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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

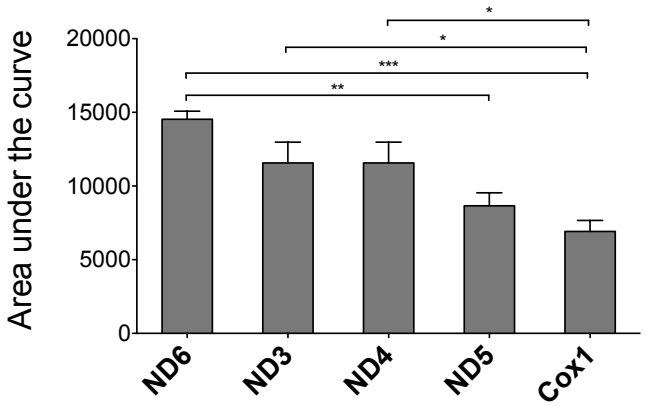
What were the major goals of the project?

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) ± 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.

What was accomplished under these goals?

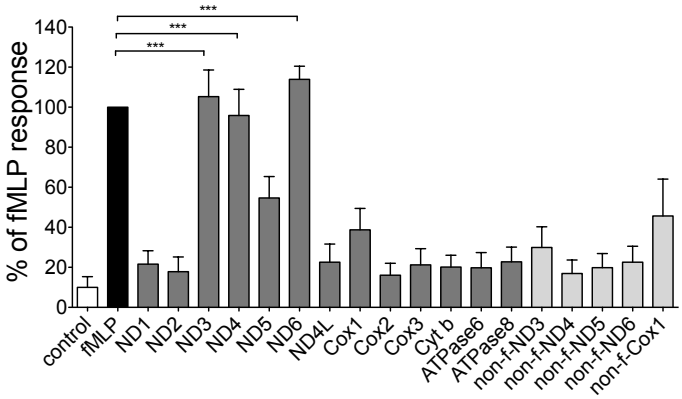
PROJECT 1 (HAUSER): Mitochondrial f-peptides and DNA released by injury suppress neutrophil function



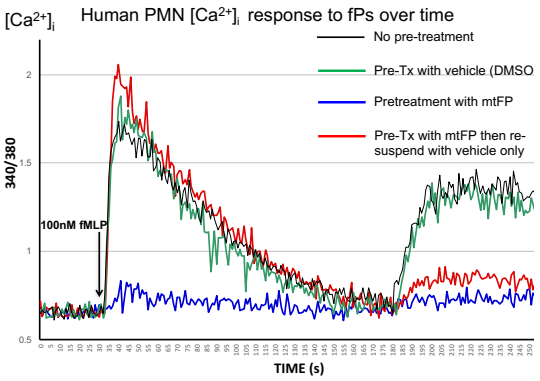
Major activities, objectives, and results

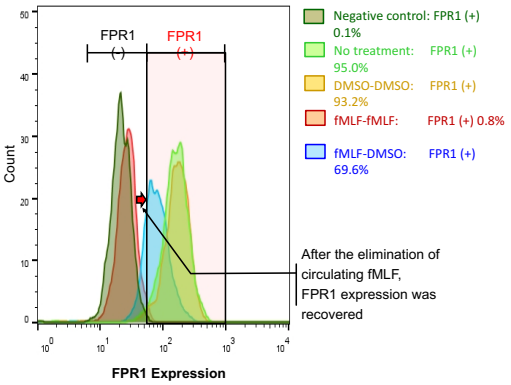
We have focused on specifying and quantifying the responses of neutrophils to the individual mitochondrial formal peptides we have shown were active. Our initial work was to assess the potency of mt-formyl peptides by Ca^{2+} mobilization assay. Human PMN were stimulated with ND6, ND3, ND4, ND5 and Cox1. Relative potency (at 100nM) is presented as the area under the Ca^{2+} response curve (AUC) in **Figure 1**, left. ND6 was the most potent formyl peptide in human MT, inducing Ca^{2+} flux similar to fMLP.

In **Figure 2** below, we see that neutrophil chemotaxis parallels Ca^{2+} mobilization by mtFPs. Again, ND6, ND3, ND4, ND5 and Cox1 cause brisk chemotaxis with responses to ND6 exceeding the response to fMLP. mtFPs that do not cause Ca^{2+} flux do not elicit chemotaxis. We also synthesized the end-terminal peptides of all of these peptides. None has any activity as a Ca^{2+} flux activator, a chemoattractant or (unfortunately) a formyl-peptide receptor (FPR) inhibitor. For Figures 1 and 2, * $p \leq 0.05$; ** $p \leq 0.005$; *** $p \leq 0.0005$.

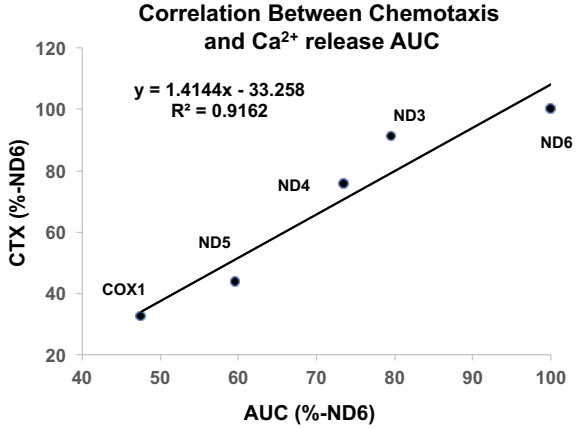


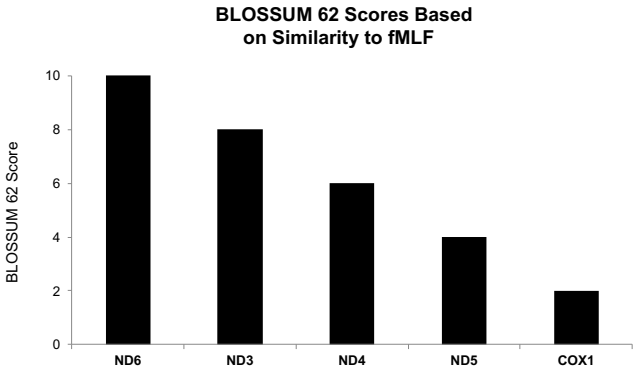
In order to understand how human neutrophils interact with circulating formal peptides under clinical circumstances we studied the suppression and re-expression of formal peptide receptors exposed to authentic formyl peptides (ie mtFPs derived from the same species. **In figure 3** below, we see that PMN rapidly become unresponsive to fMLP after pretreatment (15 min) with mtFPs (here ND6). Responses revert to normal levels rapidly (1hour) when treated PMN are re-suspended in FP free media. This effect is due to the rapid suppression and re-expression of cell-surface FPR1, as seen in **figure 4** (above, right), where human PMN are exposed to formyl peptides (here fMLF) rapidly lose surface FPR1 expression (95% → 0.8%). Expression returned to 69.6% after 1 hour with FPs absent. Thus clinical PMN responses to circulating mt-FP are expected to be a very rapid but also to be rapidly reversible.



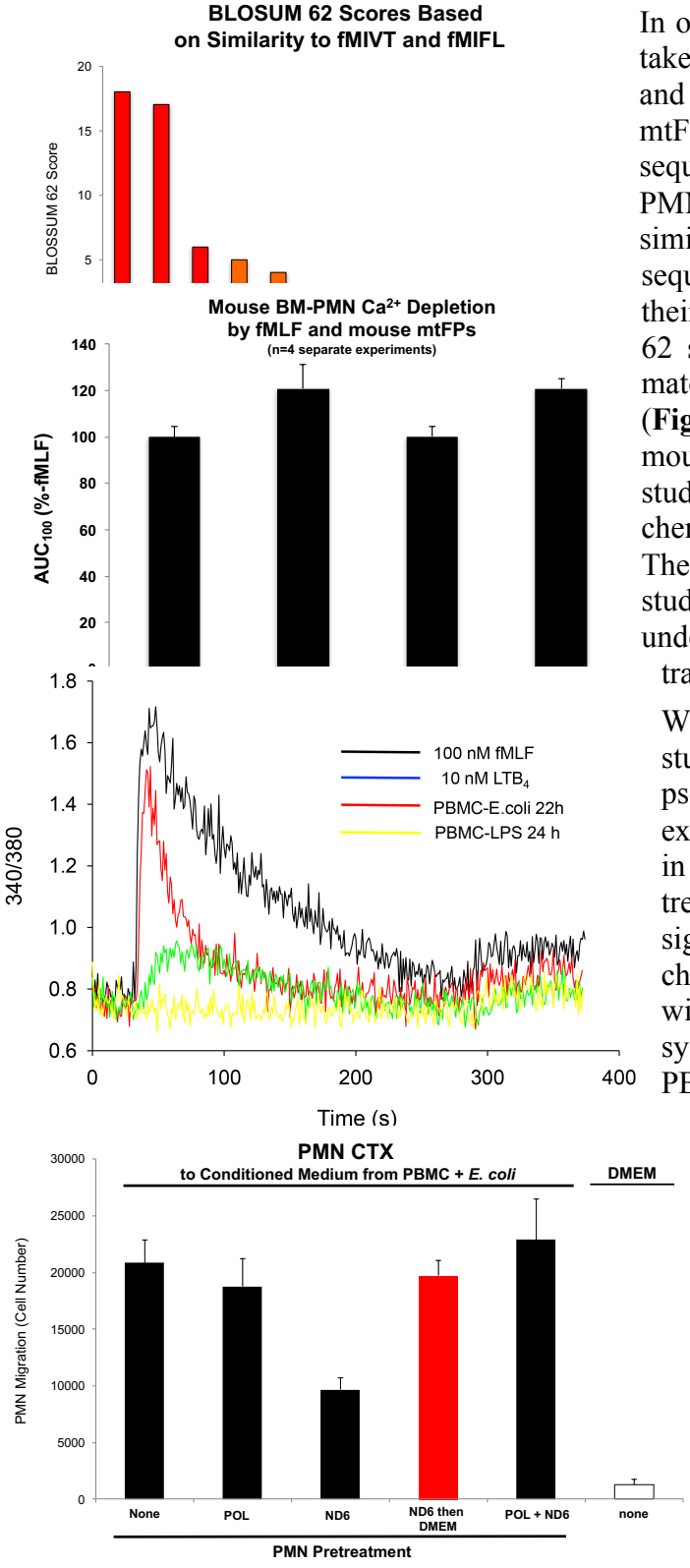


In this area, we have now begun to assess the role of formyl peptides in mouse responses to injury. We believe that it will be crucial to identify parallel responses in and humans to avoid the trap of doing mouse experiments that have no relevance to patients. Mice have long been thought to be resistant to FPs on the basis that they respond poorly to fMLP (fMLF), which is a synthetic FP thought to model bacterial peptides. We find that this experience is strain and mtFP specific. We have and Investigated this problem using the **BLOSUM62** matrix. In bioinformatics, the BLOSUM (BLOcks SUbstitution Matrix) matrix is a substitution matrix used for sequence alignment of proteins. BLOSUM matrices are used to score alignments between evolutionarily divergent protein sequences. This method gauges the





similarity of amino acid sequences based upon their component residues. We used **BLOSSUM 62** (Henikoff S, Proteins. 1993 Sep;17(1):49-61) to predict human and mouse PMN response to FPs. In **figure 5**, left we see that human mtFPs mobilize PMN calcium (and induce chemotaxis, not shown) to a degree that approximates their similarity to fMLP. Therefore also, as seen in **Figure 6**, left, human PMN chemotaxis to mtFPs (all at 100nM) is directly related to calcium mobilization by mtFPs with a very high R^2.



In order to relate mouse biology to human biology we have taken a very similar approach using mouse formyl peptides and PMN. Mice are evolutionarily distant from humans and mtFPs show rather marked differences in n-terminal sequences. Nonetheless, we see rather similar patterns of PMN responses to mtFPs where we assess their molecular similarity to fMIVT and fMIFL, which are f-peptide sequences from two pathogenic Listeria species, rather than their similarity to fMLP. In **Figure 7**, left we see BLOSUM 62 scores for mouse neutrophil. These scores show good matching with mouse PMN calcium depletion studies (**Figure 8** below) where ND1, ND4 and ND5 were the only mouse mtFPs that elicited Ca^{2+} flux from mouse MT. In early studies Ca^{2+} flux AUC showed good correlation to chemotaxis studies.

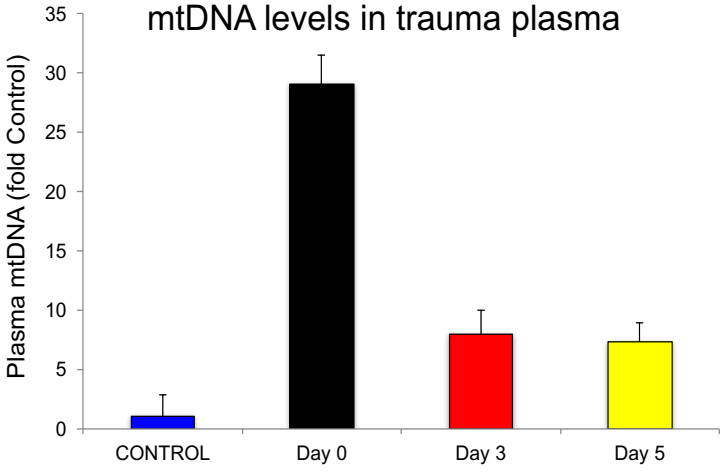
These findings give us confidence that our mechanistic studies in mice can be able be used translationally to understand the ability of PMN from clinical samples from trauma patients to respond to bacterial challenge.

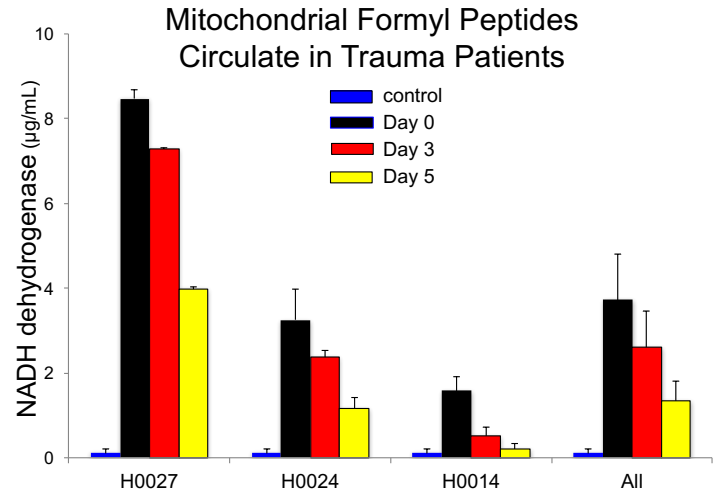
We are currently beginning to create our models for these studies. In addition to our standard Mouse liver injury and pseudo-fracture (PsFx) models, we are now developing new ex vivo models. These will use both human and mouse cells in a parallel fashion. In general, these models depend upon treating PBMC with either bacteria or a sterile bacteria-like signal (such as LPS) and then observing neutrophil chemotaxis toward PBMC-conditioned media with or without pre-treatment of the neutrophils with mtFPs. These systems can be created in a parallel fashion with human PBMC and neutrophils, or with mouse bone marrow mononuclear cells and neutrophils. After exposure to inflammatory or bacterial signals, mononuclear cells make chemoattractants, especially CXC chemokines and leukotrienes, that attract PMN. We have now shown that mtFPs suppress CXC and LT receptors. Thus prior exposure to mtFPs suppress responses to authentic mononuclear cell signals. In **Figure 9**, above left, we see that filtered conditioned media from PBMC activated by either *E.coli* or LPS causes neutrophil calcium flux (and chemotaxis).

We demonstrate this in **Figure 10** right, where we see human PMN chemotaxis to PBMC-conditioned medium is markedly suppressed by prior exposure to ND6. Suppression is prevented by pretreatment with the specific FPR1 inhibitor POL7200 (POL). There is no direct effect of POL on chemotaxis to conditioned medium, showing that chemo attraction is not the result of

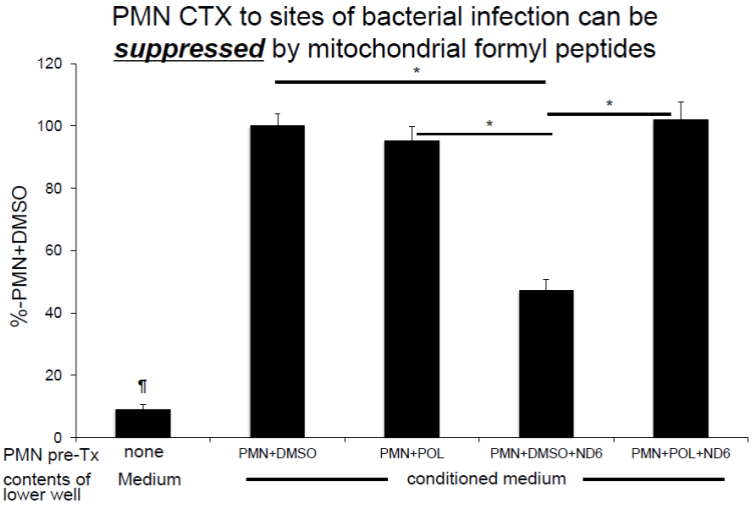
formyl peptides in the media. Similarly, washing the PMN to remove ND6 allows normal chemoattraction to non-FP chemokines. We believe that this shows neutrophil pre-activation with formal peptides prevents them from responding to authentic chemokine and leukotriene signals. These models will instruct us in how to avoid deleterious neutrophil suppression seen after injury. Testing the dependence of ND6 action on FPRs using Fpr1-/- & Fpr2-/- will be begun after these more straightforward experiments have been completed.

Since our last report, we have begun work assessing clinical mtDAMP levels in training (derivation) cohort plasma samples. These show, as we reported before (***Nature***, March 7, 2010), that plasma mtDNA is elevated immediately after injury **(Figure 11, left)**. Previously unreported however, are the findings in serial specimens that mtDNA appears to fall off rapidly after injury, then maintaining low but significant increases over time. Tracking mtDNA in trauma patients overtime is a key deliverable in this project, as it will be evaluated as a predictor of infection. The demonstration here that it can be followed using our new SOP's with a high signal-to-noise ratio is gratifying.





Additionally, as has not been shown before, mitochondrial formyl peptides (specifically the very active mtFP ND6 that we originally proposed to follow) also appear to circulate at fairly significant (µg/mL) concentrations after injury where they are almost undetectable in matched healthy controls **(Figure 12, left)**. Moreover, mtFPs seem to follow a generally similar time course as mtDNA, although mtFP levels appear to stay elevated for longer periods. Like mtDNA, tracking mtFP in trauma patients over time is a key deliverable in this project, as it will be evaluated as a predictor of infection. Moreover, it has proven difficult to create assays for these mtFP and our current results reflect a considerable amount of effort expended in generating accurate and reproducible methodologies.



In other important work we have refined our studies of the effects of mtDAMPs on neutrophil chemoattraction to mononuclear cells. To do this, we are using the conditioned supernatants from human PBMC stimulated either with bacteria or with LPS alone as shown in **Figure 10**. Both show powerful chemoattractant properties toward neutrophils. This of course is not unexpected. But we can now show that mitochondrial formyl peptides specifically inhibit the ability of neutrophils to respond to chemokine and lipid signals present in environments where PBMC are exposed to bacteria. Neutrophils arrive at infective environments on the basis of their movement toward both bacterial peptides and the chemoattractants that are generated by PBMC. These two sources of chemoattraction can be isolated, though, and we are now able to study the supernatants to

determine the exact active agents. In **Figure 13** (left) responses to infection were modeled by exposing PBMC to E. coli overnight and the conditioned medium were then collected and filtered free (culture proven) of bacteria. Fresh PMN (treated as shown) were then placed in the upper chamber of trans-wells for 90 min and CTX into media in the lower chamber was measured. Note that ND6 is the n-terminal FP of NADH-6 and POL7200 (POL) is a specific inhibitor of FP receptor 1 (FPR-1). DMSO is the solvent for POL. Here we see BAR 1: There is minimal spontaneous PMN chemotaxis ('chemokinesis') to fresh medium. BAR 2: There is vigorous PMN CTX into conditioned medium. BAR 3: POL has no direct effect on PMN movement showing CTX here depends on agonists and receptors other than FPs/FPR-1. BAR 4: ***PMN pre-treatment with ND6 significantly inhibits their CTX to non-FP agonists***, and in BAR 5: CTX to non-FP signals ***is rescued by FPR-1 blockade*** (by POL).

We believe that these experiments lend tremendous support toward the Project 1 central hypothesis that mitochondrial DAMPs suppress neutrophil antimicrobial function. Interestingly, we also show that ***blockade of the formyl peptide receptor-1 (FPR1) rescues neutrophil chemotaxis toward PBMC exposed to bacteria***. This is a key deliverable. Moreover, using this powerful system, we will now be able to study the direct effects of trauma plasma obtained serially from patients upon neutrophil migratory function and similarly, make direct assessments of whether changes in function are due to the presence of mitochondrial DNA and or proteins. We also believe that we will be able to do parallel studies in mice now that we have determined the similarities and differences of mouse mtFP and FPRs to the human species with respect to their interactions with and actions on neutrophils.

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

Major Activities, objectives and results

- Validated animal model of trauma±infection. Begun to characterize peripheral DAMP release assessed by quantitating mtDNA in serum over time after liver crush injury. (**Figures 1&2**)
- Onboarded Harvard Masters student and completed training in animal models and good lab practice. 20-02-2017
- Onboarded NIH T32 surgical fellow and completed training in animal models and in ex vivo isolation of murine bone marrow-derived neutrophils. 01-04-2017
- Plenary lecture at 1st Gasotransmitter Conference, Atlanta, GA. 22-04-2017
- Chaired gasotransmitter plenary session, Shock Society, Ft Lauderdale, FL, 05-06-2017
- Manuscript accepted in *Stroke* detailing the role of carbon monoxide and brain injury.
- First experiments completed in collaboration with **Project 5** looking at the effects of carbon monoxide on lung cell phenotypes in the presence of infection. 20-02-2017 (**Figure 4**)
- In collaboration with Project 1, tested the effects of heme as a DAMP on Ca^{++} release in human neutrophils. 19-05-2017.
- Established collaboration with neighboring medical microbiology laboratory for input on clinical relevant bacteria strains to use in the in vivo and in vitro models. Begin to establish bacteria growth and viability assays for high throughput readouts. CO Releasing Molecules (CO-RMs) are being tested 15-05-2017 (**Figure 6**).
- Generated samples in the trauma±infection for characterization of serum and bronchoalveolar lavage of the entire glycome. Preliminary data shows specific differences in protein glycosylation profiles comparing injury to injury+infection. 15-12-2016.
- Completed characterizing mouse and human PMN bacterial killing assays for mechanistic studies *in vitro* and have demonstrated that when exposed to CO, there is increased intracellular killing. 08-08-2017 **(Figure 3**).
- Complete three complete BAL CyTOF analyses comparing infection±CO that are being dissected and analyzed in collaboration with Project 5. 07-07-2017 (**Figure 4**)
- Successfully characterized PMN and macrophage depletion protocols in mice to complement CyTOF data towards understanding whether CO targets specific cell types in its augmentation of bacterial clearance. 14-09-2017 (**Figure 7-8**)
- To demonstrate the role of trauma-induced inhibition of bacterial killing in the lung, we have initiated a series of studies looking at transferring serum from an animal that undergoes trauma (presumably containing high levels of DAMPs) into a naïve mouse that is not subjected to trauma, but is challenged with a lung bacterial inoculation, Preliminary findings suggest that a circulating DAMP(s) is contributing to the poor clearance. 14-10-2017.

Key Data Sets

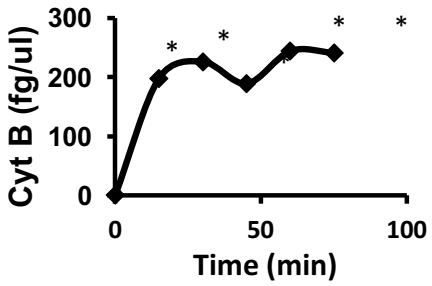


Figure 1. Kinetic of serum mtDNA appearance in male CD-1 mice after liver crush injury. n=6/time point. *p<0.001 vs baseline.

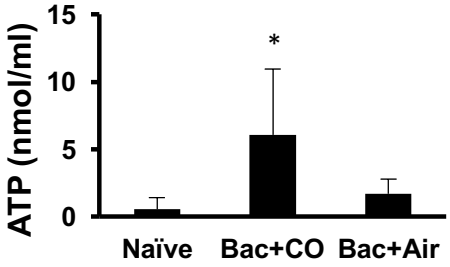


Figure 2. Male CD-1 mice were subjected to *S. aureus* infection (10^6 cfu) administered directly into the airway. 4h after bacteria mice were treated with Air or CO (0.025%, 250 ppm) for 1h. 24h later, BAL was performed and ATP levels were measured by HPLC. n=3-5/group. *p<0.05 vs naïve and Air

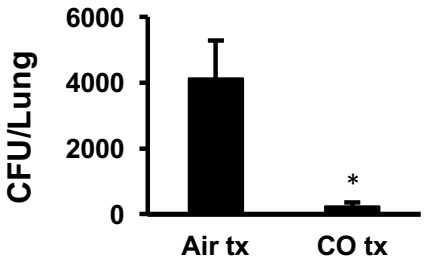


Figure 3. Male CD-1 mice were subjected to *S. aureus* infection (10^6 cfu) administered directly into the airway. 4h after bacteria mice were treated with Air or CO (0.025%, 250 ppm) for 1h. 24h later, BAL was performed and bacteria counts were performed. n=6-8/group. *p<0.001 vs Air.

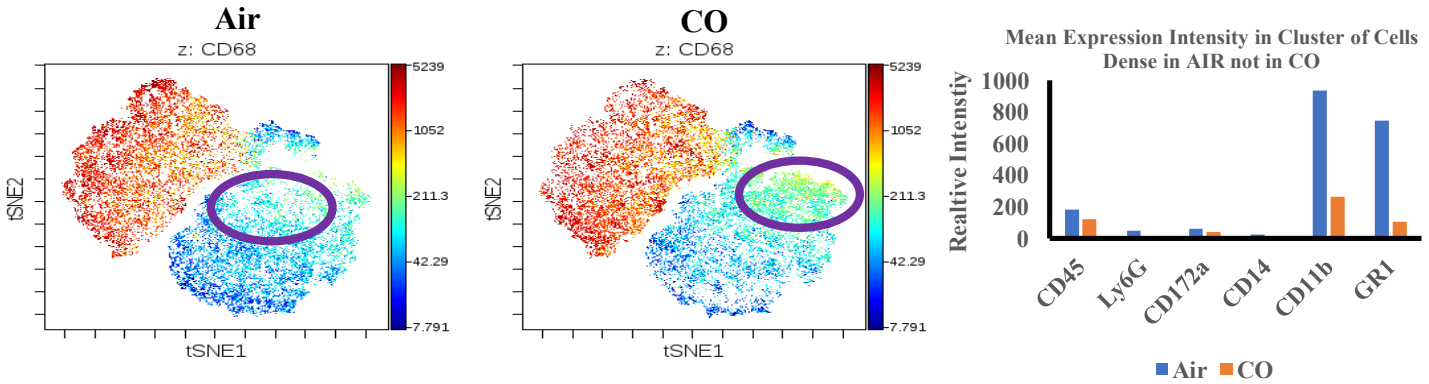
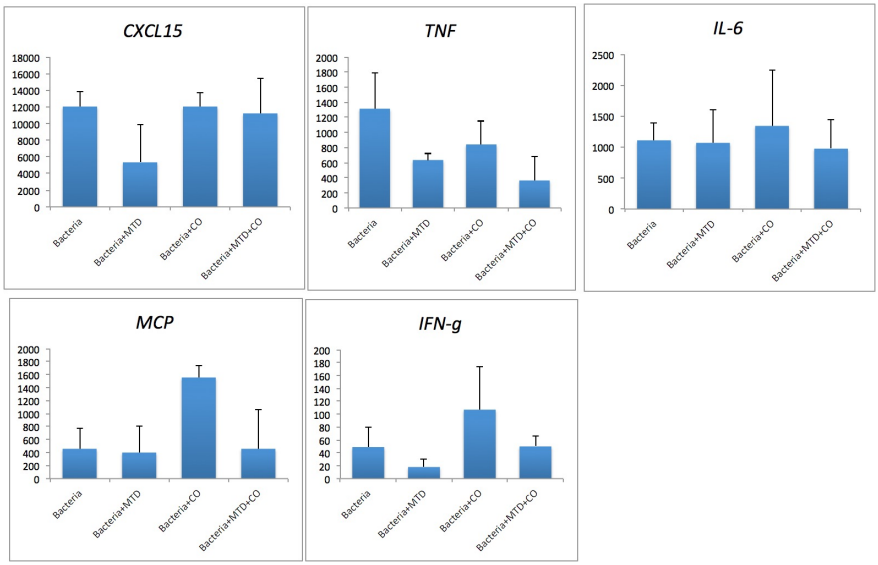


Figure 4. Male CD-1 mice were subjected to *S. aureus* infection (10^6 cfu) administered directly into the airway. 4h after bacteria mice were treated with Air or CO (0.025%, 250 ppm) for 1h and then both groups had a BAL performed and cells harvested were profiled by CyTOF using the list of antibodies in Table 1 below. The figure shows the shift in population of cell to one that is more aggressive in nature and may explain why CO treated mice have better bacterial clearance The right graph shows changes in specific surface markers. N=5 mice/group averaged. Note that CyTOF technology allows us to separate each individual mouse based on palladium coding.



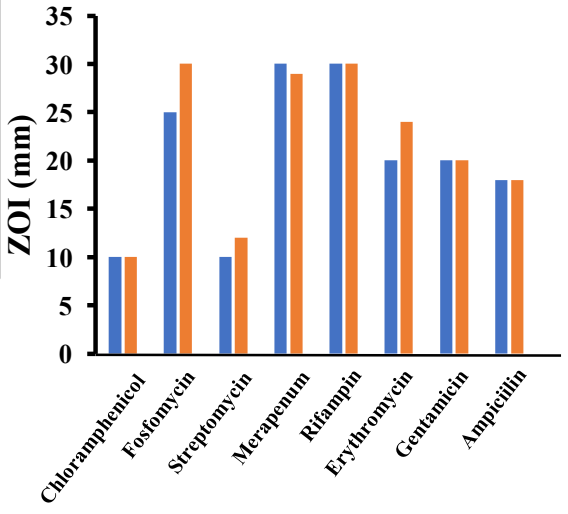


Figure 5. Male CD-1 mice were subjected to *S. aureus* infection (10^6 cfu) administered directly into the airway. 4h after bacteria mice were treated with Air or CO (0.025%, 250 ppm) for 1h. 24h later, BAL was performed and 17 different cytokines were measured by Luminex. Shown are those that were detected at 24 hr n=4/group. *p<0.05 vs Air. Note – we will be repeating this to increase the power as there were trends evident.

Figure 6. *S. aureus* were tested for their sensitivity to anitbiotics in the presence and absence of gaseous CO (250 ppm) using the disc diffusion assay**.** After overnight incubation, the zone of inhibition (ZOI) was measured. Results show that CO had no direct effects on bacterial growth. An identical assay was performed using *E coli* and similar findings were observed. This is not surprising given the known effects of CO on the host innate immune response. Results represent individual plates in duplicate.

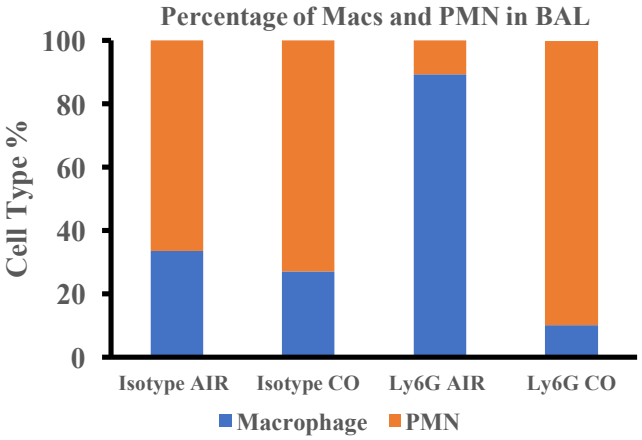


Figure 7. Male CD1 mice were infected and treated with CO as above (Figure 4) after receiving Anti-Ly6G antibody to deplete PMN. Anti-IgG was used as control. BAL was performed 5h after *S. aureus*. Low PMN recruitment is observed in the Ly6G AIR as expected. Interestingly, however, those mice treated with CO showed a mobilization of PMN into the airway. These data corresponded to bacterial clearance with CO enhancing bacterial clearance in Ly6G-treated mice (**Figure 8**). Results represent mean of 2 experiments, n=2/treatment.

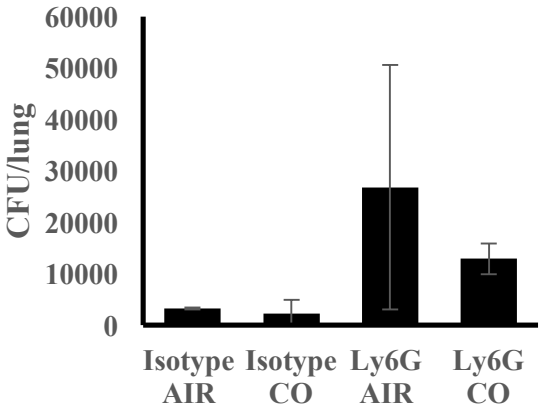


Figure 8. Male CD1 mice were infected and treated with CO as above (**Figure 4**) after receiving Anti-Ly6G antibody to deplete PMN. Anti-IgG was used as control. BAL was performed 5h after *S. aureus*. Depletion of PMN resulted in greater numbers of bacteria in the airway vs isotype control where PMN showed normal infiltration and killing. CO was able to enhance bacterial clearance in the Ly6G-treated mice likely due to the PMN recruitment rescue Results represent mean of 2 experiments, n=2/treatment. No statistical differences were noted likely due to the small number of animals in each group; n=4/group. These experiments are ongoing to increase the n.

PROJECT 3 (ROBSON): CD39 and Extracellular Nucleotide Signaling Mediate Inflammation and Immune Failure After Trauma.

Major Activities, objectives, and results

- Established and further characterized small animal models of gastrointestinal sepsis (induced by cecal ligation and puncture, CLP) and sepsis-related trauma (induced by liver crash injury) in wild type (*WT*), *Cd39* global null (*Cd39*$^{-/-}$) and targeted deletion mice. The liver crush injury model has been developed in collaboration with Projects 1 and 2. 03/04/2017
- Initiated experimental and clinical/translational studies to define interactions between purinergic signaling mediators (ATP, AMP, Ado, inosine) with oxygen, oxidative intermediates, CO and bilirubin during P2X7-mediated inflammation and sepsis. 10/01/2017.
- Probed the role of CD39 and P2X7 expression on myeloid and NKT cells as indicators of the presumptive predisposition to pneumonia. In addition, we shown direct links with purine-mediated exacerbations of inflammatory reactions in response to hyperoxia. 03/05/2017
- Commenced the identification of optimal therapeutic purinergic-based regimens in conjunction with oxygen, CO delivery and treatment of inflammatory stress in the setting of trauma and infection/sepsis. 06/05/2017
- Onboarded Dr. Dusan Hanidziar, an anesthesiology trainee, on the NIH Harvard T32-GM007592 to study the role of purinergic mediators (CD39 and P2X7) in acute lung and hyperoxic injury model; and Dr. Bynvant Sandhu, a surgical trainee, as a Fulbright Scholar to work on models of abdominal injury and pneumonia.

Made arrangements for testing expression of purinergic mediators as well as ecto-enzymatic bioactivity in human samples (bronchoalveolar lavage and blood) collected from trauma patients and controls. 03/04/2017

We have found:

- Increases in neutrophils ($Cd11b^{+}Ly6G^{+}$ cells) in the peritoneal cavity three hours after sepsis induction (**Figure 1A**)
- Reduction in neutrophil recruitment upon P2X7 receptor pharmacological inhibition or primary genetic deletion (**Figure 1A**)
- Increase in ATP and ADP hydrolysis and decrease in AMP hydrolysis in peritoneal cells from CLP septic mice (**Figure 1B**)
- Increase in the levels of IL-1, IL-6 and IL-10 in the serum and in the peritoneal fluid of *WT* and *Cd39*$^{-/-}$ mice. This increase was contained upon P2X7 receptor inhibition in all septic mice (**Figure 1C**)
- Administration of P2X7 receptor inhibitor + adenosine A2A receptor agonist protects *Cd39*$^{-/-}$ animals from liver injury during sepsis (**Figure 2A&B**) and is associated with decrease in pro-inflammatory cytokine levels as well as reduction in NFkB activation and Stat3 activation in the liver of septic mice (not shown)

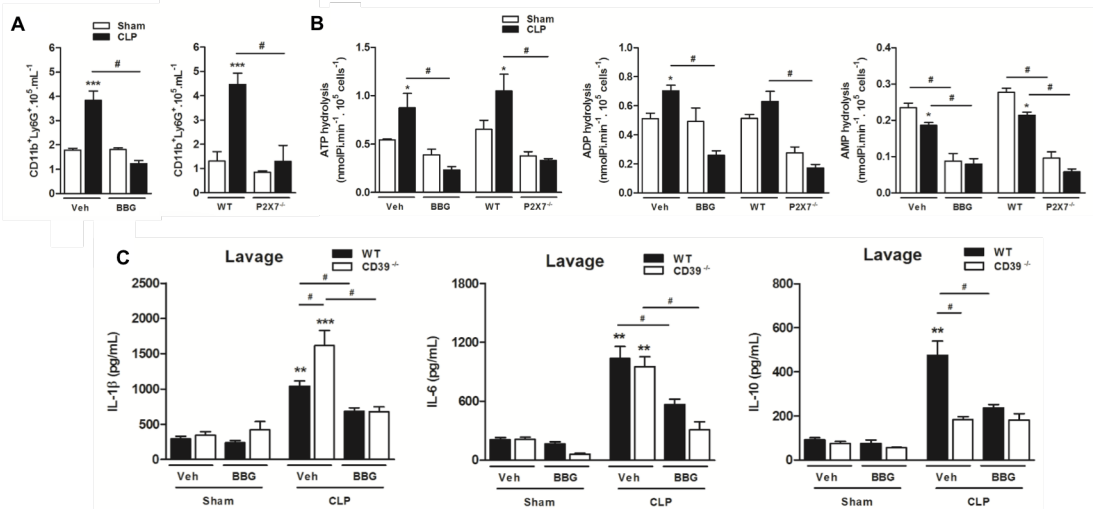


Figure 1: P2X7 receptor blockade attenuates systemic inflammatory responses with sepsis induction in WT and CD39$^{-/-}$ mice. Mice were subjected to sepsis by cecal ligation and puncture (CLP) or to laparotomy without puncture (Sham). For pharmacological inhibition of the P2X7 receptor, mice were injected i.p. with the receptor antagonist Brilliant Blue G (BBG, 45.5 mg/kg) or vehicle control (PBS), 24 hours before CLP. Peritoneal lavage fluid was collected 3 h after surgery and the **(A)** number of neutrophils ($CD11b^+Ly6G^+$) in the fluid was quantified by flow cytometry. **(B)** ATP, ADP, and AMP hydrolysis by peritoneal cavity cells from *WT* or *P2X7*$^{-/-}$or BBG-pretreated septic mice. **(C)** Cytokine production in peritoneal lavage fluid and blood from *WT* or *CD39*$^{-/-}$ BBG-pretreated or non-septic mice. Data are expressed as mean ± S.E.M. of four independent experiments (min. n=3 mice/experiment). Statistically significant differences between Sham and CLP, and between CLP groups (CLP vehicle vs. BBG, CLP WT vs. P2X7$^{-/-}$) are indicated by asterisks (*, $p< 0.05$; **, $p< 0.01$; ***, $p< 0.001$) and by the number sign (#, $p< 0.05$), respectively.

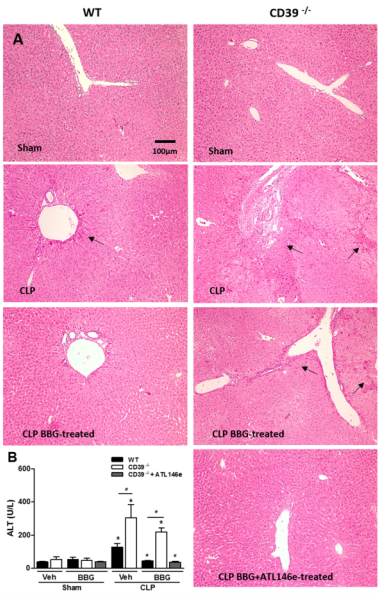


Figure 2: P2X7 receptor pharmacological inhibition and adenosine A_{2A} receptor activation completely protects the liver from *WT* and *CD39*$^{-/-}$ mice during sepsis. Mice were injected with vehicle (PBS) or BBG (45.5 mg / kg) 24 h before induction of CLP. Some animals were administered the adenosine A_{2A} receptor agonist ATL146e (1 mg/kg). The liver was harvested 24 hours after surgery and histological sections were stained with hematoxylin-eosin (H&E) for evaluation of histopathological changes. Representative photomicrographs of hepatic parenchyma in (A) *WT* and *CD39*$^{-/-}$ mice are shown. The necrotic areas (arrows) show liver damage. (B) Alanine aminotransferase (ALT) levels in septic mice treated or not with BBG or BBG+ATL146e. Data are expressed as mean ± S.E.M. of two independent experiments (n=6). Statistically significant differences between Sham and CLP, and between CLP groups (CLP vehicle vs. CLP BBG, CLP vehicle vs. CLP BBG+ATL146e) are indicated by asterisks (*, $p< 0.05$; **, $p< 0.01$; ***, $p< 0.001$) and by number sign (#, $p< 0.05$), respectively.

These investigations have constituted the experimental background for our ongoing work focused on sepsis induced by liver crash in *WT*, *Cd39* global null and conditional (in macrophages) knockout mice. During the same timeframe, we conducted parallel experiments to test the effects of hyperoxia on the immune system of humanized mice, generated upon transfer of human PBMCs into NSG immunodeficient mice.

We have found that:

- NSG immunodeficient mice have increased tolerance to hyperoxia (**Figure 3A**) and do not require early euthanasia.
- Injection of human leukocytes decreases NSG mice tolerance to hyperoxia (**Figure 3B**) and shortens time to clinical deterioration requiring euthanasia.

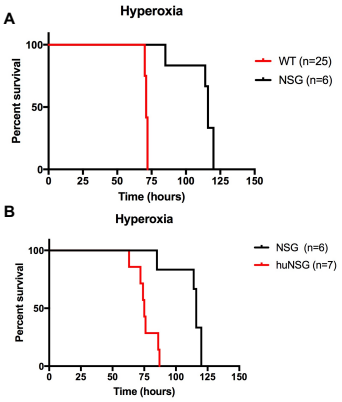


Figure 3: Effects of hyperoxia on the immune system of humanized mice. NSG immunodeficient mice were injected with 10x10^6 human peripheral blood mononuclear cells (PBMCs) and exposed to hyperoxia for up to 7 days. **(A)** Percent survival of WT and NSG mice following exposure to hyperoxia and i.e. not requiring euthanasia. **(B)** Percent survival of NSG mice to hyperoxia in the absence or presence of human PBMCs administration.

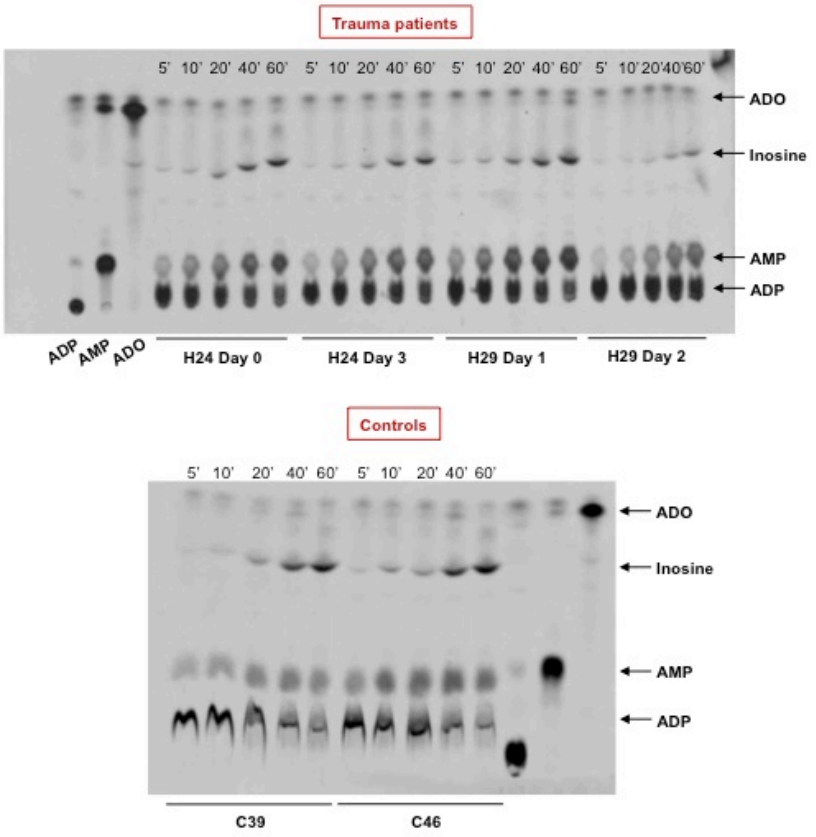


Figure 4: Thin layer chromatography of ADP phosphohydrolysis by CD39 and CD73 into derivatives. PBMC from trauma patients (top – H24 at Day 0 and day 3; H29 Day 1 and day 2) & controls (bottom – representative C39 and C46) were purified and tested for CD39 ectonucleotidase activity, manifested by C14-ADP hydrolysis to AMP. We also tested for consequent AMPase activity, as manifested by CD73. Decreases in CD39 bioactivity were observed at early time points in keeping with published changes in ATPDase activity with acute inflammatory stress in animal models and IBD. Substantive changes were also observed in adenosine deamination catalysis by ADA to inosine.

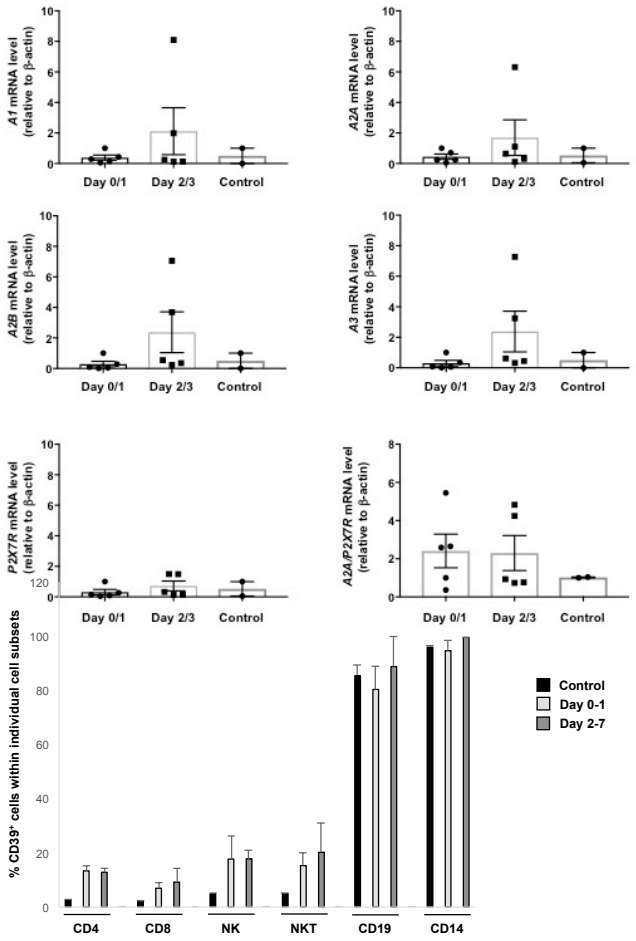


Figure 5: RT-PCR of A1, A2B &A2B with A3 ADORA-adenosine receptor levels as well as P2X7 in trauma patient cohort and controls.
Heightened levels of A1, A2A, A2B and A3 receptor RNA are noted at Days 2/3 in trauma patients. Minimal changes in P2X7 mRNA are noted. These alterations predispose to heightened adenosinergic responsiveness when we propose that CD39 activity begins to recover at the later time points (not yet demonstrated in this cohort).

Figure 6. Frequency of $CD39^+$ cells within T cell and innate immune subsets in PBMCs from trauma patients and controls. Frequency of $CD39^+$ lymphocytes within CD4, CD8, NK, NKT, CD19 and CD14 cell subsets was tested by FACS analysis in n=2 trauma patients (day 0-1 and day 2-7) and n=2 controls. Higher frequencies of $CD39^+$ cells were noted amongst CD4, CD8, NK and NKT cells from trauma patients; in CD8 and NKT subsets, $CD39^+$ cells appeared to be higher at later time points after injury.

PROJECT 4 (TALMOR): Human Subjects Core and Ventilator-Induced Injury and Lung Immune Response to Infection After Trauma

Major Activities, objectives, and results

- Obtained IRB and HRPO approval of the research protocol to enroll 1,000 patients from which to collect specimens and clinical data to support the other Projects. 26-May-2016 (IRB), 04-Nov-2016 (HRPO)
- Onboarded two clinical research assistants and completed training in good clinical practice, human subjects research, and research conduct. 11-Oct-2016; 30-May-2017
- 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
- Consented and enrolled 51 patients through 01-Oct-2017
- Collected 77 biological specimens through 01-Oct-2017

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 10/1/17 we have enrolled 58 patients.

• Trauma Cases n=23 (65%) Male, average age 43.4 years (19-87)
31 approached
23 consented
22 trauma cases from whom samples collected

• Surgical Controls n=28 (21%) Male, average age 58 years (32-79)
59 approached
28 consented
19 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.

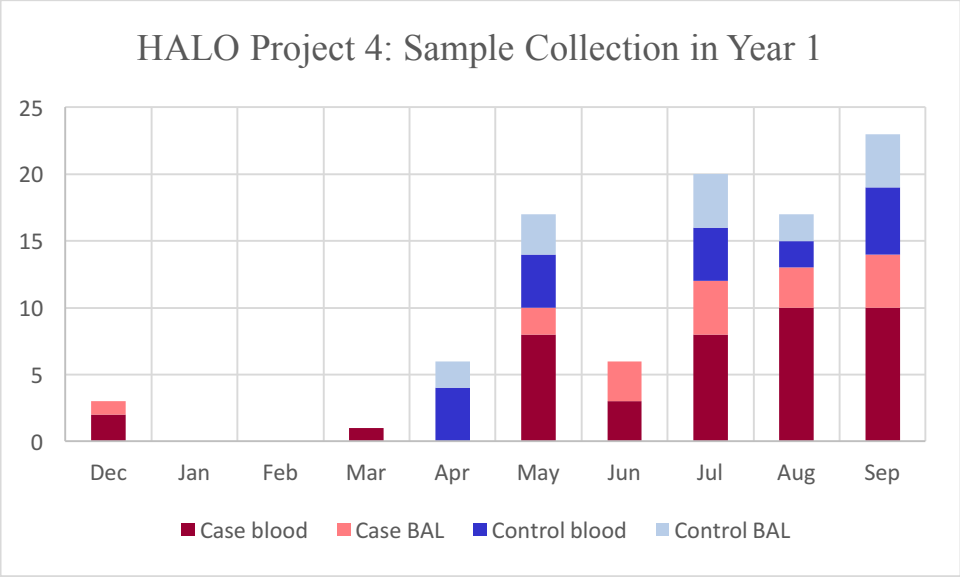
- ***Safe / correct collection of samples.***

We are collecting samples safely (no adverse events to date), and are refining our processes in conjunction with the collaborating labs to enhance optimal cell separation. From 10/1/16 through 10/1/17 we have collected 51 whole blood samples and 26 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. Figure 1 shows longitudinal sample collection over Year 1.

Figure 1: Collection of biological samples from enrolled patients over time.



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.
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.

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

We have not yet begun to address the role of PO_2.

During the first 12 months, activity has focused on securing regulatory approvals, establishing laboratory procedures, and training research staff. Enrollment rates are beginning to increase, and we anticipate soon having data to allow progress in subtasks 1 and 2.

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

Major Activities, objectives, and results

- Worked together with the HALO investigators to establish human blood isolation protocols to be used for the patient studies. This work included a streamlined SOP for preparation of plasma, peripheral blood mononuclear cells (PBMCs), and polymorphonuclear cells (PMNs, neutrophils) – 10-2016
- Validated a freeze and thaw approach to retain PBMC and PMN cell integrity and viability to be used for proposed CyTOF mass cytometry studies. 10-2016
- Designed and validated PBMC and PMN CyTOF staining panels that will be used to profile the phenotypic effects of trauma on circulating immune cell subsets at time points after traumatic injury – work to be done in collaboration with all HALO investigators, especially the computational modeling group, PI – Yaffe, 02-2017
- Harvard immunology master's student joined the lab to train and attain her Master's Degree in Immunology. Her studies focused on CpG-ODN studies in our project. Specifically, she worked on identifying and comparing the response of mouse and human PBMCs to different CpG-ODN stimulations. This work addressed the translational feasibility of using CpG-ODNs as immunotherapy for traumatic injury in humans. 09-2016, completed thesis, 05-2017
- Laura Cahill, PhD, was recruited and hired as a post-doctoral research fellow. She has been trained to gain expertise in CyTOF mass cytometry, mouse burn trauma, human immunology, and data analysis. Laura will help direct and organize a large portion of this project work. 03-2017
- Collaborative studies done with Project 2, PI Otterbein. This work involved designing and validating CyTOF antibody staining panels to study acute reactive immune cell subsets that move to the alveolar spaces in mouse lungs within 5 hours after lung bacterial infection. 02-2017, 05-2017, 06-2017
- CyTOF panel development for mouse and human immune cell profiling of blood immune cells to compare and contrast mouse vs. human reactivity to different CpG-ODN sequences to be developed for immunotherapy. CyTOF panels for mouse lung infection and tissue responses to trauma, endotoxemia, and infection. 09-2016 – 06-2017
- Four poster presentations on mouse and human trauma studies at the annual Shock Society Meeting, Ft Lauderdale, FL. Three posters described findings in mouse and human trauma studies and 1 poster described the finding on comparing mouse and human PBMC responses to CpG-ODN stimulation. 04 to 07-06-2017
- Manuscript entitled, "Phenotyping the immune response to trauma: A multiparametric systems immunology approach" published in *Critical Care Medicine*. This manuscript reported on our first CyTOF-based immune profiling of PBMCs from 10 trauma patients collected at the BWH and forms the foundation for the work to be done in this project. By error, I forgot to list the partial support by this program. 09-2017
- A second manuscript reporting on the analysis of PBMCs from a 20 trauma patient plus normal healthy controls was submitted for review and has been accepted pending minor revisions. This was a more complete cohort of patients for analysis and some statistical significance was found to indicate blood immune cell phenotype changes associated with patient outcome. The CyTOF staining and analysis was done with support from the DOD research program and is listed in the manuscript. 09-2017

We developed standard operating procedures (SOPs) for human and mouse immune cell preparation and storage by freezing. This is a methodology that will be used for the entire project. The samples that were tested and validated include blood, mouse and human bronchial alveolar lavage (BAL) cells, and dissociated cells from solid organs to include mouse lung, liver, spleen, and kidney. This is new methodology. – 10-2016 to 05-2017

During this project period, we focused our efforts on designing and validating CyTOF panels to be used to study infection and injury responses in mice. A panel to study the early response to lung infection with bacteria was designed and tested – called acute infection mouse BAL panel (AIB panel). The panel includes

markers for cell types that are known to respond to infection at 4-6 hours. Pilot experiments were done during this project period to test and refine the panels based on results. This acute mouse BAL panel will be used for our studies as well as collaborative studies done with other projects.

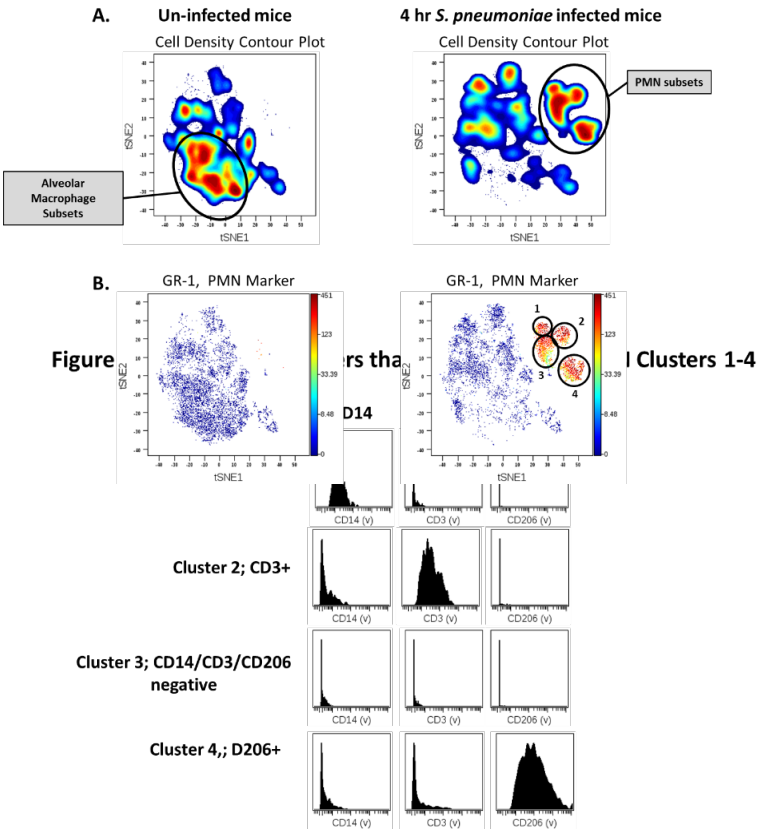
Table 1: Acute Infection BAL Panel

marker	clone	metal
EpCAM	G8.8	113In
CD4	RM4-5	115In
CD45	30-F11	141Pr
CD8a	53-6.7	142Nd
CD49b	DX5	143Nd
CXCR4	L276F12	144Nd
NKG2D	191004	145Nd
CD11c	N418	146Nd
PU.1	phpu13	147Sm
Ly6G	1A8	148Nd
CD19	6D5	149Sm
CX3CR1	SA011F11	150Nd
Ly6C	HK1.4	151Eu
CD3	145-2C11	152Sm
CD172a/SIRPa	P84	153Eu
PD-L1	10F.9G2	154Sm
CD68	FA-11	155Gd
CD14	Sa14-2	156Gd
CD205	NLDC-145	158Gd
CD206	C068C2	159Tb
Sca-1	E13-161.7	160Gd
Arginase I	Poly	161Dy
FoxP3	FJK-16s	162Dy
NK1.1	PK136	163Dy
Ki67	16A8	164Dy
CD115	460615	165Ho
CD103	2E7	166Er
SR-AI/MSR	268318	167Er
CD64	x54-5/7.1	168Er
CD11b	M1/70	169Tm
Siglec-F	E50-2440	170Er
CD279/PD-1	29F.1A12	171Yb
TCRgd	GL3	172Yb
CD69	H1.2F3	173Yb
CCR2	SA203G11	174Yb
F4/80	BM8	175Lu
GR1-Ly6G/Ly6C	RB6-8C5	176Yb
I-A/I-E	M5/114.15.2	209Bi

The finalized AIB panel is shown in Table 1. This panel includes markers specific for neutrophils and macrophage subsets as well as unique markers for functional phenotyping. In addition, CyTOF technology allows us to include a mixture of cell-surface and intracellular markers for single-cell phenotyping to allow more in depth classification of immune cell subsets. For example, we include transcription factors, PU.1, a nuclear proliferation, Ki67, and an intracellular enzyme, Arginase 1. These markers identify macrophages, proliferating cells, and macrophage subset – M2 cells. We will use this panel in our mouse burn trauma to measure the acute cellular immune response in the lungs of mice infected with *S. pneumoniae* or *S. aureus*. The effects of immunotherapy on cell-mediated immune responses to infection will be comprehensively profiled with this developed AIB panel as well as several other CyTOF panels to measure; 1) later cellular immune responses, 2) cytokine/chemokine expression in specific cell subsets, and 3) tissue immune reactions.

Figure 1 illustrates a portion of the results of our CyTOF AIB panel test done to compare un-infected and 4 hour lung immune cell response to *S. pneumoniae* bacterial infection. The aim of this study was to test the entire process for cell preparation from the lungs, freezing live cells, thawing, CyTOF staining protocol, and the AIB panel, which had been optimized during the project period. Mice were infected in the lungs by aspiration of 2 x 10^7 colony forming units (CFU) bacteria. At 4 hours after infection, bronchial alveolar lavage (BAL) cells were prepared by a washout protocol. BAL cells were stained with the AIB CyTOF panel and single-cell staining information was collected using a Helios CyTOF instrument. After acquiring the data, we analyzed the staining profiles using a series of CyTOF data analysis approaches. We use Cytobank for our CyTOF data analysis, which is cloud-based analysis platform that provides all the needed steps to analyze CyTOF staining data. Data was analyzed for staining intensity for all markers and for immune cell subset identification clusters using a dimensional reduction analysis algorithm called viSNE. ViSNE analysis organizes the multidimensional cell staining data into clusters of cells showing similar staining characteristics. In **Figure 1A**, we show density contour viSNE plots that illustrate relative cell densities of immune cell types with similar phenotype features. Comparison between un-infected and infected BAL cells indicates clear differences in cell subset density clusters. Plotting the dimensions, tSNE1 and tSNE2, on the x and y axis with individual marker staining as the coloring channel identifies the cell types within each cluster. In **Figure 1B**, the corresponding tSNE plots are colored by the

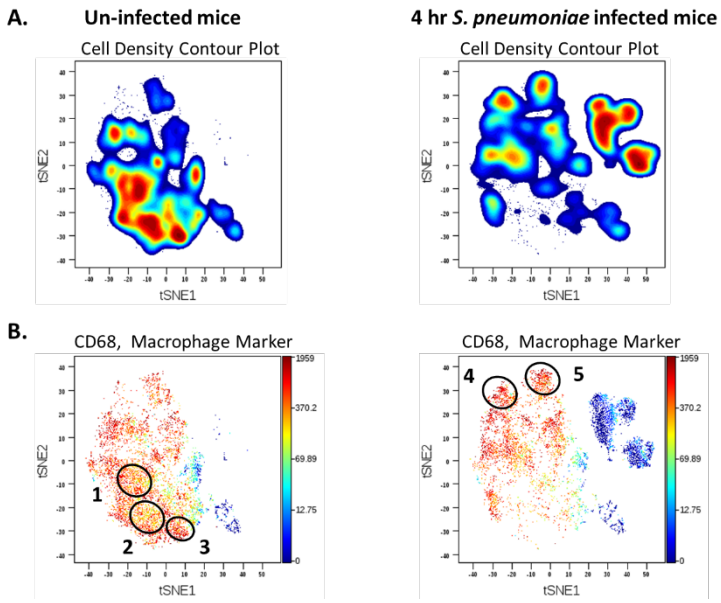
Figure 1: CyTOF Mass Cytometry Analysis of the Acute Immune Cell Response to Lung Bacterial Infection



canonical mouse neutrophil/PMN marker, GR-1. It is apparent that bacteria infection induces a specific PMN response. Furthermore, CyTOF staining with our AIB CyTOF panel was able to resolve 4 PMN subset clusters. Deeper analysis of these clusters by gating and histogram plots **(Figure 2)** reveals the unique phenotyping markers expressed on these 4 mouse PMN subsets that arrive in the lung airways at 4 hours after bacterial infection. As such, all these PMN subsets express similar levels of GR-1, but show differential expression of CD14, CD3, and CD206. These findings demonstrate our ability to use CyTOF technology to resolve specific immune cell subsets in a complex mixture of immune cells. We also demonstrate the power of using CyTOF for this type of discovery studies due to its high resolution staining without interfering signals from bacteria and debris that is found in the lung airways of mice after infection. To our knowledge, this is the first identification of these unique mouse PMN that react acutely to lung bacterial infection in mice. The markers suggest specific functions for these PMN subsets *e.g.* CD14 is part of the bacteria antigen recognition pathway, CD3 is part of the T cell receptor signaling pathway, and CD206 is a mannose receptor involved in bacteria recognition responses. PMN cluster 3, does not express any of these markers.

In **Figure 1A**, we also highlight differences in alveolar macrophage subsets found in un-infected versus infected mouse lungs. It is apparent that infection causes a change in the macrophage population that likely reflects changes in macrophage activation occurring in the lung airways in response to bacteria. In **Figure 3 A and B**, we highlight some differential marker expression profiles among macrophages in the lungs of un-infected and *S. pneumoniae* infected mice. In the viSNE plots shown in Figure 3B, the cell clusters are colored using the macrophage marker CD68. It is readily apparent that infection causes a complete change in macrophage phenotypes. The major clusters of cells are circled and numbered 1-5. The clusters (1-3) shown in un-infected mice have differential expression of CD172a, F4/80, PD-L1, and CD11c. The macrophage clusters that are highlighted in infected mice (4, 5) have differential expression of CD14 and do not express similar levels of CD11c or F4/80 as what is found in un-infected lungs.

Figure 3: Macrophage Subset Analysis

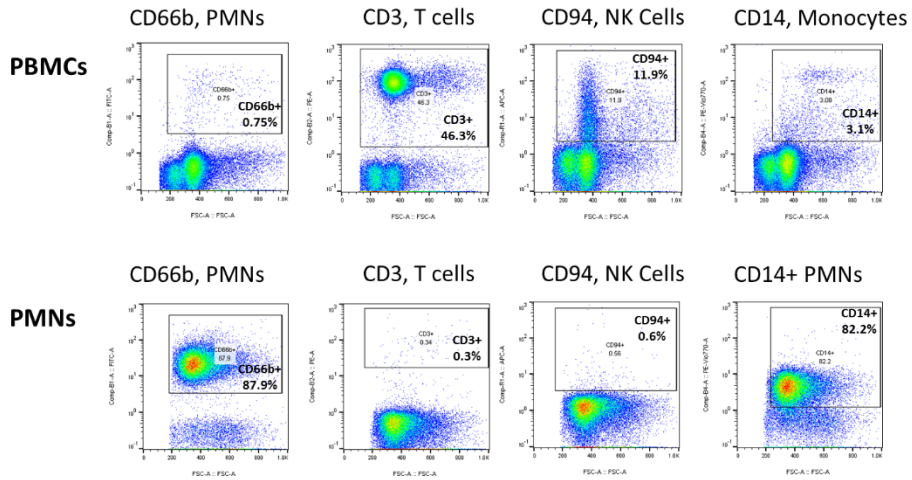


We also performed collaborative studies using this AIB CyTOF panel with Project 2. In those studies, we compared the lung airway response to *S. aureus* bacteria in mice exposed to carbon monoxide. The results of those studies identified a clear difference in macrophage-like cells that enter the lung early after infection. The results of that collaborative study are described in the report from Project 2.

Taken together, the CyTOF development and analysis work done during this project period demonstrate our ability to effectively and efficiently identify nearly all the different types of immune cells that react to bacterial infection in the lungs. We show the results from our AIB panel. We also have established CyTOF staining panels to measure lung bacterial infection responses at days 1, 3, and 5 in mice. These panels are highly similar, but use some additional CyTOF antibody reagents that recognize immune cell subsets involved in resolution of infection responses like T cell subsets, NK cells, and specialized Macrophage subsets. We will use this same systems immunology approach to precisely study the effects of trauma on the cellular immune response to lung infection. In addition, we will use this approach to study using Toll-like receptor 9 (TLR9) stimulatory CpG-oligodeoxynucleotides (ODNs) as immunotherapy for trauma-associated immune dysfunction with the ultimate aim to restore immune homeostasis and anti-microbial immunity.

An effort is ongoing to collect blood and BAL cells from trauma patients and normal controls for immune cell phenotyping studies. Our collaborative work for this Subtask has focused on developing CyTOF panels to be used for immune cell profiling blood and BAL cells. As such, we worked in collaboration with all PIs to develop SOPs for blood cell and BAL preparations. In order to feasibly perform CyTOF immune cell profiling studies, we need to bank frozen live cells that can be thawed in batches for staining with CyTOF antibody reagents. As part of this project as well as other collaborative projects, we developed and optimized blood cell preparation and storage methods. These methods have been used by us to successfully generate high-quality CyTOF staining data from peripheral blood mononuclear cells (PBMCs) and PMNs. The procedures include a rapid ficoll density centrifugation preparation for PBMCs, a RBC lysis method to prepare total blood leukocytes, or a sequential method that provides PBMCs by ficoll density centrifugation followed by a second density gradient centrifugation to prepared highly-enriched PMNs. **Figure 4** shows representative flow cytometry plots of human PBMCs and PMNs that were purified by our two-step density centrifugation method. There are few PMNs in the PBMC preparation and the appropriate immune cell subsets are identified in the PBMC preparations (T cells, NK cells, Monoctyes). In contrast, the PMN preparation shows highly-enriched PMNs with few contaminating PBMCs. Human PMNs express CD66b and low levels of CD14. The percent enrichment of PMNs by this protocol was 88%. The contaminating cell events in PMN preparations were found to be RBCs and platelets.

Figure 4: Flow Cytometry Analysis of PBMCs and PMNs Purified by HALO Human Blood Cell SOP



These different cell preparations have been frozen as live cells and then thawed for analysis by CyTOF staining without significant cell loss from the freeze/thaw procedure or loss of cell marker expression. This was determined by comparing fresh vs. frozen PBMC and PMN cell preparations. Similarly, we optimized the same freeze/thaw methods for human and mouse bronchial alveolar lavage (BAL) cells to be used for immune cell profiling by CyTOF. The complex variety of immune cells in blood justified development of multiple immune cell profiling panels. Each panel developed for this project has a particular focus. For example, the PBMC phenotyping panel are designed with T cell, B cell, or myeloid cell focus. These panels include markers that identify specific cell subsets as well as functional markers for activation and regulation of immune responses. For total leukocytes and PMNs, the panels are focused on PMN relevant markers. We also developed functional panels for cell signaling responses and cytokine expression following stimulation. **Table 2** lists some of the panels that we have developed and validated for basic human blood immune cell phenotyping.

These CyTOF antibody immune cell phenotyping panels have been developed and validated to be used to identify specific changes in abundance and activation of blood immune cells at days 1, 3, and 5 after traumatic injury in humans

Table 2: Human PBMC and PMN CyTOF Antibody Phenotyping Panels

T Cell CyTOF Panel

marker	clone	metal
CD45	HI30	141Pr
CD45RA	HI100	142Nd
CD44	IM7	143Nd
CD64	10.1	144Nd
CD4	RPA T4	145Nd
CD8a	RPA T8	146Nd
CD45RO	UCHL1	147Sm
CD28	CD28.2	148Nd
CD25	M-A251	149Sm
Tim-3	F38-2E2	150Nd
CD279/PD-1	EH12.2H7	151Eu
CD152/CTLA-4	L3D10	152Sm
CD69	FN50	153Eu
CD185/CXCR5	J252D4	154Sm
CD94	DX22	156Gd
CD3	UCHT1	158Gd
TCRα/β	IP26	159Tb
CD278/ICOS	C398.4A	160Gd
AHR	FF3399	161Dy
CD183/CXCR3	G025H7	162Dy
FoxP3 (1:50)	PCH-101	165Ho
GATA-3 (1:50)	TWAJ	166Er
Grz B	GB11	167Er
Bcl-6	IG191E/A8	168Er
PU.1	puph13	169Tm
ROR-γt (1:50)	AFKJS-9	170Er
CD127	A019D5	171Yb
KLRG1	2F1/KLRG1	172Yb
TCRγ/δ	B1	173Yb
CD62L	DREG-56	174Yb
T-bet (1:50)	4B10	175Lu
Perforin	dG9	176Yb

B Cell CyTOF Panel

marker	clone	metal
CD27	O323	141Pr
CD19	HIB19	142Nd
CD24	ML5	144Nd
CD16	B73.1	145Nd
CD1a	HI149	146Nd
CD10	HI10a	147Sm
CD20	2H7	148Nd
CD22	HIB22	149Sm
CD38	HIT2	150Nd
CD5	UCHT2	151Eu
Bcl-2	100	152Sm
IgD	IA6-2	153Eu
CD185/CXCR5	J252D4	154Sm
CD23	Bu32	155Gd
CD95/Fas	DX2	156Gd
CD3	UCHT1	158Gd
CD289/TLR9	eB72-1665	159Tb
CD307d/FcRL4	413D12	160Gd
CD267/TACI	1A1	161Dy
CD1c	L161	163Dy
Blk		164Dy
CD45	HI30	165Ho
CD40	5C3	166Er
IgA	9H9H11	168Er
CD268/BAFFR	8A7	169Tm
CD32	FUN2	170Er
CD269/BCMA	8A7	171Yb
IgM	MHM-88	172Yb
CD86	IT2.2	174Yb
CD138/Syndecan-1	MI15	175Lu
IL-10	JES3-19F1	176Yb

Myeloid Cell CyTOF Panel

marker	clone	metal
CD235a	HI264	139La
CD45	HI30	141Pr
CD11b	M1/70	142Nd
CD115	9-4D2-1E4	143Nd
CD64	10.1	144Nd
CD16	3G8	145Nd
CD8a	RPA T8	146Nd
HLA-DR	L243	147Sm
CD284/TLR4	HTA125	148Nd
CD1d	51.1	149Sm
CD193/CCR3	5E8	150Nd
CD123	6H6	151Eu
CD14	M5E2	152Sm
CD181/CXCR1	8F1/CXCR1	153Eu
CD15	MC-480	154Sm
CD4	RPA T4	155Gd
CD94	DX22	156Gd
CD3	UCHT1	158Gd
CD11c	Bu15	159Tb
CD19	HIB19	160Gd
CD303/BDCA-2	201A	161Dy
CD56/NCAM	HCD56	162Dy
CD1c/BDCA-1	L161	163Dy
CD141/BDCA-3	M80	164Dy
CD177	MEM-166	165Ho
CD80	2D10	166Er
CD287/TLR7	533707	167Er
CD1a	HI149	168Er
TCRγ/δ	B1	169Tm
CD182/CXCR2	5E8/CXCR2	170Er
CD66b	G10F5	171Yb
CD274/PD-L1	29E.2A3	172Yb
FcεRIα	AER-37	173Yb
CD86	IT2.2	174Yb
CD335/NKp46	9E2	175Lu
PD-L2	24F.10C12	176Yb

PMN CyTOF Panel

marker	clone	metal
CD3	UCHT1	115In
CD88	S5/1	141Pr
CD11b	M1/70	142Nd
CD64	10.1	144Nd
CD16	3G8	145Nd
CD14	M5E2	146Nd
CD13	WM15	147Sm
TLR4	HTA125	148Nd
CD18 (a)	MEM-148	149Sm
CD11b (a)	CBRM1/5	150Nd
CD279/PD1	EH12.2H7	151Eu
CD170	1A5	152Sm
CXCR1	8F1	153Eu
CD15	MC-480	154Sm
CD18	CBR LFA-1/2	155Gd
CD95/Fas	DX2	156Gd
Tbet	4B10	158Gd
CD11c	Bu15	159Tb
FPR1	350418	160Gd
IL-13R	SS12B	161Dy
CD85j/ILT2	GHI/75	163Dy
CD10	HI10A	164Dy
CD177	Polyclonal	165Ho
CD31	WM59	168Er
TLR6	TLR6.127	169Tm
CD54/ICAM	HA54	170Er
CD66b	G10F5	171Yb
CD274/PD-L1	29E.2A3	172Yb
CD62L	DREG-56	174Yb
CD172a/b	SE5A5	175Lu
CD302	771910	176Yb
CD45	HI30	209Bi

Immunophenotyping trauma patient blood by CyTOF mass cytometry. We used the developed panels to complete a 20 patient immunophenotyping study. The study designed was to compare blood immune cell subset changes in trauma patient blood samples collected at days 1, 3, and 5 after trauma. We completed stains on peripheral blood mononuclear cells (PBMCs) and total leukocyte preparations (for neutrophil analysis) in May, 2017. These data have been analyzed and a portion of the data was presented by Dr. Anu Seshadri at the American Association for the Surgery of Trauma meeting in September, 2017 at Baltimore, MD. The presentation focused on the mononuclear cell data from total leukocyte preparations and was also submitted as a manuscript to the Journal of Trauma – accepted for publication with minor revision. The abstract of the manuscript is shown here:

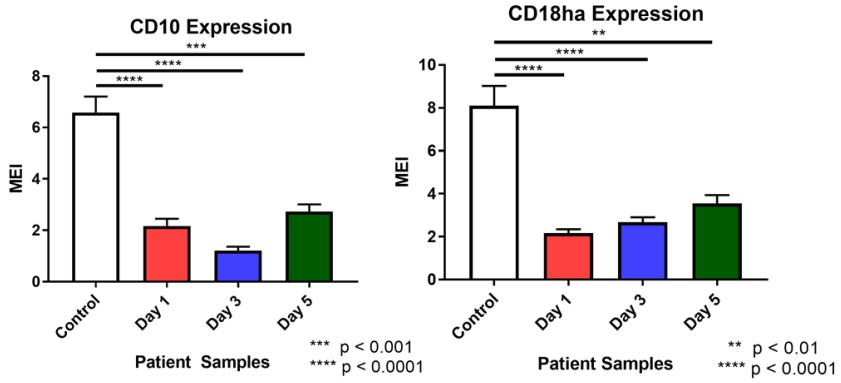
Background: Trauma induces a complex immune response, requiring a systems biology approach to capture multicellular changes. Using mass cytometry by time-of-flight (CyTOF), we evaluated time-dependent changes in peripheral blood in trauma patients to identify changes correlated with infection.
Methods: Total leukocytes were prepared via red blood cell lysis using peripheral blood samples from trauma patients with an injury severity score > 20 at days 1, 3, and 5 after injury, and from age- and gender-matched uninjured controls. Cells were stained using a 33-marker immunophenotyping CyTOF panel. Statistics were calculated using one-way ANOVA with multiple comparisons.
Results: CyTOF staining demonstrated changes in many cell subsets. The mean expression intensity (MEI) of CD86 on monocytes decreased significantly at all time points after injury. When the patients were stratified based on development of infection, there was a trend to decreased CD86 expression on monocytes of those patients that developed subsequent infection. Based on stratification, we identified significantly increased expression of CD39 on NK cells only in patients that developed an infection,
Conclusions: This study used a systems biology approach to identify novel changes in circulating immune cell subsets in trauma patients correlating with post-traumatic infection. Decreased expression of CD86, a costimulatory molecule, on monocytes demonstrates that trauma affects the innate system's ability to control

T-cell immunity. We also found that CD39 expression on NK cells increased significantly in patients with subsequent infection. CD39 is a protein that generates adenosine, which has immunosuppressive effects on several immune cell types including NK cells. In summary, our results point to pathways that may be central to second-hit infections and further study to delineate these pathways could be key to generating clinical biomarkers or targeted immune therapies for trauma patients.

The observation that CD39 expression is significantly increased on NK cells in patients that subsequently develop post-trauma infections is a significant finding worth follow-up analysis. CD39 is an ectoenzyme that converts extracellular ATP, which is a DAMP, to adenosine. Adenosine has potent counter-inflammatory and immune suppressive activity. Dr. Robson and his group (**Project 3**) are experts in CD39 biology and we are discussing how to design hypothesis-testing projects in his CD39-deficient mouse models and on human NK cells during the next year of this focused research program.

The trauma patient CyTOF dataset has been analyzed by us in more detail by several analytical methods in collaboration with **Project 6**, Dr. Yaffe and his group. We are preparing a manuscript on neutrophil and other immune cell subset changes that were identified by staining the PBMC and total leukocyte cell preparations. Some of the interesting findings included a dramatic loss of a CD10+/CD18+ neutrophil subset from the blood of trauma patients during the entire post-trauma period (**Figure 5**).

Figure 5: Significant reduction in circulating CD10+/CD18+ neutrophil in trauma patients



This subset of neutrophils has been referred to as "regulatory" neutrophils (Nregs) since CD10 functions as a metalloprotease to inactivate proinflammatory cytokines. We suspect that these cells likely leave the blood to go to the sites of tissue damage due to their expression of CD18-high affinity (ha) expression, an integrin that is intimately involved in the diapedesis of neutrophils through the vascular endothelium. The complete analysis of this CyTOF dataset is being finalized and will be submitted for publication this year.

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

- Onboarded post-doctoral fellow and completed training in animal care, flow cytometry, human subjects use and good laboratory practice. 15-12-2016
- Validated PMN priming in representative human trauma patients, and established specific protocol for assessing extent of primed state. 07-06-2017
- Initiated experiments with Hauser lab examining effects of mtDNA and DAMPs on PMN priming and Ca^{2+} mobilization (Project 1). 24-03-2017
- Demonstrated in initial experiments that complement activity in plasma of trauma patients accounts for partial priming activity at early times after trauma. 15-12-2016
- Initiated experiments examining PMN priming in mouse bone marrow-derived neutrophils examining murine mitochondrial-based DAMP peptides. 28-03-2017
- Performed experiments in collaboration with Otterbein lab demonstrating that heme is not a priming agent for PMNs (Project 2). 12-05-2017
- Initiated experiments to explore the emergence of a 'low density neutrophil' immunosuppressive population in collaboration with Lederer lab (Project 5). 28-02-2017
- Continued backcrossing NADPH oxidase p47phox knock-in mice in order to perform experiments evaluating the role of extracellular ROS on innate immune dysfunction. 29-06-2017
- Demonstrated the MK2 knock-out neutrophils show a mild decrease in ROS production but with similar kinetics as wild-type animals. 15-04-2017
- 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 17-05-2017
- Continued evaluating trauma patient blood samples for primability and ROS production. 26-07-2017
- Showed that C5a-primed PMNs from clotted blood damage endothelial cells in vitro.
- Together with Hauser lab (Project 1), demonstrated that one of the mtFP has strong agonist effect and it can de-prime neutrophils upon second stimulus. 02-10-2017

Progress Detail:

Our first sets of 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 TNFα and C5a as exogenous agents to further assess primability, and fMLP as activating agent (Figure 1). We also found that trauma whole blood is continuously releasing extracellular ROS (Figure 1a-b), which may contribute to enhanced damage 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 (Figure 2). Based on these early findings, in ongoing studies, we will explore whether trauma patients who present with high levels of basal priming on day 1 are the same ones who are then most at-risk for immunosuppression and reduced PMN function at later times.

We have now 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 naive PMNs from un-injured patients (Figures 3), but additional patient studies are needed. A likely mediator responsible for this early PMN priming activity appears to be C5a as a result of complement activation. Figure 4 shows that (1) the process of blood clotting is sufficient to prime PMNs for increased ROS release, (2) the PMN priming activity can be recapitulated using cell-free plasma which is allowed to clot in vitro; (3) the release of PMN priming mediators from clotted blood does not require significant amounts of platelets; and (4) that this type of PMN priming appears to specifically release extracellular ROS, which likely contributes to enhanced tissue damage (also included in SA2). In good agreement with this hypothesis, we have shown that naïve neutrophils pre-incubated with clotted blood are able to degrade the endothelial barrier function (Figure 5), indicating that the ROS release is the factor

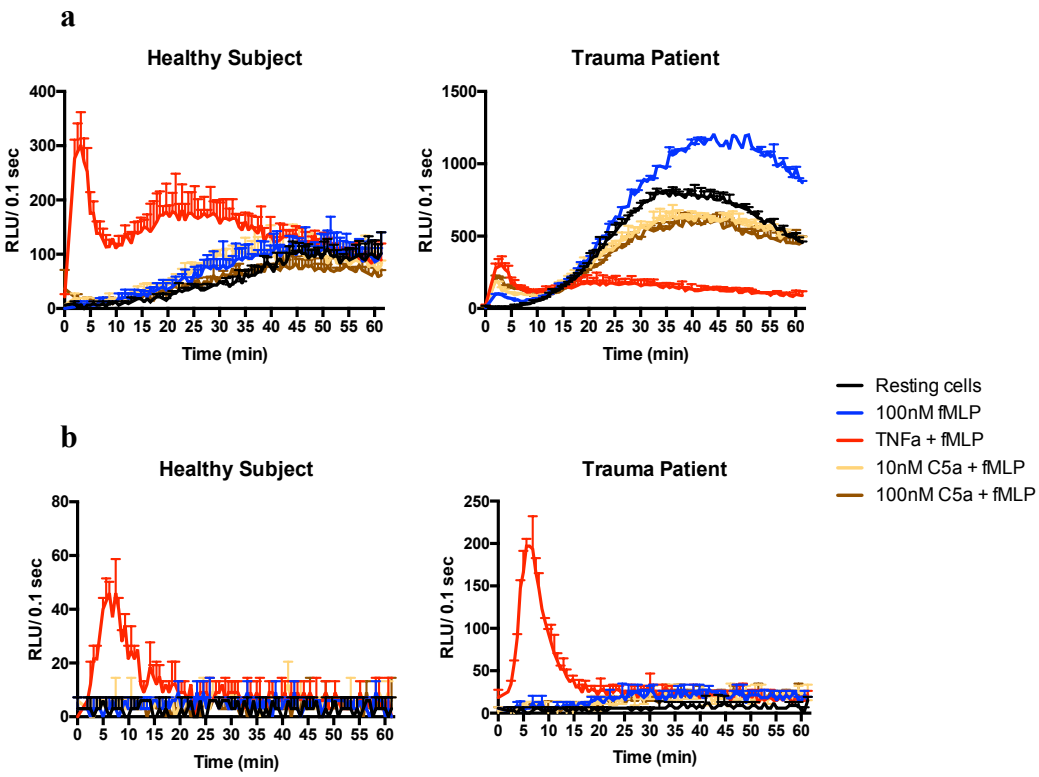


Figure 1. ROS production in whole blood collected on day 1 after trauma. (a) Total ROS. (b) Intracellular ROS.

that contributes to tissue damage. A substantial portion of the priming activity in trauma patients is suppressible at early times after injury by blocking the C5a receptor using W54011, a C5a receptor antagonist (Figure 3e and j). By 24 hours, however, the extent of suppression upon C5a antagonism is reduced, suggesting additional mediators of priming at later times after injury.

Since other priming agents and agonists may be responsible for neutrophils activation and ROS release, together with Hauser lab (Project 1) we have been examining the effects of DAMPs on PMN priming. Among the DAMPs that have been tested, there are 5 mitochondrial formyl peptides (mtFP) that induce Ca^{2+} flux similar to fMLP (Project 1). We have found that ND4 is a potent agonist for ROS release (Figure 6) that can impair ROS release after a second stimulus. Currently, the other 4 mtFP are also been tested.

One of the pathways responsible for neutrophil priming and ROS release is of p38MAPK/MK2. Our lab has created constitutive, conditional and a novel Cre-reversible knock out of MK2. In response to PMA stimulation, both wild-type and MK2-null PMNs appear to make similar amounts of ROS, with similar kinetics of ROS production. Experiments evaluating the response of these cells to other priming agents and agonists are ongoing, as well as experiments looking at cytokine release, macrophage function, and bacterial killing.

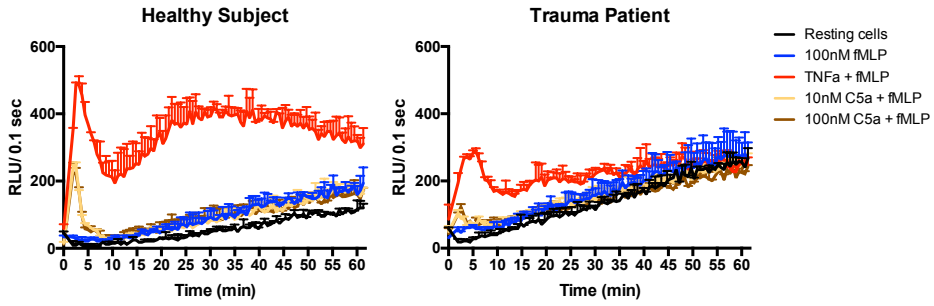


Figure 2. ROS production in whole blood collected on day 5 after trauma.

As shown in Figures 1-4, we have now shown that the early enhanced ROS release seen after neutrophil priming in response to mediators present in serum of trauma patients is primarily extracellular ROS rather than intracellular ROS, since it can be markedly inhibited by addition of SOD and catalase. We are currently evaluating the relative extent of extracellular and intracellular ROS release from primed neutrophils obtained from trauma patients at various times during the injury response. 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. We are currently in the process of backcrossing these animals into a pure C57BL/6 background, since this genetic background is required to see optimal responses in

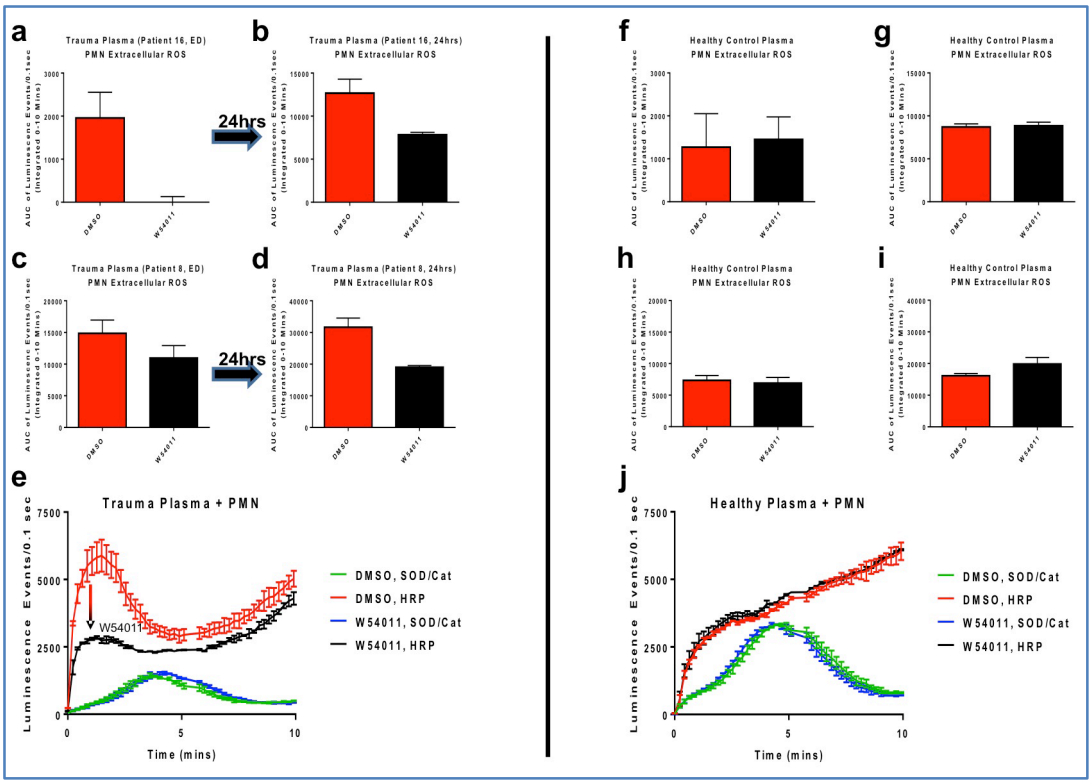


Figure 3. Plasma from trauma patients, but not uninjured controls, primes naive PMNs for early extracellular ROS production in C5a-dependent manner. Plasma from two trauma patients (a-e) and two controls (f-j) assayed for priming mediators within the first 24 hours after injury. Panels e and j show time-dependent ROS production and suppression off the early peak by the C5aR antagonist W54011.

trauma/hemorrhage models. SNP analyses showed that wild-type and p47phoxK43A mice displayed the most C57BL/6NTac character (100.0%) and can be considered congenic. p47phoxK43Q mice displayed mixed and non-C57BL/6NTac character on 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, we have breeding wild-type and p47phox$^{-/-}$ mice to generate enough number of mice to perform the experiments proposed in the original application. We are planning to start with wild-type and p47phox$^{-/-}$ mice while waiting for p47phoxK43Q mice to breed until becoming congenic.

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

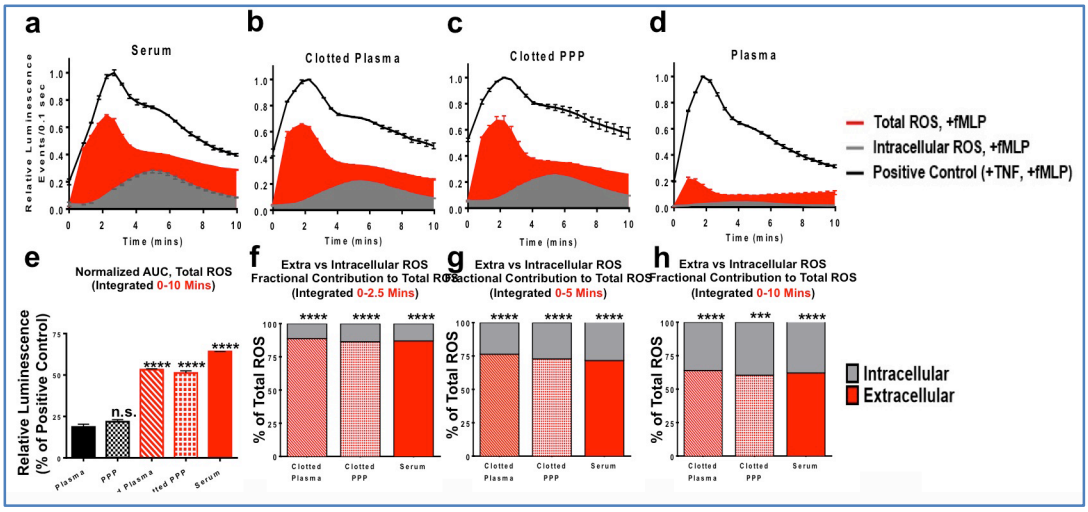


Figure 4. Neutrophil priming by products of blood coagulation. (a-d): Time course of ROS production by PMNs incubated with the indicated blood components. PPP indicates platelet poor plasma. (e-h): quantification of total intracellular and extracellular ROS production.

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 improved. Preliminary CyTOF experiments on these cells have not revealed distinguishing surface features, but the experiments are in a very early stage.

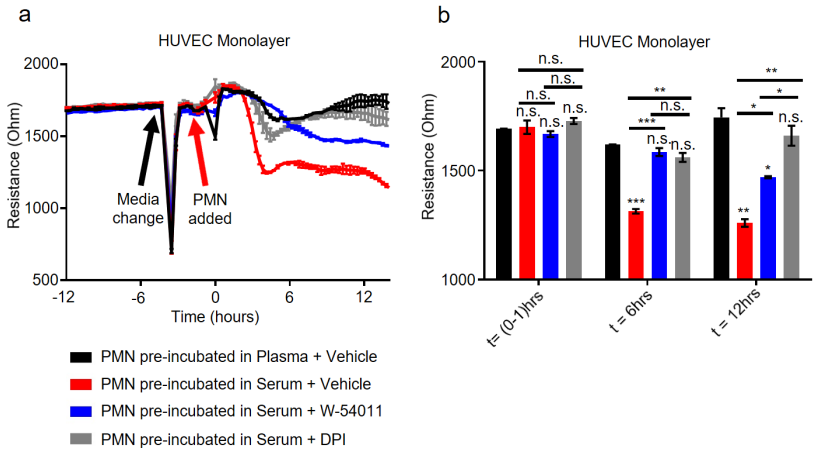


Figure 5. Neutrophil ROS released in response to C5a generated by the complement alternative pathway during coagulation degrades endothelial barrier function. (a) Kinetic curves and (b) multiple comparisons at discrete time points of Electric Cell-Substrate Impedance Sensing (ECIS) assays.

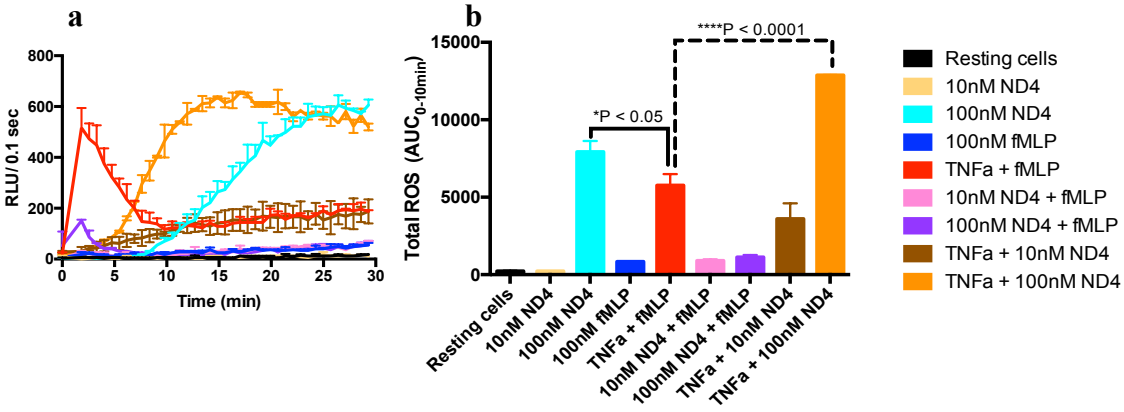


Figure 6. Effect of ND4 on neutrophil priming and ROS release. (a) Total ROS. (b) Total integrated ROS production over 30 min.

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

All of the 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 for each project)

How were the results disseminated to communities of interest?

Results of our work were presented at the following venues:

- Shock Society, Fort Lauderdale, FL
- American College of Surgeons, Baltimore, MD
- Western Trauma Association, Snow Bird, UT
- Gasotransmitter Conference, Atlanta, GA
- American Association for the Surgery of Trauma, Kona, HI
- Massachusetts Committee on Trauma, Boston, MA

Professor Charles Serhan, PhD was a visiting professor who is an authority on lipid mediators known as Resolvins. All projects were presented to him during his visit and he gave a lecture entitled "Resolving Inflammation and Infection: Novel Mediators and Mechanisms"

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, **2.**) mechanistic studies in our established animal and cell culture models, **3.**) begin building the computational modeling database and **4.**) beginning to test potential therapeutic approaches by which to decrease susceptibility to infection after trauma.

4. IMPACT

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

One of our key goals in year one was to establish the model systems and readouts in cells isolated from trauma patients and determine if this was able to be mimicked in mouse models. Each laboratory optimized their methods and in most instances collaborated successfully across disciplines in efforts to maximize the data collected.

What was the impact on other disciplines?

Nothing to report however we have begun to initiate collaborations with investigators with expertise in areas outside our network, but directly related to trauma and infection such as glycomics, complement, and small molecule medicinal chemistry.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS

Problems: Patient sample repository collection have been lower than we expected. This reflects several issues that we have recognized and now begun to overcome. First, we began our studies later than anticipated because IRB approval was slow in coming. That has now been fully resolved. Second, our patient population has turned out to have specific characteristics that often did not match up with our original expectations and tended to limit enrollment. For instance, our patient population is more likely to be intubated due to altered mental status than due to blunt tissue injury or shock. This has led to our being more dependent on next-of-kin consents and less dependent upon direct patient consenting. This is a common problem in trauma populations. Infectious risk in our

patient population was also likely more driven by early intubation then by massive blunt tissue trauma. These events have delayed our plans to assemble the initial "training set" for our computational biology aims.

We have therefore made a several significant changes in our entry criteria and have now begun recruiting patients at a much more rapid pace. These include alterations in ISS, as well as use of either intubation or transfusion as evidence of cardiorespiratory compromise. These changes are more completely documented in the human subjects core (Talmor, Project 4). In addition to those changes, we are now to planning to include a second study site, Brigham and Women's Hospital. This also has a Level 1 trauma center. Since both hospitals are within the Harvard system there will be no need for a new IRB process, and we expect the acquisition of this second site to increase our enrollment by 50 to 75% over the next few months. This should allow us to complete our training set of 100 to 150 patient samples (plus controls) in the next 4 to 6 months.

Changes that have a significant impact on expenditures:
We have decided to move to centralized assays of plasma and BALF for selected mediator content using a commercially available platform. There will be setup charges involved with creating our own unique set of assays. We believe however, that over time this approach will both save money and insure reproducibility. Moreover, by using micro assays, we will preserve our unique biologic reagents for experimental use.

Human subjects, or vertebrate animals:
There have been no significant changes in the care of human subjects or vertebrate animals. We have now made arrangements to obtain *Fpr1*$^{-/-}$ mice (Dr. Philip Murphy, NIH) and expect them to be in quarantine for 1 month

6. PRODUCTS

Publications:

1. Schallner N., Lieberum J-L, Gallo D, LeBlanc RH, Fuller PM, Hanafy, KA, Otterbein, LE; Carbon monoxide preserves circadian rhythm to reduce the severity of subarachnoid hemorrhage in mice; *Stroke.* 48; 2017; 2565-2573. Published: federal support.
2. Wegiel B, Hedblom A, Aiffin J, Gallo D, Bisht,K, Li M, Kaczmarek E, Csizmadia E, Correa-Costa M, Itagaki K, Hauser CJ, Alam J, Robson SC, Bulmer AC, Wagner K-H, Kelly VP, Mantle T, Otterbein LE; Bilivedin reductase A is a cell surface sensor for mitochondrial DNA after liver injury; Under revision for publication: federal support.
3. Correa-Costa M, Gallo D, Csizmadia E, Gomperts E, Lieberum J-L, Hauser CJ, Ji X, Wang B, Camara NOS, Robson SC, Otterbein LE. Carbon monoxide protects the kidney through the central circadian clock and CD39; Under revision for publication: federal support.
4. 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.* Accepted for publication with minor revision: federal support.
5. Diggins KE, Greenplate AR, Seshadri A, Lederer JA, Irish JM. At the Bench: Precision Medicine with Single Cell Mass Cytometry. *Journal of Leukocyte Biology.* Accepted with minor revision: federal support.
6. Barrett CD, Moore HB, Banerjee A, Silliman CC, Moore EE, Yaffe MB Human Neutrophil Elastase Mediates Fibrinolysis Shutdown Through Competitive Degradation of Plasminogen and Generation of Angiostatin. J *Trauma Acute Care Surg.* 2017 Aug 23. doi: 10.1097/TA.0000000000001685. In Press: federal support.
7. Savio LEB, de Andrade Mello P, Figliuolo VR, de Avelar Almeida TF, Santana PT, Oliveira SDS, Silva CLM, Feldbrügge L, Csizmadia E, Minshall RD, Longhi MS, Wu Y, Robson SC, Coutinho-Silva R. CD39 limits receptor inflammatory signaling and attenuates sepsis-induced liver injury. *J Hepatol.* 2017 May 26. pii: S0168-8278(17)32057-3. doi: 0.1016/j.jhep.2017.05.021. [Epub ahead of print]. federal support.

Presentations:

1. Hauser, CJ. "Application of Exogenous PMN to the Airway Rescues Bacterial Overgrowth Initiated By Trauma DAMPs." American Association for the Surgery of Trauma. 2016, Kona, HI.
2. Hauser, CJ. "Trauma-induced, DAMP-mediated remote organ injury and immunosuppression in the acutely ill patient." International DAMPs Conference 2016, Guanajuato, MX.

3. Hauser, CJ. "Sterile Inflammation and Infection after Tissue Injury." Duke University Department of Surgery, 2017, Raleigh NC.
4. Hauser, CJ. "Crush Injury: Mitochondria Matter". Society of Crit. Care Medicine. Honolulu, HI, 2017
5. Hauser, CJ. "Ownership in Trauma Care." Western Trauma Association 2017, Snowbird, UT
6. Hauser, CJ. "The Role of Danger Molecules in Susceptibility to infection: Therapeutic Opportunities." University of Strasbourg, 2017, Strasbourg, FR.
7. Hauser, CJ. "DAMPs, PAMPs and why they matter in Critical Abdominal Illness." World Society of the Abdominal Compartment Syndrome, 2017, Banff, Alberta.
8. Otterbein, LE; The social network of carbon monoxide. Gasotransmitter Conference; 2017, Atlanta, Georgia.
9. Otterbein, LE; Heme oxygenase-1 and innate defense. Agios Pharma; 2017, Cambridge, MA
10. Seshadri, A; Altered monocyte and NK cell phenotypes correlate with post-trauma infection, presented at American Association for the Surgery of Trauma, 2017, Baltimore, MD
11. Lederer, JA; Immunological consequences of Trauma, presented at Brigham and Women's Hospital, 2017, Boston, MA
12. Lederer, JA; CyTOF as an approach for deconvolution of immune cell phenotypes in the blood. Presented at Federation of Clinical Immunology Societies, 2017, Chicago, IL.
13. Barrett, Chris; Human Neutrophil Elastase Mediates Fibrinolysis Shutdown Through Competitive Degradation of Plasminogen and Generation of Angiostatin. Presented at Western Trauma Association 2017, Snowbird, UT.

Website: in development expected early 2018

Inventions, patent applications, and/or licenses
Nothing to report.

Other Products
We have established a leukocyte and serum repository that allows us to cold-store live PMN and PBMC isolated from patients and then reanimate them in cohorts for in-depth study. This is a highly unique methodology that allows us to study cohorts of patient samples simultaneously. We are using REDCap Research Electronic Data Capture) as a secure online data management software program to track patient clinical data to build and manage our participant enrollment/clinical data, sample processing, and biobanking database.

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 nosocomial pneumonia in addition to establishment of human PMN isolation methods for the entire groups. Day to day supervising roles on Dr. W-Y Kwon and Dr. Li Chen.
Funding Support: W81XWH-16-1-0464

Name: Woon Yong Kwon, MD, PhD.
Project Role: Research fellow
Researcher Identifier (e.g. ORCID ID): 0000-0002-3343-5030
Nearest person month worked: 10
Contribution to Project: Dr. Kwon is focused on the role of mtFPs in the chemotaxis exposed to mtDAMPs.
Funding Support: Seoul National University

Name: Li Chen, MD
Project Role: Research Fellow
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 10
Contribution to Project: Dr. Chen is working on Project 1 focused on neutrophil functions such as calcium mobilization, chemotaxis and response to bacterial infection after trauma.
Funding Support: China Scholarship Council: 201603170008

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

Name: Ghee Lee, BS
Project Role: Researcher: Masters Student at Harvard Medical School
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: Mr. Gallo is working on Project 2 focused on *in vivo* model characterization.
Funding Support: W81XWH-16-1-0464 & NIDDK R44DK111260-01

Project 3

Name: Simon Robson
Project Role: Co-Investigator
Researcher Identifier (e.g. ORCID ID): 0000-0001-6374-0194
Nearest person month worked: 1
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, 5P01 HL107152-07, 1R01DK108894-01A1, 5R01DK103723-03, 1R01AI132389-1, Tizona, 5R01DK104714-02

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: 4
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

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

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:	Tereza Pinkhasova
Project Role:	Clinical Research Assistant
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	Ms. Pinkhasova is working on Project 4, identifying and recruiting patients and collecting samples.
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: 10
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. Laura worked with Dr. Seshadri on the human trauma CyTOF studies and will continue working with HALO on that aspect of the project.
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 studies for CpG-ODN using human blood immune cells.
Funding Support: W81XWH-16-1-0464

Name: Yasutaka Nakahori, MD
Project Role: Research Fellow
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 4
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 a focused on the lung infection studies in our mouse burn trauma model and helps with trauma blood immunophenotyping.
Funding Support: W81XWH-16-1-046 & Osaka University General Hospital

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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:	Ingred 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:	4
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?

No change

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS QUAD CHART

See quad chart attached.

9. APPENDICES

Nothing to report.

DAMP-Mediated Innate Immune Failure After Trauma

Log# PR151953, Focused Program Award
W81XWH-16-1-0464

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

Study Aims

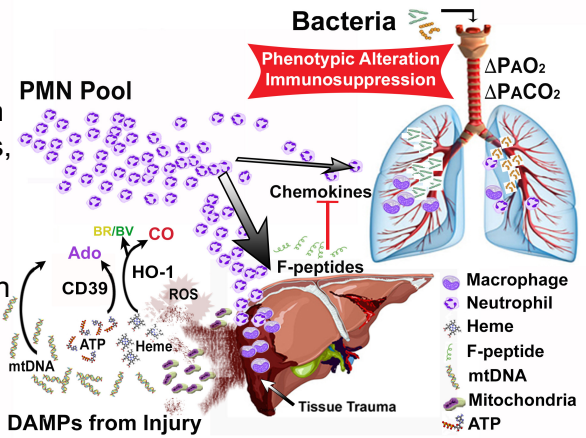
This research program seeks to identify cellular and molecular mechanisms by which wounds predispose to infection. We are focused on three primary objectives: **prevention**, **diagnosis** and **treatment** of the wounded warrior.

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 31, 2017)

Goals/Milestones

CY1: Initiate project start, obtain IACUC and IRB protocol approvals
☐Onboard personnel, establish biweekly meetings, finalize SOPs
CY2: Manuscript submission, CyTOF analyses of blood and BAL
☐Acquire human and mouse descriptive and phenotypic data sets
CY3: Establish specific DAMPs that interfere with innate immunity
☐Validate and characterize the identified DAMP(s) in model systems
CY4: Interfere with DAMP effects and restore innate immune responses.
☐Perform rescue experiments in animals and cell models.
CY5: Identify methodologies to diminish infectious morbidities of injury with new innovative treatment options for warfighters after trauma.
☐Enact a systems and computational biology approach to influence standard of care.

Comments: All projects fully operational. A revised plan for patient enrollment has been instituted to rectify with an alternative approach.
Budget Expenditure to date: $1,475,812.68 TC, All Projects.