

AWARD NUMBER: W81XWH-17-1-0058

TITLE: Manipulating the Macrophage Iron-Hepcidin Axis to Prevent Acute Lung Injury/Acute Respiratory Distress Syndrome Secondary to Severe Trauma and Hemorrhagic Shock

PRINCIPAL INVESTIGATOR: Aranya Bagchi, M.B.,B.S.

CONTRACTING ORGANIZATION: Massachusetts General Hospital

REPORT DATE: Sept 2019

TYPE OF REPORT: Final Report

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE			<i>Form Approved</i> <i>OMB No. 0704-0188</i>		
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE Sept 2019		2. REPORT TYPE Final Report		3. DATES COVERED 05/15/2017 - 05/14/2019	
4. TITLE AND SUBTITLE Manipulating the Macrophage Iron-Hepcidin Axis to Prevent Acute Lung Injury/Acute Respiratory Distress Syndrome Secondary to Severe Trauma and Hemorrhagic Shock			5a. CONTRACT NUMBER		
			5b. GRANT NUMBER W81XWH-17-10058		
			5c. PROGRAM ELEMENT NUMBER		
6. AUTHOR(S) Aranya Bagchi E-Mail: abagchi@mgh.harvard.edu			5d. PROJECT NUMBER		
			5e. TASK NUMBER		
			5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Massachusetts General Hospital Boston, MA 02114			8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Development Command Fort Detrick, Maryland 21702-5012			10. SPONSOR/MONITOR'S ACRONYM(S)		
			11. SPONSOR/MONITOR'S REPORT NUMBER(S)		
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Our hypothesis at the time of initiation of this project was that severe traumatic injury serves as a 'priming' insult, causing sequestration of iron into macrophages by upregulating hepcidin, the principal iron-regulating hormone. We therefore expected to see an exaggerated inflammatory response to our 'second hit' stimulus after trauma, intratracheal lipopolysachharide (LPS). To our surprise, the acute inflammatory response after intratracheal LPS was highly diminished compared to the response to intratracheal LPS alone. To determine the reason for the observed immune suppression we performed a hypothesis-neutral screen of the pulmonary transcriptome in our experimental groups using RNA sequencing. Analysis of the transcriptome led to the identification of Peroxisome-Proliferator Activated Receptor (PPAR)-gamma, a ligand induced transcription factor, that has well documented immunosuppressive effects. Our results suggest that severe trauma can invoke an early, specific induction of PPAR-gamma that dramatically suppresses the inflammatory response to a subsequent stimulus. Further study is required to determine whether pharmacologic modulation of PPAR-gamma signaling will be beneficial after acute trauma.					
15. SUBJECT TERMS Trauma, hemorrhage, acute lung injury, inflammation, immune suppression, Peroxisome-Proliferator Activated Receptor (PPAR)-gamma					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT Unclassified	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified			19b. TELEPHONE NUMBER (include area code)

TABLE OF CONTENTS

	<u>Page</u>
1. Introduction.....	4
2. Keywords.....	5
3. Accomplishments.....	6
4. Impact.....	15
5. Changes/Problems.....	15
6. Products.....	16
7. Participants & Other Collaborating Organizations.....	17
8. Special Reporting Requirements.....	17
9. Appendices.....	NA

INTRODUCTION

Trauma and hemorrhagic shock are the most common causes of mortality in adults under 45 years of age. Approximately 10-30% of patients with severe trauma/HS develop acute respiratory distress syndrome (ARDS), which can significantly increase mortality, morbidity and healthcare costs. A 'two-hit' model has been proposed to explain the pathophysiology of ARDS following trauma/HS – the traumatic injury acting to 'prime' the body for a subsequent infectious or inflammatory insult (the second hit) that results in ARDS. However, the immunologic basis for such a model has not been well studied. Our research group, and others, have shown that an acute inflammatory stimulus induces upregulation of hepcidin, the master iron regulatory hormone. We have also shown that hepcidin induces iron sequestration in macrophages, and that iron-loaded macrophages have a more robust inflammatory response to a stimulus such as lipopolysaccharide (LPS) compared to iron depleted macrophages. We therefore hypothesized that trauma causes hepcidin upregulation, which in turn induces iron sequestration in monocytes and macrophages, including pulmonary alveolar macrophages. These 'primed' macrophages would be expected to respond to a 'second hit' like intratracheal LPS more robustly, exacerbating lung injury. Consequently, we proposed targeting the hepcidin signaling pathway to modulate inflammation caused by a 'second hit' after an exposure to trauma. Our mouse model used two sequential injuries – first, hemorrhagic shock with cecectomy (polytrauma) and second, exposure to low dose intratracheal LPS, spaced twenty-four hours apart. To our surprise, our initial experiments showed that mice exposed to LPS after polytrauma had a substantially diminished inflammatory response compared to the appropriate controls (mice exposed to sham surgery followed by intratracheal LPS). Given this unexpected finding of trauma-induced anti-inflammatory response, we decided to conduct an unbiased, or hypothesis-neutral, screen to determine the cause of the observed immune suppression. Our experiments have provided, for the first time, strong evidence that exposure to trauma can cause immune suppression acutely (other groups have established the presence of delayed immune suppression after trauma). Our data also suggests a potential mechanism for the acute immune suppressive response – the activation of the Peroxisome-Proliferator Activated Receptor (PPAR) γ pathway. Given the availability of pharmacologic modulators of PPAR- γ signaling, our findings establish the groundwork for manipulation of PPAR- γ signaling in acute trauma.

KEYWORDS

Trauma

Hemorrhage

Inflammation

Acute Respiratory Distress Syndrome (ARDS)

Immune suppression

Transcriptomics

Peroxisome-Proliferator Associated Receptor

ACCOMPLISHMENTS

A. Major Goals of the Project during the reporting period:

Major Task 1: Characterize an in-vivo model of Trauma/Hemorrhagic Shock (HS) with secondary Lipopolysaccharide-induced Acute Lung Injury (ALI) – **Completed**.

Major Task 2: Compare the severity of ALI in a Trauma/HS + ALI model vs. sham surgery + ALI using (a) Transcriptome analysis using RNA sequencing – **Completed**. (b) Functional imaging using FDG-PET-CT scanning – not performed (please see **section F** for details)

Major Task 3: Characterize the efficacy of inhaled and IV iron chelators (Deferoxamine) in altering the severity of secondary ALI in mice – Not performed (please see **section F** for details)

B. METHODS:

1. Establishment of an in vivo model of Trauma-Hemorrhagic shock (polytrauma, PT) with secondary Lipopolysaccharide (LPS)-induced Acute Lung Injury (ALI):

- a. 6-8-week old C57Bl/6 mice of either sex were used to develop the model. As mentioned in the Annual report (June 2018), mice were anesthetized using isoflurane and maintained under anesthesia using isoflurane via a nose cone. Both femoral arteries were exposed and cannulated using PE-10 tubing. One artery was used for blood pressure monitoring (DigiMed; Micro-Med, Inc., Louisville, KY). The other was used for inducing hemorrhage and crystalloid resuscitation. The mice were bled to a mean arterial pressure (MAP) of 30 mm Hg (+/- 5 mm Hg) and maintained at that blood pressure for 60 minutes. Following the period of hemorrhage, the animals were resuscitated by the infusion normal saline at four times the volume of the shed blood. Following clinical stability after resuscitation, a 1-cm laparotomy was performed, and the cecum identified. The base of the cecum was ligated using 3-0 silk sutures, and the cecum resected. The laparotomy was then closed. The femoral arterial cannulas were removed, and arteries ligated. The mice were allowed to recover under close observation, and then transferred to a post-procedure facility. Control mice underwent anesthesia and skin incisions only (sham surgery group).
- b. Twenty-four hours after surgery, the mice were administered intratracheal LPS (5 mg/kg). Mice were anesthetized using isoflurane, and then suspended vertically with their mouth open. Their nares were gently occluded using forceps and the calculated volume of LPS dissolved in saline was deposited in their hypopharynx, which they aspirated while mouth-breathing. Control mice were administered an equivalent volume of normal saline.
- c. Twenty-four hours after LPS exposure mice were euthanized. The pulmonary circulation was flushed with Phosphate Buffered Saline (PBS) and the lungs were excised, and flash frozen in liquid nitrogen. Sections of the liver and spleen were frozen as well. Four groups of animals were studied: (1) Sham surgery/Intratracheal saline, (2) Sham surgery/Intratracheal LPS, (3) Polytrauma/Intratracheal saline, and (4) Polytrauma/Intratracheal LPS.

2. Immune response to Polytrauma with secondary ALI:

The immune response to polytrauma was assessed using RT-qPCR. Briefly, total RNA was extracted from lung tissue using TRIzol (Invitrogen, ThermoFisher Scientific). Reverse RNA transcription was accomplished using Moloney murine leukemia virus RT (Promega). Quantitative RT-PCR was performed using Applied Biosystems TaqMan master mix (ThermoFisher Scientific)

and an Eppendorf MasterCycler RealPlex2 (ThermoFisher Scientific). The level of target transcripts was normalized to the level of 18S rRNA using the relative CT method. TaqMan primer/probe sets were purchased from ThermoFisher Scientific.

3. RNA Sequencing of the lung transcriptome:

Total RNA was submitted to the Partners Translational Genomics Core Facility. The quantity and quality of the RNA was checked using an Agilent Bioanalyzer 2100 (Agilent Technologies) and Nanodrop ND-1000 spectrophotometer (Infinigen Biotechnology). Samples with an RNA Integrity Number >7 were used for library construction. RNA Sequencing was conducted using an Illumina HiSeq 2500 Sequencer. Sequences were aligned to a reference genome with TopHat, transcript abundance estimated with Cufflinks, and differential expression analysis performed with *Cuffdiff* and *CummeRbund*.

4. Ingenuity Pathway Analysis:

Output from *Cuffdiff* files was uploaded into the Ingenuity Pathway Analysis database (Qiagen) for core analysis and then overlaid with the global molecular network in the Ingenuity pathway knowledge base (IPKB). IPA was performed to identify canonical pathways and to categorize differentially expressed genes in specific diseases and functions. Heatmap and hierarchical cluster analysis were used to demonstrate the expression patterns of these differentially expressed genes. Volcano plots were constructed using 'R' statistical software environment.

5. Statistics:

P values were corrected with the Benjamini-Hochberg algorithm (false discovery rate; FDR). Genes were considered as differentially expressed if the adjusted P value was < 0.05 and absolute fold-change was ≥ 2 (Log_2 fold-change ≥ 1). Significance testing with qPCR analyses were performed using the Student's T test for experiments with two groups, and One-way ANOVA with Tukey's *post hoc* testing for experiments with 3 or 4 groups. Statistics were performed with the Graph Pad v8 software package.

C. RESULTS:

1. Polytrauma induces an inflammatory reaction at 6h, but the pro-inflammatory response returns to baseline by 24h. Polytrauma induces a robust early pro-inflammatory response. Six hours after exposure to polytrauma, lungs of mice show an increased expression of pro-inflammatory cytokines such as IL6, TNF α , and IL-1 β (**Fig 1, A-C**). The responses observed in mouse lungs reflect a systemic inflammatory response, because similar increases in inflammatory cytokines were observed in mouse livers (**Fig 1, D-F**). Our model of polytrauma was therefore successful in consistently inducing a strong pro-inflammatory response. However, this response appears to be relatively short-lived, as 24 h after polytrauma the pro-inflammatory response is no longer significantly different from that in animals subjected to sham surgery (data not shown). These data suggest that our model of polytrauma produces an early, but unsustained, pro-inflammatory response.

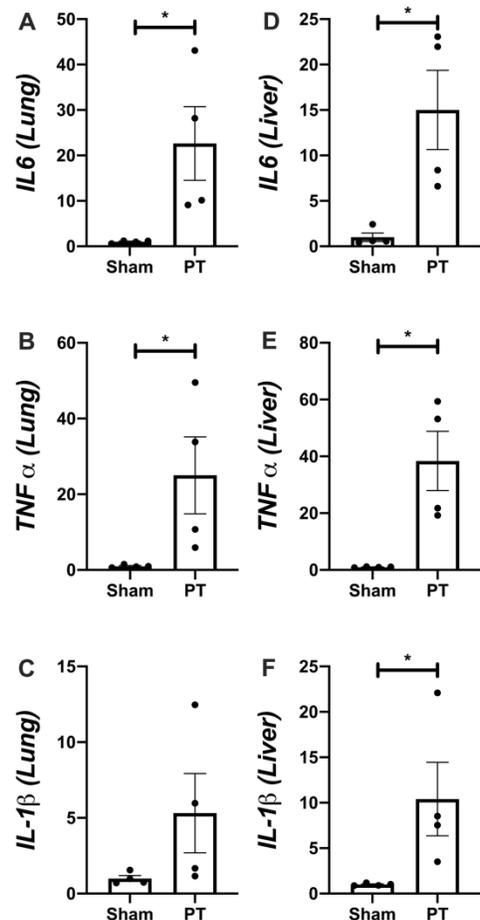


Figure 1: Early inflammatory response to polytrauma (PT) in lungs (A-C) and liver (D-E) 6 h after injury. $P < 0.05$ in all figures except C, where $p = 0.0567$ (t test)

2. Polytrauma has a strong **suppressive** effect on the inflammatory response to subsequent lung injury with lipopolysaccharide (LPS).

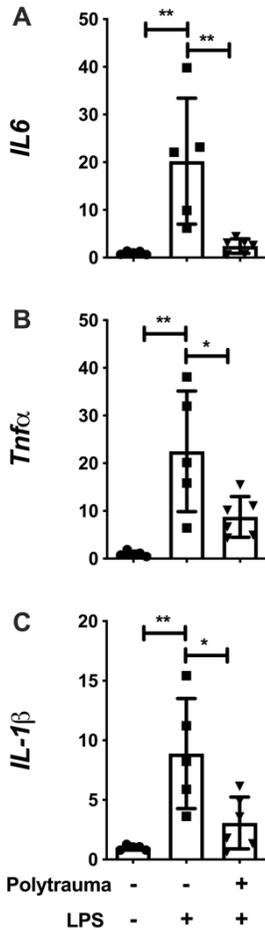


Figure 2: Exposure to polytrauma diminishes the inflammatory response to a subsequent LPS challenge (** $p < 0.01$; * $p < 0.05$, One-way ANOVA)

Our hypothesis at the time of initiating this study was that polytrauma would potentiate the inflammatory response to subsequent LPS-induced lung injury. To our surprise, we found that exposure to polytrauma 24h before intratracheal instillation of LPS severely blunted the pulmonary inflammatory response to LPS, compared to animals exposed to sham surgery followed by LPS (**Fig 2, A-C**). Since our initial hypothesis invoked an upregulation of hepcidin, the master iron regulator, following trauma as a mechanism for priming pulmonary alveolar macrophages and monocytes, we examined the expression of Hepcidin and the iron receptor protein Transferrin 1 in mouse lungs following Polytrauma. Unfortunately, in our hands **polytrauma did not induce expression of Hepcidin or Transferrin 1 – their expression was no different compared to baseline** (data not shown). To explain the unexpected immune suppression following trauma, we considered three possibilities: (1) a global suppression of gene expression following trauma at 48 h (when gene expression shown in Fig 2 was analyzed), (2) polytrauma induced a gene expression very similar to LPS exposure, resulting in a phenomenon similar to endotoxin tolerance, an LPS specific immune suppression, and (3) activation of a specific anti-inflammatory program that caused suppression of the innate immune response to a subsequent exposure to LPS. Rather than focus on preselected genes and test these hypotheses individually, we decided to proceed in a hypothesis-neutral fashion, comparing the pulmonary gene expression in four groups of mice mentioned in the methods section (**B.1.c**): (1) Sham surgery/Intratracheal saline, (2) Sham surgery/Intratracheal LPS (3) Polytrauma/Intratracheal saline, and (4) Polytrauma/Intratracheal LPS.

3. Sequential exposure to Polytrauma and Intratracheal LPS results in differing patterns of gene expression across the pulmonary transcriptome.

RNA prepared from the lungs of the four groups of mice

($n=4$ /group) were analyzed by RNA Sequencing as described in the methods section. All samples except for one (a sample from a Polytrauma/Intratracheal saline treated mouse) passed quality control measures. Our final analysis was conducted on 15 samples ($n=3$ for the Polytrauma/Intratracheal saline group, and $n=4$ for other groups). We used three methods to test whether the sequential exposure to Polytrauma and LPS resulted in differential expression of genes between the groups. In **Figure 3**, we show heatmaps demonstrating patterns of gene expression between all six possible comparisons

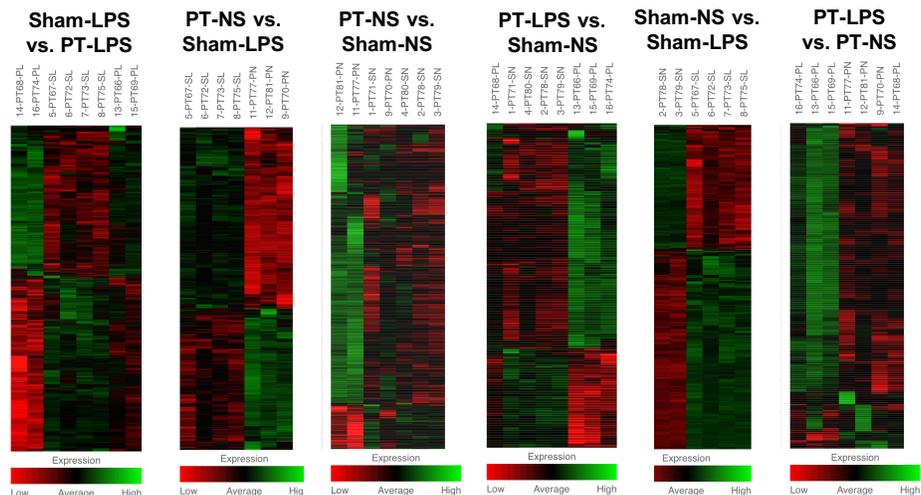


Figure 3: Heatmaps comparing Differential Gene Expression profiles across all six possible comparisons of the four experimental groups. Green denotes overexpression, red denotes reduced expression and black similar expression profiles.

we show heatmaps demonstrating patterns of gene expression between all six possible comparisons

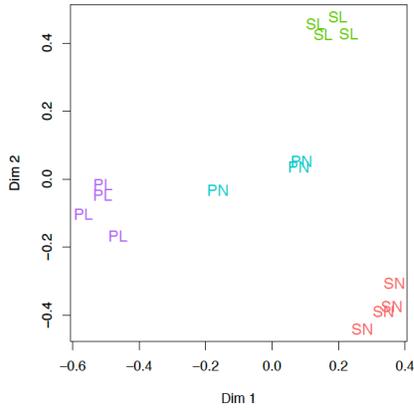


Figure 4: Multidimensional scaling showing the segregation of the individual samples and groups based on gene expression patterns. (SN- Sham surgery/ Intratracheal saline, SL-Sham surgery/Intratracheal LPS, PN-Polytrauma/Intratracheal saline, and PL-Polytrauma /Intratracheal LPS)

gene expression profiles induced by Polytrauma/Intratracheal saline and Sham surgery/Intratracheal LPS are sufficiently distinct (see the second heatmap in **Fig 3** and the middle volcano plot in **Fig 5**) that the possibility of trauma inducing an endotoxin-tolerant phenotype by means of replicating the gene expression pattern triggered by LPS appears remote.

between the four experimental groups. The heat maps confirm that patterns of gene over/under-expression were different across all intergroup comparisons, suggesting each of the four treatment groups elicited a specific gene expression profile across the transcriptome. To further compare similarities and differences in gene expression patterns across the different groups, we used **Multidimensional Scaling** to define the 'distance' between the samples scaled down to two dimensions. As seen in **Figure 4**, the four groups segregate into clearly different clusters on a two-dimensional space, confirming that (a) the individual samples within a group have a similar pattern of gene expression, and (b) the groups themselves have distinct gene expression profiles. Finally, we used volcano plots to visually demonstrate the distribution of over-and under-expressed genes in comparisons between the different groups. **Figure 5** shows representative volcano plots of three of six possible comparisons. The volcano plots confirm that comparison between each experimental group produces a set of genes that are differentially expressed between the groups. Taken together, the data shown here compellingly refute two of the three possibilities presented in section C.2. There is no evidence of global suppression of gene expression induced by polytrauma. The

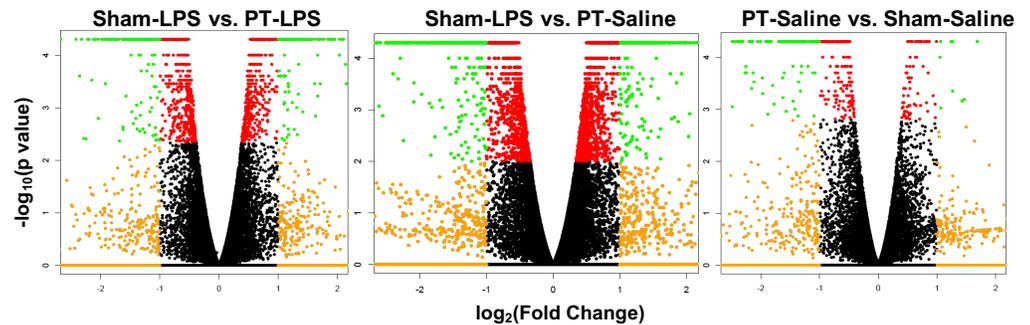


Figure 5: Representative volcano plots illustrating differences in gene expression between experimental groups. Transcripts not showing significant differences in fold expression and statistical significance are represented in black. Red is used to show transcripts that are statistically significant but are expressed at levels < 2-fold change (Log_2). Orange represents transcripts that are differentially expressed, but do not reach statistical significance, and green shows transcripts that are both differentially expressed and statistically significant.

4. Differentially expressed genes detected by RNA Sequencing were confirmed by RT-qPCR testing.

Validation of differential gene expression detected by RNA Sequencing between different groups was carried out using RT-qPCR with Taqman primer/probe sets. As shown in **Table 1**, six representative genes (3 upregulated and 3 downregulated) from the comparison of the Sham surgery/Intratracheal LPS vs. Polytrauma/Intratracheal LPS groups shows differential expression by RT-qPCR closely following differential expression estimated by RNA-Seq. Genes across other comparison groups were validated in a similar

Gene	Log_2 fold change (RNA-Seq)	Fold Change (RNA-Seq)	Fold Change (qPCR)
DOWNREGULATED GENES			
<i>IL6</i>	-2.98	0.13	0.13
<i>CXCL10</i>	-2.76	0.15	0.004
<i>SOCS1</i>	-1.76	0.25	0.39
UPREGULATED GENES			
<i>ALAS2</i>	+2.19	4.55	4.07
<i>Hbb1</i>	+1.85	3.6	3.4
<i>CD163</i>	+1.48	2.75	3.38

Table 1. A sample of upregulated and downregulated genes in comparing mice treated with Sham surgery/Intratracheal LPS and Polytrauma/Intratracheal LPS. Note that all downregulated genes shown (*IL6*, *CXCL10* and *SOCS1*) are innate immunity-related genes. Fold change values by RNA Seq are similar to those seen using RT-qPCR.

As shown in **Table 1**, six representative genes (3 upregulated and 3 downregulated) from the comparison of the Sham surgery/Intratracheal LPS vs. Polytrauma/Intratracheal LPS groups shows differential expression by RT-qPCR closely following differential expression estimated by RNA-Seq. Genes across other comparison groups were validated in a similar

fashion (data not shown). These data demonstrate that RNA sequencing was accurately able to identify differentially expressed genes across the pulmonary transcriptome.

5. *Ingenuity Pathway Analysis of gene expression across the different experimental groups shows evidence of systematic suppression of innate immune signaling pathways in mice exposed to Polytrauma.*

To begin to identify common themes across the pulmonary transcriptome in six pairs of differential gene expression analyses, we submitted the differential gene expression data from the RNA Sequencing experiments to IPA core analysis. The results were then overlaid into the manually curated dataset of Ingenuity Pathways Knowledge Base to provide a set of pathways that were predicted to be upregulated or downregulated in a given set of conditions. Finally, a comparison analysis was performed where pathways predicted to be altered were shown in a grid according to the statistical strength of the prediction using activation z-scores. The results of the comparison analysis showing the top 15 canonical pathways altered between experimental groups is shown in **Figure 6**. These data show that when the sham-surgery LPS group is compared to Polytrauma-exposed mice (either Polytrauma/Intratracheal LPS or Polytrauma/Intratracheal saline) there is a systematic downregulation of pathways associated with acute inflammation (see the first two comparisons in **Fig 6**). For example, signaling pathways including Dendritic Cell Maturation, HMGB1 signaling, IL6 signaling and Pattern Recognition Receptors in Recognizing Bacteria and Viruses are all

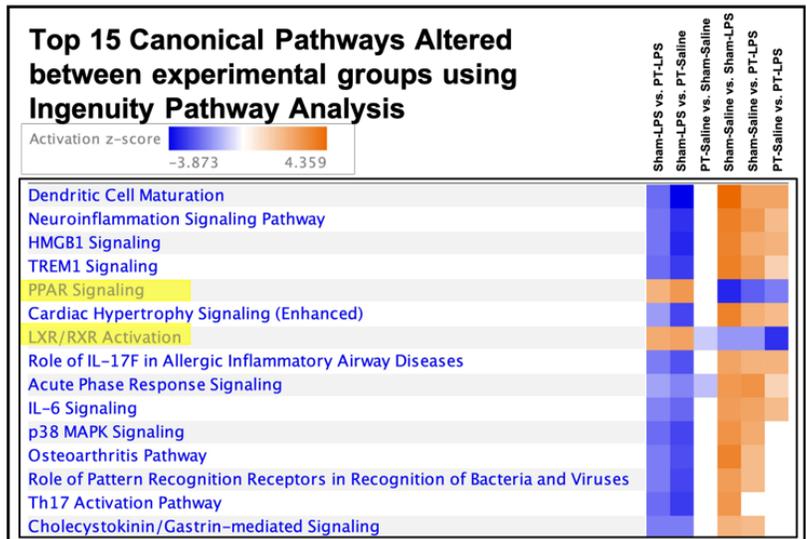


Figure 6: IPA results comparing the top 15 canonical signaling pathways altered between experimental groups. Orange indicates upregulation, blue denotes downregulation. A reciprocal relationship exists between pathways associated with acute inflammation, and pathways associated with PPAR and RXR/LXR signaling, highlighted in yellow (PPAR – Peroxisome-Proliferator Associated Receptor; LXR/RXR – Liver X Receptor/Retinoid X Receptor).

downregulated between experimental groups is shown in **Figure 6**. These data show that when the sham-surgery LPS group is compared to Polytrauma-exposed mice (either Polytrauma/Intratracheal LPS or Polytrauma/Intratracheal saline) there is a systematic downregulation of pathways associated with acute inflammation (see the first two comparisons in **Fig 6**). For example, signaling pathways including Dendritic Cell Maturation, HMGB1 signaling, IL6 signaling and Pattern Recognition Receptors in Recognizing Bacteria and Viruses are all

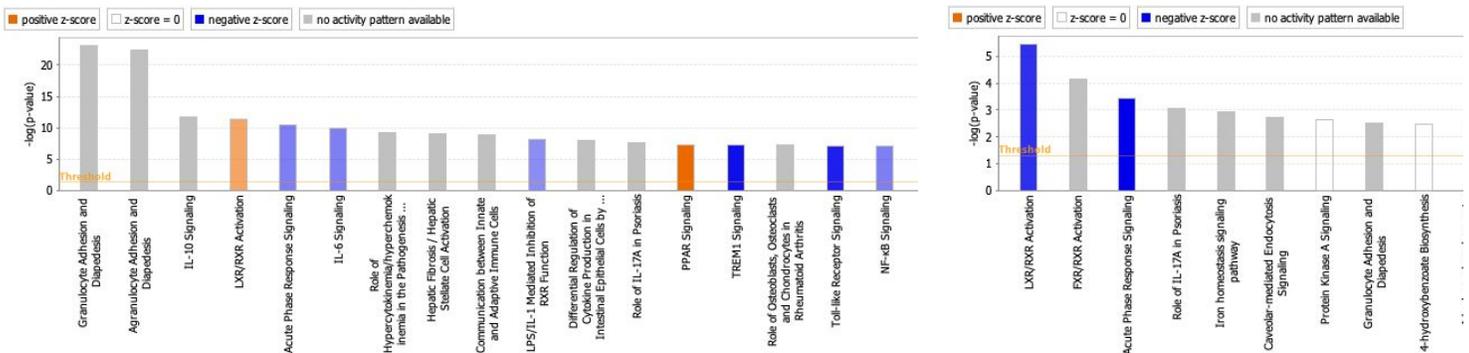


Figure 7: Canonical Pathway Analysis in IPA showing predicted up/downregulated pathways between Sham surgery/Intratracheal LPS and Polytrauma/Intratracheal LPS (Left) and Polytrauma/Intratracheal saline and Sham surgery/Intratracheal saline (Right). Pathways predicted to be upregulated are shown in orange, while those predicted to be downregulated are shown in blue. White bars denote no predicted differences in pathway activity between groups, and Grey bars represents pathways for which predictions cannot be made based on existing data.

downregulated. Interestingly, only two pathways are shown to be strongly upregulated - Peroxisome-Proliferator Associated Receptor (PPAR) signaling and Liver X Receptor/Retinoid X Receptor (LXR/RXR) signaling. **Figure 7** shows the results of IPA Canonical Pathway Analysis between the sham surgery/Intratracheal LPS and Polytrauma/Intratracheal LPS (on the left) and Polytrauma/Intratracheal saline and sham surgery/intratracheal saline (on the right). **Figure 7** again demonstrates that PPAR, and LXR/RXR signaling pathways are upregulated in mice exposed to Polytrauma prior to LPS. Conversely, LXR/RXR signaling is downregulated in Sham

surgery/Intratracheal saline mice compared to Polytrauma/Intratracheal saline mice. In other words, LXR/RXR signaling is upregulated in Polytrauma/Intratracheal saline mice compared to Sham surgery/Intratracheal Saline mice. These data suggest that exposure to polytrauma induces a systematic suppression of proinflammatory genes while upregulating specific pathways (PPAR and LXR/RXR signaling).

6. Validation of predicted upregulation of PPAR and LXR/RXR signaling pathways after exposure to Polytrauma.

Analysis of the RNA Sequencing data suggests that Polytrauma upregulates PPAR and LXR/RXR signaling while suppressing pro-inflammatory signaling. While these findings were generated in an unbiased, hypothesis-neutral fashion, a literature search showed that prior research provides an interesting biologic rationale for our findings. PPAR refers to a group of ligand-inducible transcription factors that have pleiotropic effects. PPAR proteins include PPAR- α , PPAR- β/δ , and PPAR- γ (Ahmadian M, et al. *Nature Medicine* 2019;19:557-566). Of these, PPAR- γ is strongly expressed in macrophages and monocytes and has a potent immunoregulatory effect by downregulating many components of the pro-inflammatory response, including downregulating NF- κ B, STAT and cyclo-oxygenase signaling (Chawla A, et al. *Nature Medicine* 2001;7:48-52, Odegaard JI, et al. *Nature* 2007;447: 1116-1120). In addition, PPAR- γ forms obligate heterodimers with RXR-b (Ahmadian M, et al. *Nature Medicine* 2019;19:557-566), explaining why PPAR and RXR signaling were shown to be upregulated in our analysis. To examine upregulation of mRNA associated with PPAR/RXR signaling, we tested gene expression of components of this pathway using RT-qPCR. The results of our experiments are shown in Figures 8 and 9. Figure 8 shows IL6, PPAR- γ and CD163 gene expression in mice subjected to the four experimental conditions. It is

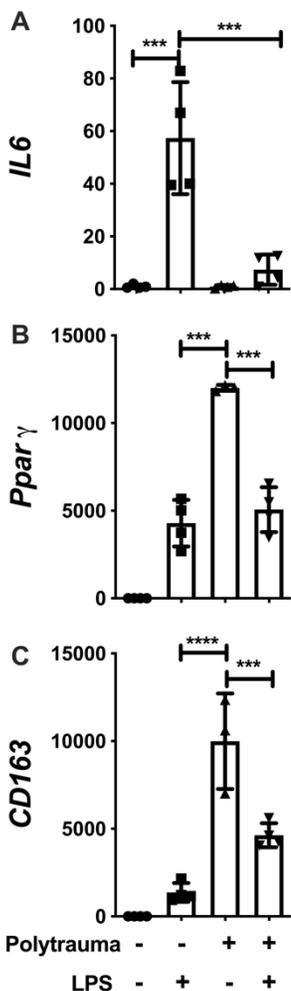


Figure 8: Polytrauma induces immune suppression as shown by reduced IL6 gene expression (A) while increasing expression of genes associated with immune suppression such as PPAR-g and CD163 (B, C). ****p<0.0001, ***p<0.001 (One-way ANOVA with Tukey's post hoc test)

evident that IL6 expression is greatly diminished in the Polytrauma/Intratracheal LPS group compared to Sham surgery/Intratracheal LPS (Fig 8A). Interestingly, PPAR- γ signaling is strongly upregulated in the Polytrauma/Intratracheal saline group compared to others (Fig 8B). CD163, a macrophage specific receptor (Etzerodt A and Moestrup SK. *Antioxidants and Redox Signaling* 2013;18:2352-2363) is also strongly upregulated in the Polytrauma/Intratracheal saline exposed mice and is significantly upregulated in Polytrauma/Intratracheal LPS treated mice compared to Sham surgery/Intratracheal LPS mice (Fig 8C). In addition, LXR-b, RXR-b expression is increased in mice following Polytrauma/Intratracheal saline as well (Fig 9 A, B). Of note, gene expression of PPAR- β/δ and RXR-a was not significantly different between groups (data not shown). Gene expression of PPAR- α followed a similar pattern as seen in PPAR- γ , although to a lesser degree. Finally, gene expression of NRF2, another transcription factor associated with PPAR/RXR signaling (Cho RL, et

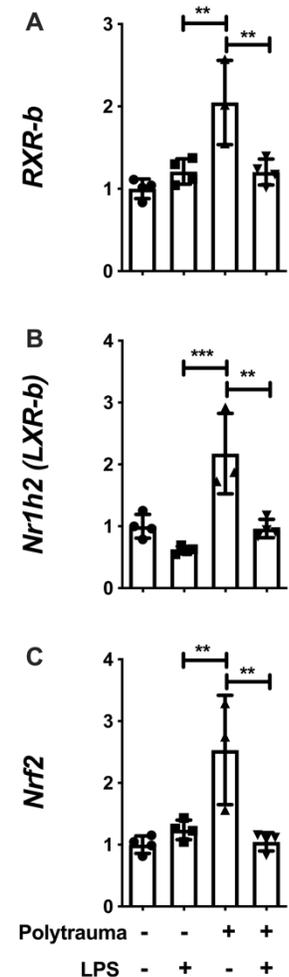


Figure 9: Polytrauma induces LXR/RXR signaling (A, B) and upregulates NRF2 gene expression (C). ***p<0.001, **p<0.01 (One-way ANOVA with Tukey's post hoc test)

al. *British Journal of Pharmacology* 2018;175:3928-3946) was also increased following Polytrauma (Fig 9C).

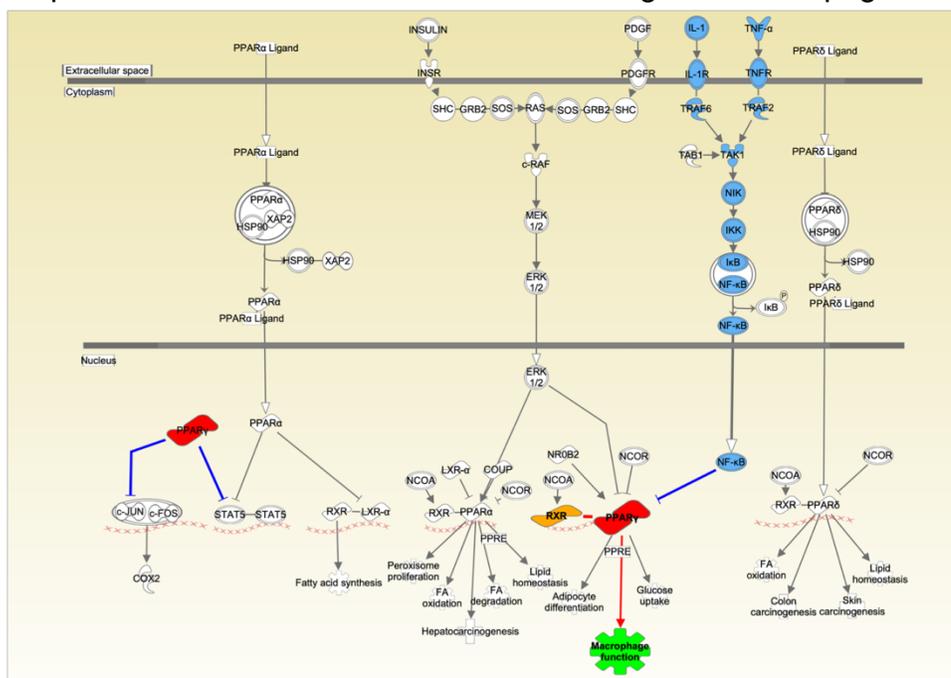
D. DISCUSSION

Major trauma is the commonest cause of death in adults under 45 (Anderson RN and Smith BL. *National Vital Statistics Report* 2005;53:1-89). Complications such as acute lung injury complicate the course of between 10 – 30% of patients with severe trauma, resulting in worse outcomes (Hudson LD, et al. *American Journal of Respiratory and Critical Care Medicine* 1995;151:293-301). The mechanism(s) through which sequential insults modulate the immune system are far from clear. One model suggests that the original trauma acts as a priming stimulus, exaggerating the response to a subsequent inflammatory stimulus, resulting in more extensive organ dysfunction. Other models have focused on the delayed immune response to major trauma. Multiple investigators have described a phenotype of depressed immune responsiveness in models of chronic trauma – often defined as PICS (Persistent Inflammation-Immunosuppressive Catabolism Syndrome) (Huber-Lang M, et al. *Nature Immunology* 2018;19:327-341). Our study contributes to this body of information in several ways:

- (1) We have described a well-defined model of sequential trauma and lung injury in mice
- (2) We have shown an acute immunosuppressive phenotype in this model, differing from prior studies that have found immune suppression in chronic models of injury
- (3) Using a hypothesis-neutral transcriptomics approach we have conducted a detailed analysis of the pulmonary transcriptome and arrived at a possible mechanistic explanation for the observed immune suppression, i.e. activation of PPAR- γ signaling in polytrauma.

1. Relevance of PPAR- γ signaling in acute inflammatory states.

Figure 10 presents an overview of PPAR- γ signaling. PPAR- γ is a member of the superfamily of ligand-inducible transcription factors. While PPAR- γ is most highly expressed in white and brown adipose tissue and is considered a master regulator of adipogenesis and whole-body lipid



metabolism, studies have also revealed an abundant distribution of PPAR- γ in monocytes and macrophages (Odegaard JI, et al. *Nature* 2007;447: 1116-1120). Multiple studies have established that PPAR- γ , together with the RXR receptor with which it forms an obligatory heterodimer has very potent anti-inflammatory effects by inhibiting a range of pro-inflammatory transcription factors, including NF- κ B, STAT1, and inhibition of Arachidonic Acid metabolite (e.g. prostaglandin) generation (Duan SZ, et al. *Circulation Research* 2008;102:283-294). Since pharmacologic and dietary modulators of PPAR signaling

Figure 10: An overview of the PPAR signaling pathway (created using the IPA pathways tool) showing the reciprocal relationship between NF- κ B signaling pathway (highlighted in blue) and PPAR- γ signaling, the relationship between RXR and PPAR- γ , and the influence of PPAR- γ signaling on macrophage function.

are available, multiple studies have examined the utility of using PPAR ligands to inhibit acute inflammation in models of LPS induced alveolar epithelial injury, in models of septic shock and

models of central nervous system injury. The discovery that our model of polytrauma results in the potent induction of PPAR- γ therefore fits into the framework of PPAR signaling as an endogenous anti-inflammatory mechanism.

2. PPAR- γ -induced induction of M2 macrophages in the lungs may provide a mechanistic explanation for the polytrauma induced immunosuppression.

Our data shows both the strong upregulation of PPAR- γ and CD163 in mouse lung mRNA (**Fig 8B and C**). CD163 is a monocyte-macrophage specific receptor that acts as a haptoglobin-free hemoglobin complex receptor. CD163 is also a well-documented marker of alternate macrophage activation (M2) phenotype. M2 macrophages are anti-inflammatory macrophages associated with tissue regeneration and repair, providing a teleologic explanation for their expression as a medium for promoting healing after trauma. The fact that our data shows a dramatic increase in CD163

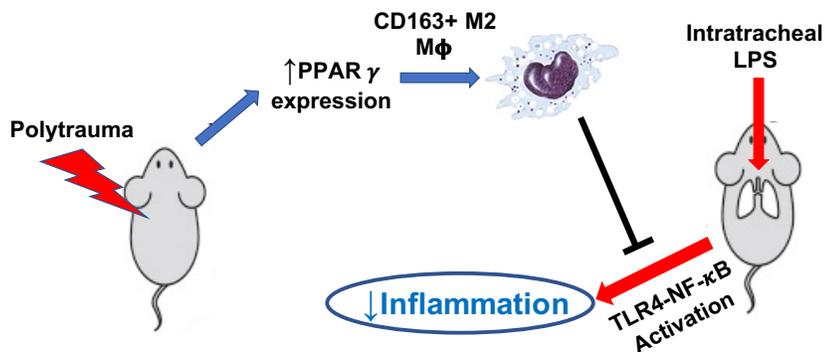


Figure 11: A schematic of the immune response to sequential insults. Polytrauma caused upregulation of PPAR- γ signaling, leading to increased numbers of CD163-expressing M2 macrophages (M ϕ). These anti-inflammatory macrophages then inhibit the TLR4-NF- κ B inflammatory response induced by LPS, causing a net anti-inflammatory response.

Behavioral Neurology 2018; doi 10.1155/2018/7646104). While further work is needed, our findings lead us to propose the following model of immune responses in acute trauma (**Figure 11**).

3. Implications of our results.

Our experiments have led to the identification of trauma-induced PPAR- γ signaling as a possible mechanistic explanation for post-traumatic immune suppression in the acute setting. Strengths of our approach include clearly defined experimental groups and unbiased analysis of the transcriptome in an end organ (the lungs) rather than in circulating blood. Multiple modulators of PPAR- γ are available, including a class of drugs (the Thiazolidinediones) that have been approved for clinical use, allowing interventional studies testing outcomes in models of polytrauma/lung injury after modulation of PPAR- γ signaling.

4. Limitations of the present work:

While we believe that our results are interesting and potentially important, our work has important limitations, perhaps most significant of which are the following: (a) we have used LPS as the 'second hit' rather than a more clinically relevant model such as a bacterial pneumonia, and (b) we have not examined whether the trauma-induced anti-inflammatory response effects outcomes. We chose to focus on LPS as a second injury rather than a bacterial challenge because we wanted to standardize what was a rather complicated model and were concerned that adding bacterial pneumonia would add another layer of variability to the model. In addition, our focus in this work was to try to attempt to define a mechanism for a phenomenon that ran counter to our hypothesis when we proposed this work. We believe that we have accomplished that. We intend to move forward to examining the effects of prior polytrauma on pneumonia induced by clinically relevant bacteria.

expression after trauma suggests the possibility that PPAR- γ induction may act to induce a M2 phenotype in pulmonary macrophages, reducing the inflammatory response to a subsequent exposure to LPS. Prior research supports a direct link between PPAR- γ activation and CD163 expressing M2 macrophage production. Wang et al. have shown that using PPAR- γ agonist monascin induces CD163 expression in a rat model of intracerebral hemorrhage, while the use of a PPAR- γ antagonist prevents CD163 expression (**Wang G, et al.**

E. CONCLUSIONS

Our data challenge conventional assumptions of acute trauma as a 'priming' injury that worsens inflammation after a secondary insult. We report a possible immunosuppressive role of PPAR- γ in the setting of sequential injuries (polytrauma and lung injury) using an unbiased approach. Further study is required to determine whether pharmacological modulation of the PPAR- γ pathway is beneficial in acute trauma.

F. STATED GOALS NOT MET:

1. Functional imaging using FDG-PET-CT scanning.

Our hypothesis called for an exaggerated immune response to intratracheal LPS following prior trauma and postulated that hepcidin induced iron sequestration in macrophages was responsible for the exaggerated inflammatory response. However, as described in section C.2., our results ran directly counter to our hypothesis – not only did we not see changes in the expression of genes associated with iron homeostasis, we found that prior exposure to polytrauma had an anti-inflammatory effect on a subsequent challenge with LPS. Under these circumstances, we decided that functional imaging of the lungs would add little to our understanding of the anti-inflammatory effect of trauma and decided to focus instead on identifying potential mechanisms for this anti-inflammatory effect.

2. Characterize the efficacy of inhaled and IV iron chelators (Deferoxamine) in altering the severity of secondary ALI in mice.

Since we did not find evidence of altered iron homeostasis in mouse lungs after trauma, and demonstrated an anti-inflammatory effect of prior trauma (rather than a proinflammatory effect) testing the effect of iron chelators on lung inflammation after trauma would not have been consistent with our results.

G. TRAINING AND PROFESSIONAL DEVELOPMENT:

Nothing to report.

H. HOW WERE THE RESULTS DISSEMINATED TO COMMUNITIES OF INTEREST?

Nothing to report.

I. PLAN DURING NEXT REPORTING PERIOD TO ACCOMPLISH GOALS

Final report – noting to report.

J. IMPACT.*1. What was the impact on the development of the principal discipline(s) of the project?*

Our project has revealed a possible mechanism for an acute anti-inflammatory effect of trauma on subsequent inflammatory insults. This is information that was hitherto unrecognized in the field, marking an advance in our understanding of the inflammatory responses to trauma. While further work is needed to define the importance of PPAR- γ signaling after acute trauma, the data generated by this project suggest avenues for further research, both in animal models of trauma as well as in human patients. Given the number of pharmaceutical agents that can modulate PPAR- γ signaling, translational uses of some of these agents may be possible in the near future to attempt to modulate the immune response to trauma.

2. What was the impact on other disciplines?

Nothing to report.

3. What was the impact on technology transfer?

Nothing to report.

4. What was the impact on society beyond science and technology?

Nothing to report.

K. CHANGES/PROBLEMS*1. Changes in approach.*

As mentioned in section G, we did not complete the functional imaging and iron chelation portions of the protocol because the results obtained during the performance of this project no longer could justify the need for those components of the project. We did complete the RNA sequencing portion of the project as described above, with the identification of mechanistic data that could explain our findings of an acute anti-inflammatory effect of trauma.

2. Changes that had a significant impact on expenditures

A no-cost extension was applied for and approved in September 2018. There were no significant impacts on expenditures.

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

Nothing to report

L. PRODUCTS

1. *Conference Presentations:*

Title: RNA Sequencing identifies Peroxisome Proliferator-Activated Receptor γ upregulation as an immunomodulator in a 'two-hit' mouse model of trauma followed by acute lung injury

Authors: Aranya Bagchi, Fumiaki Nagashima, Allyson Hindle and Fumito Ichinose

Conference: American Thoracic Society – Annual Conference 2020

Status: Submitted

Type of presentation: Abstract/oral presentation

Acknowledgement of federal support: Yes

2. *Website(s) or other Internet site(s)*

Nothing to report

3. *Technologies or techniques*

Nothing to report

4. *Inventions, patent applications, and/or licenses*

Nothing to report

5. *Other Products*

Nothing to report

M. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS*1. Individuals working on the project:*

Name:	Aranya Bagchi, M.B.,B.S.
Project Role:	Principal Investigator
Nearest Person Month	6
Contribution to the project:	Dr. Bagchi is responsible for experimental design, performing experiments, data analysis and all aspects of the project.
Funding Support:	Current Award (W81XWH-17-1-0058)

Name:	Fumiaki Nagashima, M.D.
Project Role:	Post-doctoral Researcher
Nearest Person Month	2
Contribution to the project:	Dr. Nagashima performed experiments, analyzed data and helped write manuscripts resulting from the project.
Funding Support:	Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General

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

Nothing to report.

3. What other organizations were involved as partners?

Nothing to report.

N. SPECIAL REPORTING REQUIREMENTS*1. Collaborative Awards.*

Not Applicable

2. Quad charts.

Not Applicable