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

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14. ABSTRACT During the reporting period, we have established a mouse model of polytrauma (hemorrhagic shock with laparotomy and cecectomy) that consistently induces systemic inflammation and have developed and validated a method for the non-invasive administration of intratracheal drugs, which we will use to deliver intratracheal lipopolysaccharide (LPS). Our studies in mice lacking the hepcidin gene support our principal hypothesis that increased intracellular iron levels are associated with a more robust inflammatory response. We have also tested multiple iron chelators for their anti-inflammatory properties, showing that deferiprone and pyridoxal isonicotinoyl hydrazone (PIH) are at least as effective as deferoxamine at blunting the acute inflammatory response, at lower concentrations. Taken together, our data strongly suggest a critical role for intracellular iron in acute inflammation. We are well placed to continue the project to test the efficacy of iron chelators in a 'two-hit' murine model of polytrauma followed by endotoxin induced lung injury.					
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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. Because the onset of ARDS results in worse clinical outcomes in trauma patients, it is important to explore strategies that reduce the risk of developing ARDS. Our work explores an approach for preventing the development of ARDS in trauma patients by targeting intracellular labile iron in immune cells. Our central hypothesis is that trauma and hemorrhagic shock prime monocytes and macrophages by increasing their intracellular iron content due to the effect of systemic and locally produced hepcidin. A subsequent inflammatory stimulus then induces a response that is amplified by the increased intracellular iron, resulting in ARDS. Our mouse model is designed to replicate a 'two-hit' mode of post-trauma lung injury. The studies we are currently engaged in will test the hypothesis that by chelating intracellular iron after trauma, we can blunt the severity of ARDS induced by a 'second hit'. We also intend to use FDG-PET-CT to non-invasively assess the severity of lung inflammation in mice. Increased uptake of FDG by immune cells in the lungs is strongly correlated with inflammatory activity of the immune cells. Thus, we will be able to determine the severity of lung injury as well as the effect of iron chelation on the severity of lung injury non-invasively. Finally, transcriptomic analysis will enable us to identify major pathways that are activated in post-trauma lung injury and determine how iron chelation effects these pathways.

Keywords:

Trauma

Hemorrhagic shock

Inflammation

Acute Respiratory Distress Syndrome (ARDS)

Intracellular iron

Iron Chelation

Deferoxamine

Deferiprone

[¹⁸F]fluoro-2-deoxy-D-glucose (FDG)

Positron Emission Tomography (PET)

Transcriptomics

ACCOMPLISHMENTS

Major Goals of the Project during the reporting period:

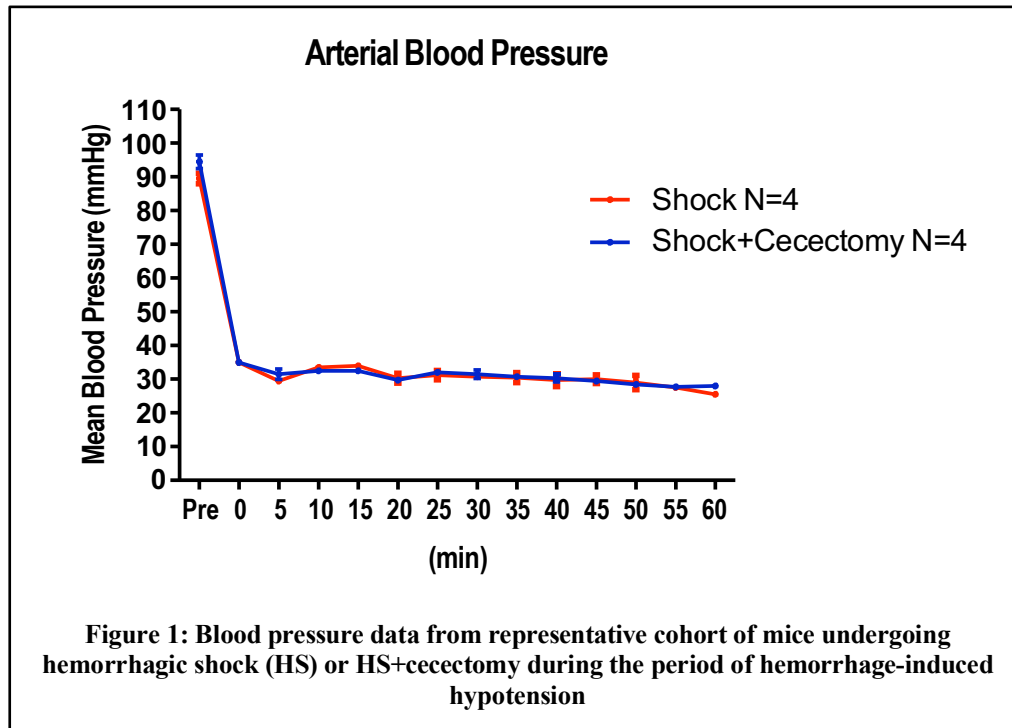
Major Task 1: Characterize an in-vivo model of Trauma/Hemorrhagic Shock (HS) with secondary LPS-induced Acute Lung Injury (ALI)

Major Task 2: Compare the severity of ALI in a Trauma/HS + ALI model vs. sham surgery+ALI

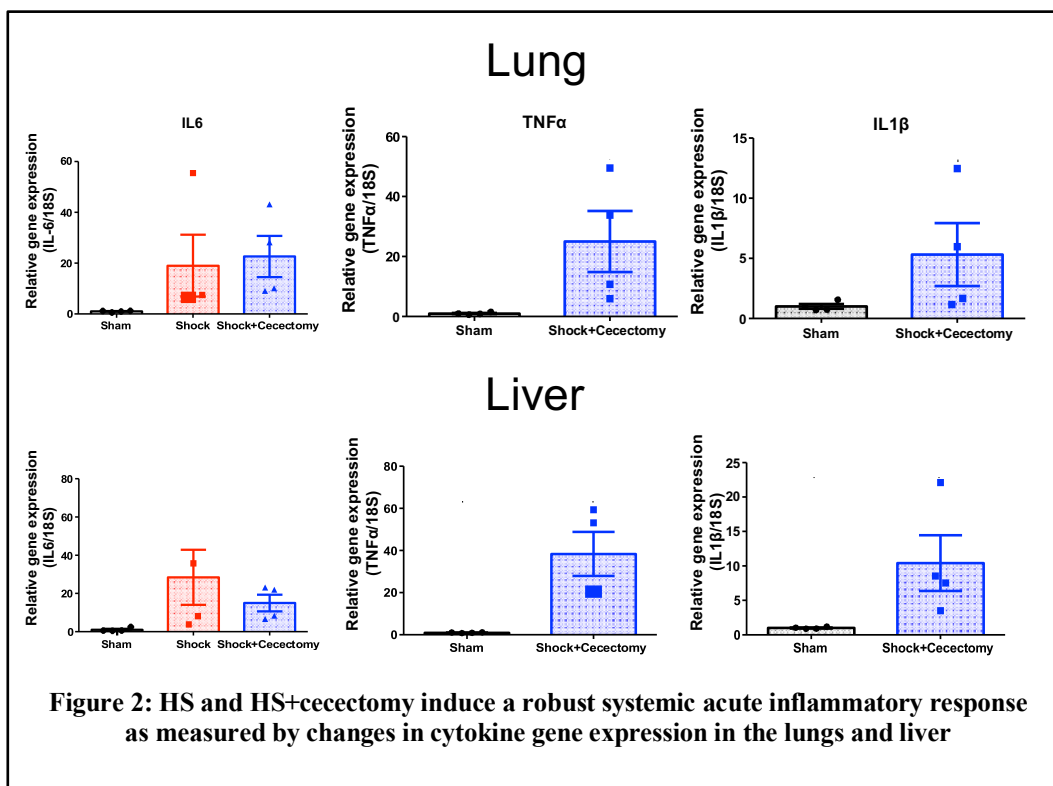
Major Activities: Please note that the animal experiments reported here have been supported by using Sundry funds from the Department of Anesthesia, Critical Care and Pain Medicine at the Massachusetts General Hospital.

1. Establishment of a model of hemorrhagic shock: C57Bl/6 mice of either sex between 6-8 weeks were used for these experiments. The 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 ($\pm$ 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.
2. Adding cecectomy to mice subjected to hemorrhagic shock: Once we were successful in establishing a murine model of hemorrhagic shock as above, we began to add the polytrauma component, consisting initially of cecectomy. 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. Table 1 and Figure 1 show representative data from a cohort of 8 mice that underwent HS only or HS+cecectomy.

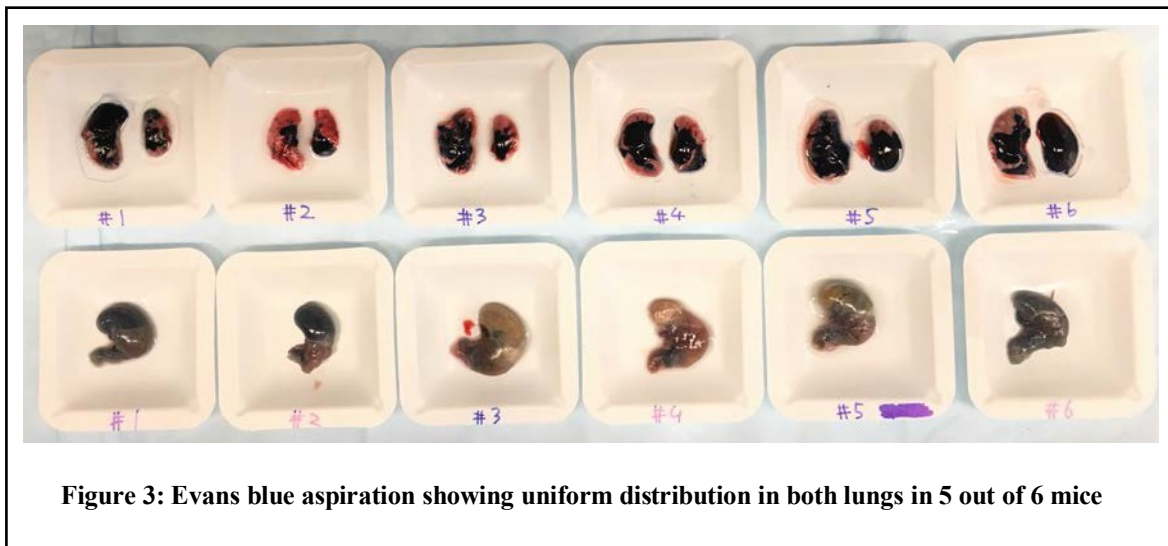
	HS (N=4)	HS + Cecectomy (N=4)
Weight (g)	29.90 $\pm$ 0.80	29.65 $\pm$ 1.25
Shed blood (μ l)	732.0 $\pm$ 36.4	717.7 $\pm$ 62.8
Shed blood/ Estimated Blood Volume (%)	31.95 $\pm$ 1.49	31.68 $\pm$ 3.20
Resuscitation volume (μ l)	2928 $\pm$ 145.4	2871 $\pm$ 251.3
Operation time (min)	143.0 $\pm$ 1.3	156 $\pm$ 1.3
Anesthesia time (min)	148 $\pm$ 0.91	163 $\pm$ 1.47
Survival of 6h after injury	100%	100%
Values are expressed as the means $\pm$ SEM		
Table 1: Data from a representative cohort of mice undergoing hemorrhagic shock (HS) or HS+cecectomy		



3. Assessing the inflammatory effect of the models of hemorrhagic shock or HS+cecectomy: In the animal models established as mentioned earlier, lungs and livers were harvested after 6h to look for markers of early inflammation. Representative data are shown in figure 2. Sham operated mice were anesthetized, had their femoral arteries cannulated, and had a 1 cm laparotomy incision, without being subjected to either hemorrhage or cecectomy. As shown in the figure, both HS and HS+cecectomy induce a significant systemic acute inflammatory response. *Consequently, we decided to not include a component of femur fracture to the model in the interest of reducing variability in the model and not subjecting mice to unnecessary procedures. As these are preliminary experiments, we chose to focus on a few genes, primarily IL6 as endpoints.*



4. Testing a reproducible method for delivering intratracheal lipopolysaccharide: With our polytrauma model established, we next turned to intratracheal lipopolysaccharide (LPS) delivery, the second component of our ‘two-hit’ model. Since we were performing survival procedures, we wanted to minimize the invasiveness of the intratracheal delivery. In our hands, methods involving endotracheal intubation of anesthetized mice were often associated with mortality and/or non-homogeneous distribution of the injected LPS (assessed by measuring lung cytokine levels 4-6 hours after injection). We therefore attempted a less invasive methods. Mice were anesthetized using isoflurane, and then suspended vertically with their mouth open. Their nares were gently occluded using forceps and 25 μ l of Evans blue dye was deposited in their hypopharynx, which they aspirated while mouth-breathing. None of the mice suffered distress or died from the use of this method. Six hours after recovery, the mice were sacrificed, and their lungs and stomachs were examined for Evans blue staining. As shown in figure 3, this method reliably deposits Evans blue dye in both lungs, with minimal gastrointestinal uptake. We are now using this method to compare the effect of intratracheal LPS in sham operated vs HS-cecectomy mice.



Supporting Data:

The data in this section was not part of the original proposal. However, we have recently acquired mouse colonies of Hepcidin knock-out mice (on a C57Bl/6 background). The hepcidin KO mice do not produce any hepcidin, and thus have high serum iron but low macrophage iron levels. These mice therefore provide an approach to testing our central hypothesis – that the presence of low intracellular iron levels will be associated with a less intense acute inflammatory response. We took the opportunity to test this hypothesis in C57Bl/6 WT and Hepcidin KO mice. Both groups of mice were injected with 5 mg/kg LPS or the equivalent volume of normal saline. The mice were sacrificed after 6 hours and hepcidin gene expression, serum iron levels, inflammatory cytokine levels and histologic sections of the lung and spleen were examined. Figure 4 shows the lack of hepcidin gene expression, the increased serum iron levels and the decreased macrophage iron levels (measured by Prussian blue staining) in the hepcidin KO mice.

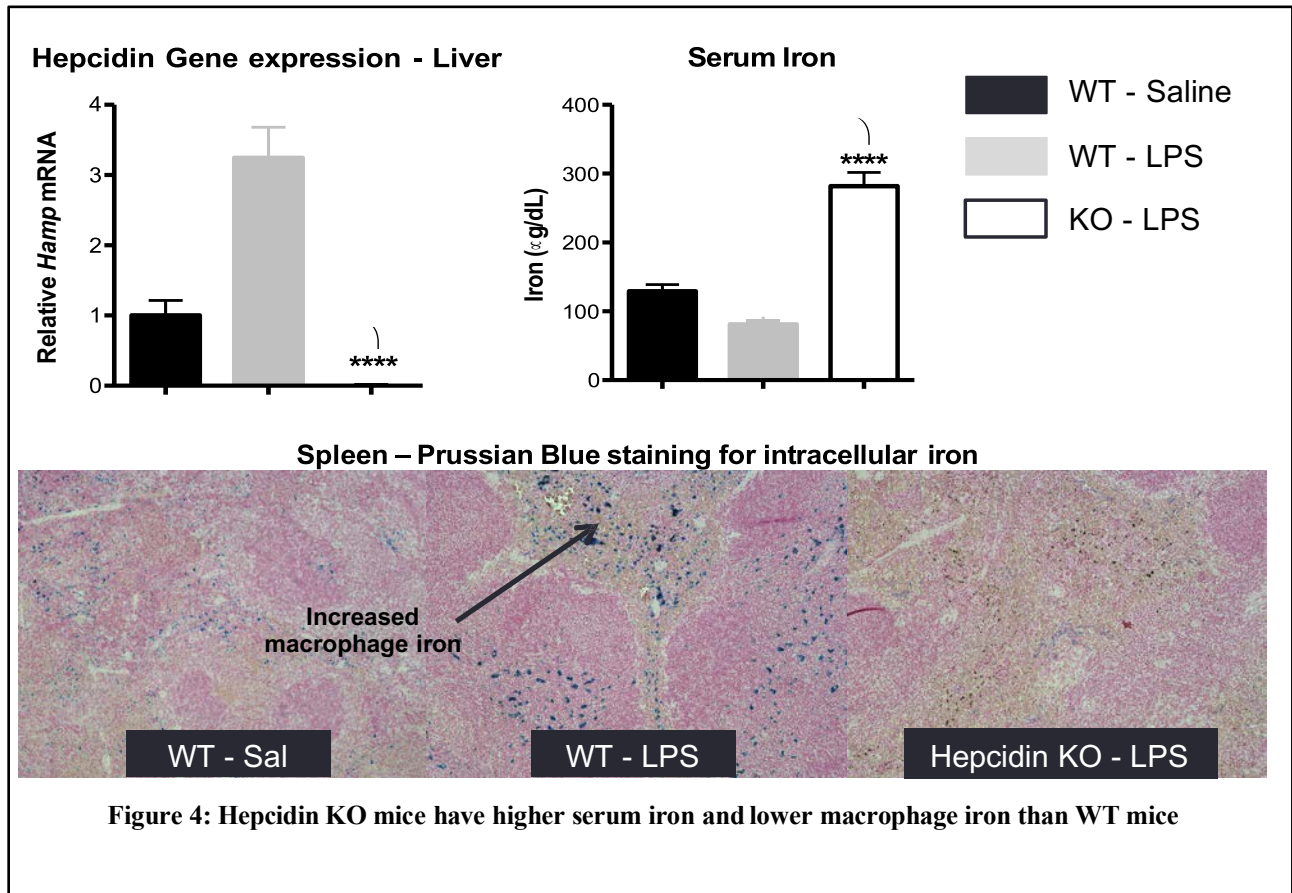
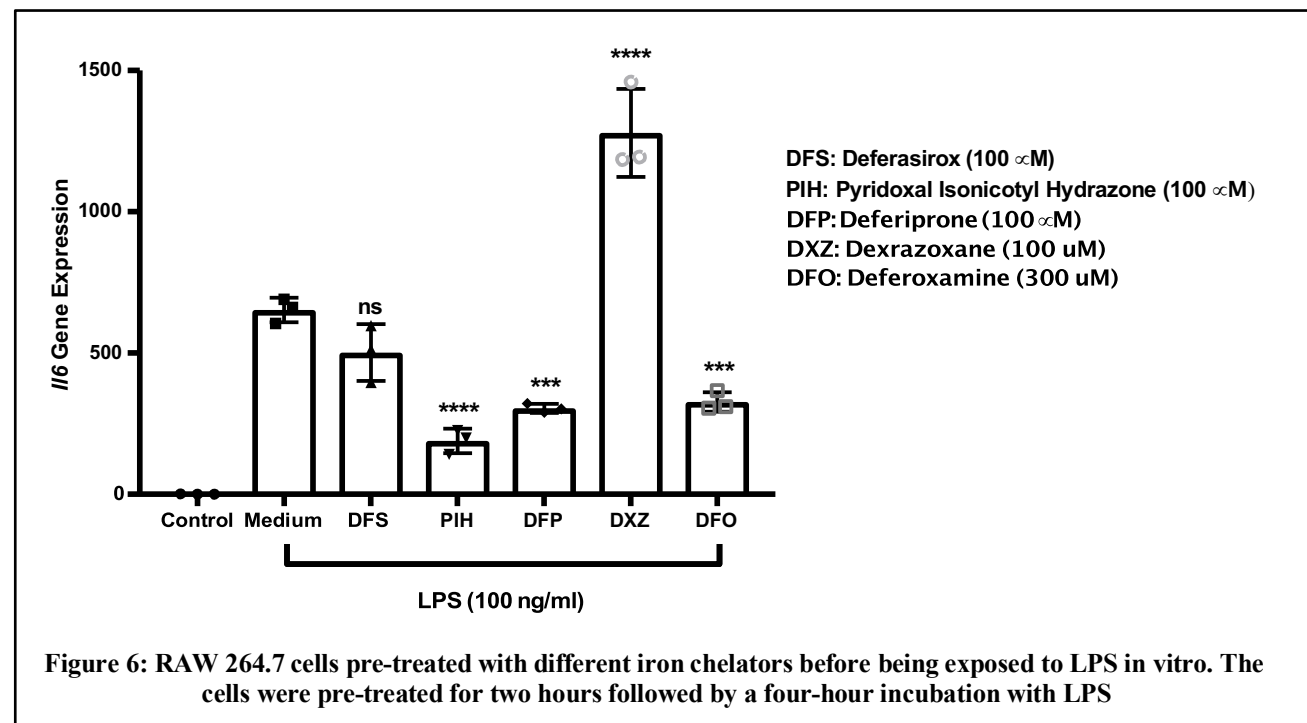
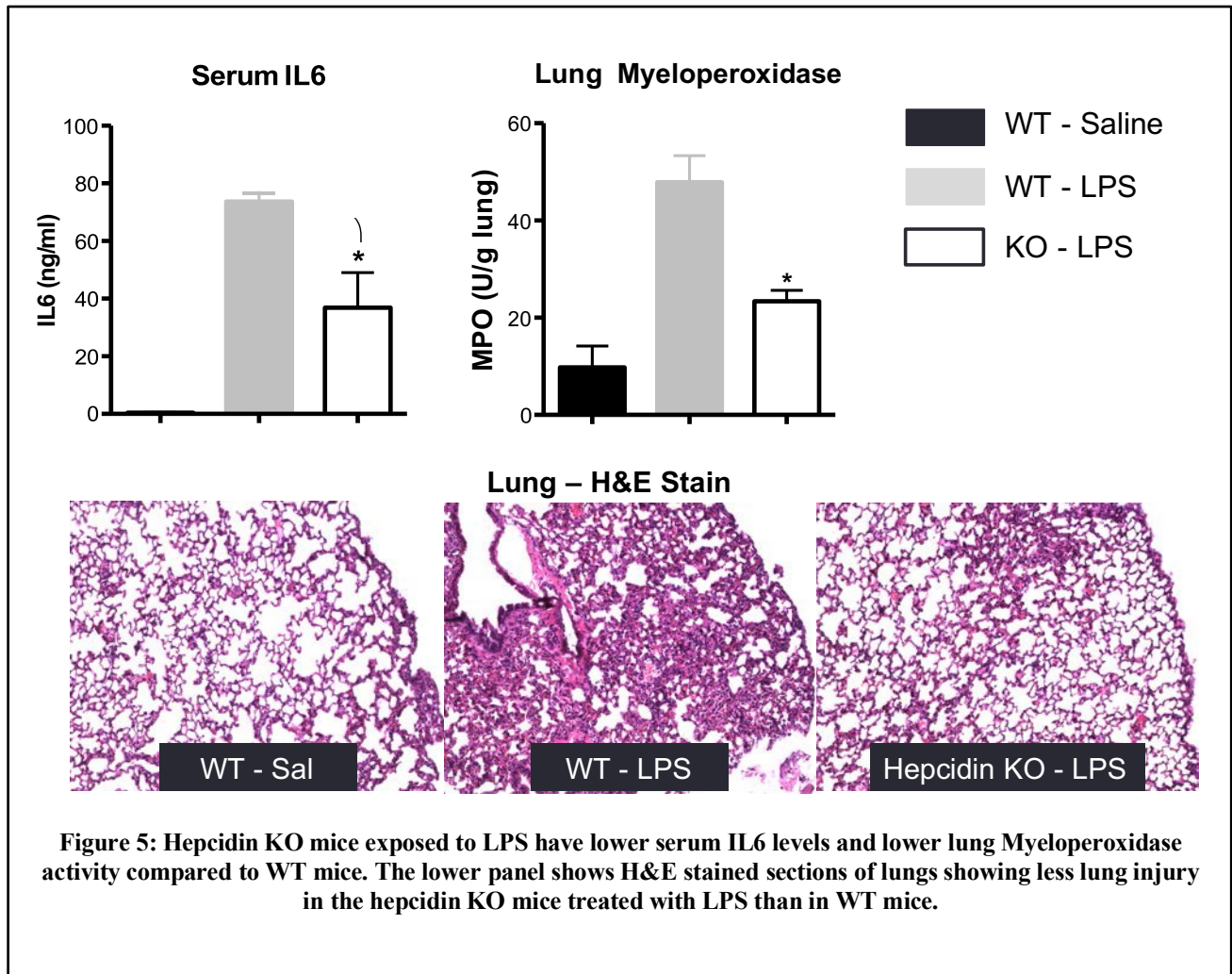


Figure 5 shows that hepcidin KO mice treated with LPS have lower serum IL6 protein levels in serum than WT mice, that the myeloperoxidase activity in the lungs (a marker for activated neutrophils) is lower in the hepcidin KO mice and that the severity of lung injury by histology is less intense in hepcidin KO mice than in the WT mice.

Our previous work has shown that iron loading WT mice increases monocyte and macrophage iron levels and results in a more intense inflammatory response to LPS (Hoeft K, et al. Anesthesiology 2017;127:121-135, please see appendix). Taken together, our data from the hepcidin KO mice and our previously published work strongly support our central hypothesis – i.e. low macrophage iron appear to be an important factor in modulating the acute inflammatory response. As such, these data provide important support for the work being undertaken as part of the current project.

Initial testing of iron chelators in vitro:

We have begun to test the ability of multiple iron chelators to blunt the effect of LPS-induced inflammation on RAW 264.7 cells, a mouse macrophage cell line. Deferoxamine, while an effective iron chelator, is not cell-membrane permeable, and we wanted to test whether a membrane permeable, FDA approved iron chelator like deferiprone would be as effective as deferoxamine. As the data shown in figure 6 indicate, deferiprone and PIH (pyridoxal isonicotinoyl hydrazone, another membrane permeable iron chelator) are both at least as effective as deferoxamine, and at lower concentrations. We will therefore test both these chelators in the therapeutic arm of our project.



Stated goals not met:

The following goals for the reporting period have not yet been completed:

1. Compare the severity of ALI in a Trauma/HS + ALI model vs. sham surgery+ALI: As described in the report above, we have developed a mouse model of polytrauma and established its pro-inflammatory effects and have developed and validated a minimally invasive method of reliably treating mice with intratracheal LPS. This took somewhat longer than we had originally planned. However, we are now in the process of continuing with this part of our goals. With the technical part of the model development having been accomplished, we anticipate that we will be able to complete this portion of the project relatively quickly.
2. FDG-PET-CT imaging of mice: We have delayed FDG-PET imaging of the mice to do preliminary experiments where we assess the severity of inflammation by gene expression first. We have done this in order to maximize the value of the PET scans, which are an expensive resource. Once we are confident that the model is reproducible, we will proceed to the PET-CT scans.

Training and Professional Development:

Nothing to report.

How were results disseminated to communities of interest?

Nothing to report.

Plan during the next reporting period to accomplish remaining goals:

As mentioned earlier in this report, a significant amount of work has been completed to develop the model of trauma/acute lung injury. Recently, a post-doctoral fellow has joined this project, and has been trained in the model. With this addition, we expect to be able to move quite quickly during the next six months to complete the project, as he will be able to work continuously on the project. Before the addition of the post-doctoral researcher, I was performing all the experiments myself, and therefore would have inevitable breaks in working on the project while I was on clinical service. I am thus confident that we will be able to move much faster now.

IMPACT

We expect that once completed, this project will have made a significant contribution to understanding the role of intracellular iron in the pathogenesis of post-traumatic lung injury, and that our work will lead to further investigation in this area with potential translational benefits to patient care. At this stage of the project however, it is too early to assess the impact of our project.

What was the impact on the development of the principal discipline of the project?

Nothing to report.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

CHANGES/PROBLEMS

Changes in approach and reasons for change:

There has been no major change in our experimental approach. However, we have supplemented the experiments in the project with experiments in Hepcidin KO mice, as described in the accomplishments section, *included as unplanned additional work*. The hepcidin KO mice provided a powerful method of more firmly establishing our rationale for doing the work described in this project, i.e. demonstrating the importance of intracellular iron in the acute inflammatory response. We believe that these experiments will provide data (examples are shown in figures 4 and 5 of the accomplishments section) that will be critical to the scientific basis of our project. Our second minor modification to our experimental plan is the comparison of two more iron chelators in addition to deferoxamine – deferiprone and pyridozal isonicotinoyl hydrazone (PIH). Our preliminary data (represented in Figure 6 of the accomplishments section) suggest that these two iron chelators are as effective anti-inflammatory agents as deferoxamine and act at lower doses, perhaps because they are membrane permeable, unlike deferoxamine. We will thus test the efficacy of these two chelators systemically, in addition to deferoxamine.

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

Till recently I have been performing most of the experiments by myself, in part to conserve resources that I am anticipating will be required in the portions of the project that are resource intensive (the PET-CT scans and transcriptomics). Since I spend approximately 50% of my time in clinical duties, that inevitably results in interruptions in my workflow, and results in delays. Recently however, a post-doctoral fellow (supported by the department of Anesthesia) has been assigned to spend some of his time on my project and has been trained in the mouse model. I therefore believe that we can make much more rapid progress now that someone besides me will be working on the project.

Changes that had a significant impact on expenditures:

Nothing to report

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

The experiments that generated the animal data in this report were performed under the following IACUC-approved animal protocols:

A: #2018N000062: The role of iron metabolism on development of brain injury after cardiac arrest and cardiopulmonary resuscitation in mice.

PI: Fumito Ichinose, MD, PhD Co-Investigator: Aranya Bagchi, MBBS

Date of approval: 04/25/2018; Funding source: Sundry funds, Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital

B: #2011N00020: Modulation of Acute Lung Injury by Bone Morphogenetic Protein-Hepcidin Signaling

PI: Aranya Bagchi, MBBS

Date of Approval: 02/09/2011; Funding Source: Sundry funds, Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital

Further experiments will be carried out under an IACUC-approved protocol explicitly written for this project:

#2018N000105: Manipulating the Macrophage Iron-Hepcidin Axis to prevent Acute Lung Injury/Acute Respiratory Distress Syndrome secondary to severe trauma/hemorrhagic shock.

PI: Aranya Bagchi, MBBS

Date of Approval: 08/16/2018; Funding Source: USAMRAA

PRODUCTS

Publications, conference papers and presentations:

Nothing to report

Website(s) or other Internet site(s):

Nothing to report

Technologies or techniques:

Nothing to report

Inventions, patent applications and/or licenses:

Nothing to report

Other products:

Nothing to report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Individuals working on the project:

Name:	Aranya Bagchi, M.B.,B.S.
Project Role:	Principal Investigator
Nearest Person Month Worked:	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 Worked:	2
Contribution to the project:	Dr. Nagashima will perform experiments, analyze data and help write manuscripts resulting from the project.
Funding Support:	Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital

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.

What other organizations are involved as partners?

Nothing to report.

SPECIAL REPORTING REQUIREMENTS

Collaborative Awards:

Not Applicable

Quad charts:

Not Applicable

RESPONSE TO REVIEWER'S QUESTIONS:

Thank you for providing this report of your progress during the 1st year of your award. I am disapproving this report primarily due to the omission of information on animal protocols. You indicated in an earlier email that your upcoming animal protocol is undergoing IACUC review at this time, and that all animal work that has been performed thus far is under a separate protocol which is paid for with other funds. I have provided a list of items that should be addressed in a revised report.

Thank you for the detailed review – our responses to your queries are indicated below. Please note that the sections of the report that have been modified have been underlined and italicized for ease of identification.

1. It appears that IL6 was the only inflammatory marker observed in all 3 groups (Figure 2)? Is this accurate, or did you just not report all findings? Please be sure to report a summary of all findings.

This is accurate – since these are preliminary experiments we have focused on a few markers of inflammation, predominantly examining IL6 as a sensitive early marker of inflammation.

2. In your proposal you state that you will induce polytrauma through cecectomy and femur fx with muscle damage. I did not see the femur fx in your report. Do you still plan to use this approach? Please provide any rationale for changing your original plan (for instance, if you found that additional injury was not necessary to elicit a robust inflammatory response).

We found that the combination of hemorrhagic shock and cecectomy induces a robust inflammatory response, as noted in figure 2. As you have suggested, we decided not to include femur fracture and muscle damage as part of our model given that additional injury did not appear necessary to elicit a reliable inflammatory response.

3. In the supporting data section, please indicate whether this work was a part of another grant, or if it was added to this grant as unplanned additional work.

We have modified our paragraph under 'Changes in approach and reasons for Change' to indicate that the data generated from the hepcidin knock out mice have been added to this grant as unplanned additional work.

4. Add information about the animal protocols to the "Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents". The directions for this section state "Also, specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates". Include the IACUC protocol # and name of research project under which animal work for this project is being performed. Also provide the IACUC # for the protocol which is pending approval for Aim 2 of this project.

This information has been added on page 13. A copy of the IACUC-Approved animal protocol (#2018N000105) has also been included as an appendix.

5. At the start of your description of animal work, please be sure to indicate that the following animal work was funded by another grant. Once you receive ACURO approval for work funded by the DoD, be sure to be clear about which work was funded through which source.

We have submitted our approved protocol for approval by ACURO, and we shall be sure to clearly define the portions of the work funded by the DoD as well as alternate sources.

Thank you for providing the details of your work with hepcidin KO mice. This nicely supports your targeting of intracellular iron.

Thank you for the comment. We agree that the data from the hepcidin KO mice provides further support for our hypothesis that reducing labile intracellular iron concentrations may help to reduce the intensity of inflammatory organ injury.

APPENDICES

Copy of Manuscript titled: “**Iron Loading Exaggerates the Inflammatory Response to the Toll-like Receptor 4 Ligand Lipopolysaccharide by Altering Mitochondrial Homeostasis**” published in the journal *Anesthesiology*, referred to in section on ‘Accomplishments, page 9’

Copy of IACUC-Approved animal protocol #2018N000105

Iron Loading Exaggerates the Inflammatory Response to the Toll-like Receptor 4 Ligand Lipopolysaccharide by Altering Mitochondrial Homeostasis

Konrad Hoefft, Cand.Med., Donald B. Bloch, M.D., Jan A. Graw, M.D., Rajeev Malhotra, M.D., M.S., Fumito Ichinose, M.D., Ph.D., Aranya Bagchi, M.B.B.S.

ABSTRACT

Background: Perioperative and critically ill patients are often exposed to iron (in the form of parenteral-iron administration or blood transfusion) and inflammatory stimuli, but the effects of iron loading on the inflammatory response are unclear. Recent data suggest that mitochondrial reactive oxygen species have an important role in the innate immune response and that increased mitochondrial reactive oxygen species production is a result of dysfunctional mitochondria. We tested the hypothesis that increased intracellular iron potentiates lipopolysaccharide-induced inflammation by increasing mitochondrial reactive oxygen species levels.

Methods: Murine macrophage cells were incubated with iron and then stimulated with lipopolysaccharide. C57BL/6 wild-type mice were intraperitoneally injected with iron and then with lipopolysaccharide. Markers of inflammation and mitochondrial superoxide production were examined. Mitochondrial homeostasis (the balance between mitochondrial biogenesis and destruction) was assessed, as were mitochondrial mass and the proportion of nonfunctional to total mitochondria.

Results: Iron loading of mice and cells potentiated the inflammatory response to lipopolysaccharide. Iron loading increased mitochondrial superoxide production. Treatment with MitoTEMPO, a mitochondria-specific antioxidant, blunted the pro-inflammatory effects of iron loading. Iron loading increased mitochondrial mass in cells treated with lipopolysaccharide and increased the proportion of nonfunctional mitochondria. Iron loading also altered mitochondrial homeostasis to favor increased production of mitochondria.

Conclusions: Acute iron loading potentiates the inflammatory response to lipopolysaccharide, at least in part by disrupting mitochondrial homeostasis and increasing the production of mitochondrial superoxide. Improved understanding of iron homeostasis in the context of acute inflammation may yield innovative therapeutic approaches in perioperative and critically ill patients. (**ANESTHESIOLOGY** 2017; 127:121-35)

IRON is an essential trace element.¹ Increased appreciation of the adverse effects of anemia in the perioperative period together with awareness of the risks of blood transfusion have led to the preoperative use of intravenous iron preparations as part of patient blood management protocols.²⁻⁴ Critically ill patients in intensive care units are also exposed to acute iron loading (defined here as an increase in intracellular iron concentration in response to iron administration) in the form of blood transfusions, as well as oral and parenteral iron treatment.^{5,6} Although patients are often exposed to both iron loading and inflammatory stimuli such as major surgery or critical illness, the effects of iron loading on the inflammatory response are incompletely understood.

Because of the ability of iron to generate reactive oxygen species (ROS) by the Fenton reaction,⁷ iron administration

What We Already Know about This Topic

- Inflammation may play a role in critical illness
- Iron can increase the formation of reactive oxygen species, potentially affecting inflammation
- Critically ill patients may be exposed to iron through transfusion

What This Article Tells Us That Is New

- In rodent and cellular models, iron loading potentiated inflammation caused by lipopolysaccharide
- Iron loading in this model increased the production of mitochondrial superoxide and disrupted mitochondrial homeostasis

might be expected to potentiate the response to subsequent inflammatory stimuli. Studies supporting a proinflammatory role for iron include those by Zager *et al.*,⁸ who showed that

Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of this article on the Journal's Web site (www.anesthesiology.org).

Submitted for publication February 21, 2016. Accepted for publication March 14, 2017. From the Anesthesia Center for Critical Care Research, Department of Anesthesia, Critical Care and Pain Medicine (K.H., D.B.B., J.A.G., F.I., A.B.), Division of Rheumatology, Allergy and Immunology, Department of Medicine (D.B.B.), and Division of Cardiovascular Medicine, Department of Medicine (R.M.), Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts; and Department of Anesthesiology, University Hospital of the RWTH Aachen, Aachen, Germany (K.H.).

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iron administration worsened *Escherichia coli*-induced sepsis, and Wang *et al.*,⁹ who reported that the iron chelator deferrioxamine blunted lipopolysaccharide-induced inflammation. However, Pagani *et al.*¹⁰ found that iron-deficient mice had a more robust inflammatory response to lipopolysaccharide than iron-replete mice, and De Domenico *et al.*¹¹ demonstrated that iron administration diminished the inflammatory response to lipopolysaccharide. The reason for the lack of agreement between these studies is unclear but may be related to differing routes and timing of iron administration.

Iron homeostasis is tightly controlled to minimize the risk of toxicity. Hcpidin is a key regulator of iron homeostasis.^{12,13} Hcpidin binds to and down-regulates ferroportin, the only known iron exporter in mammals. Acute inflammation has been shown to increase hcpidin levels,¹⁴ inducing systemic hypoferremia with an increase in intracellular iron.

Mitochondria have an important role in the acute inflammatory response. Pathogen-associated molecular patterns such as lipopolysaccharide, a Toll-like receptor 4 (TLR4) agonist, induce inflammation in part by increasing the production of mitochondrial reactive oxygen species (mtROS).¹⁵ Mitochondria are a major site of iron utilization within the cell¹⁶ and exist in a dynamic equilibrium between biogenesis and mitophagy (removal of dysfunctional mitochondria by autophagy).¹⁷ Perturbations in mitochondrial homeostasis (defined here as the balance between mitochondrial biogenesis and mitophagy) may increase the proportion of damaged or nonfunctional mitochondria, increasing mtROS production.¹⁸

In this study, we examined the effect of acute iron loading on the inflammatory response using *in vivo* and *in vitro* models of inflammation. We hypothesized that iron loading would exaggerate the proinflammatory effect of lipopolysaccharide by increasing mtROS production.

Materials and Methods

Reagents and Chemicals

Escherichia coli lipopolysaccharide (O55:B5), a TLR4 agonist, was purchased from List Biologicals (USA). The TLR2 and TLR3 agonists Pam-3-Cys (P3C) and poly(I:C) (PIC), respectively, were obtained from Invivogen (USA). Formyl peptide *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLF), iron dextran, 20% dextran, ferric ammonium citrate, deferrioxamine, antimycin, and 3-methyladenine were purchased from Sigma-Aldrich (USA). Calcein-acetoxymethyl (AM), MitoSOX, MitoTracker Deep Red, MitoTracker Green, and SYTOX Blue were purchased from Molecular Probes (USA). Antibodies, isotype controls, and reagents for flow cytometry were obtained from BD Biosciences (USA). MitoTEMPO, a mitochondria-specific antioxidant,¹⁹ was purchased from Enzo Life Sciences (USA).

Animals

The Institutional Animal Care and Use Committee at the Massachusetts General Hospital approved the animal studies. Male C57BL/6 mice (6 to 8 weeks old) were purchased

from Jackson Laboratories (USA). The mice were fed a standard, iron-replete diet and were injected intraperitoneally with one dose of iron dextran (1 g/kg in a volume of 10 μ l/g) or normal saline (control) for iron loading experiments. Pilot experiments were performed with a range of iron doses (0.5 to 2.0 g/kg), and serum iron levels and liver hcpidin messenger RNA (mRNA) were measured at various time points (1, 3, 4, and 7 days; data not shown). Mice injected with 1 g/kg iron dextran demonstrated both a sustained increase in serum iron levels and a strong induction of hcpidin after 3 days, leading us to choose this 72-h time point for further investigation. A separate group of wild-type mice was injected with normal saline or 7.5% dextran (10 μ l/g, the same volume as iron dextran). Three days later the mice were injected intraperitoneally with lipopolysaccharide (5 mg/kg) or an equal volume of normal saline (control). The mice were sacrificed 6 h later, and blood and organs (lungs and liver) were collected. Serum and organs were stored at -80°C until use.

Cell Culture

The murine macrophage cell line RAW 264.7 was obtained from American Type Cell Collection (USA). RAW 264.7 (hereafter referred to as "RAW") cells were cultured in 6-well (at 8×10^5 cells/well) or 96-well tissue culture plates (at 1.28×10^5 cells/well) in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum with glutamine, penicillin, and streptomycin. For iron supplementation experiments, the cells were incubated with ferric ammonium citrate (final concentration of elemental iron, 200 μ M) overnight and then treated with lipopolysaccharide (150 ng/ml) or medium alone for 6 h. The concentration of iron was chosen so as to approximate the serum iron concentrations used in pilot, *in vivo* dose-response experiments.

Human Monocyte Isolation

The Institutional Review Board at the Massachusetts General Hospital approved the collection of whole blood from volunteers for the purpose of monocyte isolation (Institutional Review Board protocol No. 2014P001656). Mononuclear cells were isolated from human whole blood collected in EDTA using Polymorphprep density gradient (Axis-Shield, Norway) according to the manufacturer's instructions. Mononuclear cells were incubated (at 8×10^5 cells/well) in 6-well tissue culture plates in serum-free DMEM for 4 h. The cells were then washed with serum-free DMEM to remove nonadherent cells and incubated overnight in DMEM with 10% fetal calf serum. The cells were incubated with iron and/or lipopolysaccharide as in the RAW cell experiments.

Mouse Serum Studies

Serum interleukin-6 and tumor necrosis factor (TNF) levels were measured using mouse interleukin-6 and TNF Quantikine enzyme-linked immunosorbent assay kits (R&D Systems, USA). Serum iron levels were measured using an Iron-SL assay (Japan).

Quantitative Reverse Transcription-Polymerase Chain Reaction

TaqMan primers for quantitative reverse transcription (RT)-polymerase chain reaction (PCR) were purchased from ThermoFisher Scientific (USA). SYBR Green primers were synthesized by the Massachusetts General Hospital DNA core facility. The sequences of SYBR Green and TaqMan primers used in this study are listed in supplemental table 1 (Supplemental Digital Content 1, <http://links.lww.com/ALN/B431>). Total RNA was extracted from mouse liver and lung tissues or RAW cells using TRIzol (Invitrogen, ThermoFisher Scientific, USA). Reverse RNA transcription was accomplished using Moloney murine leukemia virus RT (Promega, USA). Quantitative RT-PCR was performed using Applied Biosystems SYBR Green or 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.

Intracellular Labile Iron Measurement

Intracellular labile iron was measured as described previously.²⁰ Briefly, cells were incubated with calcein-AM, which was transported across the cell membrane by viable cells and deesterified, producing intracellular, fluorescent, free calcein. Calcein binds with intracellular labile (or free) iron, a reaction that quenches calcein fluorescence. The concentration of labile iron in a cell is inversely proportional to the intensity of calcein fluorescence. SYTOX Blue was used to identify and exclude nonviable cells, which do not take up calcein-AM, and may thereby confound results.

Flow Cytometry

Mouse whole blood was incubated in erythrocyte lysis buffer for 3 min and washed twice in flow cytometry buffer (phosphate-buffered saline with 2% fetal calf serum). The cells were then incubated with PerCP-Cy5.5-conjugated mouse monoclonal anti-CD11b antibody, allophycocyanin-conjugated mouse monoclonal anti-Ly6G antibody, or isotype controls. The cells were then incubated for 30 min with calcein-AM (0.125 μ M). RAW cells incubated with iron and/or lipopolysaccharide were treated with calcein-AM (0.125 μ M), MitoSOX (2.5 μ M), MitoTracker Deep Red (50 nM), or MitoTracker Green (50 nM) for 30 min at 37°C in DMEM. Flow cytometry was performed using a FACS Aria III machine (BD Biosciences, USA), and the results were analyzed using FlowJo software (TreeStar, USA). In all cases, the gating parameters were set to exclude doublets and nonviable cells.

Determination of the Ratio of Mitochondrial to Nuclear DNA

Total genomic DNA was isolated from RAW cells with a DNeasy blood and tissue kit (Qiagen, USA). Quantitative PCR was used to measure the amounts of cytochrome c oxidase I (CO1, a mitochondrial gene) and 18S ribosomal

DNA (18S, a nuclear gene), as previously described.²¹ The ratio of CO1 to 18S was used as a measure of the relative proportions of mitochondrial DNA (mtDNA) and nuclear DNA.

Statistics

For *in vitro* studies, the data are expressed as mean and SD of individual experiments replicated thrice. The data were tested for a normal distribution by the Shapiro–Wilk test and analyzed using Student's *t* test (or Mann–Whitney U test if the data were not normally distributed) or two-way ANOVA (iron $\times$ lipopolysaccharide). If the iron $\times$ lipopolysaccharide interaction was statistically significant, we applied all possible pairwise comparisons (Bonferroni *post hoc* tests). If the interaction was not statistically significant, we interpreted the main effects only and refrained from *post hoc* testing. For data that were not normally distributed, we used the Kruskal–Wallis test (with Dunn *post hoc* tests for all possible pairwise comparisons). For the sake of clarity, not all pairwise comparisons have been reported in the figures. Hypothesis testing was two-tailed. Values of $P < 0.05$ were considered statistically significant. Statistical analyses were performed using GraphPad Prism 7.0 (USA). Sample sizes for *in vivo* experiments were based on our prior experience with lipopolysaccharide injection without *a priori* power calculations. Conditions in the *in vivo* experiments were nonsequential, and processing of samples for the *in vivo* experiments was performed by investigators who were blinded to the experimental conditions.

Results

Iron Dextran Administration Increases Serum Iron and Intracellular Iron in Circulating Neutrophils and Monocytes

Injection of mice with iron dextran increased serum iron levels 10-fold (fig. 1A). Lipopolysaccharide injection alone reduced serum iron levels by more than 50%, consistent with previous reports.²² Mice that were treated with both iron and lipopolysaccharide had iron levels similar to those of mice injected with iron alone. Iron loading or lipopolysaccharide administration each increased hepatic hepcidin gene expression, as has been described by others²³ (fig. 1B). Intracellular labile iron levels in circulating neutrophils (Ly6G-positive cells) and circulating monocytes (CD11b-positive cells) were elevated as shown by decreased calcein fluorescence in iron-treated mice (fig. 1, C and D). These observations demonstrate that parenteral administration of iron dextran induces hepcidin production and increases intracellular iron levels in circulating neutrophils and monocytes.

Iron Administration Potentiates the Inflammatory Effects of Lipopolysaccharide

Iron administration alone did not induce inflammation in mice, as determined by the absence of increase in either

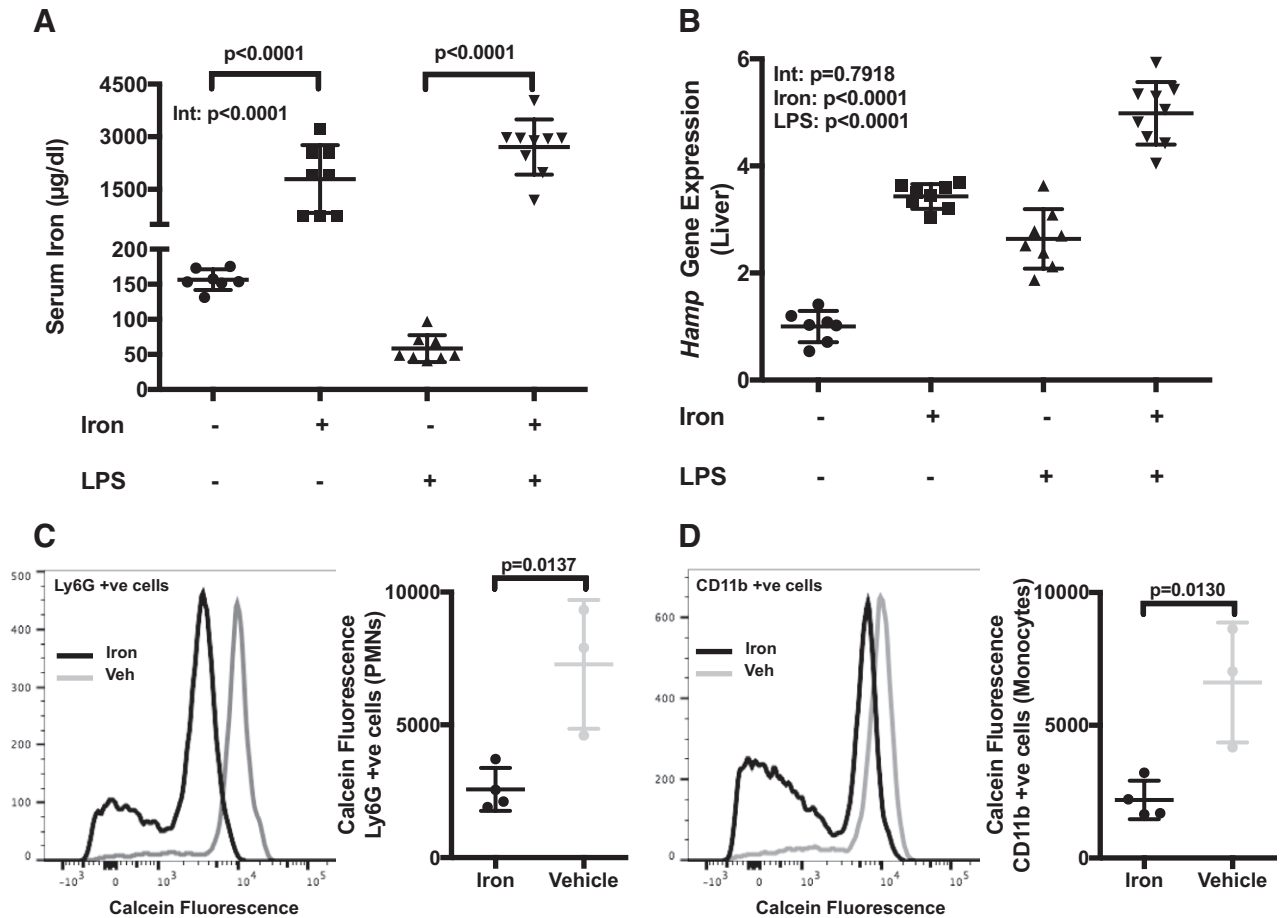


Fig. 1. Effects of parenteral iron overload in mice. (A) Mice treated with iron had significantly higher serum iron levels, while mice treated with lipopolysaccharide (LPS) alone became hypoferremic (two-way ANOVA with Bonferroni *post hoc* tests performed on $\log_{10}$ -transformed values; *P* values adjusted for all possible comparisons). (B) Iron and lipopolysaccharide independently induce hepcidin (*Hamp*) mRNA levels in the liver (two-way ANOVA, interaction between iron and lipopolysaccharide not significant, therefore only main effects reported). *N* = 7 to 9 mice/group for (A, B). (C, D) Iron injection increases intracellular labile iron in circulating neutrophils and monocytes. Both neutrophils (Ly6G-positive cells) and monocytes (CD11b-positive cells) from the iron-treated mice had lower calcein fluorescence intensities, consistent with higher intracellular iron levels. Representative histograms are from one vehicle-treated (Veh) and one iron-treated mouse. The dot plots show mean fluorescent intensities of 3 to 4 mice/group (unpaired *t* test). Int = interaction *P* value; PMN = polymorphonuclear leukocytes.

serum protein levels or mRNA levels (in liver and lungs) of the cytokines interleukin-6 and TNF α (fig. 2, A–F). A nonlethal lipopolysaccharide challenge induced a marked increase in serum interleukin-6 and TNF α levels, as well as the corresponding mRNA levels in mouse liver and lungs. Iron-treated mice challenged with lipopolysaccharide showed more than 5-fold higher serum cytokine levels than mice challenged with lipopolysaccharide alone. Similarly, the mRNA levels of *interleukin-6* and *Tnf α* in lungs and liver of mice treated with iron and lipopolysaccharide were between 1.5- and 2.5-fold greater than in mice treated with lipopolysaccharide alone. Iron treatment and subsequent lipopolysaccharide challenge did not alter mRNA levels of the antiinflammatory cytokine interleukin-10 in the lung compared to lipopolysaccharide challenge alone (supplemental fig. 1, Supplemental Digital Content 1, <http://links.lww.com/ALN/B431>). In control studies, we showed that

7.5% dextran (the vehicle for iron) did not have an independent proinflammatory effect on a subsequent stimulation with 5 mg/kg lipopolysaccharide (supplemental fig. 2, A and B, Supplemental Digital Content 1, <http://links.lww.com/ALN/B431>). Taken together, these observations indicate that parenteral iron administration strongly augments the proinflammatory response to lipopolysaccharide in mice.

Preincubation with Iron Augments Lipopolysaccharide-induced Cytokine mRNA Induction in Human Monocytes and RAW Cells

Human monocytes were found to have a more intense response to lipopolysaccharide stimulation after being iron-loaded (fig. 3, A and B). *In vitro* incubation of RAW cells with iron increased intracellular labile iron (nonferritin-bound, catalytically active iron) concentration in RAW cells. Deferoxamine, an iron chelator, was used as an assay control, demonstrating

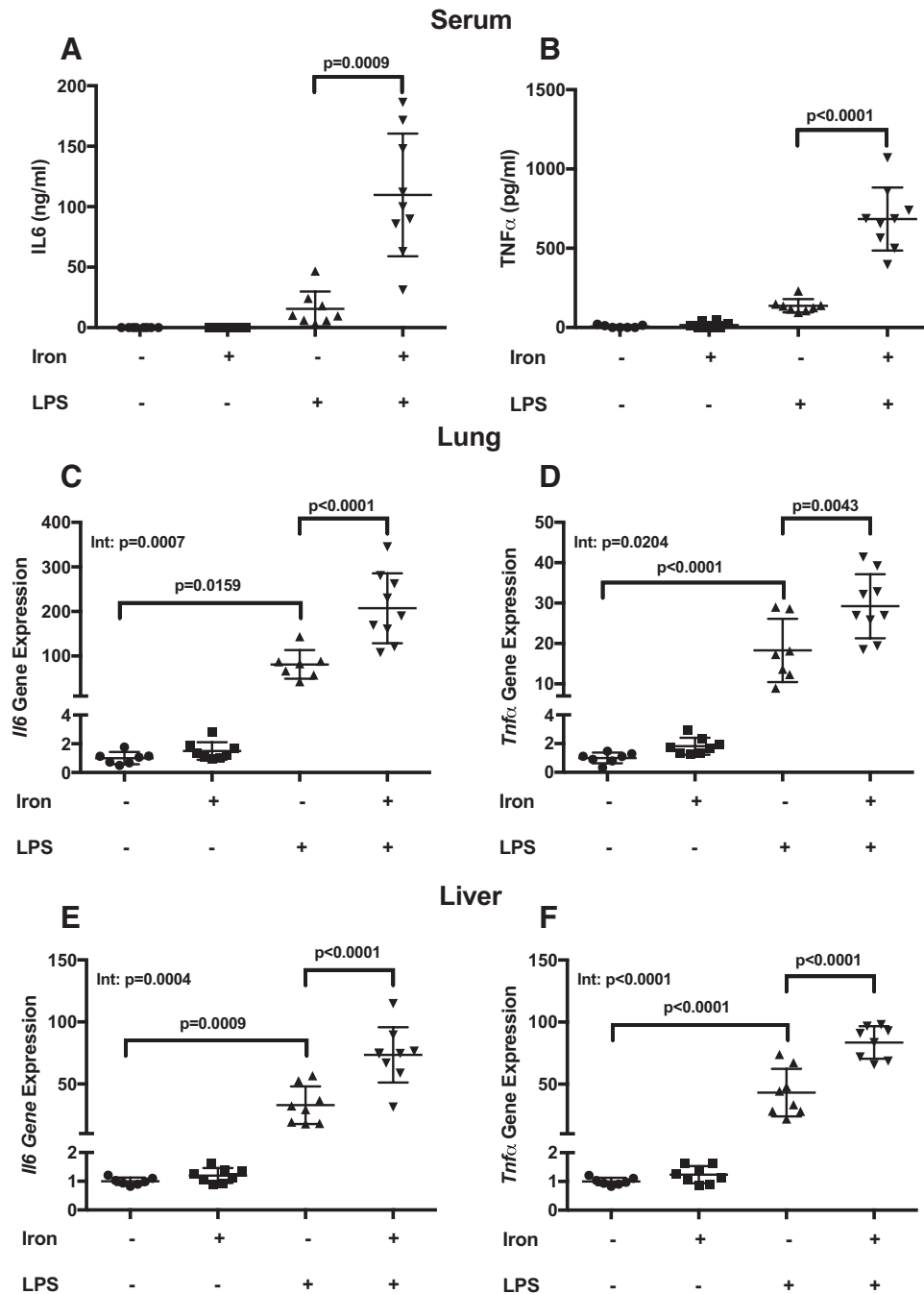


Fig. 2. Effect of parenteral iron administration on inflammatory gene expression and serum cytokine levels. Serum, lung, and liver samples were harvested from C57Bl/6 mice treated with iron and/or lipopolysaccharide (LPS) and analyzed for serum Il6 (A), serum Tnf α (B), and mRNA levels of the two cytokines in the lungs (C, D) and livers (E, F). In each case, iron supplementation by itself did not induce inflammation. Lipopolysaccharide alone induced increases in cytokine protein levels, as well as mRNA levels. Serum cytokine levels in the control and iron-treated groups were below the detection level of the assay in many cases. Iron administration significantly enhanced the effect of lipopolysaccharide-induced inflammation in the mice. $N = 7$ to 9 mice per group (Mann–Whitney U test [A, B] and two-way ANOVA with Bonferroni *post hoc* tests [C–F]; P values adjusted for all possible comparisons). Il = interleukin; Int = interaction P value; TNF, tumor necrosis factor.

the inverse relationship between cellular labile iron levels and calcein fluorescence (fig. 4A). Iron-loaded RAW cells stimulated with lipopolysaccharide had significantly higher mRNA levels of interleukin-6 and TNF α than cells treated with

lipopolysaccharide alone (fig. 4, B and C). Iron-loaded RAW cells stimulated with lipopolysaccharide also produced significantly more interleukin-6 protein than cells stimulated with lipopolysaccharide alone (supplemental fig. 3, Supplemental

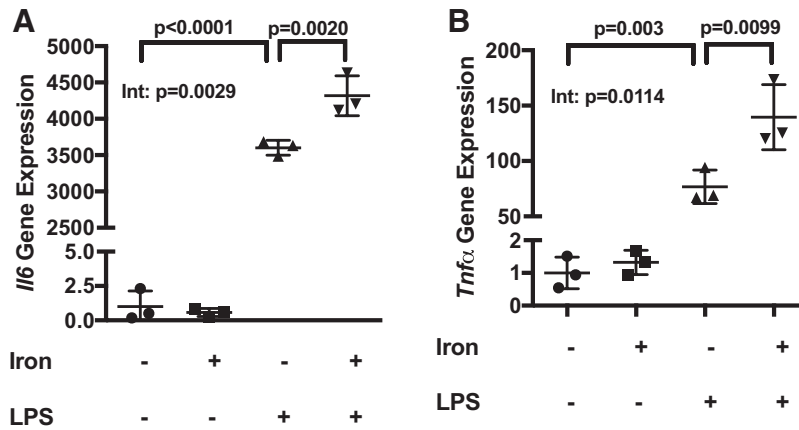


Fig. 3. Effect of iron loading on human monocytes *in vitro*. Human monocytes were isolated from peripheral blood and incubated in six-well plates. The cells were incubated with iron (200 μ M) or control overnight and then stimulated for 6 h with lipopolysaccharide (LPS; 150 ng/ml) or medium. Lipopolysaccharide alone induced *IL6* mRNA (A) and *Tnf α* mRNA (B). Iron loading significantly increased mRNA levels in response to LPS (two-way ANOVA with Bonferroni *post hoc* tests; *P* values adjusted for all possible comparisons; *n* = 3 replicates/condition). Int = interaction *P* value; IL = interleukin.

Digital Content 1, <http://links.lww.com/ALN/B431>). Iron-treated RAW cells challenged with lipopolysaccharide showed increased mRNA expression of the chemokine monocyte chemoattractant protein 1 (*Mcp1*) compared to cells treated with lipopolysaccharide alone (fig. 4D). Iron pretreatment with subsequent lipopolysaccharide reduced the expression of the antiinflammatory cytokine *interleukin-10* compared to cells treated with lipopolysaccharide alone (fig. 4E). Conversely, RAW cells preincubated with 30 μ M deferoxamine showed a blunted response to lipopolysaccharide (fig. 5, A and B). Of note, incubation of cells with 200 μ M iron had no adverse effects on cell viability, as determined by flow cytometric detection of nonviable cells (data not shown). These results demonstrate that iron loading augments the proinflammatory effect of lipopolysaccharide on RAW cells *in vitro* and that the use of an iron chelator blunts the cytokine response to lipopolysaccharide. Similarly, pretreatment of human monocytes with iron augments the proinflammatory effect of a subsequent exposure to lipopolysaccharide.

Response to Iron Loading In Vitro Differs Depending on the Type of Inflammatory Stimulus

We examined the effect of iron loading on the response of RAW cells to three additional proinflammatory mediators: P3C, PIC, and fMLF. P3C is a TLR2 agonist found in Gram-positive bacteria, PIC is a viral TLR3 agonist, and fMLF is a formylated peptide found in bacteria and mitochondria and an example of a damage-associated molecular pattern (DAMP). In contrast to the 2.5-fold increase in *interleukin-6* mRNA levels induced by iron and lipopolysaccharide, compared to lipopolysaccharide alone, iron-loaded cells stimulated with PIC did not show any significant increase in *interleukin-6* mRNA compared to cells treated with PIC alone. The combination of iron and P3C produced a 1.5-fold increase in *interleukin-6* mRNA compared to P3C alone. Iron together with the mitochondrial DAMP fMLF produced a 2.5-fold

increase in *interleukin-6* mRNA compared to DAMP alone (fig. 5C). These results suggest that the effects of iron loading on enhancing the inflammatory response are pathway specific and depend on the type of proinflammatory stimulus.

mtROS Contribute to the Proinflammatory Effect of Iron Loading

To determine the effect of iron loading on mtROS levels, RAW cells were incubated with iron and stained with MitoSOX, a fluorescent dye that specifically detects mitochondrial superoxide.²⁴ Compared to untreated RAW cells, RAW cells exposed to iron had a 40% increase in fluorescence intensity, suggesting that iron loading results in an increased level of intracellular mtROS. The combination of iron and lipopolysaccharide increased RAW cell mtROS levels by 50% compared to cells treated with lipopolysaccharide alone (fig. 6, A and B).

To consider the possibility that an antioxidant might blunt the proinflammatory effect of iron, RAW cells were incubated with MitoTEMPO (100 μ M), a mitochondria-specific antioxidant,¹⁹ before stimulation with lipopolysaccharide. Preincubation with MitoTEMPO significantly blunted (but did not abolish) the inflammatory response to lipopolysaccharide compared to similarly treated cells that were not exposed to MitoTEMPO (fig. 6, C and D), showing *interleukin-6* and *Mcp1* mRNA levels, respectively). These results demonstrate that iron induces mtROS in RAW cells and that inhibiting mtROS production diminishes the inflammatory response in iron-loaded cells treated with lipopolysaccharide. However, increased mtROS alone (as caused by iron loading) is not sufficient to increase inflammatory mRNA levels, as cells treated with iron alone did not express increased cytokine mRNA levels (fig. 4, B and C).

Iron Administration Modifies mRNA Levels of Genes Involved in Mitochondrial Homeostasis

Compared to control mice, mice treated with lipopolysaccharide had lower mRNA levels of genes associated with

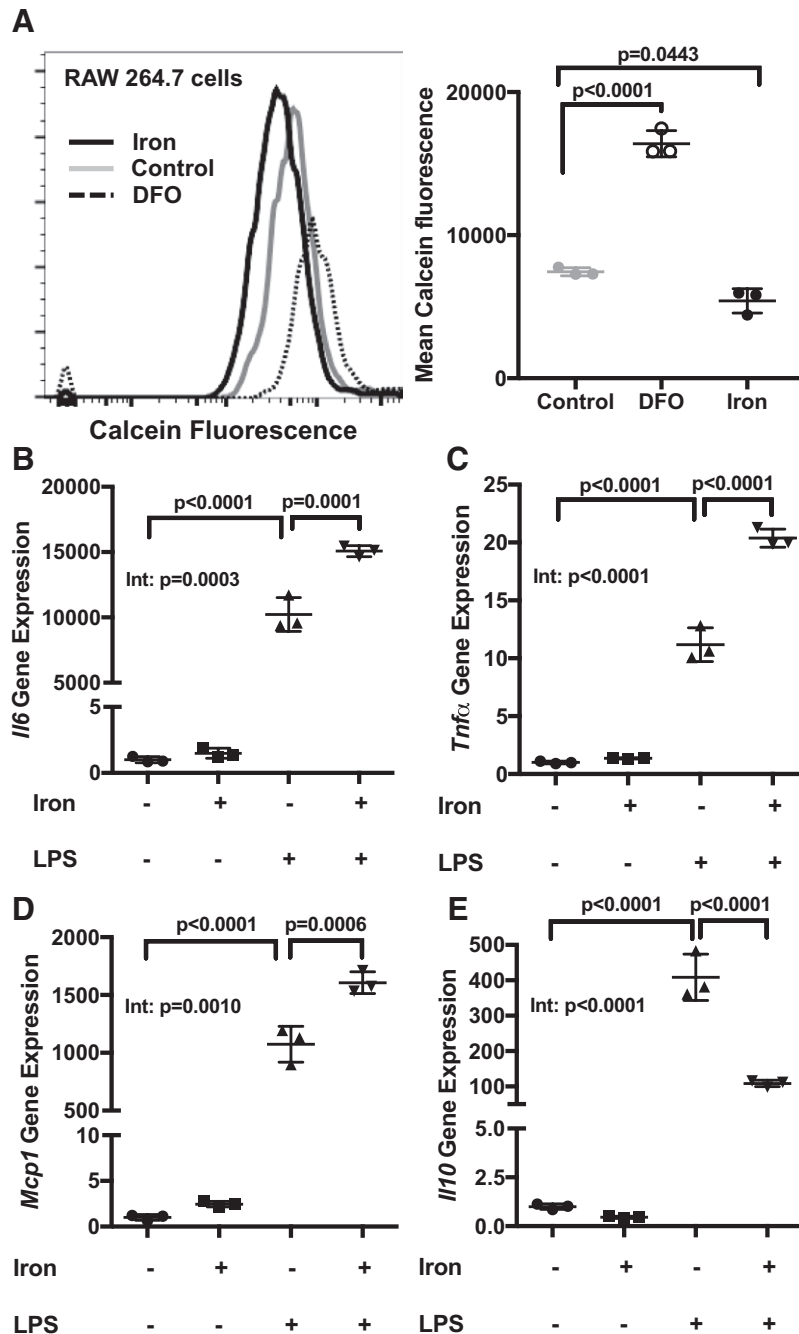


Fig. 4. Effect of iron loading on RAW 264.7 cells *in vitro*. (A) RAW cells treated with lipopolysaccharide (LPS), iron, or deferoxamine (DFO, an iron chelator) were incubated with calcein and subjected to flow cytometry, and calcein fluorescence was quantified. Calcein fluorescence decreased in iron-treated cells (one-way ANOVA with Bonferroni *post hoc* tests; *P* values adjusted for all possible comparisons). A representative histogram from one of three independent experiments is shown. The dot plots show mean fluorescent intensities of three replicates/condition. (B, C) RAW cells were incubated with iron (200 μ M) or control overnight and then stimulated for 6 h with lipopolysaccharide (150 ng/ml). Lipopolysaccharide alone induced *//6* and *Tnf α* mRNA. Iron loading significantly increased mRNA levels in response to lipopolysaccharide. (D) The combination of iron and lipopolysaccharide increased *Mcp1* mRNA levels to a greater extent than lipopolysaccharide alone. (E) Conversely, the combination of lipopolysaccharide and iron resulted in less *//10* mRNA levels than lipopolysaccharide alone (two-way ANOVA with Bonferroni *post hoc* tests [B–E]; *P* values adjusted for all possible comparisons; *n* = 3 replicates/condition). *Il* = interleukin; *Int* = interaction *P* value.

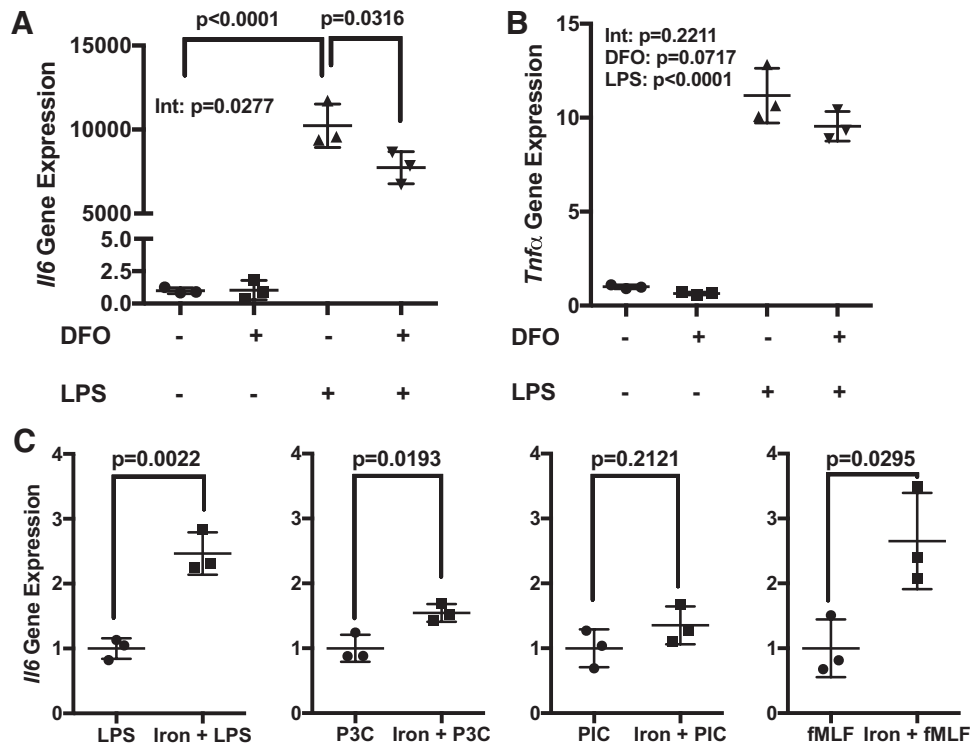


Fig. 5. (A, B) Effect of an iron chelator on RAW 264.7 cells *in vitro*. RAW cells were pretreated with 30 μ M deferoxamine (DFO, an iron chelator) and subsequently stimulated with lipopolysaccharide (LPS) for 6 h. Deferoxamine pretreatment decreases //6 mRNA induced by lipopolysaccharide significantly. There is a trend towards decreasing TNF mRNA in deferoxamine-pretreated cells that did not reach statistical significance (two-way ANOVA with Bonferroni *post hoc* tests; *P* values adjusted for all possible comparisons; *n* = 3 replicates/condition). (C) RAW cells were incubated with iron (200 μ M) or control overnight and then stimulated for 6 h with lipopolysaccharide (150 ng/ml), Pam-3-Cys (P3C; 1 μ g/ml), poly(I:C) (PIC; 25 μ g/ml), or *N*-formyl-L-methionyl-L-leuyl-L-phenylalanine (fMLF; 20 μ M). //6 gene expression was normalized to the response to lipopolysaccharide alone. The response to iron loading is not uniform across different proinflammatory agents. Iron loading augments the inflammatory response to lipopolysaccharide (a TLR4 ligand) and P3C (a TLR2 ligand), but not to PIC (a TLR3 ligand). Iron loading also augments the response to *N*-formyl-L-methionyl-L-leuyl-L-phenylalanine, a formylated peptide that is considered a damage-associated molecular pattern (unpaired *t* tests; *n* = 3 replicates/condition). // = interleukin; Int = interaction *P* value.

mitochondrial biogenesis (*Pgc-1 α* and *Ampk*; fig. 7, A and B) in the liver, but higher expression of *Lc3b*, a gene involved in mitophagy (fig. 7C). Conversely, iron-treated mice that were challenged with lipopolysaccharide had greater expression of *Pgc-1 α* and *Ampk* than mice treated with lipopolysaccharide alone (fig. 7, A–C), suggesting a shift towards either greater mitochondrial biogenesis or decreased mitophagy. We found similar changes in *Ampk* gene expression in mouse lungs and in RAW cells (supplemental fig. 4, A and B, Supplemental Digital Content 1, <http://links.lww.com/ALN/B431>). These results suggest that lipopolysaccharide alone induces a relative shift towards mitophagy, while iron pretreatment before lipopolysaccharide administration tilts the balance towards mitochondrial biogenesis.

Iron Loading Increases the Proportion of Nonfunctional to Total Mitochondria in RAW Cells

To examine the effect of iron treatment on the functional status of mitochondria, we stained RAW cells that were treated with iron and/or lipopolysaccharide with

MitoTracker Deep Red (a dye that stains functional mitochondria only) and MitoTracker Green (a dye that stains all mitochondria, functional and otherwise).¹⁸ The proportion of RAW cells stained with MitoTracker Green alone (*i.e.*, [MT Green – MT Deep Red]/MT Green) was significantly increased in RAW cells treated with iron compared to control cells and was also significantly higher in iron-loaded cells treated with lipopolysaccharide than in lipopolysaccharide-only cells (fig. 8, A and B). This suggests that iron loading increases the proportion of nonfunctional mitochondria in RAW cells.

Mitochondrial genomic DNA content, a measure of mitochondrial mass, was higher in iron-loaded RAW cells treated with lipopolysaccharide than in cells treated with lipopolysaccharide alone (fig. 8C). The mtDNA content in iron-loaded cells that were not treated with lipopolysaccharide was similar to lipopolysaccharide-treated cells that were not iron-loaded. Taken together, these data suggest that iron-loaded cells stimulated with lipopolysaccharide have both a higher mitochondrial mass and a greater proportion

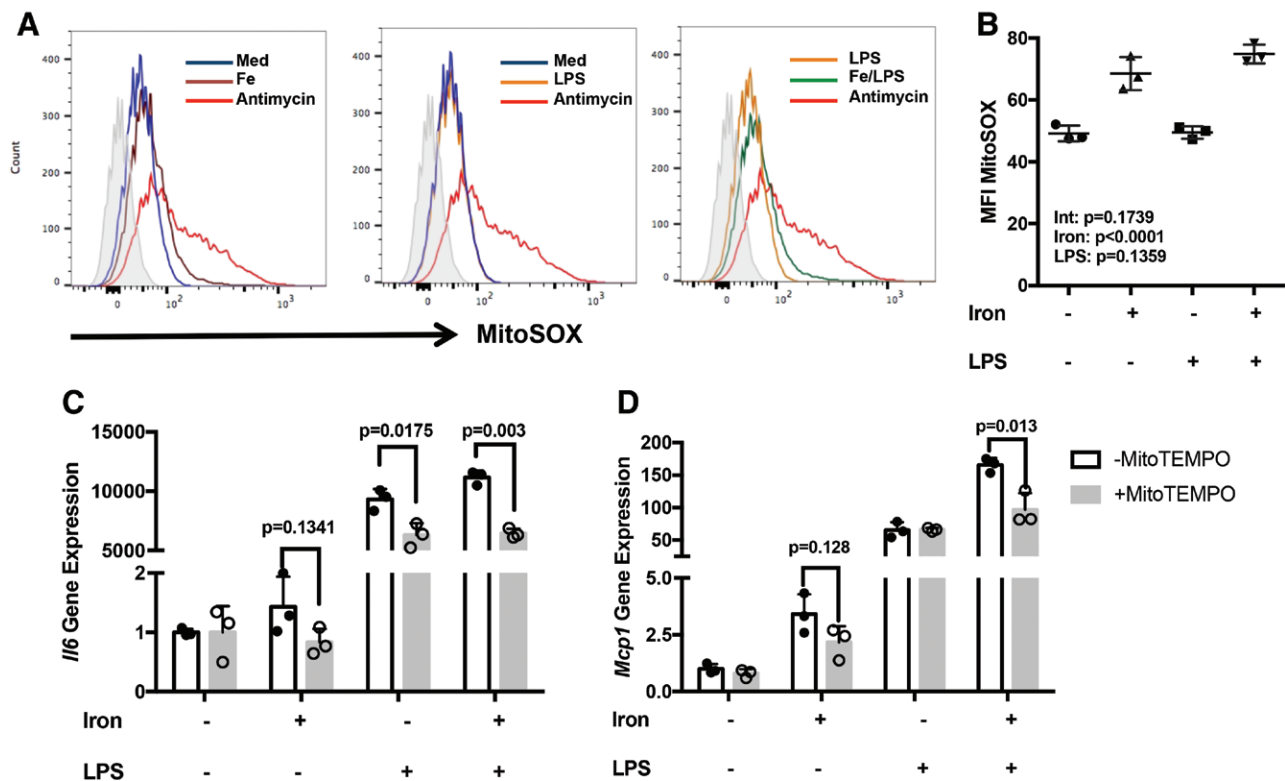


Fig. 6. The effect of iron and lipopolysaccharide (LPS) on mitochondrial reactive oxygen species production. (A) Effect of iron supplementation on mitochondrial reactive oxygen species production. The shaded histogram depicts unstained cells. The histogram in red shows the effects of antimycin (20 μ g/ml), an inhibitor of mitochondrial complex III used as a positive control. Iron-naïve cells treated with lipopolysaccharide do not show increased MitoSOX fluorescence compared to controls (*middle*). However, iron-pretreated RAW cells exposed to lipopolysaccharide have more mitochondrial reactive oxygen species production compared to cells treated with lipopolysaccharide alone (*right*), although the increase is similar to that caused by iron incubation alone (*left*). Data are representative of three experiments. (B) Summary bar graphs showing relative MitoSOX fluorescence (two-way ANOVA; interaction between iron and lipopolysaccharide not significant, therefore only main effects reported; $n = 3$ replicates/condition). (C, D) Incubating RAW cells with 100 μ M of the mitochondrion-specific antioxidant MitoTEMPO blunted the mRNA response to lipopolysaccharide (*Il6* and *Mcp1*) in iron-loaded macrophages (unpaired t tests; $n = 3$ replicates/condition). Int = interaction P value; Med = medium; MFI = mean fluorescent intensity.

of nonfunctional mitochondria than cells that were treated with lipopolysaccharide alone.

Discussion

In this study, we observed a strong proinflammatory effect of iron loading on a subsequent challenge with lipopolysaccharide, *in vivo* and *in vitro*. Parenteral iron loading increased intracellular labile iron in circulating neutrophils and monocytes and strongly increased the cytokine response to a subsequent lipopolysaccharide challenge. Similarly, the addition of iron to RAW cells increased intracellular labile iron and further augmented the lipopolysaccharide-induced increase in mRNA levels of proinflammatory genes. As with RAW cells, iron-loaded human peripheral blood monocytes also had higher inflammatory cytokine mRNA levels after lipopolysaccharide stimulation. Iron loading induced mtROS, and inhibition of mtROS formation blunted the augmented response to lipopolysaccharide in iron-loaded RAW cells. Iron loading in conjunction with lipopolysaccharide stimulation

also increased the expression of genes associated with mitochondrial biogenesis *in vivo* (in liver and lungs) and increased mitochondrial genomic DNA *in vitro* (in RAW cells), suggesting that iron loading alters mitochondrial homeostasis. Iron loading increased the proportion of nonfunctional mitochondria in RAW cells. Taken together, the data suggest that a combination of iron loading together with an inflammatory stimulus results in an increased proportion of defective mitochondria and increased mtROS production.

Anesthesiologists often provide care for patients given parenteral iron supplements before major surgery and for critically ill patients in intensive care units, who may be iron-loaded from blood transfusion or iron therapy. Thus the effect of acute iron loading on the inflammatory response is of particular relevance in perioperative medicine, especially because there are few studies that have examined the effects of iron infusions on outcomes.²⁵

Wang *et al.*²⁶ used a mouse model of hemochromatosis (*Hfe* knockout mice) to show that low intracellular labile iron in *Hfe*

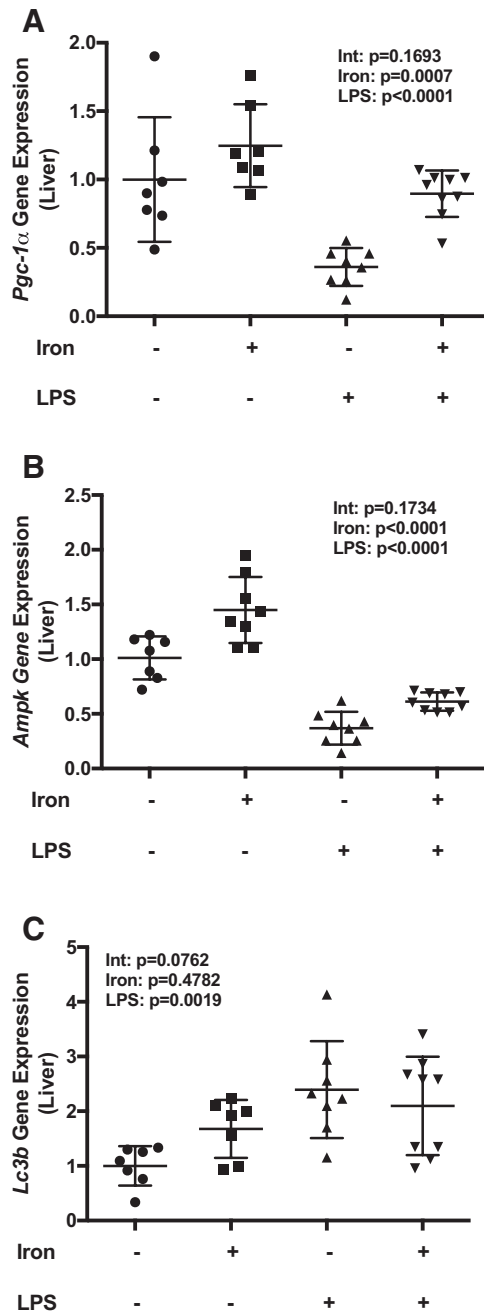


Fig. 7. Iron loading changes the balance between genes responsible for mitochondrial biogenesis and mitochondrial autophagy (mitophagy). C57Bl/6 mice were treated with iron and/or lipopolysaccharide (LPS); $N = 7$ to 9 mice per group. Total RNA was extracted from mouse liver, and quantitative reverse transcription-polymerase chain reaction was performed using primers for *Pgc-1α* (a mitochondrial biogenesis-associated gene [A]), *Ampk* (a mitochondrial biogenesis-associated gene [B]), and *Lc3b* (a mitophagy-associated gene [C]). Lipopolysaccharide alone increases *Lc3b* mRNA levels, while decreasing *Pgc-1α* and *Ampk* mRNA levels. Iron-loaded mice stimulated with lipopolysaccharide had the opposite profile, with an increase in the mRNA levels of the mitochondrial biogenesis-associated genes *Pgc-1α* and *Ampk* (two-way ANOVA; interaction between iron and lipopolysaccharide not significant, therefore only main effects reported). Int = interaction P value.

KO macrophages caused a diminished inflammatory response to lipopolysaccharide. In addition, pretreatment of macrophages from wild-type mice with an iron chelator reduced the inflammatory response to lipopolysaccharide.²⁶ Other studies showed that the iron chelator deferoxamine attenuated the inflammatory response to lipopolysaccharide in RAW cells and decreased inflammation in mouse models of endotoxemia⁹ and peritonitis.²⁷ In contrast, Pagani *et al.*¹⁰ found that iron-deficient mice (with low macrophage iron levels) had a greater inflammatory response to lipopolysaccharide compared to iron replete mice. De Domenico *et al.*¹¹ found that oral iron supplementation followed by a lipopolysaccharide challenge blunted the response of mice to lipopolysaccharide. In both of the last two studies, the authors attributed their findings to antiinflammatory effects of the iron-regulating hormone hepcidin. Iron supplementation increases hepcidin production, while low hepcidin production in iron-deficient mice induces an exaggerated response to lipopolysaccharide. However, the mechanism proposed for the presumptive antiinflammatory effect of hepcidin (activation of the janus kinase-signal transducer and activator of transcription pathway by hepcidin-ferroportin binding¹¹) has since been questioned.²⁸ In this study, we demonstrated a robust proinflammatory response to iron loading in spite of an increase in hepcidin gene expression (fig. 1B). The mode of iron supplementation (enteral and parenteral), as well as the formulation of iron, may impact the bioavailability of iron and hence intracellular iron concentrations,^{7,29} possibly accounting for differences between our study and that of De Domenico *et al.*

We found that the response to iron loading differs depending on the type of proinflammatory stimulus, suggesting the presence of specific pathways that are influenced by intracellular iron. Indeed, Wang *et al.*²⁶ found that intracellular iron influences lipopolysaccharide signaling specifically by modifying the MyD88-independent adaptor toll/interleukin-1 receptor domain-containing adaptor inducing interferon beta-related adaptor molecule-related response to lipopolysaccharide.

To further examine the mechanisms responsible for the proinflammatory effects of iron loading, we measured mtROS production. Iron loading increased mtROS production in RAW cells, and a mitochondrial-specific antioxidant (Mito-TEMPO) blunted the proinflammatory effect of iron on RAW cells, reducing cytokine (*interleukin-6*) and chemokine (*Mcp1*) mRNA levels to those of macrophages treated with lipopolysaccharide alone. These findings suggest that iron-induced mtROS may have a “priming” effect on macrophages, augmenting the response to a subsequent lipopolysaccharide challenge. In spite of the lack of a demonstrated increase in mtROS production with lipopolysaccharide, lipopolysaccharide stimulation appears to increase mtROS production, because treatment with Mito-TEMPO blunts the inflammatory response to lipopolysaccharide alone. These results are consistent with findings reported by Bulua *et al.*,³⁰ who found that mouse embryonic fibroblasts have a decreased response to lipopolysaccharide when pretreated with a different mitochondrial superoxide inhibitor, MitoQ. Bulua

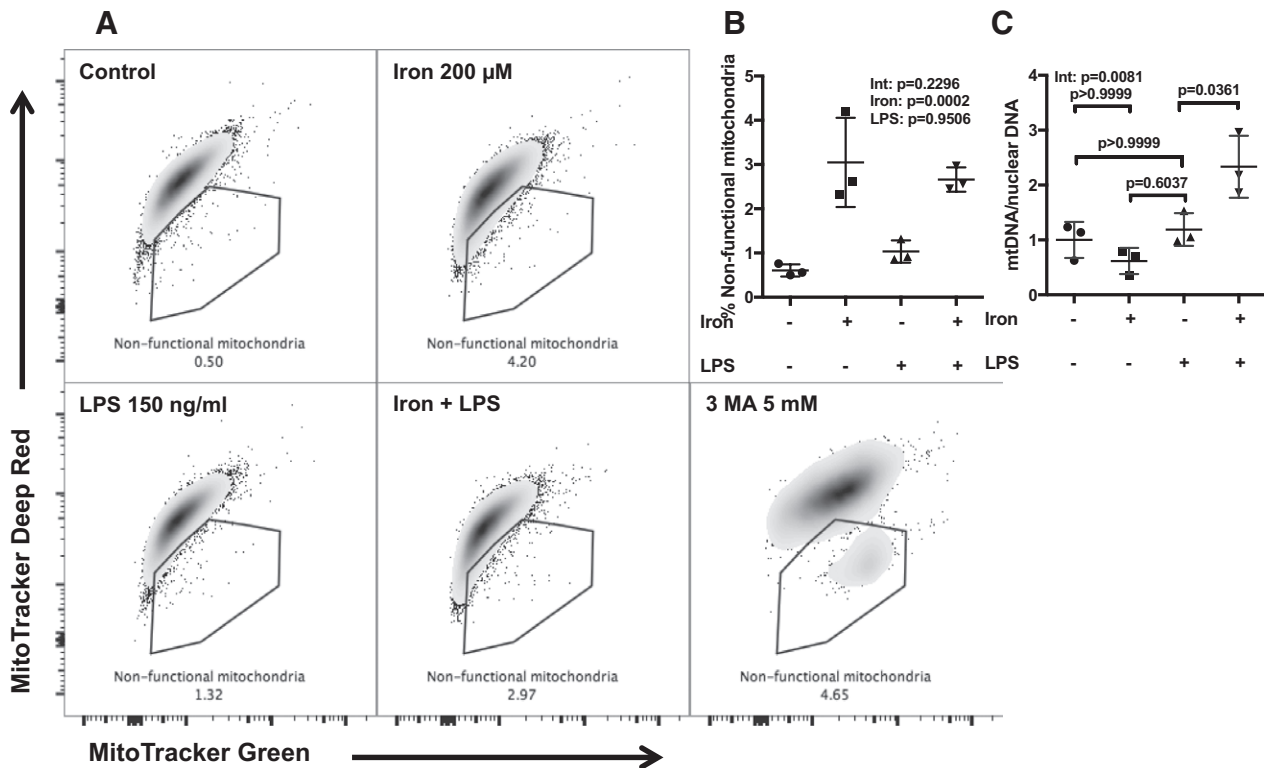


Fig. 8. Iron loading increases the proportion of nonfunctional mitochondria in RAW 26.7 cells. RAW cells were treated with iron and/or lipopolysaccharide (LPS), then incubated with MitoTracker Deep Red and MitoTracker Green, and subjected to flow cytometry. (A) Density plots for each condition, representative of three independent experiments. The gate shown contains the cells that are MitoTracker DeepRed-negative/MitoTracker Green-positive cells (nonfunctional mitochondria). 3-Methyladenine (3 MA), an inhibitor of autophagy, was used as a positive control. (B) A summary histogram of the cytometry results (two-way ANOVA; interaction between iron and lipopolysaccharide not significant, therefore only main effects reported; $n = 3$ replicates/condition). (C) Iron-loaded, lipopolysaccharide-treated RAW cells have higher mitochondrial mass defined by mitochondrial gene: nuclear gene ratio (two-way ANOVA with Bonferroni *post hoc* tests; P values adjusted for all possible comparisons; $n = 3$ replicates/condition). Int = interaction P value; mtDNA = mitochondrial DNA.

*et al.*³⁰ also did not find increased MitoSOX staining in the fibroblasts treated with lipopolysaccharide alone.³ We speculate that while lipopolysaccharide does increase mtROS production, the effects of lipopolysaccharide on mtROS levels may be opposed by increased mitophagy, resulting in no change in net mtROS levels.

Other studies have found that increased intracellular iron increased mtROS production and that mtROS was associated with increased inflammation in different cell types, such as cardiomyocytes³¹ and macrophages.³² The results of this study therefore add to the existing literature by showing that iron loading potentiates inflammation by augmenting mtROS production.

Although the *in vitro* data in our study were derived from monocytes and macrophages, it is possible that iron loading may impact ROS production by neutrophils, which are a major source of ROS *in vivo*.³³ Sampaio *et al.*³⁴ showed that iron dextran administration in a streptozotocin-induced model of diabetes in rats was associated with a strong increase in neutrophil ROS production. Iron loading may therefore be proinflammatory in both monocytes and neutrophils.

Iron-loaded cells exposed to lipopolysaccharide had a greater mitochondrial mass, as determined by the relative abundance of mitochondrial DNA to nuclear DNA. Of note, we did not find a significant difference in the proportion of mtDNA to nuclear DNA between iron-loaded cells and lipopolysaccharide-treated cells. Iron loading RAW cells increased the proportion of nonfunctional mitochondria relative to total mitochondria. Others have shown that damaged or nonfunctional mitochondria produce more mtROS.^{18,35} Mitochondrial mass can be increased by increasing mitochondrial biogenesis, decreasing mitophagy, or both. In this study, the combination of iron and lipopolysaccharide increased mRNA levels of genes involved in mitochondrial biogenesis, *Pgc-1 α* and *Ampk* *in vivo*. Lipopolysaccharide-treated mice had higher expression of hepatic *Lc3b*, a gene involved in mitophagy, which is consistent with prior data reporting that lipopolysaccharide increases mitophagy.³⁶ The data therefore suggest that the combination of iron loading and lipopolysaccharide stimulation increases mitochondrial biogenesis *in vivo*. Of note, increased mitochondrial biogenesis is not necessarily deleterious; some studies have shown

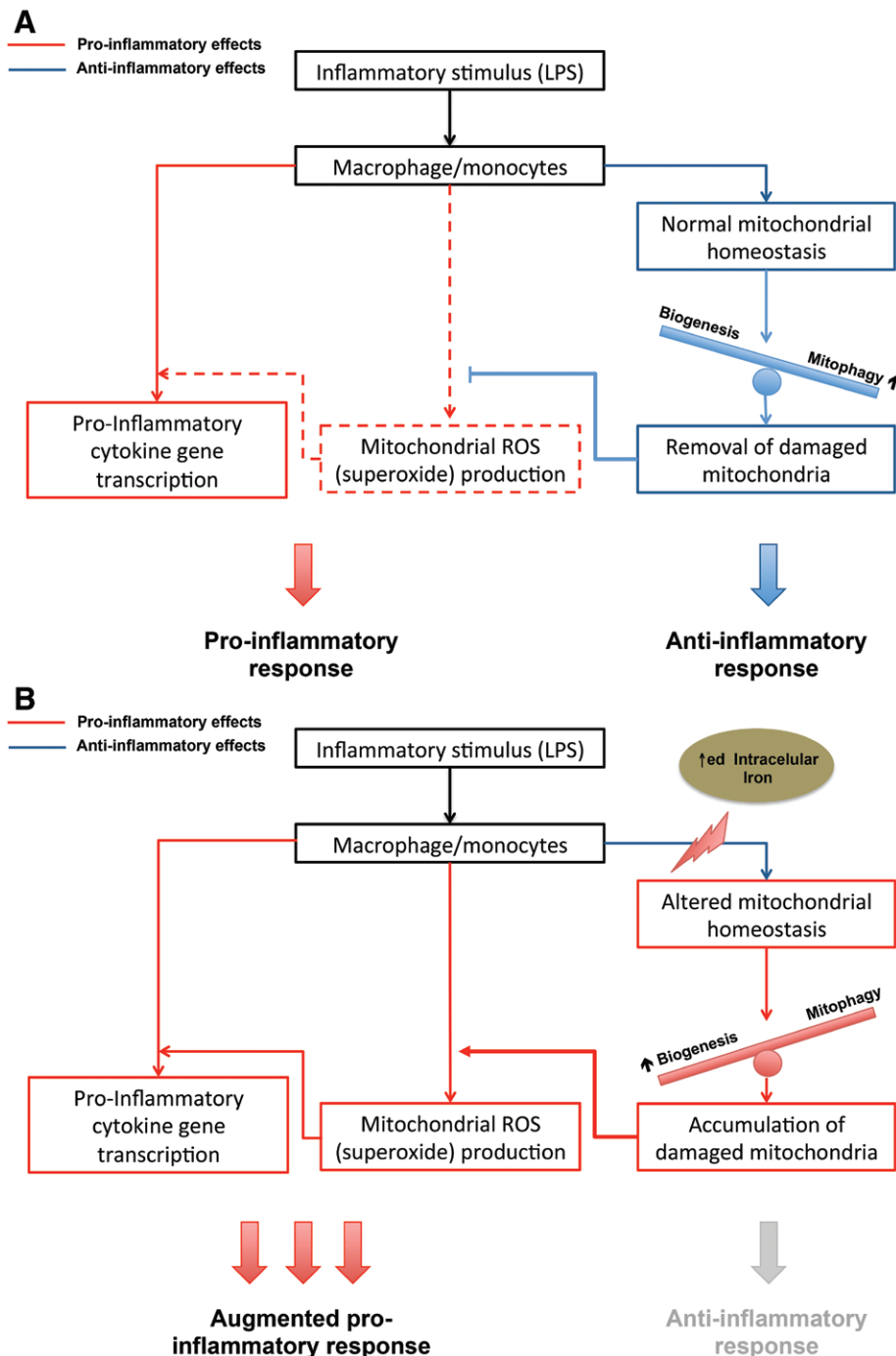


Fig. 9. A schematic view of the role of mitochondrial homeostasis (the balance between mitochondrial biogenesis and mitophagy) in the acute inflammatory response. (A) Lipopolysaccharide (LPS)-induced inflammation induces inflammatory gene transcription. Simultaneously, maintenance of mitochondrial homeostasis allows removal of damaged mitochondria by mitophagy (mitochondrial “quality control”), resulting in a blunted inflammatory response, which may be why mitochondrial reactive oxygen species (ROS) does not appear to increase in lipopolysaccharide-stimulated cells. (B) In the presence of excess intracellular iron, the balance of mitochondrial homeostasis shifts towards increased biogenesis and less effective mitophagy, resulting in an accumulation of damaged mitochondria, increased mitochondrial reactive oxygen species, and an augmented proinflammatory effect.

that mitochondrial biogenesis imparted a prosurvival phenotype in acute inflammatory states.³⁷

Studies using mouse models of defective mitophagy showed that accumulation of nonfunctional mitochondria potentiated mtROS formation and induced a more

potent inflammatory response to innate immune stimulants, including lipopolysaccharide.^{18,21,38} Duvigneau *et al.*³⁹ showed that endotoxin-induced iron accumulation in cells was associated with altered mitochondrial respiration and mitochondrial dysfunction. Lowering intracellular

iron levels using iron chelators induced mitophagy in a *Caenorhabditis elegans* model of *Pseudomonas* infection,⁴⁰ while iron loading promoted mitochondrial biogenesis in osteoclasts.⁴¹ These reports are consistent with our finding that intracellular iron levels modulate mitochondrial homeostasis.

This study has some limitations. We used iron dextran, rather than iron sucrose or iron gluconate, because iron dextran was previously shown to be the least likely to cause direct iron-induced toxicity.⁴² Because we did not test other forms of iron *in vivo*, we cannot comment on the effects of other iron formulations. A second potential limitation is that we examined the effects of increased intracellular iron on the early inflammatory response and thus cannot comment on the effect of iron loading on the temporal course of inflammation. In addition, while we have found that iron loading has a proinflammatory effect on macrophages, we did not investigate the effect of iron loading on macrophage phenotype, although others have shown that iron loading induced an M1 phenotype in macrophages.⁴³ In RAW cells, we observed a decrease in *interleukin-10* mRNA in response to iron loading, but a similar effect was not observed in murine lungs *in vivo* (supplemental fig. 1, Supplemental Digital Content 1, <http://links.lww.com/ALN/B431>). Finally, our data demonstrating the effects of iron loading on mitochondria function are limited to *in vitro* assays. Further work is needed to determine whether iron loading alters mitochondrial function *in vivo*.

The results of this study suggest that increased intracellular iron leads to an increased proinflammatory response to the TLR4 ligand lipopolysaccharide, raising the possibility of targeting intracellular iron as a therapeutic modality in acute inflammatory states. However, many questions need to be addressed before intracellular iron can be considered a viable biologic target. Critically ill patients are often hypoferremic, and iron chelators will likely exacerbate hypoferrmia. Therapy with iron chelators^{44,45} may increase the risk of bacterial infections by organisms that can extract iron from the iron-chelator complex. Finally, we do not currently have reliable assays for monitoring intracellular iron levels to guide therapy.

Conclusions

Our results suggest that iron loading alters mitochondrial homeostasis, leading to the accumulation of defective mitochondria and increasing production of mtROS. The production of mtROS primes macrophages for a “second hit,” such as exposure to lipopolysaccharide, greatly augmenting the inflammatory response to the second stimulus. Figure 9 presents a schematic of the role increased intracellular iron plays in modulating the acute inflammatory response. The data presented here highlight the proinflammatory effects of iron loading in acute inflammation and suggest that clinicians should consider the risks of treatments that result in iron loading in acutely ill patients.

Acknowledgments

We gratefully acknowledge advice on statistical methods from Timothy T. Houle, Ph.D., Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital, Boston, Massachusetts.

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Competing Interests

Dr. Bagchi is a consultant for Lungpacer Medical Inc., Burnaby, British Columbia, Canada. The other authors declare no competing interests.

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Address correspondence to Dr. Bagchi: Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital, 55 Fruit Street, White 434B, Boston, Massachusetts 02114. abagchi@mgh.harvard.edu. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

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ANESTHESIOLOGY REFLECTIONS FROM THE WOOD LIBRARY-MUSEUM

Laughing Gas in Baltimore, Hagerstown, and Smithsburg: Maryland's Dr. D. W. Crowther



Son of an English army officer, David William Crowther, D.D.S. (1834 to 1916), was born in Devonshire but relocated as a baby with his family to Drummondville, Canada. As a young man, he subsequently moved to New York and then Alabama to serve from Mobile in the Confederacy's First Alabama Battery. Following the Civil War, he trained in dentistry, earning his D.D.S. in Maryland in 1868 from the Baltimore College of Dental Surgery. Around 1874 he moved 74 miles northwest in Maryland to Hagerstown, where he established his dental practice on North Potomac Street. According to this lovely trade card from the Ben Z. Swanson Collection of the Wood Library-Museum, Dr. Crowther extracted teeth "with [laughing] gas" just a "door above Clapp's Junior Hall Store." Never forsaking Maryland professionally, Crowther moved in 1890 about 8 miles east to Smithsburg. He retired from dentistry in 1896 and passed away a decade later. All told, during his 28 yr of practice as a degreed dentist, Crowther administered laughing gas in Baltimore, Hagerstown, and finally Smithsburg, Maryland. (Copyright © the American Society of Anesthesiologists' Wood Library-Museum of Anesthesiology.)

George S. Bause, M.D., M.P.H., Honorary Curator and Laureate of the History of Anesthesia, Wood Library-Museum of Anesthesiology, Schaumburg, Illinois, and Clinical Associate Professor, Case Western Reserve University, Cleveland, Ohio. UJYC@aol.com.

Title: **Manipulating the macrophage iron-hepcidine axis to prevent acute lung injury/acute respiratory distress syndrome secondary to severe trauma/hemorrhagic shock**

Sponsor Name: **None**

PI Name: **Bagchi, Aranya**

Protocol #: **2018N000105**

Type: **Initial Review**

Species: **Mice**

Of Animals: **328**

Date Received: **July 11, 2018**

Study Staff

Name	Role	Degree	Organization
Bagchi, Aranya	Principal Investigator		MGH > Anesthesia
Hayashida, Kei	Co-Investigator	MD, Ph.D	MGH > Anesthesia
Ichinose, Fumito	Co-Investigator	MD	MGH > Anesthesia
Nagashima, Fumiaki	Administrator		MGH > Anesthesia

Response to Review 1

Principal Investigator:

Bagchi, Aranya

Protocol Number: (2014N000987)

2018N000105

Study Title:

Manipulating the macrophage iron-hepcidine axis to prevent acute lung injury/acute respiratory distr

Response to:

- ☒ Initial Review
- ☐ Amendment
- ☐ Annual Review
- ☐ Triennial Review

IACUC Review Date: (MM/DD/YYYY)

07/18/2018

Response Date: (MM/DD/YYYY)

08/10/2018

Please respond to the IACUC questions by typing your response under each question, formatted in a way that distinguishes the question from the answer.

This facilitates the review effort and makes your file very clear in the event of external review.

1. In #1 of the Abstract, please define ARDS (acute respiratory distress syndrome) when it first appears.

The following information has been added: Acute respiratory distress syndrome (ARDS) is a rapidly progressive disease occurring in critically ill patients. ARDS can result from a variety of insults such as trauma, infection and sterile inflammation. It is clinically defined as the presence of acute hypoxemia and bilateral lung infiltrates on a chest x-ray.

2. What is the ratio of the male and female C57BL/6 mice will be used in the protocol, especially in the Experiments 1 to 3? If both genders are used, and if there is a difference based on gender, your n for some experiments is very low (n=3). You could consider starting the project using one gender and add more animals by amendment at a later date.

As advised, we will start the project using male mice alone. If we need to add female mice, we will amend the protocol to include them.

3. Please include the reference of the published model of non-lethal mouse polytrauma for review. Is the method of cecectomy you describe an established model? Cecal ligation puncture seems to be a better established model compared what you are describing.

Reference is as below:

Lori F. Gentile, et al: Identification and description of a novel murine model for polytrauma and shock. Crit Care Med 2013; 41: 1075-1085

The purpose of including cecectomy in the model is to model human polytrauma, which typically includes injury to several systems, and that can be used for subacute experiments.

4. You state Mice are fixed on a surgery board, the tongue is pulled out with a forceps, and the LPS/saline mixture is placed onto the distal part of the oropharynx while the nose was gently closed. The liquid will be aspirated to the lungs by spontaneous respiration. After that, mice will be returned to their holding cage. Is this an established technique? Please provide a reference. It is likely this will just be swallowed by the mouse. Nasal instillation or intubation may be more appropriate.

A reference for this technique is provided below:

Christine Egger et al: Administration of Bleomycin via the Oropharyngeal Aspiration Route Leads to Sustained Lung Fibrosis in Mice and Rats as Quantified by UTE-MRI and Histology. PLOS ONE 2013; 8: 1-13.

Please also see appendix #1 where we show that instilling Evans Blue dye by this method results in reliable staining of both lungs.

5. You state Because of the induction of trauma/HS, moderate systemic inflammation

will occur in mice. But as mention above, it is not lethal and mice can maintain the ability to ambulate and groom as well as feed and drink. And after the 24 hours, mice will be administrated LPS via intratracheal as a second hit. Then, lung inflammation will be induced severely and continue to the next 24 hours, which is the time point of sacrifice. So most critical phase is after the intratracheal administration of LPS as a second hit. But, after the induction of trauma/HS is also critical phase, so we will continuously observe these animals after procedures. How do you plan to continuously monitor mice for 54 hours? Will they be returned to the CCM facility? Will they remain in your lab? Are you requesting lab housing? Most of surgical procedures and treatments will be performed in THR 505.

Post-surgical follow up care will be provided by Fumiaki Nagashima.

Mice after Trauma/HS will be monitored continuously in Thier Room 505 until 8 h post Trauma/HS. If mice do not recover at 8 h after Trauma/HS, mice will be euthanized. Mice survive for 8 h after Trauma/HS will be housed in Thier Room 505 (an IACUC approved satellite mouse housing facility) or Room B04 until next morning. Mice will be monitored every 8-10 hours during the post-procedure period in the storage facility. 24 h after Trauma/HS, Mice will be imaged by PET-CT. After PET-CT imaging, mice will be monitored until next 6 h in thier room 505. 6 h after PET-CT imaging, Mice will be induced lung injury by administration of LPS. Then, mice will be monitored continuously in Thier room 505 until 6 h after lung injury. If mice do not recover at 6 h after lung injury, mice will be euthanized. Mice survive for 6 h after lung injury will be housed in room Thier 505 or B04 until next morning. They will be monitored every 8-10 hours while in the storage facility. Next morning, Mice will be brought back to the room 505, and monitored continuously until sacrificed.

6. These mice will undergo PET-CT imaging in Simches Research Center, Suite 5.210. who will perform the mice transfer? Where these mice will be held, Simches or Thier building after PET-CT imaging?

We will transfer the mice to and from the Simches Research Center. These mice will be held Their building after PET-CT imaging.

7. You have checked Moribund as an Endpoint (Section 3 of the Standard Application form). Do you expect or intend any of these animals to reach a moribund state? If not, please uncheck this box. If so, please specifically designate which mice these would be, as they may need to be reclassified as Category E animals. Please discuss with the IACUC office or with your CCM clinical veterinarian if you have any questions.

We have corrected the application form to show that we do not plan to use a moribund state as an end point. We will use a modified version of the Murine Sepsis Severity Score (Ref: A robust scoring system to evaluate sepsis severity in an animal model. BMC Research Notes 2014;7:233).

The following criteria will define mice that will be euthanized to prevent undue suffering:

1. Piloerection in majority of back, or a generalized puffy appearance

2. Activity severely impaired mouse remains stationary when provoked with possible tremors.
3. Loss of righting reflex
4. Eyes closed or milky
5. Severely reduced respiration (>0.5 sec between breaths)
6. Respiration labored with intermittent gasps

8. You state you will be trained by Dr. Ichinose. He is not on this protocol. How will he train if you if he cant touch the animals on this protocol? The committee notes that there are several experienced and qualified staff members in your lab. The committee recommends you add more experienced personnel to this protocol that can assist in the procedures and monitoring of this intense model. It appears that one person is doing almost all the work and he has minimal experience with this species.

We will add Dr. Fumito Ichinose and Dr. Kei Hayashida to the protocol, both of whom have extensive experience with similarly complex models.

9. The committee recommends you meet with the Thier Veterinarian, Andrea Slate, ASLATE1@mgh.harvard.edu, before protocol is resubmitted to discuss the details of invasive procedures and your clinical endpoints as they are unclear.

We met with Dr. Slate on 7/27/18, and have incorporated her suggestions into the revised protocol

10. Please clarify if you will buprenex if pain is noted or every 8-12 hour for the duration of the study.

Mice will be closely monitored by Fumiaki Nagashima and buprenox will be planned to administrated every 8-12 hours. But, If mice exhibited signs of pain (self mutation, inability to walk, eat, or drink), additional analgesics (buprenorphinebuprenorphine 0.1 mg/kg, SC) will be administrated.

11. BCS is unlikely to change rapidly in 54 hours. A change in the animals activity level and ability to obtain food and water will be more accurate predictors on impending death.

Please see our response to point #7 for a detailed description of the metrics we will use to define pain and distress in the experimental animals.

12. Does anyone on the study staff have experience canalizing femoral arteries in mice, and performing cecectomy? How much blood will be removed?

Kei Hayashida, who has been included in the protocol, and Aranya Bagchi , PI of this project have experience canalizing femoral arteries in mice and with performing surgical procedures such as cecectomy. We will calculate estimated circulating blood

volume of mice using calculation formula 75ml/kg, and then, about 30% of blood will be removed to induce hemorrhagic shock.

13. Deferiprone is only mentioned in the Procedure form for Inhalational treatment with iron chelator and in no other place. Do you plan to use this? Please note that all non-pharmaceutical grade agents must be listed in the Pharmacologic Agents form.

We don't have plan to use deferiprone for now. If we need to use deferoprone, we will amend the protocol.

14. Please provide the funding information.

Fund # 230636. Grant title: "Manipulating the Macrophage Iron-Hepcidin Axis to Prevent Acute Lung Injury/Acute Respiratory Distress Syndrome Secondary to Severe Trauma and Hemorrhagic Shock"

When you resubmit your revised protocol submission, please use the response to review form on the forms page for your point by point response. This form includes the IACUC's review questions and concerns.

All study staff identified on this protocol application including the Principal Investigator, MUST complete the CITI training program. This training requirement must be met prior to IACUC approval of this protocol submission.
<https://www.citiprogram.org>

All study staff identified on this protocol application including the Principal Investigator, MUST be cleared by Occupational Health (OHS) to use research animals. Please contact OHS, 617-726-2217, for assistance.

In Insight / Animals / Pending Applications page, the protocol submission is unlocked to allow you to respond to the IACUC review and edit applicable protocol forms. Please reply promptly to this IACUC review notification. If you do not respond to this review notification within 90 days from the date of receipt, Insight will automatically withdraw the submission from further review at the end of the 90 day period.

Abstract

ABSTRACT INSTRUCTIONS Using non-technical terms understandable to non-medical IACUC members, provide a brief answer to each of the following questions.

1. What is the overall objective of the study?

The overall objective of the research in this application is to develop a treatment that is safe, effective and that can be provided in austere environments to patients with trauma/hemorrhagic shock, and that significantly diminishes their risk of developing ARDS (acute respiratory distress syndrome). Acute respiratory distress syndrome (ARDS) is a rapidly progressive disease occurring in critically ill patients. ARDS can result from a variety of insults such as trauma, infection and sterile

inflammation. It is clinically defined as the presence of acute hypoxemia and bilateral lung infiltrates on a chest x-ray.

2. Why is this work important/significant?

Trauma is the leading cause of death among people less than 45 years of age in the United States. Severe trauma is often accompanied by hemorrhagic shock (HS) requiring aggressive resuscitation. Between 10-30% of patients with severe trauma/HS develop acute respiratory distress syndrome (ARDS), the onset of which may increase mortality 4 fold. Intense investigation in this area has not yielded clear biologic targets for intervention, and specific therapeutic agents to modulate ARDS remain elusive. While there have been significant improvements in supportive care of patients with established ARDS, there has been very little progress in therapeutic approaches designed to prevent the onset of ARDS in patients with severe trauma/HS. The work proposed in this application is designed to explore such a preventative strategy.

3. How will the animals be used to accomplish the goals of the study?

Very briefly describe the general experimental plan.

We will adapt a published model of non-lethal mouse polytrauma in 6-8 week old C57Bl/6 mice. Briefly, mice will be anesthetized and undergo 60 minutes of shock (to a mean arterial pressure of 30 mm Hg) followed by crystalloid resuscitation. They will then be subjected to a laparotomy and cecectomy. We will use this model to define a priming injury. Thirty hours after the initiation of trauma/HS, lung injury will be induced by the intratracheal administration of a low dose of LPS(lipopolysaccharide) under anesthesia. We will thus be able to define whether the priming injury significantly exacerbates LPS-induced lung injury, including causing death.

4. How does this work build upon prior work with animals?

It is well documented that severe trauma/HS can result in increased monocyte/macrophage iron levels. Moreover, previous our study has demonstrated that acute iron loading potentiates the inflammatory response to lipopolysaccharide. However, the precise mechanisms of the effect of intracellular iron on inflammation are not completely understood. This project will characterize the effects of trauma/HS on subsequent LPS-induced lung injury. And determine the efficacy of iron chelation therapy administered after trauma/HS, but before the onset of endotoxin induced lung injury on severity of lung injury and mouse mortality.

5. What are the study endpoints for the animals and what are the expected results?

Mice will be sacrificed at time point for analysis, which is 24 hours after Intratracheal administration of Lipopolysaccharide and after PET-CT imaging. And mice will be euthanized when mice indicate severe morbidity, including the presence and absence of piloerection, inability to walk, inability to eat and drink water, diarrhea, and vomiting to minimized suffering. (Ref: A robust scoring system to evaluate sepsis severity in an animal model. BMC Research Notes 2014;7:233) Study endpoints are to characterize the effects of trauma/HS on subsequent LPS-induced lung injury. And determine the efficacy of iron chelation therapy administered after trauma/HS, but before the onset of endotoxin induced lung injury

on severity of lung injury and mouse mortality. We anticipate that iron chelation should decrease the severity of LPS induced lung injury in the post traumatic setting.

Standard Application

1. PRINCIPAL INVESTIGATOR

The Principal Investigator must have a faculty-level appointment at the selected institution and cannot be a resident or research fellow. As the PI is responsible for the welfare of the animals, the PI must meet the requirements set by the IACUC with regard to training and completion of the Occupational Health program. The PI responsible for the conduct of the animal research does not necessarily need to be the same as the PI named on the related grant.

2. ANIMAL The IACUC currently restricts protocols to a single species only. If your protocol will require xenographs, indicate the donor species (including applicable protocol number) in the written narrative of the protocol. If you will be using a species of animal that is not in the list, please contact the IACUC office for additional information.

a. Please select the species that will be used.

Mice

b. Please enter any special characteristics of the selected species: Example: If this is a rodent study, indicate the strain(s)/stock(s) including any abnormal phenotypes or behaviors exhibited.

i.e. Strain- NOD, C57Bl, Transgenic,

i.e. Stock- Swiss Webster, Sprague Dawley

i.e. Breed- New Zealand White, Yorkshire

i.e. Phenotype- animals will appear obese, exhibit tremors, grow spontaneous tumors and have respiratory difficulties as part of an induced genetic mutation

Wild-type male mice (C57BL6)

c. Please indicate what method(s) of identification will be used to track these animals.
(select all that apply)

☐ Ear tag or notch

☒ Tattoos

☐ Collar

☐ Implant

☒ Cage card

☐ Other

d. The species selected is appropriate because:

☒ The process resembles that in humans.

☒ Prior research has been done in this species.

☒ Tissues or other substances to be harvested require an animal of this size.

☒ The size or anatomy of this species is best or uniquely suited to the procedure to be done.

- ☒ Tissues or substances needed are best or uniquely provided by this species.
- ☒ Species lower on the phylogenetic scale cannot be used.
- ☐ Other

e. Total number of animals requested for project.

The total number here must be the same as listed in the Potential pain or distress section as well as in the schema/flow diagram(s).

This number should include only animals ordered/obtained for this 3-year approval. This number is composed of:

any animals to be ordered (adults, males, females, pregnant females, pups, etc); animals born in-house (include those that may be culled due to genotypes) and; animals obtained from other researchers.

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If the experimental goals of the proposed studies are accomplished with fewer animals than the number approved, any "EXTRA" animals ARE NOT to be used.

The number of animals requested must be based on the amount needed to answer a specific scientific question. Animals are not to be justified on the basis of how many experiments can be physically performed by a certain number of researchers in a given period of time.

The number of experimental animals should be the minimum necessary to produce valid results. To determine minimum sample size, refer to Sample Size Calculations in Animal Research by Wayne W. LaMorte, M.D., Ph.D., M.P.H., Boston University Medical Center.

EXAMPLE 1: Sample size needed to study differences in the means of two or more populations. Indicate expected variability, mean, "p-value", and power level supporting requested group/sample size.

EXAMPLE 2: Sample size needed to study difference in frequency of an event. Indicate expected frequency, "p-value", and power level supporting requested group/sample size.

EXAMPLE 3: Sample/group size needed to perform a pilot study or to provide procedural training. Justify the number requested.

EXAMPLE 4: If the number of animals requested is based upon the need for detailed analyses of certain tissues, provide a detailed breakdown of assays to be performed, amount of material needed for each assay and amount of material available from each animal.

f. Provide the sample size calculation or other justification for sample and group size.

Be sure to confirm the numbers with your flow chart and double check the math.

For Aim 1, we will examine the effects of trauma/HS on subsequent LPS-induced lung injury. Define in detail a mouse model of trauma/HS (priming injury) followed by endotoxin induced lung injury (secondary injury) using PET-CT imaging to determine extent of pulmonary inflammation and metabolism of immune cells (Experiment 1), markers of lung injury and ROS production (Experiment 2), effect of Trauma/HS on serum iron, monocyte and macrophage intracellular iron, and mapping signaling and metabolic pathways altered by both priming and secondary injuries using next generation sequencing technology (RNA-Seq) to evaluate the circulating leucocyte and pulmonary transcriptome (Experiment 3).

We propose to study 4 groups of mice in Aim 1

Group1. trauma/HS with intratracheal LPS

Group2. sham surgery with intratracheal PBS

Group3. sham surgery with intratracheal LPS

Group4. trauma/HS with intratracheal PBS.

To maintain 80% power and a type I error rate of 5%

We will need 6 mice in each group. Therefore, $6 \times 4 \times 3 = 72$ mice will be needed.

For Aim 2, We will determine the efficacy of iron chelation therapy with deferoxamine (DFO) administered after trauma/HS, but before the onset of endotoxin induced lung injury on severity of lung injury (primary outcome) and mouse mortality (secondary outcome). Our primary outcome will be severity of lung injury, based on the lung injury score (Experiment 4). In addition to the LIS, severity of lung injury will be assessed by FDG-PET imaging (Experiment 5) and markers of lung injury and ROS production (Experiment 6), as specified in Aim 1. Our secondary outcome will be the effect of iron chelation therapy on mortality (Experiment 7).

We propose to study 4 groups of mice in Aim 2

Group1. trauma/HS with intratracheal LPS and Intravenous DFO

Group2. trauma/HS with intratracheal PBS and Intravenous DFO

Group3. trauma/HS with intratracheal LPS and inhaled DFO

Group4. trauma/HS with intratracheal PBS and inhaled DFO

To maintain 80% power and a type I error rate of 5%

We will need 16 mice in each group. Therefore, $16 \times 4 \times 4 = 256$ mice will be needed.

g. Does the number of animals requested include extra animals to cover anticipated failures or to familiarize the staff with the procedures described?

☒ Yes ☐ No

Discuss anticipated failure rate and steps to be taken to minimize this rate. If some animals will be used to train staff with the procedures to be used, describe that process here.

We included a animals to cover a 10% failure rate

3. SPECIAL FORMS CONSIDERATIONS The protocol involves the following (please check all that apply):

- ☐ Ascites or Antibody Production
- ☐ Tumor implants or growth
- ☐ Death as an endpoint studies (i.e. LD50)
- ☐ Moribund as an endpoint
- ☒ Burns or trauma

MGH only: The current minimal analgesia requirement for full thickness burns is a pre-emptive systemic analgesia administration in addition to a topical analgesic used at the burn margins.

- ☐ Breeding
- ☐ Device evaluation
- ☐ Restraint (using either conventional or novel devices) of any duration
- ☐ Transportation outside institution
- ☒ Transportation within institution
- ☐ Housing animals at facilities other than institution
- ☒ Anesthetic Agents (either injectable or inhalants)

- ☒ Survival Surgical Procedure
- ☒ Procedures including minor manipulations, injections, nonsurvival surgery, behavior observations, etc.
- ☐ Multiple survival operative procedures
- ☐ Surgery performed at a facility not part of the institution
- ☐ Paralytic agents
- ☐ Food or water deprivation other than required for pre-op
- ☐ Study of pain, distress or stress
- ☐ Unalleviated pain or distress

4. HAZARDOUS MATERIALS USE Will any radioisotope, biological agent, hazardous chemicals or carcinogens be used in animals under this protocol?

- ☐ Yes ☒ No

Potential Pain and Distress

Each animal must be assigned to a pain category based on the most invasive procedure or procedure that has the greatest potential to cause pain or distress. The total number of animals assigned in all categories must equal the total number of animals requested.

Pain Category B is defined as Animals being bred, conditioned, or held for use in teaching, testing, experiments, research or surgery, but not yet used for such purposes).

B. Does this protocol involve teaching, research experiments or tests that involve only breeding or housing of animals with no planned euthanasia?

- ☐ Yes ☒ No

Pain category C is defined as animals upon which teaching, research, experiments, or tests will be conducted involving no pain, distress, or use of pain-relieving drugs.

C. Does the protocol involve teaching, research experiments or tests conducted that potentially induce no more than momentary pain or distress and do not involve the use of pain relieving drugs.

For example: routine injections or imaging, euthanasia by compressed CO2 gas for rodents.

- ☐ Yes ☒ No

Pain category D is defined as animals upon which experiments, teaching, research, surgery, or tests will be conducted involving accompanying pain or distress to the animals and for which appropriate anesthetic, analgesic, or tranquilizing drugs will be used.

D. Does the protocol involve teaching, research experiments or test conducted that potentially induce more than momentary pain or distress, and for which appropriate anesthetic, analgesic or tranquilizing drugs will be used. For example: retroorbital blood draw, tail snips after day 21, euthanasia by exsanguination under general anesthesia, survival and non-survival surgical procedures.

- ☒ Yes ☐ No

How many animals will be assigned to Category D?

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Pain category E is defined as animals upon which teaching, experiments, research, surgery, or tests will be

conducted involving accompanying pain or distress to the animals and for which the use of appropriate anesthetic, analgesic, or tranquilizing drugs will adversely affect the procedures, results, or interpretation of the teaching, research, experiments, surgery, or tests.

E. Does the protocol involve teaching, research experiments, surgery or tests that involve PAIN or DISTRESS, and for which appropriate anesthetic, analgesic or tranquilizing drugs are NOT used because they would adversely affect the results or interpretation of the data. For example: LD50 studies, observing an animal until a moribund condition is reached, creating a debilitating condition or phenotype, pain studies.

- ☐ Yes ☒ No

Literature Search- Refine

A. Pain and/or Distress Refinements

A literature search for alternative procedures must be performed for each procedure that has the potential to cause pain or distress, including prolonged restraint devices.

Please select all that apply.

- ☒ The literature search conducted indicates that there are no alternative procedures that would involve less pain or distress
- ☐ There are alternative procedures, however they cannot be used for these experiments

B. Details of Literature Search i) Indicate the date the literature search was completed.

2/14/2018

ii) Indicate the database(s) searched.

- ☒ Medline
- ☐ Agricola
- ☐ Biosis
- ☒ Pubmed
- ☐ ALTWEB
- ☐ Animal Welfare Institute
- ☐ Other

iii) Indicate time period surveyed (i.e. 1960-2008)

1960-2018

iv) Indicate the procedure and keyword(s) used for each potentially painful or distressful procedure described in this protocol.

Please use the format of the following EXAMPLE:

Procedure 1: Tail snip

Keywords: Tail snip, Mice, genotyping, alternatives

Procedure 1: Polytrauma and hemorrhagic shock

Keywords: polytrauma, laparotomy, cecectomy, hemorrhagic shock, inflammation, mortality, lung

Procedure 2: cannulation for femoral arterial

Key words: catheterization, cannulation, femoral, artery

Procedure 3: lung injury induced by endotoxin and iron chelation therapy

Key words: endotoxin, LPS, lung injury, iron chelator, deferoxamine(DFO), intratracheal, intravenous, inhale, mortality

C. Alternative Animal Models

The scientific reliance on live animals should be minimized. Alternative models, such as mathematical, computer simulation or in vitro biological systems can sometimes be used to replace animals. Explain why the use of animals is necessary for this experiment.

- ☐ Mathematical models are not a suitable alternative to live animals
- ☐ Computer simulation is not a suitable alternative to live animals
- ☐ In vitro biological systems are not a suitable alternative to live animals
- ☒ Other, explain below

Polytrauma and hemorrhagic shock has complex physiology involving several organ systems, such as the muscle, blood and liver, that cant be replicated in vitro or in silico. In addition, important clinical outcomes for human patients with polytrauma and hemorrhagic shock are preventing systemic inflammatory response and ARDS, both of which require mice and cannot be simulated in vitro or in silico.

D. Unnecessary Duplication of Research Unnecessarily duplicative research should be avoided for scientific and ethical reasons. Have the results fulfilling the experimental goals of this study been published in medical or veterinary journals?

- ☐ Yes ☒ No

Detailed Research Plan

Please answer all questions completely. DO NOT DUPLICATE GRANT APPLICATION LANGUAGE. A grant application is not considered a protocol. Limit the discussion of methods to experiments involving animals or animal tissues.

A. STUDY GOALS:

State the broad long-term objectives and concisely describe the hypothesis to be tested.

The overall objective of the research proposed in this application is to rigorously test the efficacy of intracellular iron chelation in reducing the severity of ARDS in a mouse model of severe trauma/HS. We hypothesize that iron chelation prevent the development of ALI in Trauma/HS patients.

B. BACKGROUND and SIGNIFICANCE

Briefly sketch the background to the present proposal (include a summary of any preliminary findings). State concisely the importance of the research.

Trauma is the leading cause of death among people less than 45 years of age in the United States. Severe trauma is often accompanied by hemorrhagic shock (HS) requiring aggressive resuscitation. Between 10-30% of patients with severe trauma/HS develop acute respiratory distress syndrome (ARDS), the onset of which may increase mortality 4 fold. ARDS after trauma/HS is generally understood to be a two-hit process, with the trauma/HS and/or massive transfusion acting as the initial

priming agent followed by a second hit, often an infectious or inflammatory stimulus. Intense investigation in this area has not yielded clear biologic targets for intervention, and specific therapeutic agents to modulate ARDS remain elusive. While there have been significant improvements in supportive care of patients with established ARDS, there has been very little progress in therapeutic approaches designed to prevent the onset of ARDS in patients with severe trauma/HS. The work proposed in this application is designed to explore such a preventative strategy. Severe trauma/HS can result in increase monocyte/macrophage iron levels through multiple mechanisms. The first involves hepcidin, a hormone that is the master regulator of iron homeostasis. Although predominantly produced in the liver, hepcidin is also produced by macrophages in response to inflammatory stimuli such as lipopolysaccharide (LPS), an inflammatory agent that acts on the Toll-like receptor 4 (TLR4). Macrophage-produced hepcidin has autocrine and paracrine, but not systemic effects. Hepcidin increases macrophage intracellular iron levels by degrading ferroportin, the only known iron exporter in mammals. Traumatic injuries have been shown to trigger endogenous danger signals known as Damage Associated Molecular Patterns (DAMPs). There is evidence that many DAMPs are TLR agonists as well. We hypothesize that exposure to DAMPs will increase macrophage hepcidin production, raising intracellular iron levels. In addition, trauma/HS results in local bleeding as well as blood transfusion that leads to increased monocytic phagocytosis of senescent/damaged red blood cells and free hemoglobin. Metabolism of hemoglobin in macrophages results in the generation of increased amounts of intracellular iron. While the precise mechanisms of the effect of intracellular iron on inflammation are not completely understood, it is likely that iron exerts its pro-inflammatory effect, at least in part, by its effects on the production of reactive oxygen species (ROS) and free radicals. We anticipate that iron chelation therapy administered after trauma/HS, but before the onset of endotoxin induced lung injury prevent the development of ALI in Trauma/HS patients. These hypotheses will be examined using a well-established mouse model of trauma/HS.

C. EXPERIMENTAL DESIGN and METHODS: Describe the methods and procedures to be used on the animals.

Describe the overall research plan, outline the time-course of the project indicating each activity and any potential adverse effects. Describe each step of the project and how it relates to an animal enrolled in this study. There is not a need to include surgical and nonsurgical procedure description here.

Induction of trauma/HS: Mice will be anesthetized with isoflurane at FiO₂ 0.21 from a non-rebreathing circuit via a nose cone. Heparinized polyethylene-10 tubing is used to cannulate both femoral arteries. While one arterial line is used for blood withdrawal and resuscitation the other served to continuously monitor arterial blood pressure. Anesthetized mice are bled over 10 min to a mean arterial blood pressure (MAP) of 30 mmHg. MAP is maintained at 30 mmHg for 60 min by additional blood withdrawal or replacement. After 60 min of hemorrhagic shock, mice will be resuscitated over 30 min with a transfusion of crystalloid. They will then be subjected to a laparotomy, Cecectomy. After injury, catheters will be removed, wounds will be surgically closed in a single layer using 5-0 nylon suture and all mice will be administered buprenorphine prior to arousal from anesthesia and every 8-12 hours afterward until killing and 0.25% bupivacaine will be infiltrated around incisional wound 2h after Trauma/HS or Sham operation before mice are allowed to wake up. During surgical procedure, body temperature will be maintained between 36.0 to 37.0 by a heating pad and monitored by a rectal thermocouple probe.

This model has been shown to be non-lethal and to cause a strong pro-inflammatory response 24 after injury. We will use this model to define a priming injury. Thirty hours after the initiation of trauma/HS, lung injury will be induced by the intratracheal administration of a low dose of LPS (5mg/kg in 1ul/g PBS, a second hit, or secondary injury) under anesthesia. We will thus be able to define whether the priming injury significantly exacerbates LPS-induced lung injury, including causing death. Control groups will include sham surgery with intratracheal PBS, sham surgery with intratracheal LPS, and trauma/HS with intratracheal PBS. In this model of priming and secondary injuries, we will characterize the influence of the priming injury on the secondary injury using the following techniques.

We propose to study 4 groups of mice:

Group1. trauma/HS with intratracheal LPS

Group2. sham operation with intratracheal PBS

Group3. sham operation with intratracheal LPS

Group4. trauma/HS with intratracheal PBS.

Sham operation: Sham operation will be done exactly the same with above except for the induction of hemorrhagic shock and cecectomy

Experiment 1: In vivo FDG-PET-CT imaging

Imaging with [¹⁸F]fluoro-2-deoxy-D-glucose positron emission tomography (FDG-PET) has been well studied in the context of acute lung injury in both humans and animal models. Mice anesthetized with isoflurane will be injected with a bolus of FDG (5-6 MBq) and will be imaged in a Siemens Inveon PET-CT scanner. Each mouse will be imaged twice first, 24h after trauma/HS or sham surgery, and second 24h after intratracheal LPS/PBS challenge. Data from the scans will be analyzed using both static (Standardized Uptake Values) and dynamic (obtaining the influx rate constant Ki through compartmental modeling) approaches.

Experiment 2: Markers of lung injury, ROS production and Iron parameters in mice

After the second PET-CT imaging the mice will be sacrificed and serum, BAL fluid and lungs will be harvested for analysis. Four pieces of lung tissue will be used for RT-qPCR, histology, myeloperoxidase assay and frozen section for dihydroethidium (DHE) staining. Serum and BAL will be used for measuring iron level.

Experiment 3: Unbiased evaluation of alterations in signaling pathways using next generation sequencing

Mice will be anesthetized with isoflurane. Mice will be heparinized and whole blood (0.5ml) will be collected by cardiac puncture. After that, lung will be perfused with sterile PBS and harvested. RNA will be extracted from blood and lungs and will be using for the test. Gene expression pattern will be compared between sham operated, trauma/HS and trauma/HS with lung injury mouse group. Differences in gene expression between circulating white cells and lung tissue will also be compared within each group. Functional pathway analysis will be performed using Ingenuity Pathway Analysis to identify signaling and metabolic pathways enriched in each experimental condition.

Aim 2: Determine the efficacy of iron chelation therapy administered after trauma/HS, but before the onset of endotoxin induced lung injury on severity of lung injury (primary outcome) and mouse mortality (secondary outcome).

Experiment 4:

We will compare the efficacy of intravenous DFO to inhaled DFO (both at 100 mg/kg, based on doses typically used clinically) on both the severity of inflammation and effectiveness of iron chelation. Our primary outcome will be severity of lung injury, based on the lung injury score.

Experiment 5: severity of lung injury will be assessed by FDG-PET imaging and markers of lung injury, as specified in Experiment 1

Experiment 6: Evaluation of the effects of trauma/HS on subsequent LPS-induced lung injury using markers of lung injury and ROS production, as specified in Experiment 2

Experiment 7: Evaluation of the effect of iron chelation therapy on mortality.

D. FLOW CHART A schema or flow chart diagramming each experiment, giving the overall picture of the study design and treatment groups must be included with this submission (please see the Attachments tab for instructions). The flow diagram should include all experimental groups, the number of animals per group, the procedures performed on the animal, and the time course of the experiment from beginning until end.

Duration, Clinical Signs, Endpoints and Euthanasia

1. Study Duration

Please indicate the length of time the animals are expected to survive for this study.

Mice will be sacrificed at 54h after Trauma/HS for analysis.

2. Follow-up Care: Note: Please review services provided and available by CCM staff prior to initiating any research project to confirm roles and responsibilities of all parties.

A. Describe the investigator's responsibilities during the post-surgical and/or post-experimental period, including frequency of examination and monitoring.

Post-surgical follow-up care will be provided by Dr. Fumiaki Nagashima. The mice will be treated every 8-12 hours afterward until killing with buprenorphine for adequate analgesia until killing and 0.25% bupivacaine will be infiltrated around incisional wound 2h after Trauma/HS or Sham operation before mice are allowed to wake up. The condition of the mice will be monitored closely and mice weight will be measured every day. The mice will be euthanized with pentobarbital IP and counted as death if they meet pre-specified indicators of severe morbidity, including the presence and absence of piloerection, inability to walk, inability to eat and drink water, diarrhea, and vomiting to minimize suffering.

B. Will CCM staff be responsible for post procedure care?

☐ Yes ☒ No

3. Health Status

A. Describe the health status of the animals during the study period. If the health of an animal is to be compromised by a surgical procedure, the introduction of disease, tumor, or by administration of a toxic agent (including biologic and hazardous material), describe the course of the disease or expected response.

Impact of Trauma/HS:

We will adapt a published model of non-lethal mouse polytrauma in 6-8 week old

C57Bl/6 mice. According to this paper, none of the murin models was lethal, and the animals were able to maintain the ability to ambulate and groom as well as feed and drink. None of the groups of mice lost more than 20% of their body weight. There were no obvious infectious complications.

Because of the induction of trauma/HS, moderate systemic inflammation will occur in mice. But as mention above, It is not lethal and mice can maintain the ability to ambulate and groom as well as feed and drink. And after the 24 hours, Mice will be administrated LPS via intratracheal as a second hit. then, Lung inflammation will be induced severely and continue to the next 24 hours, which is the time point of sacrifice. So most critical phase is after the intratracheal administration of LPS as a second hit. But, induction of trauma/HS is also critical, so we will continuously observe these animals after procedures.

B. Would you like to pre-authorize any parenteral or supportive care that CCM can provide to these animals?

☐ Yes ☒ No

4. Pain Management Unless scientifically justified, means for alleviating animal pain, distress or discomfort must be instituted. In general, conditions that cause more than momentary pain, distress or discomfort in humans will have the same adverse effects on animals. Such conditions that may cause pain or discomfort include; surgery, excessive inflammation, necrosis, drug or radiation toxicity, noxious stimuli without escape, functional impairments. Such conditions that may cause distress include; restraint of the unadapted animal, restraint for more than 4-8 hours, abnormal diet or environment.

Analgesia should be provided to all animals undergoing surgical procedures. Methods that can be used include; infiltration of the incision site and/or regional nerve block with long-acting anesthetics, and/or systemic analgesics. By administering analgesics pre-operatively, the agents block or suppress the pain cycle and in many cases, minimize the need for analgesics post-operatively. For major surgical procedures, the committee generally recommends analgesics given pre-operatively and then on a scheduled basis for 72 hours. After that time, PRN administration is usually acceptable.

Please describe the frequency the animals will be assessed for pain management and indicate the clinical criteria to be used to determine that analgesics are needed.

0.25% bupivacaine will be applied directory onto the open incision before closure.

Buprenorphine will be administered IP prior to arousal from anesthesia.

Mice will be closely monitored by Fumiaki Nagashima and buprenox will be planned to administrated every 8-12 hours. But, If mice exhibited signs of pain (self mutation, inability to walk, eat, or drink), additional analgesics (buprenorphine 0.1 mg/kg, SC) will be administrated.

5. Endpoints

What clinical criteria will be used to remove animals from the study for euthanasia?

Endpoint of the study are to determine the efficacy of iron chelation therapy administered after trauma/HS, but before the onset of endotoxin induced lung injury on severity of lung injury (primary outcome) and mouse mortality (secondary outcome). Every effort will be made to find whether iron chelation therapy effect on severity of lung injury and mortality and if we find severe morbidity, mice will be euthanized with Pentobarbital i.p and counted as death.

We will use a modified version of the Murine Sepsis Severity Score (Ref: A robust scoring system to evaluate sepsis severity in an animal model. BMC Research Notes 2014;7:233). The following criteria will define mice that will be euthanized to

prevent undue suffering:

1. Piloerection in majority of back, or a generalized puffy appearance
2. Activity severely impaired mouse remains stationary when provoked with possible tremors.
3. Loss of righting reflex
4. Eyes closed or milky
5. Severely reduced respiration (>0.5 sec between breaths)
6. Respiration labored with intermittent gasps

6. Euthanasia Method How will euthanasia be performed (include agent(s), route of administration and dose). A method must be indicated even if the procedure is not terminal, in the event of an emergency.

Inhalational anesthesia with isoflurane followed by exanguination for mice that complete the study. In the event of severe pain and distress requiring euthanasia, we will use pentobarbital 200 mg/kg IP.

Housing

A. HOUSING LOCATIONS Please check all applicable housing areas that will be used.

- ☐ 114 CNY - 1
- ☐ 149 CNY - 8
- ☐ 149 CNY - 9
- ☐ 400 Technology Square
- ☐ 75 CNY
- ☐ Cox 7
- ☐ Edwards 6
- ☐ Shriner's Hospitals for Children
- ☐ Simches 8
- ☐ Thier 8
- ☒ Thier B/1
- ☐ x- Other

B. LABORATORY HOUSING Housing in a laboratory area requires inspection and approval by both the IACUC and the Director, Center for Comparative Medicine. Is it proposed to house animals in a research lab for more than 12 hours?

- ☒ Yes ☐ No

i) Please select the building(s) where the animals will be housed in the lab

- ☐ 400 Technology Square
- ☐ 65 Landsdowne Street
- ☐ Bartlett Hall
- ☐ Bartlett Hall Extension
- ☐ Blake
- ☐ Bullfinch

- ☐ CNY-114
- ☐ CNY-149
- ☐ CNY-75
- ☐ CNY-79/96
- ☐ Cox Research
- ☐ Edwards Research
- ☐ Ellison
- ☐ Founders House
- ☐ Grafton
- ☐ Gray/ Bigelow
- ☐ Jackson
- ☐ Shriners Hospitals for Children
- ☐ Simches Research Center
- ☐ Southbridge
- ☒ Thier Research

Please indicate the room(s) in Thier that will be used

505

- ☐ Vincent/ Burnham
- ☐ Warren
- ☐ White
- ☐ Yawkey

ii) Why will animals be housed in the lab for more than 12 hours?

We would like to monitor mice closely and continuously after procedure in Thier room 505 (an IACUC approved satellite mouse housing facility).

iii) When was the housing space last approved by the IACUC?

January 2018

C. Will the animals on this study require any special handling, husbandry or housing requirements? This includes:

anything outside of normal routine husbandry/handling services utilized by CCM defined in the species specific SOPs (i.e. alterations in bedding types, cage change frequencies, housing densities, special diets/fluids, deviations from currently approved IACUC policies, etc)single housing must be scientifically justified and included in the applicable box below

Note: Exemptions that are defined and approved by the IACUC Policy on Environmental Enrichment, Social housing and Exercise of Laboratory Animals and do not need to be described in the protocol

include:Research attrition of cage/pen matesAggressive or incompatible conspecifics as determined by CCM veterinary services staffAcute studies (<90 days)Breeding and weaning situationsPerioperative period of up to 14 days

A flow chart detailing the policy is available to assist in the determination if the single housing planned is covered by the policy.

- ☐ Breeding (i.e. delayed weaning requirements, harem breeding strategies, etc)
- ☐ Immunocompromised
- ☐ Genetically modified animals (includes knock-outs, knock-ins, transgenics)
- ☐ Other/ Social Housing Exceptions

Pharmacological Agents

A pharmaceutical grade compound is defined as a drug, biologic, or reagent that is approved by the Food and Drug Administration (FDA) or for which a chemical purity standard has been established by the United States Pharmacopeia-National Formulary (USP-NF), or British Pharmacopeia. Reagents are not drugs.

Drugs are manufactured by a pharmaceutical producer under Good Manufacturing Practices and approved by the FDA.

The IACUC requires that all chemical compounds administered to any animal species be of pharmaceutical grade, if that agent is available in pharmaceutical grade. Specific permission must be obtained to administer any non-pharmaceutical chemical compound. Such compounds must be sterile, maintained in a sterile container and labeled to provide the name and concentration of the compound as well as its expiration date.

1. Agents:

Please select the pharmacological agents that will be used on this protocol. This includes any anesthetics, analgesics, test substances or other agents to be administered to the animal. If the agent to be used is not in the list, please enter it below.

Format shown is Agent name, Schedule, Pharmaceutical grade (Y,N)

,0.9% Sodium Chloride,0,PGC(Y)

,18F-FDG,0,PGC(Y)

,Euthasol,0,PGC(Y)

Isoflurane,Forane,0,PGC(Y)

,bupivacaine,0,PGC(Y)

desferoxamine,desferral,1,PGC(Y)

Heparin,Heparin,0,PGC(Y)

,buprenorphine,0,PGC(Y)

Lipopolysaccharides,LPS,0,PGC(Y)

2: New Agents:

Add new Pharmacological Agent(s):

Please be sure you have included the route, volume, and concentration of the agents indicated by this form in the appropriate section(s) of the protocol (procedure forms, anesthesia regimen form, or detailed research plan).

3. Non-Pharmaceutical Grade Justification and Preparation:

A. At least one of the agents selected is a non-pharmaceutical grade agent. Please provide justification for the use of those selected agent(s).

As a reminder, cost in itself is not typically considered an adequate reason to use non-pharmaceutical grade compounds.

OLAW has specifically addressed the use of non-pharmaceutical grade agents in a Frequently Asked Question on their website.

B. Describe how the non-pharmaceutical grade compound(s) will be prepared, stored and disposed. Be sure to address the following: Grade/ Purity (e.g. HPLC grade, life science grade) Sterility/ Pyrogenicity (e.g. micron filtration, sterile diluent) Formulation/pH range Stability/ Compatibility Labeling/Storage (e.g. container type, temperature) Expiration/Disposal

More information on the MGH IACUCs Expired Materials Policy for additional information with regards to storage and labelling.

More information on the McLean IACUCs Expired Materials Policy.

Anesthesia Regimen: Anesthesia for inhalational iron chelator therapy

Please assign a label for this method of anesthesia. It will be helpful to refer to this label in other sections to reduce duplication.

(i.e. Surgical Option A, Minor Procedure Option A, Ketamine Option, etc)

Anesthesia for inhalational iron chelator therapy

1. Please enter the agent(s) that will be used for this regimen. Be sure to include the dose/concentration and route and frequency of administration for each agent. (Example: Ketamine, 50-60 mg/kg, IM, once) The IACUC approved preferred formulary for the most commonly used species can be found here Note: To open the link above, you must be inside the Partners firewall/network.

Ketamine 85mg/kg + Xylazine 10mg/kg, IP, once

2. The procedure(s) using this regimen is[are] expected to last:

- ☐ Less than 20 minutes ☒ 20 minutes or longer

Please indicate how long the procedure(s) is[are] expected to take.

30 minutes

3. Please indicate the person(s) responsible for anesthetizing and monitoring the animal during the procedure. For details of current IACUC requirements for anesthetic monitoring, please refer to the Anesthesia and Analgesia Policy.

Nagashima, Fumiaki

4. The adequacy or depth of anesthesia will be monitored by:(check all that apply)

- ☒ Respiration Rate
☐ ECG
☐ Toe pinch
☐ Blood gas
☐ Corneal or Palpebral reflex
☐ Blood pressure

- ☐ Heart Rate
- ☒ Tail pinch
- ☐ Jaw laxity
- ☒ Muscle Relaxation
- ☐ Pulse oximetry
- ☐ Respirometer
- ☐ Other

5. How frequently will the depth of anesthesia be assessed?

Continuous

6. How frequently will this assessment be documented? Documentation is required for procedures that will last 20 minutes or greater. For guidance to the recommended documentation frequency, please review the Anesthesia and Analgesia Policy.

once at the end of Procedure

Anesthesia Regimen: anesthesia for PET CT imaging, intratracheal administration of LPS, induction of trauma/HS

Please assign a label for this method of anesthesia. It will be helpful to refer to this label in other sections to reduce duplication.

(i.e. Surgical Option A, Minor Procedure Option A, Ketamine Option, etc)

anesthesia for PET CT imaging, intratracheal administration of LPS, induction of trauma/HS

1. Please enter the agent(s) that will be used for this regimen. Be sure to include the dose/concentration and route and frequency of administration for each agent. (Example: Ketamine, 50-60 mg/kg, IM, once) The IACUC approved preferred formulary for the most commonly used species can be found here Note: To open the link above, you must be inside the Partners firewall/network.

Isoflurane, 1.5-3% continuous

2. The procedure(s) using this regimen is[are] expected to last:

- | | |
|----------------------------|-------------------------------------|
| ☐ Less | ☒ 20 |
| than 20 | minutes |
| minutes | or longer |

Please indicate how long the procedure(s) is[are] expected to take.

For PET CT imaging: 1 hours, For intratracheal administration of LPS: 5 minutes, For Induction of trauma/HS: 3 hours

3. Please indicate the person(s) responsible for anesthetizing and monitoring the animal during the procedure. For details of current IACUC requirements for anesthetic monitoring, please refer to the Anesthesia and Analgesia Policy.

Nagashima, Fumiaki

4. The adequacy or depth of anesthesia will be monitored by:(check all that apply)

- ☒ Respiration Rate
- ☐ ECG
- ☐ Toe pinch
- ☐ Blood gas
- ☐ Corneal or Palpebral reflex
- ☐ Blood pressure
- ☐ Heart Rate
- ☒ Tail pinch
- ☐ Jaw laxity
- ☒ Muscle Relaxation
- ☐ Pulse oximetry
- ☐ Respirometer
- ☐ Other

5. How frequently will the depth of anesthesia be assessed?

Continuous

6. How frequently will this assessment be documented? Documentation is required for procedures that will last 20 minutes or greater. For guidance to the recommended documentation frequency, please review the Anesthesia and Analgesia Policy.

once at the end of procedure

Procedures: Intratracheal administration of LPS

Complete this form for each procedure to be performed.

A procedure is any manipulation of an animal for an experimental application, for examination purposes or for treatment of an induced or spontaneous disease or condition. For clarity of definition the IACUC uses the terms 'procedure' or 'surgery' to describe all manipulations performed here at MGH.

"Procedure" is used to describe injections, bandaging or casting, imaging, antibody production, collection of blood and other clinical samples, non-invasive physiological monitoring, breeding, behavior observations, euthanasia, etc. Procedures may or may not require the use of a sedative or anesthetic, and may or may not require the use of analgesics.

"Surgery" usually involves an incision and exposure of a tissue for an operative method or the operative manipulation of physiologic or physical parameters to create a model of a clinical disease process or condition and/or treatment of a disease or condition. Surgery usually requires anesthesia, and is further subclassified as major or minor, and survival or non-survival.

Please enter a label for this procedure.

Intratracheal administration of LPS

A. What is the type of manipulation?

- | | |
|-----------------------|----------------------------------|
| ☐ | ☒ |
| Surgery | Procedure |

Please select the procedure from the list.(Select the item that best represents the manipulation or the approach used).If you cannot find your procedure listed, please enter new one below.

Intratracheal Administration of Agents

Please enter new procedure name.

Please indicate how long it will take to complete the procedure.

5min

B. Please indicate the person(s) performing the procedure.

Nagashima, Fumiaki

C. Please indicate the building where the procedure will be performed.

Thier Research

Please indicate the room number.

505

D. Will anesthesia be used for this procedure?

☒ Yes ☐ No

Please enter the anesthetic regimen(s) that will be used for this procedure.
(Enter the labels defined by anesthetic regimen form completed on the forms tab).

Anesthesia for intratracheal administration of LPS

E. The procedure is:

☒ Survival ☐ Non-survival

WRITTEN DESCRIPTION OF PROCEDURE

Describe the procedure, including the approach used. Describe how pain and distress will be minimized during the procedure (include name, dose, route and duration of effect of sedative, anesthetic, and/or analgesic used). Describe the immediate post-procedural care and monitoring plan; what criteria will be used to determine the animals are stable and have recovered from anesthesia before being returned to the animal facility.

Procedure:

Mice are anesthetized with 3% isoflurane in an anesthetic chamber and LPS (2.5mg/kg) in 1 micro litter for 1g of body weight of Saline will be administered via oropharyngeal aspiration using a micropipette. Mice are fixed on a surgery board, the tongue is pulled out with a forceps, and the liquid is placed onto the distal part of the oropharynx while the nose was gently closed. the liquid will be aspirated to the lungs by spontaneous respiration. after that, mice will be returned to their holding cage.

1. Indicate the staff responsible for post-procedure care.

Nagashima, Fumiaki

2. In the event of an emergency, indicate the person to contact and provide beeper/phone numbers

Fumiaki Nagashima 617-460-0115

3. Indicate the frequency of post-procedure observations.

Mice will be observed every hour after intratracheal administration of LPS up to 8 h after intratracheal administration of LPS and every 8-12h thereafter.

4. Length of time animal survives post procedure:

24h

5. Will antibiotics be administered?

☐ Yes ☒ No

6. Will any other miscellaneous post-procedural medications be used?

☐ Yes ☒ No

F. Will analgesics be used for this procedure?

☐ Yes ☒ No

Procedures: FDG-PET-CT imaging

Complete this form for each procedure to be performed.

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"Procedure" is used to describe injections, bandaging or casting, imaging, antibody production, collection of blood and other clinical samples, non-invasive physiological monitoring, breeding, behavior observations, euthanasia, etc. Procedures may or may not require the use of a sedative or anesthetic, and may or may not require the use of analgesics.

"Surgery" usually involves an incision and exposure of a tissue for an operative method or the operative manipulation of physiologic or physical parameters to create a model of a clinical disease process or condition and/or treatment of a disease or condition. Surgery usually requires anesthesia, and is further subclassified as major or minor, and survival or non-survival.

Please enter a label for this procedure.

FDG-PET-CT imaging

A. What is the type of manipulation?

☐ Surgery ☒ Procedure

Please select the procedure from the list.(Select the item that best represents the manipulation or the approach used).If you cannot find your procedure listed, please enter new one below.

Imaging, PET/CT

Please enter new procedure name.

Please indicate how long it will take to complete the procedure.

70 minutes

B. Please indicate the person(s) performing the procedure.

Nagashima, Fumiaki

Bagchi, Aranya

C. Please indicate the building where the procedure will be performed.

Simches Research Center

Please indicate the room number.

Suite 5.210

D. Will anesthesia be used for this procedure?

☒ Yes ☐ No

Please enter the anesthetic regimen(s) that will be used for this procedure.

(Enter the labels defined by anesthetic regimen form completed on the forms tab).

Anesthesia for PET-CT imaging

E. The procedure is:

☒ Survival ☐ Non-survival

WRITTEN DESCRIPTION OF PROCEDURE

Describe the procedure, including the approach used. Describe how pain and distress will be minimized during the procedure (include name, dose, route and duration of effect of sedative, anesthetic, and/or analgesic used). Describe the immediate post-procedural care and monitoring plan; what criteria will be used to determine the animals are stable and have recovered from anesthesia before being returned to the animal facility.

Procedure:

Mice will be anesthetized with isofluren (3% for induction; 1.5% for maintenance) at FiO2 0.21 from a non-rebreathing circuit via a nose cone. Mice will be injected with bolus of FDG (5-6MBq) via lateral tail vein injection. Mice will be awakened and returned to individual holding cages. Precisely 60min post FDG injection, re-anesthetized mice will be placed prone in a heated (36C) multimodality chamber in the PET scanners gantry and will be imaged in a

Siemens Inveon PET-CT scanner. Each mouse will be imaged twice-first, 24 after trauma/HS or sham surgery, and second 24 h after intratracheal LPS/PBS challenge.

-
1. Indicate the staff responsible for post-procedure care.

Nagashima, Fumiaki

2. In the event of an emergency, indicate the person to contact and provide beeper/phone numbers

Fumiaki Nagashima 617 460 0115

3. Indicate the frequency of post-procedure observations.

Mice will be observed every hour after PET CT imaging up to 8 h after PET CT imaging and every 8-12h thereafter.

4. Length of time animal survives post procedure:

30h

5. Will antibiotics be administered?

☐ Yes ☒ No

6. Will any other miscellaneous post-procedural medications be used?

☐ Yes ☒ No

-
- F. Will analgesics be used for this procedure?

☐ Yes ☒ No

Procedures: induction of trauma/HS

Complete this form for each procedure to be performed.

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"Procedure" is used to describe injections, bandaging or casting, imaging, antibody production, collection of blood and other clinical samples, non-invasive physiological monitoring, breeding, behavior observations, euthanasia, etc. Procedures may or may not require the use of a sedative or anesthetic, and may or may not require the use of analgesics.

"Surgery" usually involves an incision and exposure of a tissue for an operative method or the operative manipulation of physiologic or physical parameters to create a model of a clinical disease process or condition and/or treatment of a disease or condition. Surgery usually requires anesthesia, and is further subclassified as major or minor, and survival or non-survival.

Please enter a label for this procedure.

induction of trauma/HS

- A. What is the type of manipulation?



Surgery



Procedure

1. Please select the surgical manipulation to be performed from the list.(Select the item that best represents the manipulation or the approach used).

Trauma Induction

Please enter new Surgery Name

2. Aseptic technique will be maintained by:

- ☒ Clipping/shaving fur around incision site
- ☒ Surgical soap scrub/ alcohol rinse/ final application of solution over incision site
- ☐ Draping of the animal
- ☒ Clean lab coat, masks, surgical gloves (used for rodent surgeries)
- ☐ Sterile gown, hat, booties, mask, surgical gloves (required for large animals)
- ☒ Sterile instruments

Instruments will be made sterile by:

- ☒ Autoclave
- ☒ Glass bead sterilizer
- ☐ Ethylene oxide sterilizer
- ☐ Plasma sterilizer
- ☐ Chemical sterilent

3. Preoperative Procedures:

Describe preoperative procedures such as the withholding of food and/or water, administration of antibiotics, sedatives, or other medications prior to the induction of anesthesia (include name, dose, route of administration and duration of effect of premedication).

none

Please indicate how long it will take to complete the procedure.

3h

B. Please indicate the person(s) performing the procedure.

Nagashima, Fumiaki

C. Please indicate the building where the procedure will be performed.

Thier Research

Please indicate the room number.

505

D. Will anesthesia be used for this procedure?

- ☒ Yes ☐ No

Please enter the anesthetic regimen(s) that will be used for this procedure.
(Enter the labels defined by anesthetic regimen form completed on the forms tab).

Anesthesia for induction of Trauma/HS

E. The procedure is:

- ☒ Survival ☐ Non-survival

WRITTEN DESCRIPTION OF PROCEDURE

Describe the procedure, including the approach used. Describe how pain and distress will be minimized during the procedure (include name, dose, route and duration of effect of sedative, anesthetic, and/or analgesic used). Describe the immediate post-procedural care and monitoring plan; what criteria will be used to determine the animals are stable and have recovered from anesthesia before being returned to the animal facility.

Procedure:

Mice will be anesthetized with isofluren at FiO2 0.21 from a non-rebreathing circuit via a nose cone. Both side femoral arteries will be exposed via midline thigh incision. Heparinized polyethylene-10 tubing will be used to cannulate both femoral arteries. While one arterial line is used for blood withdrawal and resuscitation the other served to continuously monitor arterial blood pressure. Then, anesthetized mice will be bled over 10 minutes to a mean arterial blood pressure (MAP) of 30 mmHg. MAP is maintained at 30 mmHg for 60 min by additional blood withdrawal or replacement. After 60 min of hemorrhagic shock, mice will be resuscitated over 30 min with a transfusion of crystalloid. They will then be subjected to a laparotomy, cecectomy, the cecum will be ligated twice with 3-0 silk suture and resected. The edge of cecum will not be cleaned up, so spillage of contaminated material into the abdomen. the abdomen will be closed in layers using 5-0 nylon suture.

After injury, catheters will be removed, wounds will be surgically closed in a single layer using 5-0 nylon suture and all mice will be administered buprenorphine prior to arousal from anesthesia and every 8-12 hours afterward until killing. During surgical procedure, body temperature will be maintained between 36.0 to 37.0 by a heating pad and monitored by a rectal thermocouple probe.

1. Indicate the staff responsible for post-procedure care.

Nagashima, Fumiaki

2. In the event of an emergency, indicate the person to contact and provide beeper/phone numbers

Fumiaki Nagashima 617-460-0115

3. Indicate the frequency of post-procedure observations.

Mice will be observed every hour after trauma/HS surgery up to 8 h after trauma/HS surgery and every 8-12h thereafter.

4. Length of time animal survives post procedure:

54h

5. Will antibiotics be administered?

☐ Yes ☒ No

6. Will any other miscellaneous post-procedural medications be used?

☐ Yes ☒ No

F. Will analgesics be used for this procedure?

☒ Yes ☐ No

1. Please indicate the agent, dose, route, frequency and duration of administration.

all mice will be administered buprenorphine (0.1mg/kg) prior to arousal from anesthesia and every 8-12 hours afterward until killing and 0.25% bupivacaine will be infiltrated around incisional wound 2h after Trauma/HS or Sham operation before mice are allowed to wake up.

Procedures: Intravenous treatment with Iron chelator

Complete this form for each procedure to be performed.

A procedure is any manipulation of an animal for an experimental application, for examination purposes or for treatment of an induced or spontaneous disease or condition. For clarity of definition the IACUC uses the terms 'procedure' or 'surgery' to describe all manipulations performed here at MGH.

"Procedure" is used to describe injections, bandaging or casting, imaging, antibody production, collection of blood and other clinical samples, non-invasive physiological monitoring, breeding, behavior observations, euthanasia, etc. Procedures may or may not require the use of a sedative or anesthetic, and may or may not require the use of analgesics.

"Surgery" usually involves an incision and exposure of a tissue for an operative method or the operative manipulation of physiologic or physical parameters to create a model of a clinical disease process or condition and/or treatment of a disease or condition. Surgery usually requires anesthesia, and is further subclassified as major or minor, and survival or non-survival.

Please enter a label for this procedure.

Intravenous treatment with Iron chelator

A. What is the type of manipulation?

☐ Surgery ☒ Procedure

Please select the procedure from the list.(Select the item that best represents the manipulation or the approach used).If you cannot find your procedure listed, please enter new one below.

Tail vein injections

Please enter new procedure name.

Please indicate how long it will take to complete the procedure.

5 minutes

B. Please indicate the person(s) performing the procedure.

Nagashima, Fumiaki

C. Please indicate the building where the procedure will be performed.

Thier Research

Please indicate the room number.

505

D. Will anesthesia be used for this procedure?

☐ Yes ☒ No

E. The procedure is:

☒ Survival ☐ Non-survival

WRITTEN DESCRIPTION OF PROCEDURE

Describe the procedure, including the approach used. Describe how pain and distress will be minimized during the procedure (include name, dose, route and duration of effect of sedative, anesthetic, and/or analgesic used). Describe the immediate post-procedural care and monitoring plan; what criteria will be used to determine the animals are stable and have recovered from anesthesia before being returned to the animal facility.

Procedure:

Set up a restraining device and syringe (1ml syringe with 30G needle) with iron chelator(deferoxamine). Remove mouse from the cage by its tail and place mouse onto the barrel of the restrainer. Insert the plunger and push it far enough to make that mouse cannot move. Locate one of the two lateral vein of tail. Hold the tail and restrainer at the same time. Line up the needle exactly in line with vein and administrate 250 micro L of 50mg/kg Deferoxamine.

1. Indicate the staff responsible for post-procedure care.

Nagashima, Fumiaki

2. In the event of an emergency, indicate the person to contact and provide beeper/phone numbers

617-460-0115

3. Indicate the frequency of post-procedure observations.

Mice will be observed every hour after injection of iron chelator up to 8 h after inhalation of iron chelator and every 8-12h thereafter

4. Length of time animal survives post procedure:

24

5. Will antibiotics be administered?

☐ Yes ☒ No

6. Will any other miscellaneous post-procedural medications be used?

☐ Yes ☒ No

F. Will analgesics be used for this procedure?

☐ Yes ☒ No

Procedures: Inhalational treatment with iron chelator

Complete this form for each procedure to be performed.

A procedure is any manipulation of an animal for an experimental application, for examination purposes or for treatment of an induced or spontaneous disease or condition. For clarity of definition the IACUC uses the terms 'procedure' or 'surgery' to describe all manipulations performed here at MGH.

"Procedure" is used to describe injections, bandaging or casting, imaging, antibody production, collection of blood and other clinical samples, non-invasive physiological monitoring, breeding, behavior observations, euthanasia, etc. Procedures may or may not require the use of a sedative or anesthetic, and may or may not require the use of analgesics.

"Surgery" usually involves an incision and exposure of a tissue for an operative method or the operative manipulation of physiologic or physical parameters to create a model of a clinical disease process or condition and/or treatment of a disease or condition. Surgery usually requires anesthesia, and is further subclassified as major or minor, and survival or non-survival.

Please enter a label for this procedure.

Inhalational treatment with iron chelator

A. What is the type of manipulation?

☐ Surgery ☒ Procedure

Please select the procedure from the list. (Select the item that best represents the manipulation or the approach used). If you cannot find your procedure listed, please enter new one below.

Inhalation of agent

Please enter new procedure name.

Please indicate how long it will take to complete the procedure.

30 minutes

B. Please indicate the person(s) performing the procedure.

Nagashima, Fumiaki

C. Please indicate the building where the procedure will be performed.

Thier Research

Please indicate the room number.

505

D. Will anesthesia be used for this procedure?

☒ Yes ☐ No

Please enter the anesthetic regimen(s) that will be used for this procedure.

(Enter the labels defined by anesthetic regimen form completed on the forms tab).

Anesthesia for inhalational iron chelator therapy

E. The procedure is:

☒ Survival ☐ Non-survival

WRITTEN DESCRIPTION OF PROCEDURE

Describe the procedure, including the approach used. Describe how pain and distress will be minimized during the procedure (include name, dose, route and duration of effect of sedative, anesthetic, and/or analgesic used). Describe the immediate post-procedural care and monitoring plan; what criteria will be used to determine the animals are stable and have recovered from anesthesia before being returned to the animal facility.

Procedure:

Mice will be anesthetized with a single intraperitoneal injection of ketamine (85 mg/kg) + xylazine (10 mg/g). Their eyes will be coated with Refresh Lacri-lube (Allergan) to prevent drying. The mice will then be inserted into a nose cone that is attached to a nebulizer (Aeroneb, Kent Scientific). The nebulizer will be dosed with the iron chelator (deferroxamine 100mg/kg) dissolved in 5 ml vehicle. The mice will be exposed to the nebulization till the 5ml of liquid is exhausted, and then allowed to recover from anesthesia. The duration of nebulization will be less than 30 minutes.

1. Indicate the staff responsible for post-procedure care.

Nagashima, Fumiaki

2. In the event of an emergency, indicate the person to contact and provide beeper/phone numbers

Fumiaki Nagashima 617-460-0115

3. Indicate the frequency of post-procedure observations.

Mice will be observed every hour after inhalation of iron chelator up to 8 h after inhalation of iron chelator and every 8-12h thereafter.

4. Length of time animal survives post procedure:

24h

5. Will antibiotics be administered?

☐ Yes ☒ No

6. Will any other miscellaneous post-procedural medications be used?

☐ Yes ☒ No

F. Will analgesics be used for this procedure?

☐ Yes ☒ No

Transportation

The transportation of animals must conform to IACUC Policies and federal regulations.

Does the transportation involve facilities outside the institution?

☐ Yes ☒ No

Does the transportation only involve moving within or between institution campuses?

☒ Yes ☐ No

Describe how the animals will be transported through the campus. Be sure to include a description of the transport device or containment method to be utilized, as well as travel route that will be used to ensure minimal patient/visitor area exposure, or note that transfers will be performed by animal facility staff.

In order to PET-CT imaging, We need to transfer mice to Shimches Research center. We will transfer the mice to and from the Shimches Research Center.

Sponsor Funding: U.S. Army Medical Research Acquisition Activity

Select the appropriate sponsor / funding type:

- ☒ Government / Foundation / Other Non-Profit
- ☐ Corporate Sponsor
- ☐ Institutional Award
- ☐ Department Funds
- ☐ None

Indicate application type:

- ☒ Grant / Contract (direct award to BWH, MGH or SHC)
- ☐ Subcontract (from another Institution to BWH, MGH or SHC)

Indicate the applicant institution:

- ☐ BWH
- ☒ MGH
- ☐ SRH
- ☐ McLean
- ☐ Faulkner
- ☐ Other

Enter Principal Investigator name (if different):

225

Enter title of proposal (if different):

Manipulating the macrophage iron-hepcidin axis to prevent and ameliorate acute lung injury secondary to severe trauma

Enter Project / Grant # (if known):

W81XWH-17-1-0058

Enter the InfoEd proposal number (for example 2010A000123):

2016D005404

Has the project been awarded at the time of this submission?

- ☒ Yes ☐ No

NOTE: For PHS-funded research: The Institutional Animal Care and Use Committee(IACUC) requires a comparison of the grant application with the protocol when there is a direct award to the institution.

For guidance, refer to the IACUC website for more information.

Is this the primary sponsor?

- ☒ Yes ☐ No ☐ Not applicable

Will the primary sponsor cover all animal study-related drugs, devices, procedures and tests?

- ☐ Not applicable
(no

- ☒ Yes ☐ No animal study-related costs)

Initial Survey

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE In accordance with federal regulations and hospital policies, all animal research conducted at or funded through the Massachusetts General Hospital (MGH), the Shriners Hospitals for Children-Boston (SHC), Brigham and Women's Hospital (BWH), or McLean Hospital (MCL) must be reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) prior to initiation of the study. This policy applies to any vertebrate animal used for any type of research, teaching, or testing. The IACUC has the sole authority to approve, require modifications (in order to secure approval), or withhold approval of research protocols involving the use of animals at the selected Institution. Protocols can be approved for a maximum of three years, subject to satisfactory annual reviews. The IACUC also must review and approve in advance any changes or modifications to previously approved protocols.

The questions below will help to identify if IACUC approval is required prior to initiation of your research project.

A. Which Institution will the research be conducted at?

- ☒ MGH ☐ BWH ☐
 or SHC McLean

B. The proposed research project will involve the following:

- ☒ The entire animal research project will be conducted at the selected institution.
☐ Only a portion of the animal research project will be conducted at the selected Institution. This includes but is not limited to housing, surgery procedures, behavior assessments, imaging sessions, etc.
☐ The entire animal research project will be completed elsewhere, however, the work will be funded in part or whole through the selected Institution.

C. The proposed study involves the use of:

- ☒ Any live animal (ie. mouse, rat, rabbit, dog, cat, swine, sheep, nonhuman primate, etc)
☐ Animal tissues (whole dead animal included) or products, not otherwise approved by the IACUC as part of the investigator's own animal research protocol.
☐ Animal blood or blood products, obtained from a licensed biological supply company

Attachments

Name	Mode
Flowchart	Electronic
Flowchart_02(Flowchart)	Electronic
Preliminary Review Letter July 2018(Other)	Electronic
Appendix#1(Other)	Electronic
Point by Point Response_01(Point by Point Response)	Electronic

Flow Chart

Experiment 1: Evaluation of the effects of trauma/HS on subsequent LPS-induced lung injury. Define in detail a mouse model of trauma/HS (priming injury) followed by endotoxin induced lung injury (secondary injury) using PET-CT imaging to determine extent of pulmonary inflammation and metabolism of immune cells.

Day	6 Wild-type mice	6 Wild-type mice	6 Wild-type mice	6 Wild-type mice
0	Trauma/HS	Sham Surgery	Sham Surgery	Trauma/HS
1	1) PET imaging 2) Intratracheal LPS	1) PET imaging 2) Intratracheal PBS	1) PET imaging 2) Intratracheal LPS	1) PET imaging 2) Intratracheal PBS
2	1) PET imaging 2) Euthanasia	1) PET imaging 2) Euthanasia	1) PET imaging 2) Euthanasia	1) PET imaging 2) Euthanasia

Experiment 2: Evaluation of the effects of trauma/HS on subsequent LPS-induced lung injury using markers of lung injury and ROS production

Day	6 Wild-type mice	6 Wild-type mice	6 Wild-type mice	6 Wild-type mice
0	Trauma/HS	Sham Surgery	Sham Surgery	Trauma/HS
1	Intratracheal LPS	Intratracheal PBS	Intratracheal LPS	Intratracheal PBS
2	1) Euthanasia 2) Organ harvest	1) Euthanasia 2) Organ harvest	1) Euthanasia 2) Organ harvest	1) Euthanasia 2) Organ harvest

Experiment 3: Evaluation of the effect of Trauma/HS on serum iron, monocyte and macrophage intracellular iron, and mapping signaling and metabolic pathways altered by both priming and secondary injuries using next generation sequencing technology (RNA-Seq) to evaluate the circulating leucocyte and pulmonary transcriptome.

Day	6	6	6	6
-----	---	---	---	---

	Wild-type mice	Wild-type mice	Wild-type mice	Wild-type mice
0	Trauma/HS	Sham Surgery	Sham Surgery	Trauma/HS
1	Intratracheal LPS	Intratracheal PBS	Intratracheal LPS	Intratracheal PBS
2	1) Euthanasia 2) Organ harvest	1) Euthanasia 2) Organ harvest	1) Euthanasia 2) Organ harvest	1) Euthanasia 2) Organ harvest

Experiment 4: Evaluation of the efficacy of iron chelation therapy with deferoxamine (DFO) on severity of lung injury, assessed by markers of lung injury and ROS production

Day	16 Wild-type mice	16 Wild-type mice	16 Wild-type mice	16 Wild-type mice
0	Trauma/HS surgery	Trauma/HS surgery	Trauma/HS surgery	Trauma/HS surgery
1	1) IV DFO 2) IT LPS	1) IT DFO 2) IT LPS	1) IV DFO 2) IT PBS	1) IT DFO 2) IT PBS
2	1) Euthanasia 2) Organ harvest	1) Euthanasia 2) Organ harvest	1) Euthanasia 2) Organ harvest	1) Euthanasia 2) Organ harvest

Experiment 5: Evaluation of the efficacy of iron chelation therapy with deferoxamine (DFO) on severity of lung injury, assessed by PET-CT imaging.

Day	16 Wild-type mice	16 Wild-type mice	16 Wild-type mice	16 Wild-type mice
0	Trauma/HS surgery	Trauma/HS surgery	Trauma/HS surgery	Trauma/HS surgery
1	1) PET imaging 2) IV DFO 3) IT LPS	1) PET imaging 2) IT DFO 3) IT LPS	1) PET imaging 2) IV DFO 3) IT PBS	1) PET imaging 2) IT DFO 3) IT PBS
2	1) PET imaging 2) Euthanasia	1) PET imaging 2) Euthanasia	1) PET imaging 2) Euthanasia	1) PET imaging 2) Euthanasia

Experiment 6: Evaluation of the efficacy of iron chelation therapy with deferoxamine (DFO) on severity of lung injury, assessed by markers of lung injury and ROS production

Day	16 Wild-type mice	16 Wild-type mice	16 Wild-type mice	16 Wild-type mice
0	Trauma/HS surgery	Trauma/HS surgery	Trauma/HS surgery	Trauma/HS surgery
1	1) IV DFO 2) IT LPS	1) IT DFO 2) IT LPS	1) IV DFO 2) IT PBS	1) IT DFO 2) IT PBS
2	1) Euthanasia 2) Organ harvest	1) Euthanasia 2) Organ harvest	1) Euthanasia 2) Organ harvest	1) Euthanasia 2) Organ harvest

Experiment 7: Evaluation of the effect of iron chelation therapy with deferoxamine (DFO) on mortality.

Day	16 Wild-type mice	16 Wild-type mice	16 Wild-type mice	16 Wild-type mice
0	Trauma/HS	Trauma/HS	Trauma/HS	Trauma/HS
1	1) IV DFO 2) IT LPS	1) IT DFO 2) IT LPS	1) IV DFO 2) IT PBS	1) IT DFO 2) IT PBS
2	1) Euthanasia 2) Organ harvest	1) Euthanasia 2) Organ harvest	1) Euthanasia 2) Organ harvest	1) Euthanasia 2) Organ harvest

Flow Chart

Experiment 1: Evaluation of the effects of trauma/HS on subsequent LPS-induced lung injury. Define in detail a mouse model of trauma/HS (priming injury) followed by endotoxin induced lung injury (secondary injury) using PET-CT imaging to determine extent of pulmonary inflammation and metabolism of immune cells.

Day	6 Wild-type mice	6 Wild-type mice	6 Wild-type mice	6 Wild-type mice
0	Trauma/HS	Sham Surgery	Sham Surgery	Trauma/HS
1	1) PET imaging 2) Intratracheal LPS	1) PET imaging 2) Intratracheal PBS	1) PET imaging 2) Intratracheal LPS	1) PET imaging 2) Intratracheal PBS
2	1) PET imaging 2) Euthanasia	1) PET imaging 2) Euthanasia	1) PET imaging 2) Euthanasia	1) PET imaging 2) Euthanasia

0 min: Anesthesia will be induced with isoflurane 1-3% in room air via a nose corn.
 Insertion of both side arterial catheter.
 Blood pressure, rectal temperature will be continuously monitored and recorded.
 30 min: Mice will be bled over 10 min to mean arterial blood pressure of 30 mmHg.
 40 min: Maintain blood pressure at 30 mmHg for 60 min.
 100 min: Resuscitation with crystalloid will be started over 30 min.
 130 min: Laparotomy and Cecectomy will be started.
 140 min: Catheters will be removed, wounds will be surgically closed using nylon suture.
 200 min: 0.25% bupivacaine 2mg/kg will be infiltrated around incisional wound and Buprenorphine 0.1mg/kg will be administrated IP. Returned to mouse cage for follow up.
 12h: Buprenorphine 0.1mg/kg will be administrated IP.
 24h: mice will be anesthetized with 1.5-3% isoflurane
 PET CT imaging and Buprenorphine 0.1mg/kg will be administrated IP.
 30h: mice will be anesthetized 3% isoflurane and will be intratracheal administration of LPS or PBS.
 36h: Buprenorphine 0.1mg/kg will be administrated IP.
 48h: Buprenorphine 0.1mg/kg will be administrated IP.
 54h: mice will be anesthetized with 1.5-3% isoflurane
 PET CT imaging
 55h: mice will be euthanized.

Experiment 2: Evaluation of the effects of trauma/HS on subsequent LPS-induced lung injury using markers of lung injury and ROS production

Day	6 Wild-type mice	6 Wild-type mice	6 Wild-type mice	6 Wild-type mice
0	Trauma/HS	Sham Surgery	Sham Surgery	Trauma/HS
1	Intratracheal LPS	Intratracheal PBS	Intratracheal LPS	Intratracheal PBS
2	1) Euthanasia 2) Organ harvest	1) Euthanasia 2) Organ harvest	1) Euthanasia 2) Organ harvest	1) Euthanasia 2) Organ harvest

0 min: Anesthesia will be induced with isoflurane 1-3% in room air via a nose cone.
Insertion of both side arterial catheter.
Blood pressure, rectal temperature will be continuously monitored and recorded.
30 min: Mice will be bled over 10 min to mean arterial blood pressure of 30 mmHg.
40 min: Maintain blood pressure at 30 mmHg for 60 min.
100 min: Resuscitation with crystalloid will be started over 30 min.
130 min: Laparotomy and Cecectomy will be started.
140 min: Catheters will be removed, wounds will be surgically closed using nylon suture.
200 min: 0.25% bupivacaine 2mg/kg will be infiltrated around incisional wound and Buprenorphine 0.1mg/kg will be administered IP. Returned to mouse cage for follow up.
12h: Buprenorphine 0.1mg/kg will be administered IP.
24h: Buprenorphine 0.1mg/kg will be administered IP.
30h: mice will be anesthetized 3% isoflurane and will be intratracheal administration of LPS or PBS.
36h: Buprenorphine 0.1mg/kg will be administered IP.
48h: Buprenorphine 0.1mg/kg will be administered IP.
54h: mice will be sacrificed.

Experiment 3: Evaluation of the effect of Trauma/HS on serum iron, monocyte and macrophage intracellular iron, and mapping signaling and metabolic pathways altered by both priming and secondary injuries using next generation sequencing technology (RNA-Seq) to evaluate the circulating leucocyte and pulmonary transcriptome.

Day	6 Wild-type mice	6 Wild-type mice	6 Wild-type mice	6 Wild-type mice
0	Trauma/HS	Sham Surgery	Sham Surgery	Trauma/HS
1	Intratracheal LPS	Intratracheal PBS	Intratracheal LPS	Intratracheal PBS
2	1) Euthanasia 2) Organ harvest	1) Euthanasia 2) Organ harvest	1) Euthanasia 2) Organ harvest	1) Euthanasia 2) Organ harvest

0 min: Anesthesia will be induced with isoflurane 1-3% in room air via a nose corn.
 Insertion of both side arterial catheter.
 Blood pressure, rectal temperature will be continuously monitored and recorded.
 30 min: Mice will be bled over 10 min to mean arterial blood pressure of 30 mmHg.
 40 min: Maintain blood pressure at 30 mmHg for 60 min.
 100 min: Resuscitation with crystalloid will be started over 30 min.
 130 min: Laparotomy and Cecectomy will be started.
 140 min: Catheters will be removed, wounds will be surgically closed using nylon suture.
 200 min: 0.25% bupivacaine 2mg/kg will be infiltrated around incisional wound and Buprenorphine 0.1mg/kg will be administered IP. Returned to mouse cage for follow up.
 12h: Buprenorphine 0.1mg/kg will be administered IP.
 24h: Buprenorphine 0.1mg/kg will be administered IP.
 30h: mice will be anesthetized 3% isoflurane and will be intratracheal administration of LPS or PBS.
 36h: Buprenorphine 0.1mg/kg will be administered IP.
 48h: Buprenorphine 0.1mg/kg will be administered IP.
 54h: mice will be sacrificed.

Experiment 4: Evaluation of the efficacy of iron chelation therapy with deferoxamine (DFO) on severity of lung injury, assessed by markers of lung injury and ROS production

Day	16 Wild-type mice	16 Wild-type mice	16 Wild-type mice	16 Wild-type mice
0	Trauma/HS surgery	Trauma/HS surgery	Trauma/HS surgery	Trauma/HS surgery
1	1) IV DFO 2) IT LPS	1) IT DFO 2) IT LPS	1) IV DFO 2) IT PBS	1) IT DFO 2) IT PBS
2	1) Euthanasia 2) Organ harvest	1) Euthanasia 2) Organ harvest	1) Euthanasia 2) Organ harvest	1) Euthanasia 2) Organ harvest

0 min: Anesthesia will be induced with isoflurane 1-3% in room air via a nose corn.
 Insertion of both side arterial catheter.
 Blood pressure, rectal temperature will be continuously monitored and recorded.
 30 min: Mice will be bled over 10 min to mean arterial blood pressure of 30 mmHg.
 40 min: Maintain blood pressure at 30 mmHg for 60 min.
 100 min: Resuscitation with crystalloid will be started over 30 min.
 130 min: Laparotomy and Cecectomy will be started.
 140 min: Catheters will be removed, wounds will be surgically closed using nylon suture.
 200 min: 0.25% bupivacaine 2mg/kg will be infiltrated around incisional wound and Buprenorphine 0.1mg/kg will be administered IP. Returned to mouse cage for follow up.
 12h: Buprenorphine 0.1mg/kg will be administered IP.
 24h; Buprenorphine 0.1mg/kg will be administered IP.

25h: IV DFO or IT DFO administration
 30h: mice will be anesthetized 3% isoflurane and will be intratracheal administration of LPS or PBS.
 36h: Buprenorphine 0.1mg/kg will be administrated IP.
 48h: Buprenorphine 0.1mg/kg will be administrated IP.
 54h: mice will be sacrificed.

Experiment 5: Evaluation of the efficacy of iron chelation therapy with deferoxamine (DFO) on severity of lung injury, assessed by PET-CT imaging.

Day	16 Wild-type mice	16 Wild-type mice	16 Wild-type mice	16 Wild-type mice
0	Trauma/HS surgery	Trauma/HS surgery	Trauma/HS surgery	Trauma/HS surgery
1	1) PET imaging 2) IV DFO 3) IT LPS	1) PET imaging 2) IT DFO 3) IT LPS	1) PET imaging 2) IV DFO 3) IT PBS	1) PET imaging 2) IT DFO 3) IT PBS
2	1) PET imaging 2) Euthanasia	1) PET imaging 2) Euthanasia	1) PET imaging 2) Euthanasia	1) PET imaging 2) Euthanasia

0 min: Anesthesia will be induced with isoflurane 1-3% in room air via a nose corn.
 Insertion of both side arterial catheter.
 Blood pressure, rectal temperature will be continuously monitored and recorded.
 30 min: Mice will be bled over 10 min to mean arterial blood pressure of 30 mmHg.
 40 min: Maintain blood pressure at 30 mmHg for 60 min.
 100 min: Resuscitation with crystalloid will be stared over 30 min.
 130 min: Laparotomy and Cecectomy will be started.
 140 min: Catheters will be removed, wounds will be surgically closed using nylon suture.
 200 min: 0.25% bupivacaine 2mg/kg will be infiltrated around incisional wound and Buprenorphine 0.1mg/kg will be administrated IP. Returned to mouse cage for follow up.
 12h: Buprenorphine 0.1mg/kg will be administrated IP.
 24h: mice will be anesthetized with 1.5-3% isoflurane
 PET CT imaging and Buprenorphine 0.1mg/kg will be administrated IP.
 25h: IV DFO or IT DFO administration
 30h: mice will be anesthetized 3% isoflurane and will be intratracheal administration of LPS or PBS.
 36h: Buprenorphine 0.1mg/kg will be administrated IP.
 48h: Buprenorphine 0.1mg/kg will be administrated IP.
 54h: mice will be anesthetized with 1.5-3% isoflurane
 PET CT imaging
 55h: mice will be euthanized.

Experiment 6: Evaluation of the efficacy of iron chelation therapy with deferoxamine (DFO) on severity of lung injury, assessed by markers of lung injury and ROS production

Day	16 Wild-type mice	16 Wild-type mice	16 Wild-type mice	16 Wild-type mice
0	Trauma/HS surgery	Trauma/HS surgery	Trauma/HS surgery	Trauma/HS surgery
1	1) IV DFO 2) IT LPS	1) IT DFO 2) IT LPS	1) IV DFO 2) IT PBS	1) IT DFO 2) IT PBS
2	1) Euthanasia 2) Organ harvest	1) Euthanasia 2) Organ harvest	1) Euthanasia 2) Organ harvest	1) Euthanasia 2) Organ harvest

0 min: Anesthesia will be induced with isoflurane 1-3% in room air via a nose corn.
Insertion of both side arterial catheter.
Blood pressure, rectal temperature will be continuously monitored and recorded.
30 min: Mice will be bled over 10 min to mean arterial blood pressure of 30 mmHg.
40 min: Maintain blood pressure at 30 mmHg for 60 min.
100 min: Resuscitation with crystalloid will be started over 30 min.
130 min: Laparotomy and Cecectomy will be started.
140 min: Catheters will be removed, wounds will be surgically closed using nylon suture.
200 min: 0.25% bupivacaine 2mg/kg will be infiltrated around incisional wound and Buprenorphine 0.1mg/kg will be administrated IP. Returned to mouse cage for follow up.
12h: Buprenorphine 0.1mg/kg will be administrated IP.
24h: Buprenorphine 0.1mg/kg will be administrated IP.
25h: IV DFO or IT DFO administration
30h: mice will be anesthetized 3% isoflurane and will be intratracheal administration of LPS or PBS.
36h: Buprenorphine 0.1mg/kg will be administrated IP.
48h: Buprenorphine 0.1mg/kg will be administrated IP.
54h: mice will be euthanized.

Experiment 7: Evaluation of the effect of iron chelation therapy with deferoxamine (DFO) on mortality.

Day	16 Wild-type mice	16 Wild-type mice	16 Wild-type mice	16 Wild-type mice
0	Trauma/HS	Trauma/HS	Trauma/HS	Trauma/HS
1	1) IV DFO 2) IT LPS	1) IT DFO 2) IT LPS	1) IV DFO 2) IT PBS	1) IT DFO 2) IT PBS
2	1) Euthanasia	1) Euthanasia	1) Euthanasia	1) Euthanasia

	2) Organ harvest	2) Organ harvest	2) Organ harvest	2) Organ harvest
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0 min: Anesthesia will be induced with isoflurane 1-3% in room air via a nose cone.
 Insertion of both side arterial catheter.
 Blood pressure, rectal temperature will be continuously monitored and recorded.
 30 min: Mice will be bled over 10 min to mean arterial blood pressure of 30 mmHg.
 40 min: Maintain blood pressure at 30 mmHg for 60 min.
 100 min: Resuscitation with crystalloid will be started over 30 min.
 130 min: Laparotomy and Cecectomy will be started.
 140 min: Catheters will be removed, wounds will be surgically closed using nylon suture.
 200 min: 0.25% bupivacaine 2mg/kg will be infiltrated around incisional wound and Buprenorphine 0.1mg/kg will be administered IP. Returned to mouse cage for follow up.
 12h: Buprenorphine 0.1mg/kg will be administered IP.
 24h: Buprenorphine 0.1mg/kg will be administered IP.
 25h: IV DFO or IT DFO administration
 30h: mice will be anesthetized 3% isoflurane and will be intratracheal administration of LPS or PBS.
 36h: Buprenorphine 0.1mg/kg will be administered IP.
 48h: Buprenorphine 0.1mg/kg will be administered IP.
 54h: mice will be euthanized and organ harvest.

**MASSACHUSETTS GENERAL HOSPITAL
INSTITUTIONAL ANIMAL CARE
AND USE COMMITTEE**

TO: Aranya Bagchi, MBBS, MD

FROM: Heidi Langendoen, D.V.M.
Senior Protocol Review Specialist
Institutional Animal Care and Use Committee (IACUC)
Research Administration, CNY-149-5260, PH: 724-3567, 724-2912
FAX #: 4-2475
Email addresses for questions:
hlangoen@mgh.harvard.edu, jawinter@mgh.harvard.edu iacuc@mgh.harvard.edu

DATE: July 6, 2018

RE: Manipulating the macrophage iron-hepcidine axis to prevent acute lung injury/acute respiratory distress syndrome secondary to severe trauma/hemorrhagic shock (Mice) (2018N000105)

Your research proposal has been received for review by the MGH Institutional Animal Care and Use Committee, **July** meeting. For your study to be included on the July meeting agenda, we need to receive your point-by-point response to the preliminary review questions along with a revised application and other requested documents by the deadline listed below. Please also note that we will only be able to accommodate your application if there is room on the July agenda. Upon preliminary review, the following additional information is needed:

To help guide you through the questions below, page numbers refer to pages on the PDF version of your application form. You can see the PDF version by clicking the button “View Full Application” under the “Pending Applications” heading >> type in the protocol number >> “Search” >> click on the title for this version of the application >> “Workflow” heading (NOT under the “Workflow History” column).

IMPORTANT NOTICE: Please be advised that the MGH Pharmacy will no longer fill prescriptions for non-clinical use (including for research animals) after September 30, 2013. Any questions, please see the information on the IACUC website: <http://is.partners.org/aniweb/controlled.htm>

URGENT NOTICE: As of January 6, 2014: Please note that **all** members of your Study Staff **MUST** complete the CITI training program. For more information, please go to: <http://is.partners.org/aniweb/Training/Training.html>

1. In #5 of the *Abstract*, please include the study endpoints for the animals (e.g. upon reaching prescheduled ending time points, or upon reaching predetermined physiologic criteria).
2. Please clarify if you will be using both males and females in this study. If you will be using only one gender, please explain and justify why both genders cannot be used in Section 2b of the *Standard Application*. Note that some funding agencies now require this information.
3. In Section C (Experimental Design and Methods) of the *Detailed Research Plan*:
 - a. Please note that braided material such as silk are not recommended for skin closure due to the possibility of capillary action (“wicking”). A monofilament material is preferred.
 - b. Please note that doses of anesthetics are **ONLY** needed in the *Anesthesia Regimen* forms, and the doses of analgesics are **ONLY** needed in Line F of the *Procedure* forms. This helps reduce inconsistencies throughout the application. It is helpful to include the analgesic agent and duration of administration in the other sections (be sure the information is consistent throughout the application). You have listed a dose for buprenorphine that is higher than the recommended dose in mice. Please consult with your CCM Veterinarian. **
 - c. Femur fracture is mentioned [as an exception] in the “Sham operation” group (page 9) and is listed in the *Literature Search* for the polytrauma, but this procedure is not included anywhere else in the application. Please remove this from these two areas of your application to avoid confusion.
4. When describing the analgesic regimen, it is recommended to give a range, such as every 8 – 12 hours. This reduces the chance that the frequency could be considered insufficient (for example, giving analgesics at 8 am and 4 pm is BID, but 4 pm to 8 am is too long for certain analgesics in use to cover pain). Also, when you state that analgesics will be given every 12 hours, this dosing regimen must be exact to every 12 hours. Including a range is easier to ensure compliance.

5. In the *Duration, Clinical Signs, Endpoints* form:
 - a. In Section 2 (Follow-up Care) please indicate how often you will weigh the animals, as weight loss is a criterion for intervention (treatment, supportive care or euthanasia). Note that weight loss used as a criterion for intervention (treatment, supportive care or euthanasia) should be compared to normal age-matched animals, or growth charts, not based on initial weights, especially for young, growing animals.
 - b. In Sections 4 (Pain management) and 5 (Endpoints), please include more information regarding criteria will be used to determine if an animal should be provided with additional analgesics OR if an animal should be euthanized prior to the scheduled end point of the study (“humane endpoints”). Use quantitative criteria whenever possible. Note that using a body condition score (BCS) can also be helpful, with a BCS of 2 (out of 5) being an indication for euthanasia.
6. Please clarify what you mean in Section C of the *Housing* form (“perioperative period of up to 7 days) as this is unclear.
7. In the *Anesthesia Regimen* form for isoflurane (on page 15), please include the dose ranges for induction and maintenance in Line 1. You have three separate anesthesia forms for isoflurane, each is different. This is confusing. Note that you do not need a separate anesthetic form for each procedure.
8. In the *Procedure* forms:
 - a. You describe the anesthesia as being delivered “into a box”. It is not clear what you mean by this. Please check with the CCM staff, as anesthetic vaporizers are available.
 - b. Is it necessary for the animals to be anesthetized for the nebulizer treatment? Why can’t this be delivered to awake animals in enclosed cages? CCM staff may be able to help arrange special caging.
 - c. Please include more details to describe the trauma surgery. Describe the surgical approach, the techniques used for cecectomy and whether this involves spillage of contaminated material into the abdomen, and the method of closure of the cecectomy. As indicated in #3a, above, braided material such as silk is not recommended for skin closure.
 - d. For the IV Iron chelator treatment, please include the dose and volume to be administered, and the size (gauge) needle to be used.
9. In the *Morbidity and Mortality* form, you state that you will continuously observe animals during the most critical phase of the experiment, but no “critical” phase has been identified. Please describe in Section 3 (Health status) of the *Duration, Clinical Signs, Endpoints* form. Describe the health status of the animals during this phase, including the severity during progression of clinical signs and relative time course.
10. Just as an FYI: Please note that templates are available that include standard language for routine procedures and can be found at: <http://is.partners.org/aniweb/Forms/Templates.html>
11. Please expand the *Flow Chart* so it is clear when procedures, including analgesic administration and follow-up care, are done. Also indicate when anesthesia will be administered. It should also be clear which animals undergo a procedure and proceed to the next time-point and procedure, and which animals undergo a terminal procedure. This information should also be consistent with the information in the *Detailed Research Plan* (Experimental Design and Methods).
12. Just as a reminder: Please be sure **all study staff members** have completed the OHS and CITI training requirements. Each staff member must complete their own training and they can do this anytime, but if any staff member has not completed these requirements by the time your application is ready to be approved, the **staff member will be removed** from the project so the IACUC office can approve your submission at that time. The staff member would then need to complete the training requirements and can be added by amendment. It would be much easier for all to be up to date with these requirements by the time your application is ready for approval. **Incomplete staff information may delay resubmission** of this application. For modules that are missing in the **CITI training**, you can hover your mouse over the red “No” under the Staff and Access tab; this should show what modules need to be completed. For the **OHS requirements**, please contact OHS 726-2217. For the CITI program, more details are available here: <http://is.partners.org/aniweb/Training/Training.html>. If you have any questions regarding these requirements, please feel free to contact Diane McCabe (724-9718), Marilyn Grant (643-8395), or via email to iacuc@mgh.harvard.edu. (No response needed)
13. Please also be sure every member of the **Study Staff “signs off”** on their participation on this project. Once your study is accepted for a meeting agenda, each staff person will receive an email from Insight and a reminder every 7 days while the protocol is under review notifying the individual to complete activity in Insight to agree to participate in the research or decline to participate in the research. If an individual does not complete the Insight activity by the time the application is ready for approval, **Insight automatically withdraws the individual from the protocol** and you will have to submit an amendment to add the individual(s) back to the protocol. You can check the status and see if any staff members still need to complete this sign-off process by looking in the Submission History / Detailed View. It is **your responsibility** to remind your staff to complete this step. (No response needed)

14. Please note that some of the *Study Staff Qualifications* forms may include incomplete or outdated information. F. Nagashima's form indicates that he will be trained, however he is the only person listed on several of the forms, including the only person listed as performing anesthesia and procedures in some instances. Please have him update his form to include current experience, training and qualifications for this species and the procedures in this project. Please see separate paragraph below:

REGARDING STUDY STAFF: For Your Information – No response necessary: The eIACUC process requires staff to certify that they have read the protocol and know the rules and policies for working with animals, as well as to provide information related to experience/qualifications or training and Occupational Health Services Program participation. If any study staff have not completed their certification or training requirements prior to the protocol being approved, the IACUC will remove their name from the study staff list. The staff member can then be added to the study at a later time through an amendment. **PLEASE ALSO NOTE** that each individual should be sure that their information is current (within 2 years) in their Study Staff Qualifications page and describes their experience, training and qualifications with THIS SPECIES and with the procedures described in this application. This can be done as follows: The person can update their own qualifications at any time using the Admin tab in Insight. (1. Log into Insight; 2. Click on 'Admin' tab; 3. Select your name; 4. There are several tabs present where information can be updated. This is where a department/division can be updated, an email address, "Qualifications and Experience" forms, etc.) **IMPORTANT:** Your staff form will not "update" to the current date unless you make some changes and hit "SAVE". Please be sure to do so.

HOW TO ADD A GRANT / FUND TO YOUR IACUC APPLICATION:

For an Initial Review (IR): Under "Forms", click on "Add Sponsor / Funding" under the orange "Sponsor / Funding" heading. This will bring up another form. Enter any known information in the form (e.g. Agreement number, Fund number, etc.) in the appropriate box, then click on the **Search** button. Select the correct grant / fund and click on the **Add** button. The rest of the information should auto-populate. **NOTE:** If the correct grant is "grayed out" it may mean that the grant has already been entered for this IACUC protocol. Your grants administrator can help you determine the Agreement # for the grant.

For a Triennial Review (TR): For TRs, you may need to indicate in Question #3 of the Triennial Review form that you will be revising or adding a funding source. This will allow you to access the Sponsor / Funding forms. Then follow instructions as above.

For an Amendment (AME): In the second question (after selecting **No** for staff changes), click **Yes** to indicate that there will be a change in the Sponsor / Funding Source AND click the first option ("New/Additional Sponsor or Funding Source will be used"). Next click Save at the bottom of the form. This will bring you to the Sponsor / Funding forms. Follow instructions as above.

TO ACCESS FORMS IN THE TRIENNIAL REVIEW SUBMISSION THAT ARE LOCKED OR

'UNAVAILABLE': You will need to go into the "Forms" tab and locate the "Triennial Review" form. In this form, review all questions regarding existing forms (procedure forms, Abstract, Detailed Research Plan, ETC.) and you may need to change your response to any form you need to access to indicate that changes ARE needed in the form.

***IF YOU SEEK ASSISTANCE IN RESPONDING TO ANY QUESTIONS RAISED BY THE IACUC:** If you would like assistance responding to the study design questions raised by the IACUC, please contact your Facility Veterinarian. <http://intranet.massgeneral.org/ccm/animal-health/veterinary-coverage/index.asp>. The clinical veterinarians are available to provide input in experimental design and methods, clinical signs and endpoints, anesthesia/analgesia/euthanasia and other questions. Questions regarding the IACUC review process, application review status, IACUC policy, funding or study staff (CITI training/Occupational health status, etc.) can be addressed with the IACUC Office.

NOTE: Please include a point-by-point response (this can be included as an attachment) and revisions of your application in Insight. Please be sure to proofread the application and eliminate spelling and typographical errors. Please remember to hit "SUBMIT"! This action electronically 'signs' the form.

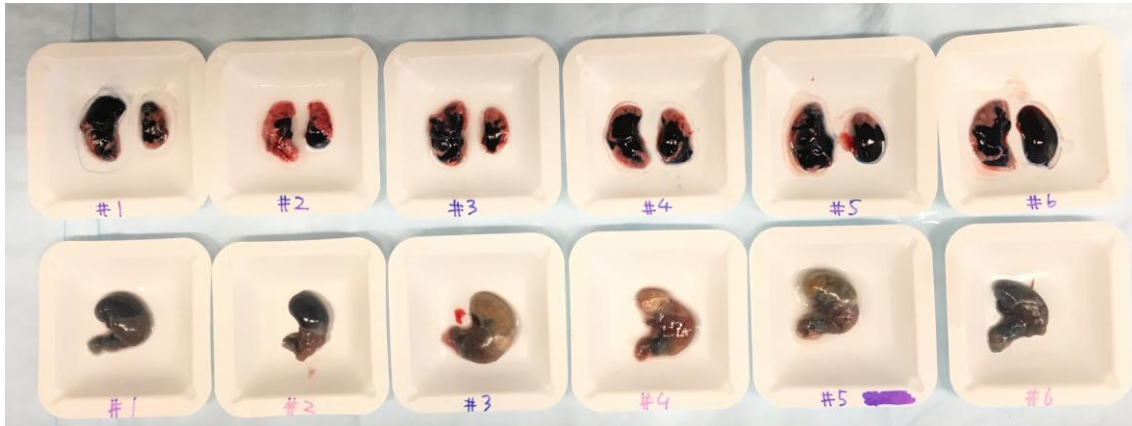
Preliminary review of your protocol submission is being done to ensure that the reviewers have all the necessary information to review your proposal. Hopefully, by identifying missing documents or need for clarification, unnecessary delays in the review process can be eliminated. Your response to this request for information is intended to facilitate IACUC review, not guarantee IACUC approval. Please note that your responses will be included in your protocol submission file. Additional questions may be raised at the meeting.

Please submit your response by **10 AM on Monday, July 16th, 2018 with point-by-point response** so that the reviewers will have sufficient time to review it before the meeting. If your response is received after the date

noted your application will be considered incomplete and review will be postponed until next month's meeting. If you have any questions, contact the Institutional Animal Care and Use Committee Office at 726-3495.

Appendix #1

Result of instilling Evans Blue dye by this method



**MASSACHUSETTS GENERAL HOSPITAL
INSTITUTIONAL ANIMAL CARE
AND USE COMMITTEE**

TO: Aranya Bagchi, MBBS, MD

FROM: Heidi Langendoen, D.V.M.
Senior Protocol Review Specialist
Institutional Animal Care and Use Committee (IACUC)
Research Administration, CNY-149-5260, PH: 724-3567, 724-2912
FAX #: 4-2475

Email addresses for questions:

hlangendoen@mg.harvard.edu, jawinter@mg.harvard.edu iacuc@mg.harvard.edu

DATE: July 6, 2018

RE: Manipulating the macrophage iron-hepcidine axis to prevent acute lung injury/acute respiratory distress syndrome secondary to severe trauma/hemorrhagic shock (Mice) (2018N000105)

Your research proposal has been received for review by the MGH Institutional Animal Care and Use Committee, **July** meeting. For your study to be included on the July meeting agenda, we need to receive your point-by-point response to the preliminary review questions along with a revised application and other requested documents by the deadline listed below. Please also note that we will only be able to accommodate your application if there is room on the July agenda. Upon preliminary review, the following additional information is needed:

To help guide you through the questions below, page numbers refer to pages on the PDF version of your application form. You can see the PDF version by clicking the button "View Full Application" under the "Pending Applications" heading >> type in the protocol number >> "Search" >> click on the title for this version of the application >> "Workflow" heading (NOT under the "Workflow History" column).

IMPORTANT NOTICE: Please be advised that the MGH Pharmacy will no longer fill prescriptions for non-clinical use (including for research animals) after September 30, 2013. Any questions, please see the information on the IACUC website: <http://is.partners.org/aniweb/controlled.htm>

URGENT NOTICE: As of January 6, 2014: Please note that all members of your Study Staff **MUST** complete the CITI training program. For more information, please go to: <http://is.partners.org/aniweb/Training/Training.html>

1. #5 of the *Abstract*, please include the study endpoints for the animals (e.g. upon reaching prescheduled ending time points, or upon reaching predetermined physiologic criteria).

I revised that as below.

Mice will be sacrificed at time point for analysis, which is 24 hours after Intratracheal administration of Lipopolysaccharide and after PET-CT imaging. Mice will be euthanized when signs indicate severe morbidity, including the presence of piloerection, inability to walk, inability to eat and drink water, diarrhea, and vomiting to minimize suffering. Study endpoints are to characterize the effects of trauma/HS on subsequent LPS- induced lung injury, and to determine the efficacy of iron chelation therapy administered after trauma/HS, but before the onset of endotoxin induced lung injury on severity of lung injury and mouse mortality. We anticipate that iron chelation should decrease the severity of LPS induced lung injury and improve survival.

2. Please clarify if you will be using both males and females in this study. If you will be using only one gender, please explain and justify why both genders cannot be used in Section 2b of the *Standard Application*. Note that some funding agencies now require this information.

We will use both males and females in this study.

3. In Section C (Experimental Design and Methods) of the *Detailed Research Plan*:

a. Please note that braided material such as silk are not recommended for skin closure due to the possibility of capillary action (“wicking”). A monofilament material is preferred.

I have revised the relevant section, and will use nylon suture instead of silk.

b. Please note that doses of anesthetics are ONLY needed in the *Anesthesia Regimen* forms, and the doses of analgesics are ONLY needed in Line F of the *Procedure* forms. This helps reduce inconsistencies throughout the application. It is helpful to include the analgesic agent and duration of administration in the other sections (be sure the information is consistent throughout the application). You have listed a dose for buprenorphine that is higher than the recommended dose in mice. Please consult with your CCM Veterinarian. **

I revised that and dose of buprenorphine was changed to 0.1mg/kg every 8-12h.

c. Femur fracture is mentioned [as an exception] in the “Sham operation” group (page 9) and is listed in the *Literature Search* for the polytrauma, but this procedure is not included anywhere else in the application. Please remove this from these two areas of your application to avoid confusion.

I revised that and I have removed this.

4. When describing the analgesic regimen, it is recommended to give a range, such as every 8 – 12 hours. This reduces the chance that the frequency could be considered insufficient (for example, giving analgesics at 8 am and 4 pm is BID, but 4 pm to 8 am is too long for certain analgesics in use to cover pain). Also, when you state that analgesics will be given every 12 hours, this dosing regimen must be exact to every 12 hours. Including a range is easier to ensure compliance.

I added the information as every 8-12 hours.

5. In the *Duration, Clinical Signs, Endpoints* form:

a. In Section 2 (Follow-up Care) please indicate how often you will weigh the animals, as weight loss is a criterion for intervention (treatment, supportive care or euthanasia). Note that weight loss used as a criterion for intervention (treatment, supportive care or euthanasia) should be compared to normal age-matched animals, or growth charts, not based on initial weights, especially for young, growing animals.

I added the information as I will weight animals every day.

b. In Sections 4 (Pain management) and 5 (Endpoints), please include more information regarding criteria will be used to determine if an animal should be provided with additional analgesics OR if an animal should be euthanized prior to the scheduled end point of the study (“humane endpoints”). Use quantitative criteria whenever possible. Note that using a body condition score (BCS) can also be helpful, with a BCS of 2 (out of 5) being an indication for euthanasia.

I added the information as below.

We will use Body Condition Score (BCS) as a criteria for intervention and Euthanasia. If a mouse indicate with BCS of 2 out of 5, we will euthanize the mouse.

6. Please clarify what you mean in Section C of the *Housing* form (“perioperative period of up to 7 days) as this is unclear.

I revised that and we don’t need special handling, husbandry or housing requirement.

7. In the *Anesthesia Regimen* form for isoflurane (on page 15), please include the dose ranges for induction and maintenance in Line 1. You have three separate anesthesia forms for isoflurane, each is different. This is confusing. Note that you do not need a separate anesthetic form for each procedure.

I revised that.

8. In the *Procedure* forms:

a. You describe the anesthesia as being delivered “into a box”. It is not clear what you mean by this. Please check with the CCM staff, as anesthetic vaporizers are available.

I revised that as follows.

Mice are anesthetized with 3% isoflurane in an anesthetic chamber and LPS (2.5mg/kg) in 1 micro liter for 1g of body weight of Saline will be administered via oropharyngeal aspiration using a micropipette. Mice are fixed on a surgery board, the tongue is pulled out with a forceps, and the liquid is placed onto the distal part of the oropharynx while the nose was gently closed. the liquid will be aspirated to the lungs by spontaneous respiration. after that, mice will be returned to their holding cage.

b. Is it necessary for the animals to be anesthetized for the nebulizer treatment? Why can't this be delivered to awake animals in enclosed cages? CCM staff may be able to help arrange special caging.

We don't have access to a inhalation chamber for rodents for aerosol administration, and consequently are using the apparatus described. If CCM does have an aerosol administration chamber, we will modify our protocol as necessary and administer the aerosol in the chamber without anesthesia.

c. Please include more details to describe the trauma surgery. Describe the surgical approach, the techniques used for cecectomy and whether this involves spillage of contaminated material into the abdomen, and the method of closure of the cecectomy. As indicated in #3a, above, braided material such as silk is not recommended for skin closure.

I added the information as below.

Mice will be anesthetized with isoflurane at FiO₂ 0.21 from a non-rebreathing circuit via a nose cone. Both femoral arteries will be exposed via midline incisions on either thigh. Heparinized polyethylene-10 tubing will be used to cannulate both femoral arteries. While one arterial line is used for blood withdrawal and resuscitation the other served to continuously monitor arterial blood pressure. Then, anesthetized mice will be bled over 10 minutes to a mean arterial blood pressure (MAP) of 30 mmHg. MAP is maintained at 30 mmHg for 60 min by additional blood withdrawal or replacement. After 60 min of hemorrhagic shock, mice will be resuscitated over 30 min with a transfusion of crystalloid. They will then be subjected to a laparotomy, cecectomy, the cecum will be ligated twice with 3-0 silk suture and resected. The edge of cecum will not be cleaned up, so spillage of contaminated material into the abdomen. the abdomen will be closed in layers using 5-0 nylon suture. After injury, catheters will be removed, wounds will be surgically closed in a single layer using 5-0 nylon suture and all mice will be administered buprenorphine prior to arousal from anesthesia and every 8-12 hours afterward until killing. During surgical procedure, body temperature will be maintained between 36.0 to 37.0 by a heating pad and monitored by a rectal thermocouple probe.

d. For the IV Iron chelator treatment, please include the dose and volume to be administered, and the size (gauge) needle to be used.

I added the information as below.

We intend to administer 250 micro L of 50mg/kg Deferoxamine via tail vein, using a 30G needle.

9. In the *Morbidity and Mortality* form, you state that you will continuously observe animals during the most critical phase of the experiment, but no “critical” phase has been identified. Please describe in Section 3 (Health status) of the *Duration, Clinical Signs, Endpoints* form. Describe the health status of the animals during this phase, including the severity during progression of clinical signs and relative time course.

I added the information as below.

Because of the induction of trauma/HS, moderate systemic inflammation will occur in mice. But as mention above, It is not lethal and mice can maintain the ability to ambulate and groom as well as feed and drink. And after the 24 hours, Mice will be administered LPS via intratracheal as a second hit. then, Lung inflammation will be induced severely and continue to the next 24 hours, which is the time point of sacrifice. So most critical phase is after the intratracheal administration of LPS as a second hit. But, after the induction of trauma/HS is also critical phase, so we will continuously observe these animals after procedures.

10. Just as an FYI: Please note that templates are available that include standard language for routine procedures and can be found at: <http://is.partners.org/aniweb/Forms/Templates.html>

11. Please expand the *Flow Chart* so it is clear when procedures, including analgesic administration and follow-up care, are done. Also indicate when anesthesia will be administered. It should also be clear which animals undergo a procedure and proceed to the next time-point and procedure, and which animals undergo a terminal procedure. This information should also be consistent with the information in the *Detailed Research Plan* (Experimental Design and Methods).

Please see modified flowchart in the protocol

12. Just as a reminder: Please be sure **all study staff members** have completed the OHS and CITI training requirements. Each staff member must complete their own training and they can do this anytime, but if any staff member has not completed these requirements by the time your application is ready to be approved, the **staff member will be removed** from the project so the IACUC office can approve your submission at that time. The staff member would then need to complete the training requirements and can be added by amendment. It would be much easier for all to be up to date with these requirements by the time your application is ready for approval. Incomplete staff information may delay resubmission of this application. For modules that are missing in the **CITI training**, you can hover your mouse over the red “No” under the Staff and Access tab; this should show what modules need to be completed. For the **OHS requirements**, please contact OHS 726-2217. For the CITI program, more details are available here: <http://is.partners.org/aniweb/Training/Training.html>. If you have any questions regarding these requirements, please feel free to contact Diane McCabe (724-9718), Marilyn Grant (643-8395), or via email to iacuc@mgc.harvard.edu. (No response needed)

13. Please also be sure every member of the Study Staff ‘signs off’ on their participation on this project. Once your study is accepted for a meeting agenda, each staff person will receive an email from Insight and a reminder every 7 days while the protocol is under review notifying the individual to complete activity in Insight to agree to participate in the research or decline to participate in the research. If an individual does not complete the Insight activity by the time the application is ready for approval, **Insight automatically withdraws the individual from the protocol** and you will have to submit an amendment to add the individual(s) back to the protocol. You can check the status and see if any staff members still need to complete this sign-off process by looking in the Submission History / Detailed View. It is **your responsibility** to remind your staff to complete this step. (No response needed)

14. Please note that some of the *Study Staff Qualifications* forms may include incomplete or outdated information. F. Nagashima’s form indicates that he will be trained, however he is the only person listed on several of the forms, including the only person listed as performing anesthesia and procedures in some instances. Please have him update his form to include current experience, training and qualifications for this species and the procedures in this project. Please see separate paragraph below:

I have revised that.

REGARDING STUDY STAFF: For Your Information – No response necessary: The eIACUC process requires staff to certify that they have read the protocol and know the rules and policies for working with animals, as well as to provide information related to experience/qualifications or training and occupational Health Services Program participation. If any study staff have not completed their certification or training requirements prior to the protocol being approved, the IACUC will remove their name from the study staff list. The staff member can then be added to the study at a later time through an amendment. **PLEASE ALSO NOTE** that each individual should be sure that their information is current (within 2 years) in their Study Staff Qualifications page and describes their experience, training and qualifications with THIS SPECIES and with the procedures described in this application. This can be done as follows: The person can update their own qualifications at any time using the Admin tab in Insight. (1. Log into Insight; 2. Click on 'Admin' tab; 3. Select your name; 4. There are several tabs present where information can be updated. This is where a department/division can be updated, an email address, “Qualifications and Experience” forms, etc.) **IMPORTANT:** Your staff form will not “update” to the current date unless you make some changes and hit “SAVE”. Please be sure to do so.

HOW TO ADD A GRANT / FUND TO YOUR IACUC APPLICATION:

For an Initial Review (IR): Under “Forms”, click on “Add Sponsor / Funding” under the orange “Sponsor / Funding” heading. This will bring up another form. Enter any known information in the form (e.g. Agreement number, Fund number, etc.) in the appropriate box, then click on the **Search** button. Select the correct grant / fund and click on the **Add** button. The rest of the information should auto-populate. **NOTE:** If the correct grant is “grayed out” it may mean that the grant has already been entered for this IACUC protocol. Your grants administrator can help you determine the Agreement # for the grant.

For a Triennial Review (TR): For TRs, you may need to indicate in Question #3 of the Triennial Review form that you will be revising or adding a funding source. This will allow you to access the Sponsor / Funding forms. Then follow instructions as above.

For an Amendment (AME): In the second question (after selecting **No** for staff changes), click **Yes** to indicate that there will be a change in the Sponsor / Funding Source AND click the first option (“New/Additional Sponsor or Funding Source will be used”). Next click Save at the bottom of the form. This will bring you to the Sponsor / Funding forms. Follow instructions as above.

TO ACCESS FORMS IN THE TRIENNIAL REVIEW SUBMISSION THAT ARE LOCKED OR ‘UNAVAILABLE’: You will need to go into the “Forms” tab and locate the “Triennial Review” form. In this form, review all questions regarding existing forms (procedure forms, Abstract, Detailed Research Plan, ETC.) and you may need to change your response to any form you need to access to indicate that changes ARE needed in the form.

***IF YOU SEEK ASSISTANCE IN RESPONDING TO ANY QUESTIONS RAISED BY THE IACUC:** If you would like assistance responding to the study design questions raised by the IACUC, please contact your Facility Veterinarian. <http://intranet.massgeneral.org/ccm/animal-health/veterinary-coverage/index.asp>. The clinical veterinarians are available to provide input in experimental design and methods, clinical signs and endpoints, anesthesia/analgesia/euthanasia and other questions. Questions regarding the IACUC review process, application review status, IACUC policy, funding or study staff (CITI training/Occupational health status, etc.) can be addressed with the IACUC Office.

NOTE: Please include a point-by-point response (this can be included as an attachment) and revisions of your application in Insight. Please be sure to proofread the application and eliminate spelling and typographical errors. Please remember to hit “SUBMIT”! This action electronically ‘signs’ the form.

Preliminary review of your protocol submission is being done to ensure that the reviewers have all the necessary information to review your proposal. Hopefully, by identifying missing documents or need for clarification, unnecessary delays in the review process can be eliminated. Your response to this request for information is intended to facilitate IACUC review, not guarantee IACUC approval. Please note that your responses will be included in your protocol submission file. Additional questions may be raised at the meeting.

Please submit your response by **10 AM on Monday, July 16th, 2018 with point-by-point response** so that the reviewers will have sufficient time to review it before the meeting. If your response is received after the date noted your application will be considered incomplete and review will be postponed until next month’s meeting. If you have any questions, contact the Institutional Animal Care and Use Committee Office at 726-3495.

Name: **Bagchi, Aranya**

Degree:

Mice Qualifications and Experience

Date: May 19, 2017

Study Staff Relevant Qualifications and Experience This form is used to describe the qualifications, training and experience you have with the species described by this protocol. Please answer all questions completely. The IACUC will review this form prior to granting approval for staff to perform the procedures described using the species indicated in a protocol.

Phone/Pager/Beeper:

617-840-2941/15421

Email Address:

abagchi@partners.org

Species for this protocol:

Mice

Do you have experience working with this species of animal?

Yes

Provide information regarding the specific qualifications, experience and training with respect to the species selected above which enable you to perform the procedures you are responsible for, as described by the protocol.

Credentials alone are not sufficient qualifications.

(i.e., Dr. Jane Doe has 4 years experience with rats performing subcutaneous injections, intravenous tail vein injections, and genotyping by ear punch. Dr. Jane Doe has also shown proficiency in performing left pneumonectomy surgeries, in which she has three years experience.)

I have more than nine years experience working with mice. This work has included administering IV and IP injections, drawing blood, euthanizing mice and removing tissue sampling, and in vivo hemodynamic monitoring. I have also completed the CITI training program on 02/08/2014.

Name: **Hayashida, Kei**

Degree: **MD, Ph.D**

Mice Qualifications and Experience

Date: May 19, 2017

Study Staff Relevant Qualifications and Experience This form is used to describe the qualifications, training and experience you have with the species described by this protocol. Please answer all questions completely. The IACUC will review this form prior to granting approval for staff to perform the procedures described using the species indicated in a protocol.

Phone/Pager/Beeper:

617-935-4215

Email Address:

khayashida@mgh.harvard.edu

Species for this protocol:

Mice

Do you have experience working with this species of animal?

Yes

Provide information regarding the specific qualifications, experience and training with respect to the species selected above which enable you to perform the procedures you are responsible for, as described by the protocol.

Credentials alone are not sufficient qualifications.

(i.e., Dr. Jane Doe has 4 years experience with rats performing subcutaneous injections, intravenous tail vein injections, and genotyping by ear punch. Dr. Jane Doe has also shown proficiency in performing left pneumonectomy surgeries, in which she has three years experience.)

Kei Hayashida has 8 years of experience performing this type of surgery on mice and rats.

Name: **Ichinose, Fumito**

Degree: **MD**

Mice Qualifications and Experience

Date: May 06, 2016

Study Staff Relevant Qualifications and Experience This form is used to describe the qualifications, training and experience you have with the species described by this protocol. Please answer all questions completely. The IACUC will review this form prior to granting approval for staff to perform the procedures described using the species indicated in a protocol.

Phone/Pager/Beeper:

617-643-0987

Email Address:

fichinose@partners.org

Species for this protocol:

Mice

Do you have experience working with this species of animal?

Yes

Provide information regarding the specific qualifications, experience and training with respect to the species selected above which enable you to perform the procedures you are responsible for, as described by the protocol.

Credentials alone are not sufficient qualifications.

(i.e., Dr. Jane Doe has 4 years experience with rats performing subcutaneous injections, intravenous tail vein injections, and genotyping by ear punch. Dr. Jane Doe has also shown proficiency in performing left pneumonectomy surgeries, in which she has three years experience.)

Dr. Fumito Ichinose has more than 25 years of experience performing a various types of surgery and experiments on mice. He also has experience in using MPTP, neurobehavioral disease monitoring, and animals housed in chambers.

Name: **Nagashima, Fumiaki**

Degree:

Mice Qualifications and Experience

Date: July 10, 2018

Study Staff Relevant Qualifications and Experience This form is used to describe the qualifications, training and experience you have with the species described by this protocol. Please answer all questions completely. The IACUC will review this form prior to granting approval for staff to perform the procedures described using the species indicated in a protocol.

Phone/Pager/Beeper:

6174600115

Email Address:

fnagashima@mgh.harvard.edu

Species for this protocol:

Mice

Do you have experience working with this species of animal?

Yes

Provide information regarding the specific qualifications, experience and training with respect to the species selected above which enable you to perform the procedures you are responsible for, as described by the protocol.

Credentials alone are not sufficient qualifications.

(i.e., Dr. Jane Doe has 4 years experience with rats performing subcutaneous injections, intravenous tail vein injections, and genotyping by ear punch. Dr. Jane Doe has also shown proficiency in performing left pneumonectomy surgeries, in which she has three years experience.)

Dr. Ichinose has trained me to handle this animal.