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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 are developing novel therapeutic treatment strategies and deliverables that will reduce the incidence of acute lung injury and promote recovery from trauma.

2. KEYWORDS

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

3. ACCOMPLISHMENTS

What were the major goals of the project?

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

Minor goals: we have developed animal models (trauma and burn) $\pm$ infection that we are working toward mimicking what is observed in the patients that have been recruited. In addition to the functional readouts *in vivo* in mice, we are now able to compare murine leukocytes (neutrophils, macrophages, Tcells) in blood and bronchoalveolar lavage with those cells collected from patients with trauma. These methodologies were a large focus for this year ensuring that all readouts were validated and reproducible.

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

Major activities, objectives, and results.

- Evaluated all 13 of human mitochondrial formyl peptides (mtFPs, synthesized) for calcium modulation and chemotaxis. Only five (ND6>ND3>ND4>ND5>Cox I) induced both activities against human PMN. This order correlates very well with BLOSUM 62 based on similarity to fMLF. Results were presented at Western Trauma Conference (Hauser) and published in Journal of Trauma and Acute Care Surgery.
- Analyzed plasma samples from trauma patients (Day 0,3, and 5) and healthy volunteers for mitochondrial proteins ND6 and NADH Dehydrogenase I by ELISA and qPCR to detect mtDNA (**Figure 1**)
- Established conditioned medium that mimic PMN migration to bacteria infected lung after trauma by exposing PBMC to *E. coli*, *S. aureus*, or LPS. PMN migration and PMN calcium modulation by conditioned medium were evaluated by pre-exposing PMN to mtDAMPs to mimic injury (**Figure 2**).
- We identified which chemokines/cytokines play important roles in conditioned medium to induce PMN migration toward bacteria infected lung by using various inhibitors for chemotaxis (**Figures 4-10**).
- We then demonstrated that the endogenous mitochondrial formyl peptide NADPH dehydrogenase 6 (ND6) regulates leukotriene B₄ (LTB₄) mediated PMN chemotaxis (**Fig. 7**). This finding has translational importance since ND6 is released by injury and LTB₄ is a critical high-affinity chemoattractant that

mononuclear cells use to recruit neutrophil to sites of infection.

- We then showed that the ND6 effect on LTB₄ mediated signaling and chemoattraction reflects internalization of leukotriene receptors, and that such internalization can be rescued by FPR1 blockade using the "designer drug" POL 7200 (**Fig. 9**).
- We then demonstrated that the (ND6) similarly regulates CXCL1 (GRO- α) mediated PMN chemotaxis. This finding has translational importance since CXCL1 is a critical high-affinity CXC chemokine that that is vital to the process of recruit neutrophil to the lung (**Fig. 8**).
- We also showed that the ND6 effect on CXCL1-mediated chemoattraction reflected internalization of PMN CXCR2. Again, such internalization was rescued by FPR1 blockade using POL 7200 (**Fig. 10**).
- In critical and novel work, we then showed that mitochondria are actively released (as exosomes or microparticles) by PBMC after exposure to mtDAMPs as well as other inflammatory stimuli. This process increases the ability of cultured media from stimulated PBMC to attract PMN and appears to reflect an important and previously undescribed process by which tissue mononuclear cells recruit PMN.
- To further understand the source of extracellular nucleic acids we have developed the data necessary to assess the sources of nucleic acids at various times after injury. As expected, trauma patients have significantly more circulating DNA then healthy volunteers.
- **Figure 13.** Plasma mtDNA and nuclear DNA in addition however, we have looked for changes in the relationship between plasma mtDNA and nuclear DNA after injury. the study show that there are specific patterns of the ratio between nuclear DNA (nDNA) vs mtDNA in trauma and volunteer. After establishing our methodology, we studied this issue using qPCR and specific primers (**Figure 12**). of great interest, there was found to be a time several days after injury where the ratio of mtDNA to nDNA became markedly higher. We believe that this is a reflection of the active secretion of mitochondria from immune cells as described above.
- We have assayed the concentrations of 65 chemokines and cytokines (luminol assay, **Figure 3**) produced by mononuclear cells in response to multiple inflammatory conditions including both exposure to DAMPs and PAMPs. this work is extremely productive and complex, but it has already led to multiple are important observations.
- We show mitochondrial budding from PBMC after exposure to LPS by electron microscopy as well as in video where mitochondria were stained with mito-tracker (**Figure 11**). These data show that release of mitochondria at later time points is not due to cell death but due to active release.
- We discovered that mtDAMPs-induced receptor desensitization will induce internalization of all other chemokine receptors leading to limited PMN migration toward bacteria (**Figure 5**)
- We established qPCR methods to determine mtDNA copy number per cell since mtDNA copy number in mononuclear cells decreases in sepsis patients and correlates to survival rates (**Figure 6**)
- We established a method to evaluate mtDNA and nuclear DNA by qPCR from trauma and volunteer plasma since increasing number of publications suggest correlations between severity of various types of illness and these DNA levels in plasma (**Figure 7**)
- Using mouse bone-marrow PMN with knockout of FPR-1 and FPR-2 (supplied by collaborators at the NIH) we demonstrated that heterologous suppression of leukotriene and chemokine receptors by mitochondrial formal peptides specifically depends on formal peptide receptor-1 (**Fig. 14**). Since "designer" inhibitors for the human FPR-1 receptor are available this suggests potential therapy to diminish post-traumatic infection.

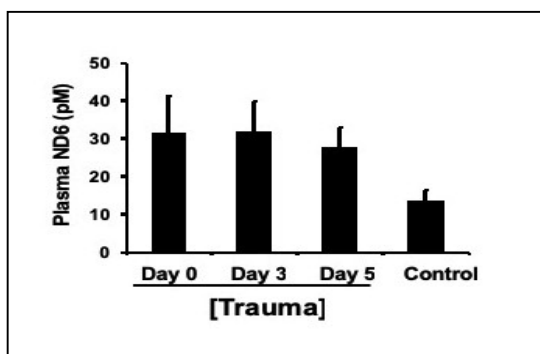


Figure 1. Plasma ND6 from Day 0,3, and 5 patients and control-volunteers

Trauma patients (13) and Volunteers (8) were used to evaluate mtFP-ND6 levels by ELISA. We plan to increase number of samples and examine the correlations with injury severity and plasma mtDNA levels determined by qPCR. Hyperacute samples (collected in 4h) will be studied separately. Mean and SE values are shown from three different experiments.

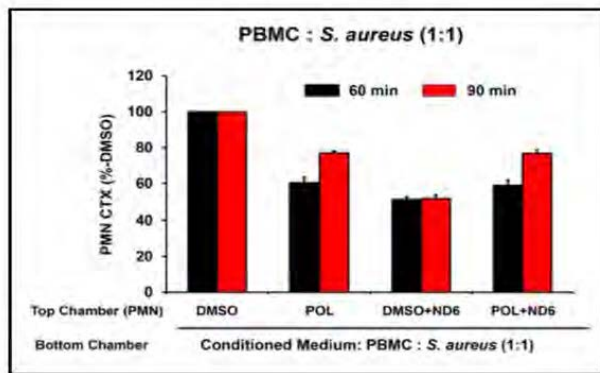


Figure 2. PMN Chemotaxis to Conditioned media by PBMC: *S. aureus* (1:1) incubation Chemotaxis was done for 60 (Black) and 90 (Red) min. Pre- exposure to the mitochondrial formyl peptide **ND6** reduced PMN migration. That was rescued by treatment with POL (an inhibitor of FPR1). In addition, POL treated PMN showed less than DMSO (vehicle) treated PMN, showing involvement of FPR1 reacting chemoattractants in this conditioned media. 60 min chemotaxis showed limited migration with POL treatment. Mean and SE values are shown from three different experiments.

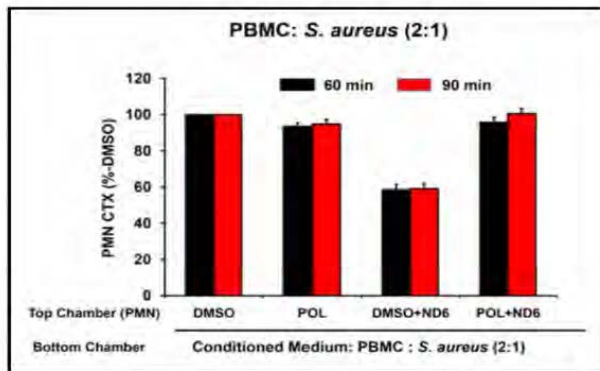


Figure 3. PMN Chemotaxis to Conditioned media by PBMC: *S. aureus* (2:1) incubation Similar to Figure 2. Increased numbers of *S. aureus* mimic more severe or prolonged bacterial infection *in vivo*. Unlike Figure 2, POL treated PMN showed similar chemotaxis to DMSO-treated PMN suggesting more chemokines were present in this conditioned media than only FPR1 reacting chemoattractants. Mean and SE values are shown from three different experiments.

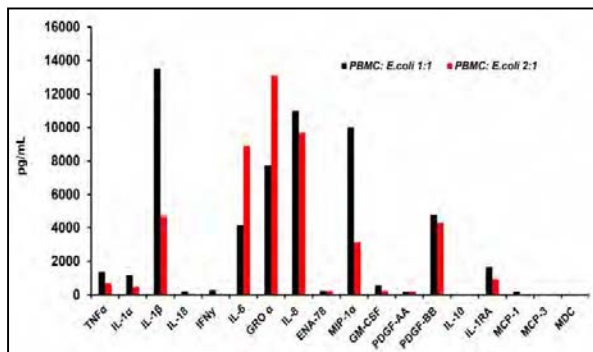


Figure 4. Effects of various inhibitors on PMN migration to conditioned media (PBMC: *E. coli* 2:1) Inhibitors to FPR1 (POL), CSCR1/2 (SCH), BLT1/2 (LY), and LTB4 (U-7) were applied separately and all together (+All). When applied together, 60% of PMN migration was inhibited. Mean and SE values are shown from three different experiments.

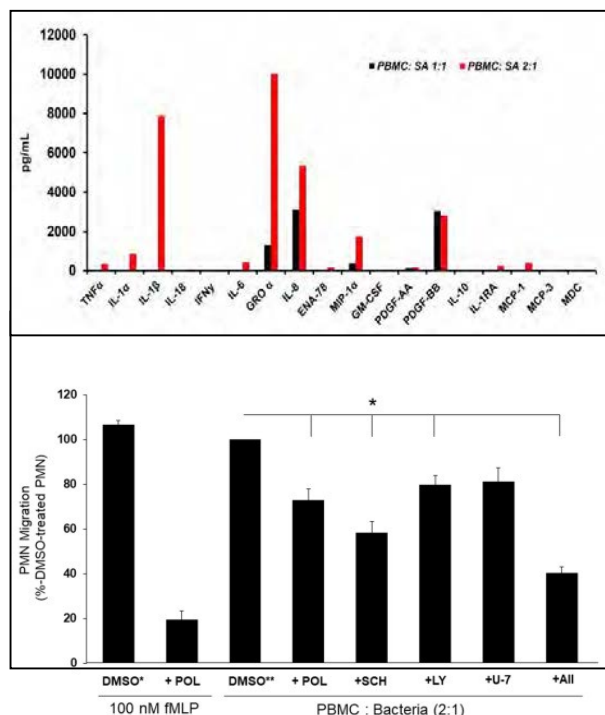


Figure 5. Determination of chemokines/ cytokines involved in conditioned medium PBMC: *E. coli*, 1:1 and 2:1

We evaluated 65 chemokines/cytokines by luminex assay by Eve Technology. Here we list the most concentrated. Assays were done in duplicates - these were almost identical. In addition, we examined LTB4 and ND6 separately. These showed high concentrations proving monocytes produce LTB4 and actually release mitochondria in response to infection.

Figure 6. Determination of chemokines/ cytokines involved in conditioned medium PBMC: *S. aureus*, 1:1 and 2:1.

Similar to Figure 5, however PBMC were incubated with *S. aureus*. In addition, we examined LTB4 and ND6 that showed high concentrations. LTB4 was produced in much greater amounts by monocytes exposed to the gram(+) *S. aureus* than the Gram(-) *E. coli*.

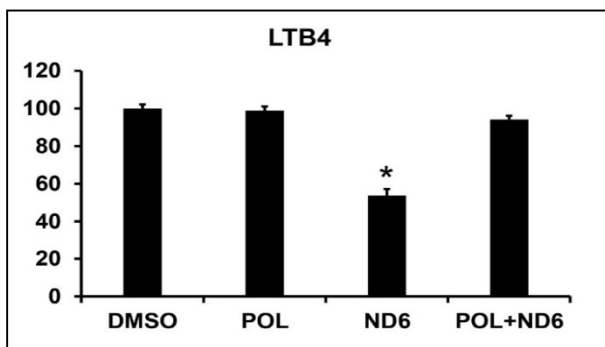


Figure 7. Pre-exposure to ND6 reduced GRO- α -induced PMN chemotaxis. We found increased amount of GRO- α in conditioned media thus we evaluated whether pre-exposure to ND6 (mtDAMPs) reduced chemotaxis. ND6 reduced chemotaxis by 60%, however POL rescued it to control level. Mean and SE values are shown from three different experiments.

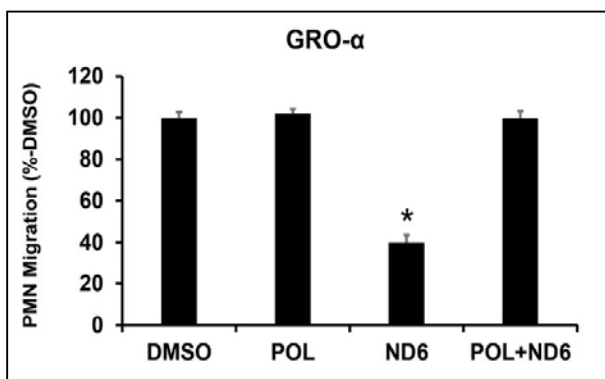


Figure 8. Pre-exposure to ND6 reduced LTB4- induced PMN chemotaxis We found increased amount of LTB4 in conditioned media thus we evaluated whether pre-exposure to ND6 (mtDAMPs) reduced chemotaxis. ND6 reduced chemotaxis by 40%, however POL rescued it to control level. Mean and SE values are shown from three different experiments

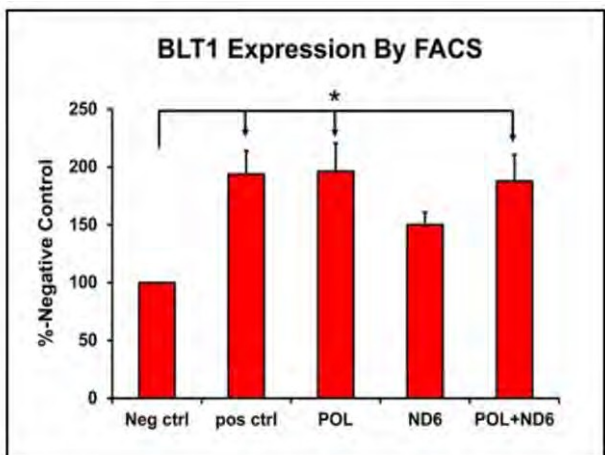


Figure 9. Pre-exposure to mtDAMPs reduces BLT1 expression on PMN. Human PMN were treated in various ways as described left. When treated with ND6 (mtDAMPs) PMN plasma membrane expression of BLT1 was significantly reduced. However, POL treatment rescued this heterologous receptor desensitization / internalization. Mean and SE values are shown from three different experiments.

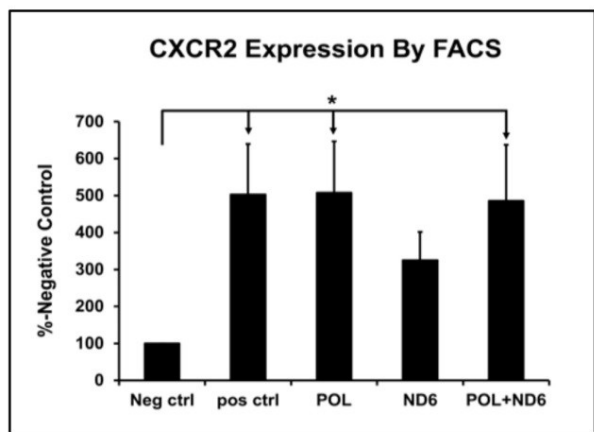


Figure 10. Pre-exposure to mtDAMPs reduces CXCR2 expression on PMN. Human PMN were treated in various ways as described left. When treated with ND6 (mtDAMPs) PMN plasma membrane expression of CXCR2 was significantly reduced. However, POL treatment rescued this heterologous receptor desensitization / internalization. Mean and SE values are shown from three different experiments.

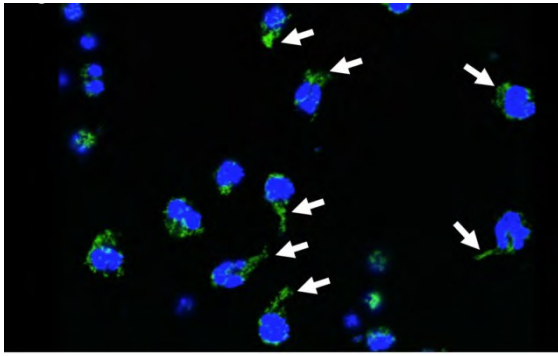


Figure 11. Mitochondria were actively released from PBMC without cell death by exposure to mtDAMPs

Human PMN were loaded with DAPI (blue for nuclei) mito-tracker that specifically label mitochondria in green. External solution contained propidium iodide that will stain dead cells in red. This is a picture from video that shows green tail-like mitochondria from live cells activated by 1 μ M LPS. No dead cells were detected.

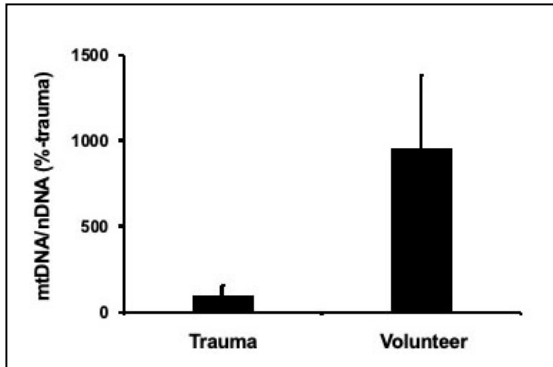


Figure 12. mtDNA copy numbers per PMN

We have isolated DNA from pure human PMN and used nuclear DNA-specific and mtDNA-specific primers to determine mtDNA copy numbers per cell. PMN however, have a variable number of nuclei. Thus this number is not exact mtDNA copy per cell. Nonetheless, using the same protocol we should be able to determine mtDNA copy numbers from mono-nuclear cells such as PBMC. We have determined that there are significant differences between trauma patients and healthy volunteers.

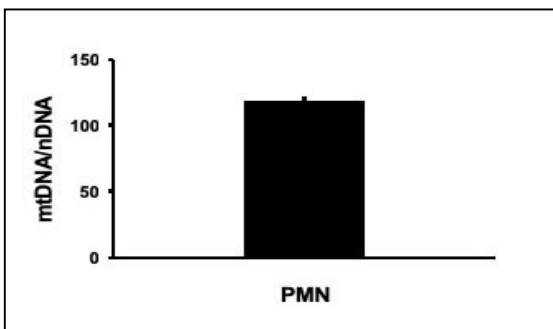


Figure 13. Plasma mtDNA and nuclear DNA ...

We also developed a protocol for determination of mtDNA and nuclear DNA in plasma after purification of total DNA. Here we show ratio of nuclear DNA (nDNA) vs mtDNA in trauma and volunteer. The method is established using qPCR and specific primers as described in Figure 12. These trauma patients did not contain increased level of mtDNA thus this data may not reflect reality. However, this protocol for sure can be used in our future study. We sampled n=4 each from patients and volunteers. Mean and SE values are shown.

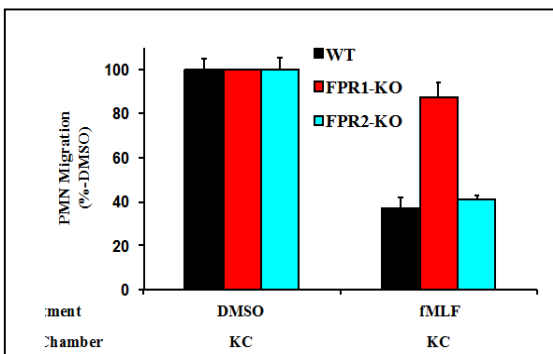


Figure 14: chemokine receptor suppression depends on FPR-1.

Using bone marrow PMN from FPR-1 and FPR-2 knockout mice (kind gift of NIH) we see that in the presence of FPR-1 (i.e. in wild type and FPR-2 KO mice) response to the CXC chemokine "KC" is markedly attenuated by prior exposure to mtFPs. In the absence of FPR-1, response to KC is unaffected by prior exposure to FPs.

What was accomplished under these goals?

Summary: Our results suggest that conditioned medium that we established by incubating PBMC with *E.coli*, *S. aureus*, Formyl peptides (not shown), and LPS (not shown) mimics bacteria infected lungs and contain various chemokines and cytokines. PMN migration toward this conditioned medium after mtDAMPs treatment will mimic in vivo condition where seriously injured trauma patients. PMN will be first exposed to mtDAMPs released from injured tissue/cells then receptor desensitization will happen thus PMN cannot migrate toward bacteria infected lungs where we showed decreased PMN migration toward conditioned medium after exposure to ND6. However, POL treatment rescued PMN migration to control level. Thus, POL treatment in vivo may increase PMN migration toward bacteria infected lungs after injury. In addition, role of mtDNA will be further investigated through our qPCR assay by detecting mtDNA and nDNA in plasma and per cell.

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

Major Activities, objectives and results

- We sought to complete the animal model and ready it for publication.
- Further study the role of heme as a danger molecule and the relationship with Heme Oxygenase-1 (HO-1) and Carbon Monoxide (CO) in injury and infection. Characterize conditional HO-1 knockout mice to better understand the specific cell types involved.
- We continue to develop CO towards clinical application as a deliverable from this project for eventual use by the injured warfighter. We studied mechanisms of action of CO in the neutrophil and more broadly how CO maintains tissue homeostasis and imparts therapeutic effects for traumatic injury and infection.
- Fostered collaborations with projects 3,5, and 6 to understand the relationship of heme, nucleotides, ROS and alterations in cell phenotypes to correlate with tissue function.

What was accomplished under these goals?

- Completed data sets of the animal model, being formatted into manuscript. (**Figure 1**), 01-09-2018
- Graduated successful Harvard Masters student with honors. Thesis on CyTOF analysis of BAL fluid in mice after lung infection showing enhanced bacterial clearance. Thesis titled “The Effect of Carbon Monoxide on the Innate Immune Response to Bacterial Lung Infection” 15-04-2018
- Completed NIH T32 fellow training with final report to T32 committee. Data shows effects of CO on PMN free radical formation in collaboration with Project 6 will be incorporated into manuscript 05-01-2018
- Plenary lecture at American Chemical Society, New Orleans, LA. 18-03-2018
- Poster presentation at Military Health System Research Symposium 22-08-2018
- Manuscript accepted in *PNAS* detailing the role of CO and kidney injury 03-03-2018
- Experiments completed in collaboration with Project 5 looking at the effects of liver crush trauma on immune cell phenotypes. 08-01-2018 (**Figure 2-3**)
- In collaboration with Project 5, tested the effects of heme as a DAMP on human PBMC and PMN showing a remarkable activation of cells in response to heme. 21-06-2018 (**See Project 5**).
- Characterized the tamoxifen-regulated-Hmox-1 deficient mice driven by the ROSA26 cre-recombinase and the LyzM-cre-HO-1^{fl/fl} mice measuring Bilirubin and CO levels. 07-07-2018.
- Show human and mouse PMN as more efficient at bacterial phagocytosis and killing and that CO increases reactive oxygen species through NADPH oxidase 15-04-2018 (**Figure 4**).
- Showed that heme exacerbates susceptibility to infection, which is further exaggerated in hemopexin-deficient mice 09-09-2018 (**Figure 5**)
- Serum transfer experiments were finalized. Serum from an animal subjected to trauma (containing high levels of DAMPs, **Figure 5**) were injected intravenously into naïve mice and show similar sensitivity to infection as mice that are subjected to liver crush injury prior to bacterial inoculation. 05-10-2018.
- Onboarded Janick Weber from the Univ of Freiburg, Germany who led the TBI project 22-06-2018.
- Compared PMN killing in healthy human PMN vs PMN from trauma patients with and without CO and showed that CO is as effective in augmenting killing capabilities in both (**Figure 6**) 08-04-2018
- Initiated and characterized a model of Traumatic Brain Injury (TBI) for another project that will be used in future years as an additional trauma model (**Figure 7**). Show that mice that lack HO-1 specifically in myeloid cells have remarkable sensitivity to TBI. 10-08-2018.
- Initiated collaboration with Beth Israel Deaconess Medical Center for Glycomics to begin to profile plasma and cell N- and O-glycosylation profiles from our patient repository. 10-10-2018.
- Mentored two Career Development Award Grant Submissions for Dr. Shahzad Shaefi as an NIH K08 application and Dr. Rodrigo Souza as an American Heart Association proposal. Both proposals focus on the role of heme and CO on the injury response 15-10-2018.
- Funded by an NIH SBIR grant (R43GM125430-01) to evaluate an oral formulation (HBI-002) for delivery of CO via the gastrointestinal tract. Data showing pharmacokinetics in mice was published in *PLoS One*, Oct, 2018, PMID 30308028. And this formulation will be evaluated in the liver crush trauma model and also in a model of hemorrhagic shock as part of a collaborative effort with the University of Pittsburgh trauma center. 20-09-2018.

Key Data Sets

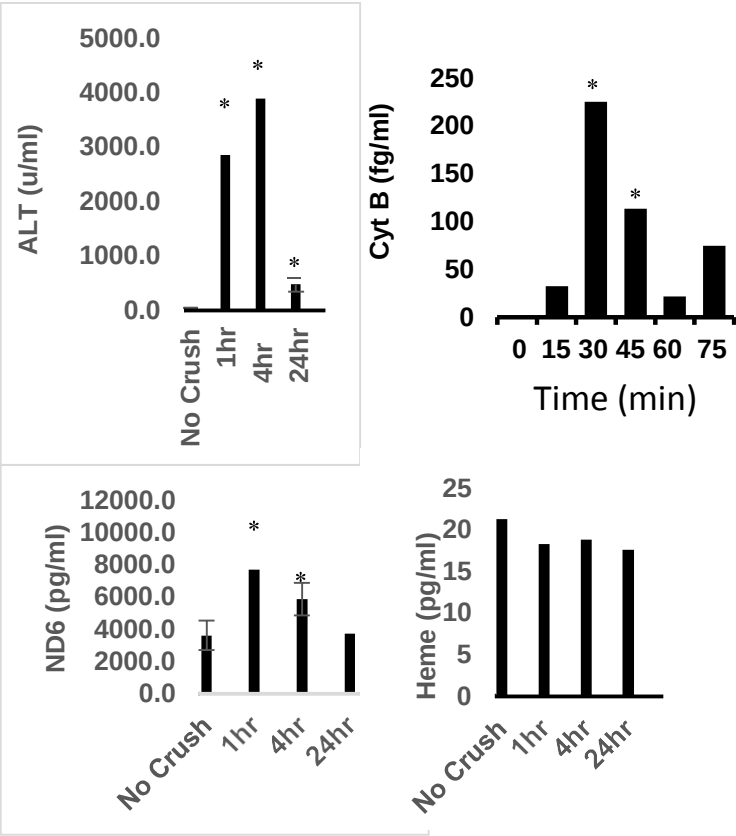


Figure 1. Liver crush trauma leads to increases in blood levels of mtDAMPS. Male CD-1 mice were subjected to a 10% liver crush injury and blood was collected over time. Serum was analyzed for the listed Danger Associated Molecular Patterns (DAMPS). N=4-6 mice/group. Injury resulted in elevations in alanine aminotransferase (ALT) with an IDEXX chem analyzer, mtDNA as measured by PCR of Cytochrome B message, the formyl peptide ND6 by ELISA and total heme colorimetrically. Significant differences were noted in ALT, CytB (mtDNA), ND6, $p < 0.03$ vs control. Heme levels were not different likely due to limited assay sensitivity.

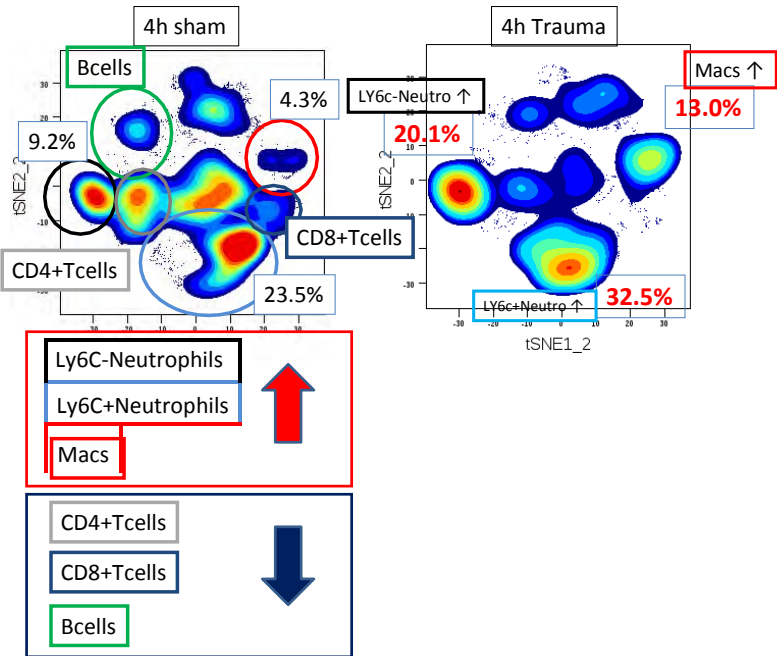


Figure 2. Liver crush injury results in dramatic changes in neutrophil infiltration into the liver. Mice were treated as above using the same liver crush injury. Cell infiltrates into the liver were assessed by CyTOF. Significant increases in percent LY6 neutrophils (PMN) was observed at 4h comparing sham vs injury (32.5% LY6c+ and 20.1% LY6c-) (n=5/group). Increases in macrophages was also observed at 13%. In contrast there was a decrease in T and B cell populations at this time point.

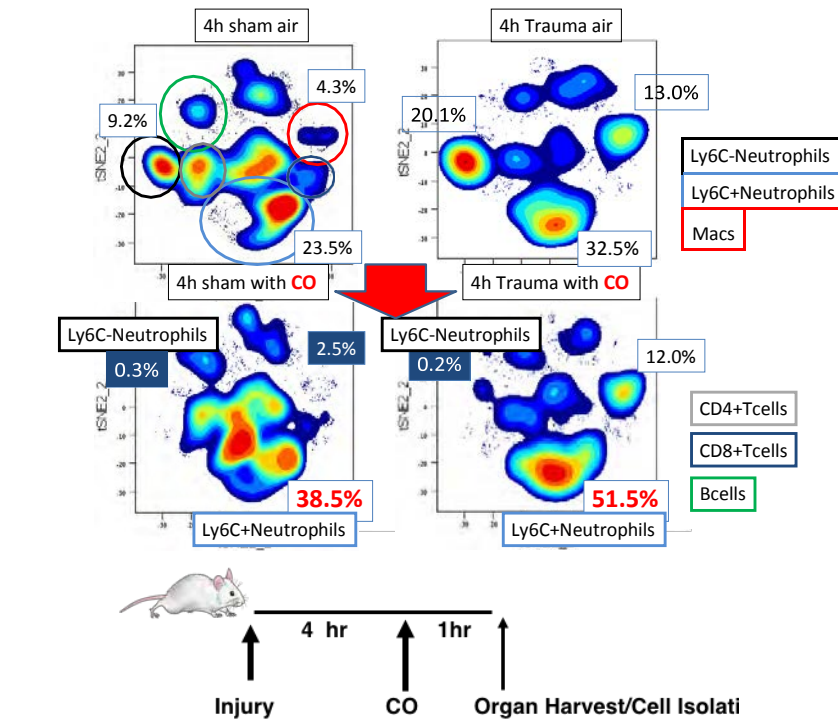


Figure 3. CO further increase PMN infiltration into the liver after crush injury. Mice were treated as above using the same liver crush injury. Cell infiltrates into the liver were assessed by CyTOF. We observed a 51.5% increase in infiltrating LY6C+ neutrophils (PMN) in CO treated mice vs air controls both with and without trauma. LY6C- PMN were reduced in CO treated mice with and without trauma. Macrophages, Tcells and Bcells were not significantly changes. Mice were treated as depicted in the diagram. Liver tissue was harvested at 5h in mice treated with CO for 1h (250ppm), starting 4h after injury. N=5 CD-1 mice/group.

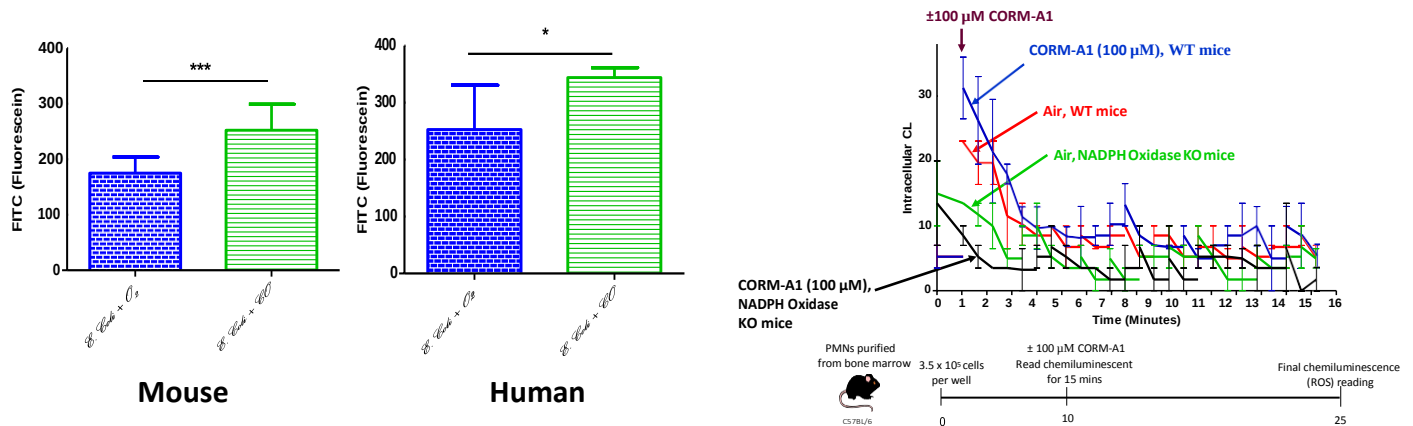


Figure 4. CO enhances PMN phagocytosis and enhances reactive oxygen species (ROS) generation. **A.** Fresh primary PMN (Human or mouse) were treated killed *E. coli* (K-12 strain) cells loaded with the fluorescent dye fluorescein for 1hr and then cells were treated with *E. coli* and CO simultaneously. Fluorescence intensity was measured by a plate reader (Ex/Em ~480/520 nm) 1h later and is directly related to the amount of *E. coli* that have been phagocytosed. Note that CO significantly enhanced fluorescence vs air (O_2) treated PMN, * $p < 0.05$, *** $p < 0.01$. Results represent mean $\pm$ SD of 3 experiments in triplicate. **B.** ROS generation was measured by chemiluminescence in mouse PMN isolated from WT mice and *p91*^{-/-} mice (NADPH oxidase nulls) $\pm$ treatment with CO. In this experiment, a CO Releasing Molecule (CORM-A1) was used to be able to add CO directly to the luminometer. Note that CO increases ROS generation in WT PMN but not from *p91*^{-/-} PMN and *p91*^{-/-} PMN do not generate ROS as expected. Collectively, these data support NADPH oxidase as a potential target for CO and may be related to increased phagocytosis. Results represent mean—SD of 5 experiments performed in triplicate. The diagram below depicts the treatment regimen used.

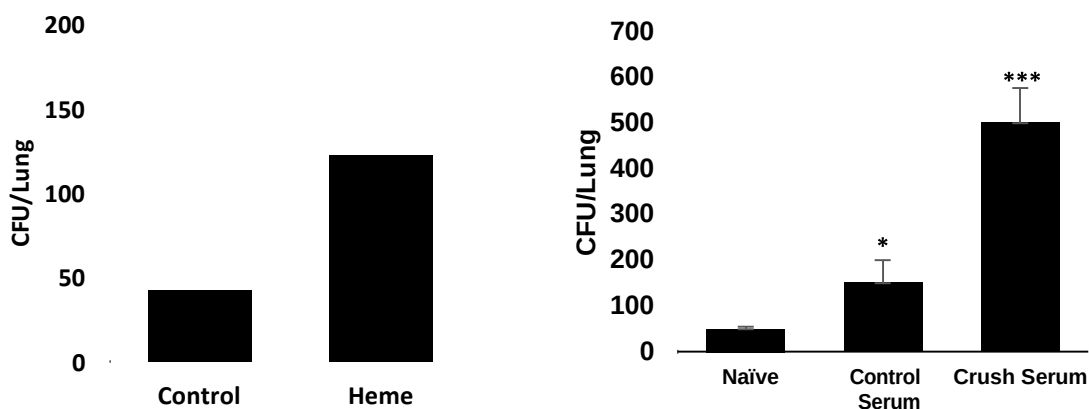


Figure 5. Heme exacerbates bacterial lung infection likely contributing in part as a serum DAMP. **A.** Male CD-1 mice were treated with heme (15mg/kg, i.p. simulating liver crush) at time 0, 2 and 8hr after inoculation with *S. aureus* (10^{10} cfu/mouse). A bronchoalveolar lavage (BAL) was performed 24h after bacteria and bacteria counts were determined using a standard agar plate assay. Results represent mean $\pm$ SD of 4 mice/group, $p=0.1$. **B.** Male CD-1 mice were subjected to a 10% liver crush or laparotomy control and the serum collected 4hr later. This serum was then injected (0.5ml) into naïve mice which were then infected 24h later and a BAL performed 4h after inoculation with *S. aureus* as above. Results show that serum contains a soluble mediator that results in increased susceptibility to infection. $N=8-10$ mice/group. * $p<0.02$, ** $p<0.01$ vs naïve. The elevation in control serum are likely DAMPS released from the surgical procedure.

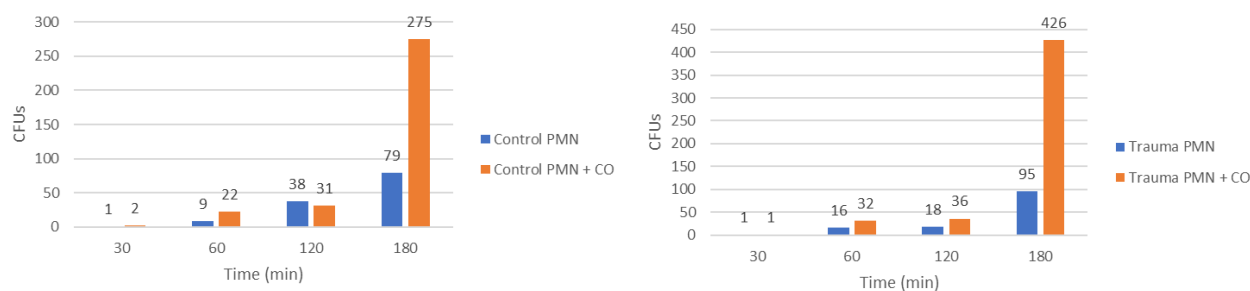


Figure 6. CO enhances the ability of both healthy volunteer and trauma patient PMN to clear *S. aureus*. PMN were purified from whole blood of control patients or Day 1 trauma patients and treated with 1 MOI *S. aureus*. PMN were treated with bacteria and immediately placed into CO exposure (250ppm) or air. At each indicated time point PMN were collected and lysed and the number of bacteria present in cell lysates by agar plate assay. Results represent mean CFU I triplicate samples and suggest that PMN can clear bacteria to the same extent whether from healthy control or trauma patients.

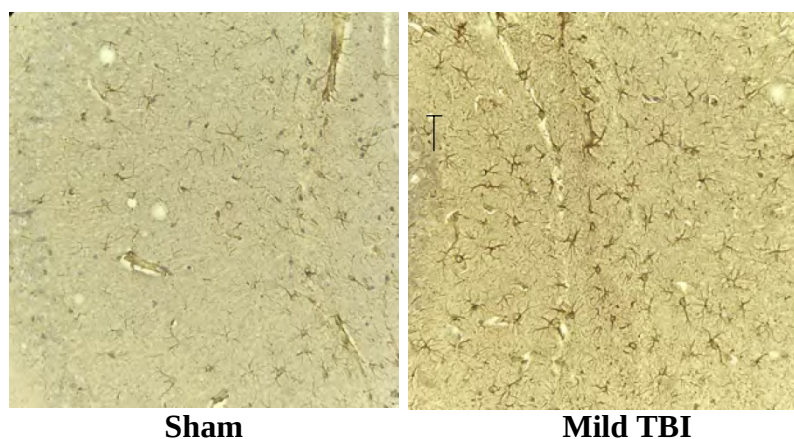


Figure 7. Traumatic Brain Injury (TBI) Model. Male CD-1 mice were anesthetized and subjected to a single mild TBI to the right side of the head (weight; 30g, height, 30 inches). 5 days later the brains were harvested and stained for GFAP as a marker of cell stress. Shown to the left are representative images of the right hippocampus stained with anti-mouse GFAP. Note the greater intensity of GFAP positivity vs sham control. Images are representative of 10-20 sections/mouse from 10 mice/group. Mag = 20X

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 study how type-1 purinergic receptor (P1R) signaling by adenosine modulates the innate immune response to infection after trauma, in experimental trauma
- To characterize and correct kinetics of aberrant immune purinergic responses within blood and alveolar micro-environment of trauma patients

What was accomplished under these goals?

- Completed studies on the interactions between hypoxia, purinergic mediators and drug transporters in Th17 cells differentiated from controls and patients impacted by chronic inflammatory disorders.
- Defined optimal purinergic-based regimens in conjunction with oxygen and treatment of inflammatory stress in the setting of hyperoxia and trauma models.
- Continued testing and measurement of intracellular and extracellular heme derivatives in T cell subsets to further define the interactions between purinergic signaling mediators with oxygen, oxidative intermediates and hydrocarbon pathway elements.
- Defined purinergic mediator profile as well as ectoenzymatic activity in human samples (peripheral blood and bronchoalveolar lavage, BAL) collected from controls and trauma patients at different time points.
- Continued flow cytometry characterization of human samples (peripheral blood and BAL) collected from controls and trauma patients.
- Isolated liver mitochondria and started injection into WT mice to induce systemic inflammation.
- Onboarded Research Fellow (Dr. Shilpa Tiwari-Heckler, MD).
- Characterized distinct subsets and molecular phenotype of pulmonary macrophages under normoxia and hyperoxia in mouse models of hyperoxic lung injury.

During this time frame, we have been testing the purinergic profile of mononuclear cells isolated from the peripheral blood of trauma patients and their respective controls. Measurement of adenosine receptor status and P2X7 receptor expression was performed by qPCR using peripheral blood mononuclear cells (PBMCs) obtained from 14 trauma patients and 10 controls. In trauma patients, PBMCs were obtained at day 0 or 1

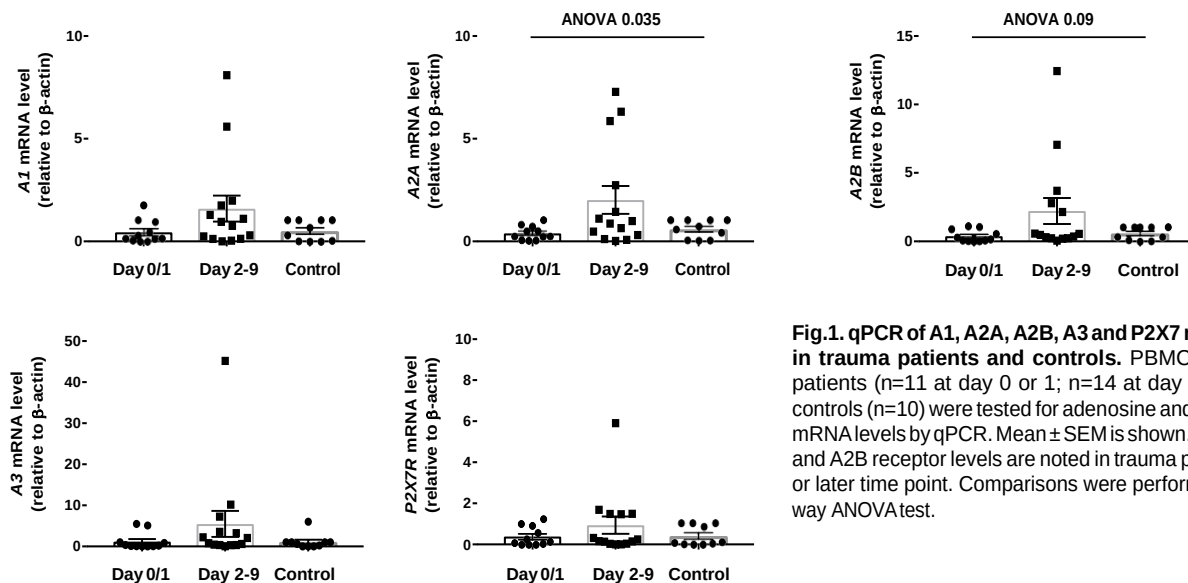


Fig.1. qPCR of A1, A2A, A2B, A3 and P2X7 receptor levels in trauma patients and controls. PBMCs from trauma patients (n=11 at day 0 or 1; n=14 at day 2 or later) and controls (n=10) were tested for adenosine and P2X7 receptor mRNA levels by qPCR. Mean $\pm$ SEM is shown. Increased A2A and A2B receptor levels are noted in trauma patients at day 2 or later time point. Comparisons were performed using One-way ANOVA test.

after trauma (n=11), or at day 2 or later timepoint (n=14). We note increases in the levels of A2A and A2B adenosine receptor expression at day 2 and later, when compared to day 0 or 1 after trauma and controls (**Fig. 1**). These changes predispose, at least in part, to increased adenosinergic responsiveness, possibly associated with recovering CD39 activity at later time points and predisposing to “immune type paralysis”.

We have been analyzing levels of ectonucleotidase and adenosine deaminase activity in PBMCs and BAL samples in patients; and have determined the impact of the trauma event on extracellular nucleotide flux. PBMCs were obtained from 7 controls and 9 trauma patients; BAL was obtained from 5 controls and 2 trauma patients. Ectonucleotidase and adenosine deaminase activities were determined by thin layer chromatography (TLC) upon incubation of peripheral blood and BAL-derived mononuclear cells with ¹⁴C-labelled ADP and will be further validated by HPLC.

We have noted decreases in CD39 and adenosine deaminase activity in trauma patients at day 2 (**Fig. 2**). These changes would predispose to increases in local ADP and possibly preserving local adenosine levels. In contrast, when analyzing BALs, we have observed “compartmentalization” of responses – with increases in both CD39 and adenosine deaminase activity and decreases in CD73 activity, at these same time points on day 2 (**Fig. 2**).

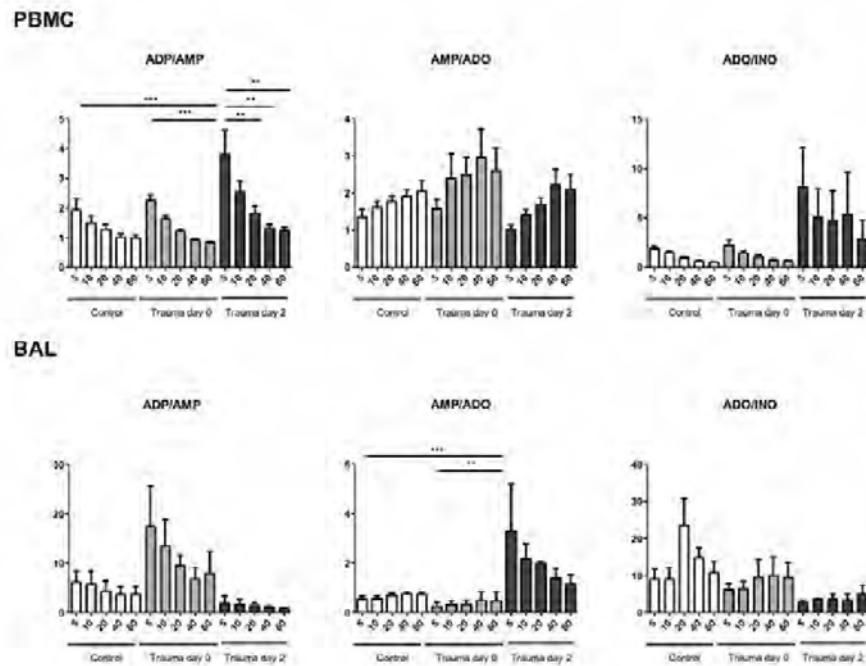


Fig. 2. Ectonucleotidase and adenosine deaminase activity in PBMC and BAL samples. PBMCs and BALs from controls (n=7 for PBMCs and n=5 for BAL) and trauma patients (n=9 for PBMCs and n=2 for BAL) were tested for CD39 activity, manifested by ¹⁴C-ADP hydrolysis to AMP. We also tested for consequent AMPase activity, manifested by CD73. CD39, CD73 and adenosine deaminase activities were expressed as ADP/AMP, AMP/adenosine and adenosine/inosine ratios. Bars show Mean ± SEM. In PBMCs, there is decrease in CD39 and adenosine deaminase activity at day 2; while in BAL, at the same time point, we observe increase in CD39 and adenosine deaminase activity but decrease in CD73 activity.

In parallel experiments, we have measured the proportion of double positive CD39⁺CD73⁺ lymphocytes in innate immune cells and T lymphocytes subsets within PBMCs obtained from controls (n=5) and trauma patients at day 1 (n=5) or 1 and 2 (n=4). We note increases in the proportion of CD39⁺CD73⁺ cells amongst

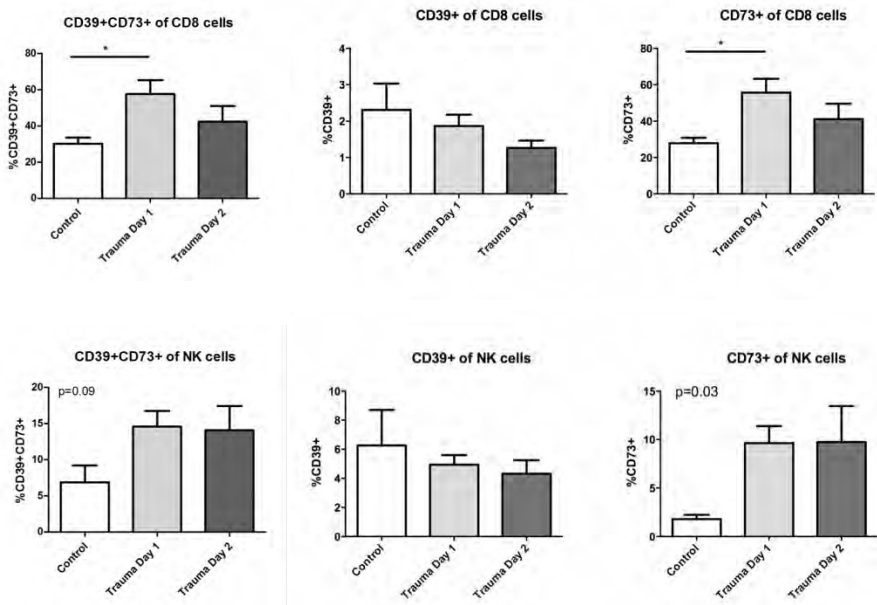


Fig. 3. Frequency of CD39⁺CD73⁺ cells amongst CD8 and NK cell subsets in trauma patients and controls. The frequency of double positive CD39⁺CD73⁺ and single positive CD39⁺ and CD73⁺ cells within the CD8 and NK subsets was determined by flow cytometry in PBMCs obtained from controls (n=5) and trauma patients at day 1 (n=5) and day 2 (n=4). We note that increase in the fraction of CD39⁺CD73⁺ cells reflects that of CD73⁺ single positive cells in both CD8 and NK subsets.

increases, as displayed by CD37⁺ cells in both subsets (**Fig. 3**).

To further characterize the phenotype of CD8 and NK cells during trauma, we assessed the expression of Granzyme B and IFN as well as that of inhibitory molecules like Tim-3 and PD1 in both subsets. When analyzing CD8 cells, we note decrease in the levels of Granzyme B (Fig. 4) and an increase in the proportion of PD-1⁺Tim-3⁺ double positive lymphocytes within the CD39⁺ CD8 subset (Fig. 5) of trauma patients, when compared to controls. A similar pattern of Granzyme B, PD-1 and Tim-3 expression is observed also when testing the CD39⁺ fraction of NK cells (data not shown).

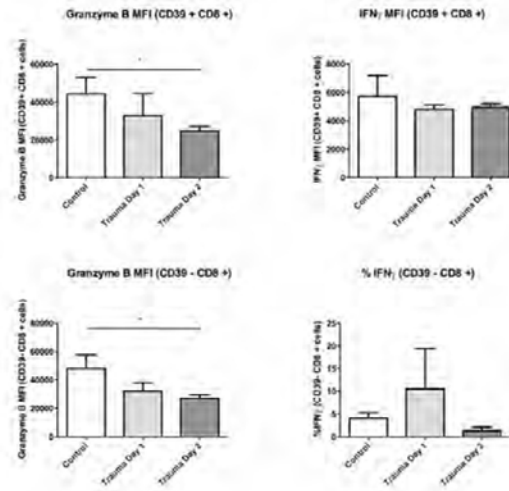


Fig. 4. Granzyme B and IFN γ mean fluorescence intensity of CD39⁺ and CD39⁻ CD8⁺ cell subsets. Granzyme B and IFN γ mean fluorescence intensity (MFI) of the CD39⁺CD8⁺ and CD39⁻CD8⁺ cell fractions was tested by flow cytometry intracellular staining in PBMCs from controls (n=5) and trauma patients at day 1 (n=5) and day 2 (n=4). We note decrease in the levels of Granzyme B in both CD8⁺ cell fractions while no significant changes are noted in the levels of IFN γ in both CD8⁺ T cell subsets of trauma patients. A similar trend was noted also when testing the CD39⁺ and CD39⁻ cell fractions of NK cells (data not shown).

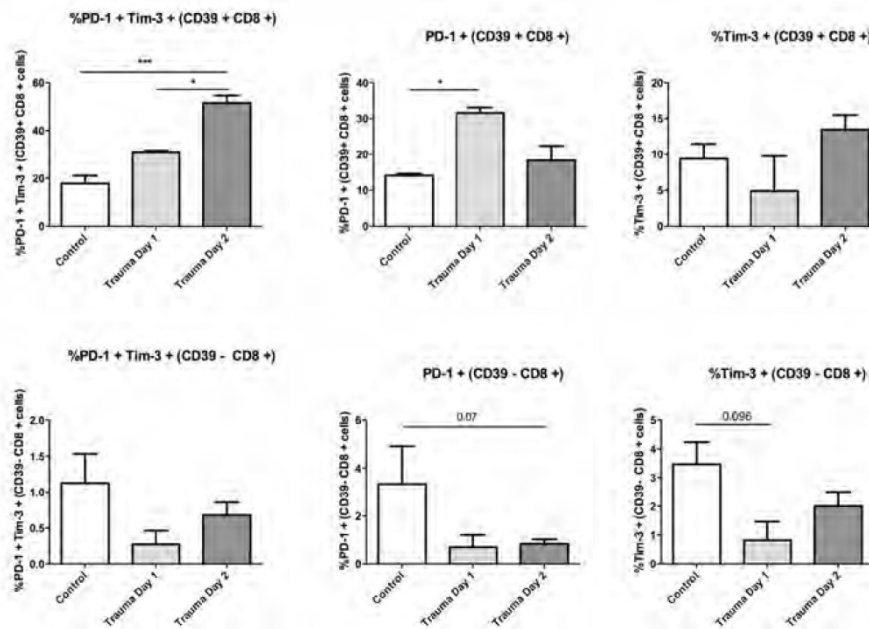


Fig. 5. Frequency of PD-1⁺Tim-3⁺ cells amongst CD8 cells in trauma patients and controls. The frequency of double positive PD-1⁺Tim-3⁺ and single positive PD-1⁺ and Tim-3⁺ cells within the CD39⁺ and CD39⁻ CD8⁺ cell subset was determined by flow cytometry in PBMCs obtained from controls (n=5) and trauma patients at day 1 (n=5) and day 2 (n=4). We note increase in the proportion of PD-1⁺Tim-3⁺ double positive cells within the CD39⁺ subset and a trend to decrease in the frequency of these cells in the CD39⁻ CD8⁺ subset. A similar pattern was noted also when testing the CD39⁺ and CD39⁻ cell fractions of NK cells (data not shown).

In this timeframe we have also performed isolation of mitochondria from mouse liver (C57BL6 mice) with the view of injecting these organelles to induce systemic inflammation. In Fig. 6, we depict morphology of isolated mitochondria and noted the staining patterns with Mito Tracker Green FM.

In the context of experiments aimed at defining mechanisms of experimental hyperoxic acute lung injury, we have utilized mass cytometry-CyTOF and appropriate systems biology analytic algorithms (viSNE, CITRUS) to characterize molecular phenotypes of pulmonary CD45⁺ immune cells under normoxia (controls) and in hyperoxic acute lung injury in mice (after 48 hours exposure to FiO₂>95%). Unbiased identification of spatially distinct immune cell subsets in whole lung parenchyma and bronchoalveolar space is depicted in Figure 7. The immune lineage of distinct clusters is described in Table 1 and Table 2.

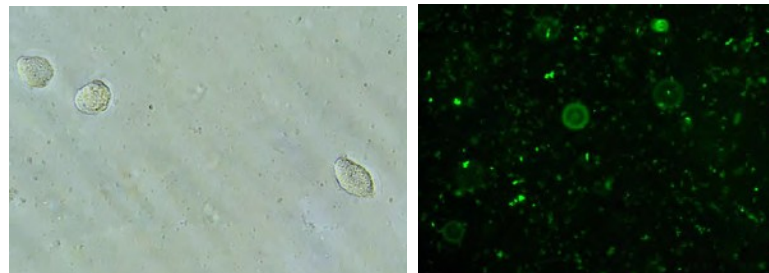


Fig. 6. Isolation of mouse mitochondria. Mitochondria were isolated from the liver of C57BL6 mice, stained with Mito Tracker Green FM and then visualized under fluorescence microscope. A representative of 4 independent experiments is shown.

PROJECT 4 (TALMOR): Human Subjects Core and 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 24 we have enrolled 61 trauma patients and 73 volunteer controls.

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

We have completed this task and ensure continuous ongoing compliance with all regulatory requirements.

SA2: Collect and process data on enrolled patients

Subtask 1 (months 3-60): Collect relevant patient and outcomes data.

Data are collected and entered on all enrolled patients within approximately seven days.

Subtask 2 (months 3-60): Safe / correct collection of samples.

We have completed this task. Through month 24 we have collected 276 samples. There was one adverse event, transient laryngospasm during bronchoscopy for BAL collection.

SA3: Physiochemical effects on airway innate immunity

Subtask 1 (months 3-15): Evaluate role of PO₂ in alveolar immune environment

We have designed an interventional protocol that will allow us to test this, and are securing the necessary regulatory approval prior to launch.

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

Subtask 1 (months 3-60): identify variations in biological signaling that may impact clinical care

We have not yet begun work on this task

Subtask 2 (months 3-60): design and execute Phase 0 RCTs as pilot studies to identify simple interventions that may prevent PNA

We are designing a Phase 0 RCT that will enable us to examine the impact of normoxia, hyperoxia, normocarbica, and hypercarbia on neutrophil and macrophage activation, purine metabolism, cytokines and biomarkers of inflammation.

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)
- Onboarded two clinical research assistants and two research fellows and completed training in good clinical practice, human subjects research, and research conduct. 11-Oct-2016 TP; 30-May-2017 JL; 01-Nov-2017 AB; 01-Sep-2018 IL.
- 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 134 patients through 01-Oct-2018
- Collected 276 biological specimens through 01-Oct-2018

Progress Detail:

Obtained IRB/HRPO approval.

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

Patient enrollment

From 10/1/16 through 10/1/18 we have enrolled 134 patients.

- Trauma Cases n=61 (70.5%) Male, average age 43.4 years (19-89)
127 approached
61 consented
60 trauma cases from whom samples collected
- Surgical Controls n=73 (53.9%) Male, average age 60 years (32-79)
167 approached
73 consented
47 surgical controls from whom samples collected

Regulatory requirements are in place.

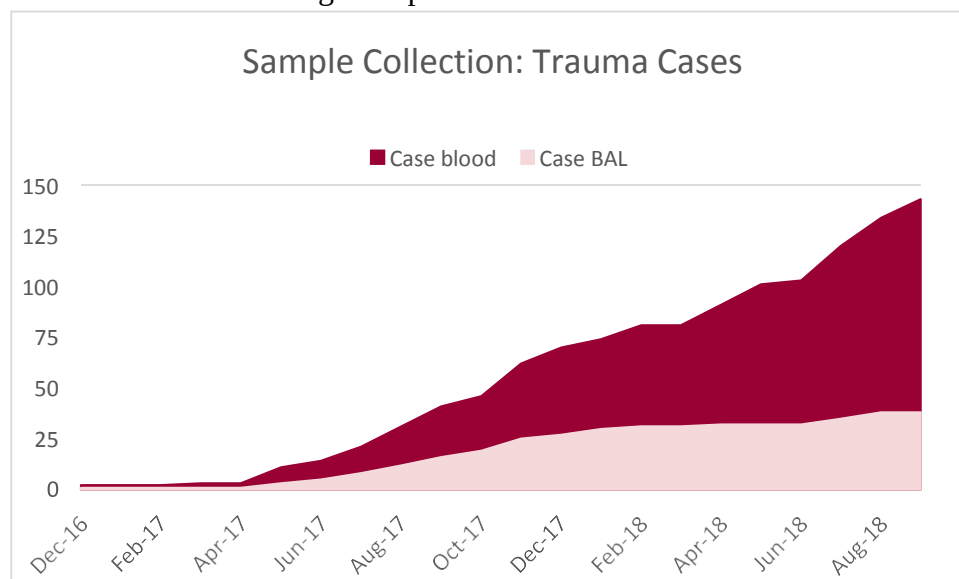
Complete regulatory files are maintained and audited monthly. Study staff maintain current research regulatory training at all times. Subject consenting is periodically monitored to ensure high level of quality.

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 10/1/18 we have collected 193 whole blood samples and 83 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. Figures 1 and 2 shows longitudinal sample collection to date in both groups.

Figure 1. Sample collection from Trauma Cases (serial sampling)

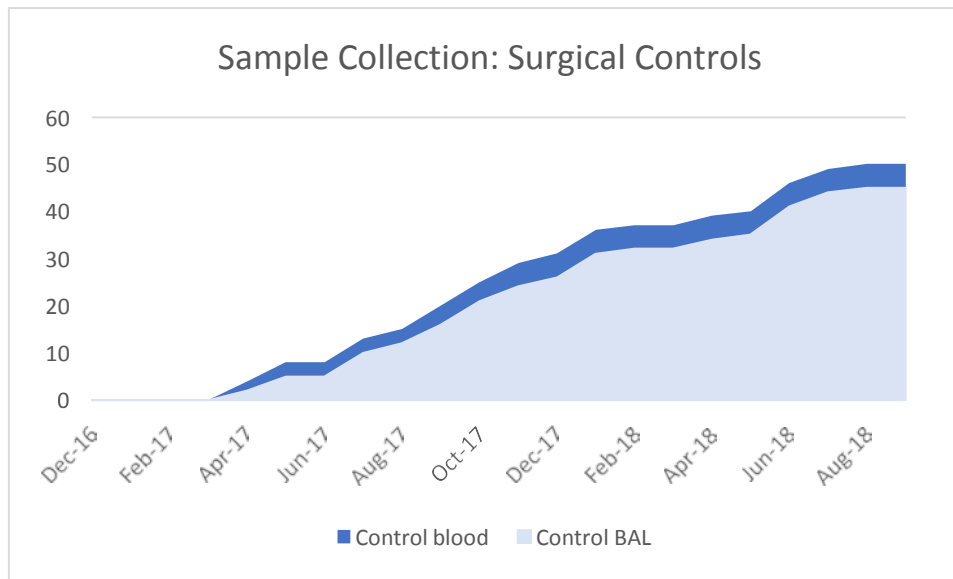


Figure 2. Sample collection from Surgical Controls (Day of surgery only)

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

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

- ***Evaluate role of PO_2 in alveolar immune environment***

We have designed a third study group, intubated critically ill patients, who will undergo ventilator manipulation and serial sampling. This will allow investigation of the role of PO_2 . Local IRB approval was obtained in Aug 2018; HRPO approval and Clinicaltrials.gov registration is in process.

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

Major activities, objectives, and results

Major activities: To define the phenotypic influences of trauma on cell-mediated immune responses to lung bacterial infections and to characterize the cellular and molecular features responsible for beneficial immune and anti-microbial function induced by CpG-ODN treatment in injured mice

- Performed immune profiling studies to identify how trauma alters the anti-microbial immune response to lung infections by profiling lung airway cellular immune response to *S. pneumoniae* infection, 10-2017 to 1-2018
- Performed experiments to characterize the significant protective effect of using synthetic CpG DNA molecules (CpG-ODN) as an immunotherapy to increase cell-mediated anti-microbial immune function in burn trauma mice, 11-2017, 12-2017
- CyTOF data analysis pipeline to determine cellular immune response changes for the cellular phenotyping studies, 12-2017, 1-2018
- Optimized protocols for digesting lung tissue and other organs to be used for single-cell CyTOF immune profiling studies, 1-2018, 2-2018
- Tested tissue extraction methods for lung tissue and optimized a lung tissue CyTOF antibody staining panel, 2-2018
- Established a new experimental approach to measure the effects of trauma plasma on normal human PBMCs, 2-2018
- Repeated studies to test trauma plasma activity on normal human PBMCs, 2-2018, 3-2018, 4-2018
- Re-initiated burn trauma two-hit mouse model studies to study the inflammatory response initiated by trauma in an animal model, LPS-induced mortality and cytokine experiments were performed, 3-2018
- Performed several experiments to test whether CpG-ODN immunotherapy reduces the inflammatory response and trauma two-hit response phenotype, 5-2018, 6-2018
- Profiled spleen and lung immune cell phenotype in our trauma two-hit response model including CyTOF antibody panel optimization and studies on the influence of CpG-ODN immunotherapy on the two-hit cellular immune response, 6-2018, 7-2018, 8-2018

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

- Worked with Project 3 group on lung tissue preparation for hyperoxia studies in mice to measure immune cellular changes associated with hyperoxia by CyTOF mass cytometry, 3-2018, 4-2018
- Luminex cytokine assays for mouse and human trauma studies, lung BAL infection cytokines, plasma cytokines, tissue cytokines, 3-2018, 4-2018, 7-2018
- Developed and tested lung hyperoxia CyTOF antibody staining panel in collaboration with Project 3, 4-2018
- Performed pilot liver crush injury studies to measure tissue injury infiltrating immune cells in collaboration with Project 2 group, 5-2018
- Repeated studies to measure lung hyperoxia immune response in mice by CyTOF mass cytometry including data analysis, 6-2018, 7-2018, 8-2018
- Analyzed CyTOF staining data from repeat lung hyperoxia project, lung bacterial infection model, and trauma two-hit response to prepare figures for manuscript drafts, 7-2018, 8-2018, 9-2018
- Panel design and testing for trauma PBMC, neutrophil, and BAL studies, 5-2018, 8-2018, 9-2018
- Human trauma PBMC CyTOF antibody panels and batch staining design finalized for mid-project immune profiling study, 10-2018

Other accomplishments

- Revised a manuscript for Journal of Trauma that reports on immune profiling 20 trauma patients, which is now accepted for publication, 8-2018

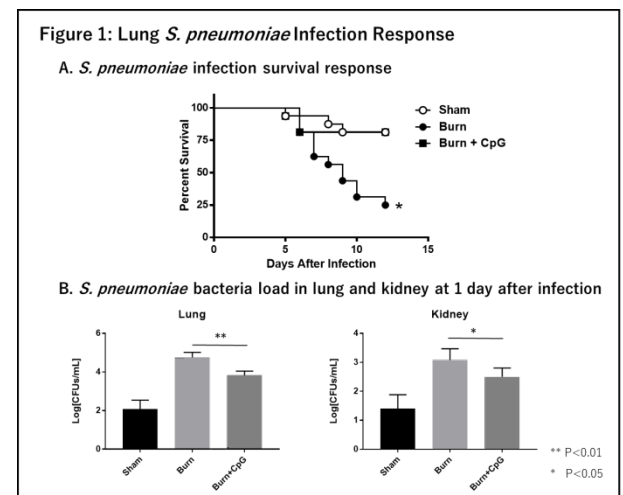
- Three presentations at the 2018 Shock Society meeting in Scottsdale to present the results of our mouse studies on trauma infection, trauma two-hit response, and human PBMC reactivity to trauma plasma

Results relevant to Specific Aims 1 and 2: To define the phenotypic influences of trauma on cell-mediated immune responses to lung bacterial infections and to identify cellular and molecular features responsible for beneficial immune and anti-microbial function induced by IRM treatment in injured mice.

During year 2, we continued to work towards identifying how trauma alters the anti-microbial immune response and the mechanisms-of-action for the significant protective effect of using synthetic CpG DNA molecules (CpG-ODN) as an immunotherapy for trauma. We addressed this work by focusing on two basic mechanisms of actions for CpG-ODN activity: 1) the biological effects of CpG-ODN treatment on the cellular immune response to lung bacterial infection and 2) the modulatory activity of CpG-ODN resuscitative immunotherapy on the systemic inflammatory response phenotype that occurs after traumatic injuries and the “two-hit” response phenotype. The results of these 2 aspects of our work were presented orally at the Shock Society Meeting that was held in Scottsdale, Arizona in June, 2018

Mechanism 1: We used our established mouse burn trauma model and lung bacterial infection approach to study how CpG-ODN therapy influences the anti-microbial cellular immune response in mice. The technologies used included CyTOF mass cytometry to profile the immune cell response to lung bacterial infection and cytokine assays by Luminex technology. In brief, mice underwent sham or burn injury and were treated 2 hours after injury with the class A CpG-ODN sequence 2336 or saline. At 1 day after injury, mice were infected in the lungs with *S. pneumoniae* bacteria (2×10^6 CFU). Importantly, in this trauma and lung pneumonia model, burn trauma mice are highly susceptible to bacterial pneumonia. To address how post-trauma CpG-ODN immunotherapy enhances survival, we prepared lung bronchoalveolar lavage fluid from mice to comprehensively measure differences in the immune cell response to lung infection between un-injured (sham) mice and burn trauma mice. The influence of CpG-ODN treatment was determined by comparing cellular immune responses between treated and untreated groups of mice. The anti-microbial cellular and cytokine immune responses were measured using CyTOF mass cytometry and Luminex cytokine assays. Lung and kidney bacterial counts were performed to assess the level of anti-bacterial immunity in mice. The summary of results indicated:

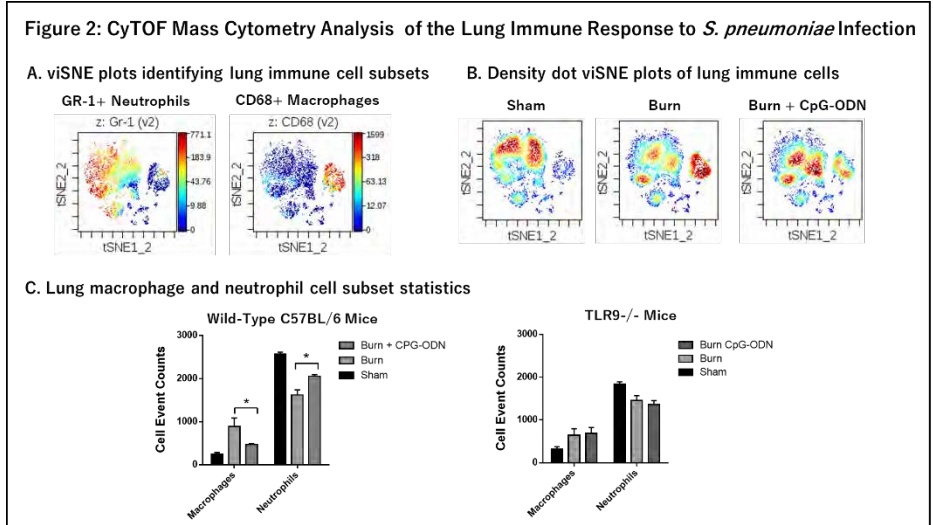
1. Burn trauma mice showed a significant mortality when challenged with *S. pneumoniae* as compared to sham mice;
2. CpG-ODN treatment given at 2 hours after trauma demonstrated a significant increase in pneumonia survival response;
3. specific differences in cell-mediated immune responses to lung infection between sham and burn mice were identified by CyTOF mass cytometry – burn trauma mice showed a marked deficiency in neutrophil subset response to lung bacterial infection;
4. CpG-ODN treatment at 2 hours after trauma reversed the suppressed neutrophil response to lung infection;
5. the beneficial activity of CpG-ODN immunotherapy for trauma did not occur in mice with Toll-like receptor 9 (TLR9) deficiency – TLR9 is one of the known receptors for CpG DNA;
6. improved survival in CpG-ODN treated burn mice was associated with reduced lung and kidney bacteria burden.



Data to support some of the above listed conclusions are presented here in this report (Figures 1 and 2). In Figure 1A, we demonstrate the survival response in sham, burn, and burn CpG-ODN treated mice that were infected at 1 day after injury. Sham and burn mice showed significant differences in survival. Treatment with CpG-ODN 2336 at 2 hours after burn trauma, significantly enhanced the survival response. In Figure 1B, the

colony forming units (CFU) of *S. pneumoniae* in lung and kidney homogenates are plotted as mean +/- SEM for *S. pneumoniae* bacteria detected in these organs.

In Figure 2, CyTOF mass cytometry analysis of immune cell subsets identified in the lungs of sham, burn, and burn CpG-ODN treated mice are shown. These data are plotted using a dimensional reduction method called viSNE to allow visualization of immune cell subsets (cell identification) and changes in immune cell densities (density contour plots). In A), the location of lung airway neutrophils are identified in viSNE plots by GR-1 marker expression and macrophages are identified by CD68 marker expression. In B), these viSNE density dot plots show the changes in immune cell subset densities in lung airways at 1 day after infection of sham, burn, or burn CPG-ODN treated mice. There is an obvious difference in macrophage and neutrophil cell densities between the experimental groups. The plots in Figure 2C summarize the relative differences in lung macrophage and neutrophil cell numbers between experimental mouse groups and compare CpG-ODN treatment activity in wild-type (normal mice) and TLR9 gene deficient mice (TLR9^{-/-}). Sham wild-type mice show a dominant neutrophil increase, burn mice showed a higher macrophage response and lower neutrophil numbers. Burn trauma mice treated with CpG-ODN showed a significant decrease in macrophages and an increase in neutrophils. Mice that are genetically deficient in TLR9 did not demonstrate any changes with CpG-ODN treatment, which indicates that the immunotherapeutic activity of CpG-ODN requires functional TLR9 expression.

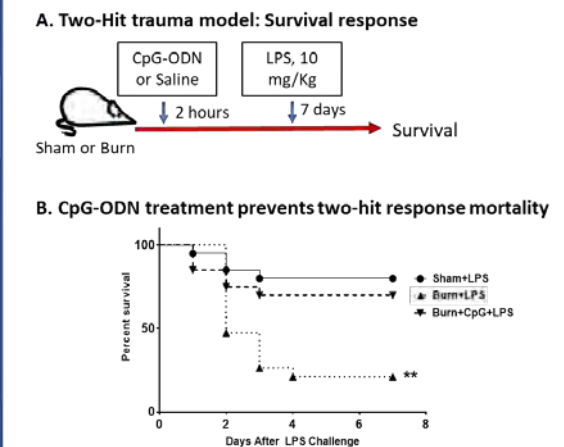


Summary: The data generated in this study provides the first complete profile of trauma-induced cell-mediated immune deficiencies that contribute to poor anti-microbial immunity following trauma. By comparing the anti-microbial immune response in mice given CpG-ODN immunotherapy, we also demonstrate which immune cell responses are beneficial. The reduction in CD68+ macrophages and increase in neutrophil response to infection in CpG-ODN treated mice was significant. Importantly, the multi-dimensional single-cell analysis capability of CyTOF mass cytometry showed that the type of neutrophils that respond to lung bacterial infection in burn trauma mice are different. As illustrated in Figure 2B, there are 2 dominant neutrophil clusters in sham mice and 3 dominant clusters in burn trauma mice. Treatment with CpG-ODN did not revert the burn trauma neutrophil response to the sham neutrophil response phenotype. Thus, this study revealed new insights into how trauma changes neutrophil subset reactivity to infection.

This research study was presented at the 2018 Shock Society Meeting by Takeshi Wada, MD. He received a top-rated abstract award for this work. A manuscript to report these findings has been drafted and will be finalized to submit for peer review and publication during year 3.

Mechanism 2: One of our hypotheses is that trauma primes the innate immune system to be more pro-inflammatory, which in turn can enhance reactivity to bacterial infections to trigger the two-hit response. Post-trauma inflammation and the two-hit response can induce septic shock, organ injury, and chronic critical illnesses in severely injured people. Thus, we extended our mechanisms-of-action studies for CpG-ODN

Figure 3: CpG-ODN treatment prevents two-hit mortality response in mice



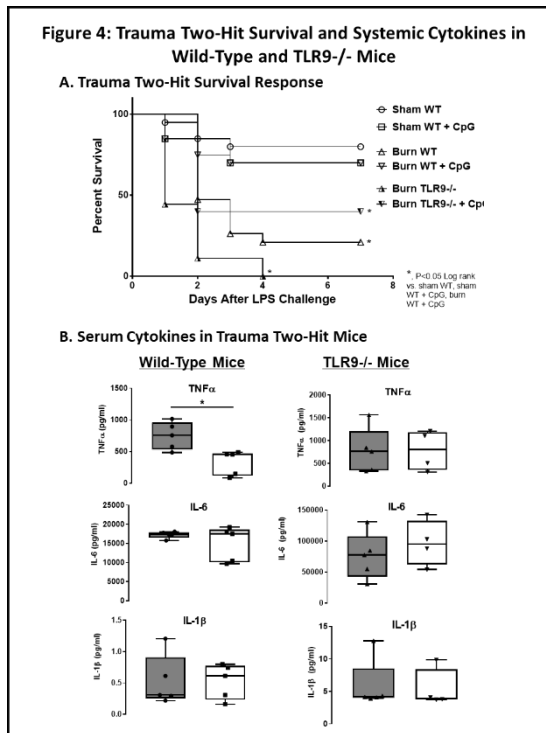
immunotherapy to test the hypothesis that CpG-ODN treatment may alter the inflammatory response to burn trauma.

To test this hypothesis, we used an established mouse two-hit trauma mortality model that involves performing burn trauma and then following with a second hit challenge at 1 week post injury by injecting mice with a bacterial toxin called lipopolysaccharide (LPS). This second hit is thought to mimic the septic shock response to bacterial infections that can occur in people that are injured and develop a subsequent bacterial infection. The infection rate post-trauma can be as high as 45% when injury severity is high. During year 2, we found that CpG-ODN 2336 treatment at 2 hours after burn trauma reversed the two-hit response mortality in this mouse model (Figure 3B). This discovery suggests that CpG-ODN immunotherapy may reduce the severity of inflammatory complications in people that develop infections after trauma. Additional mechanistic studies were performed to study organ immune cell changes (spleen and lung) that are altered by burn injury and that are modulated by CpG-ODN treatment. We used CyTOF mass cytometry as an approach to study which LPS-reactive cells are increased by burn trauma and which cells showed heightened reactivity to *in vivo* LPS challenge. Luminex cytokine assays were used to measure systemic cytokine levels in LPS-challenged mice. A summary of the results indicated that:

1. Treating mice with CpG-ODN at 2 hours after trauma significantly improved survival to LPS challenge in burn-injured mice (Figure 3B);
2. the results of matched LPS challenge survival experiments in Toll-Like Receptor 9 gene deficient (TLR9 $-/-$) mice indicated that CpG-ODN protective activity depends on functional TLR9 expression;
3. analysis of tissues in LPS-challenged mice by CyTOF mass cytometry revealed that Ly6C $^{+}$ macrophages may be the primary LPS-reactive cell subset in burn trauma mice
4. results of cytokine staining of spleen and lung cells from burn and burn CpG-ODN treated mice indicated that CpG-ODN treatment significantly reduced TNF α and IL-6 expression by the Ly6C $^{+}$ macrophage subset.

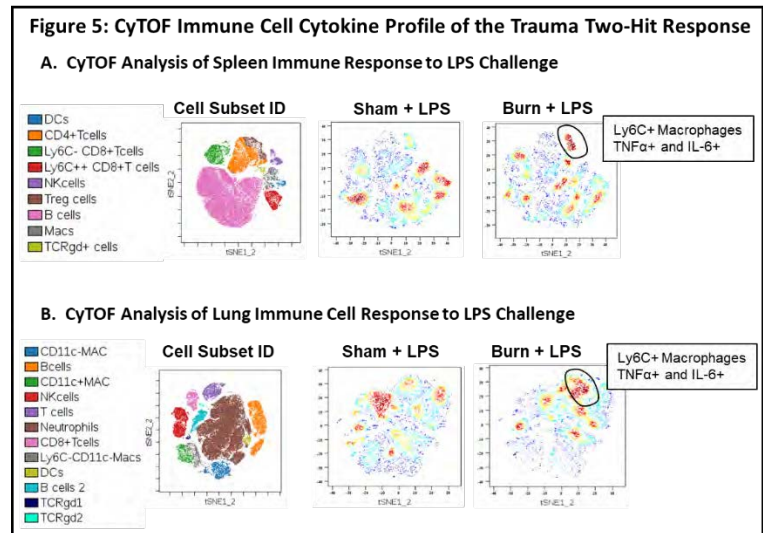
Collectively, the results of this research supports the hypothesis that trauma induces an enhanced two-hit inflammatory phenotype that can trigger post-trauma mortality. The observation that CpG-ODN treatment at 2 hours after trauma can significantly reduce the two-hit response supports the idea that CpG-ODN may alter the clinical trajectory by reducing post-trauma inflammation. The feasible use of CpG-ODN as a resuscitative immunotherapy is extremely important for military use, but also useful for civilian use in trauma and critical care programs. The 2 hour post-trauma treatment was pursued for these reasons. Later treatment at 1 day post-

trauma was also found to be protective in our mouse trauma model.



We used our mouse burn trauma model and challenged mice at 7 days post trauma with *E. coli* bacteria lipopolysaccharide (LPS) (10 mg/Kg) to mimic the shock response from systemic bacterial infection. We report that burn trauma mice demonstrated a significantly higher mortality response to LPS challenge. CpG-ODN treatment at 2 hours after burn trauma significantly reduced two-hit mortality to sham mice levels and reduced systemic cytokine levels (Figure 4). Working with TLR9 $-/-$ mice allowed us to validate that functional TLR9 expression is required for the therapeutic activity of CpG-ODNs (Figure 4). CyTOF mass cytometry was used to identify that Ly6C $^{+}$ macrophages are the major immune cell subset that mediates the two-hit response and are the primary source of TNF α and IL-6. Data to support this conclusion is shown in Figure 5.

The findings from this study were presented as a talk at the 2018 Shock Society Meeting by Yasutaka Nakahori. He was awarded a travel award to present this work at the meeting based on the quality of his abstract. There was significant interest in the results that were presented because they are among the first to suggest that an immunotherapy for trauma can protect traumatically-injured mice, and possibly people, from developing severe post-trauma inflammation and shock from bacterial infections. A manuscript to report the results of this research work has been drafted and will be submitted for peer review during year 3.



Summary: Taken together, the results of these pre-clinical studies support developing class A CpG-ODNs as an immunotherapy to protect severely-injured patients and warfighters from developing infections and infectious complications after civilian or battlefield injuries.

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

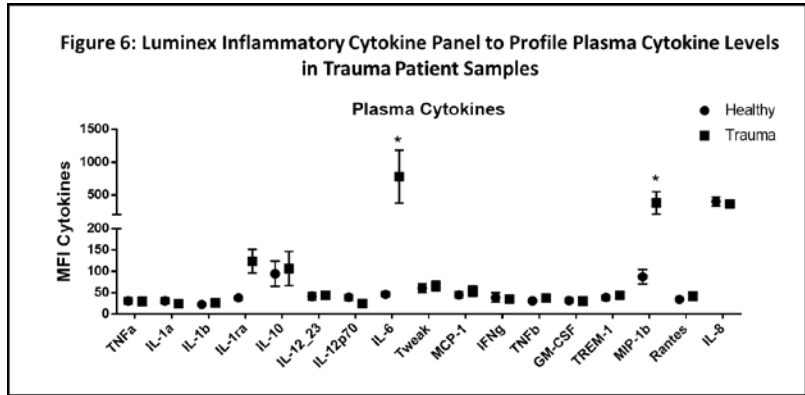
We worked in collaboration with Projects 2 and 3 on using CyTOF mass cytometry technology to study how CO treatment or hyperoxia alters the immune system. Specifically, we developed and validated panels to identify lung immune system changes in mice that were 1) Project 2, exposed to CO and challenged with bacteria in the lungs or 2) Project 3, exposed to hyperoxia condition. We completed the lung bronchial alveolar lavage (BAL) CyTOF immune profiling study for Project 2. In that study, we worked with them to identify the effects of CO on lung bacterial infection responses. The Project 2 group is in the process of performing flow cytometry work to validate some of the novel subsets that were identified by CyTOF.

We performed an additional collaborative liver crush injury CyTOF immune cell subset profiling study that is mutually relevant to Projects 2 and 5. The aim for project 2 was study how CO exposure acutely affects the immune cell types that respond to tissue injuries. The aim for project 5 was to identify the immune cells that travel to a tissue injury site. These CyTOF data have been analyzed and are currently being interpreted for subsequent validation studies by CyTOF or flow cytometry. Differences were observed in the neutrophil and macrophage response to liver crush injury in the CO exposure model. Comparison between uninjured and injured liver tissue allowed the project 5 group to identify which neutrophil and macrophage subsets react acutely to tissue injury. It is hoped that the liver crush model will serve as a model to understand the unique nature of the immune cell response to tissue damage and danger associated molecular patterns (DAMPs).

Collaborative work with Project 3 (Robson) was completed during year 2. A research fellow from his group worked directly with my lab to profile lung immune cell changes in mice exposed to hyperoxia condition. After developing and validating a specific CyTOF antibody panel, we performed a series of repeated studies. A manuscript has been fully drafted and will be submitted soon. A detailed description of this work is provided in the accomplishment report for project 3 (Robson).

Luminex Cytokine Assays: Mouse and human Luminex cytokine assays are in use for all projects and openly available to project investigators. In Project 5, we used the mouse Luminex cytokine assays to measure systemic and organ cytokine levels that arise at 90 minutes and 6 hours after LPS challenge in our trauma two-hit mouse model as described in this report. Project 5 also used Luminex assay technology to profile cytokine and chemokine levels in the lung airway after infection by measuring BAL fluid. Human Luminex cytokine assays have been used to profile plasma cytokine levels in samples from healthy vs trauma patients. We identified that IL-6 and MIP1 β levels are significantly higher in patients at day 1 after trauma as compared to normal healthy controls using this approach, n=9 patients and controls (Figure 6). We plan an interim Luminex cytokine

profiling analysis of all control and trauma patient samples that have been collected to date for the HALO project. This data will be analyzed by Project 6 to determine correlative and predictive models using clinical patient data.



markers based on discussions with the project 1 and 6 investigators (Hauser and Yaffe). To improve the quality of all CyTOF staining data, we developed a compensation strategy for 39 marker antibody panels based on a recent published method. The approach will prevent crosstalk among antibody detection markers and involves a series of analytical methods for subsequent data analysis. This refinement will be important for trauma patient studies due to the inherent complexing of the response.

Figure 7: CyTOF Antibody Staing Panels to Profile Human PBMCs for the HALO Project

Innate			Adaptive		
Marker	Clone	Metal	Marker	Clone	Metal
CD20	2H7	113In	CD20	2H7	113In
CD3	UCHT1	115In	CD3	UCHT1	115In
CD33	WM53	141Pr	CD33	WM53	141Pr
CD11b	M1/70	142Nd	CD45RA	HI100	142Nd
CD115	9-4D2-1E4	143Nd	CD279/PD-1	EH12.2H7	143Nd
CD39	A1	144Nd	CD39	A1	144Nd
CD163	GHI/61	145Nd	CD258/LIGHT	115520	145Nd
CD8	RPAT8	146Nd	CD8	RPAT8	146Nd
CD86	IT2.2	147Sm	CD45RO	UCHL1	147Sm
TLR4 (CD284)	HTA125	148Nd	CXCR3	G025H7	148Nd
autotaxin	polyclonal	149Sm	CD25	M-A251	149Sm
Arginase I	Polyclonal	150Nd	CD11c	Bu15	150Nd
85d (ILT4)	421D	151Eu	TCR Vd1	REA173	151Eu
CD14	M5E2	152Sm	CD152/CTLA-4	L3D10	152Sm
CXCR1	8F1/CXCR1	153Eu	IL-17F	SHLR17	153Eu
CD15	W6D3	154Sm	CD15	W6D3	154Sm
CD4	RPA T4	155Gd	CD4	RPA T4	155Gd
CD73	AD2	156Gd	CD73	AD2	156Gd
CD16	3G8	157Gd	HO-1	HO-1-1	157Gd
T-bet	4B10	158Gd	CD71	CY1G4	158Gd
CD11c	Bu15	159Tb	CCR7	G043H7	159Tb
FPR1	350418	160Gd	CD278 (ICOS)	C398-4A	160Gd
Ki-67	8D5	161Dy	Ki-67	8D5	161Dy
CD56	NCAM16.2	162Dy	CD56	NCAM16.2	162Dy
CCR5	J418F1	163Dy	CCR5	J418F1	163Dy
CD141	M80	164Dy	CD161	HP-3G10	164Dy
CXCR2	5E8/CXCR2	165Ho	FoxP3	PCH-101	165Ho
CD80	2D10	166Er	CD80	2D10	166Er
PD-L2	MIH18	167Er	Grz B	GB11	167Er
HO-1	HO-1-1	168Er	CD196 (CCR6)	G034E3	168Er
CD88	S5/1	169Tm	CD95/FAS	DX2	169Tm
CX3CR1	2A9-1	170Er	CD127	eBioRDR5	170Er
CCR2	K036C2	171Yb	TCR Vd2	123R3	171Yb
PD-L1	293.2AE	172Yb	CD38	HIT2	172Yb
C3AR	hC3aRZ8	173Yb	TIGIT	MBSA43	173Yb
HLA-DR	L243	174Yb	HLA-DR	L243	174Yb
CD172ab	SE5A5	175Lu	Tbet	4B10	175Lu
CD68	Y1/82A	176Yb	RORγT	AFKJS-9	176Yb
CD45	HI30	209Bi	CD45	HI30	209Bi

incorporate clinical outcomes information with immune cell phenotype changes.

Human Blood Immune Cell Profiling by CyTOF Mass Cytometry: Blood samples from trauma and control patients continue to be collected and cryopreserved for immune profile analysis by CyTOF mass cytometry or other approaches. We refined our thawing and stabilization protocols for blood neutrophils by adding a unique fixation step to prevent clumping and lysis by these fragile cells during the CyTOF staining process. In addition, we expanded our blood neutrophil panel by several

We are in the process of starting an interim, mid-project analysis of all peripheral blood mononuclear cell samples from trauma patients and case controls that have been cryopreserved. We decided to approach these samples for analysis using two different 39-marker CyTOF antibody staining panels that focus on innate or adaptive immune cell populations. These panels are shown in Figure 7. The innate panel includes markers to identify major immune cell subsets and other unique innate immune cell markers that are relevant to the aims of the HALO project. The adaptive panel similarly includes markers for major immune cell subsets and markers relevant to adaptive immune cell activation and differentiation. Prior knowledge was used to develop these CyTOF antibody panels. The objective of our CyTOF immune cell profiling work for HALO is to provide unbiased profiling of time-dependent abundance and phenotype immune cell changes that occur in trauma patients.

There are nearly 100 cryopreserved PBMC samples that will be profiled by CyTOF using these panels. We have set up our batch staining strategy, which will be limited to doing a maximum of 60 stained samples per day. The trauma patient samples will be randomized into 3 batch staining days using barcoding strategies. This approach is necessary to reduce batch effects in these large clinical studies. Our analysis program for CyTOF data has advanced to a series of steps that will allow us to efficiently analyze multi-dimensional single-cell CyTOF staining data. The CyTOF stain data will be analyzed by us to identify significant cell changes. We will then work with the computational experts in Project 6 to

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

- Continued evaluating trauma patient blood samples for primability and ROS production. 15-12-2017.
- Together with Lederer lab (Project 5), continued to explore a variety of systems-based models that capture the emergence of various immune populations following human trauma using CyTOF data. 01-04-2018.
- Published in Journal of Trauma and Acute Care Surgery showing the role of mitochondrial formyl peptides on impairing human neutrophils functionality (together with projects 1 and 2). 4-05-2018.
- Established two mouse colonies, *p47phox*^{-/-} and *p47phox*^{K43A/K43Q}, and continued backcrossing *p47phox*^{K43Q/K43Q} mice in order to perform experiments evaluating the role of extracellular ROS on innate immune dysfunction. 24-08-2018.
- Published in Clinical & Experimental Immunology demonstrating that Blood clotting and traumatic injury with shock mediates complement-dependent neutrophil priming for extracellular ROS, ROS-dependent organ injury and coagulopathy. 01-10-2018.
- Onboarded vet technician and completed training in animal care and good lab practice. 01-10-2018.
- Obtained ACURO approval for animal use in the liver crush injury model. 12-10-2018.
- Continued to prepare samples to evaluate the presence and characterization of a low-density neutrophil immunosuppressive population using CyTOF and in collaboration with Project 5. 10-10-2018.
- Performed initial experiments using liver crush injury in *p47phox*^{-/-} mice. 20-10-2018.

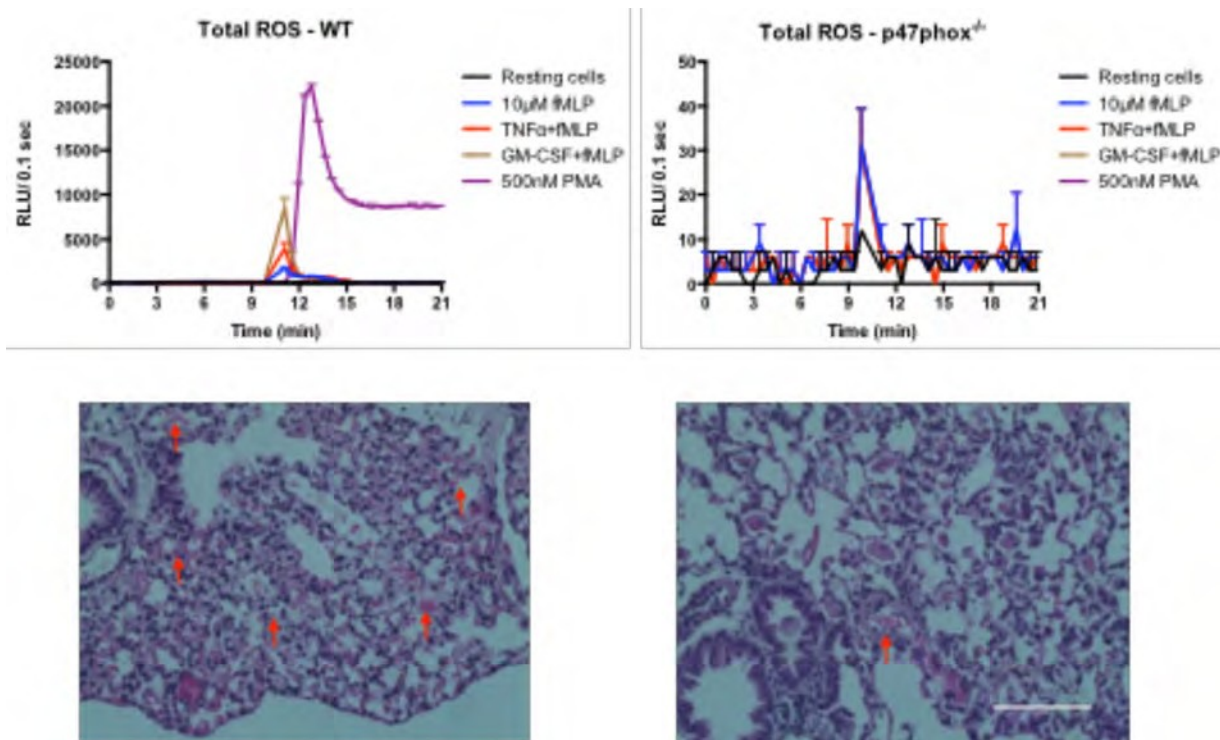
What was accomplished under these goals?

Our studies on human trauma patients whose blood was collected 1, 3, and 5 days after trauma demonstrated significant pre-existing priming in the basal state by day 1 after trauma, when assayed using TNF α and C5a as exogenous agents to further assess primability, and fMLP as the activating agent. We also found that trauma whole blood is continuously releasing extracellular ROS, which may contribute to enhanced damage of tissue. On the other hand, there was reduced basal priming at later days, as well as some indications that these cells may be further resistant to exogenous priming agents. We are continuing to evaluate ROS, priming and depriming of neutrophils from trauma patients in different day points.

In particular, we validated that a substantial portion of priming activity is present in the plasma of human trauma patients early after injury, but not in plasma from uninjured controls, when assayed against naïve PMNs from un-injured patients. Much of this priming activity appears to be the result of C5a generated as a consequence of complement activation. C5a production appears to result, at least in part, from the process of blood clotting and trauma coagulopathy, and this type of PMN priming appears to specifically release extracellular ROS, which likely contributes to enhanced tissue damage. We further validated that naïve neutrophils pre-incubated with clotted blood are able to compromise endothelial barrier function, indicating that the ROS release is the factor that contributes to tissue damage, which was validated in a murine trauma hemorrhage model. Importantly, we showed that organ injury and endothelial barrier function compromise was attenuated in *p47phox* knock-out animals. This work was published this year in *Clinical and Experimental Immunology*. Since other priming agents and agonists may be responsible for neutrophils activation and ROS release, together with Hauser lab (Project 1) we examined the effects of all N-formyl mitochondrial DAMPs on PMN priming and activation. Among the DAMPs that have been tested, there are 5 mitochondrial formyl peptides (mtFP) that induce Ca²⁺ flux similar to fMLP, of which ND6 was the most potent for ROS production and chemotaxis. This work was published this year in the Journal of Trauma and Acute Care Surgery.

As detailed in the original proposal, we have created specific *p47phox* mutant knock-in mice that generate reduced (*p47phox*-K43A) and increased (*p47phox*-K43Q) amounts of extracellular ROS in order to specifically address the importance of extracellular ROS in tissue injury and innate immune dysfunction after trauma. Recently we established *p47phox*^{-/-} and *p47phox*K43A mouse colony in pure C57BL/6 background, since this genetic background is required to see optimal responses in trauma/hemorrhage models. SNP analyses showed that wild-type and *p47phox*K43A mice displayed the most C57BL/6NTac character (100.0%) and can be considered congenic. *p47phox*K43Q mice displayed mixed and non-C57BL/6NTac character on

chromosomes 5, 9, 14 and 17. *p47phox*^{-/-} mice displayed mixed and non-C57BL/6NTac character on chromosome 5. To have these mice (*p47phox*^{-/-} and *p47phoxK43Q*) congenic, we have been backcrossing them with pure C57BL/6, and have generated pure *p47phox*^{-/-} mice in this background. *p47phoxK43Q* mice have been more difficult to breed and need to go through two more generations before being congenic. Meanwhile, after receiving ACURO approval for the use of those mice in the liver crush injury model, we performed a liver crush injury pilot experiment comparing *p47phox*^{-/-} and wild-type mice. As expected, *p47phox*^{-/-} neutrophils do not produce significant levels of ROS in response to fMLP or PMA, even after priming with GM-CSF or TNF α , as shown below. Interestingly, there was less accumulation of immune cells in the lungs of *p47phox*^{-/-} mice.



Finally, we have begun initial experiments in collaboration with the Lederer lab (Project 5) to investigate whether a previously undocumented population of neutrophils – so called ‘low-density neutrophils’ with markedly immunosuppressive properties emerges as a function of time after trauma. This neutrophil population was recently described in the cancer literature (c.f. Sagiv et al., Cell Reports 2015, 10:562-573; Scapini et al., Immun. Rev. 2016, 273:48-60) as showing a reduced oxidative burst and phagocytosis. A portion of these cells appears to be distinct from myeloid- derived suppressor cells, emerging instead from mature previously high-density neutrophils. To date no one has reported on the existence or creation of these cells after trauma, but if present, they could significantly explain the development of an immunosuppressive state after trauma. We have now been able to document the existence of these cells in both human and murine peripheral blood, but the separation protocol described in the literature needs to be improved. Preliminary CyTOF experiments on these cells have not revealed distinguishing surface features, but the experiments are in a very early stage.

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?

- Dr. Hauser was invited to give an oral presentation at Western Trauma conference. The data were published at Journal of Trauma and Acute Injury.
- Dr. Itagaki was invited to give an oral presentation results at 3rd Japan-US Technical Information Exchange Forum on BLAST INJURY (JUFBI2018) at Shinjuku, Tokyo organized by the National Defense Medical College.
- Dr. Kwon presented data at Longwood Area Department of Surgery meeting and received 1st prize as the best poster presentation.
- Dr. Lederer presented at Shock Society, Scottsdale, AZ
- Dr. Yaffe, publications, and presentations at the Western Association of Trauma, and the Annual Congress of the American College of Surgeons.
- Dr. Otterbein presented at the American Chemical Society, New Orleans, LA
- Dr. Otterbein presented at the Military Health System Research Symposium, Orlando, FL
- Dr. Otterbein was a visiting professor at University of California/Scripps Oceanography Institute, San Diego, CA.
- Ghee Lee presented at the Longwood Area Department of Surgery meeting
- Dr. Robson presented at the Physiological Society, London. UK.
- Dr. Robson presented at Purines2018 in Igazu, Brazil
- Dr. Robson presented at Beijing University, 6th joint conference, Beijing China

Professor Eric Elster, MD was a visiting professor from the Uniformed Services University. He is the Department of Surgery Chairman who has similar interests regarding the wounded warfighter and identifying deliverables for military and civilian use. All projects were presented to him during his visit and he gave a lecture entitled “From Battlefield to Bedside: Bringing Precision Medicine to Critical Care”.

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

Plans for next reporting period include: 1.) continuing to accumulate patient specimens in collaboration with Dr. Elster at USUHS and Dr. Rennick at Boston University Medical Center, 2.) continuing to dissect mechanisms of action in our established animal and cell culture models, 3.) begin to analyze the big data to perform the computational modeling and 4.) evaluate therapeutic approaches by which to decrease susceptibility to infection after trauma, e.g. oral CO delivery. Overall, there will be no major deviations from the original plan. We provide brief summaries of project plans.

PROJECT 1

We plan to study mtDNA and mtFPs in plasma collected trauma patients and volunteers to apply our techniques established during the past two years. Also, we will investigate animal injury models using wild type and FPR1/2 KO animals.

PROJECT 2

We will further explore the role of heme as a Danger molecule released during trauma and seek to understand how it is released from the cell, circulates and ultimately recognized and metabolized by the cell generating CO. Efforts are underway supporting the clinical testing of CO using a novel oral formulation.

PROJECT 3

Define clinical role of P2X7 and Adenosine Receptors expressed by CD8+CD39+ cells and monocyte macrophages in blood and BAL of patients post trauma. CYTOF final analyses pending – Projects #2 and #5. Define clinical role of Ectonucleotidases/CD39 and Adenosine Deaminases in trauma associated

immunosuppression and link this to infections in clinical studies followed by modeling in mouse experimentation using mitochondrial derived ATP and DAMPS. Defining Monocyte-Macrophage-dependent purinergic mechanisms post trauma and associated immunosuppression. Ongoing extension of project to evaluate purinergic cell signaling in myeloid cells. Defining Monocyte-Macrophage-dependent inflammatory pathways of hyperoxic acute lung injury in experimental mouse models – manuscript in preparation.

PROJECT 4

We will continue to submit all necessary reports to the local IRB and HRPO and continue screening and recruiting trauma and surgical control patients. We are actively exploring collaborations with Boston University Medical Center and Uniformed Services University to increase our accrual numbers. We are now designing a third study group of intubated critically ill patients, who will undergo ventilator manipulation and serial sampling. We continue to work closely with consortium team members to review laboratory findings and run analyses to uncover variations correlated with clinical outcomes. Based on the results of analyses described above, we have begun to formulate study design capsules for pilot studies

PROJECT 5

Perform mid-project CyTOF-based immune profiling studies for patient and control cryopreserved blood immune cells and lung airway lavage cells. Analyze CyTOF staining results and work with the computational group (Project 6) to develop models that use cellular information and clinical data. Continue working on mechanisms-of-action and translational studies to support using CpG-ODNs as an immunotherapy for traumatic injuries. Develop more extensive Luminex biomarker assays to include additional DAMPs and relevant cytokines.

PROJECT 6

Continue to focus on examining the relative priming and primability of PMNs obtained from the blood, peritoneum, and lungs (BAL) from wild type and p47phox knock-out and K43A or K43Q knock-in mice using the liver crush model. We will examine the extent to which p47phox function, and the function of the stress kinase MK2 contributes to priming induced release of neutrophil granule contents that may influence immune function, as well as the extent to which MK2 contributes to the development of immunosuppressive macrophages and neutrophils that link DAMP release to subsequent immune dysfunction after trauma. These studies are all done in collaboration with Projects 1,2 and 3. Finally, we will continue to explore the emergence of immunosuppressive neutrophil and macrophage subsets using CyTOF in collaboration with Project 5.

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

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

One of our key goals in year two was to continue to expand our knowledge of the model systems and readouts in cells isolated from trauma patients and determine if this was able to be mimicked in mouse models. We are confident that we are able to compare the effects we observe in mice, in human cells. Each laboratory has optimized their methods and in most instances collaborated successfully across disciplines in efforts to maximize the data collected. All projects were highly productive in year 2 with additional grant support, publications, and national and international presentations.

What was the impact on other disciplines?

In Project 5, the development of a reliable approach to digest tissues for CyTOF mass cytometry single cell has led to several important collaborations with cancer biology and immunology research groups. We are using approaches gained from this project to profile immune cell reactivity to different types of brain tumors, head and neck cancers, and in controlled mouse cancer immunology studies to test the impact of immune modulation on the immune response. The impact of our work in trauma immunology will have impact through collaboration with cancer biologists. Our efforts to expand our collaborative efforts was successful in leveraging our tissue

repository for exploratory studies in glycomics and microbiome studies. These will be directly compared with our animal models to allow us to move findings identified in human cells into more mechanistic studies in mice.

What was the impact on technology transfer?

Interactions with Dr. Eric Elster from the Uniformed Services University has led to a potential opportunity to develop specific biomarker assays to be used in a clinical prediction model and project that he has developed for battlefield and civilian trauma diagnostics. These would be important and exciting deliverables.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS

Changes in approach and reasons for change

Nothing to report.

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

Nothing to report.

Changes that had a significant impact on expenditures

None to report.

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

No deviations or changes other than standard amendments. Project 6 at MIT was approved by ACURO to use the liver injury and infection model in mice in October, 2018.

SIGNIFICANT CHANGES IN USE OR CARE OF HUMAN SUBJECTS:

No significant changes to report.

SIGNIFICANT CHANGES IN USE OR CARE OF VERTEBRATE ANIMALS:

No significant changes to report.

SIGNIFICANT CHANGES IN USE OF BIOHAZARDS AND/OR SELECT AGENTS:

No significant changes to report.

6. PRODUCTS

Publications, conference papers, and presentations

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

- 1) Xie, A., Robles, R.J., Mukherjee, S., Zhang, H., Feldbrugge, L., Csizmadia, E., Wu, Y., Enjyoji, K., Moss, A.C., Otterbein, L.E., Quintana, F.J., Robson, S.C. & Longhi, M.S. HIF-1 α -induced xenobiotic transporters promote Th17 responses in Crohn's disease. *Journal of autoimmunity* (2018), Aug 8. pii: S0896-8411(18)30340-8. doi: 10.1016/j.jaut.2018.07.022. yes
- 2) Correa-Costa M, Gallo D, Csizmadia E, Gomperts E, Lieberum JL, Hauser CJ, Ji X, Wang B, Câmara NOS, Robson SC, Otterbein LE. Carbon monoxide protects the kidney through the central circadian clock and CD39. *PNAS USA*. 2018 Mar 6;115(10):E2302-E2310. doi: 10.1073/pnas.1716747115. Epub 2018 Feb 20. yes
- 3) Mukherjee S, Hanidziar D. More of the Gut in the Lung: How Two Microbiomes Meet in ARDS. *Yale J Biol Med*. 2018 Jun 28;91(2):143-149. 2018. Review . yes

- 4) Review being finalized describing the role of heme degradation products (biliverdin and bilirubin) together with adenosine in the modulation of innate immune responses. This is co-authored with Dr. Leo Otterbein and members of Project #2. yes
- 5) Shankar P, Robson SC, Otterbein LE, Shaefi S. 2018. Clinical Implications of Hyperoxia. *International Anesthesiology Clinics*, 56(1):68-79. PMID: 29227312. Yes.
- 6) Kaczmarek E., Hauser CJ., Kwon W.Y., Rica I., Li C., Sandler N., Otterbein LE., Campbell Y., Cook CH., Yaffe MB., Marusich MF., and Itagaki K. A subset of five human mitochondrial formyl peptides mimics bacterial peptides and functionally deactivates human neutrophils.. *J Trauma Acute Care Surg*, Vol 85, Number 5, p936-43. 2018. W81XWH-16-1-0464. Yes.
- 7) Aswani A, Manson J, Itagaki K, Chiazza F, Collino M, Wupeng WL, Chan TK, Wong WSF, Hauser CJ, Thiemermann C, Brohi K. Scavenging Circulating Mitochondrial DNA as a Potential Therapeutic Option for Multiple Organ Dysfunction in Trauma Hemorrhage. *Front Immunol*. 2018 May 8;9:891. doi: 10.3389/fimmu.2018.00891. eCollection 2018. PMID: 29867926, W81XWH-16-1-0464. Yes.
- 8) Hauser CJ, Otterbein, LE. Danger signals from mitochondrial DAMPS in trauma and post-injury sepsis. *Eur J Trauma Emerg Surg*. 2018 Jun;44(3):317-324. doi: 10.1007/s00068-018-0963-2. 24. PMID: 29797026. W81XWH-16-1-0464. Yes.
- 9) Sandler N, Kaczmarek E, Itagaki K, Zheng Y, Otterbein L, Khabbaz K, Liu D, Senthilnathan V, Gruen RL, Hauser CJ. Mitochondrial DAMPs Are Released During Cardiopulmonary Bypass Surgery and Are Associated With Postoperative Atrial Fibrillation. *Heart Lung Circ*. 2018 Jan;27(1):122-129. doi: 10.1016/j.hlc.2017.02.014. Acknowledgement of federal support, W81XWH-16-1-0464. Yes.
- 10) Seshadri A, Brat GA, Yorkgitis BK, Giangola M, Keegan J, Nguyen JP, Li W, Nakahori Y, Wada T, Hauser CJ, Salim A, Askari R, Lederer JA. Altered Monocyte and NK Cell Phenotypes Correlate with Post-Trauma Infection. *Journal of Trauma*; in press. 2018: yes.
- 11) Ledderose C, Liu K, Kondo Y, Slubowski CJ, Dertnig T, Denicolo S, et al., Purinergic P2X4 receptors and mitochondrial ATP production regulate T cell migration. *J Clin Invest*; 128, 3583- 94. 2018: yes
- 12) Donlin LT, Rao DA, Wei K, Slowikowski K, McGeachy MJ, Turner JD, et al., Methods for high-dimensional analysis of cells dissociated from cryopreserved synovial tissue. *Arthritis Res Ther* 20, 139. 2018: yes
- 13) Barrett CD, Hsu AT, Ellson CD, Y Miyazawa B, Kong YW, Greenwood JD, Dhara S, Neal MD, Sperry JL, Park MS, Cohen MJ, Zuckerbraun BS, Yaffe MB. Blood clotting and traumatic injury with shock mediates complement-dependent neutrophil priming for extracellular ROS, ROS-dependent organ injury and coagulopathy. *Clin Exp Immunol* 2018 Oct;194(1):103-117. yes
- 14) Zheng Y, Ji X, Yu B, Ji K, Gallo D, Csizmadia E, Zhu M, Choudhury MR, De La Cruz LKC, Chittavong V, Pan Z, Yuan Z, Otterbein LE, Wang B. Enrichment-triggered prodrug activation demonstrated through mitochondria-targeted delivery of doxorubicin and carbon monoxide. *Nature Chemistry* 2018 May 14. yes
- 15) Hopper CP, Meinel L, Steiger C, Otterbein LE. Where is the Clinical Breakthrough of Heme Oxygenase-1 / Carbon Monoxide Therapeutics? *Curr Pharm Des*. 2018 Jul 23. yes

Books or other non-periodical, one-time publications.

“Nothing to report”

Other publications, conference papers, and presentations.

LOCAL

Presented by Dr. Kwon.

- Clowes Research Symposium at BIDMC
- *FPR1 Blockade Rescues the Chemotaxis of Human Neutrophils toward Secondary Infective Sites after Injury

Presented by Dr. Hauser

- “Sepsis and SIRS after Trauma and Surgery – Seed or Soil” Department of Anesthesia Grand Rounds, Beth Israel Deaconess Medical Center.
- “DANGER signals in Surgery – Changing our understanding, for the better”. Department of Surgery Grand Rounds, Beth Israel Deaconess Medical Center.
- “SIRS in injury and infection” Anesthesia Fellowship Lecture. Department of Anesthesia, Beth Israel Deaconess Medical Center
- “Neutrophils, inflammation and Immunity after injury”, Beth Israel Deaconess Medical Trauma Center Conference.
- “Danger Signaling After Tissue Injury”, Novartis Institute of Biomedical Research - Seminars in Basic Science.
- “Why hip fractures lead to pneumonia” Surgery Grand Rounds, Mount Auburn Hospital, Cambridge, MA.

Presented by Dr. Lederer

- “Development of a Normal Flora Mouse Facility for Translational Immunology Research” Massachusetts Innovation Labs, 2018, Cambridge, MA
- “Technical Innovations for Trauma Research” Stepping Strong Trauma Symposium, 2018, Boston, MA

Presented by Ghee Lee (Project 2)

- “Effects of Carbon Monoxide on the Innate Immune Response to Infection”. Harvard Surgery Day, Boston, MA.

Presented by Dr. Longhi

- “Effects of unconjugated bilirubin and oxygen on Th17 cell immunity in IBD”, BIDMC, Boston, MA.
- “CD39 and Purinergic Signaling: Conditioning of Immune Responses in Inflammatory Diseases”. UMASS Memorial Medical Center, Worcester, MA

NATIONAL

Presented by Dr. Hauser.

- *Western Trauma Association: A Subset of Five Human Mitochondrial Formyl Peptides Mimic Bacterial Peptides and Functionally Deactivate Human Neutrophils(PMN)
- “Crush Injuries: Mitochondria Matter”. The Society of Critical Care Medicine, Critical Care Congress: Honolulu, Hawaii.
- *American Association for the Surgery of Trauma Webinar: Danger Signaling: An Important New Principle in Trauma and Surgical Injury.

Presented by Dr. Lederer

- “Radiation Injuries to the Immune System” Radiation Research Society, 2018, Chicago, IL
- “Cellular Therapy for Radiation and Traumatic Injuries” NIH-NIAID, 2018, Rockville, MD

Presented by Dr. Otterbein

- “Beneficial effects of Carbon Monoxide in the Acute Liver Injury” American Chemical Society, New Orleans, LA
- “Carbon Monoxide Enhances Bacterial Clearance in the Lung”. Military Health System Research Symposium, Orlando, FL
- “Heme Oxygenase-1 and the Stress Response”. University of California/Scripps Oceanography Institute, San Diego, CA.

Presented by Dr. Longhi

- “Hypoxia boosts Th17 cell responses in inflammatory bowel disease through increased efflux of ligands reactive with the aryl hydrocarbon receptor”, American Association of Immunologists.
- “Regulatory T cells in autoimmune liver disease: loss, gain and (un)friendly environment”, Children’s Hospital Cincinnati, OH

INTERNATIONAL**Presented by Dr. Itagaki**

- “Receptor desensitization by mitochondrial DAMPs may play a key role in the development of nosocomial pneumonia after trauma” 3rd Japan-US Technical Information Exchange Forum on BLAST INJURY (JUFBI2018) at Shinjuku, Tokyo organized by National Defense Medical College.

Presented by Dr. Hauser

- “DAMPs in injury-induced multi-organ failure. The role of danger molecules in susceptibility to infection and therapeutic opportunities. Keynote lecture: Symposium on DAMP-mediated remote organ failure and immunosuppression in the acutely ill patient: Where Trauma meets Sepsis. Faculté de Médecine, Strasbourg, France.
- “DAMPs and PAMPs and why they matter in Critical Intra-abdominal Injury/Illness”. WSACS – the Abdominal Compartment Society; Banff, Alberta, Canada.
- “Activation of Critical Inflammation by Mitochondrial DAMPs”. Queen Mary Hospital Trauma Science Colloquium. Chichely Hall, London, England.

Presented by Dr. Robson

- “Role of CD39-Adenosinergic Axis in Pathophysiology and Transplantation”, Physiological Society, London. UK.
- “Therapeutic potential of CD39”. Purines2018, Igazu, Brazil
- “Novel purinergic check point inhibitor targets in cancer and immune exhaustion”, Beijing University, 6th joint conference, Beijing China

Website(s) or other Internet site(s)

N/A

Technologies or techniques

N/A

Inventions, patent applications, and/or licenses

N/A

Other Products

N/A

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Project 1

Name: Carl Hauser, MD
Project Role: Co-Investigator
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: W81XWH-16-1-0464

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 establishment of human PMN isolation methods for the entire day supervising roles on Dr. W-Y Kwon and Dr. B. Vlkova, Mr. C. Bucin and Ms. Q. Zhang.
Funding Support: W81XWH-16-1-0464

Name: Woon Yong Kwon, MD, PhD.
Project Role: Research fellow
Researcher Identifier (e.g. ORCID ID): 0000-0002-334-5030
Nearest person month worked: 7
Contribution to Project: Dr. Kwon is focused on the role of mtFPs in the chemotaxis exposed to mtDAMPs.
Funding Support: Seoul National University

Name: Barbora Vlkova, PhD
Project Role: Research Fellow
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked 6
Contribution to Project: Dr. Vlkova is working on Project 1 focused on neutrophil functions such as calcium mobilization, chemotaxis and response to bacterial infection after trauma. She also established how to measure mtDNA copy number per cell.
Funding Support: Slovak Scientific Funding

Name: Canberk Bucin, BS
Project Role: Research Student, summer
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked 2
Contribution to Project: Mr. Bucin is working on human PMN isolation and chemotaxis.
Funding Support: Self supported

Name: Quanzhi Zhang, MS
Project Role: Research PhD Student
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 3
Contribution to Project: Ms. Zhang is working on human PMN purification and chemotaxis. She also learned all techniques from Dr. Vlkova.
Funding Support: W81XWH-16-1-0464

Name: James Harbison, BS
Project Role: Research Technician Researcher
Identifier (e.g. ORCID ID):
Nearest person month worked: 12 *Mr. Harbison's effort is shared among all projects
Contribution to Project: Mr. Harbison is working on clinical sample preparations for entire project, which includes human neutrophil, PBMC, plasma, and platelets.
Funding Support: W81XWH-16-1-0464

Project 2

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

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

Name: Mahtab Fakhari, MD
Project Role: Researcher/T32 trainee Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 4
Contribution to Project: Ms. Fakhari is working on Project 2 focused on effects of CO on neutrophil function and response to bacterial infection after trauma.
Funding Support: NIH T32GM103702-02/ W81XWH-16-1-0464

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

Name: Rodrigo Alves Souza, PhD
Project Role: Research Fellow
Nearest person month work: 6
Contribution to Project: Mr. Alves
Funding Support: Self Funded, Brazil

Name: Janick Weber
Project Role: Research Student Intern
Identifier (e.g. ORCID ID):
Nearest person month work: 6
Contribution to Project: Developed and characterized TBI model in mice
Funding Support: DFG Scholar

Project 3

Name: Simon Robson
Project Role: Co-Investigator
Researcher Identifier (e.g. ORCID ID): 0000-0001-6374-0194
Nearest person month worked: 2
Contribution to Project: Dr. Robson is the Principal Investigator of Project 3. He oversees the work and directs the research to fulfill the goals of the specific aims.
Funding Support: W81XWH-16-1-0464, 5P01HL107152-07, 1R01DK108894-01A1, 5R01DK103723-03, 1R01AI132389-1, Tizona, 5R01DK10471402

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: W81XWH-16-1-0464, 1R01DK108894-01A1

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

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

Name:	Dusan Hanidziar
Project Role:	Postdoctoral/ Anesthesiologist
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	Studies on the role of NKT cell-autotaxin-lysophosphatidic acid pathway in alveolar injury induced by hyperoxia.
Funding Support:	T32 GM007592-40

Project 4

Name:	Daniel Talmor, MD MPH
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	
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:	W81XWH-16-1-0464 & 1U01HL123022

Name:	Shahzad Shaefi, MD
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	
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:	W81XWH-16-1-0464 & FAER&BIDMC CAO

Name:	Valerie Banner-Goodspeed, MPH
Project Role:	Researcher/Clinical Research Manager
Researcher Identifier (e.g. ORCID ID):	
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:	W81XWH-16-1-0464, 1U01HL123022

Name: Julia Larson
Project Role: Clinical Research Assistant
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 2
Contribution to Project: Ms. Larson is working on Project 4, identifying and recruiting patients, collecting samples and collecting data.
Funding Support: W81XWH-16-1-0464 & 1U01HL123009

Name: Ariel Mueller, MA
Project Role: Biostatistician II
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1
Contribution to Project: Ms. Mueller is working on Project 4, serving as database architect and manager and overseeing data integrity.
Funding Support: W81XWH-16-1-0464 & FAER

Name: Andres Brenes Bastos, MD
Project Role: Research Fellow
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1
Contribution to Project: Dr. Bastos is working on Project 4 identifying and recruiting patients, collecting samples and collecting data.
Funding Support: 1U01HL123009

Name: Isabel Londono, MD
Project Role: Research Fellow
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1
Contribution to Project: Dr. Londono is working on Project 4, identifying and recruiting patients, collecting samples and collecting data.
Funding Support: W81XWH-16-1-0464, 1U01HL123009

Project 5

Name: James A. Lederer, PhD
Project Role: Project 5 Leader
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 2
Contribution to Project: Dr. Lederer is the director of project 5. He oversees the individuals working in his laboratory on this project. He also coordinates the collaborative work among the project groups relevant to CyTOF mass cytometry and other systems immunology technologies.
Funding Support: W81XWH-16-1-0464/NIH U01 AI107360

Name: Laura Cahill, PhD
Project Role: Research Fellow
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 4
Contribution to Project: Dr. Cahill is working on project 5 as a CyTOF mass cytometry expert. She is responsible for training HALO collaborators and for implementing human and mouse CyTOF experiments. She also helps with administrative work in the laboratory.
Funding Support:.. W81XWH-16-1-0464 /NIH T32GM103702

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

Name: Yasutaka Nakahori, MD
Project Role: Research Fellow
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 5
Contribution to Project: Dr. Nakahori is a trauma surgeon from Japan and is working on the mechanisms-of-action studies for CpG-ODN immunotherapy. He is focused on the lung infection studies and on the two hit response experiments. He also works on all aspects of CyTOF mass cytometry in our lab including trauma blood immunophenotyping studies
Funding Support: W81XWH-16-1-046 & Osaka University General Hospital

Name: Takeshi Wada, MD
Project Role: Research Fellow
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 10
Contribution to Project: Dr.Wada is also a trauma surgeon from Japan. He worked on the lung infection immunology studies for the project. His studies focused primarily on the mechanisms-of-action for the CpG-ODN immunotherapy for lung bacterial infections. He worked collaboratively with Dr. Nakahori on all aspects of the lung infection and injury mouse studies that were performed.
Funding Support: Hokkaido University

Project 6

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

Name: Ingrid Rica, Ph.D,
Project Role: Post-Doctoral Fellow
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 12
Contribution to Project: Post-doctoral research on project 6
Funding Support: W81XWH-16-1-0464

Name: Brian Joughin, Ph.D,
Project Role: Staff Scientist
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 3
Contribution to Project: Computational Scientist for project 6 and the Computational Core
Funding Support: W81XWH-16-1-0464

Name: Yuliana Hernandez
Project Role: Technician
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 4
Contribution to Project: Technician for project 6
Funding Support: W81XWH-16-1-0464

Name: Shohreh Varmeh
Project Role: Senior Scientist
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 4
Contribution to Project: Staff scientist for project 6
Funding Support: W81XWH-16-1-0464

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

Nothing to report.

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

QUAD CHARTS: see attached

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

SCOPE OF WORK: see attached

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

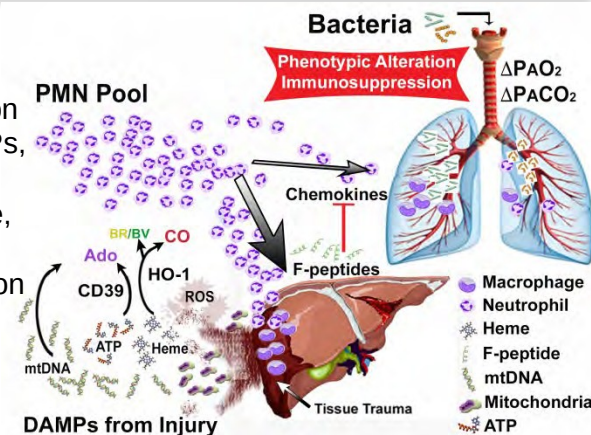
This research program seeks to identify cellular and molecular mechanisms by which wounds predispose to infection. We are focused on three primary objectives: **prevention**, **diagnosis** and **treatment** of the wounded warrior.

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: (Oct 30, 2018)

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
DAcquire human and mouse descriptive and phenotypic data sets
CY3: Establish specific DAMPs that interfere with innate immunity
DValidate and characterize the identified DAMP(s) in model systems
CY4: Interfere with DAMP effects and restore innate immuneresponses.
DPerform 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.
DEnact 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: \$ 3,261,161 TC

STATEMENT OF WORK – October 28, 2015

PROPOSED START DATE September 30, 2016

Site 1: Beth Israel Deaconess Medical Center 300 Brookline Ave, Boston MA, PI Dr. Hauser

Site 2: Brigham and Women's Hospital, 75 Francis Street, Boston MA, Partnering PI Dr. Lederer

Site 2: Massachusetts Institute of Technology, 77 Massachusetts Ave, Cambridge MA Partnering PI Dr. Yaffe

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

<i>Milestone #6: Manuscript on chloroquine as prophylaxis in trauma. Presentation at meeting.</i>	36	Hauser			0
<i>Milestone #7: State of the art review manuscript by Group</i>					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
<i>Milestone #8: HRPO and IACUC approval received</i>	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
<i>Milestone #9: Establish statistical parameters for powering prospective studies</i>	18	Hauser Talmor			0
<i>Milestone #10: Initial manuscript describing human DAMPs kinetics</i>		Hauser Talmor			100
Subtask 5: Recruit, consent, and enroll 400 patients / subjects to pre-clinical study. Conduct prospective study of mtDAMPs in trauma. Conduct prospective study of innate Mφ/PMN phenotypes in trauma.	18-60 18-60	Talmor Hauser Hauser/ Otterbein/ Robson	Yaffe	Lederer	25 25 0
<i>Milestone #11: Mid-term manuscript(s) describing human DAMPs kinetics</i>	40	ALL	Yaffe	Lederer	0
<i>Milestone #12: Initial manuscript(s) describing human Mφ/PMN phenotypes in trauma</i>	40-48	ALL	Yaffe	Lederer	20
<i>Milestone #13: Final manuscript(s) describing human DAMPs time course</i>	60	ALL	Yaffe	Lederer	0
<i>Milestone #14: Mid-term manuscript(s) on DAMPs and clinical infection</i>	48-60	ALL	Yaffe	Lederer	0
<i>Milestone #15: Final manuscripts describing DAMPs kinetics</i>	60	ALL	Yaffe	Lederer	0
<i>Milestone #16: Final manuscripts describing human Mφ/PMN phenotypes in trauma</i>	50-60	ALL	Yaffe	Lederer	0
<i>Milestone #17: Final manuscript(s) on DAMPs and clinical infection</i>		ALL	Yaffe	Lederer	0
<i>Milestone #18: New grant submissions (NIH, DoD, Foundations)</i>	30-60	ALL	Yaffe	Lederer	0

Project 2: Determine the role of innate immune responses to Heme in the increased susceptibility to infection after trauma	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	90
Subtask 2: Establish Dr. Lederer's burn model from BWH at BIDMC	6-12	Otterbein		Lederer	25
Subtask 3: Evaluate CO-releasing molecule 3 (CORM-3)	30-36	Otterbein		Lederer	75
Subtask 4: Optimize CO dosing	12-36	Otterbein	Yaffe	Lederer	75
<i>Milestone #1: mid-term manuscript(s) describing role of heme as a DAMP. Manuscript(s) describing lung phenotyping in liver crush</i>	12-15	Otterbein Hauser	Yaffe	Lederer	50
Project 2, SA2: Determine mechanisms by which CO augments the innate immune response to bacteria in the lung <i>in vitro</i> and <i>in vivo</i> . Participating Projects: 1, 3, 4, 5, 6					
Subtask 5: Describe effects of CO on Mφ and PMN <i>in vivo</i>	30-42	Otterbein Hauser	Yaffe	Lederer	30
Subtask 6: Study effects of heme on Mφ and PMN phenotype and signaling <i>in vitro</i>	42-48	Otterbein Hauser	Yaffe	Lederer	10
<i>Milestone #2: subsequent manuscript(s) describing effects of heme and CO on Mφ and PMN phenotype and signaling, presentation at meetings.</i>	24-30	Otterbein Hauser	Yaffe	Lederer	30
<i>Milestone #3: Manuscript on effects of purines on inflammasome activation</i>	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	10
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	75
<i>Milestone #4: Final manuscript(s) describing the effects of heme and CO on primary human cells from trauma patients and volunteers</i>	54-60	ALL			0
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	50
Subtask 2: Define role of MyD88/mtDNA in P2x7 signaling	9-24	Robson/ Otterbein/ Hauser	Yaffe	Lederer	50
Subtask 3: Is aberrant CD39 scavenging of ATP, globally or targeted on myeloid cells, critical for hyper inflammation and associated suppression of responses to bacteria after injury.	12-24	Robson/ Otterbein/ Hauser	Yaffe	Lederer	75
Subtask 4: Determine how P2x7 signaling in PMN affects the innate immune response to PNA	24-36	Robson/ Otterbein/ Hauser/ Junger	Yaffe	Lederer	10
Subtask 5: Determine how P2x7 signaling in Monocyte-Macrophages affects the innate immune response to PNA	12-36	Robson/ Otterbein/ Hauser	Yaffe	Lederer	50
<i>Milestone #1: Initial manuscript(s) describing role of purinergic signaling in association with mitochondrial DAMPs in trauma/sepsis.</i>	12-36	Otterbein / Robson	Yaffe	Lederer	50
<i>Milestone #2: Mid-term manuscript defining role of purinergic signaling in myeloid cells in trauma/sepsis models.</i>	24-48	Otterbein / Robson	Yaffe	Lederer	75
<i>Milestone #3: Later manuscript defining ATP-purines-heme in inflammasome activation</i>	42-48	Otterbein / Robson	Yaffe	Lederer	25
Project 3, SA2: To study how type-1 purinergic receptor (P1R) signaling by adenosine modulates the innate immune response to infection after trauma, in experimental models. Participating Projects: 1, 2, 3, 5, 6.					
Subtask 1: Optimize dosing of soluble CD39 and exogenous apyrase in liver crush injury and sepsis/PNA.	0-24	Robson	Yaffe	Lederer	75
Subtask 2: Test impacts of elective adenosinergic receptor agonists vs. antagonists in liver injury and sepsis. Review impacts of CO + adenosine in this system.	9-36/60	Robson/ Otterbein/ Hauser	Yaffe	Lederer	50
Subtask 3: Test combinations of pharmacological P2X7 antagonists and optimized adenosinergic drugs in liver injury and PNA.	12-36	Robson/ Otterbein/ Hauser	Yaffe	Lederer	85
Subtask 4: Determine depletion of intracellular ATP in immune cells in the liver injury and PNA.	24-60	Robson/ Otterbein/ Hauser/ Junger	Yaffe	Lederer	50
Subtask 5: Test salutary role of oxygen (O ₂) tension and HIF-1	12-48/60	Robson/	Yaffe	Lederer	

targeting in liver injury and sepsis. Review additional impacts of CO in this system.		Otterbein/ Hauser			50
<i>Milestone #4: Early mid-term manuscript(s) describing salutary effects of early CD39 administration in trauma/PNA.</i>	18-24	ALL	Yaffe	Lederer	50
<i>Milestone #5: Mid-term manuscript(s) describing role of adenosinergic signaling and CO in trauma/sepsis models.</i>	36-48	ALL	Yaffe	Lederer	75
<i>Milestone #6: Final manuscript(s) describing depletion of intracellular ATP in immune cells in the liver injury and sepsis.</i>	40-60	ALL	Yaffe	Lederer	25
<i>Milestone #7: New grant submissions (NIH, DoD, Foundations)</i>	30-60	ALL	Yaffe	Lederer	0
Project 3, SA3: To characterize and correct kinetics of aberrant immune purinergic responses within blood and alveolar micro-environment of trauma patients. Participating Projects: 1, 2, 3, 5, 6					
Subtask 1: Determine evidence for immediate – day 0-1 - early clinical phase ATP-mediated P2X7 responses.	12-24	Robson/ Talmor	Yaffe	Lederer	90
Subtask 2: Study delayed phase - day 2-5 - hyperadenosinergic responses in blood and BAL.	9-36	Robson/ Otterbein/ Hauser/ Talmor	Yaffe	Lederer	90
Subtask 3: Study kinetics and determine evidence for immediate decreases vs. late depletion of intracellular ATP in blood cells and BAL.	12-36	Robson/ Otterbein/ Hauser/ Talmor	Yaffe	Lederer	75
Subtask 4: Correct aberrant P2X7 signaling and adenosinergic responses by modulating CD39 ectonucleotidase activity	24-60	Robson/ Otterbein/ Hauser/ Junger/ Talmor	Yaffe	Lederer	50
Subtask 5: Restore intracellular ATP (energy charge) in immune cells	12-48	Robson/ Otterbein/ Hauser/ Talmor/ Junger	Yaffe	Lederer	25
Subtask 6: In vitro O ₂ and CO studies	24-48	Robson/ Otterbein/ Talmor	Yaffe	Lederer	15
<i>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</i>	18-24	ALL	Yaffe	Lederer	50-75
<i>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</i>	36-48	ALL	Yaffe	Lederer	10-25

Milestone #9: Final manuscript(s) on Purines and DAMPs as modulators of inflammation and clinical infection	40-60	ALL	Yaffe	Lederer	10-25
Shared Milestone #10: New grant submissions (NIH, DoD, Foundations)	30-60	ALL	Yaffe	Lederer	0
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	3-6	Talmor/ Hauser			100 11/2016
Subtask 2: enroll 100 patients and 100 volunteer controls	3-18				67
Subtask 3: regulatory requirements in place	0-60				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				13.4
Milestone #2: complete the enrollment	60				13.4
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				99.6
Milestone #5: Review of monitoring reports activities monitored weekly	monthly				100 continous
Project4, SA3: Physicochemical effects on airway innate immunity Participating Projects: 1, 2, 3, 4, 5, 6					
Subtask 1: Evaluate role of PO₂ in alveolar immune environment protocol written, IRB/HRPO approved as of 12/17/18.	3-15	Talmor/ Hauser/ Otterbein	Yaffe	Lederer	10
Subtask 2: Evaluate role of PCO₂ in alveolar immune Environment protocol written, IRB/HRPO approved as of 12/17/18.	15-30	Talmor/ Hauser/ Otterbein	Yaffe	Lederer	10
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	10
	15-24	Talmor/	Yaffe	Lederer	

Milestone #6: manuscript on PO ₂ and alveolar immunity		Hauser/ Otterbein			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	0
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	20
Subtask 3: Collaborative CyTOF and Luminex studies with Projects 1,2,3, and 6 as part of the Immune Profiling Core	1-60	Robson	Yaffe	Lederer	25

(IPC)					
<i>Milestone #3: Manuscripts reporting CyTOF findings from mouse studies performed in other projects. Reports based on IPC and CMC activities.</i>	36-60	Otterbein / Robson/ Hauser	Yaffe	Lederer	25
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
<i>Milestone #4: Manuscript reporting comparative CpG-ODN treatment findings</i>	24-30	ALL		Lederer	25
Subtask 2: CyTOF and Luminex phenotyping studies for beneficial CpG-ODNs	30-60	Otterbein / Robson/ Hauser	Yaffe	Lederer	25
<i>Milestone #5: Manuscript reporting systems data from CpG-ODN treatment studies</i>	36-60	ALL	Yaffe	Lederer	25
Subtask 3: Functional studies examining roles of IL-12, IL-17, PD-L1, $\gamma\delta$ T cells, TLR9	24-48			Lederer	30
<i>Milestone #6: Manuscript describing results of functional studies and presentation at scientific meetings</i>	48-60	ALL	Yaffe	Lederer	0
Project 5, SA#3: To use CyTOF and Luminex technologies to generate systems immunology data from trauma patients and research samples Participating Projects: 1, 2, 3, 4, 5, 6					
Subtask 1: Establish and validate human adaptive and immune cell CyTOF panels for blood and BAL cells	12	ALL	Yaffe	Lederer	100 9/2018
<i>Milestone #7: Validation data showing single-cell phenotyping data by CyTOF for a training sample set (10 patients and normals)</i>	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.					60
<i>Milestone #8: Validation data showing single-cell phenotyping data by CyTOF for cell preparations for mouse research samples</i>	12-24	Otterbein / Robson/ Hauser	Yaffe	Lederer	100 9/2018
<i>Milestone #9: Establishment of project specific Luminex assay panels for cytokines, chemokines, and DAMPs</i>	12-36	ALL	Yaffe	Lederer	50
Subtask 3: Trauma patient immune profiling by CyTOF and Luminex					60
<i>Milestone #10: Manuscript describing outcome of patient immune profiling data generated by IPC and analyzed by CMC</i>	48-60	ALL	Yaffe	Lederer	0
<i>Shared Milestone #11: New grant submissions (NIH, DoD, Foundations)</i>	30-60	ALL	Yaffe	Lederer	0
Project 6: The Role of Neutrophil Priming, ROS Release, and MK2 Signaling in the Innate Immune Response	Timeline	Site 1	Site 2	Site 3	%

after Trauma.		(BID)	(MIT) PI: Yaffe	(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	95
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	25
<i>Milestone #1: First Manuscript describing the effects of DAMP priming on PMN function</i>	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	85
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	25
<i>Milestone #2: manuscript on PMN priming after trauma and the sensitivity to lung infection.</i>	24-36	Hauser/ Otterbein	Yaffe	Lederer	0
<i>Milestone #3: presentation at Shock Meeting and DoD</i>	24-36	ALL	Yaffe	Lederer	0
<i>Milestone #4: Review article describing the role of PMN priming on susceptibility to infection after trauma.</i>	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 dysfunction.					
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	75
Subtask 2: Study <i>Mk2^{-/-}</i> mice in the liver crush+infection model expecting resistance to infection in the absence of MK2.	24-60	Hauser/ Otterbein	Yaffe	Lederer	25
<i>Milestone #5: Manuscript describing role of MK2 signaling in PNA after trauma</i>	48-53	Hauser/ Otterbein	Yaffe	Lederer	0
<i>Milestone #6: presentation at Shock Meeting</i>	48-60		Yaffe	Lederer	0
<i>Milestone #7: Review article summarizing how trauma influences susceptibility to PNA based on the priming state of</i>	48-60	ALL	Yaffe	Lederer	0

<i>the PMN</i>					
<i>Milestone #11: New grant submissions (NIH, DoD, Foundations)</i>	30-60	ALL	Yaffe	Lederer	0

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

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

Total 5 year Enrollment : 500 Volunteers and 500 trauma patients