Continuation/Research Progression Projects Form (7)

Required for projects that are a continuation/progression in the same field of study as a previous project.
This form must be accompanied by the previous year's abstract and Research Plan/Project Summary.

Student's Name(s) Sari Strizik

To be completed by Student Researcher: List all components of the current project that make it new and different from previous research. The information must be on the form; use an additional form for previous year and earlier projects.

Components	Current Research Project	Previous Research Project: Year: 2018
1. Title	Neuronal HMGB1 Facilitates the Inflammatory Response via Increased of Proinflammatory Cytokines	Differential Regulation of Disulfide HMGB1 Proinflammatory Activity in RAW 264.7 Macrophages Treated with LPS and Sulfonyl HMGB1
2. Change in goal/ purpose/objective	Objective is to examine the role of Neuronal HMGB1 throughout the body during inflammation	Objective was to examine different role played by various isoforms of HMGB1 (Disulfide/Sulfonyl)
3. Changes in methodology	Used Western Blots, ELISAs	Used ELISAs, Uptake/Microscopy
4. Variable studied	Cytokine levels in various mice tissues	Disulfide HMGB1 and TNF levels in aritificial macrophage cell line
5. Additional changes		

Attached are:

☑ Abstract and Research Plan/Project Summary, Year 2018

I hereby certify that the above information is correct and that the current year Abstract & Certification and project display board properly reflect work done only in the current year.

Sari Strizik	Sari Strizik	1/26/19
Student's Printed Name(s)	Signature	Date of Signature (mm/dd/yy)

18-19 ABSTRACT

OFFICIAL ABSTRACT and CERTIFICATION

Differential Regulation of Disulfide HMGB1 Proinflammatory Activity in RAW 264.7 Macrophages Treated with LPS and Sulfonyl HMGB1

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Inflammation is an important part of the immune response to harmful stimuli. Upon recognition of these stimuli, the inflammatory response begins, which involves the release of cytokines. Cytokines call immune cells to the site of infection. This inflammatory response may become over exaggerated and spread to parts of the body where inflammation is unnecessary, resulting in organ damage as blood flow can be cut off to the organs leading to fatalities. HMGB1 is a proinflammatory cytokine found in high quantity in inflammatory conditions, specifically in its Disulfide isoform. Sulfonyl HMGB1 is another isoform of HMGB1, which has not been shown to have any inflammatory effects. LPS is a bacteria found in many infections. Two pathways were tested in order to determine the effects of the combination of Disulfide HMGB1 with LPS and Sulfonyl HMGB1 - (1) TLR4, a receptor pathway that releases proinflammatory cytokines, and (2) RAGE, a pathway that works via endocytosis. The TLR4 pathway was tested by the use of a TNF ELISA, testing the amount of TNF released for the different combinations. The RAGE pathway was tested by measuring the Uptake of Alexa-555 labeled HMGB1 by macrophages. In the presence of LPS, Disulfide HMGB1's Uptake was increased and the amount of TNF released was increased. In the presence of Sulfonyl HMGB1, the Uptake results suggested the Sulfonyl HMGB1 had no effect, but the TNF release was inhibited. If the mechanism behind these results could be better understood, treatments could be designed to possibly treat various inflammatory conditions.

Category
Pick one only—
mark an "X" in box
at right

Animal Sciences	☐
Behavioral & Social Sciences	☐
Biochemistry	☐
Biomedical & Health Sciences	■
Biomedical Engineering	☐
Cellular & Molecular Biology	☐
Chemistry	☐
Computational Biology & Bioinformatics	☐
Earth & Environmental Sciences	☐
Embedded Systems	☐
Energy: Chemical	☐
Energy: Physical	☐
Engineering Mechanics	☐
Environmental Engineering	☐
Materials Science	☐
Mathematics	☐
Microbiology	☐
Physics & Astronomy	☐
Plant Sciences	☐
Robotics & Intelligent Machines	☐
Systems Software	☐
Translational Medical Sciences	☐

1. As a part of this research project, the student directly handled, manipulated, or interacted with (check ALL that apply):

   ☐ human participants ☐ potentially hazardous biological agents

   ☐ vertebrate animals ☐ microorganisms ☐ rDNA ☐ tissue

2. I/we worked or used equipment in a regulated research institution or industrial setting: ■ Yes ☐ No

3. This project is a continuation of previous research. ☐ Yes ■ No

4. My display board includes non-published photographs/visual depictions of humans (other than myself): ☐ Yes ■ No

5. This abstract describes only procedures performed by me/us, reflects my/our own independent research, and represents one year's work only ■ Yes ☐ No

6. I/we hereby certify that the abstract and responses to the above statements are correct and properly reflect my/our own work. ■ Yes ☐ No

This stamp or embossed seal attests that this project is in compliance with all federal and state laws and regulations and that all appropriate reviews and approvals have been obtained including the final clearance by the Scientific Review Committee.

18-19 RESEARCH PLAN

Research Plan

a. Rationale

Although inflammation serves to help the body protect against harmful stimuli, it may malfunction. Its normally beneficial role in the body can become detrimental if the inflammation ends excessive in magnitude and/or duration.[1] This is the cause of many inflammatory diseases and conditions. The inflammation spreads to healthy organs and tissue, preventing them from performing their normal functions. During Sepsis, the inflammatory response initially follows its normal path of releasing chemicals into the bloodstream to combat an infection. These released chemicals trigger the inflammatory response as it normally would progress. However, during Sepsis, the inflammation causes the blood to clot and blood vessels to leak. This impairs blood flow throughout the body, often times cutting off a specific organ. When an organ does not receive blood flow, it becomes deprived of the oxygen and nutrients it needs to perform its basic functions.

Cytokines play a key role in the inflammatory response as they call immune cells to the site of infection. One specific cytokine is HMGB1, which was discovered fairly recently in 1973. HMGB1 is found extracellularly after either passive release via necrosis or active transport out of the cell.[2] Extracellularly, Disulfide HMGB1 binds to the co-receptor for TLR4, MD2. This allows for cytokine stimulation, which promotes inflammation.[3,4] Disulfide HMGB1 is found in high quantities in patients with various inflammatory conditions. Sulfonyl HMGB1 is another isoform, which has been shown to have no immune function, and LPS (Lipopolysaccharide) is a molecule found in many infections. Disulfide HMGB1 has been shown to be receptive to

interactions with other molecules, specifically as a way of inhibiting or enhancing its inflammatory effects. This may provide a way to treat the exaggerated inflammatory response in conditions such as Sepsis.

b. Research Questions

The following questions will be used to test the hypothesis:

How does LPS affect Disulfide HMGB1's inflammatory effects along the TLR4 and RAGE Pathways?

- TNF Release inhibited or enhanced?
- HMGB1 Uptake by macrophages inhibited or enhanced?

How does Sulfonyl HMGB1 affect Disulfide HMGB1's inflammatory effects along the TLR4 and RAGE Pathways?

- TNF Release inhibited or enhanced?
- HMGB1 Uptake by macrophages inhibited or enhanced?

Hypothesis/Expected Outcomes:

I hypothesize that LPS will enhance Disulfide HMGB1's inflammatory effects along both pathways. It is a molecule found in infections, thus it should trigger an inflammatory response of its own. When combines with Disulfide HMGB1, this response should be enhanced. It should have the same effect along both pathways, since both pathways are important parts of the typical inflammatory response in which both molecules take part. I hypothesize Sulfonyl HMGB1 will inhibit Disulfide HMGB1's inflammatory effects along both pathways. Sulfonyl HMGB1 has not

been shown to have any immune effects. Thus, there would be no reason to believe it would provide any enhancement to the inflammatory effects of Disulfide HMGB1. It would most likely inhibit Disulfide HMGB1's effects, since its lack of a role in the TLR4 and RAGE pathways may prevent Disulfide HMGB1 from carrying out all of the processes it needs.

c. Procedures

Cell Culture

All experiments will be done using RAW 264.7 cells, an immortalized macrophage cell line. Cells will be maintained at approximately 10^5/mL in RPMI 1640 Media. The LPS used will be from Escherichia Coli 011:B4 from Sigma Aldrich. The Disulfide HMGB1 and Sulfonyl HMGB1 will be extracted and purified from mice located in the Feinstein Institute. For the TNF ELISA and Disulfide HMGB1 Uptake, cells will be treated with different molecules, specifically Disulfide HMGB1, LPS, and Sulfonyl HMGB1.

TNF ELISA

Cells will be treated with varying amounts of Disulfide HMGB1, Sulfonyl HMGB1, and/or LPS will be stimulated for two days in Opti-MEM Medium. The R&D Systems ELISA Kit will be used to perform a sandwich ELISA. The 96-well plates will be coated with 100 μL/well of Mouse TNF-α Capture Antibody, which will be diluted in PBS to a working concentration of 0.8 μg/mL. The plates will sealed and left to incubate overnight at room temperature. Each well will then be washed three times with ELISA wash buffer. The plates will be blocked by adding 300 μL of Reagent Diluent (1% BSA in PBS) to each well and incubated

for a minimum of one hour at room temperature. Each well will then be washed three times with ELISA wash buffer. 100 µL of sample will be added to each well in the first three rows of the plate (diluted in Reagent Diluent if necessary). Mouse TNF-α Standard will be added in wells A7 - H8 in two-fold serial dilution in Reagent Diluent, with a high standard of 2,000 pg/mL. All other wells will serve as blanks and will be filled with 100 µL of Reagent Diluent. The plates will be sealed and left to incubate at room temperature for two hours. They will then be washed with ELISA wash buffer three times. 100 µL of Streptavidin-HRP will be added to each well. The plates will be sealed and incubated for 20 minutes. They will then be washed five times in ELISA wash buffer. 100 µL of the Substrate Solution (1:1 mixture of H_2O_2 and Tetramethylbenzidine) will be added to each well. The plates will not be placed in direct light and will then be incubated for ten minutes. 50 µL of Stop Solution (N_2SO_4) will be added to each well.

Disulfide HMGB1 Uptake

The RAW 264.7 Cells will be plated on glass coverslips which will be placed in six wells of a 24-well plate. They will be left to incubate overnight, and then the excess medium will be removed. DMEM Medium without Phenol Red will be added instead to reduce background. Each coverslip will then be given one of the six treatments including a combination of Alexa-555 Labeled Disulfide HMGB1, LPS, and/or Sulfonyl HMGB1. The treated cells will then be left to incubate in the dark for two hours at 37℃ in a 5% CO_2 incubator. They will then be rinsed three times with PBS. The cells will then be fixed using 4% Paraformaldehyde (300 µL per well) and

left to incubate for 30 minutes at room temperature. They will be rinsed three times with PBS and then mounted on slides using DAPI mounting medium.

Risks and Safety

I will be working with RAW 264.7 cells, Lipopolysaccharide, and multiple isoforms of HMGB1. Nonbiological hazards that I will be working include 4% PFA, various buffers and antibodies from the TNF ELISA kits, cell media, PBS, and UV rays from the fume hood. I will follow all proper safety precautions in order to prevent any damage from occurring. This includes wearing a lab coat, gloves, goggles, long pants, and close toed shoes at all times in the laboratory. All surfaces will be cleaned with the use of ethanol spray in order to prevent contamination. Experimentation with the live cells will only be performed in a fume hood, which will be kept in highly sterile conditions. Any hazardous chemical work will be performed in a fume hood as well, and all harmful wastes will be disposed of properly.

Data Analysis

Once the ELISA plates are treated with the stop solution (N_2SO_4), they will be brought immediately to the Magellan ELISA Reader. The plates will be read at 450 nm and a wavelength of 570 nm.

After being plated with DAPI Mounting Medium, the Uptake slides will sit in a cool, dark place for 24 - 48 hours. Then, images will be taken on the Carl Zeiss fluorescence microscope with the 40x objective using the black and white camera.

d. Bibliography

1. Okin D, Medzhitov R. (2013, September). Evolution of inflammatory diseases. Curr Biol. 2012;22:R733–R740.
2. Pisetsky, D. S., Gauley, J., & Ullal, A. J. (2011, October). HMGB1 and Microparticles as Mediators of the Immune Response to Cell Death. Antioxid Redox Signal. 15(8): 2209–2219.
3. Yang, H., Wang, H., Chavan, S. S., & Andersson, U. (2015, October). High Mobility Group Box Protein 1 (HMGB1): The Prototypical Endogenous Danger Molecule. Mol Med. 21 Suppl 1:S6-S12.
4. Li J., Kokkola R., et al. (2003, January). Structural basis for the proinflammatory cytokine activity of high mobility group box 1. Mol Med. 9(1-2):37-45.
5. Andersson, U., Yang, H., & Harris, H. (2018, March). Extracellular HMGB1 as a therapeutic target in inflammatory diseases. Expert Opin Ther Targets. 22(3):263-277.

1. Human Participants Research

N/A

2. Vertebrae Animal Research

N/A

3. Potentially Hazardous Biological Agents Research

The RAW 264.7 cells are an immortalized macrophage cell line, which will be used in all of the experiments. They will be maintained at approximately 10^5/mL in RPMI 1640 Media. They will be stored in a 5% CO_2 incubator at 37℃. When experimenting with these cells, all procedures will be performed in a highly sterile fume hood. All materials used in these procedures will be kept sterile through cleaning via ethanol. A lab coat and gloves will be worn at all times while working with these cells as well.

4. Hazardous Chemicals, Activities, and Devices

The LPS used will be from Escherichia Coli 011:B4 from Sigma Aldrich. The Disulfide HMGB1 and Sulfonyl HMGB1 will be extracted and purified from mice located in the Feinstein Institute. These molecules will be stores at 4℃. They are potentially hazardous upon ingestion and/or contact with skin/eyes. They will only be handled while wearing a lab coat, gloves, goggles, long pants, and close toe shoes.

PBS, PFA, Ethanol, the R&D Systems Antibodies/Diluents, and ELISA Wash Buffer may be hazardous upon contact with the skin, eyes, and/or flames. They will only be handled while wearing a lab coat, gloves, goggles, long pants, and close toe shoes. After use of them is complete, they will be disposed of carefully.

– NO ADDENDUMS EXIST –