Neuronal HMGB1 Facilitates the Inflammatory Response via Increased Release of Proinflammatory Cytokines

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Introduction

Inflammation is part of the immune system's biological response to harmful stimuli, such as infection. It can be initiated by a variety of factors. Typically, the immune system recognizes damaged cells, irritants, and/or pathogens, and begins to undergo its healing process as part of this defense mechanism. At the tissue level, this inflammatory response often consists of redness, swelling, pain, immobility, and heat at the site of infection. These symptoms attempt to return the tissue to a state of homeostasis.[1] Although this inflammation serves to help the body protect against harmful stimuli, it may malfunction. More specifically, its normally beneficial role in the body can become detrimental if the inflammation ends excessive in magnitude and/or duration.[2] 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. The immune system begins to fight itself instead of the true infection. This will initially lead to organ damage and in some cases organ failure, which can result in death.[3] These inflammatory diseases can be fatal if not treated quickly. Sepsis is an inflammatory disease that is the leading cause of hospitalized death in the Western World. 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. Further, Sepsis leads to organ failure, which can result in death if a

significant organ is not functioning. For example, Septic Shock occurs when blood flow is cut off to the heart, resulting in acute circulatory failure.[4]

All response pathways for inflammation share a common mechanism that can be summarized in the following four steps: (1) receptors on cells' surfaces recognize harmful stimuli, (2) the inflammatory pathway to respond to this stimuli is activated, (3) inflammatory markers are released, and (4) inflammatory cells are called to action. One specific type of immune cell is a macrophage. During this immune response, macrophages, and other immune cells such as lymphocytes and monocytes, release cytokines. Cytokines play a key role in the inflammatory response as they call immune cells to the site of infection. Pro- and anti-inflammatory cytokines inhibit and induce inflammation, respectively. They serve as modulators of the immune response pathways via complex interactions.[7] However, excessive amounts of cytokines result in the previously discussed inflammatory conditions. Research involving cytokines and their signaling pathways may allow for better understanding of these inflammatory conditions and the development of treatments for them.[5]

One specific cytokine is HMGB1, which was discovered by a team of researchers in Europe in 1973. HMGB1 stands for High Mobility Group Box 1. It is a protein that has been shown to assist in binding chromatin and is widely expressed in all nucleated mammalian cells. The name HMGB1 comes from the molecule's high electrophoretic mobility on polyacrylamide gels.[10] HMGB1 is the most abundant of the HMGB protein family which includes HMGB1, HMGB2, HMGB3, and HMGB4. HMGB1 expresses 214 amino acids that are arranged in two consecutive HMG box domains along with a C-terminal tail that has a stretch of 30 continuous glutamic and aspartic acids.[11] The order of the large amount of positively and negatively charged

amino acids makes HMGB1 have a bipolar charge.[12] HMGB1 is found extracellularly after either passive release via necrosis or active transport out of the cell.[8]

Of the fourteen receptor systems claimed to be receptors for HMGB1, only TLR4 and RAGE are definitively documented.[17, 18, 19] For the TLR4 system, Disulfide HMGB1 binds to the co-receptor for TLR4, MD2. This then results in the signaling of proinflammatory cytokines.[18, 20] For the RAGE system, when Disulfide HMGB1 binds to the RAGE molecule, there is an uptake via endocytosis of HMGB1. Once HMGB1 is present in the cytosol, proinflammatory mediators are released.[6, 21]

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 was taken by knocking out the molecule entirely. However, this proved to be not a viable option, as the knockout was deadly in mice (REF)[6], 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)[7]. 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.

Our objectives were to test the success of the neuronal HMGB1 knockout model and measure the inflammation levels in non-neuronal tissue. If the knockout is successful, we can

examine the role the neuronal HMGB1 plays in non-neuronal tissue by observing these inflammation levels.

Materials and Methods

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 recognizes two LoxP sites and cuts the HMGB1 in between them. A neuronal-specifc promoter known as Synapsin is linked to the Cre. This allows the neuronal specific HMGB1 to be recognized and knocked out of the brain, spinal cord, and dorsal root ganglia. All operations were performed by lab faculty. A diagram detailing this process is shown in Figure 1.

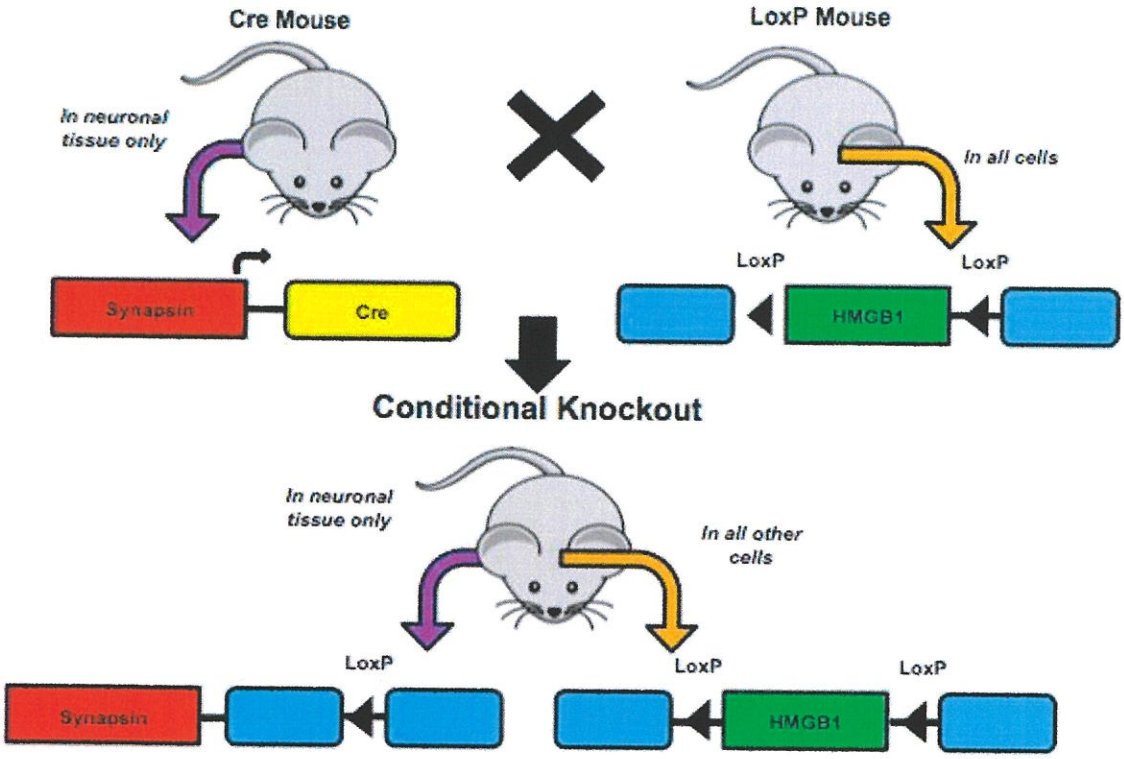


Figure 1: Student-generated diagram detailing the Syn-Cre breeding technique utilized.

Chronic Constriction Injury (CCI) of the Sciatic Nerve

The CCI model as shown in Figure 2 is used to promote inflammation in the mice so that HMGB1 levels can be observed. In this procedure, all mice are anesthetized and opened, in order to control any possible outside effects the stress of the procedure could cause. The CCI mice have their right sciatic nerve exposed. Three loose knots are applied to it, and then the mice's skin is clamped closed. This causes damage to the nerve without cutting it, as this would eliminate inflammation entirely. All operations were performed by lab faculty.

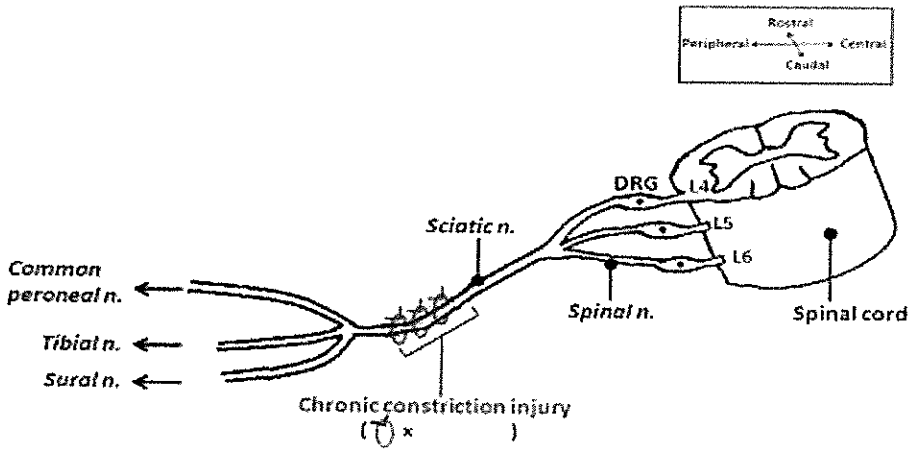


Figure 2: An image of the right sciatic nerve in the CCI model. (Source: Babic, et al.)

Western Blot

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

ELISA

The R&D Systems ELISA Kit was used to perform a sandwich ELISA on the mouse tissue from the wild type and Syn-Cre mice. The 96-well plates were coated with 100 μL/well of

Mouse TNF-α/CXCL1/IL-18 Capture Antibody, which was diluted in PBS to a working concentration of 0.8 µg/mL. The plates were sealed and left to incubate overnight at room temperature. Each well was then washed three times with ELISA wash buffer. The plates were 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 was then washed three times with ELISA wash buffer. 100 µL of sample was 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 was 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 served as blanks and were filled with 100 µL of Reagent Diluent. The plates were sealed and left to incubate at room temperature for two hours. They were then washed with ELISA wash buffer three times. 100 µL of Streptavidin-HRP was added to each well. The plates were sealed and incubated for 20 minutes. They were then washed five times in ELISA wash buffer. 100 µL of the Substrate Solution (1:1 mixture of H_2O_2 and Tetramethylbenzidine) was added to each well. The plates were not placed in direct light and incubated for ten minutes. 50 µL of Stop Solution (N_2SO_4) was added to each well. The plates were then read at 450 nm and a wavelength of 570 nm.

Results

One objective of this project was to determine the success of the neuronal HMGB1 knockout model. Figure 3 shows the results from the Western Blot, showing the HMGB1 was knocked out of the neuronal tissue. The other objective involved observing the effects this model

had on inflammation. Figures 4 and 5 show a Western Blot and ELISAs that show the lack of neuronal HMGB1 lower the inflammatory response.

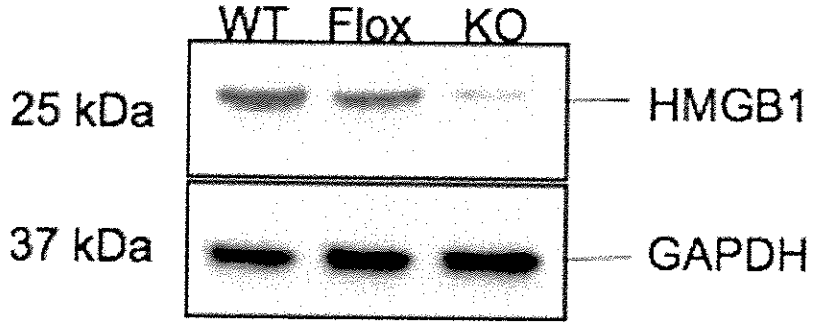


Figure 3: Data from Western Blot showing that neuronal HMGB1 is knocked out in the Dorsal Root Ganglia of the knockout mice.

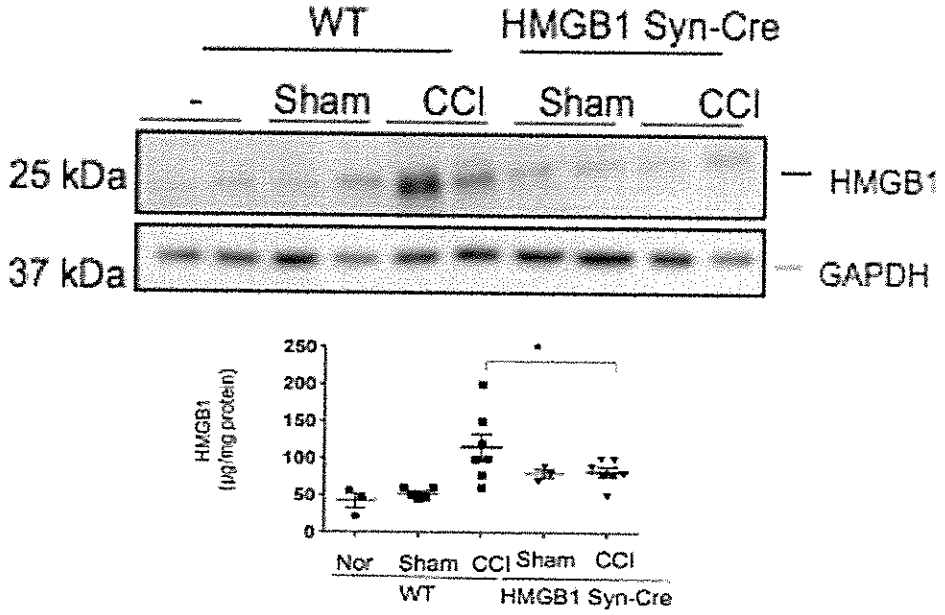
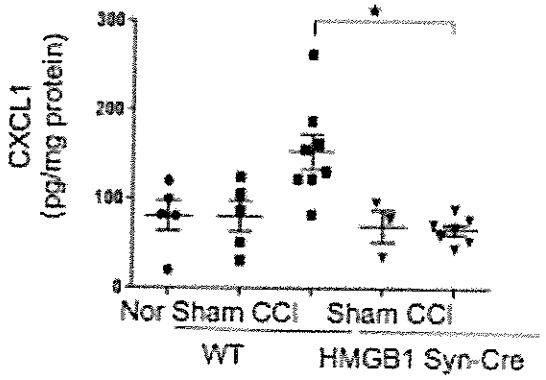
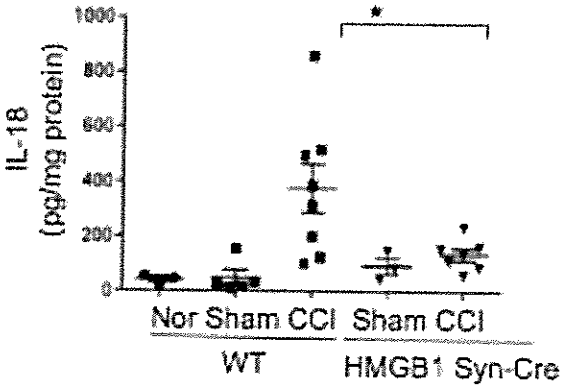


Figure 4: Data from Western Blot and ELISA showing that HMGB1 is inhibited in non-neuronal tissue (paw) in CCI model of knockout mice.



CXCL1 ELISA



IL-18 ELISA

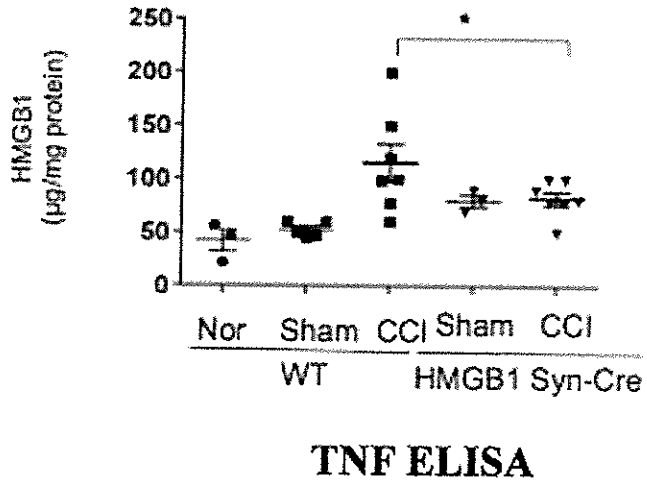


Figure 6: ELISA's showing that inflammation is decreased in non-neuronal tissue (paw) in CCI model of knockout mice

Discussion

The neuronal HMGB1 Knockout model was tested via a Western Blot. The Western Blot showed that the knockout model had lower levels of HMGB1 compared to both the wildtype and flox groups, showing that the knockout model worked as intended.

The CCI model in the neuronal HMGB1 knockout mice had reduced levels of HMGB1 compared to the wild type and flox groups. There were also decreased levels of CXCL1, IL-18, and TNF. Thus, inflammation was lowered. The change in inflammation can be attributed to the lack of neuronal HMGB1.

Conclusion

In conclusion, the neuronal HMGB1 knockout model was generated successfully. This was shown in dorsal root ganglia tissue, a type of neuronal tissue. Thus, this model can be used for neuropathic pain or inflammation models, as it can provide insight into the effects of neuronal HMGB1. The CCI knockout model showed that when inflammation is present, neuronal HMGB1 plays a role in promoting it. This was shown not only in a western blot, but also in multiple ELISAs of different known proinflammatory cytokines.

To continue this, we would like to repeat this experiment, as it could confirm our findings. Additionally, we would like to look into the specific pathway of HMGB1 in neuronal inflammation. To do this, we would look into the receptor signaling pathways of HMGB1, specifically TLR4 and RAGE. We would also study the neuronal HMGB1 knockout in other models, such as the formal nociception test, diabetic peripheral neuropathy (DPN), and collagen antibody induced arthritis (CAIA). Ideally, these experiments will allow us to understand the mechanism behind these results in order to design a treatment to lower inflammation utilizing neuronal HMGB1.

Works Cited

1. Medzhitov, R. (2010, March). Inflammation 2010: new adventures of an old flame. Cell, 140, 771-776.
2. Okin D, Medzhitov R. (2013, September). Evolution of inflammatory diseases. Curr Biol. 2012;22:R733–R740.
3. Babic Z.M., Zunic F.Z., et al. (2018, July). IL-33 receptor (ST2) deficiency downregulates myeloid precursors, inflammatory NK and dendritic cells in early phase of sepsis. J Biomed Sci. 12;25(1):56.
4. Gossez M., Rimmelé T., et al. (2018, November). Proof of concept study of mass cytometry in septic shock patients reveals novel immune alterations. Sci Rep. 23;8(1):17296
5. Linlin Chen, Huidan Deng, et al. (2018, January). Inflammatory responses and inflammation-associated diseases in organs. Oncotarget. 9(6): 7204–7218.
6. 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.
7. Jia, X., Hu, X., et al. Increased M1 macrophages in young miR-15a/16-/- mice with tumor grafts or dextran sulfate sodium-induced colitis. Scand J Immunol. 88(3):e12703.
8. 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.

9. 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.

10. Goodwin GH, Sanders C., Johns EW. (1973, September). A new group of chromatin-associated proteins with a high content of acidic and basic amino acids. Eur J Biochem. 38(1):14-19.

11. Stros M. (2010, January). HMGB proteins: interactions with DNA and chromatin. Biochim Biophys Acta. 1799(1-2):101-113.

12. Merenmies J., Pihlaskari R., Latinen J., et al. (1991, September). 30-kDa heparin-binding protein of brain (amphoterin) involved in neurite outgrowth. Amino acid sequence and localization in the filopodia of the advancing plasma membrane. J Biol Chem. 266(25):16722-16729

13. 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.

14. Venereau E., Casalgrandi M., et al. (2012, August). Mutually exclusive redox forms of HMGB1 promote cell recruitment or proinflammatory cytokine release. J Exp Med. 209(9):1519-1528.

15. Schiraldi M., Raucci A., Munoz L.M., et al. (2012, March). HMGB1 promotes recruitment of inflammatory cells to damaged tissues by forming a complex with CXCL12 and signaling via CXCR4. J Exp Med. 209(3):551-563.

16. Yang, H., Wang H., Ju Z., et al. (2012, March). Redox modification of cysteine residues regulates the cytokine activity oh high mobility group box-1 (HMGB1). Mol Med. 18:250-259

17. Kang R., Chen R., et al. (2014, December). HMGB1 in health and disease. Mol Aspects Med. 40:1-116.

18. Yang H., Wang H., Ju Z., et al. (2015, January). MD-2 is required for disulfide HMGB1-dependent TLR4 signaling. J Exp Med. 212(1)5-14.

19. Hori O., Brett J., Slattery T. et al. (1995, October). The receptor for advanced glycation end products (RAGE) is a cellular binding site for amphoterin. Mediation of neurite outgrowth and co-expression of rage and amphoterin in the developing nervous system. J Biol Chem. 270(43):25752-25761.

20. Yang H., Hreggvidsdottir H.S., et al. (2010, June). A critical cysteine is required for HMGB1 binding to Toll-like receptor 4 and activation of macrophage cytokine release. Proc Natl Acad Sci USA. 107(26):11942-11947.

21. Ramasamy R., Yan S.F., et al. (2011, December). Receptor for AGE (RAGE): signaling mechanisms in the pathogenesis of diabetes and its complications. Ann NY Acad Sci. 1243:88-102.