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] HMGB1 is released from a variety of cells, as inflammation can occur throughout the body. However, once it is extracellular, HMGB1 can play a variety of roles depending on the tissue it is released from. In order to look at the role played by HMGB1 throughout the body with more clarity, a genetic approach can be taken by knocking out the molecule entirely. However, this has already been proved not a viable option, as the knockout was deadly in mice

(REF)[5], due to HMGB1's role in transcription. With this, researchers looked to develop a tissue-specific HMGB1 knockout to eliminate this problem as well as examine the difference in impact of HMGB1 from a variety of tissues. This tissue-specific knockout has been generated successfully for heart, liver, and myeloid tissue (REF)[4]. With this, researchers turned to look at the role of HMGB1 from neuronal cells, as it has not been determined. Neuronal HMGB1 has been found to be released, but its impact remains unclear.

Thus, in order to further examine the role the neuronal HMGB1 plays in non-neuronal tissue by observing these inflammation levels, we can examine the success of the neuronal HMGB1 knockout model and utilize it in a model of neuropathic inflammation. 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:

Is neuronal HMGB1 successfully knocked out of neuronal tissue in the Syn-Cre mice?

- Are HMGB1 levels lower in the Syn-Cre mice compared to the wild type and flox?

How does neuronal HMGB1 affect non-neuronal inflammation?

- Are cytokine levels (HMGB1, TNF, CXCL1, IL-18) higher or lower in the Syn-Cre mice compared to the wild type and flox?

Hypothesis/Expected Outcomes:

I hypothesize that neuronal HMGB1 will promote inflammation in non-neuronal tissue via the increased release of proinflammatory cytokines. I expect the HMGB1 neuronal knockout model to be successful, as Cre-Lox breeding has successfully knocked out HMGB1 in other tissues. Cytokine levels should be lowered once neuronal HMGB1 is knocked out, as it most likely plays a role beyond its specific tissue.

c. Procedures

Cre-Lox Breeding

Cre-Lox is a breeding technique that allows for the knockout of specific molecules. It allows researchers to have control over gene expression location and timing. Cre is a recombinase enzyme for LoxP, which is a site on bacteriophage P1. In this model, the Cre will recognize two LoxP sites and cut the HMGB1 in between them. A neuronal-specifc promoter known as Synapsin will be linked to the Cre. This will allow the neuronal specific HMGB1 to be recognized and knocked out of the brain, spinal cord, and dorsal root ganglia. All operations will be performed by lab faculty.

Chronic Constriction Injury (CCI) of the Sciatic Nerve

The CCI model as shown is used to promote inflammation in the mice so that HMGB1 levels can be observed. In this procedure, all mice will be anesthetized and opened, in order to control any possible outside effects the stress of the procedure could cause. The CCI mice will

have their right sciatic nerve exposed. Three loose knots will be applied to it, and then the mice's skin will be clamped closed. This will cause damage to the nerve without cutting it, as this would eliminate inflammation entirely. All operations will be performed by lab faculty.

Western Blot

Western Blots will be used to analyze the levels of HMGB1. First, gel electrophoresis will be used to separate the molecules based on size. The proteins in the gel will then be transferred to a PVDF membrane using a current. They will be bound non-specifically. These non-specific interactions will be suppressed using BSA or dry milk to wash the membrane. The membrane will then be treated with primary antibody, which will bind only to the protein of interest. Secondary antibody will be added, which will recognize the primary antibody and is attached to an enzyme necessary for detection. The protein-antibody complex will form on the membrane and be detected so that images can be taken.

ELISA

The R&D Systems ELISA Kit will be used to perform a sandwich ELISA on the mouse tissue from the wild type and Syn-Cre mice. The 96-well plates will be coated with 100 µL/well of Mouse TNF-α/CXCL1/IL-18 Capture Antibody, which will be diluted in PBS to a working concentration of 0.8 µg/mL. The plates will be 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/CXCL1/IL-18 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 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 incubated for ten minutes. 50 μL of Stop Solution (N_2SO_4) will be added to each well. The plates will then be read at 450 nm and a wavelength of 570 nm.

Risks and Safety

I will be working with mouse tissue and disulfide HMGB1. Nonbiological hazards that I will be working include various buffers/washes/antibodies for ELISAs and Western Blots, PBS, 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 any hazardous chemicals will only be performed in a fume hood, which will be kept in highly sterile conditions. All harmful wastes will be disposed of properly.

Data Analysis

After being treated with detector solution, the Western Blots will be imaged immediately using the BioRad Gel Doc reader. 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.

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.

No Addendums exist