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14. ABSTRACT

Injury resulting from combat trauma dramatically impacts the host immune response. Combat wounds release "Damage Associated Molecular Patterns" (DAMPs) and importantly infection of the host is more common after injury. This program studies how DAMPs alter the immune response to trauma through the study of specific patient populations, highly correlative animal models, computational modeling and statistics and molecular mechanisms of action, all focused toward identifying therapeutic targets. We have assembled a group of laboratories that have each previously studied individual DAMPs and their impact on innate immunity. Project 1 studies mitochondrial formyl peptides and DNA. Project 2 studies heme and its gasotransmitter product, carbon monoxide. Project 3 studies extracellular purinergic signaling. Project 4 studies the alveolar physicochemical environment. Project 5 studies oligonucleotides and global immune phenotypic alterations by injury. Project 6 studies the roles of complement and reactive oxygen species and computational modeling. Each laboratory has made tremendous progress in their own project, which has been amplified through integrated collaborative efforts. Year one allowed us: 1. to establish a biorepository that collected nearly 100 clinical

15. SUBJECT TERMS

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 .

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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 have identified novel therapeutic treatment strategies that we believe 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

Major goals: We have studied the effects of DAMPs released by injury and translated basic discoveries into treatment strategies that will improve outcomes of injured warfighters, particularly related to infection. DAMPs including heme, formyl peptides, DNA, nucleotides and reactive oxygen species contribute to an immunosuppression that sets the host up for invasive infection. As the DAMPS are released by tissue trauma they hamper innate immune surveillance of pathogens at barrier sites like the lung. Our goal has been to identify means to restore normal host responses to bacterial challenge after trauma. In recent work the molecular targets we have identified in our discovery work directed us to test known drugs and repurpose them. They are highly effective at rescuing a poor immune response caused by traumatic injury.

Minor goals: We have developed a pig model of injury ± infection that we believe mimicks what is observed in patients that have been recruited in our trauma center where >20% show increased rates of infection. We have begun analysis of our tissue repository specimens in high throughput Luminex analyses and CyTOF profiling. We have published our work that details the novel 2-hit mouse model and formed the basis for our novel pig model.

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

Major activities, objectives, and results:

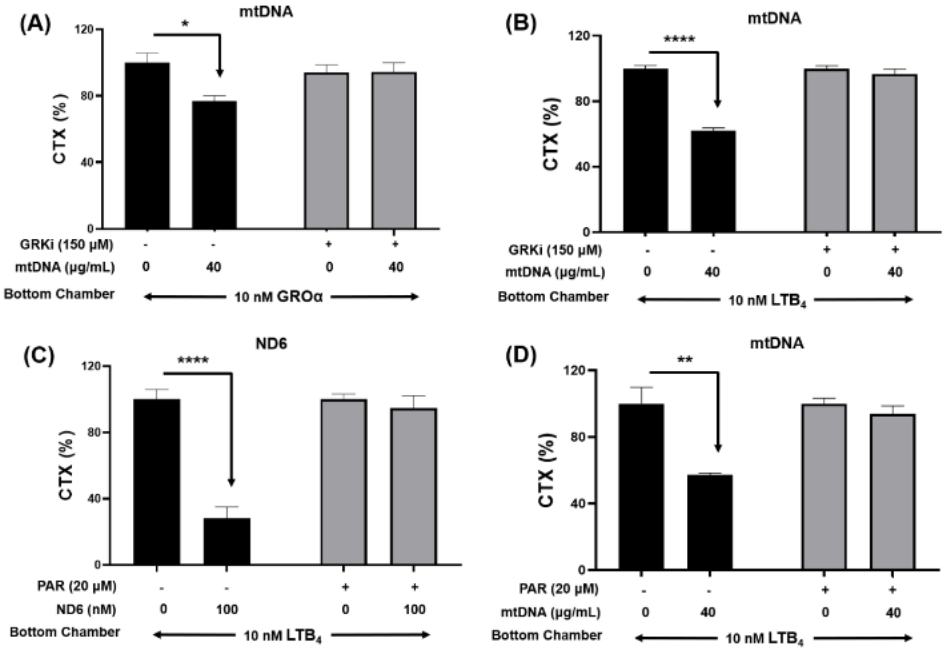
- During this year we have come back strongly from the prior slowdown due to the global pandemic of SARS-CoV-19. We have focused our efforts on broad goals related to interpretation of data originally obtained earlier in the project, especially towards understanding the cell signaling events that lead from the sensation of danger signals at the cell surface to altered immune cellular phenotypes subsequent susceptibility to infection. These are key accomplishments and represent critical deliverables for the project as a whole that we believe can lead directly to improved outcomes for injured warfighters.
- Since this was the major overriding goal of the entire project, we have been very gratified to see our work leading to a novel therapeutic approaches that can benefit injured Warfighters. This work has now been incorporated into a provisional patent (#63256054) entitled **"Methods for treating a trauma patient".** This patent application addresses the use of valproate and paroxetine to diminish the risk of infection in injured warfighters by intervening in trauma-activated cell-signaling pathways that suppress antimicrobial immunity.
- We previously showed that mtDNA circulates after injury and in sepsis. We also showed in the last period that mtDNA is actively released in micro-particles and exosomes. Although we did not find mtDNA suppress his respiratory burst, we did find that it suppresses chemotaxis. Moreover, we showed that this occurs to a novel mechanism involving TLR-9 mediated activation of G-protein coupled receptor kinase 2 (GRK2). Rather than suppressing surface receptor expression, this mechanism acts via histamine deacetylases (HDACs) to impede cytoskeletal re-organization. Thus it impedes both cellular migration and bacterial phagocytosis. We showed this in a series of studies. Moreover we found that pharmacologic inhibition of either GRK2 or HDACs has beneficial effects on neutrophil function in the setting of trauma. However, these benefits were only seen with pre-therapy, which is impractical in Trauma.
- In a subsequent group of experiments we showed that GRK2 inhibition (using several inhibitors, but most of our work was done with paroxetine, because it is a well-known, safe generic drug) as well as inhibition of HDACs (using valproate, another safe generic drug) reversed the effects of trauma on neutrophil (PMN) anti-microbial function even in post-therapy.
- We then showed in-vivo in a murine model of injury (liver crush) followed by tracheal injection of staph. aureus that the loss of normal anti-microbial function generated by the liver crush was fully mitigated by the combination of valproate and paroxetine (VALPAR) even when given ***after*** trauma. We believe that this approach will be therapeutic in the injured Warfighter and we are currently moving ahead two large animal (porcine) models and have applied through the CDMRP expansion award program for funding to begin early phase human safety trials.

What was accomplished under these goals?

During this period we continued to focus on our ground breaking translational studies of the role of G-protein receptor kinases in mediating the effects of danger signals and subsequent SIRS-mediators on neutrophil responses to bacterial infection.

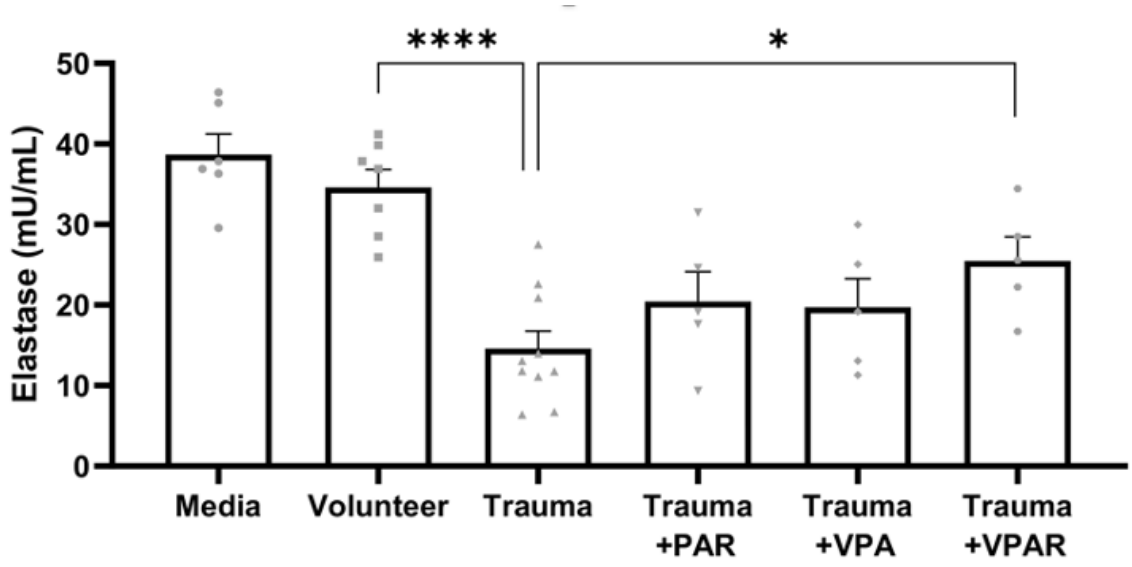
- Because of our success with small animal (mouse) trauma models we have initiated development of pig studies (in collaboration with Project 2) that recapitulate the pulmonary infection-after-injury paradigm. Here, we have now developed methods for bronchoscopic inoculation of Staph Aureus into specific pig bronchi, and have now begun to calibrate this model for subsequent use with VAL-PAR. This will be reported out subsequently.

- Continuing our work from the prior period, we showed that mtDNA and mtFPs induced CTX suppression via activation of GRK2.



In **Figure 1** above, we see that mtDNA induced suppression of PMN CTX to both (**A**) GROα and (**B**) LTB_4 were rescued by a pre-clinical GRK2 inhibitor (GRK*i*). Suppression of PMN CTX to LTB_4 after exposure to (**C**) ND6 and suppression of CTX to (**D**) LTB4 by mtDNA were also both rescued by the widely used GRK2 inhibitor Paroxetine (PAR).

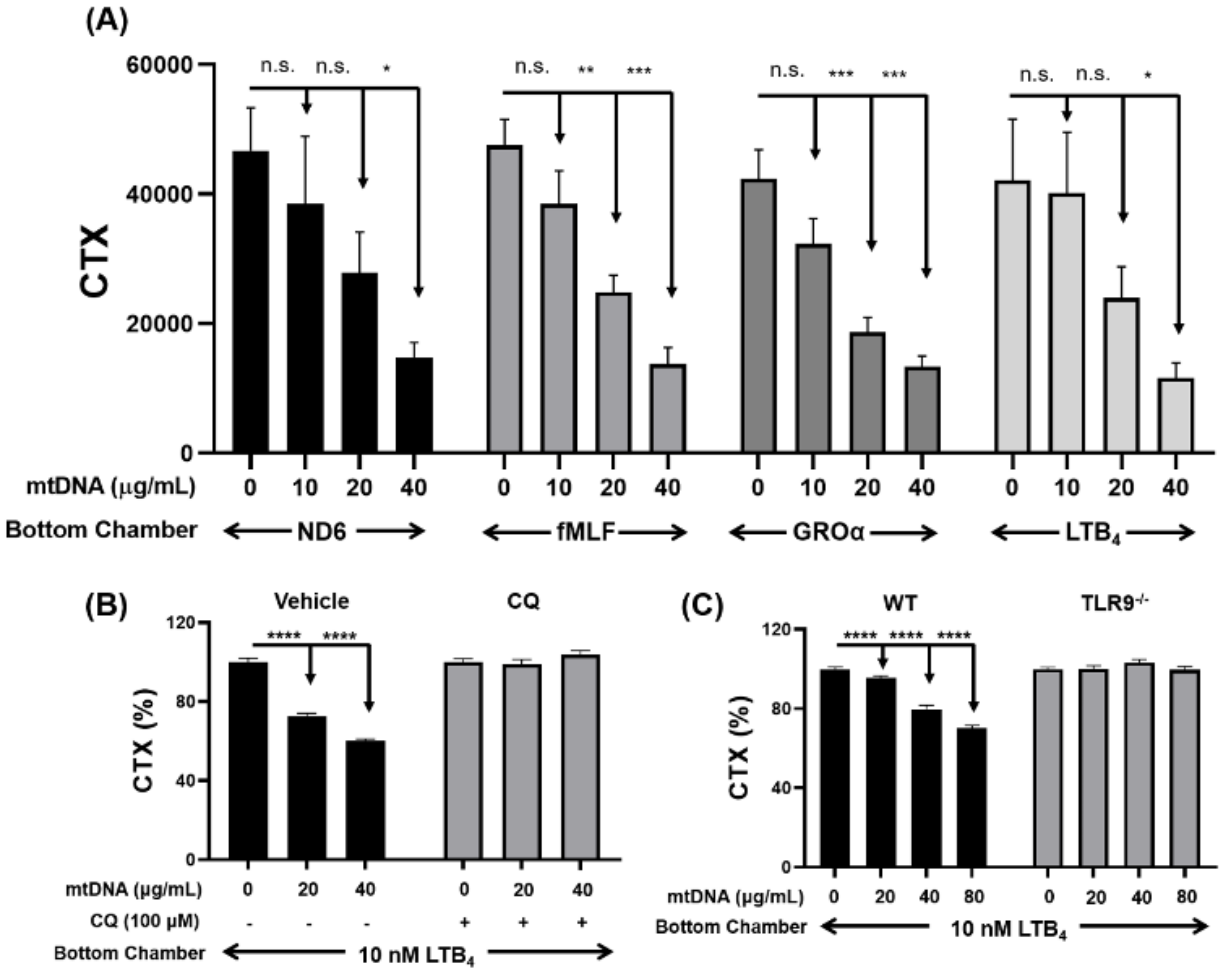
- Subsequently, we have confirmed that that plasma and wound fluids from trauma patients markedly reduce induction of neutrophil extracellular traps (NETs) and that this effect is reversed by VAL-PAR.



Here **(Figure 2)** we see NET production (NETosis, by elastase assay) of human PMN incubated with 10% plasma from healthy volunteers (n=7), from trauma patients (n=10) or in RPMI media only (n=6) for 25 min at room temperature. NeETosis was initiated with 100nM PMA. In some applications, we applied 20 μM paroxetine (PAR), valproic acid (VAR), or VAL + PAR (VPAR) for 30 min at room temperature prior to PMA exposure. As we had shown in the prior period, tretreatment of PMN with 10% trauma patients' plasma

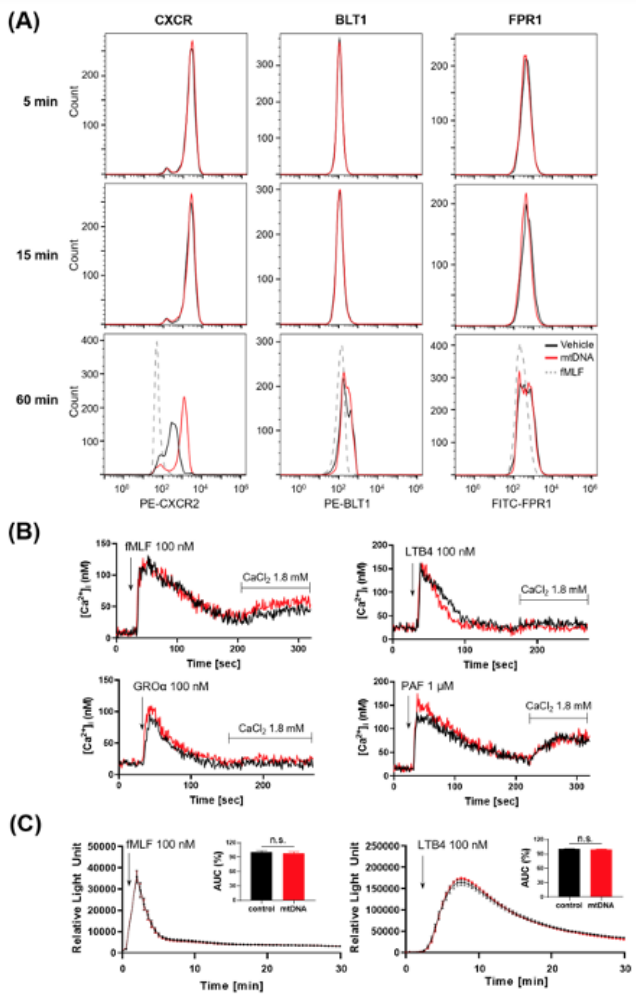
significantly reduced NETs production by PMA. Here, we see that VPAR, even in post treatment, significantly rescues that important PMN anti-microbial effector function.

- Following up on our work in the last reporting period, we now proved specifically that mtDNA suppresses PMN chemotaxis via an endosomal mechanism that involves toll-like receptor (TLR)-9.



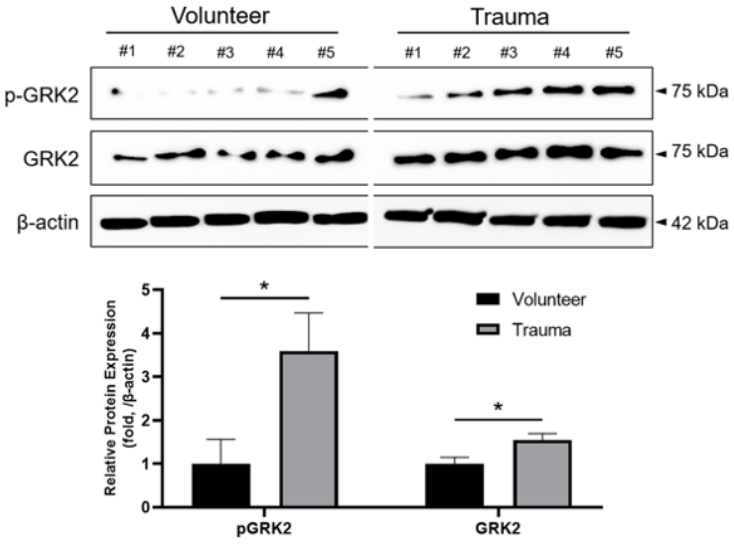
In **Figure 3**, we see (**A**) mtDNA suppresses chemotaxis to multiple GPCR stimuli including formyl peptides (ND6, fMLF) chemokines (GROα) and lipid agonists (LTB_4) in a dose-dependent fashion. (**B**) The suppressive effect of mtDNA is blocked by chloroquine, showing dependence on endosomal acidification. (**C**) The suppressive effect of mtDNA on chemotaxis is completely absent in PMN from $TLR9^{-/-}$ mice.

- Further evaluating the mechanisms by which DAMPs change, we demonstrated that mtDNA does not change GCPR expression or receptor bias in human PMN. It is crucial in showing that it is not due to the typical β-arrestin mechanism expected, but rather reflects a new previously unknown pathway.



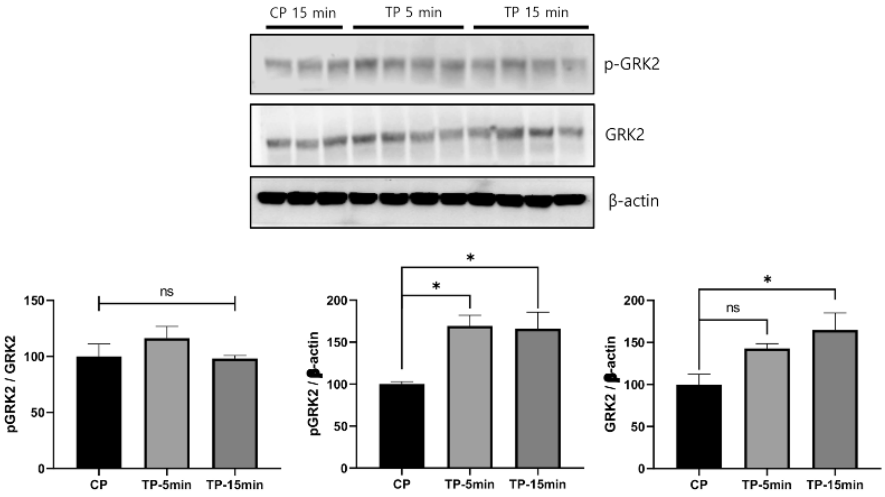
In **Figure 4** (left), we see that (**A**) mtDNA fails to suppress surface expression of FPR1, BLT1 and CXCR2 at 5 and 15 minutes. At 60 minutes there is actually a slight increase in CXCR2 expression after PMN exposure to mtDNA. All of these receptors are regulated by fMLF (third row). (**B**) Here, we see that cytosolic calcium ($[Ca^{2+}]_i$) responses to fMLF, LTB_4, GROα and PAF in Ca^{2+}-free (then followed by Ca^{2+}-replete) media are identical before (black trace) and after (red trace) exposure to mtDNA. In (**C**), we note that receptor- dependent respiratory burst (RB) responses to fMLF and LTB4 are unaffected by mtDNA.

- Next, we restudied and clearly confirmed that Trauma induces phosphorylation and expression of PMN GRK2 in human trauma patients.



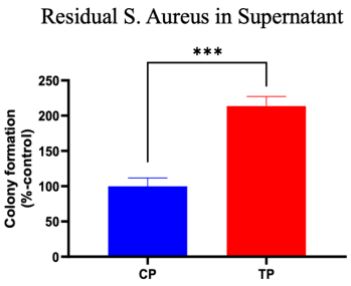
In **Figure 5** (left) we see that trauma clearly induces phosphorylation and expression of PMN GRK2 in humans. PMN were isolated from the peripheral blood of trauma patients (day 0-1 post injury, ISS>15) and from pre-op elective surgery patients. Cells were banked and stored as part of our ongoing biorepositry program. Here, PMN from patients chosen at random (n=5 from each group) were unfrozen and thawed. Cells were then lysed and western blots were performed for GRK2 and $pGRK2^{Ser670}$ using beta-actin for housekeeping. * indicates $P<0.05$ by unpaired t-test.

- Next, in order to continue our mechanistic work on induction of GRK activity, we demonstrated that plasma from trauma patients clearly causes both activation (phosphorylation) and increased expression of GRK2.

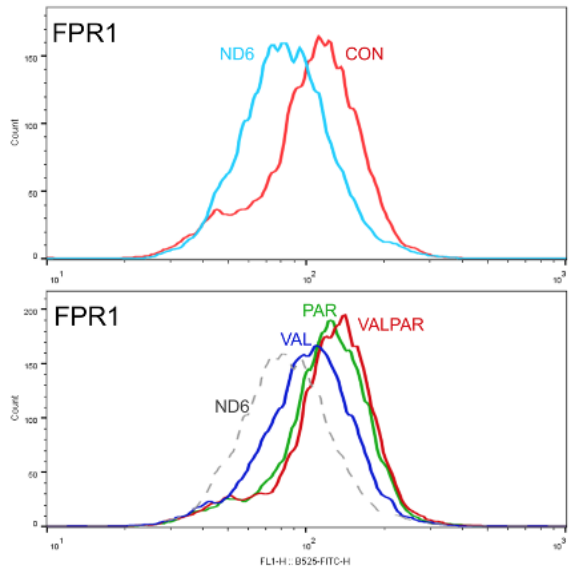


In **Figure 6 (left)** we examined p-GRK2 and GRK2 expression in PMN from healthy volunteers after incubating those cells with healthy volunteer plasma or trauma patients' plasma (n=3-4/group). PMN GPR2 and p-GRK expression were both significantly increased after treatment with trauma patients' plasma. The p-GRK2/GRK2 ratio did not change.

- Seeking to create models with which to demonstrate the ability of VAL-PAR to reverse post-traumatic neutrophil deficits, we next demonstrated incubation of normal PMN in trauma plasma decreases their ability to kill Staphylococcus aureus.



In Figure 7 (left) volunteer PMN were incubated with 10% volunteer or trauma plasma for 25 min and then washed and incubated with S. Aureus for another 30 min. Incubation in trauma plasma markedly increased the number of residual bacteria in the supernatant, indicating that the trauma plasma environment suppresses phagocytosis and killing of bacteria.



- To evaluate the effect of the trauma environment on neutrophil receptor expression and determine whether treatment with VAL and PAR can rescues receptor suppression, we next challenged PMN with the mitochondrial formyl peptide ND6

(Figure 8, left), and then examined surface expression of the formyl peptide receptor (FPR1) with and without VAL, PAR and VALPAR by flow cytometry. In the upper panel, we see that normal PMN expression of FPR1 (red trace) is markedly suppressed by exposure to ND6 (blue trace). Post-treatment with PAR and VAL (**after** ND6 exposure) brought expression back towards normal density. VAL+PAR returned expression to normal levels.

- Mitochondrial DNA is a potent suppressor of PMN migration. But although mtDNA is present at high concentrations immediately after injury, it is subsequently cleared and then levels are only moderate in the plasma. We therefore wanted to confirm that trauma patient's plasma decreased PMN migration. In

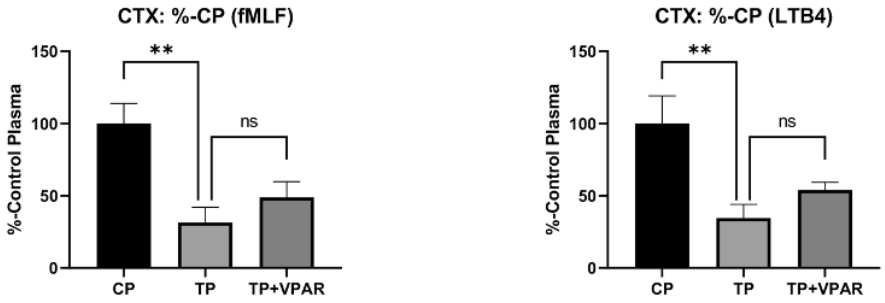
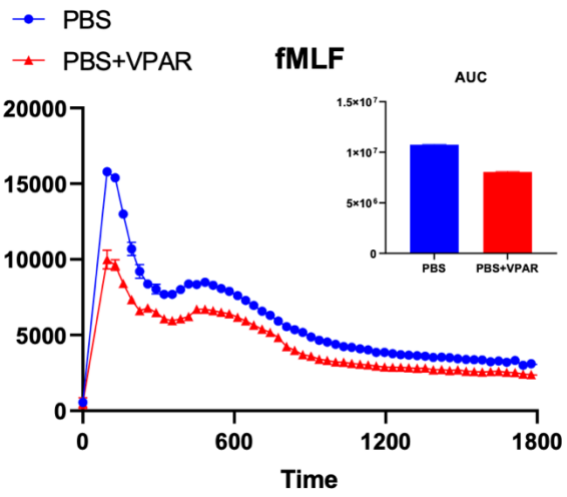


Figure 9 (left) we see PMN from healthy volunteers that were treated with healthy volunteer versus trauma patient plasma show suppression of chemotaxis to both fMLF (left) and LTB_4 (right) by trauma plasma (TP). Post-treatment with VAL+PAR for 30 min showed a trend towards increasing PMN chemotaxis to both agonists, although it did not reach statistical significance in this small experiment. This needs to be repeated.

- Last, to use VAL and PAR safely in clinical trauma patients suspected of infective risk, we should be certain these drugs will not cause inflammatory "overshoot" of PMN function that might lead to organ failure. In **Figure 10 (left)** we therefore studied treatment of normal PMN with VAL and PAR, and showed that they do not intrinsically cause an increase in respiratory burst. Here, cells were incubated in buffer only and then treated with fMLF to initiate RB. VALPAR did not increase RB (the slight decrease was not significant). We then found similar results when PMN were pre-incubated in healthy volunteer plasma, and whether the terminal stimulation was with fMLF or PMA. Suppression of RB by trauma plasma is reversed by the VALPAR combination.

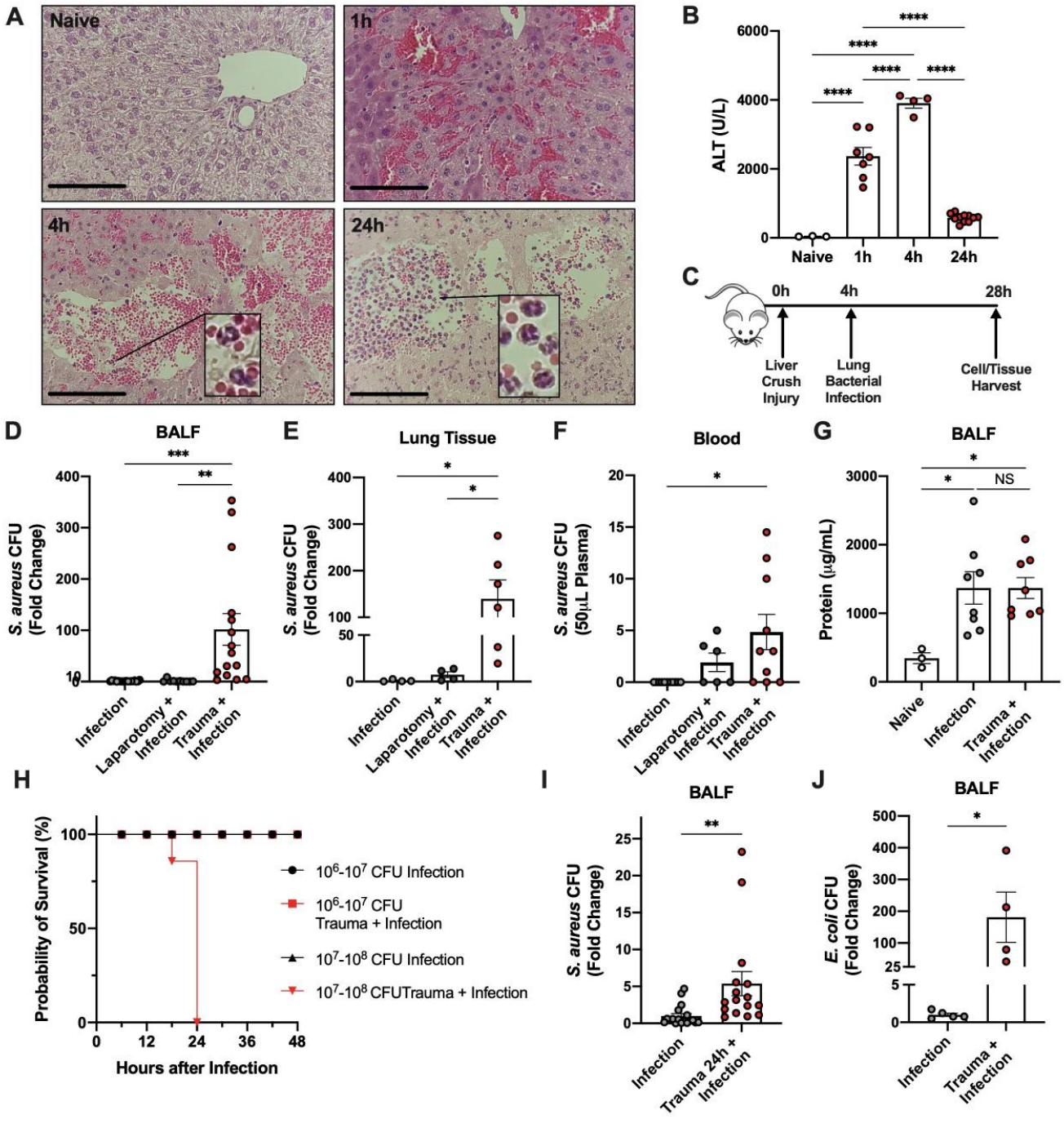


PROJECT 2 (OTTERBEIN): Heme Metabolism and The Innate Response to Trauma and Infection

Major activities, objectives, and results.

- Since liver is the solid organ that is damaged most commonly as a result of blunt trauma, we developed and standardized a model of non-lethal blunt liver trauma in mice. One hour after liver crush, clotting was observed both macroscopically (not shown) and microscopically (**Fig. 1A**). Four hours after liver crush, PMN rise in the circulation and accumulate in the injured liver (**Fig. 1A**). Standardization of the severity of the crush injury was confirmed by measuring serum alanine aminotransferase (ALT) levels over time (**Fig. 1B**).
- To test whether liver injury alters host bacterial clearance from the lung, mice were inoculated with 10^6-10^7 colony forming units (CFU) of *S. aureus* into the lungs 4 hours after liver crush injury as depicted in **Fig. 1C**. *S. aureus* is a clinically relevant pathogen that commonly causes early post-traumatic pneumonia in at-risk trauma subject. Twenty-four hours after inoculation, animals were euthanized and a bronchoalveolar lavage (BAL) was performed to measure cell and bacteria counts.- Mice without surgery and mice with laparotomy only, cleared bacteria effectively from the lung at 24h. Mice subjected to liver crush however, showed 100-fold more bacteria in the BAL and the lung tissues at 24h when compared to sham controls (**Fig. 1D**-**E**). Similarly, higher *S. aureus* counts were detected in the blood of infected mice with liver crush injury (**Fig. 1F**). Lung infected mice showed increased accumulation of protein in the BAL as a marker of lung injury, but no significant differences were observed between infected mice with or without liver injury (**Fig. 1G**). However, mice challenged with a higher dose of *S. aureus* in the presence of liver crush all died while all survived the high dose inoculation in the absence of liver crush (**Fig. 1H**).
- When bacterial inoculation was delayed 24h post-trauma we found similar deficiencies in animals' ability to clear bacteria as compared with controls (**Fig. 1I**). We also noted that liver injury had the same effect on Gram-negative *E. coli* clearance as *S. aureus* (**Fig. 1J**). Taken together, the results show that an increased susceptibility to bacterial infection similar to that observed in human trauma can be simulated by liver trauma in mice.
- Traumatic injury can lead to bleeding and release of heme. Under these circumstances, heme can act as a potent DAMP and can contribute to further cellular injuy. Healthy humans have very low or undetectable levels of free heme in the plasma (<1µM, **Fig 2A**). In contrast, plasma from trauma patients collected one day after traumatic injury showed a 10-fold rise in free serum heme (**Fig. 2A**). Variability may reflect the time the first blood sample was collected relative to the time the trauma occurred. Similarly, mice subjected to a liver crush injury showed a time-dependent rise in circulating free heme from a baseline of 25µM that peaked at >75µM within 30 minutes after injury and returned to baseline by 4h (**Fig. 2B**). The difference in peak heme levels between human and mouse may be attributed to the type of injury and/or differences in physiological and metabolic response times between human and mice. Given the unpredictability of human trauma, it is challenging to generate a specific kinetic of heme release in trauma patients and thus we grouped plasma collected from trauma patients into batches. It is likely that peak plasma heme levels occur early after injury depending on the severity and cause. Hemopexin (Hpx) is a serum protein whose principal role is to scavenge and bind free heme. After liver crush, Hpx levels in plasma increased and peaked at 24h (**Fig. 2C**). Elevated Hpx levels most likely reflect an acute phase mechanism to clear free heme and thus limit its activity as a DAMP. Heme is well known to be metabolized by heme oxygenases. So next we measured inducible HO-1 as a response to the increase in free heme elicited by liver crush injury. HO-1 expression increased in both the injured and uninjured liver lobes at 4h at the mRNA level, but was significantly higher in tissue from the injured lobe (**Fig. 2D**). HO-1 protein expression peaked at 24h in the injured liver lobes. (**Fig. 2E**). Additionally, both circulating WBC and bone marrow (BM) PMN showed increased HO-1 expression 24h after liver crush (**Fig. 2F-G**). *Hpx*$^{-/-}$ mice were far worse at clearing bacteria from the lung after liver crush than were wild-type injury plus infection mice (**Fig. 2I**). Injured mice, inoculated with bacteria and treated with Hpx showed that mice cleared bacteria to levels equivalent to infected mice without a liver crush injury (**Fig. 2J**). Data not shown describe in detail the role of heme on TLR2 expression as a mechanism of action (Lee et al *JCI Insight* 2021).

Figure 1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Trauma + Infection
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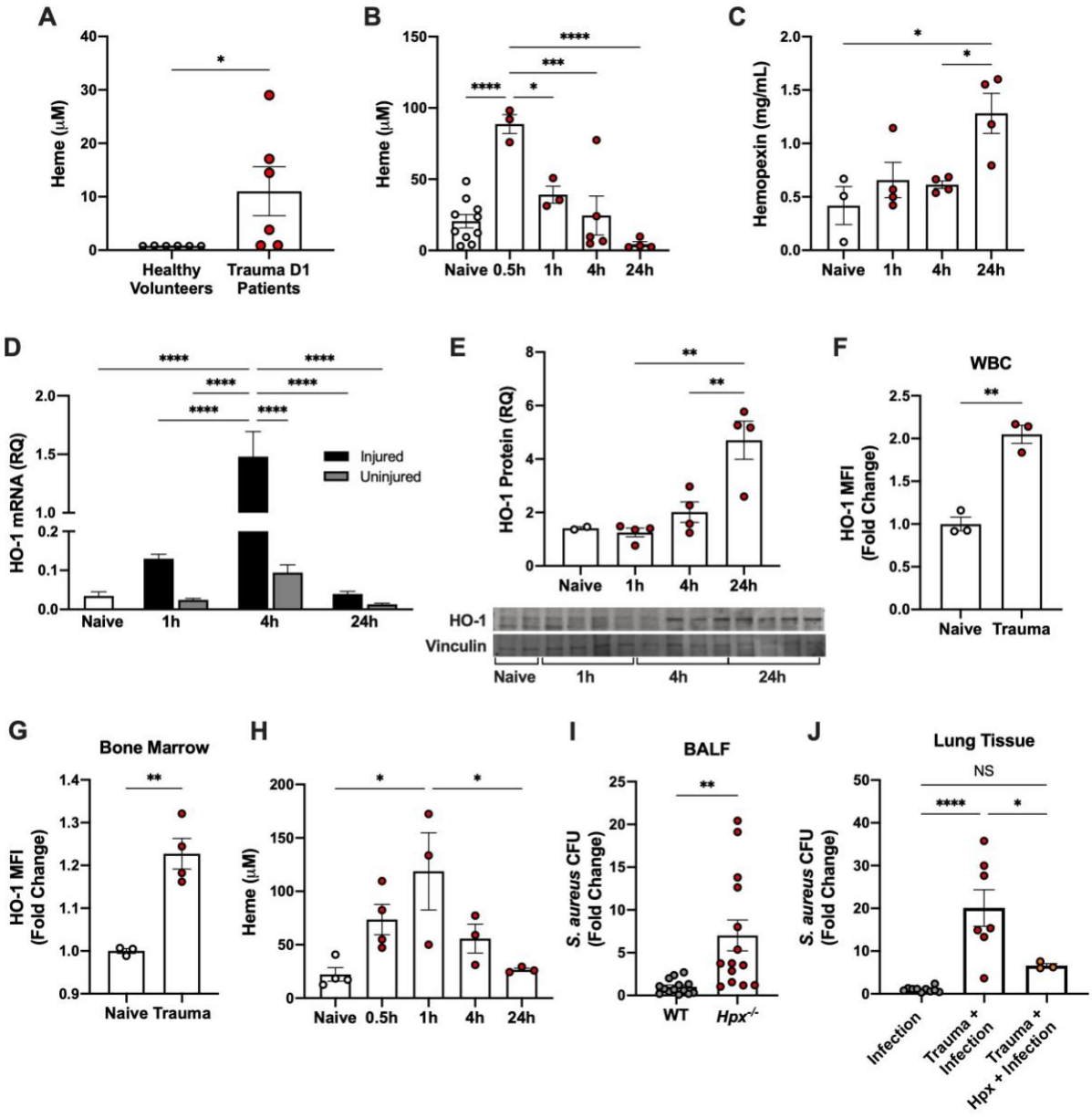
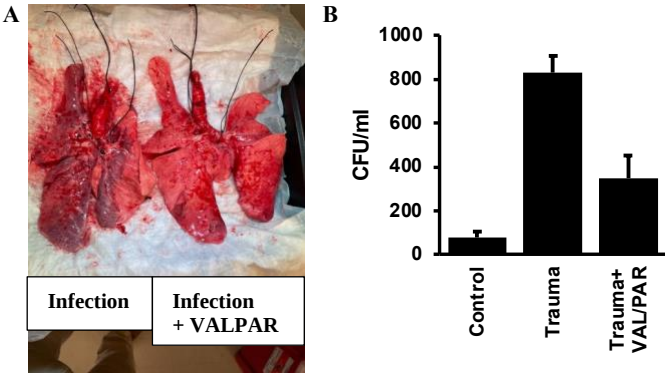
Figure 2



- We have now developed a pig model of pseudo trauma (liver slurry)+*S. aureus* infection that mimics that observed in the mice. Preliminary data show increased susceptibility to lung infection after trauma compared to infection alone and in collaboration with project 1, we have begun testing of VALPAR and exciting early data suggest a benefit of treatment (**Figure 3A-B**). Fig A shows gross images of pigs infected with S. Aureus (10^{12} cfu) after trauma vs pigs treated with VALPAR after trauma and before infection (n=2/group, *p<0.05).



What was accomplished under these goals?

The work presented above (Figure 1 and 2) are some of data that resulted in a high impact publication in Lee et al., JCI Insight; 2021 Oct 22;6(20):e150813. Figure 3 shows where the next body of work for the next year that will provide the preclinical data in that will help design the clinical trial.

PROJECT: #3 (ROBSON): CD39 and extracellular nucleotide signaling mediate inflammation and immune failure after trauma

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 models.
  To complete studying a model of trauma where liver-derived mitochondria have been injected into wild-type and CD39 KO mice and systemic and local immune responses have been assessed before and after bacterial instillation.
- To study how type-1 purinergic receptor (P1R) signaling by adenosine modulates the innate immune response to infection after trauma, in experimental models.
  To complete the studies aimed at defining the impact of CD39 gene knockout on disease outcome in mice injected with liver-derived mitochondria before and after bacterial instillation.
- To characterize and correct kinetics of aberrant immune purinergic responses within blood and alveolar micro-environment of trauma patients.
  To complete testing and analysis of peripheral blood and BAL-derived mononuclear cells of trauma patients and correlate these parameters with indices of disease activity.
- To study nature of liver injury in a mouse model of hyperoxia.

What was accomplished under these goals?

During this timeframe, we have completed testing the *in vitro* effects of liver-derived mitochondria on peripheral blood-derived CD8 T cells. We have previously noted that CD8 cells, obtained from the peripheral blood of wild type (WT) mice, upregulate exhaustion markers including CD39 and PD-1 when exposed to liver-derived mitochondria. To define whether these effects were mediated by ATP, which we have previously shown being produced by liver mitochondria, we exposed PBMCs from WT mice to anti-CD3/CD28 *in vitro* in the presence of ATP, adenosine or intact mitochondria. Exposure to mitochondria or ATP resulted in a comparable increase in the frequency of $CD39^+$, Granzyme B^+ and Tim-3^+ CD8 cells. Exposure to liver-derived mitochondria resulted also in increased frequencies of PD-1^+TIM-3^+ and IL-10^+ cells among CD8 T lymphocytes. These effects were not observed after PBMCs exposure to adenosine. We have also noted decreases in the frequency of PD-1^+ cells among circulating CD8 lymphocytes in the presence of liver-derived mitochondria, ATP or adenosine (**Figure 1A-F).**

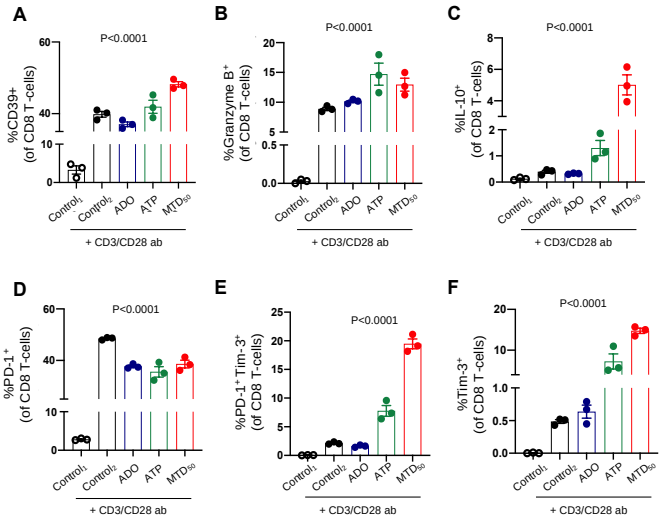


Figure 1. Effects of ATP and liver-derived mitochondria on CD8 T cell immune phenotype. Peripheral blood-derived CD8 cells from WT mice (n=3) were exposed to anti-CD3/CD28 beads and their phenotype in the presence of vehicle, adenosine, ATP and liver-derived mitochondria was assessed 24 hours later. Analysis was performed using One-way ANOVA test.

Increase in the frequency of Granzyme$^+$, IL-10$^+$, Tim-3$^+$/PD-1$^+$ and Tim-3$^+$ CD8 T cells noted in the presence of liver-derived mitochondria was abrogated, at least to some extent, upon addition of apyrase (**Figure 2B-C, E-F**). Decrease in PD-1$^+$ CD8 T lymphocytes observed in the presence of mitochondria was limited upon addition of apyrase (**Figure 2D**).

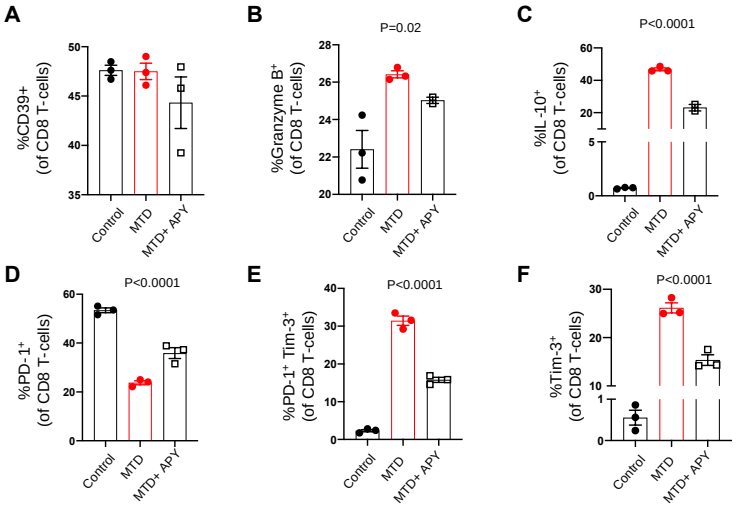


Figure 2. Effects of apyrase and liver-derived mitochondria on CD8 T cell immune phenotype. Peripheral blood-derived CD8 cells from WT mice (n=3) were exposed to anti-CD3/CD28 beads and their phenotype in the presence of vehicle, apyrase (APY) and liver-derived mitochondria was assessed 24 hours later. Analysis was performed using One-way ANOVA test.

Overall, these data indicate that ATP obtained from liver-derived mitochondria could induce CD8 T cells with 'exhausted' immune phenotype.

During this reporting period we have also completed characterizing the kinetics of immune purinergic responses within blood and alveolar microenvironment of trauma patients.

We have previously noted that CD8 cells, obtained from the peripheral blood of trauma patients, are characterized by impaired cytotoxicity (i.e., low Granzyme B expression levels) and display features of immune exhaustion, as reflected by high proportions of PD1$^+$Tim-3$^+$ cells. In this reporting period we analyzed the phenotype of circulating CD8 T cells derived from trauma patients, based on the development of injury-mediated SIRS (**Figure 3A**). We found that the frequency of CD39$^+$ cells amongst CD8$^+$ lymphocytes obtained from trauma patients with SIRS was significantly elevated, compared to control patients (**Figure 3B**). Notably, CD8$^+$ T cells obtained from trauma patients with SIRS exhibited increased proportion of PD-1$^+$Tim-3$^+$ cells and reduced frequency of Granzyme B$^+$ cells, when compared to controls (**Figure 3C-D**). These findings suggest that dysfunctional CD8$^+$ T cells, characterized by an exhausted immune phenotype and reduced cytotoxic capacity, are associated with injury-induced SIRS. This state of immune dysfunction might increase the susceptibility to infection and thus prolong the critical illness seen in trauma patients.

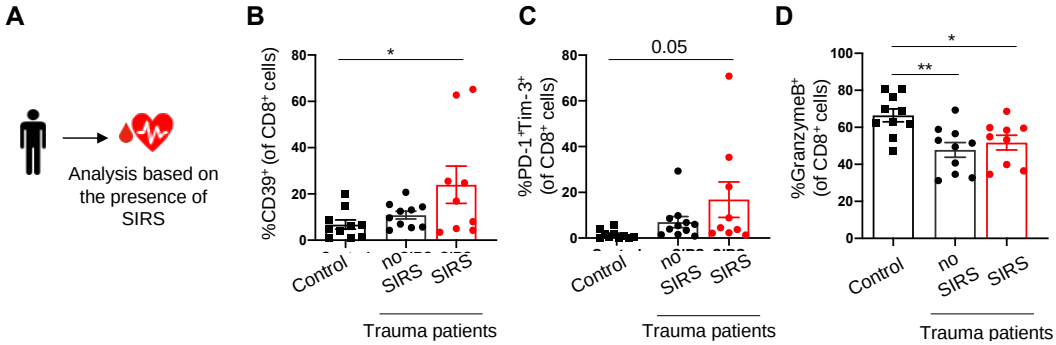


Figure 3. Frequency of CD39$^+$, PD1$^+$Tim-3$^+$ and Granzyme B$^+$ cells was measured by flow cytometry in peripheral blood-derived CD8 cells from controls and trauma patients without and with SIRS. Analysis was performed using One-way ANOVA test. * P≤0.05.

A manuscript reporting the above described data has been submitted; and is currently in revision at *Thorax*.

Distinct hepatic phenotypes develop in a mouse model of hyperoxic lung injury. We have investigated whether mice exposed to prolonged hyperoxia develop hepatic dysfunction and injury. We found that C57BL/6 mice exposed to hyperoxia (72 hours, FiO2 >95%), develop mixed steatosis, preferentially in hepatic acinar zone 1 **(Figure 4)**. No immune cell infiltrates or areas of necrosis were found. Statistically significant elevations of AST (p=0.002) and LDH (p=0.007) were found in serum samples from hyperoxia group, indicating the presence of tissue injury **(Figure 5)**.

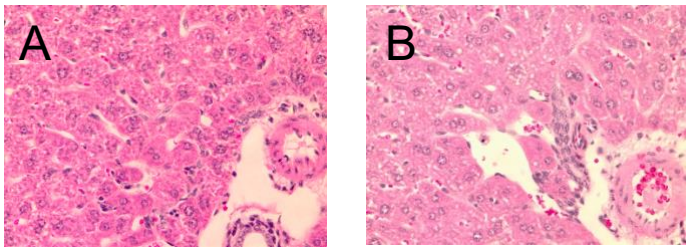


Figure 4. Periportal areas of the liver in normoxia and hyperoxia. A) normal parenchyma B) mixed steatosis. H&E staining, magnified X400

Figure 5. Serum levels of AST and LDH in normoxia (n=10) and hyperoxia (n=10). A) AST elevation in hyperoxia (p=0.002) B) LDH elevation in hyperoxia (p=0.007)

PROJECT # 4 (TALMOR) Ventilator-Induced Injury and Lung Immune Response to Infection After Trauma

Major Activities, objectives, and results
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 48 we have enrolled 102 trauma patients and 114 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.
Subtask 4 (months 0-60): Enroll a total of 500 trauma patients and 500 volunteer controls.
Through month 48 we have enrolled 102 trauma patients and 114 volunteer controls.

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 48 we have collected approximately 440 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 and launched an interventional protocol that will allow us to test this.
Subtask 2 (months 15-30): Evaluate role of PCO2 in alveolar immune environment
We have designed and launched an interventional protocol that will allow us to test this.
Subtask 3 (months 30-48): Ventilator strategies and the alveolar immune environment.
We have designed and launched an interventional protocol that will allow us to test this.

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 begun investigating cell signaling and timing of infection.
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.

What was accomplished under these goals?

- 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)
- Oriented multiple clinical research assistants and research fellows, including training in good clinical practice, human subjects research, and research conduct. 30-Oct-2020 (AL), 30-Oct-2020 (AT).
- 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 231 patients through 11-Mar-2020. Closed to enrollment.
  Collected 453 biological specimens through 11-Mar-2020. Closed to enrollment.

Progress Detail:

- ***Obtained IRB/HRPO approval.***
  - Local IRB approval for primary project granted 26-May-2016; HRPO approval granted 04-Nov-2016.
  - Local IRB approval for ancillary project collecting discarded samples approved 09-Mar-2020; HRPO approval granted 14-Jul-2021.

- ***Patient enrollment.***

  From 10/1/16 through 10/30/2021 we have enrolled 232 patients.

  • Trauma Cases n=109 (73.4%) Male, average age 53.2 years (18-94)
  241 approached
  109 consented
  95 trauma cases from whom samples were collected

  • Surgical Controls n=122 (52.6%) Male, average age 59.4 years (28-83)
  261 approached
  122 consented
  71 surgical controls from whom samples were collected

  • Phase 0 n=1
  1 approached
  1 consented
  1 surgical controls from whom samples were 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 10/30/21, we have collected 538 whole blood samples and 117 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 #2, enroll first 500 patients in each group by 60 months. However, the number of eligible trauma patients has been lower than projected. Steps were taken to widen our enrollment pool, and increase catchment rates. In March 2020, all non-COVID human subjects research was suspended at BIDMC. Due to subsequent erratic COVID-19 surges, the decision was made to close the protocol to enrollment. Current focus is on analysis of the specimens collected to date, as well as developing collaborations with other groups with complementary biorepositories.

- ***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. One patient has been enrolled to date. The study was put on hold in March 2020 due to the COVID-19 pandemic, and has since been closed to enrollment.

PROJECT 5: (LEDERER): Systems Immunology Studies on Immunotherapy for Trauma-Associated Immune Dysfunction

- Note: Project 5 was redefined as a Core for assistance with immune profiling of repository samples.

PROJECT #6: (YAFFE) :The Role of Neutrophil Priming, ROS Release, and MK2 Signaling in the Innate Immune Response after Trauma

Major activities, objectives and results

- 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.
- Examine the importance of extracellular ROS released from PMNs at the site of trauma-induced injury as a modulator of innate immune dysfunction.
- 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 dysfunction.

What was accomplished under these goals?

- Evaluated ROS production, intra and extracellularly, by $MK2^{-/-}$ neutrophils in response to fMLP and PMA. Their primability was also analyzed after incubation with GM-CSF (**Figure 1**).
- Measured intra and extracellular ROS production by $MK2^{-/-}$ neutrophils in response to C5a, a potent neutrophil activator (**Figure 2**).
- Examined chemotactic activity of $MK2^{-/-}$ neutrophils towards established chemoattractants (**Figure 3**).
- Evaluated calcium influx in the $MK2^{-/-}$ neutrophils, once stimulated by priming agents and agonists of ROS production and chemotaxis (**Figure 4**).
- In collaboration with Project 2, $MK2^{-/-}$ mice were submitted to the liver crush injury model followed by bacterial intratracheal instillation and bacterial clearance in the lungs was evaluated 24h later (**Figure 5**).
- Reran CyTOF analysis for identification of potential markers that could differentiate immunosuppressive low-density neutrophils in trauma patients (**Figure 6**).

Neutrophil priming refers to the ability of certain cytokines such as TNF, IL-1alpha and beta, and platelet activating factor (PAF) to enhance ROS production by the neutrophils NADPH oxidase when the neutrophils receive a second 'activating' stimulus such as fMLP or CD11b/CD18 engagement. Neutrophil priming by itself, can cause release of variable amounts of the metalloproteinase MMP-9, however, in the absence of an activating stimulus, it does not result in ROS release in solution-phase neutrophils. Consequently, it has been proposed that this 'silent' priming event corresponds to the first 'hit' in the 2-hit model of multi-system organ failure, with the second hit corresponding to an activating stimulus that leads to neutrophil adherence to tissue endothelial cells, massive ROS release, and secondary tissue injury. We have shown previously that neutrophil priming by TNF-alpha and IL-1 results from activation of the p38MAPK-MK2 pathway, while priming by PAF results from activation of the Erk1/2MAPK pathways. Furthermore, we showed that neutrophil activation by fMLP could be inhibited by ~70% using p38MAPK inhibitors, while activation by CD11b/CD18 engagement could not. This observation has important therapeutic implications for targeting the p38MAPK- MK2 pathway by small molecule inhibitors in warfighters to limit trauma-induced innate immune dysfunction since mt-FP may be a critical DAMP molecule responsible for this phenotype.

Knowing the potential importance of MK2 pathway for trauma-related immune dysfunction, and the major role that neutrophils play in immune surveillance, we hypothesized that MK2 is responsible for many of the neutrophil functions, and it is directly implicated in trauma-related neutrophil dysfunction. To test this hypothesis, we start characterizing murine bone marrow MK2$^{-/-}$ neutrophils. One of the main neutrophil functions is to release reactive oxygen species (ROS). In order to evaluate the role of MK2 in ROS production by neutrophils, we isolated bone marrow neutrophils from wild type (WT) and MK2$^{-/-}$ mice, and measured their ROS production after priming (GM-CSF + fMLP) or after potent agonist stimulation (PMA). The lack of MK2 reduces primability and ROS production by neutrophils (**Figure 1**).

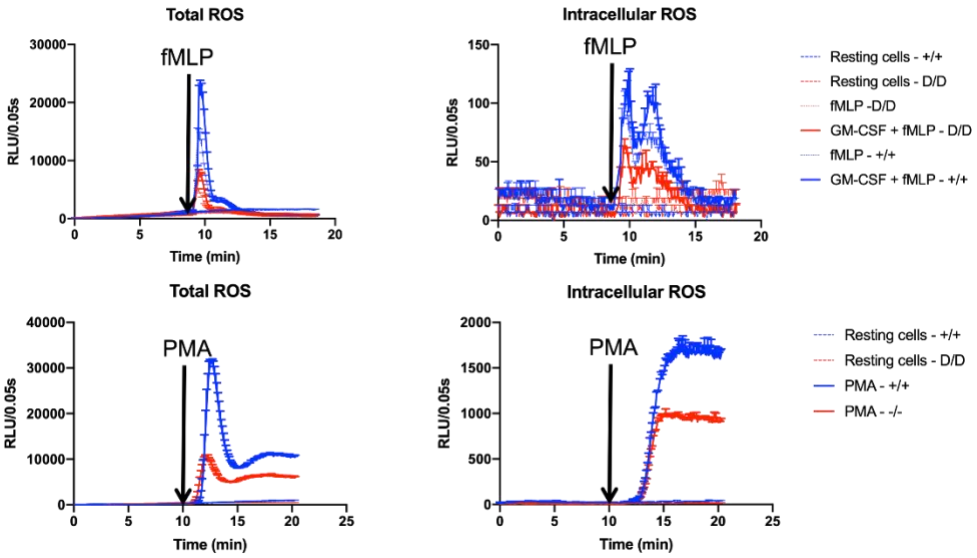


Figure 1. GM-CSF primed bone-marrow MK2$^{-/-}$ neutrophil ROS production after fMLP stimulation. Primary mouse BMNs from WT and MK2$^{-/-}$ mice were primed with GM-CSF (24 ng/mL) for 10 min and incubated with 10 μM fMLP or stimulated with 500 nM PMA. HRP was added to the wells to detect total ROS; or superoxide dismutase (SOD) and catalase, but without HRP to detect intracellular ROS. Luminol-dependent chemiluminescence was followed over time.

A substantial portion of priming activity is present in the plasma of trauma patients early after injury, but not in plasma from uninjured controls. A likely mediator responsible for this early PMN priming activity appears to be C5a as a result of complement activation. Here, we tested the effect of C5a on ROS production by MK2 -proficient or deficient neutrophils (**Figure 2**). C5a acted as potent agonist in both concentrations tested. MK2$^{-/-}$ neutrophils consistently produced less intracellular and total ROS compared to WT neutrophils.

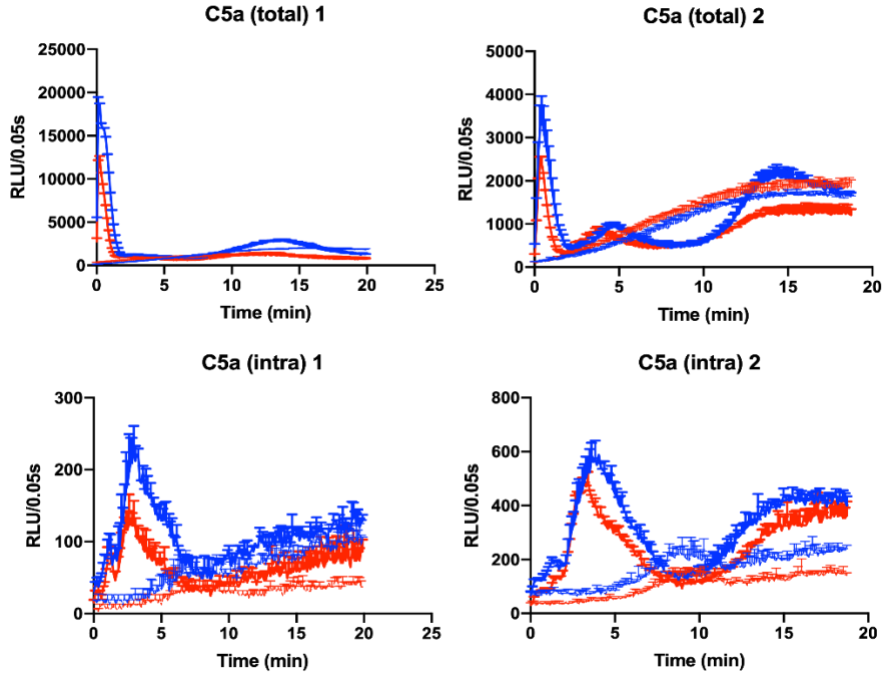


Figure 2. Bone-marrow MK2$^{-/-}$ neutrophil ROS production after C5a stimulation. Primary mouse BMNs from WT and MK2$^{-/-}$ mice stimulated with 10 or 100 nM C5a. HRP was added to the wells to detect total ROS; or superoxide dismutase (SOD) and catalase, but without HRP to detect intracellular ROS. Luminol-dependent chemiluminescence was followed over time.

Neutrophils are the first responder cells to arrive at the local of tissue injury. To do so, one of their important functions is their capability to sense and migrate towards chemoattractants. We next compared the capability of chemotaxis between WT and MK2$^{-/-}$ neutrophils towards two potent chemoattractants, fMLP and LBT4 (**Figure 3**). MK2$^{-/-}$ neutrophils migrate less towards fMLP and LTB4 compared to WT neutrophils.

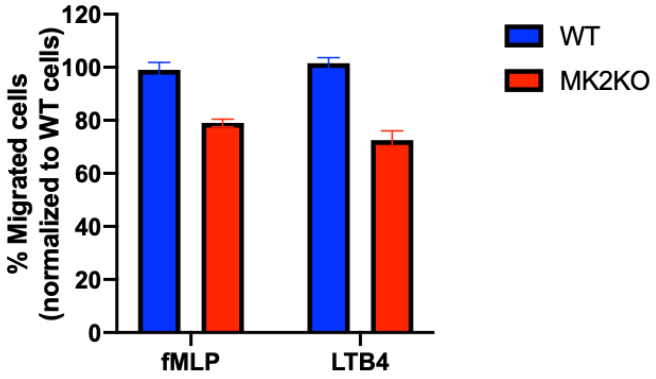


Figure 3. Chemotactic activity of MK$^{-/-}$ neutrophils. Primary mouse BMNs from WT and MK2$^{-/-}$ mice were added to the top chamber of transwells and exposed to fMLP and LTB4 in the media of the lower chamber. After 90 min, the number of migrated cells in the lower chamber was assessed by measurement of DNA content against a standard curve.

Most of neutrophil functions involve activation of signaling pathways dependent on Ca^{2+} mobilization from RE to the cytosol. Therefore, we investigated whether the reduction in ROS release and chemotaxis in MK2$^{-/-}$ neutrophils could be explained by a reduction in Ca^{2+} mobilization. Interestingly, despite the marked reduction of neutrophil functionality when MK2 is lacking, no differences were observed between MK2$^{-/-}$ and WT neutrophils in terms of Ca2+ mobilization (**Figure 4**).

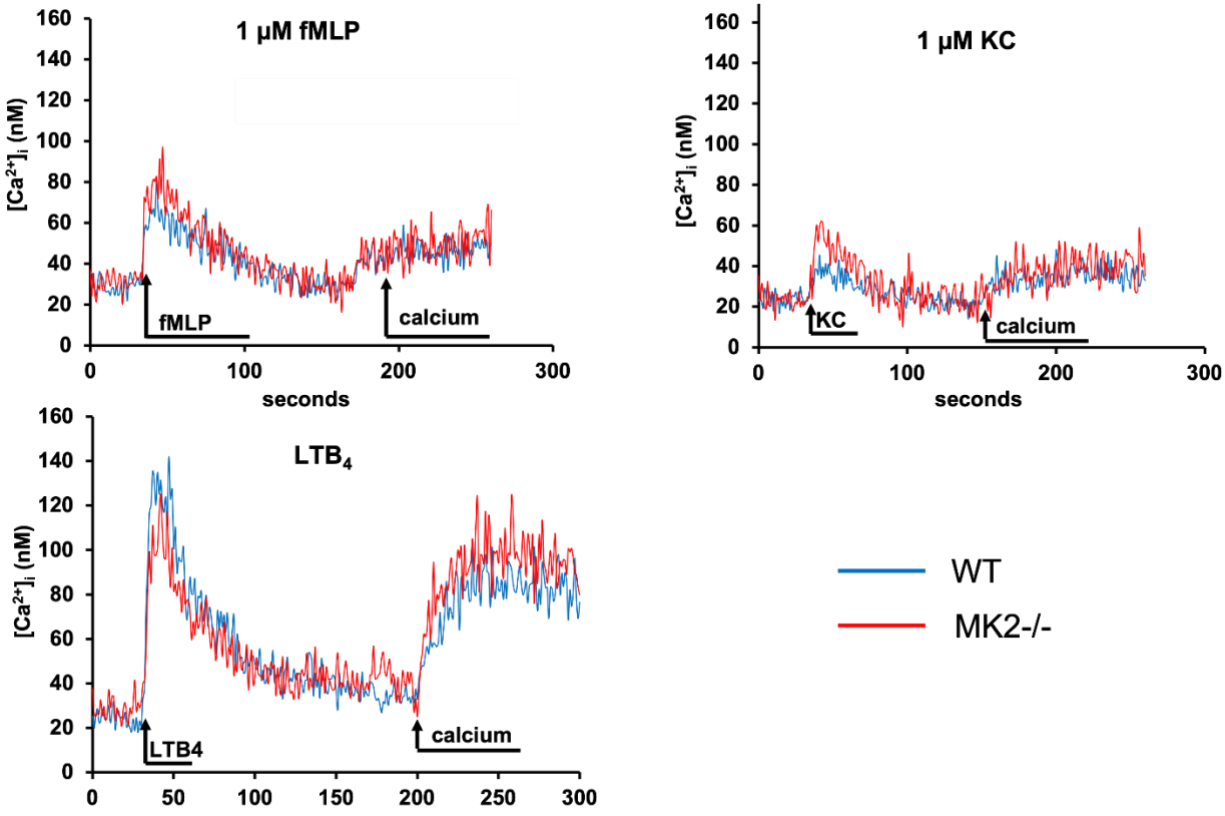


Figure 4. Calcium mobilization in bone-marrow MK2$^{-/-}$ neutrophils. BM neutrophils were stimulated with, fMLP, KC and LTB4 to investigate changes in intracellular Ca^{2+}. Arrows indicate times when agonists were added.

In collaboration with Project 2, we next tested if the lack of MK2 would affect bacterial clearance in lungs of mice submitted to the liver crush injury model. WT and MK2$^{-/-}$ mice had their livers crushed 4h prior to intratracheal bacterial instillation. 24h later, bronchoalveolar fluid (BALF) was collected and CFU was counted in the BALF to assess bacterial growth. As shown in Figure 5 and in collaboration with Project 2 in the characteized mouse model of trauma+infection, MK2$^{-/-}$ mice showed more bacterial clearance without liver crush, but the liver injury did not protect those mice from bacterial growth.

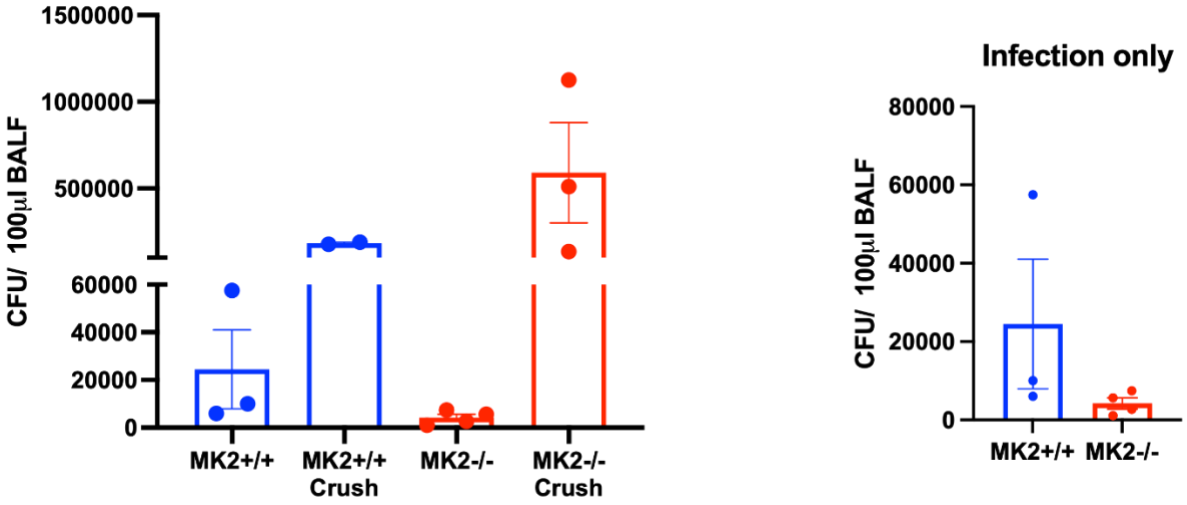


Figure 5. MK$^{-/-}$ mice were submitted to the liver crush injury model. WT and MK2$^{-/-}$ mice had their livers crushed 4h prior to intratracheal bacterial instillation. 24h later, bronchoalveolar fluid

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.

How were the results disseminated to communities of interest?

Nothing to report

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

As we enter the approved extension of the project we will be entirely focused two areas: 1.) the large animal model and testing of VALPAR and, time permitting carbon monoxide and 2.) completion of our immune profiling of our repository samples and the effects of the wounded warrier plasma provided by the SC2i program from USUHS.

Project 1

- We will continue to study the role of global importance of G-protein receptor kinases (GRKs) in the immune suppression ('anergy', 'tolerance') characteristic of Trauma. We will use both small and large animal (pig) models to further define mechanisms of effect and develop both pre-clinical and clinical approaches to their use in care of the injured warfighter.
- We will continue to perform PMN functional studies using bio-repository specimens from the HALO Project, focusing on the suppression of antimicrobial function by injury. We have moved ahead with bio-repository-wide assessments of GRK2 activity in PMN and this huge effort should bear fruit soon.
- We will continue to assess the association of PMN GRK activation with functional suppression in bio-repository specimens from the HALO Project and correlate those findings with clinical infections as well as organ dysfunction syndromes in the source patients.
- Continue to pursue translational studies using porcine models in collaboration with Dr. Otterbein in order to test therapeutics defined in HALO Project 1 for use in subsequent programs targeting systemic infections.
- We will pursue translational studies using murine and porcine models in collaboration with Dr. Otterbein in order to test the therapeutics defined in HALO Project 1 for use in subsequent programs targeting wound infections.
- We have begun CyTOF evaluation of the cellular bio repository. This long-term effort is now beginning to bear fruit.

Project 2

- Large animal model characterization and formal testing of VALPAR and carbon monoxide (second lead) to rescue immune failure after traumatic injury, We are using a modified pseudotrauma model (data shown above, Projects 1&2) where liver homogenate is injected into the peritoneum. This will be converted to a true liver injury where 20% of the liver is crushed prior to inoculation with bacteria. Gram positive and Gram negative bacteria will be tested. We will perform standard dose ranging with VALPAR and/or carbon monoxide as well as altering the timing of bacterial inoculation relative to the injury.
- We will assist with the human repository sample processing with FAC/flow validation studies.

Project 3

- Continue studies on the role of CD39 in exosome formation and modulation of inflammation by differential generation of miRNA. Continue studies of mitochondrial dysfunction and cellular exhaustion.
- Continue to study involvement of B cells in hyperoxic lung injury.
- Continue to study involvement of complement and innate immunity in hyperoxic lung injury.

- Extend studies of CD39-CD73 soluble recombinant proteins in cecal ligation and puncture models as well as extending studies to liver crush plus infection model. If results are encouraging, the plan is to scale up production of recombinant proteins and perform further testing in large animals (swine).
- Completion of human trauma CyTOF studies; with Projects 1, 2 and 5.

Project 4

SOW Project 4 – Specific Aim 1: Identify and recruit trauma patients with, or at risk for critical illness.

Subtask 3 (months 0-60): Regulatory requirements in place.

- We will continue to submit all necessary reports to the local IRB and HRPO.

Subtask 4: enroll a total of 500 volunteers and 500 trauma patients

- Given changes in surgical case scheduling and trauma patient presentation during the SARS-CoV-2 pandemic, the study was closed early. Current focus is on analysis of the specimens collected to date, as well as developing collaborations with other groups with complementary biorepositories.

SOW Project 4 – Specific Aim 2: Collect and process data on enrolled patients

Subtask 1: Collect relevant patient and outcomes data

Subtask 2: Safe/correct collection of samples

- Data collection and specimen collection has been completed.

SOW Project 4 – Specific Aim 3: Physicochemical effects on airway innate immunity

Subtask 1: Evaluate role of PO2 in alveolar immune environment

- To address subtask1, we designed a third study group, intubated critically ill patients, who will undergo ventilator manipulation and serial sampling. This protocol is approved by the local IRB and by the HRPO, and the protocol is posted publicly (https://clinicaltrials.gov/ct2/show/NCT03993002). Enrollment began in the 4th quarter of year 3, but has since been closed due to COVID-19.

SOW Project 4 – Specific Aim 4: Develop preclinical studies as a foundation for subsequent clinical trials

Subtask 1: identify variations in biological signaling that may impact clinical care

- We will continue to work closely with consortium team members to review laboratory findings and run analyses to uncover variations correlated with clinical outcomes.

Subtask 2: design and execute phase 0 RCTs as pilot studies to identify simple interventions that may prevent PNA

- Based on the results of analyses described above (Project 4, SA4, subtask 1), we have begun to formulate study design capsules for pilot studies.

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.

4. IMPACT:

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

Our work has had considerable generalized impact upon current understanding of human immunobiology

- **What was the impact on other disciplines?**

Nothing to report

- **What was the impact on technology transfer?**

- We have initiated a collaboration with NICOYA industries. This company builds detectors based upon surface plasmon resonance that can simultaneously detect and analyze multiplexed groupings of biologic mediators. We believe that this approach can lead to miniaturized (hand held) detectors that will be useful in far forward applications for the purposes of detecting immunologically important conditions like SIRS and sepsis. Also, we are also trying to extend our current experience with sepsis and SIRS to include detection and prediction of prognosis in COVID-19 as well as influenza and potentially other emerging infections.
- A provisional patent was submitted on the VALPAR concept.

- **What was the impact on society beyond science and technology?**

Nothing to report.

5. CHANGES/PROBLEMS:

- **Changes in approach and reasons for change**

For Year 5, we discontinued project 4 and have changed project 5 to a fee for service program. Owing to the decrease in trauma patients associated with the COVID-19 pandemic and also to the difficulty in enrolling patients with current IRB limitations due to the pandemic, we no longer plan to enroll new patients at BIDMC, but rather to focus on working with live cells stimulated with plasma and wound fluids supplied by the SC2i group.

Project 5 has had difficulty in achieving the systems biology deliverables in the setting of the COVID pandemic and has lost key fellows who were involved in the project. These personnel will be also be slow to be replaced due to travel restrictions. The program directors therefore decided that the collaborative can fulfill its original deliverables best using the Lederer laboratory as a resource focusing on acquisition of "big data" results using Luminex and CyTOF Technologies. That way we can also divert funds to commercial laboratories to complete deliverables as needed.

- **Actual or anticipated problems or delays and actions or plans to resolve them…from YR4**

See above – the pandemic had a significant impact on productivity by all projects and has resulted in refocusing of the program as a whole as we move our preclinical findings into large animals and plans for human application.

- **Changes that had a significant impact on expenditures** None noted

- **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:

Journal publications.

Other publications, conference papers, and presentations.

- Lee GR, Gallo D, Alves de Souza RW, Tiwari-Heckler S, Csizmadia E, Harbison JD, Shankar S, Banner-Goodspeed V, Yaffe MB, Longhi MS, **Hauser CJ**, Otterbein LE. Trauma-induced heme release increases susceptibility to bacterial infection. JCI Insight. 2021 Oct 22;6(20):e150813. doi: 10.1172/jci.insight.150813. PMID: 34520397.

- Sandler N, Ho H, Draxler DF, Bain C, Smith JA, **Hauser CJ**, Gruen RL, Myles PS, Medcalf RL. Characterisation of Plasma Mitochondrial DNA, MMP-9 and Neutrophil Elastase in Patients Undergoing Coronary Artery Bypass Grafting: Effects of Tranexamic Acid and Postoperative Pneumonia. Heart, Lung and Circulation (2021)

- Longhi MS, Vuerich M, Wang N, Kalbasi A, Moss AC, Cheifetz AS, Robson SC. 'Unconjugated bilirubin modulates Th17-cell immunity by curbing glycolysis-related genes'. Oral and poster presentation at presentation at Virtual IMMUNOLOGY2021. 14-MAY-2021 (presentation date); abstract published in *J Immunol*, 2021, 206 (1 Supplement) 109.02.

- Longhi MS, Wang N, Kalbasi A, Vuerich M, Manickas-Hill ZJ, Hecht J, Shaefi S, Robson SC. Aberrant CD39 levels and regulation mark COVID-19 late stage'. Oral and poster presentation at Virtual IMMUNOLOGY2021. 12-MAY-2021 (presentation date); abstract published in *J Immunol*, 2021, 206 (1 Supplement) 62.02.

- Wang N, Vuerich M, Kalbasi A, Shaefi S, Robson SC, Longhi MS. Limited TCR repertoire and aberrant CD39 regulation mark late-stage COVID-19. Oral presentation at The Liver Meeting 2021. 12-15-NOV-2021.

- Graham JJ, Wang N, Kalbasi A, Bonder A, Patwardhan V, Robson SC, Longhi MS. Selective inhibition of estrogen receptor alpha restores regulatory T cell functional phenotype in autoimmune hepatitis. Late-breaking abstract The Liver Meeting 2021. 12-15-NOV-2021.

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- Savio LEB, Robson SC, Longhi MS. Ectonucleotidase modulation of lymphocyte function in gut and liver. Front Cell Dev Biol, 2021; 8: 621760. Acknowledged Federal Support.

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- Biofluids From Trauma Patient Suppress Neutrophil Netosis and Associated Respiratory Burst. Jinbong Park, Hyo In Kim, Leo E. Otterbein, Kiyoshi Itagaki, Carl J. Hauser. Podium presentation at the 44th annual conference on SHOCK.

Website(s) or other Internet site(s)
N/A

Technologies or techniques
N/A

Inventions, patent applications, and/or licenses

- During this period we filed provisional patent number 63256054 entitled **"Methods for treating a trauma patient".** This patent addresses the use of valproate and paroxetine to diminish the risk of infection in injured patients, such as war fighters.

Other Products
N/A

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Project 1

Name**:**	**Carl Hauser, MD**
Project Role:	PD/PI
Researcher Identifier (e.g. ORCID ID):	
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:	No changes

Name:	**Kiyoshi Itagaki, PhD**
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	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:	No changes

Name:	**Jinbong Park, PhD**
Project Role:	Research Fellow
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked	11
Contribution to Project:	Dr. Park is working on human PMN purification, chemotaxis, and ROS induced by biological materials and mtFPs. He was supervised by Dr. Itagaki.
Funding Support:	No changes

Name:	**Hyo In Kim, PhD**
Project Role:	Research Fellow
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked	3
Contribution to Project:	Ms. Ho is working on human PMN purification and chemotaxis and ROS induced by biological materials and mtFPs. She was supervised by Dr. Itagaki.
Funding Support:	No changes

Name:	**Sidharth Shankar, BS**
Project Role:	Research Technician
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	*Mr. Shankar's effort is shared among all projects- he is working on clinical sample preparations for entire project,which includes human neutrophil,PBMC, plasma,

Funding Support: No changes

Project 2

Name: **Leo E. Otterbein, PhD**
Project Role: Principal Investigator
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 2.5
Contribution to Project: Dr. Otterbein oversees all of project 2 and is co-director of The overall FPA with Dr. Hauser
Funding Support: R01DK108894, Agios, NFL, R01DK119202, R01HL142758, R41DK123933, R41HL150889, R01GK119202 (Completed: R44DK111260, R43GM125430)

Name: **David Gallo, BS**
Project Role: Researcher/Lab
Manager Researcher Identifier (e.g. ORCID ID):
Nearest person month work: 1
Contribution to Project: Project 2 focused on *in vivo* model characterization.
Funding Support: No changes

Name: **Sidharth Shankar, BS**
Project Role: Research Technician
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 6
Contribution to Project: *Mr. Shankar's effort is shared among all projects- he is working on clinical sample preparations for entire project,which includes human neutrophil,PBMC, plasma, and platelets.
Funding Support: No changes

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: (NEW) 1R01 DK 125846-01, (NEW) R21AG 065923-01, (NEW) 1R01 DK 126674-01A1, R01-DK108894-04, 1R01-AI132389-04, 5R01DK104714-05 (NCE) , 5 R01 DK 120862-02, 5 R01 HL 147095-02, 1 R01 DK 119202-02, 1 R01 DK 124408-01 and Tizona SRA.

Name: **Maria Serena Longhi**
Project Role: Research Associate
Researcher Identifier (e.g. ORCID ID): 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: No changes

Name: **Dusan Hanidziar**
Project Role: Postdoctoral/
Anesthesiologist Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1-unfunded
Contribution to Project: Studies on the role of NKT cell-autotaxin-lysophosphatidic acid pathway in alveolar injury induced by hyperoxia.
Funding Support: No changes

Name: **Haohai Zhang**
Project Role: Postdoctoral/ Anesthesiologist
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 9
Contribution to Project: Testing CD39 and purinergic markers of cells derived from healthy controls and patients after trauma and other inflammatory diseases. Studies on the immunological role of CD39 in end-organ injury.
Funding Support: No changes

Name: **Lili Feng**
Project Role: Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 3
Contribution to Project: Performed molecular biology studies and mouse experimentation and genotyping
Funding Support:

Name: **Vilmosne Csizmadia**
Project Role: Technician
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 2
Contribution to Project: Provided technical aspects, tissue preparation assistance and immunopathoogy
Funding Support:

Name: **David Melton**
Project Role: Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1
Contribution to Project: Provided technical aspects, animal experimentation and surgery
Funding Support:

Project 4

Name:	**Daniel Talmor, MD MPH**
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0002-7239-8068
Nearest person month worked:	1
Contribution to Project:	Dr. Talmor is working on Project 4, examining ways in which alveolar damage and release of DAMPs drive severity of illness and pneumonia risk.
Funding Support:	(NEW) 3U01 HL 123009, (NEW) 5OT2 HL 156812, 5 U01 HL 123022, 5 R01 AG 065554

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, testing the hypothesis that physicochemical alterations in the airway environment are associated with lung danger signaling, lead to production of DAMPs and alter innate immunity.
Funding Support:	No changes

Name:	**Valerie Banner-Goodspeed, MPH**
Project Role:	Researcher/Clinical Research Manager
Researcher Identifier (e.g. ORCID ID):	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 that can form the foundation for subsequent clinical trials.
Funding Support:	No changes

Name:	**Lauren Kelly, MPH**
Project Role:	Biostatistician-II
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	Ms. Kelly is working on Project 4, serving as database architect and manager, and overseeing data integrity.
Funding Support:	No Changes

Name:	**Krystal Capers, MPH**
Project Role:	Clinical Research Coordinator
Researcher Identifier (e.g. ORCID ID):	0000-0003-3499-7921
Nearest person month worked:	1
Contribution to Project:	Ms. Capers is working on Project 4, collecting and cleaning data.
Funding Support:	No changes

Name:	**Andre deSouza Licht**
Project Role:	Clinical Research Coordinator

Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	Mr. Licht Capers is working on Project 4, collecting and cleaning data.
Funding Support:	No changes

Name:	**Sean O'Connor**
Project Role:	Clinical Research Assistant
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	2
Contribution to Project:	Mr. O'Connor is working on Project 4, examining characteristics of PBMCs in patents with sepsis and trauma at interval times during injury, as well as examining the effect of hyperoxic and normoxic conditions post trauma and sepsis insults in pre-clinical models.
Funding Support:	No changes

Project 6

Name:	**Michael Yaffe M.D., Ph.D.**
Project Role:	Leader, Project 6
Researcher Identifier (e.g. ORCID ID):	0000-0002-9547-3251
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:	No changes

Name:	**Ingred Rica, Ph.D,**
Project Role:	Post-Doctoral Researcher
Identifier (e.g. ORCID ID):	0000-0001-5644-1153
Nearest person month worked:	12
Contribution to Project:	Post-doctoral research on project 6
Funding Support:	No changes

Name:	**Brian Joughin, Ph.D,**
Project Role:	Staff Scientist
Researcher Identifier (e.g. ORCID ID):	0000-0003-1022-9450
Nearest person month worked:	2
Contribution to Project:	Computational Scientist for project 6 and the Computational Core
Funding Support:	No changes

Name:	**Samantha Rosenberg**
Project Role:	Technician
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2
Contribution to Project:	Technician for project 6
Funding Support:	No changes

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

8. SPECIAL REPORTING REQUIREMENTS

QUAD CHARTS: updated for YR5

9. APPENDICES: SOW table updated

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

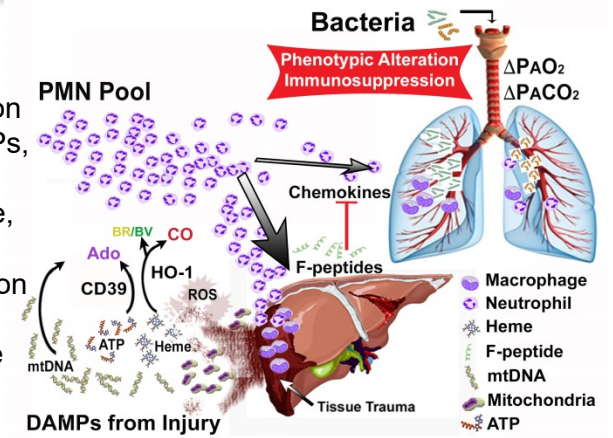
This research program seeks to identify cellular and molecular mechanisms by which wounds predispose to infection. We are focused on three primary objectives: **prevention**, **diagnosis** and **treatment** of the wounded warrior.

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, 2021)

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: $7,497,935 TC

STATEMENT OF WORK

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 3: 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	100 100
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			100 02/2020
Milestone #2: Manuscript on mtFP effects on innate immune phenotypes. Presentation at meetings	18	Hauser Otterbein	Yaffe	Lederer	100 09/2020
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	100 100 02/2020
Milestone #3: Manuscript on mtFP biological effects in context of other DAMPs.	36-60	Hauser/ Otterbein / Robson	Yaffe	Lederer	100 02/2020
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	100 100 100 25
Subtask 2: Assess mtDNA as a biomarker of injury in mice	1-36	Hauser			100
Milestone #4: Manuscript on mtDNA as a biomarker.	12	Hauser/ Otterbein		Lederer	100 01/2018
Subtask 3: role mtDNA in bacterial clearance after trauma	12-48				90
Determine relationship of mtDNA to infective risk	12-24				90
Study role of chloroquine	24-36				75

Milestone #5: Manuscript on mtDNA as a predictor of infection. Presentation.	30	Hauser			100 09/2020
Milestone #6: Manuscript on chloroquine as prophylaxis in trauma. Presentation at meeting.	36	Hauser			100 11/2018
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 10/2020
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	50 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	50
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	25
Milestone #18: New grant submissions (NIH, DoD, Foundations)	30-60	ALL	Yaffe	Lederer	50

Project 2: Determine the role of innate immune responses to Heme in the increased susceptibility to infection after trauma	**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		**PI: Otterbein**			
Subtask 1:Characterize Heme as a DAMP in PNA	0-12	Otterbein Hauser	Yaffe	Lederer	100 06/21
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	100 09/20
Subtask 4: Optimize CO dosing	12-36	Otterbein	Yaffe	Lederer	100 03/20
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	100 07/21
Subtask 6:Study effects of heme on Mϕ and PMN phenotype and signaling *in vitro*	42-48	Otterbein Hauser	Yaffe	Lederer	100 07/20
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	100 09/20
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	95
Milestone #4: Final manuscript(s) describing the effects of heme and CO on primary human cells from trauma patients and volunteers	54-60	ALL			50

Project 3: CD39 and Extracellular Nucleotide Signaling Mediate Inflammation and Immune Failure After Trauma.	**Timeline**	**Site 1** (BID)**PI: Robson**	**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	80
Subtask 2: Define role of MyD88/mtDNA in P2x7 signaling	9-24	Robson/ Otterbein/ Hauser	Yaffe	Lederer	100 Mid-2020
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	100 End-2019
Subtask 4: Determine how P2x7 signaling in PMN affects the innate immune response to PNA	24-36	Robson/ Otterbein/ Hauser/ Junger	Yaffe	Lederer	70
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	100 Under review - Mid-2021
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	55
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	90

Subtask 2: Test impacts of elective adenosinergic receptor agonists vs. antagonists in liver injury and sepsis. Review impacts of adenosine in this system.	9-36/60	Robson/ Otterbein/ Hauser	Yaffe	Lederer	100 08-2021
Subtask 3: Test combinations of pharmacological P2X7 antagonists and optimized adenosinergic drugs in liver injury and PNA.	12-36	Robson/ Otterbein/ Hauser	Yaffe	Lederer	100 04-2021
Subtask 4: Determine depletion of intracellular ATP in immune cells in the liver injury and PNA.	24-60	Robson/ Otterbein/ Hauser/ Junger	Yaffe	Lederer	70
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	100 06-2021
Milestone #5: Mid-term manuscript(s) describing role of adenosinergic signaling and CO in trauma/sepsis models.	36-48	ALL	Yaffe	Lederer	90
Milestone #6: Final manuscript(s) describing depletion of intracellular ATP in immune cells in the liver injury and sepsis.	40-60	ALL	Yaffe	Lederer	75
Milestone #7: New grant submissions (NIH, DoD, Foundations)	30-60	ALL	Yaffe	Lederer	50
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	100 Mid-2021
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	85
Subtask 4: Correct aberrant P2X7 signaling and adenosinergic responses by modulating CD39 ectonucleotidase activity	24-60	Robson/ Otterbein/ Hauser/ Junger/	Yaffe	Lederer	80

		Talmor			
Subtask 5: Restore intracellular ATP (energy charge) in immune cells – mitochondrial bioenergetics	12-48	Robson/ Otterbein/ Hauser/ Talmor/ Junger	Yaffe	Lederer	75
Subtask 6: In vitro O_2 and CO studies	24-48	Robson/ Otterbein/ Talmor	Yaffe	Lederer	55
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	95
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	75
Milestone #9: Final manuscript(s) on Purines and DAMPs as modulators of inflammation and clinical infection	40-60	ALL	Yaffe	Lederer	75
Shared Milestone #10: New grant submissions (NIH, DoD, Foundations)	30-60	ALL	Yaffe	Lederer	50
Project 4: Ventilator-Induced Injury and Lung Immune Response to Infection After Trauma	**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 100 11/2019 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				23
Milestone #2: complete the enrollment	60				23
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 continuous
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	20
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	20
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	20
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	30-36	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	**Timeline**	**Site 1** (BIDMC)	**Site 2** (MIT)	**Site 3** (BWH)	
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	50
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	75
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	60
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	70
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	75
Subtask 3: Functional studies examining roles of IL-12, IL-17, PD-L1, γδ T cells, TLR9	24-48			Lederer	30
Milestone #6: Manuscript describing results of functional studies and presentation at scientific meetings	48-60	ALL	Yaffe	Lederer	50
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.					100 09/2020
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	100 09/2020
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.	**Timeline**	**Site 1** (BID)	**Site 2** (MIT) **PI: Yaffe**	**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	100 03/2020
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	80
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	100 03/2020

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	70
Milestone #2: manuscript on PMN priming after trauma and the sensitivity to lung infection.	24-36	Hauser/ Otterbein	Yaffe	Lederer	50
Milestone #3: presentation at Shock Meeting and DoD	24-36	ALL	Yaffe	Lederer	100 06/2019
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	95
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	50
Milestone #5: Manuscript describing role of MK2 signaling in PNA after trauma	48-53	Hauser/ Otterbein	Yaffe	Lederer	30
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	90

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