Interactions between High Mobility Group Box 1 (HMGB1) Protein and Toll-Like Receptor 4 (TLR4) And Their Roles in Inflammation Responses in the Brain and Liver: A Scientific Review

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INTRODUCTION

Inflammation is a response process of the immune system, which is designed to alert the body to some damage or invasion, and recruit the necessary components of the immune system to the area of danger in order to rectify the problem. In order to initiate an inflammatory response, some initial signal is needed in order to alert immune cells in the immediate vicinity to the danger. The signal is usually a molecule recognized by the body as only being present in a dangerous situation. When the molecule is derived from a foreign invader, it is called a pathogen-associated molecular pattern (PAMP). When the molecule is released from a host cell in response to danger, it is called a danger-associated molecular pattern (DAMP). PAMPs and DAMPs are recognized by pattern recognition receptors (PRRs) on innate immune cells, including a class of molecules known as toll-like receptors (TLRs). Signaling through a TLR pathway can signal an innate immune cell, such as a dendritic cell, monocyte, macrophage, or granulocyte, to release factors that can begin the immune response. Inflammation, in appropriate amounts, is beneficial to the body, as it allows for the immune system to do its job in clearing out danger. However, excessive inflammation can cause damage to the body, as many factors used by the immune system to clear pathogens or create inflammation are toxic to body cells when used in large amounts or when left in the body for long periods of time.

High Mobility Group Box 1 (HMGB1) Protein

High mobility group box 1 (HMGB1) protein is a DNA-binding protein, which "stabilizes nucleosomes and facilitates transcription" of DNA when present in the cell (Park *et al*., 2006). When released from the cell, however, HMGB1 can serve as a DAMP, by signaling through several toll-like receptors, as well as other signaling pathways, and lead to inflammatory responses (Park *et al*., 2006). HMGB1 is excreted by the cell in response to creation of reactive oxygen species in the cell, usually as a result of TLR4 signaling (van Golen *et al*., 2012). This activation pathway is shown in Figure 1. A cell may release HMGB1 purposely as a danger signal (Park *et al.,* 2006), or it may be released as a result of cell death, indicating necrosis (van Golen *et al*., 2012). No matter the reason or mechanism behind its release, once HMGB1 is in the extracellular environment it can act as a danger signal, by signaling through various receptors, including TLR3 (Qin and Crews, 2012), TLR2, TLR4, and Receptor for Advanced Glycation End Products (RAGE) (Mazarati et al., 2011). These interactions, especially those between TLR4 and HMGB1, have been implicated in inflammation responses, over-responses, and injuries; particularly in the liver and brain. Among these injuries are alcoholic and non-alcoholic fatty liver disease, alcoholic cirrhosis, ischemic and hemorrhagic stroke, memory impairment, sepsis, and heatstroke. The investigations and discoveries about the mechanism of these interactions are discussed herein.

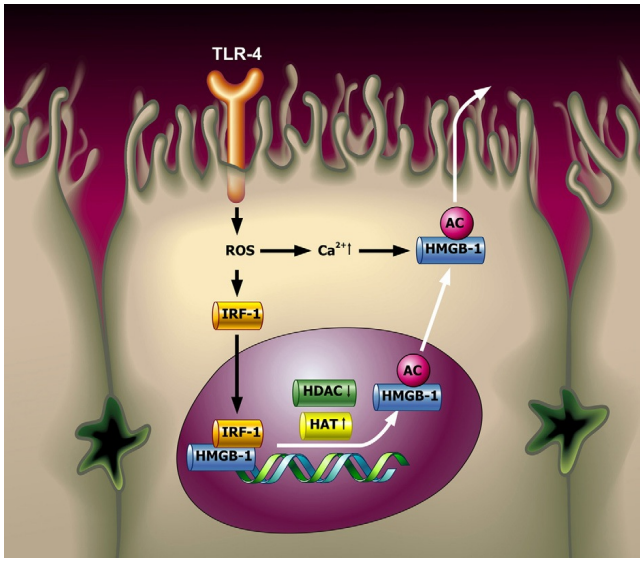

Figure 1: Here the mechanism for induced extracellular release of HMGB1 is demonstrated. The binding of TLR4's target leads the hepatocyte to produce reactive oxygen species, which then allows IRF-1, a transcription factor, to cross into the nucleus. This leads to a decrease in histone deacetylase (HDAC) activity and an increase in histone acetyltransferase (HAT) activity. Combined, these two factors cause HMGB1 to be acetylated, which allows it to exit the nucleus, and then exit the cell. (van Golen *et al.,* 2012)

Principal Approaches

Gene-Silenced Model Organisms: Most of the investigations into the HMGB1-TLR4 interactions and their role in inflammation make use of gene-silenced model organisms. In these experiments, a well-known model organism has a particular gene of interest silenced, usually either the TLR4 gene or HMGB1 gene. The most common model organisms for use in these experiments are C57/Bl6 mice. However several other organisms have also been utilized, including Sprague–Dawley rats (He *et al*., 2012), Wistar rats (Zhu *et al*., 2013), New Zealand white rabbits (Wang *et al*., 2013), and imprinting control region (ICR) mice (Kim *et al*., 2012). For the majority of these experiments, the model organism, with its gene of interest silenced, would be subjected to some form of liver or brain injury, including, but not limited to: heatstroke, crush injury, cirrhosis, or an inflammatory drug. Following injury, the inflammation responses would be measured using various methods, described later. Some experiments included attempts to treat the inflammation with various drugs. In some cases the effects of the inflammation were the main target for testing, however some experiments sought to determine levels of HMGB1 protein produced in response to injury (these did not use gene silenced animals).

Real-Time Reverse-Transcription Polymerase Chain Reaction (Real-Time RT-PCR): Experiments which relied on the measurement of gene expression utilized real-time reverse-transcription polymerase chain reaction. RNA would be isolated from cells or tissues, which would then be reverse transcribed into cDNA. This cDNA would then be replicated by real-time PCR, which would allow for relative levels of gene expression to be determined. In this way, different genes could be analyzed for their expression levels in response to various experimental conditions.

Histological Staining: Several papers sought to examine the organs for damage caused by inflammation. In order to study the organs, the researchers used histological staining methods. This process involves labeling slides containing thin slices of the organ of interest with some stain that would label an area or areas of interest, such as cell surface molecules or inflammation markers. This could be a simple color stain, or an antibody specific for a certain protein, which has been fluorescently labeled. After staining, these slides could be examined under a light or fluoresce microscope for changes.

Western Blot: A common method for determining protein presence is the use of a Western Blot. A protein sample is first treated with a detergent which breaks down its tertiary and secondary structures, leaving only the linear primary structure, or amino acid sequence. The sample is then placed into the well of a SDS-PAGE gel, and the proteins are run out on the gel using electric current. The proteins are then transferred from the gel to a nitrocellulose film. The film is then treated with fluorescently- or radioactively-labeled antibodies specific for the protein of interest, allowing them to be visualized.

Serum Analysis: In live-animal experiments, the blood of the organism is often subject to analysis. Some studies involved obtaining blood samples from the organism after experimental conditions were established. The plasma of the blood would then be isolated and the proteins present would be analyzed by various methods, including Western Blot and ELISA. The investigators would be interested in various proteins, including cytokines, HMGB1, and various indicators of inflammation. A blood panel may also be performed to check for indicators of liver damage, such as bilirubin.

Present Knowledge

Inflammation Caused by HMGB1-TLR4 Interactions are Responsible for Several Types of Liver Injury
When extracellular HMGB1 is recognized by cells through their TLR4 molecules, they begin to create and excrete inflammatory molecules and recruitment cytokines, which attract immune cells and lead to increased inflammation (van Golen *et al*., 2012). This increased inflammation is capable of creating or exacerbating hepatic damage. Liver injuries caused or exacerbated by inflammation include ischemia/reperfusion injury, hepatitis, hepatomas, steatohepatitis, sepsis, and heatstroke injuries.

One interaction responsible for exacerbation of liver injury is HMGB1-TLR4 interaction leading to the activation of caspase-1, which has been shown to allow hepatocellular carcinomas to metastasize (Yan *et al*., 2012). Yan and her colleagues demonstrated that HMGB1 is overexpressed in hepatocellular carcinoma cells; additionally, hypoxia conditions release the HMGB1 from the cells, allowing them to activate caspase-1 (Yan *et al*., 2012). Caspase-1, like

HMGB1, is an inflammatory factor, and has been demonstrated to aid in metastasis of the tumor (Yan *et al*., 2012). Caspase activation, along with apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and interleukin-beta 1 (IL-β1) have also been shown to induce an inflammation response by a HMGB1 mechanism, leading to damage in ischemia/reperfusion liver injuries (Kamo *et al.,* 2013). ASC knockout mice were shown to have reduced HMGB1 activity, which led to decreased liver damage as a result of ischemia/reperfusion injury (Kamo *et al.,* 2013). Ischemia/reperfusion injury results from cell death in the liver caused by lack of blood flow (Kamo *et al*., 2013). Restoration of blood flow allows toxins and other compounds to be washed into the bloodstream, resulting in systemic inflammation (Kamo *et al.,* 2013). The role of TLR4 in ischemic liver injury, at a cellular level, was determined by Nace and his colleagues, who showed that TLR4 is required by parenchymal hepatocytes for a sterile immune response (Nace *et al*., 2013). Furthermore they demonstrated that TLR4 knockout mice had significantly lower levels of extracellular HMGB1 (Nace *et al.*, 2013),

HMGB1 has also been implicated in other common liver injuries, including those induced by fatty liver. Non-alcoholic fatty liver disease is caused by fat buildup in the liver as a result of a high fat diet (Zhang *et al.*, 2013). One of the results of this fat buildup is an increase in extracellular HMGB1 in the liver, which leads to increased signaling of TLR4, which causes inflammation that is a component of fatty liver damage (Zhang *et al.,* 2013). One treatment for this condition is betatine, which lowered expression of TLR4 and HMGB1 (and other proinflammatory cytokines) in a rat model (Zhang *et al*., 2013). Another compound, quercetin, has been shown to also reduce inflammation and liver damage as a result of fatty liver (Marcolin *et al.,* 2012). Quercetin administered to mice with nonalcoholic steatohepatitis (NASH) decreased liver damage, as observed by immunohistochemical staining (Marcolin *et al.,* 2012). In addition to decreased liver damage, it was also demonstrated that HMGB1 gene expression was downregulated by the quercetin, which was likely partially responsible for the reduction in damage (Marcolin et al., 2012). Another drug, curcumin, was shown to reduce inflammation following induction of hepatic fibrosis by CCl_4, by downregulating HMGB1, TLR2, and TLR4 (Tu *et al.,* 2012).

In addition to liver injury resulting from inflammation, HMGB1 has also been implicated in liver dysfunction, including acute on chronic liver failure (ACLF), which is acute liver failure arising from an already existing chronic liver problem (Li *et al*., 2013). As has already been discussed, HMGB1 is known to signal through TLR4 to produce inflammatory cytokines. Li and his colleagues demonstrated that anti-HMGB1 monoclonal antibodies are able to significantly reduce chemically-induced ACLF in a rat model (Li *et al.,* 2013). Blocking HMGB1 allowed for a decrease in inflammation response (Li *et al*., 2013). Another type of liver failure, fulminant hepatic failure (FHF), has also been shown to be involved with HMGB1 (Laliena *et al.,* 2012). Acute liver failure induced by rabbit hemorrhagic disease virus (RHDV) in New Zealand rabbits was shown to increase expression of HMGB1 and TLR4 (Laliena *et al*., 2012). The same study demonstrated that melatonin reduces inflammation in the liver, and downregulates both HMGB1 and TLR4 (Laliena *et al*., 2012). Another drug, a traditional Chinese preparation, called Qinggan Huoxue, has been shown to increase liver function in rats suffering from chemically-induced acute liver failure, by downregulating TLR4 and HMGB1 (Zhu *et al., 2013).*

HMGB1-TLR4 Interactions Leading to Brain Injury and Memory Impairment

The brain has also been demonstrated to be vulnerable to the inflammatory effects of HMGB1-TLR4 interactions. The body of research relating to HMGB1 and its role in brain injury has made it a target for anticonvulsant therapy research (Vezzani *et al.*, 2011), as well as for therapy following ischemic stroke (Shichita, Ago*, et al.,* 2012). HMGB1-TLR4 signaling, along with other HMGB1 pathways, is responsible for many different types of inflammation-related brain injury, as well as memory disorders. In addition to its already well-understood inflammation pathways, HMGB1 released from neurons following ischemic stroke also “increases vascular permeability and promotes BBB breakdown” (Shichita, Sakaguchi, *et al*., 2012). Several studies have attempted to elucidate the mechanism by which HMGB1 participates in brain injury, and to discover ways in which it may be stopped.

One brain injury that has been observed to have a HMGB1 inflammation mechanism is hemorrhage, or bleeding in the brain cavity. A subarachnoid brain hemorrhage results in an upregulation of TLR4 and HMGB1, leading to an increase in inflammation, which exacerbates an already critical situation (Sun *et al.*, 2013). It was hypothesized that inhibiting the activity of HMGB1 could reduce inflammation following hemorrhagic brain injury, thereby leading to improved patient outcomes (Sun *et al.,* 2013). One drug that was proposed to inhibit HMGB1 activity was glycyrrhizic acid, which was shown to prevent release of HMGB1 from both damaged cells and from immune cells,

while having few deleterious side effects (Sun *et al.,* 2013). Another drug that could aid in reducing inflammation after hemorrhage is melatonin (Wang *et al.*, 2013). Melatonin has been shown to reduce inflammation following subarachnoid hemorrhage, while downregulating TLR4 and HMGB1, as well as several other inflammatory proteins (Wang *et al.,* 2013). It is possible that the antioxidant activity of melatonin would inhibit the creation of reactive oxygen species, which would not only prevent inflammation directly, but would also inhibit the release of HMGB1, as shown in Figure 1 (Wang *et al.,* 2013). Another method for inhibiting HMGB1, a monoclonal anti-HMGB1 antibody, has been shown to be effecting in treating HMGB1 related complications of traumatic brain injuries in rats (Okuma *et al.,* 2012).

HMGB1 has also been shown to impair memory function in mice (Mazarati *et al.,* 2011). Mazarati and colleagues demonstrated that intracranial HMGB1 in wild-type mice impaired the formation of new memories, as determined by the novel object recognition test (NORT) (Mazarati *et al.*, 2011). Memory formation was also impaired in TLR4 -/- mice and RAGE -/- mice (Mazarati *et al*., 2011). When both TLR4 and RAGE were knocked out, however, memory impairment ceased (Mazarati *et al.,* 2011). This indicated that HMGB1 signals through both TLR4 and RAGE in order to cause memory impairment, likely as a result of inflammation (Mazarati *et al*., 2011).

Liver and Brain Damage Caused by HMGB1-TLR4 Interactions in Other Disease Systems

Beyond the brain and liver specific diseases already mentioned, some other systemic disease states cause damage to the liver and brain by means of a HMGB1-TLR4 pathway. For example, heatstroke injuries of the brain and liver rely on a HMGB1-TLR4 interaction leading to over-inflammation (Dehbi *et al.,* 2012). Knockout of the TLR4 gene led to an increase in survival in mice exposed to heatstroke conditions (Dehbi *et al.,* 2012). Sepsis, or overactive inflammation of the body, also relies on a HMGB1-TLR4 pathway, and this pathway can be stopped by the drug genipin, again by halting production of TLR4 and HMGB1 (Kim *et al.,* 2012). Finally, these interactions have been implicated in crush injury in a rat model (Shimazaki *et al.,* 2012). Crushing of the limbs of rats led to substantial increase in plasma HMGB1, which was responsible for multiple organ failure that is common in crush injuries (Shimazaki *et al*., 2012). Sequestration of HMGB1 by monoclonal anti-HMGB1 antibody in that experiment improved rat survival (Shimazaki *et al*., 2012).

Conclusion

HMGB1 is a naturally occurring protein found in the nucleus of all cells. However, when released into the extracellular environment, either deliberately by an immune cell or as a result of cell necrosis, it can act as a DAMP, and signal to immune cells through TLR4. This signaling, shown as a result of cell lysis in Figure 2, leads to an induction of an immune response. Furthermore, HMGB1 can signal through TLR4 on non-immune cells, which leads to the production of IFN-β, which can recruit immune cells and aid in inflammation (van Golen *et al.,* 2012). Often times, during times of injury or disease, the HMGB1-TLR4 interaction produces too much inflammation, leading to complications.

Many pathologies include a HMGB1 inflammation component. Among these are fatty liver disease, cirrhosis, hepatic fibrosis, brain trauma, ischemic stroke, memory impairment, hemorrhage, heatstroke, and sepsis. It has been demonstrated that certain drugs are able to interfere with the inflammation responses in these diseases by inhibiting HMGB1-TLR4 interactions, usually by downregulating one or both of these compounds. Inhibition of the inflammation response is critical in order to treat many of these conditions.

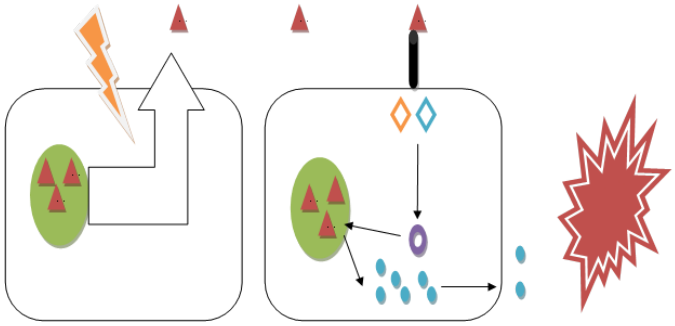

Figure 2: Demonstrated here is the mechanism for HMGB1 inflammation as a result of cell damage. The cell on the left has been lysed (orange bolt), which has released the HMGB1 (red triangle) from the nucleus (green circle). The protein has moved over to a neighboring cell, where it has activated TLR4 (black cylinder). The TLR4 signals a cascade, including factors TRAM and TRIF (orange and blue outlined diamonds), which activate transcription factor IRF-3 (purple doughnut), which enters the nucleus. This leads to upregulation of various inflammatory compounds, including IFN-β which exit the cell and recruit and activate immune cells (red burst). (adapted and expanded from van Golen *et al.,* 2012).

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