an anti-inflammatory therapeutic and potentially obviate the need for devices or new drug modalities and may prove useful for the medic in theater.

PROJECT #: 3
TITLE: CD39 and extracellular nucleotide signaling mediate inflammation and immune failure after trauma
PI: Simon C. Robson

Major activities, objectives, and results.

- *To examine how loss of CD39 bioactivity, as a consequence of oxidative stress, triggers excessive type-2 purinergic receptor (P2R) signaling, in experimental model.*
  As part of these studies, to develop and test a model of trauma where liver-derived mitochondria were injected into wild-type and CD39 KO mice and presence of lung and systemic damage assessed before and after bacterial instillation.
- *To study how type-1 purinergic receptor (P1R signaling by adenosine modulates the innate response to infection after trauma, in experimental models.*
  As part of these investigations, to test the effects of CD39 gene deletion on disease outcomes in mice administered exogenous liver-derived mitochondria in the absence or presence of consequent, delayed bacterial instillation.
- *To characterize and correct kinetics of aberrant immune purinergic responses within blood and alveolar micro-environment of trauma patients.*
  To test immunophenotypes and determine ectoenzymatic activities of peripheral blood and BAL-derived mononuclear cells of trauma patients, obtained at defined time points after trauma.

What was accomplished under these goals?

- Completed studies on the protective role of CD39 overexpression in the context of chronic inflammatory models.
- Tested the beneficial effects of APT102, an exogenous ATPase/ADPase on immunoregulation *in vitro* and in chronic inflammatory models.
- Assessed the *in vitro* effects of heme derivatives and hypoxia on T cell metabolism in health and chronic inflammatory disorders (Nanostring and Seahorse).
- Completed flow cytometry characterization of CD8 cell subsets in the peripheral blood of trauma patients at different time points.
- Optimized isolation of mitochondria from mouse liver and performed injections into WT and $CD39^{-/-}$ mice to induce systemic inflammation. As part of these investigations, we assessed peripheral blood mononuclear cell immunophenotype and cytokine production after liver-derived mitochondria injections.
- Demonstration of extracellular (e)ATP generation by injected mitochondria are being tested using Luciferase-transgenic mice. Modulatory impacts of exogenous apyrase supplementation and eATP scavenging are to be determined.
- Tested the effects of liver mitochondria injections and bacteria (*S. Aureus*) instillations in WT recipients; assessed CFU and BAL cell immunophenotype by flow cytometry.
- Tested mitochondria purity and ATP production in vitro using an ATP-specific probe.
- Tested the *in vitro* effects of liver mitochondria on WT peripheral blood mononuclear cell immunophenotype at different time points.
- Started testing possible causes of ectonucleotidase alterations in mononuclear cells from trauma patients.

During this timeframe, we have completed the immunophenotype analysis of $CD39^{+}CD8^{+}$ and $CD39^{-}CD8^{+}$ T cell subsets, obtained from the peripheral blood of trauma patients (n= 4 at day 1 and n=6 at day 2) and controls (n=6). We have noted heightened proportions of cells positive for both PD1 and Tim-3 in $CD39^{+}CD8^{+}$ cells of trauma patients at day 2, when compared to trauma patients at day 1 and controls (**Figure 1A**). When considering single positive PD1 or Tim-3 subsets (**Figure 1, B-C**), we have observed higher frequencies of Tim-3^{+} cells within the $CD39^{+}CD8^{+}$ subpopulation (**Figure 1C**) and lower percentages of $PD1^{+}$ cells within the $CD39^{-}CD8^{+}$ cell compartment of trauma patients at day 2 (**Figure 1E**).

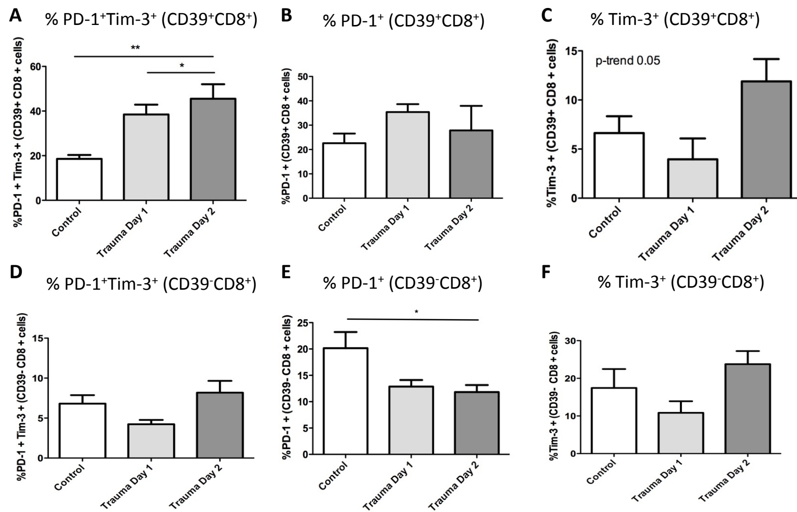


Figure 1: Frequency of cells positive for PD-1 and/or Tim-3 within $CD39^+CD8^+$ and $CD39^-CD8^+$ T cell subsets in trauma patients. Frequency of cells positive for PD1 and/or Tim-3 within the $CD39^+$ and $CD39^-$ CD8 T cell subsets was assessed by flow cytometry in the peripheral blood of trauma patients at day 1 (n=4), day 2 (n=6) and in controls (n=6). *: $P<0.05$; **: $P<0.01$.

Furthermore, in trauma patients at day 2, there were higher frequencies of $CD45RO^+$ lymphocytes within the $CD39^+CD8^+$ cell subset (**Figure 2B**) and lower proportions of CTLA-4^+ lymphocytes within the $CD39^-CD8^+$ T cell compartment (**Figure 2C**). Functional analysis of $CD39^+$ and $CD39^-$ $CD8^+$ T cell subpopulations indicated increased proportions of Granzyme B^+ lymphocytes amongst the $CD39^+CD8^+$ subset in trauma patients at day 2; and lower frequencies of $IFNg^+$ cells within the $CD39^-$ $CD8^+$ subset at the same time point (data not shown).
These experiments were complemented by CyTOF analysis (carried out in collaboration with Dr. Lederer, PI of Project 5).

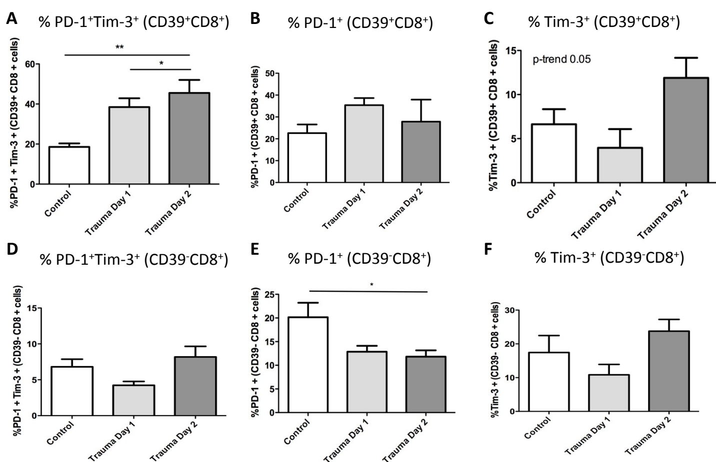


Figure 2: Immunophenotype of $CD39^+CD8^+$ and $CD39^-CD8^+$ T cell subsets in trauma patients. Frequency of cells positive for CTLA-4 or CD45RO within the $CD39^+$ and $CD39^-$ CD8 T cell subsets was assessed by flow cytometry in the peripheral blood of trauma patients at day 1 (n=4), day 2 (n=6) and in controls (n=6). *: $P<0.05$.

During this reporting period, we have also isolated mitochondria from mouse liver (C57BL6 mice) and started injection into C57BL6 and $CD39^{-/-}$ recipients to induce systemic inflammation. In Figure 3, we depict the morphology of the isolated mitochondria (**Figure 3A**). Purity of mitochondria isolate was verified by staining with Mito Tracker (**Figure 3B**); ATP generation was assessed using ATP probe (**Figure 3C**). ATP generating mitochondria are shown in **Figure 3D** as double stained cells.

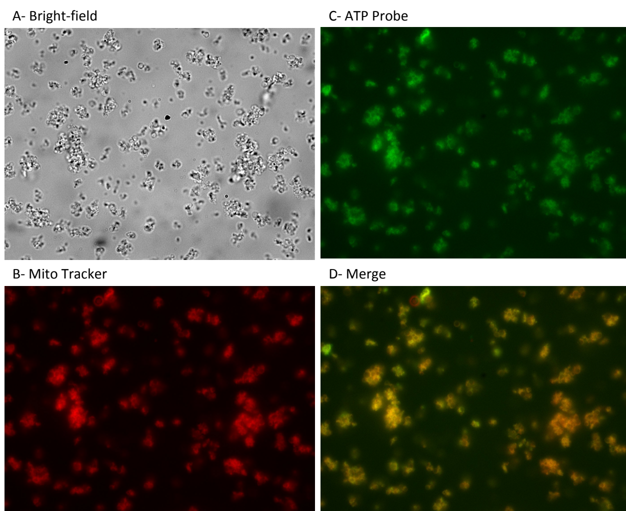


Figure 3. Isolation and staining of liver-derived mitochondria. Mitochondria were isolated from the liver of C57BL6 mice, stained with Mito Tracker Green FM and then visualized under fluorescence microscope. ATP generation was assessed using an ATP probe. **A**: bright field; **B**: Staining with Mito Tracker; **C**: staining with ATP probe; **D**: merge. A representative of 4 independent experiments is shown.

In a subsequent series of experiments, we stained liver-derived mitochondria from WT mice with Mito Tracker Red CMXRos and verified the presence of injected mitochondria in the liver and lung, where mitochondria were respectively taken up by hepatocytes and by alveolar macrophages (**Figure 4A-B**).

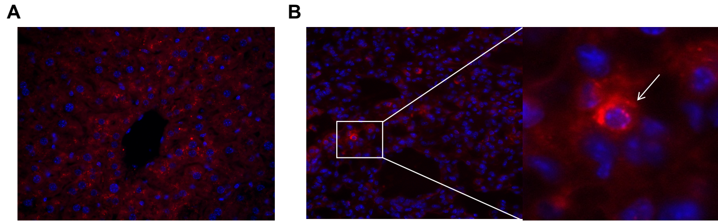


Figure 4. Mitochondria were isolated from the liver of WT mice, stained with Mito Tracker Red CMXRos and injected i.p. at 20% of liver weight into WT recipients. Mitochondria uptake was verified 1 hour after the injection by immunofluorescence on **(A)** hepatic and **(B)** lung frozen tissue sections. A representative of 3 independent experiments is shown.

Four and 24-hour after mitochondria injection into WT mice, we have noted increases in the proportion of CD39$^+$ cells within the CD8 subset (**Figure 5A**) and heightened frequencies of PD1$^+$ Tim-3$^+$ CD8 T lymphocytes over the 24-hour period (**Figure 5B**).

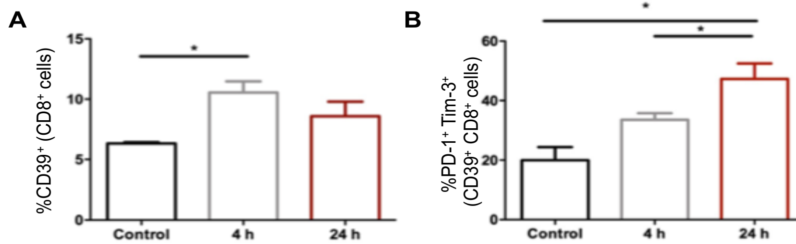


Figure 5. Liver derived mitochondria were injected i.p. into WT recipients at 20% of liver weight. Mice were harvested 4 (n=8) and 24 (n=8) hours later. Immunophenotypes of peripheral blood CD8 T cells was determined by flow cytometry. *P: ≤ 0.05.

Analysis of the CD8 cytokine profile revealed increases in the proportion of IL-10 producing lymphocytes, both when considering the CD39$^+$ and CD39$^-$ CD8 cell fractions (**Figure 6**).

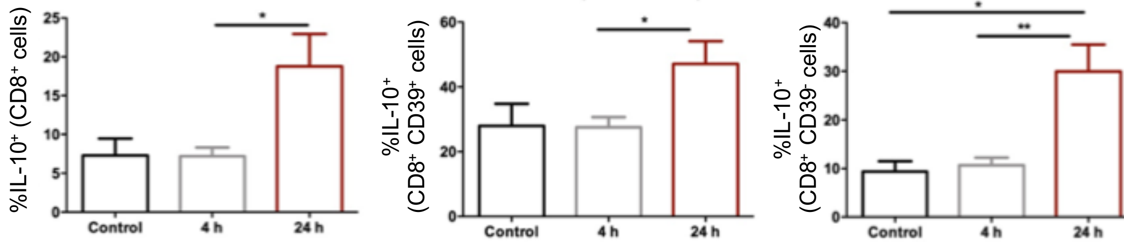


Figure 6. Frequencies of IL-10 producing CD8 T cells in the peripheral blood of WT mice, injected with liver mitochondria and harvested at 4 (n=8) and 24 (n=8) hours. *P: ≤ 0.05.

Overall, these results show that mitochondrial injection into WT mice results in increase in CD8 cells with exhausted phenotype; these findings recapitulate the immunophenotypic pattern observed after trauma injury in patients. Injection of liver mitochondria in CD39 KO recipients resulted in a significant increase in the proportion of CD8 cells expressing the CD73 ectoenzyme, as compared to WT mice; no differences were, however, noted in the frequencies of PD1$^+$ Tim-3$^+$, IFN $^+$ and IL-10$^+$ CD8 T cells 24 hours after mitochondria injection between WT and CD39KO recipients (**Figure 7**).

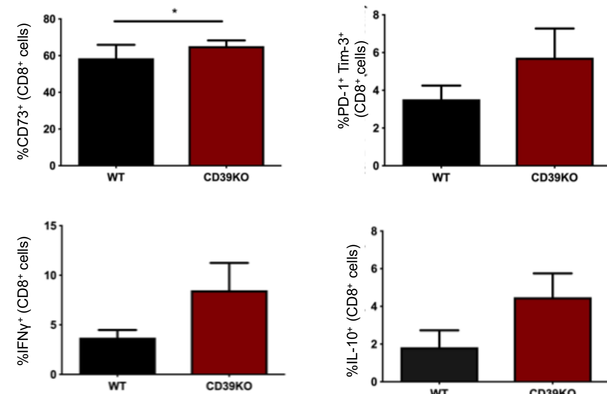


Figure 7. Liver-derived mitochondria were injected i.p. into WT (n=4) and CD39 KO (n=8) recipients at 20% of liver weight. Mice were harvested 24 hours later and immunophenotype of peripheral blood-derived CD8 T lymphocytes assessed by flow cytometry. *P: ≤ 0.05.

We have then tested the effects of liver-derived mitochondria and bacteria (*S. aureus*) instillations in WT recipient mice. In these experiments, mitochondria obtained from the liver of C57BL6 mice (20% of liver weight) were injected i.p. into WT recipients (n=8). Four hours later mice were intra-tracheally instilled with *S.aureus* (7x10^8 CFU/50 ul) and harvested 20 hours later. Prior to harvest, BAL was collected and assessed for CFU as well as mononuclear cell phenotype by flow cytometry. As shown in **Figure 8**, there were higher CFU in mice receiving mitochondria injections and then bacteria instillation.

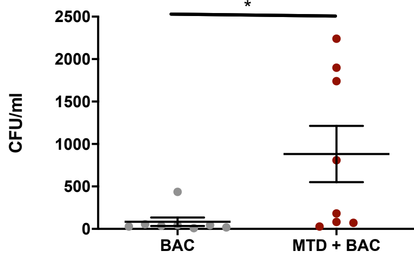


Figure 8. Mitochondria were isolated from the liver of WT mice and injected i.p. into n=8 recipients. Four hours later mice were administered *S. aureus* intra-tracheally and BAL was collected 20 hours later. Mice receiving both mitochondria (MTD) and bacteria (BAC) displayed higher CFU/ml compared to recipients injected with BAC only. *: P≤ 0.05.

Flow cytometry analysis of BALs collected at 24 hours prior to harvesting showed an enrichment in $CD11b^+LY6G^+$ cells in mice receiving both mitochondria and bacteria, when compared to controls receiving bacteria only (**Figure 9A-B**).

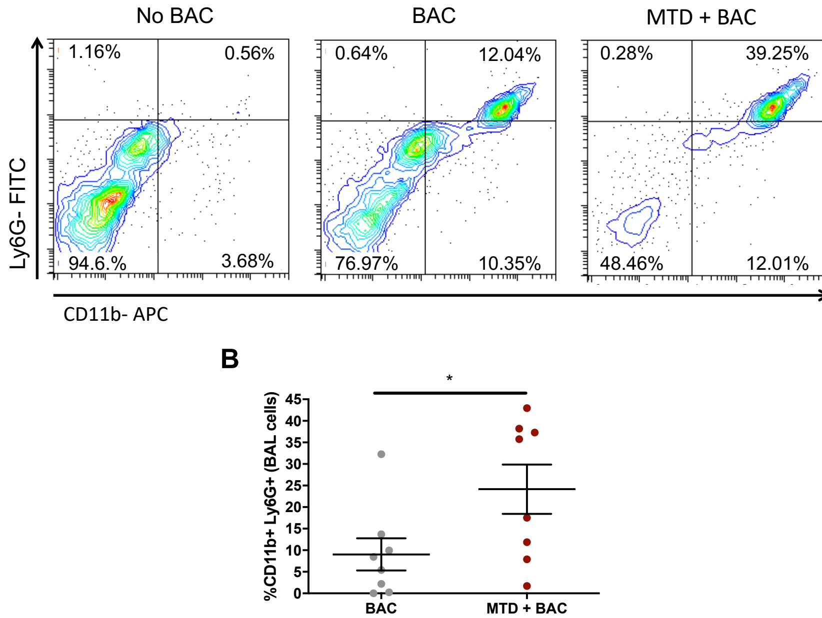


Figure 9. Liver-derived mitochondria were injected i.p. into WT recipients (n=8) at 20% of liver weight. Four hours later mice were intra-tracheally instilled with *S. aureus* at $7x10^8$ CFU/50 l. BALs were collected 20 hours later. **(A)** Dot plots of CD11b (X axis) and LY6G (Y axis) fluorescence in one representative control (No BAC), one BAC instilled and one MTD plus BAC instilled mouse. **(B)** Mean ± SEM frequency of $CD11b^+LY6G^+$ cells in BAL 24 hours after MTD injection (n=8). *P: ≤ 0.05.

Importantly, while no differences were noted in the percentage of BAL $CD11b^+$ cells between mice instilled with bacteria only and mice receiving both mitochondria and bacteria (**Figure 10A**), we did observe increased frequencies of $CD39^+$ cells and CD39 MFI in the $CD11b^+$ subset derived from the BAL of mice receiving both mitochondria and bacteria (**Figure 10B-C**).

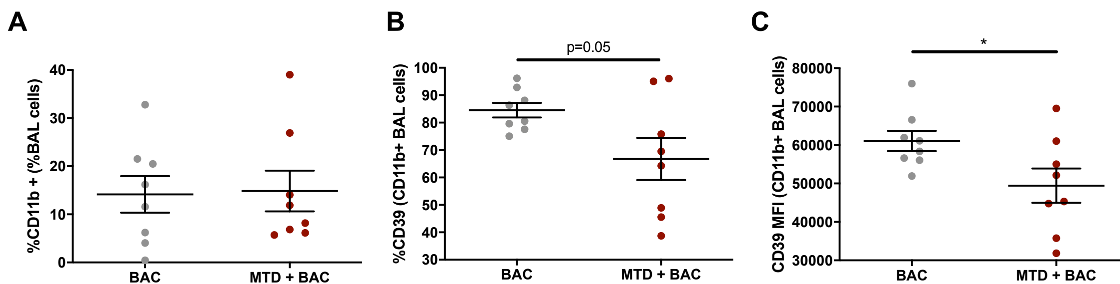


Figure 10. BAL were collected from bacteria instilled (BAC) and mitochondria and bacteria treated (MTD + BAC) mice and analyzed by flow cytometry. Mean ± SEM frequency of **(A)** $CD11b^+$ cells; **(B)** $CD39^+$ lymphocytes within the CD11b subset; and **(C)** CD39 MFI in $CD11b^+$ lymphocytes in BAC and MTD + BAC treated mice. *P: ≤ 0.05

Experiments are currently ongoing to test the effects of mitochondria injections and bacterial instillation in $CD39^{-/-}$ mice; and to determine role of eATP scavenging and/or adenosine supplementation.

The *in vitro* effects of mitochondria on the immunophenotype of circulating CD8 cells obtained from WT mice showed decreased expression of CD39 along with reduced frequencies of PD-1 and Tim-3 two hours after treatment

(Figure 11). This supports our initial hypothesis that the first phase of trauma - here recapitulated *in vitro* by exposure of cells to mitochondria - is characterized by an hyperinflammatory phase.

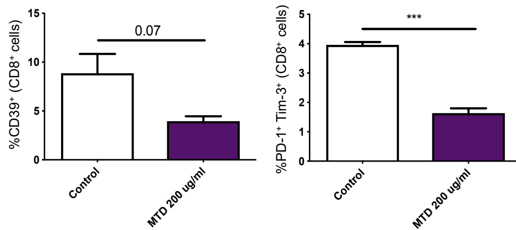


Figure 11. Peripheral blood mononuclear cells were obtained from peripheral blood and subjected to treatment with 200 ug/ml liver-derived mitochondria. Immunophenotype of untreated or mitochondria-treated cells was determined by flow cytometry. *: P≤ 0.05.

PROJECT # 4
TITLE: Human Subjects Core and Ventilator-Induced Injury and Lung Immune Response to Infection After Trauma
PI: Daniel Talmor

Major Activities, objectives, and results

- Obtained IRB and HRPO approval of the research protocol to enroll 1,000 patients from which to collect specimens and clinical data to support the other Projects. 26-May-2016 (IRB), 04-Nov-2016 (HRPO)
- Onboarded multiple clinical research assistants and research fellows, including training in good clinical practice, human subjects research, and research conduct. 11-Oct-2016 TP; 30-May-2017 JL; 01-Nov-2017 AB; 01-Sep-2018 IL; 14-Feb-2019 BH; 19-Aug-2019 MK.
- Built REDCap database to house clinical data and specimen data. 01-Feb-2017
- Established collaboration with human subjects research group in Emergency Medicine conducting complementary protocol with trauma patients. Co-enrollment approved by the local IRB. 02-Mar-2017
- Piloted and modified specimen collection methods in conjunction with consortium labs to enhance cell extraction. Ongoing
- Designed sub-study to investigate impact of mechanical ventilator settings on gas exchange and alveolar stretch. Obtained IRB approval 24-Aug-2018
- Consented and enrolled 196 patients through 30-Sep-2019
- Collected 391 biological specimens through 30-Sep-2019

Progress Detail:

- ***Obtained IRB/HRPO approval.***

Local IRB approval granted 05/26/2016; HRPO approval granted 11/04/2016.

- ***Patient enrollment***

From 10/1/16 through 09/30/19 we have enrolled 196 patients.

- Trauma Cases n=94 (75.5%) Male, average age 50.6 years (18-89)
  208 approached
  94 consented
  86 trauma cases from whom samples collected
- Surgical Controls n=102 (52.9%) Male, average age 59.4 years (28-83)
  230 approached
  102 consented
  57 surgical controls from whom samples collected

Regulatory requirements are in place

Complete regulatory files are maintained and audited monthly. Study staff maintain current research regulatory training at all times. All informed consent documents are monitored to ensure high level of quality.

Collect relevant patient and outcomes data

We are collecting clinical and outcomes data on all enrolled patients.

Safe / correct collection of samples

We are collecting samples safely, and have refined our processes in conjunction with the collaborating labs to enhance optimal cell separation. From 10/1/16 through 9/30/19, we have collected 286 whole blood samples and 105 bronchoalveolar lavage samples.

Initial recruitment was deliberately slow as the trial database was built and tested, and sample collection and processing procedures were tested and refined. By month 9, staffing was secure, collaborations for enrollment were established, and we increased the pace of enrollment.

Based on screening data there are adequate numbers of surgical control patients to meet Project 4, SA1, milestone #1, enroll first 100 patients by 15 months. However, the number of eligible trauma patients has been lower than projected. To address this, we have modified the inclusion criteria to capture more cases. Furthermore, we have identified several additional methodologies that we can employ as needed to increase enrollment:

1. Continue to adjust our criteria for injury severity to permit enrollment of patients with lower ISS. We would then stratify our analysis based on severity of injury and conduct experiments on specific subsets of patients. This was done in 2017.
2. Add additional team members to recruit and consent. This was done in 2018, which has resulted in an increase in our surgical control enrollment rates.
2. Collaborate with a neighboring institution that admits a larger number of penetrating traumas with high ISS. Initial exploratory conversations with potential co-investigators have been promising.
3. We have completed negotiations to share specimen with another research group.

Evaluate role of PO_2 in alveolar immune environment

We have designed a third study group, intubated critically ill patients, who will undergo ventilator manipulation and serial sampling. This will allow investigation of the role of PO_2. The local IRB approved the revised protocol on 24-Aug-2018; HRPO approved the amendment on 07-Nov-2018. The protocol was registered on Clinicaltrials.gov 19-Jun-2019 under NCT03993002.

SA1: Identify and recruit trauma patients

Subtask 1 (months 3-6): Obtain IRB/HRPO approval.
We have completed this task.

Subtask 2 (months 3-18): Enroll 100 patients and 100 volunteer controls.
Through month 36 we have enrolled 94 trauma patients and 102 volunteer controls.

Subtask 3 (months 0-60): Regulatory requirements in place.
We have completed this task and ensure continuous ongoing compliance with all regulatory requirements.

SA2: Collect and process data on enrolled patients

Subtask 1 (months 3-60): Collect relevant patient and outcomes data.
Data are collected and entered on all enrolled patients within approximately seven days.

Subtask 2 (months 3-60): Safe / correct collection of samples.
We have completed this task. Through month 36 we have collected 391 samples. There was one adverse event, transient laryngospasm during bronchoscopy for BAL collection.

SA3: Physiochemical effects on airway innate immunity

Subtask 1 (months 3-15): Evaluate role of PO2 in alveolar immune environment
We have designed an interventional protocol that will allow us to test this, and are securing the necessary regulatory approval prior to launch.

SA4: Develop preclinical studies as a foundation for subsequent clinical trials

Subtask 1 (months 3-60): identify variations in biological signaling that may impact clinical care
We have not yet begun work on this task

Subtask 2 (months 3-60): design and execute Phase 0 RCTs as pilot studies to identify simple interventions that may prevent PNA
We designed a Phase 0 RCT that will enable us to examine the impact of normoxia, hyperoxia, normocarbia, and hypercarbia on neutrophil and macrophage activation, purine metabolism, cytokines and biomarkers of inflammation. This study is registered on clinicaltrials.gov and is currently open for enrollment.

PROJECT # 5
TITLE: Systems Immunology Studies on Immunotherapy for Trauma-Associated Immune Dysfunction
PI: James Lederer

Major activities: To define the phenotypic influences of burn trauma in a mouse model on cell-mediated immune responses and to characterize the cellular and molecular features involved in restoring immune responses in CpG-ODN treated mouse burn trauma model.

- Optimized in vivo method to examine phenotypic cytokine expression changes in immune cells from sham and burn trauma mice treated with CpG-ODN. 4-2019, 5-2019
- Performed immune profiling studies in our mouse burn trauma model to measure functional phenotype of immune cell populations from the lymph nodes and spleen of mice that were treated with CpG-ODN. Time points included days 1 and 7 post-injury and cells were stimulated with PMA/Ionomycin to induce intracellular cytokine expression. 6- 2019, 8-2019
- Analysis of cytokine CyTOF data from studies to prepare manuscript for publication. 8-2019, 9-2019

Major activities: To use CyTOF and Luminex technologies to generate systems immunology data from trauma patients and research samples.

- Manuscript revised extensively and resubmitted that describes our work in collaboration with Project 3 on lung hyperoxia injury cellular immune response, Scientific Cell Reports, 7-2019, 10-2019
- Human Luminex cytokine assay panel finalized for HALO studies to assess cytokine levels in plasma and other biological samples generated by stimulating human blood immune cells with plasma or specific stimuli such as ND6 peptide, CpG-DNA, liver slurry, ATP, Heme, mitochondria, or LPS. 3-2019, 4-2019, 5-2019, 8-2019
- Completed data collection of interim CyTOF mass cytometry analysis of PBMCs from controls, day 1, 3, and 5 trauma patients using 2 validated CyTOF antibody panels to detect innate and adaptive immune cell subsets. 11-2018
- Initial computational analysis of innate immune cell panel data for presentation at the DOD milestone meeting in Falls Church, VA. 1-2019

- Computational analysis of innate and adaptive immune cell CyTOF mass cytometry data for manuscript preparation and to share with Project 6 for computational modeling of immune cell changes with clinical characteristics of trauma patients. 5-2019, 7-2019, 9-2019
- Completion of trauma plasma PBMC activation studies with preparation of manuscript. This work involved CyTOF mass cytometry analysis of normal PBMCs that were activated in vitro with plasma from healthy donors or trauma patients from days 1, 3, or 5. Also, Luminex analysis of cytokines that were present in these same plasma samples using the validated HALO panel. 7-2019, 8-2019, 9-2019, 10-2019
- Validation of bronchioaleveolar lavage (BAL) CyTOF panel for analysis of BAL samples from trauma patients and controls.

Other accomplishments:

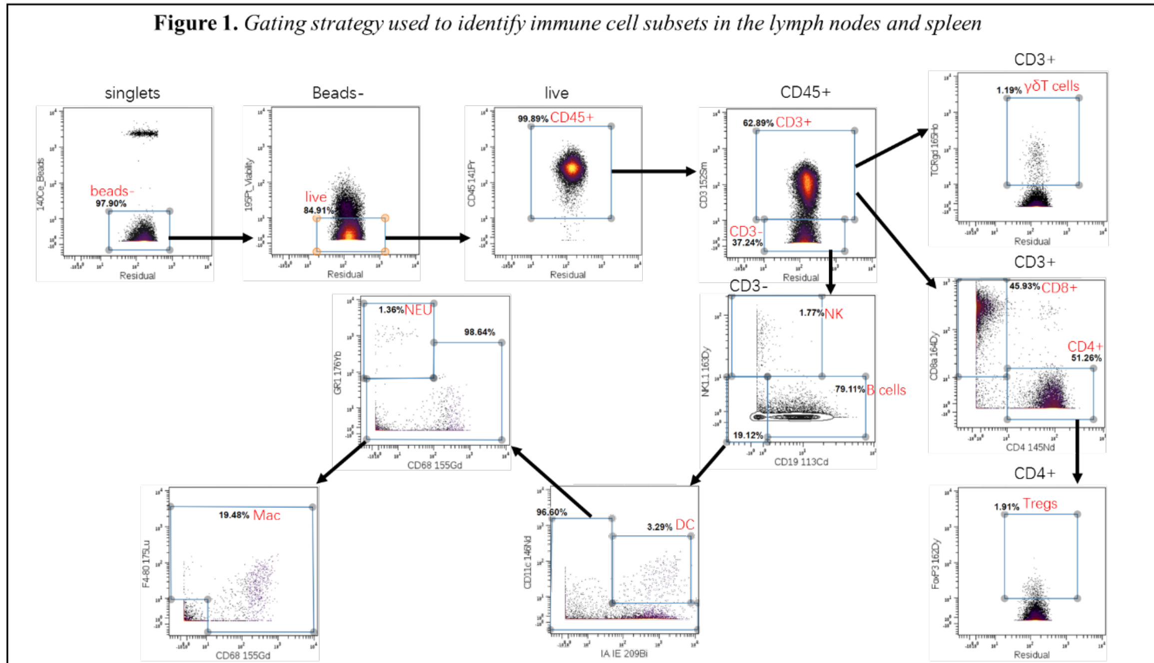
- Manuscript published in Journal of Trauma and Critical Care Surgery that reports on immune profiling 20 trauma patients by CyTOF, J Trauma Acute Care Surg. 2019 Aug;87(2):337-34, 8-2019
- Several presentations on research findings at the 2019 Shock Society meeting at Coronado, CA, 6-2019
- Presentation of trauma patient PBMC innate immune cell CyTOF mass cytometry findings presented as a talk at the Military Health System Research Symposium (MHSRS) at the 2019 annual meeting, 8-2019

Studies relevant to Specific Aims 1 and 2: To define the phenotypic influences of trauma on cell-mediated immune responses to lung bacterial infections and to identify cellular and molecular features responsible for beneficial immune and anti-microbial function induced by IRM treatment in injured mice.
During year 3, we continued to work towards identifying how trauma alters immune system and the mechanisms-of-action for the significant protective effect of using synthetic CpG DNA molecules (CpG-ODN) as an immunotherapy for trauma. We focused on studying the effects or burn trauma on cytokine expression by immune cell populations from the lymph nodes and spleens of sham, burn, and burn + CpG-ODN treated mice. We used CyTOF technology to comprehensively assess cytokine expression in adaptive immune cell subsets from mice that were treated with CpG-ODN or left untreated. We focused on days 1 and 7 after burn trauma for these studies. The results of this work was presented at the 2019 Shock Society meeting held at Coronado, CA.
Results: We used our established mouse burn trauma model to study how CpG-ODN therapy changes the immune cell subset reactivity to polyclonal stimulation by PMA/Ionomycin (PI). This stimulation provides receptor independent cell activation and results in significant cytokine production by many immune cell types. After preparing lymph node or spleen cells from mice, we stimulated the cells with PI and added a golgi block drug to prevent cytokine release. After 4 hours, the cells were stained with a CyTOF antibody panel to detect immune cell subsets and to simultaneously detect cytokine expression in single cells. Our objective was to determine how CpG-ODN changes the functional cytokine response profiles of multiple immune cell subsets. Comparisons included sham, burn trauma, and burn trauma with CpG-ODN treatment at 2 hours after injury. Cells from these groups of mice were harvested at 1 and 7 days later for cytokine analysis by CyTOF.

All CyTOF data was analyzed using our current analytical workflow as follows: 1) data cleaning by gaussian parameters, 2) normalization, 3) deconvolution by barcode signatures, 4) data upload into Cytobank to gate live single cell populations and major subsets, 5) viSNE dimensional reduction analysis to organize cells into single cell grouping with similar marker expression profiles, 6) cluster determination by k-nearest neighbor analysis, 7)SPADE cluster using estimated cluster numbers, 7) statistical and qualitative interpretation of cluster abundances and phenotypes. After analysis, the data was plotted for visualization and interpretation.

Figure 1 shows the gating strategy that was used to identify immune cell subset populations from the lymph nodes or spleen of mice. Cells were gated manually be these parameters so that we could identify the source of cytokines following their PI stimulation *ex vivo*. **Figure 2** presents heatmaps to illustrate the breadth of cytokine expression profiles gained by CyTOF technology in this study. These heatmaps show changes in cytokine expression at day 1 and 7 in the indicated lymph node cell subsets. Stimulation with PI (label "S") showed strong induction of multiple

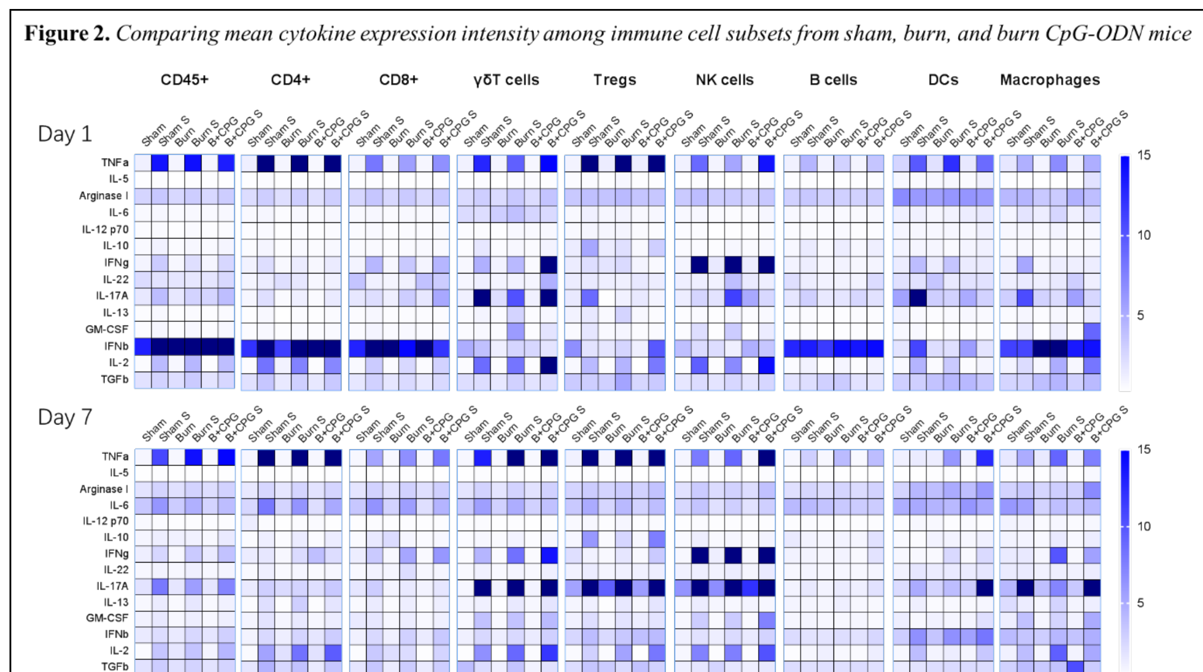
Figure 1. *Gating strategy used to identify immune cell subsets in the lymph nodes and spleen*



cytokines. By gating the CD45+ cells into subsets, it is possible to identify the immune cell subsets that are modulated by burn trauma or by CpG-ODN treatment. From this overview analysis, we identified that γδ T cells were most affected by CpG-ODN treatment showing higher levels of IFNγ, IL-17A, and IL-2 than untreated burn trauma mice. At day 7, we observed a similar effect of CpG-ODN treatment on γδ T cells, but Tregs also showed some differences in IL-17A and IL-2 as well as unique increase in IL-10 expression.

Statistical analysis of these data was consistent with the heatmap visualization, but other significant findings were observed in other immune cell subsets besides γδ T cells.

Figure 2. *Comparing mean cytokine expression intensity among immune cell subsets from sham, burn, and burn CpG-ODN mice*



As shown in **Figure 3**, we identified that CpG-ODN treatments had significant effects on cytokine expression in CD8 T cells, NK cells, and Tregs. In CD8 T cells, CpG-ODN treatment increased TNFα and IFNγ expression. In NK cells we observed increased TNFα, IFNγ, and IL-17A expression. And in Tregs, CpG-ODN treatment increased IL-10 expression. Identical type cytokine expression profiling was done using spleen cells from these same groups of mice. The findings in the spleen were not as striking and there were less differences in cytokine expression levels among different immune cell subsets. However, γδ T cells and NK cells were affected by CpG-ODN treatment as compared to other immune cell subsets (**Figure 4**). These cell subsets showed significantly higher IFNγ expression levels in the burn trauma group, but CpG-ODN treatment did not enhance expression levels like what was observed in the lymph nodes.

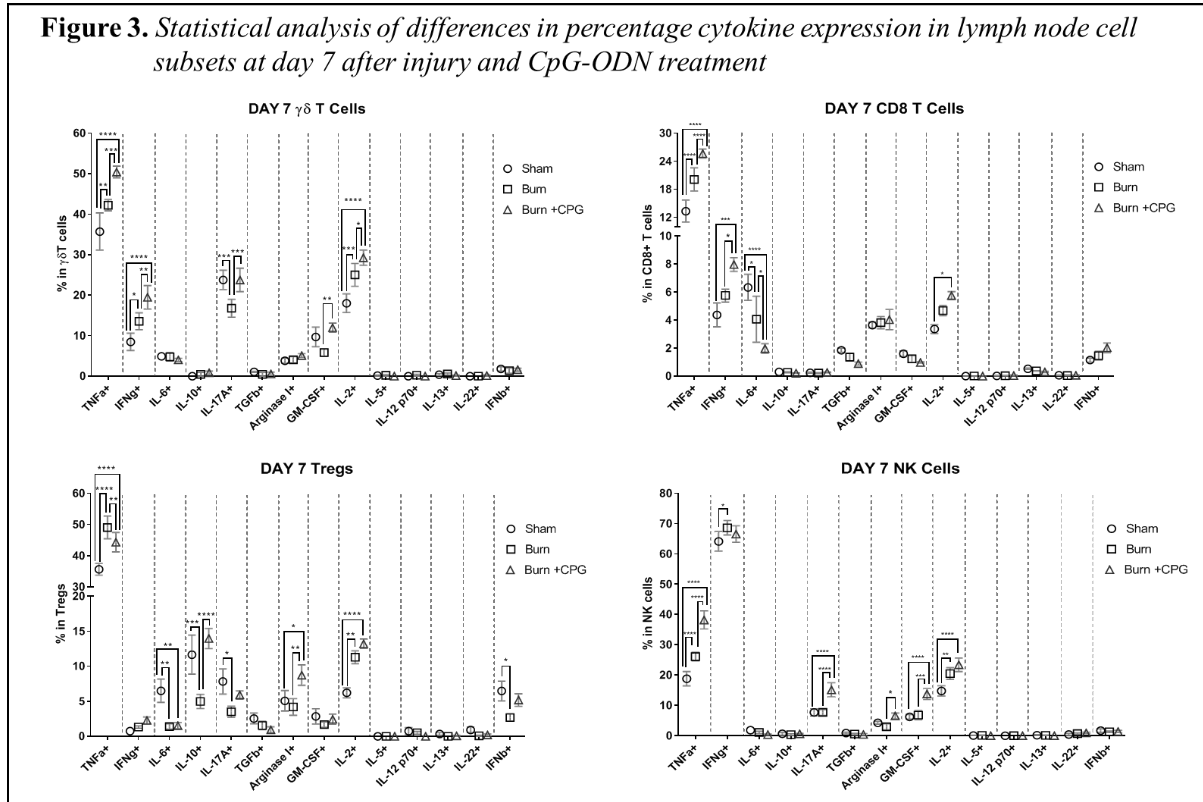


Figure 3. *Statistical analysis of differences in percentage cytokine expression in lymph node cell subsets at day 7 after injury and CpG-ODN treatment*

We completed analysis of these studies by unbiased cluster analysis using SPADE, an artificial intelligence (AI) method that organizes CyTOF staining data into clusters based on multiple marker expression levels on cells. By this method, we validated our findings that used manual gating of immune cell subsets. Accordingly, clustering by SPADE showed that lymph node γδ T cells, Tregs, and NK cells were modulated by CpG-ODN treatment and that these same cell subsets in the spleen showed significant changes in cytokine expression by burn trauma but no significant changes from CpG-ODN treatment.

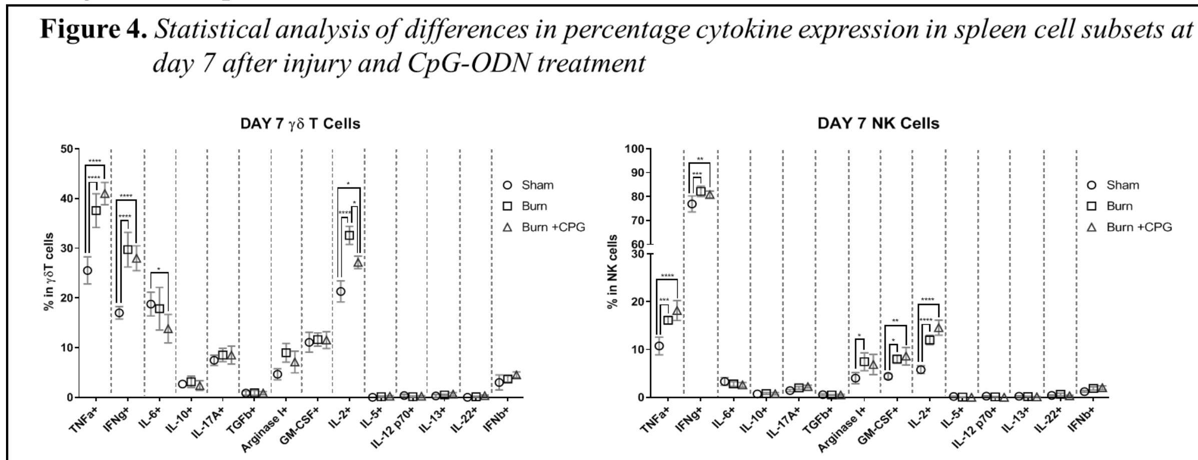


Figure 4. *Statistical analysis of differences in percentage cytokine expression in spleen cell subsets at day 7 after injury and CpG-ODN treatment*

Summary: The data generated in this study provides a clear vision of these immune cell subsets that are affected by burn trauma and by CpG-ODN immunotherapy in our mouse burn trauma model. In brief, we know that the CpG-ODN treatment given at 2 hours after burn trauma can significantly improve anti-microbial immune function and significantly improve the survival of burn trauma mice that are infected in the lungs with *S. pneumoniae.* Here, we demonstrate that γδ T cells and NK cells are the primary immune cell subsets showing differences in effector cytokine production following burn trauma and CpG-ODN treatment. We also observed an effect of CpG-ODN treatment on Tregs. In contrast to increased IFNγ and IL-17A expression, they showed higher IL-10 and arginase 1 expression following CpG-ODN treatment, which suggests that CpG-ODN enhances their counter-inflammatory function. Moreover, comparisons made between day 1 and day 7 after burn trauma indicated that the effects of early CpG-ODN treatment following burn trauma persists and even shows enhanced cytokine expression phenotype changes at 7 days. The effects of CpG-ODN immunotherapy on cytokine expression levels were significant in the lymph nodes and not the spleen. The compartmentalization of the CpG-ODN activity to enhance lymph node immune cell responses suggests that the immune modulatory behavior of CpG-ODN is most pronounced on immune cells that are affected by injury and less effective on distal or systemic immune cells. In closing, the results of this study provide new insights into the mechanisms-of-action for CpG-ODN treatment to promote immune system recovery. This study focuses on changes in cytokine expression phenotypes and contributes new understanding of how CpG-ODN treatment affects adaptive and innate lymphoid cells. A manuscript is in preparation to be submitted for peer review next month, 11-2019.

Results relevant to Specific Aim 3: To use CyTOF and Luminex technologies to generate systems immunology data from trauma patients and research samples.

Human Blood Immune Cell Profiling by CyTOF Mass Cytometry: Blood samples from trauma and control patients have been collected and cryopreserved for immune profile analysis by CyTOF mass cytometry or other approaches. We performed an interim, mid-project analysis of all peripheral blood mononuclear cell samples from trauma patients and case controls that have been cryopreserved. We decided to approach these samples for analysis using two different CyTOF antibody staining panels that focus on innate or adaptive immune cell populations. The innate panel includes markers to identify major immune cell subsets and other unique innate immune cell markers that are relevant to the aims of the HALO project. The adaptive panel similarly includes markers for major immune cell subsets and markers relevant to adaptive immune cell activation and differentiation. Prior knowledge was used to develop these CyTOF antibody panels. The objective of our CyTOF immune cell profiling work for HALO is to provide unbiased profiling of time-dependent abundance and phenotype immune cell changes that occur in trauma patients. We then will use these data in collaboration with Project 6 to determine correlations and associations between changes in PBMC single-cell phenotypes and patient clinical features. The full analysis of these data is completed and a manuscript is in preparation to include the CyTOF cell staining results and clinical feature information with computational analyses.

Figure 5. *Overview of interim CyTOF analysis of PBMCs from trauma patients from HALO*

- 39-marker panels that detects cell-surface, intracytoplasmic, and nuclear markers (proteins)
- Marker information is used to identify specific immune cell subsets
- High-dimensional cell staining data can identify both known and novel immune cell subsets

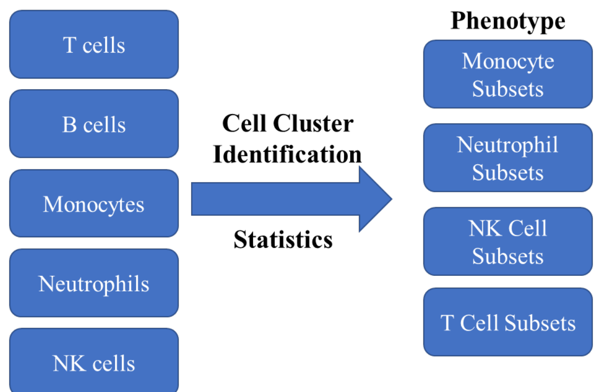


Figure 5 illustrates the CyTOF staining scheme and workflow that was used for our studies. As shown, we had samples from 114 individuals that passed quality control for CyTOF analysis (enough cell numbers, >85% viability). We collected multiple time points from patients to include day 1, days 2-3, and days 4-6 after trauma. Control samples were collected from consented

patients that underwent minor procedures. Demographic information was collected for all controls and trauma patients. Clinical outcomes such as time on ventilation, injury severity scores, and post-trauma infection were collected for all patients and recorded in REDCap.

Cell samples were randomized to include samples from controls and patients on each CyTOF analysis staining day. Batches for each run day included 20 patient or control samples with 2 CyTOF antibody panels. Cells were plated at 5 x 10^5 per well and stained using our standard operating protocols. After staining, single-cell CyTOF staining data was collected on a Helios CyTOF instrument (Fluidigm). Once all batches were completed, the CyTOF staining data was analyzed using our data analysis workflow as follows; 1) data cleaning by gaussian parameters, 2) normalization, 3) deconvolution by barcode signatures, 4) data upload into Cytobank to gate live single cell populations and major subsets, 5) viSNE dimensional reduction analysis to organize cells into single cell grouping with similar marker expression profiles, 6) cluster determination by k-nearest neighbor analysis by Phenograph in R Cytofkit package, 7) SPADE cluster using estimated cluster numbers, 7) statistical and qualitative interpretation of cluster abundances and phenotypes.

A large amount of data is generated by CyTOF technology, so it is imperative that unbiased computational approaches are used to identify significant changes in immune cell phenotypes caused by traumatic injuries. Moreover, the single-cell staining data from CyTOF is multi-dimensional since cells can simultaneously express multiple protein markers to define their phenotype. So, to visualize the outcome, we had to perform dimensional reduction methods to visualize the data and clustering methods to identify cell phenotypes. The most popular approach for dimensional reduction of CyTOF single-cell staining data is viSNE, an analytical approach that organizes the data in two-dimensions using the t-Distributed Stochastic Neighbor Embedding (t-SNE) algorithm. The 2D viSNE maps generated by this approach provide visual representations of the single-cell data in high-dimensional space. The viSNE maps can also be colored in a third dimension to identify gradients of cellular densities or marker expression levels and profiles within the viSNE maps. **Figure 6** provides a visual overview of viSNE density, cell subset, and cluster analysis of CyTOF staining data from our innate immune cell subset antibody panel (39-markers). This figure shows that there are visual changes in monocyte, NK cell and CD4+ T cell densities/numbers in trauma patients as compared to controls. In **Figure 7**, we plotted the immune cell subset abundance data from individual control and trauma patient samples for statistical analysis. We identified that trauma causes significant reductions in circulating CD4+ T cells and NK cells, and significant increases in monocytes at all time points after traumatic injury. These data provide the first comprehensive analysis of immune cell subset changes that occur following traumatic injury in humans.

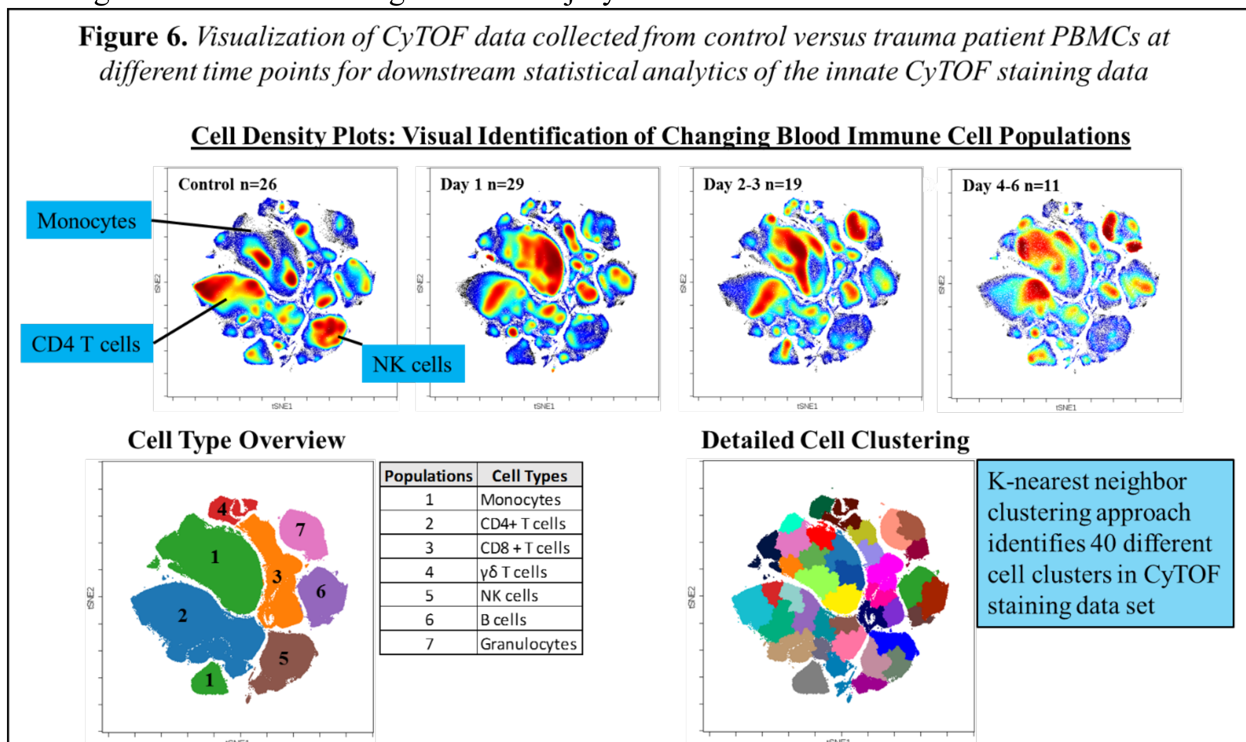
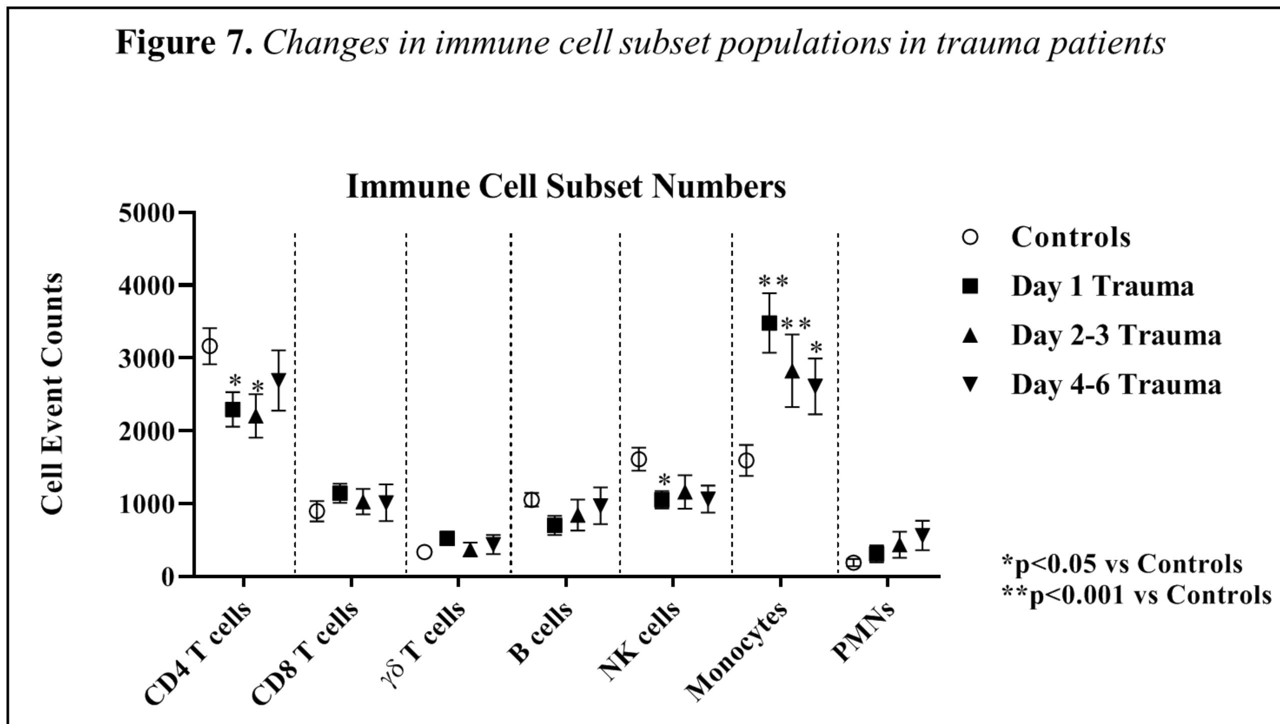


Figure 6. *Visualization of CyTOF data collected from control versus trauma patient PBMCs at different time points for downstream statistical analytics of the innate CyTOF staining data*

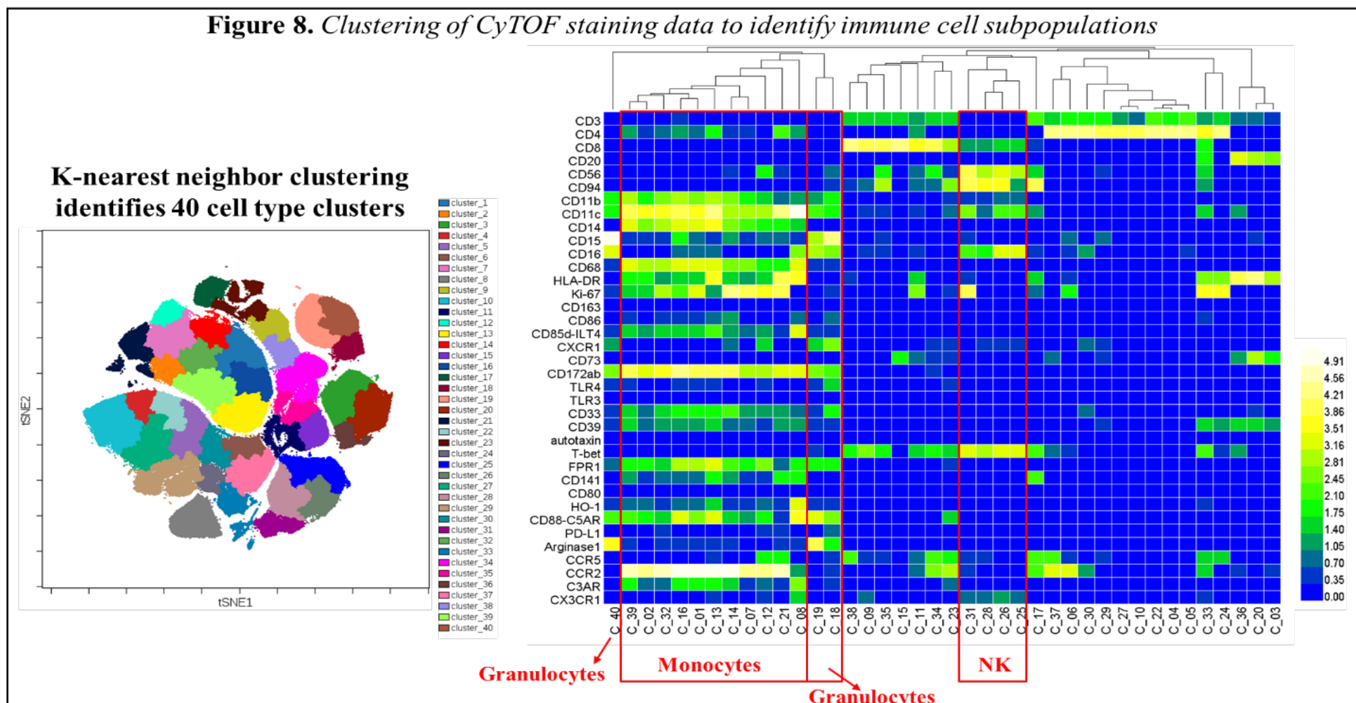
Figure 7. *Changes in immune cell subset populations in trauma patients*



Statistical analysis of cell clusters provides detailed information on subpopulations within major immune cell subsets that were identified as unique by multiple marker expression profiles. As anticipated, we estimated at least 40 different immune cell clusters in PBMCs from controls and trauma patients. By grouping or concatenating all the CyTOF staining files, called FCS files, we use clustering and marker expression patterns to phenotypically identify immune cell subpopulations (**Figure 8**).

In addition, we performed statistical analysis of changes in abundance of the 40 different clusters between control and trauma patient samples at different time points. These analytical methods provide an unbiased way to

Figure 8. *Clustering of CyTOF staining data to identify immune cell subpopulations*



determine which immune cells change in a time-dependent manner in trauma patients. We identified 5 monocyte, 3 NK cell, and 3 granulocyte clusters that are significantly changed by traumatic injury (**Figure 9**). Monocytes and

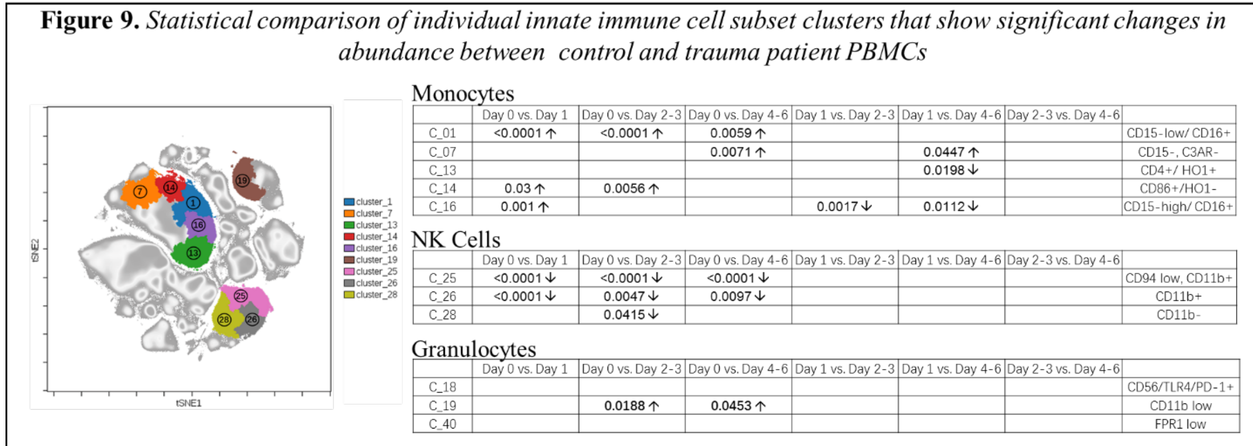


Figure 9. *Statistical comparison of individual innate immune cell subset clusters that show significant changes in abundance between control and trauma patient PBMCs*

Monocytes

	Day 0 vs. Day 1	Day 0 vs. Day 2-3	Day 0 vs. Day 4-6	Day 1 vs. Day 2-3	Day 1 vs. Day 4-6	Day 2-3 vs. Day 4-6	
C_01	<0.0001 ↑	<0.0001 ↑	0.0059 ↑				CD15-low/ CD16+
C_07			0.0071 ↑		0.0447 ↑		CD15-, C3AR-
C_13					0.0198 ↓		CD4+/ HO1+
C_14	0.03 ↑	0.0056 ↑					CD86+/HO1-
C_16	0.001 ↑			0.0017 ↓	0.0112 ↓		CD15-high/ CD16+

NK Cells

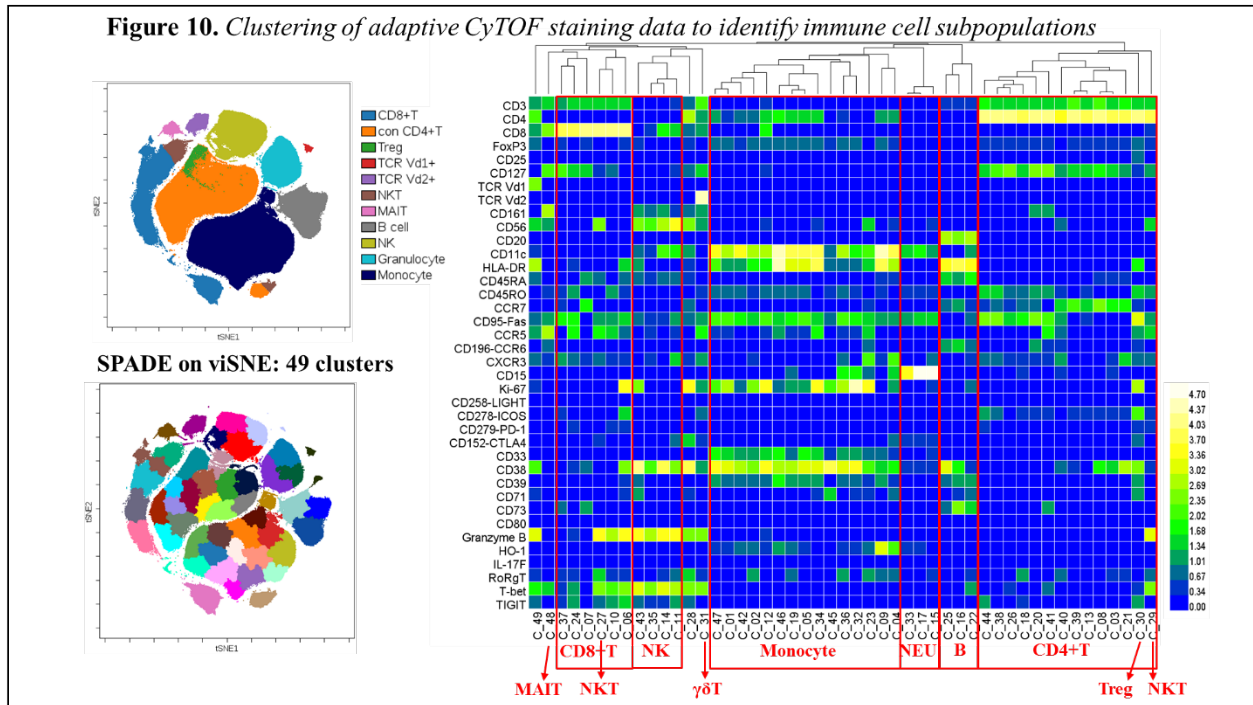
	Day 0 vs. Day 1	Day 0 vs. Day 2-3	Day 0 vs. Day 4-6	Day 1 vs. Day 2-3	Day 1 vs. Day 4-6	Day 2-3 vs. Day 4-6	
C_25	<0.0001 ↓	<0.0001 ↓	<0.0001 ↓				CD94 low, CD11b+
C_26	<0.0001 ↓	0.0047 ↓	0.0097 ↓				CD11b+
C_28		0.0415 ↓					CD11b-

Granulocytes

	Day 0 vs. Day 1	Day 0 vs. Day 2-3	Day 0 vs. Day 4-6	Day 1 vs. Day 2-3	Day 1 vs. Day 4-6	Day 2-3 vs. Day 4-6	
C_18							CD56/TLR4/PD-1+
C_19		0.0188 ↑	0.0453 ↑				CD11b low
C_40							FPR1 low

granulocytes showed increased abundance, while NK cells showed decreased abundance in the circulation of trauma patients at time points after injury. These data indicate that NK cells likely leave the circulation and are not replenished significantly. In contrast, monocytes and granulocytes showed higher levels in the circulation, which could reflect mobilization from the bone marrow in response to trauma, infection, or stress. The increased expression of Ki67, a proliferation marker, and expression of CCR2 on these monocyte clusters strongly support that the increase in circulating monocytes is related to bone marrow mobilization. Granulocytes that were increased at days 4-6 after trauma did not express Ki67 proliferation antigen or chemokine receptors but did show marker expression profiles consistent with them being low-density granulocytes that are enriched for during purification of PBMCs by density centrifugation.

Figure 10. *Clustering of adaptive CyTOF staining data to identify immune cell subpopulations*



Analysis of these same PBMC samples using a CyTOF panel to detect adaptive immune cell types was also performed. We confirmed identical changes in major immune cell subset populations that are shown in Figure 7. Using the same workflow as used for the innate cell CyTOF data analysis, we identified 4 clusters of CD4+ T cells that were significantly reduced in abundance in trauma patient blood (**Figures 10** and **11**). There were other changes that overlapped with what was observed in the innate CyTOF staining data – increased monocytes and reduced NK cells.

The complete results of CyTOF staining data were analyzed by the computational group in Project 6 – Yaffe. They assessed how each cluster identified by CyTOF changed in association with clinical measurements for each trauma patients. Those results are summarized in the report by Project 6.

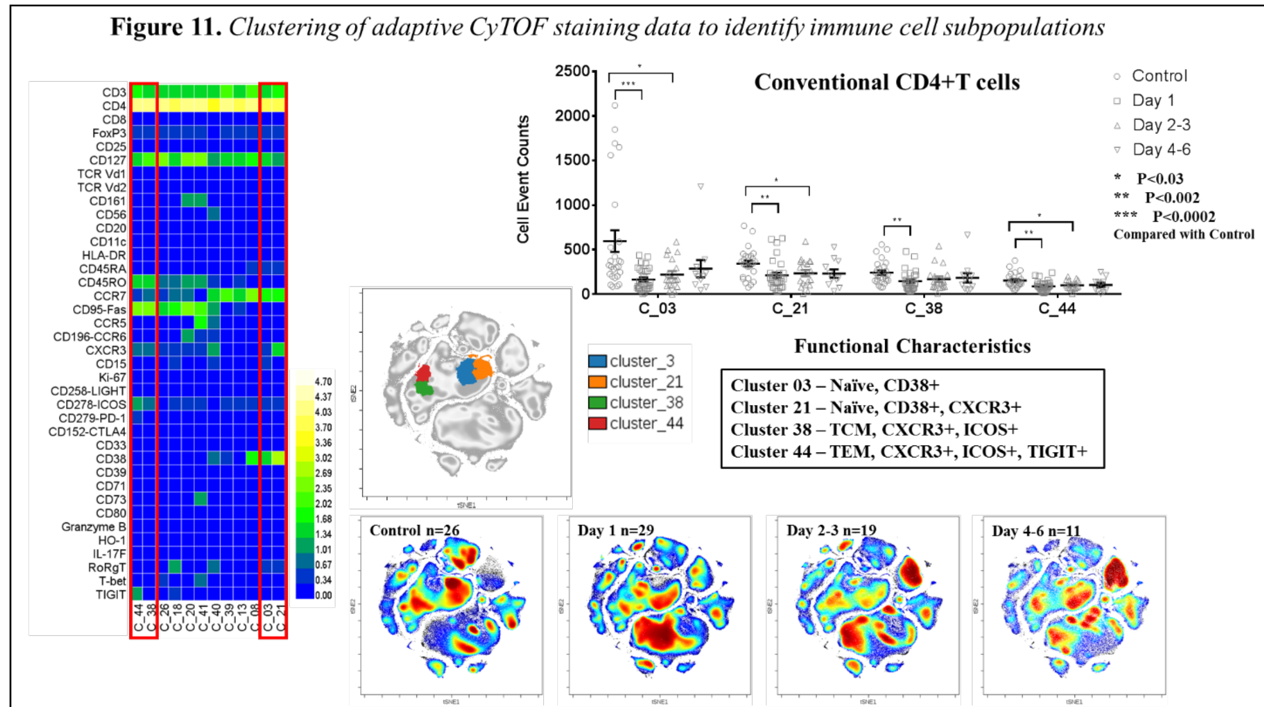


Figure 11. *Clustering of adaptive CyTOF staining data to identify immune cell subpopulations*

We are currently preparing a manuscript to report the findings form this interim analysis of trauma patient PBMCs. This paper will represent the first comprehensive analysis of changes in circulating immune cell population in trauma patients. This report will also provide new insights into what immune cell types are most affected by traumatic injury. As such, we and others will use this information to “reverse translate” this information in mouse trauma models to study tissue changes that cannot be done in humans. For example, bone marrow mobilization of monocytes and trafficking of NK cells or CD4+ T cells from the circulation into tissues. We will submit this manuscript during year 4 and subsequent CyTOF and other systems analyses such as cytokine profiling by Luminex and DAMP marker detections will build upon the findings and conclusions of this interim analysis.

PROJECT #: 6
TITLE: The Role of Neutrophil Priming, ROS Release, and MK2 Signaling in the Innate Immune Response after Trauma
PI: Michael B. Yaffe

Major activities, objectives and results

- Prepared more than 20 samples for CyTOF analysis to evaluate the presence and characterization of a low-density immunosuppressive population that we believe evolves after priminmg-induced injury. 19-NOV-2018, 27-NOV-2018, 28-NOV-2018, 06-FEB-2019, 08-FEB-2019, 22-27-FEB-2019, 06-MAR-2019, 05-08- APR-2019, 15-17-MAY-2019, 27-JUN-2019, 11-JUL-2019.
- Together with Lederer lab (Project 5) ran a CyTOF pilot and it showed that trauma patients have three times more low-density neutrophils than healthy subjects (**Figure 1**). Moreover, there is a remarkable difference between the markers expressed by trauma and healthy neutrophils (**Figure 2**). 01-NOV-2018.
- Established collaboration with Dr. Brahm Segal to investigate functionality of low-density neutrophils. 15-16-AUG-2019.

- Showed that LysMCre MK2 $^{-/-}$ neutrophils produce more intracellular ROS compared to WT neutrophils when stimulated with PMA but not when primed and then stimulated with fMLP (**Figure 3**). 10-JAN-2019, 25-JAN-2019, 07-FEB-2019.
- In collaboration with Project 1, showed that fMLP modulates intracellular calcium levels in both LysMCre MK2 $^{-/-}$ and WT neutrophils in a similar manner (**Figure 4**), however chemotaxis differs depending on the chemoattractant used (**Figure 5**). 06-MAY-2019, 20-MAY-2019.
- Performed trauma model in p47phoxK43$^{A/-}$ mice. 14-AUG-2019, 09-SEP-2019.
- Onboarded new Postdoctoral Fellow Dr. Xueyang Yu. 01-MAY-2019.
- Analyzed data obtained by CyTOF over all the samples gathered. 04-OCT-2019.

What was accomplished under these goals?
We have been collecting and preparing blood samples for analysis and characterization of a previously undocumented population of neutrophils – so called 'low-density neutrophils' with markedly immunosuppressive properties that emerges as a function of time after trauma. This neutrophil population was recently described in the cancer literature (c.f. Sagiv et al., Cell Reports 2015, 10:562-573; Scapini et al., Immun. Rev. 2016, 273:48-60) as showing a reduced oxidative burst and phagocytosis. A portion of these cells appears to be distinct from myeloid-derived suppressor cells, emerging instead from mature previously high- density neutrophils. To date no one has reported on the existence or creation of these cells after trauma, but if present, they could significantly explain the development of an immunosuppressive state after trauma. In collaboration with the Lederer lab (Project 5) we have begun the analysis of those neutrophils by CyTOF. Preliminary studies have shown that trauma patients have 3 times more low-density neutrophils than healthy subjects. Moreover, there is a remarkable difference in markers expression between neutrophils from trauma patients and healthy subjects. In order to characterize their immunosuppressive properties, we have partnered with Dr. Brahm Segal from Roswell Park Cancer Institute and we intent to assess the ability of the low-density neutrophils to suppress Tcell proliferation.

Our studies on the role of MK2 on neutrophil function and innate immune dysfunction demonstrated that LysMCre MK2$^{-/-}$ neutrophils don't differ from control neutrophils in terms of ROS production nor calcium modulation. Interestingly, LysMCre MK2$^{-/-}$ neutrophils seem to migrate less towards some chemoattractants, indicating a role for MK2 in the neutrophil function. We are continuing to evaluate the role of MK2 on the neutrophil function, by characterizing phagocytosis, granule exocytosis and apoptosis in the MK2$^{-/-}$ neutrophils. As detailed in the original proposal, we have created specific p47phox mutant knock-in mice that generate reduced (*p47phox-K43A*) and increased (*p47phox- K43Q*) amounts of extracellular ROS in order to specifically address the importance of extracellular ROS in tissue injury and innate immune dysfunction after trauma. Recently we are established *p47phox-/-* and *p47phoxK43A* mouse colony in pure C57BL/6 background, since this genetic background is required to see optimal responses in trauma/hemorrhage models. SNP analyses showed that wild-type and *p47phoxK43A* mice displayed the most C57BL/6NTac character (100.0%) and can be considered congenic. *p47phoxK43Q* mice displayed mixed and non-C57BL/6NTac character on 25 chromosomes 5, 9, 14 and 17. *p47phox-/-* mice displayed mixed and non-C57BL/6NTac character on chromosome 5. To have these mice (*p47phox-/-* and *p47phoxK43Q*) congenic, we have been backcrossing them with pure C57BL/6, and have generated pure p47phox-/- mice in this background. p47phoxK43Q mice have been more difficult to breed and need to go through two more generations before being congenic.

Previously, we performed liver crush injury experiments comparing *p47phox-/-* and wild-type mice. As expected, p47phox/- neutrophils do not produce significant levels of ROS in response to fMLP or PMA, even after priming with GM-CSF or TNFα, as shown below. Interestingly, there was less accumulation of immune cells in the lungs of *p47phox-/-* mice. Now, we are performing the trauma model in p47phoxK43$^{A/-}$ and analyses are still ongoing.

Gated PMN Population in PBMC

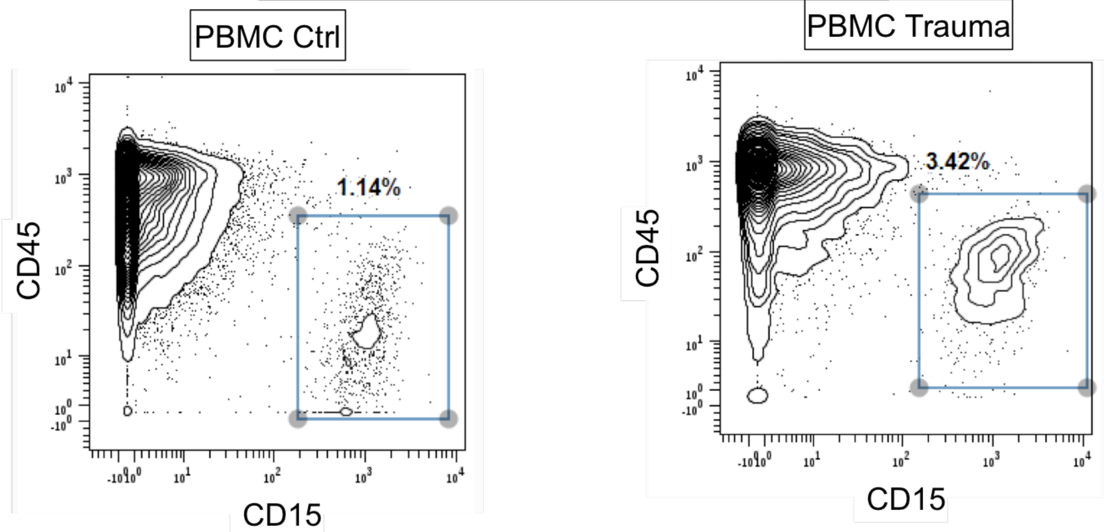


Figure 1. Analysis of neutrophils at the low-density fraction (PBMC) by CyTOF. Trauma patients show 3 times more neutrophils at the low-density fraction compared to healthy subjects.

Heat Map of Markers in PBMC PMN Population

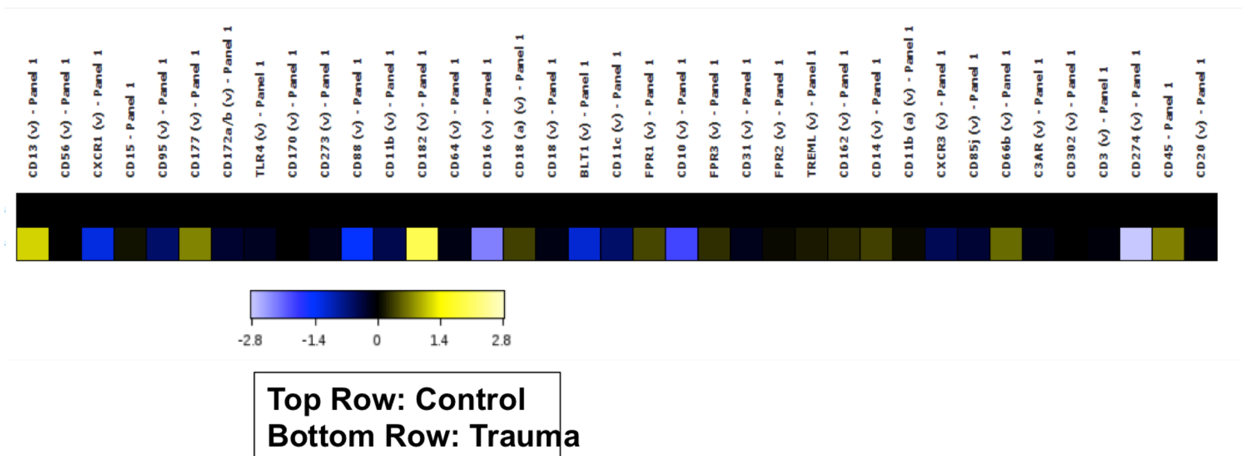


Figure 2. Heat Map of markers analyzed in neutrophils at the low-density fraction. Trauma neutrophils showed remarkable differences in markers expression compared to healthy neutrophils.

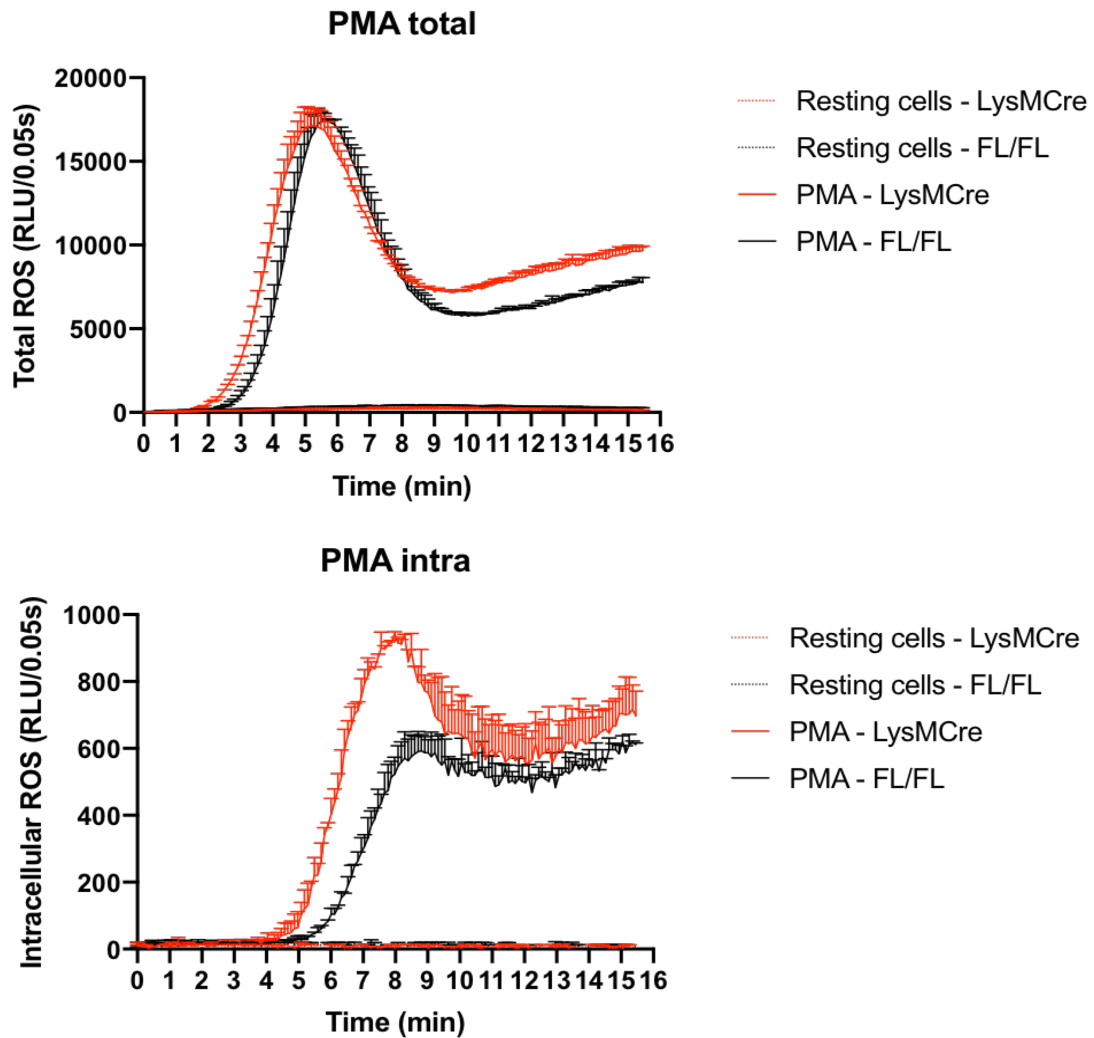


Figure 3. ROS measurement after stimulation with 500nM PMA. LysMCre MK2 $^{-/-}$ neutrophils produce more intracellular ROS compared to WT neutrophils when stimulated with PMA but not when primed and then stimulated with fMLP (data not shown).

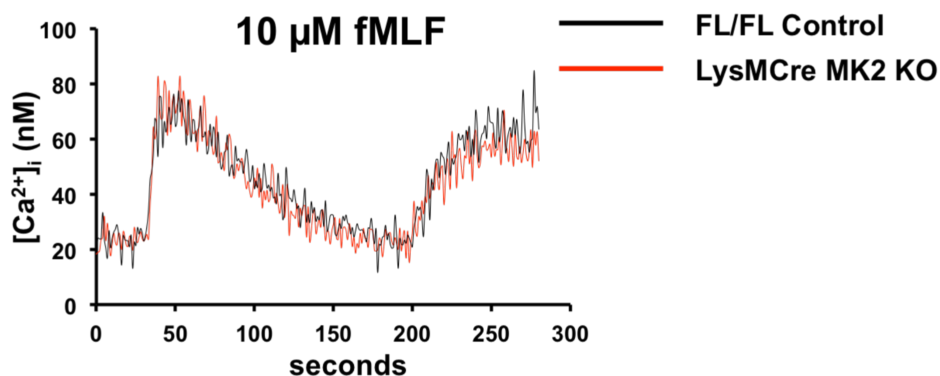


Figure 4. Calcium modulation in neutrophils after stimulation with 10 uM fMLP. fMLP modulates intracellular calcium levels in both LysMCre MK2 $^{-/-}$ and WT neutrophils in a similar manner.

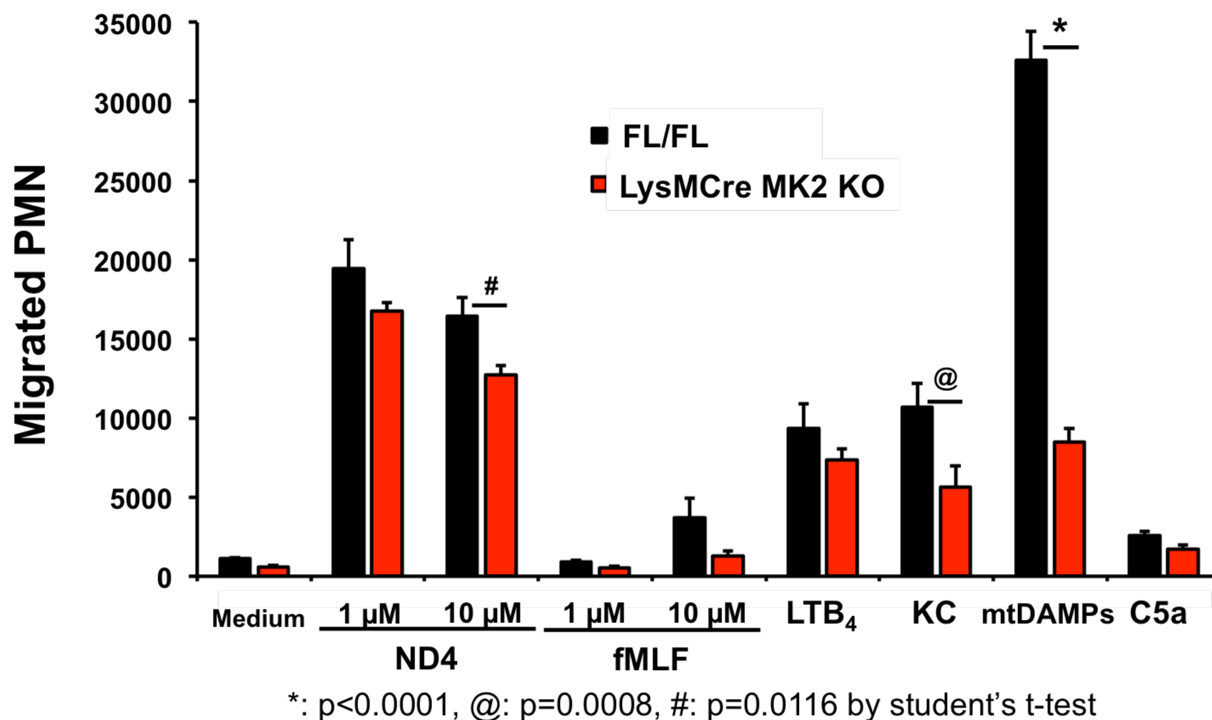


Figure 5. Neutrophil chemotaxis to various chemoattractants. LysMCre MK2 $^{-/-}$ neutrophils migrate less towards ND4, KC, mtDAMPS and fMLP compared to WT neutrophils.

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

All projects employed trainees including undergraduate, masters and doctoral students, surgical residents, training grant fellows, postdoctoral fellows and junior faculty. (see below for list of participants on each project. We had two invited speaker seminars this project period:

Richard D. Cummings, Ph.D.
S. Daniel Abraham Professor of Surgery
Beth Israel Deaconess Medical Center
Harvard Medical School
Director, HMS Center for Glycoscience

Brahm Segal, MD
Chair, Department of Internal Medicine
Chief, Division of Infectious Diseases
Professor of Oncology

How were the results disseminated to communities of interest?

Dr. Itagaki presented at the Wide River Institute of Immunology, College of Medicine of Seoul National University Hospital, Seoul, Korea.
Dr. Hauser presented as a Visiting Professor at the University of Miami School of Medicine, Miami FL. Department of Immunology.

Dr. Hauser presented at the Military Health Sciences Research Meeting, Kissimmee, FL.
Dr. Hauser presented Grand Rounds as the Nicole E. Herman Visiting Professor in Acute Care Surgery at the University of Florida, Gainesville.
Dr. Hauser presented at the 43rd Int. Congress on Military Medicine, Basel Switzerland.
Quanzhi Zhang presented at the Clowes Research Symposium. BIDMC
Dr. Hauser presented at Grand Rounds as a visiting professor at the Ryder Trauma Center, Miami FL.
Jan 18, 2019: PRMRP FY15 FPA Project Milestone Meeting in Falls Church, VA
Leo Otterbein presented at the 2nd annual Gasotransmitter Conference at the University of Oregon
Ghee Lee presented at the Military Health System Research Symposium in Orlando, FL
Leo Otterbein chaired a session at the 10th Heme Oxygenase conference in Seoul, South Korea
Dusan Hanidziar had an oral presentation at Association of University Anesthesiologists meeting and at International Anesthesia Research Society meeting. The abstract scored among top 7 in the basic science category and received the Kosaka award - indicating best of the meeting abstract finalist. Montreal, Canada
Dusan Hanidziar had an oral presentation at the American Society of Anesthesiologists, Orlando, FL
Serena Longhi, presented at the AAI meeting, San Diego, CA
Dr. Robson presented at IARS 2019 Annual Meeting and International Science Symposium, Montreal, Canada
Carl Hauser presented a poster at the Military Health System Research Symposium, Orlando, FL
Drs. Cahill and Guo presented their related studies at the 2019 Shock Society meeting in Coronado, CA
Dr. Yaffe presented at the 42nd Annual Conference on Shock, Coronado, CA, 2019.

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

Overall project plans for next reporting period include: 1.) continuing to accumulate patient specimens in collaboration with Dr. Elster at USUHS and Dr. Segal at Roswell Park Cancer Institute, 2.) continuing to dissect mechanisms of action in our established animal and cell culture models, 3.) continue the big data analysis from all specimens collected do date (>100) to perform the computational modeling and 4.) evaluate therapeutic approaches by which to decrease susceptibility to infection after trauma, e.g. oral CO delivery. Overall, there will be no major deviations from the original plan. We provide brief summaries of the project plans below.

Project 1

Evaluate the effects of mtDNA on neutrophil antimicrobial function using ex-vivo human PMN and our mouse injury plus pneumonia model. We will Evaluate chloroquine as a protectant against mitochondrial release-related susceptibility to infection in mice and use Luminex platforms to evaluate differential humoral environments in injured and infected humans. We will Define the effects of environmental thermal stress on PMN Ca^{2+} fluxes and antimicrobial function and then study potential means to restore related functional deficits. We will utilize our newly acquired functional FPR-1 knockout mice and begin studies.

Project 2

We will continue to evaluate the mechanism by which CO affects PMN function looking at the impact on human and mouse PMN mitochondrial dysfunction and how this impacts bacterial killing. Further PMN profiling comparing PMN harvested from marrow, blood and site of action, e.g. infected lung. As CO enters clinical trials, we are testing new modalities of delivery and critical pharmacology that can be used by medic to treat wounded warriors in theater.

Project 3

We are continuing to test the effects of mitochondria injections and bacterial instillations on the phenotype of peripheral blood and BAL-derived mononuclear cells of WT and $CD39^{-/-}$ mice. We will assess the effects of both mitochondria injections and bacterial instillations in luciferase mice and conduct histological assessment of lungs, liver and spleen. Test liver-derived mitochondria metabolic products and profiles by Seahorse and define the mechanisms resulting in CD39 down and upregulation in the hyperinflammatory and immunosuppressive phases of trauma. Finally, we will clarify differential roles of eATP scavenging vs. adenosine supplementation in these phases.

Project 4

We will continue to submit all necessary reports to the local IRB and HRPO. To complete subtask 4 we will continue screening and recruiting trauma patients and surgical control patients. We are actively exploring collaborations with other investigators to increase our accrual numbers. We will continue enrolling patients and refining strategies to collect all approved samples at the relevant time points. Data abstraction and entry will continue in conjunction with enrollment.
We have launched a third study group, intubated critically ill patients, who will undergo ventilator manipulation and serial sampling. We will continue to work closely with consortium team members to review laboratory findings and run analyses to uncover variations correlated with clinical outcomes. Based on the results of analyses described above we have begun to formulate study design capsules for pilot studies.

Project 5

Our research studies will focus on our mouse burn trauma research and immunotherapy with TLR9 agonists called CpG-ODN. We completed some of the work, but now will repeat the studies in male and female mice. We will perform human BAL immune cell profiling studies to add to our growing set of systems biology data for this project. These data will be incorporated into the computational modeling platform that is part of Project 6. We will work with project 6 to identify low-density granulocyte neutrophil populations from trauma versus control patients. We will refine the human PBMC profiling panels based on findings from interim analysis to run additional blood PBMC samples prepared by the HALO group. We will complete plasma biomarker analysis using our 30 marker Luminex panel to help with the systems biology nature of the project.

Project 6

During the next year we will continue to focus great effort on identifying and uncovering the role of this unique class of low-density, immune-suppressive neutrophils that we believe evolve after priming by injury-induced cytokines, and likely account for the increased susceptibility of patients to pneumonia after trauma. This will be accomplished using CyTOF technology in collaboration with Project 5, and by direct measurements of the effect of these low-density neutrophils on T-cell priming *in vitro*. We will also investigate whether wound fluids after trauma (peritoneal fluid from abdominal trauma, pleural fluid from blunt chest trauma, and/or drain fluid from orthopedic trauma) is able to induce this immune-suppressive state. In addition, we are investigating whether we can use single cell RNA sequencing to better characterize this key neutrophil population.

We will continue to explore the role of MK2 in PMN function, focusing largely on its role in cytoskeletal-mediated events that might account for the observed increase in ROS in the myeloid-specific MK2 knock-outs. The observation that PMN from constitutive MK2 null animals make less ROS suggests that MK2 function in the bone marrow microenvironment may be important for PMN maturation, which we will explore by characterizing markers of PMN maturity in the bone marrow of constitutive MK2 knock-out animals.

We will complete the murine trauma studies using the p47phox knock-in constructs that differentially regulate intracellular versus extracellular ROS. We are considering instituting an alternative model of aspiration pneumonia using intra-tracheal installation of LPS or HCl to investigate the effects of these p47phox mutants on lung injury, since this form of pneumonia is quite common in trauma patients, and recent data suggests that extracellular ROS may play a particularly important role in this type of lung damage after trauma.

4. IMPACT:

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

One of our key goals in year three was to continue to expand our knowledge of the model systems and readouts in cells isolated from trauma patients and identify specific profiles that are predictive of susceptibility to infection. We are confident that we are able to compare the effects we observe in mice, in human cells. Each laboratory continues to collaborate successfully across disciplines to maximize the data collected. All projects were highly productive in year 3 with additional grant support, publications, and national and international

presentations. Our interim FPA Project Milestone Meeting in Falls Church, VA was met with high praise for our success and achievements.

What was the impact on other disciplines?

In Project 5, the development of a reliable approach to digest tissues for CyTOF mass cytometry single cell has led to several important collaborations with cancer biology and immunology research groups. We are using approaches gained from this project to profile immune cell reactivity to different types of brain tumors, head and neck cancers, and in controlled mouse cancer immunology studies to test the impact of immune modulation on the immune response. The impact of our work in trauma immunology will have impact through collaboration with cancer biologists. Our efforts to expand our collaborative efforts was successful in leveraging our tissue repository for exploratory studies in glycomics and microbiome studies. These will be directly compared with our animal models to allow us to move findings identified in human cells into more mechanistic studies in mice.

What was the impact on technology transfer?

Based on the invited seminar speakers, we have entered an agreement with Dr. Segal to share human ascites fluid that will allow us to determine the phenotype of a suppressive neutrophil. This will then be translated to the PMN we continue to collect from human trauma patients with infection. Collaborations are also underway with Dr. Cummings where we are moving toward identifying novel serum markers based on glycomic analyses, that we anticipate will be rapid assessment of the presence of bacteria in the body.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES AND PROBLEMS:

Changes in approach and reasons for change

Nothing to report.

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

Nothing to report.

Changes that had a significant impact on expenditures

None to report.

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

No deviations or changes other than standard amendments.

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.”

Nothing to Report

Publications, conference papers, and presentations

Journal publications

- A Subset of Five Human Mitochondrial Formyl Peptides Mimic Bacterial Peptides and Functionally Deactivate Circulating Human Neutrophils After Trauma. Kaczmarek E, **Hauser CJ**, **Kwon WY**, **Riça I**, Chen L, Sandler N, **Otterbein LE**, Campbell Y, Cook CH, **Yaffe MB**, Marusich MF, **Itagaki K**. J *Trauma Acute Care Surg*. 2018 Nov;85(5):936-943. PMID: 29787548. yes
- Altered monocyte and NK cell phenotypes correlate with post-trauma infection. Seshadri A, Brat GA, Yorkgitis BK, Giangola M, Keegan J, Nguyen JP, Li W, Nakahori Y, Wada T, **Hauser C**, Salim A, Askari R, Lederer JA. *J Trauma Acute Care Surg*. 2019. doi: 10.1097/TA.0000000000002264. yes

- FPR1 blockade prevents receptor regulation by mitochondrial DAMPs and preserves neutrophil function after trauma. **Itagaki K**, Kaczmarek E, Kwon WY, Chen L, Vlkova B, Zhang Q, **Riça I**, **Yaffe MB**, Gong WH, Wang JM, Gao J, Jung F, Douglas G, Campbell Y, Marusich MF, **Otterbein LE** and **Hauser CJ**. *Crit Care Med* in press. yes
- Trauma-Induced, DAMP-Mediated Remote Organ Injury and Immunosuppression in the Acutely Ill Patient. Pottecher J, Meyer A, Wenceslau CF, Timmermans K, Hauser CJ and Land WG. *Front. Immunol.*, 20 August 2019. https://doi.org/10.3389/fimmu.2019.01971. yes
- **Otterbein LE**. Conquering Radicals with a Sense of Humor. *Cell Chem Biol*. 2019 Oct 17;26(10):1335-1337. doi: 10.1016/j.chembiol.2019.10.001. PubMed PMID: 31626781. yes
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- Belcher JD, Gomperts E, Nguyen J, Chen C, Abdulla F, Kiser ZM, Gallo D, Levy H, **Otterbein LE**, Vercellotti GM. Oral carbon monoxide therapy in murine sickle cell disease: Beneficial effects on vaso-occlusion, inflammation and anemia. *PLoS One*. 2018 Oct 11;13(10):e0205194. doi: 10.1371/journal.pone.0205194. eCollection 2018. PubMed PMID: 30308028; PubMed Central PMCID: PMC6181332. yes
- Vuerich M, Harshe RP, **Robson SC, Longhi MS**. Dysregulation of adenosinergic signaling in systemic and organ-specific autoimmunity. *Int J Mol Sci* 2019; 20: 528-543. yes
- Vuerich M, **Robson SC, Longhi MS**. Ectonucleotidases in intestinal and hepatic inflammation. *Front Immunol* 2019; 10: 507. yes
- Robles RJ, Mukherjee S, Vuerich M, Xie A, Harshe R, Cowan PJ, Csizmadia E, Wu Y, Moss AC, Chen R, **Robson SC, Longhi MS**. Modulation of CD39 and exogenous APT102 correct immune dysfunction in experimental colitis and Crohn's disease. Under minor revision. yes
- **Hanidziar D**, Nakahori Y, Cahill LA, Gallo D, Keegan J, Nguyen JP, **Otterbein LE**, **Lederer JA**, **Robson SC**. Characterization of pulmonary immune responses to hyperoxia by high-dimensional mass cytometry analyses. *Sci Rep* 2019, revised manuscript under review. yes
- Bocharnikov AV, Keegan J, Wacleche VS, Cao Y, Fonseka CY, Wang G, Muise ES, Zhang KX, Arazi A, Keras G, Li ZJ, Qu Y, Gurish MF, Petri M, Buyon JP, Putterman C, Wofsy D, James JA, Guthridge JM, Diamond B, Anolik JH, Mackey MF, Alves SE, Nigrovic PA, Costenbader KH, Brenner MB, **Lederer JA**, Rao DA. PD-1hiCXCR5- T peripheral helper cells promote B cell responses in lupus via MAF and IL-21. *JCI Insight*. 2019 Oct 17;4(20). doi: 10.1172/jci.insight.130062. PubMed PMID: 31536480. yes
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- Seshadri A, Brat GA, Yorkgitis BK, Giangola M, Keegan J, Nguyen JP, Li W, Nakahori Y, Wada T, Hauser C, Salim A, Askari R, **Lederer JA**. Altered monocyte and NK cell phenotypes correlate with posttrauma infection. *J Trauma Acute Care Surg*. 2019 Aug;87(2):337-341. doi: 10.1097/TA.0000000000002264. PubMed PMID: 31008865. yes
- Arazi A, Rao DA, Berthier CC, Davidson A, Liu Y, Hoover PJ, Chicoine A, Eisenhaure TM, Jonsson AH, Li S, Lieb DJ, Zhang F, Slowikowski K, Browne EP, Noma A, Sutherby D, Steelman S, Smilek DE, Tosta P, Apruzzese W, Massarotti E, Dall'Era M, Park M, Kamen DL, Furie RA, Payan-Schober F, Pendergraft WF 3rd, McInnis EA, Buyon JP, Petri MA, Putterman C, Kalunian KC, Woodle ES, **Lederer JA**, Hildeman DA, Nusbaum C, Raychaudhuri S, Kretzler M, Anolik JH, Brenner MB, Wofsy D, Hacohen N, Diamond B. The immune cell landscape in kidneys of patients with lupus nephritis. *Nat Immunol*. 2019 Jul;20(7):902-914. doi: 10.1038/s41590-019-0398-x. Epub 2019 Jun 17. PubMed PMID: 31209404; PubMed Central PMCID: PMC6726437. yes

acknowledgement of federal support (yes/no).

- **Books or other non-periodical, one-time publications.**
- Kuo A, **Hanidziar D**, Aldrich M. Critical Care Medicine. In: Wiener-Kronish J., Editor. Miller's Anesthesia, 9th Edition. 2019.

- **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.*

  All listed above

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Project 1

Name:	**Carl Hauser, MD**
Project Role:	PD/PI
Researcher Identifier	
Nearest person month worked:	2.4
Contribution to Project:	Dr. Hauser is the Director of the Program and Principal Investigator of Project 1. He oversees the work with Dr. Itagaki.
Funding Support:	W81XWH-16-1-0464

Name:	**Kiyoshi Itagaki, PhD**
Project Role:	Co-Investigator
Researcher Identifier:	orcid.org/0000-0002-6033-1122
Nearest person month worked:	12
Contribution to Project:	Dr. Itagaki is working on effects of human and mouse mitochondrial formyl peptides on PMN functions (*in vitro*) and on nosocomial pneumonia in addition to establishment of human PMN isolation methods for the entire groups. Day to day supervising roles on Ms. Q. Zhang.
Funding Support:	RO3 NIAID/NIH, W81XWH-16-1-0464

Name: **Barbora Vlkova, PhD.**
Project Role: Research Fellow
Researcher Identifier
Nearest person month worked 1
Contribution to Project: She was involved in PMN isolation, chemotaxis, mtDNA copy numbers per cell, determination of plasma levels of mtDNA and ND6 by qPCR and ELISA. She was supervised by Dr. Itagaki.
Funding Support: W81XWH-16-1-0464

Name: **Quanzhi Zhang, MS**
Project Role: Research PhD Student
Researcher Identifier
Nearest person month worked 9
Contribution to Project: Ms. Zhang is working on human PMN purification and chemotaxis. She performed experiments including *in vivo* mouse injury models and *in vivo* experiments of qPCR for mtDNA, nuclear DNA, and bacterial DNA and ELISA for ND6.
She was supervised by Drs. Vlkova and Itagaki.
Funding Support: W81XWH-16-1-0464

Name: **Sarena Ho**
Project Role: Summer Intern
Researcher Identifier:
Nearest person month worked 3
Contribution to Project: Ms. Ho is working on human PMN purification and chemotaxis. She also established bacterial growth rate for *E. coli*, *S. aureus*, and *P. aeruginosa*. She was supervised by Ms. Q. Zhang.
Funding Support: RO3 NIAID/NIH, W81XWH-16-1-0464

Name: **James Harbison, BS**
Project Role: Research Technician Researcher
Identifier:
Nearest person month worked: 11 *Mr. Harbison's effort is shared among all projects
Contribution to Project: Mr. Harbison is working on clinical sample preparations for entire project, which includes human neutrophil, PBMC, plasma, and platelets.
Funding Support: W81XWH-16-1-0464

Name: **Sidharth Shankar,** BS
Project Role: Research Technician
Identifier:
Nearest person month worked: 1
Contribution to Project: *Mr. Shankar's effort is shared among all projects- he replaced Mr. Harbison's position and is working on clinical sample preparations for entire project, which includes human, neutrophil, PBMC, plasma, and platelets.
Funding Support: W81XWH-16-1-0464

Project 2

Name:	**Leo E. Otterbein, PhD**
Project Role:	Principal Investigator
Researcher Identifier:	
Nearest person month worked:	2.4
Contribution to Project:	Dr. Otterbein oversees all of project 2 and is co-director of the overall FPA with Dr. Hauser
Funding Support:	W81XWH-16-1-0464,R01 1420108894-01, R44DK111260-01, R43GM125430-01

Name:	**Ghee Lee, MS**
Project Role:	Research Technician
Researcher Identifier:	
Nearest person month worked:	5
Contribution to Project:	Ms. Lee is working on Project 2 focused on the animal model of pneumonia and profiling the cell infiltrates by CyTOF ± CO treatment.
Funding Support:	. Harvard Medical School/ W81XWH-16-1-0464

Name:	**David Gallo, BS**
Project Role:	Researcher/Lab Manager
Researcher Identifier:	
Nearest person month work:	6
Contribution to Project:	Project 2 focused on *in vivo* model characterization.
Funding Support:	W81XWH-16-1-0464 & NIDDK R44DK111260-01

Name:	**Rodrigo Alves Souza, PhD**
Project Role:	Research Fellow
Researcher Identifier (e.g. ORCID ID):	
Nearest person month work:	6
Contribution to Project:	Mr. Alves participates in the animal modeling
Funding Support:	Self-Funded, Brazil

Project 3

Name:	**Simon Robson**
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0001-6374-0194
Nearest person month worked:	2
Contribution to Project:	Dr. Robson is the Principal Investigator of Project 3. He oversees the work and directs the research to fulfill the goals of the specific aims.
Funding Support:	W81XWH-16-1-0464, 1R01DK108894- 03, 5R01DK103723-04, 1R01AI132389-3, Tizona, 5R01DK104714-04, 5 R21 CA221702-02, 1 R21 AI130800-02, 1 R01 DK 119202-01

Name:	**Maria Serena Longhi**
Project Role:	Assistant Professor of Anesthesia
Researcher Identifier:	0000-0002-4510-1249
Nearest person month worked:	4
Contribution to Project:	Dr. Longhi is responsible for the conduct of human clinical research evaluating the role of CD39 in trauma related immunosuppression.
Funding Support:	W81XWH-16-1-0464, 1R01DK108894-03, 1R21 CA 221702-02, 1 R01 DK 119202-01

Name:	**Yan Wu**
Project Role:	Research Associate
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3
Contribution to Project:	Dr. Wu is responsible for the conduct of animal research evaluating the role of CD39 in trauma related immunosuppression.
Funding Support:	W81XWH-16-1-0464, 5P01HL107152-07, Antagen, 1R01AI132381, Tizona, 5R01CA186566-03

Name:	**Shilpa Tiwari-Heckler, MD**
Project Role:	DFG Scholar
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	Examination of the role of CD39 expression on CD8 and other immune cells in clinical acute lung injury post trauma. Pivotal role in studies of parenteral administration of purified mitochondria, the associated ATP fluxes in provoking systemic inflammation and lung injury
Funding Support:	DFG-T1988-1

Name:	**Dusan Hanidziar, MD**
Project Role:	Postdoctoral/ Anesthesiologist
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	Studies on the role of NKT cell-autotaxin-lysophosphatidic acid pathway in alveolar injury induced by hyperoxia.
Funding Support:	1K08HL141694-01A1

Name:	**Paola de Andrande Mello, PhD**
Project Role:	Research Fellow
Researcher Identifier:	
Nearest person month worked:	3
Contribution to Project:	Dr. de Andrade Mello has become responsible for the conduct of animal research evaluating the role of CD39 in trauma related immunosuppression, taking over responsibilities of Dr. Wu.
Funding Support:	W81XWH-16-1-0464, Antagen, 5 R21 CA221702-02

Project 4

Name:	**Daniel Talmor, MD MPH**
Project Role:	Co-Investigator
Researcher Identifier:	0000-0002-7239-8068
Nearest person month worked:	1
Contribution to Project:	Dr. Talmor is working on Project 4, studying alveolar damage and how DAMPs drive severity of illness and pneumonia risk.
Funding Support:	W81XWH-15-PRMRP-FPA & 1U01HL123022

Name:	**Shahzad Shaefi, MD**
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0002-6832-3282
Nearest person month worked:	1
Contribution to Project:	Dr. Shaefi is working on Project 4, studying the physicochemical alterations in the airway environment and lung danger signaling.
Funding Support:	W81XWH-15-PRMRP-FPA & 1K08GM134220

Name:	**Valerie Banner-Goodspeed, MPH**
Project Role:	Researcher/Clinical Research Manager
Researcher Identifier:	0000-0002-7644-2521
Nearest person month worked:	2
Contribution to Project:	Ms. Banner-Goodspeed is working on Project 4, overseeing subject enrollment and developing preclinical studies.
Funding Support:	W81XWH-15-PRMRP-FPA & 1U01HL123022

Name:	**Ariel Mueller, MA**
Project Role:	Biostatistician-II
Researcher Identifier:	0000-0002-8420-1904
Nearest person month worked:	0.84
Contribution to Project:	Ms. Mueller is working on Project 4, serving as database architect and manager, and overseeing data integrity.
Funding Support:	W81XWH-15-PRMRP-FPA & FAER

Name:	**Julia Larson**
Project Role:	Clinical Research Coordinator
Researcher Identifier:	0000-0003-3499-7921
Nearest person month worked:	2
Contribution to Project:	Ms. Larson is working on Project 4, identifying and recruiting patients, collecting samples and collecting data.
Funding Support:	W81XWH-15-PRMRP-FPA & 1U01HL123009

Name:	**Benjamin Hoenig**
Project Role:	Clinical Research Assistant
Researcher Identifier:	
Nearest person month worked:	2
Contribution to Project:	Ms. Larson is working on Project 4, identifying and recruiting patients, collecting samples and collecting data.
Funding Support:	W81XWH-15-PRMRP-FPA & W81XWH-17-PRMRP-IIRA & 1U01HL123009 & 1R01HL144624 & 1K01HL141637

Project 5

Name:	**James A. Lederer, PhD**
Project Role:	Project 5 Leader
Researcher Identifier:	
Nearest person month worked:	2
Contribution to Project:	Dr. Lederer is the director of project 5. He oversees the individuals working in his laboratory on this project. He also coordinates the collaborative work among the project groups relevant to CyTOF mass cytometry and other systems immunology technologies. As director, he is responsible for administrative details to include quarterly reports, ACURO animal approvals, and report of findings at meetings or by publication.
Funding Support:	W81XWH-16-1-0464

Name:	**Laura Cahill, PhD**
Project Role:	Research Fellow
Researcher Identifier:	
Nearest person month worked:	4
Contribution to Project:	Dr. Cahill is working on project 5 as a CyTOF mass cytometry expert. She is responsible for training HALO collaborators and for implementing human and mouse CyTOF experiments. She also helps with administrative work in the laboratory.
Funding Support:	W81XWH-16-1-0464 /NIHT32GM103702

Name:	**Fei "Sally" Guo, PhD**
Project Role:	Research Fellow
Researcher Identifier:	
Nearest person month worked:	12
Contribution to Project:	Dr. Guo is working on the CpG-ODN immune-therapeutics part of the project. She is responsible for the overseeing the mouse CpG-ODN treatment studies. Her focus is on phenotyping the bone marrow and immune cell changes caused by trauma and how CpG-ODN modulates these responses. Dr. Guo also works on translational human CyTOF clinical studies.
Funding Support:	W81XWH-16-1-0464

Project 6

Name:	**Michael Yaffe M.D., Ph.D.**
Project Role:	Leader, Project 6
Researcher Identifier:	
Nearest person month worked:	1
Contribution to Project:	Dr. Yaffe is the director of project 6. He oversees the individuals working in his laboratory on this project.
Funding Support:	W81XWH-16-1-0464

Name:	**Ingred Rica, Ph.D**
Project Role:	Post-Doctoral
Researcher Identifier:	
Nearest person month worked:	12
Contribution to Project:	Post-doctoral research on project 6
Funding Support:	W81XWH-16-1-0464

Name:	**Brian Joughin, Ph.D**
Project Role:	Staff Scientist
Researcher Identifier:	
Nearest person month worked:	2
Contribution to Project:	Computational Scientist for project 6 and the Computational Core
Funding Support:	W81XWH-16-1-0464

Name:	**Samantha Rosenberg**
Project Role:	Technician
Researcher Identifier:	
Nearest person month worked:	2
Contribution to Project:	Technician for project 6
Funding Support:	W81XWH-16-1-0464

Name:	**Xueyang Yu**
Project Role:	Post-Doctoral
Researcher Identifier:	
Nearest person month worked:	6 (She started in May 2019)
Contribution to Project:	Post-doctoral research on project 6
Funding Support:	W81XWH-16-1-0464

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to report.

What other organizations were involved as partners?
Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS QUAD CHARTS:
see attached

9. APPENDICES
Scope of Work

DAMP-Mediated Innate Immune Failure After Trauma

Log# PR151953, Focused Program Award
W81XWH-16-1-04

PI: Hauser, Carl **Org**: Beth Israel Deaconess Medical Center **Award Amount:** $10,000,000

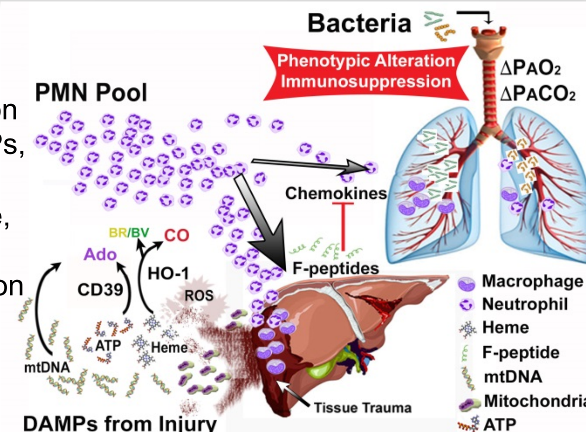
Study Aims

This research program seeks to identify cellular and molecular mechanisms by which wounds predispose to infection. We are focused on three primary objectives: **prevention**, **diagnosis** and **treatment** of the wounded warrior.

Approach

Working as a group we are leveraging the expertise of each member to increase our knowledge and understanding of the innate immune origins of infection. We will dissect the overall cell and molecular biology of post-traumatic infection. Collectively we will: 1) utilize clinically relevant animal models; 2) examine specific cell signaling pathways; 3) employ powerful computational and systems biology methodologies and 4) validate that the stimuli, responses, and cognate mechanisms active in cells and animals parallel that observed in human trauma patients.

Schematic Summary



Proposed mechanism describing the association between injury, mtDAMPs, and susceptibility to infection. In this example, liver crush releases DAMPs into the circulation that influence PMN mobilization. Each of the DAMPs listed elicits a specific response including: **i.** heme catabolism generating CO, **ii.** ATP to adenosine (Ado) **iii.** Formyl (f) peptides and **iv.** ROS generation. The result is immunosuppression in the lung that becomes predisposed to bacterial infection. Six integrated projects.

Progress Timeline and Cost (DC+IDC)

PROJECT		CY1	CY2	CY3	CY4	CY5
1	SA1					
	SA2					
	SA3					
2	SA1					
	SA2					
	SA3					
3	SA1					
	SA2					
	SA3					
4	SA1					
	SA2					
	SA3					
	SA4					
5	SA1					
	SA2					
	SA3					
6	SA1					
	SA2					
	SA3					
Estimated Budget ($M)		**2.04**	**1.99**	**1.99**	**1.99**	**1.99**

Updated: (September 30, 2019)

Goals/Milestones

CY1: Initiate project start, obtain IACUC and IRB protocol approvals

☑Onboard personnel, establish biweekly meetings, finalize SOPs

CY2: Manuscript submission, CyTOF analyses of blood and BAL

☑Acquire human and mouse descriptive and phenotypic data sets

CY3: Establish specific DAMPs that interfere with innate immunity

☑Validate and characterize the identified DAMP(s) in model systems

CY4: Interfere with DAMP effects and restore innate immune responses.

☐Perform rescue experiments in animals and cell models.

CY5: Identify methodologies to diminish infectious morbidities of injury with new innovative treatment options for warfighters after trauma.

☐Enact a systems and computational biology approach to influence standard of care.

Comments: All projects fully operational. A revised plan for patient enrollment has been instituted to rectify with an alternative approach.

Budget Expenditure to date: $5,018,370 TC

STATEMENT OF WORK
PROPOSED START DATE
Site 1: Beth Israel Deaconess Medical Center 300 Brookline Ave, Boston MA, PI Dr. Hauser
Site 2: Brigham and Women's Hospital, 75 Francis Street, Boston MA, Partnering PI Dr. Lederer
Site 2: Massachusetts Institute of Technology,77 Massachusetts Ave, Cambridge MA Partnering PI Dr. Yaffe

PROJECT 1: Determine the role of mtFPs in increased susceptibility to pneumonia (PNA) after trauma.	**Timeline**	**Site 1** (BID)	**Site 2** (MIT)	**Site 3** (BWH)	**% Done**
Project 1, Specific Aim 1: Identify which FPs modulate innate immune function. Participating Projects: 1, 2, 3, 5, 6		**PI: Hauser**			
Subtask 1: Study Ca^{2+} dependent responses to mtFPs, cytokine production, bacterial phagocytosis and killing after FP exposure. Assess effect of mtFPs on a) ROS and degranulation, b) Marker expression and cell kinase phosphorylation Assess susceptibility of *Fpr1*$^{-/-}$ and *Fpr2*$^{-/-}$ mice to pneumonia	1-18 1-24 24-36	Hauser Hauser Hauser Hauser /Otterbein /Robson	Yaffe	Lederer Lederer	75 50
Milestone #1: Manuscript on signaling by endogenous mtFPs.	12	Hauser			100 04/2018
Subtask 2: Assess mtFPs as biomarkers of injury in mice.	1-36	Hauser			0
Milestone #2: Manuscript on mtFP effects on innate immune phenotypes. Presentation at meetings	18	Hauser Otterbein	Yaffe	Lederer	100
Subtask 3: Assess the effects of ND6 on bacterial clearance after trauma Test dependence of ND6 action on FPRs using *Fpr1*$^{-/-}$ & *Fpr2*$^{-/-}$	12-30	Hauser	Yaffe	Lederer	20 100
Milestone #3: Manuscript on mtFP biological effects in context of other DAMPs.	36-60	Hauser/ Otterbein/ Robson	Yaffe	Lederer	50
Project 1, SA2: Investigate how mtDNA modulates lung protection Participating Projects: 1, 2, 3, 5, 6					
Subtask 1: study Mϕ/PMN responses to mtDNA Chemotactic responses ROS, degranulation Expression profiling	6-48 1-12 1-18 12-48	Hauser Hauser Hauser/ Otterbein	Yaffe	Lederer	75 75 75 25
Subtask 2: Assess mtDNA as a biomarker of injury in mice	1-36	Hauser			50
Milestone #4: Manuscript on mtDNA as a biomarker.	12	Hauser/ Otterbein		Lederer	25
Subtask 3: role mtDNA in bacterial clearance after trauma	12-48				25
Determine relationship of mtDNA to infective risk	12-24				25
Study role of chloroquine	24-36				10
Milestone #5: Manuscript on mtDNA as a predictor of infection. Presentation.	30	Hauser			25
Milestone #6: Manuscript on chloroquine as prophylaxis in trauma. Presentation at meeting.	36	Hauser			0

Milestone #7: State of the art review manuscript by Group					0
Project 1, SA3: Define role of mtDAMPs in PNA after clinical trauma Participating Projects: 1, 2, 3, 4, 5, 6	1-60				
Subtask 1: Submit documents for local IRB review.	-3 - Start	Talmor Hauser			100 12/2017
Subtask 2: Submit IRB approval and necessary documents for HRPO review.	0-3	Talmor			100 12/2017
Subtask 3: Submit necessary documents for DoD IACUC approval.	-3 - Start	Hauser Otterbein Robson	Yaffe	Lederer	100 03/2018
Milestone #8: HRPO and IACUC approval received	3-6	Talmor Hauser			100 03/2018
Subtask 4: Recruit, consent, and enroll 100 patients for preliminary study of mtFPs and mtDNA in trauma plasma. Conduct initial study of mtDAMPs concentrations in trauma	6-18 6-18	Talmor/ Hauser Hauser			100 06/2018 100 10/2018
Milestone #9: Establish statistical parameters for powering prospective studies	18	Hauser Talmor			50
Milestone #10: Initial manuscript describing human DAMPs kinetics		Hauser Talmor			100
Subtask 5: Recruit, consent, and enroll 400 patients / subjects to pre-clinical study. Conduct prospective study of mtDAMPs in trauma. Conduct prospective study of innate Mϕ/PMN phenotypes in trauma.	18-60 18-60	Talmor Hauser Hauser/ Otterbein/ Robson	Yaffe	Lederer	25 25 25
Milestone #11: Mid-term manuscript(s) describing human DAMPs kinetics	40	ALL	Yaffe	Lederer	25
Milestone #12: Initial manuscript(s) describing human Mϕ/PMN phenotypes in trauma	40-48	ALL	Yaffe	Lederer	40
Milestone #13: Final manuscript(s) describing human DAMPs time course	60	ALL	Yaffe	Lederer	0
*Milestone #14: Mid-term manuscript(s) on DAMPs and **clinical infection***	48-60	ALL	Yaffe	Lederer	25
Milestone #15: Final manuscripts describing DAMPs kinetics	60	ALL	Yaffe	Lederer	0
Milestone #16: Final manuscripts describing human Mϕ/PMN phenotypes in trauma	50-60	ALL	Yaffe	Lederer	0
*Milestone #17: Final manuscript(s) on DAMPs and **clinical infection***		ALL	Yaffe	Lederer	0
Milestone #18: New grant submissions (NIH, DoD, Foundations)	30-60	ALL	Yaffe	Lederer	0

Project 2: Determine the role of innate immune responses to Heme in the increased susceptibility to infection after trauma. PI: Otterbein	**Timeline**	**Site 1** (BID)	**Site 2** (MIT)	**Site 3** (BWH)	**% Done**
Project 2, SA1: Define cellular and molecular mechanisms by which heme regulates mouse innate immune responses and influences susceptibility to infection. Participating Projects: 1, 2, 3, 5, 6					
Subtask 1:Characterize Heme as a DAMP in PNA	0-12	Otterbein Hauser	Yaffe	Lederer	95
Subtask 2: Establish Dr. Lederer's burn model from BWH at BIDMC	6-12	Otterbein		Lederer	25
Subtask 3: Evaluate CO-releasing molecule 3 (CORM-3)	30-36	Otterbein		Lederer	75
Subtask 4: Optimize CO dosing	12-36	Otterbein	Yaffe	Lederer	100
Milestone #1: mid-term manuscript(s) describing role of heme as a DAMP. Manuscript(s) describing lung phenotyping in liver crush	12-15	Otterbein Hauser	Yaffe	Lederer	90
Project 2, SA2: Determine mechanisms by which CO augments the innate immune response to bacteria in the lung *in vitro* and *in vivo*. Participating Projects: 1, 3, 4, 5, 6					
Subtask 5: Describe effects of CO on Mϕ and PMN *in vivo*	30-42	Otterbein Hauser	Yaffe	Lederer	75
Subtask 6:Study effects of heme on M*ϕ* and PMN phenotype and signaling *in vitro*	42-48	Otterbein Hauser	Yaffe	Lederer	50
Milestone #2: subsequent manuscript(s) describing effects of heme and CO on Mϕ and PMN phenotype and signaling, presentation at meetings.	24-30	Otterbein Hauser	Yaffe	Lederer	75
Milestone #3: Manuscript on effects of purines on inflammasome activation	42-48	Otterbein Robson		Lederer	20
Project 2, SA3: Test whether innate immune function of human innate immune cells from trauma patients can be rescued with CO treatment. Participating Projects: 1, 3, 4, 5, 6					
Subtask 7: Determine the effects of heme on bacterial killing by human Mϕ and PMN. Repeat Subtasks 5-6 in human cells	12-60	Otterbein Talmor Hauser	Yaffe	Lederer	50
Subtask 8: Determine the effects of CO on bacterial killing by human Mϕ and PMN. Repeat Subtasks 5-6 in human cells	12-60	Otterbein Talmor Hauser	Yaffe	Lederer	90
Milestone #4: Final manuscript(s) describing the effects of heme and CO on primary human cells from trauma patients and volunteers	54-60	ALL			0

Project 3: CD39 and Extracellular Nucleotide Signaling Mediate Inflammation and Immune Failure After Trauma. PI: Robson	**Timeline**	**Site 1** (BID)	**Site 2** (MIT)	**Site 3** (BWH)	**% Done**
Project 3, SA1: To examine how loss of CD39 bioactivity, as a consequence of oxidative stress, triggers excessive type-2 purinergic receptor (P2R) signaling, in experimental models. **Participating Projects: 1, 2, 3, 5, 6.**					
Subtask 1: Define role of P2x7 in trauma associated PNA	0-24	Robson	Yaffe	Lederer	60
Subtask 2: Define role of MyD88/mtDNA in P2x7 signaling	9-24	Robson/ Otterbein/ Hauser	Yaffe	Lederer	50
Subtask 3: Is aberrant CD39 scavenging of ATP, globally or targeted on myeloid cells, critical for hyper inflammation and associated suppression of responses to bacteria after injury.	12-24	Robson/ Otterbein/ Hauser	Yaffe	Lederer	85
Subtask 4: Determine how P2x7 signaling in PMN affects the innate immune response to PNA	24-36	Robson/ Otterbein/ Hauser/ Junger	Yaffe	Lederer	20
Subtask 5: Determine how P2x7 signaling in Monocyte-Macrophages affects the innate immune response to PNA	12-36	Robson/ Otterbein/ Hauser	Yaffe	Lederer	60
Milestone #1: Initial manuscript(s) describing role of purinergic signaling in association with mitochondrial DAMPs in trauma/sepsis.	12-36	Otterbein / Robson	Yaffe	Lederer	50
Milestone #2: Mid-term manuscript defining role of purinergic signaling in myeloid cells in trauma/sepsis models.	24-48	Otterbein / Robson	Yaffe	Lederer	75
Milestone #3: Later manuscript defining ATP-purines-heme in inflammasome activation	42-48	Otterbein / Robson	Yaffe	Lederer	45
Project 3, SA2: To study how type-1 purinergic receptor (P1R) signaling by adenosine modulates the innate immune response to infection after trauma, in experimental models. Participating Projects: 1, 2, 3, 5, 6.					
Subtask 1: Optimize dosing of soluble CD39 and exogenous apyrase in liver crush injury and sepsis/PNA.	0-24	Robson	Yaffe	Lederer	75
Subtask 2: Test impacts of elective adenosinergic receptor agonists vs. antagonists in liver injury and sepsis. Review impacts of CO + adenosine in this system.	9-36/60	Robson/ Otterbein/ Hauser	Yaffe	Lederer	75
Subtask 3: Test combinations of pharmacological P2X7 antagonists and optimized adenosinergic drugs in liver injury and PNA.	12-36	Robson/ Otterbein/ Hauser	Yaffe	Lederer	85
Subtask 4: Determine depletion of intracellular ATP in immune cells in the liver injury and PNA.	24-60	Robson/ Otterbein/ Hauser/ Junger	Yaffe	Lederer	60
Subtask 5: Test salutary role of oxygen (O_2) tension and HIF-1 targeting in liver injury and sepsis. Review additional impacts of CO in this system.	12-48/60	Robson/ Otterbein/ Hauser	Yaffe	Lederer	80
Milestone #4: Early mid-term manuscript(s) describing salutary effects of early CD39 administration in trauma/PNA.	18-24	ALL	Yaffe	Lederer	50

Milestone #5: Mid-term manuscript(s) describing role of adenosinergic signaling and CO in trauma/sepsis models.	36-48	ALL	Yaffe	Lederer	75
Milestone #6: Final manuscript(s) describing depletion of intracellular ATP in immune cells in the liver injury and sepsis.	40-60	ALL	Yaffe	Lederer	25
Milestone #7: New grant submissions (NIH, DoD, Foundations)	30-60	ALL	Yaffe	Lederer	15
Project 3, SA3: To characterize and correct kinetics of aberrant immune purinergic responses within blood and alveolar micro-environment of trauma patients. Participating Projects: 1, 2, 3, 5, 6					
Subtask 1: Determine evidence for immediate – day 0-1 - early clinical phase ATP-mediated P2X7 responses.	12-24	Robson/ Talmor	Yaffe	Lederer	90
Subtask 2: Study delayed phase - day 2-5 - hyperadenosinergic responses in blood and BAL.	9-36	Robson/ Otterbein/ Hauser/ Talmor	Yaffe	Lederer	90
Subtask 3: Study kinetics and determine evidence for immediate decreases vs. late depletion of intracellular ATP in blood cells and BAL.	12-36	Robson/ Otterbein/ Hauser/ Talmor	Yaffe	Lederer	75
Subtask 4: Correct aberrant P2X7 signaling and adenosinergic responses by modulating CD39 ectonucleotidase activity	24-60	Robson/ Otterbein/ Hauser/ Junger/ Talmor	Yaffe	Lederer	60
Subtask 5: Restore intracellular ATP (energy charge) in immune cells	12-48	Robson/ Otterbein/ Hauser/ Talmor/ Junger	Yaffe	Lederer	35
Subtask 6: In vitro O_2 and CO studies	24-48	Robson/ Otterbein/ Talmor	Yaffe	Lederer	35
Milestone #7: Mid-level manuscript(s) describing the effects of ATP and Adenosine on DAMP-mediated activation of primary human cells from trauma patients and volunteers	18-24	ALL	Yaffe	Lederer	50-75
Milestone #8: Mid-level manuscript(s) describing the effects of CD39 and hypoxemia/CO on activation of primary human cells from trauma patients and volunteers	36-48	ALL	Yaffe	Lederer	30-55
Milestone #9: Final manuscript(s) on Purines and DAMPs as modulators of inflammation and clinical infection	40-60	ALL	Yaffe	Lederer	30-55
Shared Milestone #10: New grant submissions (NIH, DoD, Foundations)	30-60	ALL	Yaffe	Lederer	15

Project 4: Ventilator-Induced Injury and Lung Immune Response to Infection After Trauma. D. Talmor	Timeline	**Site 1** (BID) **PI:Talmor**	**Site 2** (MIT)	**Site 3** (BWH)	**% Done**
Project4, SA1: Identify and recruit trauma patients with, or at risk for critical illness. Participating Projects: 1, 2, 3, 4, 5, 6					
Subtask 1: obtain IRB/HRPO approval **Subtask 2:** enroll 100 patients and 100 volunteer controls **Subtask 3:** regulatory requirements in place	3-6 3-18 0-60	Talmor/ Hauser			100 11/2016 96 100 continuous
Milestone #1: enroll first 100 patients	15-18				100 5/2018
Subtask 4: enroll a total of 500 volunteers and 500 trauma patients	60				20
Milestone #2: complete the enrollment	60				20
Milestone #3: Initial manuscript(s) describing patient data sets	15-18				0
Milestone #4: Subsequent manuscript(s) describing all patient sets	50-60				0
Project4, SA2: Collect an process data on enrolled patients Participating Projects: 1, 2, 3, 4, 5, 6					
Subtask 1: Collect relevant patient and outcomes data	3-60	Talmor			100 continuous
Subtask 2: Safe/correct collection of samples (275 of 276 samples collected without errors; no patient injury)	3-60				99.8
Milestone #5: Review of monitoring reports activities monitored weekly	monthly				100 continous
Project4, SA3: Physicochemical effects on airway innate immunity Participating Projects: 1, 2, 3, 4, 5, 6					
Subtask 1: Evaluate role of PO_2 in alveolar immune environment protocol written, IRB/HRPO approved as of 12/17/18.	3-15	Talmor/ Hauser/ Otterbein	Yaffe	Lederer	15
Subtask 2: Evaluate role of PCO_2 in alveolar immune Environment protocol written, IRB/HRPO approved as of 12/17/18.	15-30	Talmor/ Hauser/ Otterbein	Yaffe	Lederer	15
Subtask 3: Ventilator strategies and the alveolar immune environment protocol written, IRB/HRPO approved as of 12/17/18.	30-48	Talmor/ Hauser/ Otterbein	Yaffe	Lederer	15
Milestone #6: manuscript on PO_2 and alveolar immunity	15-24	Talmor/ Hauser/ Otterbein	Yaffe	Lederer	0

Milestone #7: manuscript on PCO_2 and alveolar immunity	3 0-36	Talmor/ Hauser/ Otterbein	Yaffe	Lederer	0
Milestone #8: manuscript on ventilator strategies and DAMPS	48-54	Talmor/ Hauser/ Otterbein	Yaffe	Lederer	0
Milestone #9: clinical review of Physicochemical effects on airway innate immunity	54-60	ALL	Yaffe	Lederer	0
Milestone 10: New grant submissions (NIH, DoD, Foundations) 1 submission (Shaefi)					0
Project 4, SA#4: Develop preclinical studies as a foundation for subsequent clinical trials Participating Projects: 1, 2, 3, 4, 5, 6					
Subtask 1: identify variations in biological signaling that may impact clinical care	3-60	ALL	Yaffe	Lederer	0
Subtask 2: design and execute phase 0 RCTs as pilot studies to identify simple interventions that may prevent PNA	3-60	ALL	Yaffe	Lederer	15
Milestone # 11: identify biomarkers suggesting new clinical strategies	12-60	ALL	Yaffe	Lederer	0
Milestone #12: Final manuscript(s) in collaboration with other projects	48-60	ALL	Yaffe	Lederer	0
Milestone #13: New grant submissions (NIH, DoD, Foundations)	54-60	ALL	Yaffe	Lederer	0
PROJECT 5: Systems Immunology Studies on Immunotherapy for Trauma-Associated Immune Dysfunction **PI: Lederer**	**Timeline**	**Site 1** (BIDMC)	**Site 2** (MIT)	**Site 3** (BWH)	**% Done**
Project 5: SA#1: Define the phenotypic influences of trauma on cell-mediated immune responses to lung bacterial infections **Participating Projects: 1, 2, 3, 5, 6**				**PI: Lederer**	
Subtask 1: Establish and validate CyTOF immune profiling panels for mouse lung bacterial infection models to be used for all mouse studies	1-12		Yaffe	Lederer	100 9/2018
Milestone #1: First manuscript comparing time-series lung infection response in mouse burn injury model by CyTOF.	18	ALL	Yaffe	Lederer	70
Subtask 2: Computational model development from burn injury + infection with the Computational Modeling Core (CMC).	18		Yaffe	Lederer	60
Milestone #2: Manuscript reporting computational work	18	Otterbein / Robson/ Hauser	Yaffe	Lederer	20

Subtask 3: Collaborative CyTOF and Luminex studies with Projects 1,2,3, and 6 as part of the Immune Profiling Core (IPC)	1-60	Robson	Yaffe	Lederer	25
Milestone #3: Manuscripts reporting CyTOF findings from mouse studies performed in other projects. Reports based on IPC and CMC activities.	36-60	Otterbein / Robson/ Hauser	Yaffe	Lederer	50
Project 5, SA#2: Identify cellular and molecular features responsible for beneficial immune and anti-microbial function induced by IRM treatment in injured mice **Participating Projects: 1, 2, 3, 5, 6**					
Subtask 1: Screen CpG-ODNs for beneficial activity in mouse burn injury and infection models.	18-24			Lederer	75
Milestone #4: Manuscript reporting comparative CpG-ODN treatment findings	24-30	ALL		Lederer	50
Subtask 2: CyTOF and Luminex phenotyping studies for beneficial CpG-ODNs	30-60	Otterbein / Robson/ Hauser	Yaffe	Lederer	50
Milestone #5: Manuscript reporting systems data from CpG-ODN treatment studies	36-60	ALL	Yaffe	Lederer	50
Subtask 3: Functional studies examining roles of IL-12, IL-17, PD-L1, γδ T cells, TLR9	24-48			Lederer	20
Milestone #6: Manuscript describing results of functional studies and presentation at scientific meetings	48-60	ALL	Yaffe	Lederer	0
Project 5, SA#3: To use CyTOF and Luminex technologies to generate systems immunology data from trauma patients and research samples **Participating Projects: 1, 2, 3, 4, 5, 6**					
Subtask 1: Establish and validate human adaptive and immune cell CyTOF panels for blood and BAL cells	12	ALL	Yaffe	Lederer	100 9/2018
Milestone #7: Validation data showing single-cell phenotyping data by CyTOF for a training sample set (10 patients and normals)	12-24	ALL	Yaffe	Lederer	100 9/2018
Subtask 2: Establish and validate specific Project investigator CyTOF and Luminex assay panels for research samples from all mouse projects.					75
Milestone #8: Validation data showing single-cell phenotyping data by CyTOF for cell preparations for mouse research samples	12-24	Otterbein / Robson/ Hauser	Yaffe	Lederer	100 9/2018
Milestone #9: Establishment of project specific Luminex assay panels for cytokines, chemokines, and DAMPs	12-36	ALL	Yaffe	Lederer	80
Subtask 3: Trauma patient immune profiling by CyTOF and Luminex					70
Milestone #10: Manuscript describing outcome of patient immune profiling data generated by IPC and analyzed by CMC	48-60	ALL	Yaffe	Lederer	60
Shared Milestone #11: New grant submissions (NIH, DoD, Foundations)	30-60	ALL	Yaffe	Lederer	0

Project 6: The Role of Neutrophil Priming, ROS Release, and MK2 Signaling in the Innate Immune Response after Trauma. PI: Yaffe	**Timeline**	**Site 1** (BID)	**Site 2** (MIT)	**Site 3** (BWH)	**% Done**
Project 6, SA1: Determine the specific roles of mtFP, mtDNA, ATP and heme on neutrophil priming versus NADPH oxidase activation at injury sites, in the circulation, and in the lung microenvironment **Participating Projects: 1, 2, 3, 5, 6**					
Subtask 1: Characterize the effects of DAMPs on mouse and human PMN priming.	1-18	Hauser/ Otterbein	Yaffe	Lederer	98
Subtask 2: Isolate PMN from injured animals and trauma patients and assess priming and de-priming by measuring cytokines/ROS Examine serum and BAL fluid for soluble priming/depriming mediators	1-36 12-48	Hauser/ Otterbein/ Talmor	Yaffe	Lederer	50
Milestone #1: First Manuscript describing the effects of DAMP priming on PMN function	12-24	Hauser/ Otterbein/ Talmor	Yaffe	Lederer	100 10/2018
Project 6, SA2: Examine the importance of extracellular ROS released from PMNs at the site of trauma-induced injury as a modulator of innate immune dysfunction					
Subtask 1: Determine the ROS generating complexes in PMN activation and function.	1-24	Hauser/ Otterbein	Yaffe	Lederer	95
Subtask 2: Assess the role of NADPH oxidase in mice with or without NADPH oxidase activity and the ability to clear infection after trauma	24-54	Hauser/ Otterbein	Yaffe	Lederer	50
Milestone #2: manuscript on PMN priming after trauma and the sensitivity to lung infection.	24-36	Hauser/ Otterbein	Yaffe	Lederer	20
Milestone #3: presentation at Shock Meeting and DoD	24-36	ALL	Yaffe	Lederer	50
Milestone #4: Review article describing the role of PMN priming on susceptibility to infection after trauma.	36-42	ALL	Yaffe	Lederer	0
Project 6, SA3: Evaluate the specific role of the p38/MK2 pathway, a master regulator of inflammatory cytokine production, on neutrophil function and injury-induced pulmonary innate immune dyfunction.					
Subtask 1: Test the role of MK2 as a signaling kinase in mouse PMN involved in tissue injury and PMN priming	1-24	Hauser/ Otterbein	Yaffe	Lederer	85
Subtask 2: Study *Mk2*$^{-/-}$ mice in the liver crush+infection model expecting resistance to infection in the absence of MK2.	24-60	Hauser/ Otterbein	Yaffe	Lederer	35
Milestone #5: Manuscript describing role of MK2 signaling in PNA after trauma	48-53	Hauser/ Otterbein	Yaffe	Lederer	0
Milestone #6: presentation at Shock Meeting	48-60		Yaffe	Lederer	0

Milestone #7: Review article summarizing how trauma influences susceptibility to PNA based on the priming state of the PMN	48-60	ALL	Yaffe	Lederer	0
Milestone #11: New grant submissions (NIH, DoD, Foundations)	30-60	ALL	Yaffe	Lederer	0

Projected Quarterly Enrollment for year 1 duplicated each year through year 5

	Yearly Projected Enrollment			
Target Enrollment (per quarter)	**Q1**	**Q2**	**Q3**	**Q4**
BIDMC	50	50	50	50
Target Enrollment (cumulative)		**100**	**150**	**200**

Total 5 year Enrollment : 500 Volunteers and 500 trauma patients