

High Mobility Group Box Protein 1 (HMGB1): The Prototypical Endogenous Danger Molecule

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High mobility group box protein 1 (HMGB1) is an evolutionary ancient nuclear protein that exerts divergent biological tasks inside and outside of cells. The functions of HMGB1 depend on location, binding partners and redox states of the molecule. In the nucleus, HMGB1 organizes DNA and nucleosomes and regulates gene transcription. Upon cell activation or injury, nuclear HMGB1 can translocate to the cytoplasm, where it is involved in inflammasome activation and pyroptosis, as well as regulation of the autophagy/apoptosis balance. When actively secreted or passively released into the extracellular milieu, HMGB1 has cytokine, chemokine, neuroimmune and metabolic activities. Thus, HMGB1 plays multiple roles in the pathogenesis of inflammatory diseases and mediates immune responses that range from inflammation and bacterial killing to tissue repair. HMGB1 has been associated with divergent clinical conditions such as sepsis, rheumatoid arthritis and atherosclerosis. HMGB1 initiates and perpetuates immune responses during infectious and sterile inflammation, as the archetypical alarmin and damage-associated molecular pattern (DAMP) molecule. We here describe advances in the understanding of HMGB1 biology with focus on recent findings of its mission as a DAMP in danger sensing and as a therapeutic target in inflammatory diseases.

Online address: <http://www.molmed.org>

doi: 10.2119/molmed.2015.00087

INTRODUCTION

During terminal sickness, patients present shared signs and symptoms irrespective of the primary insult being an infection or an injury. Exogenous bacterial toxins such as endotoxin (lipopolysaccharide [LPS]), a cell wall component in all gram-negative bacteria that may cause inflammation and septic shock, are cleared from circulation within minutes after exposure, whereas inflammation persists for many days (1). This puzzling temporal dichotomy initiated our original studies to elucidate whether inflammation and death during terminal sickness could be due to over-

production of endogenous endotoxin-like molecules (2). If confirmed, it would explain why patients look the same regardless of the absence or presence of foreign invaders expressing PAMPs (pathogen-associated molecular pattern molecules). We unexpectedly identified the endogenous nucleoprotein high mobility group box protein 1 (HMGB1), present in all cells, as a molecule released to the extracellular environment, where it acts as a critical proinflammatory mediator in endotoxemia. Furthermore, HMGB1 even operates via the identical cellular receptor complex (toll-like receptor 4 [TLR4]) as

LPS to generate inflammation (3). Subsequent studies revealed that HMGB1 occupies a crucial functional global role as a signaling molecule that informs other cells that damage or invasion has occurred. The principle established is that products of cellular injury activate fundamental defense mechanisms that are indistinguishable from responses activated by molecules from pathogens. We here update research progress made in the HMGB1 field during the past 15 years, with special emphasis on the role of HMGB1 as a prototypic damage-associated molecular pattern (DAMP) in inflammation.

HMGB1 AS AN ENDOGENOUS ENDOTOXIN-LIKE MEDIATOR OF INFECTION

The discovery of HMGB1 in 1999 as a secreted protein highlighted the role of extracellular HMGB1 in inflammation and infection. In these studies, macrophages were activated *in vitro* by LPS, and the supernatants were screened for macrophage-derived molecules,

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Submitted April 13, 2015; Accepted for publication April 30, 2015; Published Online (www.molmed.org) October 27, 2015.

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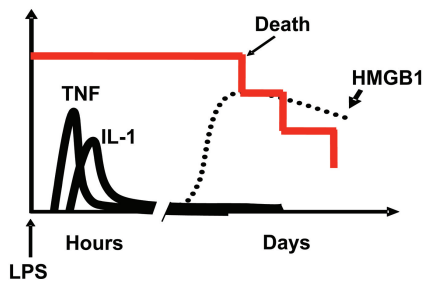


Figure 1. Early versus late mediators of endotoxin lethality. Vertebrates treated with lethal doses of LPS succumb at latencies of up to several days, long after serum TNF and IL-1 β have returned to basal levels. The timing for HMGB1 release is delayed and parallels the onset of lethality in endotoxemia and sepsis. Reprinted with permission of the American Thoracic Society. Copyright © 2015 American Thoracic Society. Wang H, Yang H, Czura CJ, Sama AE, Tracey KJ. (2001) HMGB1 as a late mediator of lethal systemic inflammation. *Am. J. Respir. Crit. Care Med.* 164(10 Pt 1):1768–73 (cited as reference (49) in the current review).

which resulted in the detection of extracellular HMGB1 as a delayed mediator after LPS stimulation. Systemic HMGB1 levels *in vivo* in mice started by 8 h and increased substantially from 16 to 32 h after LPS administration (2). HMGB1 thus appears remarkably late compared with other proinflammatory molecules, which are generally released within a few hours after initiation of sepsis (Figure 1) (2). The delayed HMGB1 release widens the temporal window of treatment opportunity with HMGB1 antagonists for sepsis patients in a noticeably unique way. This strategy has not yet been tested in clinical trials, but has proven highly successful in multiple preclinical settings (reviewed in [4]). The molecule is 99% identical in all mammals, a fact that may underpin the predictive clinical value of HMGB1 antagonist results observed in animal experiments. The exposure of cultured human monocytes to HMGB1 stimulated the release of multiple proinflammatory cytokines including tumor necrosis factor (TNF), interleukin (IL)-1, IL-6, IL-8 and macrophage inflammatory protein (MIP)-1. The kinetic re-

sponses for HMGB1- and LPS-mediated TNF release are distinctly different. HMGB1-induced TNF release is biphasic with a delayed second wave, whereas LPS-mediated TNF release only occurs in a monophasic early mode (5). The biological role of extracellular HMGB1 has been extensively studied in mice with gram-negative bacteria sepsis induced by cecal ligation and puncture (CLP) (reviewed in [4]). Treatment with neutralizing monoclonal anti-HMGB1 antibodies ameliorates tissue injury and reduces lethality in this sepsis model (6). Recent studies confirmed that siRNA-specific knockdown of HMGB1 in macrophages and dendritic cells suppressed HMGB1 release, reduced the cytokine storm and rescued humanized mice from CLP sepsis-induced lethality (7).

Mice that survive their CLP sepsis develop significant, persistent impairments in learning and memory, and anatomic changes in the hippocampus associated with a loss of synaptic plasticity (8). Serum HMGB1 levels are increased in these animals at least 8 wks after initiation of CLP. Administration of neutralizing anti-HMGB1 monoclonal antibodies to survivors, on d 9–11 after onset of peritonitis when no obvious signs or symptoms of ongoing inflammation occurred, significantly improved memory impairments and brain pathology. Administration of recombinant HMGB1 to naive mice recapitulated the memory impairments. This observation is interesting, since up to 25% of patients surviving severe sepsis are cognitively impaired. One may speculate whether HMGB1-blocking treatment might prevent this disabling late complication in patients, too.

In addition to being engaged in bacterial infections, HMGB1 and the closely related molecules HMGB2 and HMGB3 act as universal sensors for cytosolic nucleic acids during virus infections. Tian *et al.* (9) first demonstrated that HMGB1 is involved in DNA-containing complex-mediated immune responses via TLR9. Later, Yanai *et al.* (10) confirmed that HMGB1 binds to all immunogenic nucleic acids examined and mediates im-

mune responses by stimulating the transcription of type 1 interferons, IL-6 and RANTES from immune cells or embryonic fibroblasts. Extracellular HMGB1 carrying nucleic acids binds to the receptor for advanced glycation end products (RAGE) and gets internalized via dynamin-dependent endocytosis (11), which enables the transported nucleic acids to interact with intracellular receptors to mediate interferon and cytokine responses. HMGB1-deficient cells mount greatly diminished immune responses when stimulated with viral DNA or RNA compared with wild-type control cells. Synchronized knockdown of all three HMGB proteins inhibits the response to viral nucleic acid stimulation compared with solitary HMGB1 knockdown, indicating that HMGB proteins share antiviral functionality (10). Thus, HMGB proteins play an essential role as universal sentinels in nucleic acid-activated innate immune responses.

HMGB1 AS A MEDIATOR OF STERILE INJURY

The understanding of DAMP came from original work by Matzinger (12), who in 1994 suggested that the innate immune system detects and reacts to “danger” via release of host-derived mediators, what we now call “DAMP molecules.” This idea was brave, since the prevailing dogma at that time predicted that the function of the immune system was to recognize non-self-molecules. DAMPs are in general nuclear and cytosolic endogenous proteins exerting well-defined intracellular roles in the absence of cellular stress. When released extracellularly after tissue damage or injury, these molecules promote innate and adaptive immune responses and do not maintain their previous intracellular activities. HMGB1 is one of the first identified members of the DAMP molecular family. Bianchi and coworkers made the seminal observation that necrotic cells from HMGB1-deficient mice, in contrast to wild-type mice, have a markedly decreased ability to induce TNF release from cocultured macrophages. Apoptotic

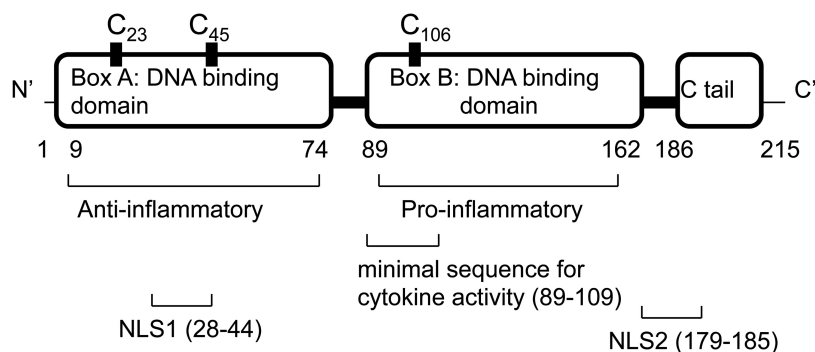


Figure 2. Structure of HMGB1 and its functionality. HMGB1 has 215 amino acids, including two DNA binding domains (box A and box B) and an acidic tail. Box A acts as an HMGB1 antagonist, whereas box B exerts the cytokine-inducing function of HMGB1. The minimal sequence for the proinflammatory activity of box B resides at aa 89–109. HMGB1 has three cysteines with two located at positions 23 and 45 in box A and one at position 106 in box B. HMGB1 contains two NLSs with one located in box A (aa 28–44) and another located in the linker region between box B and the C tail (aa 179–185).

cells from any source retains their nuclear HMGB1 strongly bound to the chromatin located within cell membrane-encircled cell fragments and do not induce TNF production in macrophage cocultures (13). Ombrellino *et al.* (14) provided the original clinical report of a case of sterile injury that generated increased HMGB1 serum levels. In the absence of infection, hemorrhagic shock stimulated a systemic release of HMGB1. Intestinal epithelial cells were recently identified as the main source for the detrimental HMGB1 release in an experimental model of hemorrhagic-induced systemic inflammation (15). Stroke patients also express increased circulating HMGB1 levels within hours after the ischemic tissue damage (16). Studies from Billiar's group confirmed that HMGB1 may act as an early mediator of inflammation and organ damage in hepatic ischemia/reperfusion injury (17). Systemic HMGB1 levels were increased during liver ischemia/reperfusion injury as early as 1 h after reperfusion and then increased in a time-dependent manner up to 24 h. Inhibition of extracellular HMGB1 activity with neutralizing antibody significantly decreased liver damage after ischemia/reperfusion in mice, whereas administration of recombinant HMGB1 worsened the injury (17). In-

creased extracellular HMGB1 expression has also been observed in several additional sterile injury models, including collagen-induced arthritis or during the spontaneous development of arthritis in mice (18,19). Systemic administration of anti-HMGB1 antibodies significantly ameliorated these autoimmune diseases, indicated by reduced weight loss and diminished cartilage/bone destruction in arthritic joints (18). Taken together, these studies establish extracellular HMGB1 as a critical mediator of both sterile and infectious inflammation.

REDOX REGULATION OF HMGB1 PROINFLAMMATORY ACTIVITIES

HMGB1 contains 215 amino acids (aa) with two folded DNA binding motifs called box A (aa 9–79) and box B (aa 95–163) and an acidic C tail (aa 186–215) (20). HMGB1 has two nuclear localization sequences (NLSs) located in box A (aa 28–44) and between box B and the C tail (aa 179–185). Truncated mutants of HMGB1 identified that HMGB1 box B protein preserves the cytokine activity of HMGB1 (21), whereas recombinant box A protein acts as an antagonist of HMGB1 (22). HMGB1 receptor usage and subsequent biological activities depend on the redox state of each of its three cysteines (C23, C45 and C106; Figure 2). Fully re-

duced HMGB1, which expresses three cysteine thiol residues, exerts chemotactic activity by forming a hetero-complex with CXCL12, which binds to the CXCL12-reciprocal receptor CXCR4 and initiates chemotaxis in a synergistic fashion compared with CXCL12 alone. The cytokine-stimulating activity of HMGB1 requires C23 and C45 to form a disulfide link, whereas C106 must express a thiol group (23). This distinctive molecular conformation enables HMGB1 to bind and signal via the TLR4/myeloid differentiation factor-2 (MD-2) complex to induce cytokine release (3,23,24). The fully reduced form of HMGB1 with or without CXCL12 cannot activate the TLR4/MD-2 signaling pathway, and the disulfide HMGB1 cannot activate the CXCL12/CXCR4 pathway (25). HMGB1 with any of the cysteine terminally oxidized (sulfonyl HMGB1) has no identified immunomodulating activity (Figure 3). Together, these studies reveal that posttranslational modifications of HMGB1 determine its role in inflammation and immunity.

EXTRACELLULAR HMGB1 RELEASE

HMGB1 release occurs during tissue injury or microbial invasion via two major pathways: one passive and the other active. Passive release, which occurs in the context of necrotic cell death, is nearly instantaneous. Active HMGB1 secretion is a much slower process that requires two essential steps: the initial step is to translocate nuclear HMGB1 to the cytoplasm, which depends on JAK-STAT signaling that will generate hyperacetylation of critical lysine residues located in the two NLS sites (26). This molecular modification of HMGB1 prevents the continuous bidirectional shuttle of HMGB1 between the cytoplasm and the nucleus and leads to cytoplasmic accumulation of hyperacetylated HMGB1. The second step involves a gradual induction of programmed, proinflammatory cell death (pyroptosis) that allows cytoplasmic HMGB1 to reach the extracellular space (27,28) or alternatively via exocytosis of secretory lysosomes that deliver

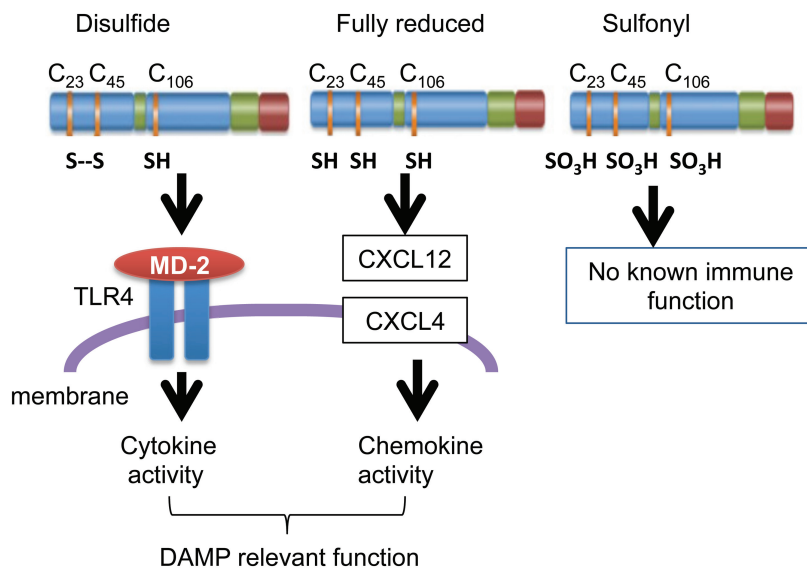


Figure 3. Redox-dependent regulation of HMGB1 cytokine activity. Disulfide HMGB1 interacts with MD-2 to facilitate the TLR4/MD-2 complex formation to elicit inflammatory responses. Fully reduced HMGB1 complexes with CXCL12 to induce recruitment of immune cells and other cells meant for repair. Terminal oxidation of any cysteine residue (sulfonyl HMGB1) prevents HMGB1 from expressing any cytokine or chemotactic activity.

HMGB1 outside cells (29). However, the intracellular signal pathways that control the sequestration of cytoplasmic HMGB1 in secretory lysosomes are not fully revealed yet. Pyroptosis requires the function of the intracellular enzyme caspase-1 that is activated by the inflammasome system. Inflammasomes are intracellular multiprotein complexes that promote the secretion of the important proinflammatory mediators IL-1 β , IL-18 and HMGB1. Genetic deletion of both IL-1 β and IL-18 did not confer any protection against lethal endotoxemia, although caspase-1 gene-deficient mice were totally protected. Most transgenic caspase-1 knockout mice also lack caspase-11 and consequently have a reduced capacity for induction of pyroptosis. Remarkably enough, neutralization via HMGB1-specific antibodies significantly prevented endotoxemia-induced lethality (27). Inflammasome-induced HMGB1 release leads to extracellular, hyperacetylated HMGB1, which is thus a novel biomarker for pyroptotic cell death (28). In contrast, necrotic as well as apoptotic cell deaths do not gen-

erate hyperacetylated HMGB1. The redox state of HMGB1 released after pyroptosis is generally in the disulfide form, after necrosis in the fully reduced or disulfide forms and after apoptosis in the fully oxidized form (sulfonyl HMGB1).

It was recently demonstrated that HMGB1 release is controlled by a neurotransmitter-mediated signaling pathway (30). Acetylcholine, from neurons as well as lymphocytes, inhibits inflammasome activation via $\alpha 7$ nicotinic acetylcholine receptors ($\alpha 7$ nAChR) present on the mitochondrial surface. Acetylcholine may penetrate the outer cell membrane in the presence of extracellular ATP. Mitochondrial $\alpha 7$ nAChR stimulation stabilizes the surface membrane of the organelles and attenuates the release of mitochondrial nucleic acids after stress-induced damage. The inflammasome system can be readily activated by intracellular DNA and RNA. The acetylcholine-mediated reduction of the cytoplasmic presence of mitochondrial nucleic acids thus prevents further inflammasome activation. Genetic dele-

tion of $\alpha 7$ nAChR significantly enhances inflammasome activation.

The accumulation of cytoplasmic HMGB1 is important for additional key cellular functions such as promoting mitophagy/autophagy, self-protective processes that remove damaged mitochondria and clear intracellular microbial intruders. Cytoplasmic HMGB1 has the capacity to initiate these events by binding to Beclin-1, required for the formation of autophagosomes. Manipulations to regulate intracellular HMGB1 traffic may on one hand diminish extracellular HMGB1 release, but at the same time interfere with autophagy capacity with hazardous consequences for the host (31). Future therapeutic strategies to pacify excessive extracellular HMGB1 activity should consider the fact that HMGB1 location is critical for HMGB1 functions.

HMGB1 RECEPTORS

HMGB1 signals via multiple seemingly unrelated receptors, all previously identified for their capacity to interact with well-characterized exogenous or endogenous ligands (9,10,25,32–42). To date, at least 11 different HMGB1 receptors have been described and are outlined in Table 1. Two of them (TLR4 and RAGE) will be discussed here, since there are important recent observations that are helpful for a better understanding of HMGB1 biology.

TLR4/MD-2 is a mandatory HMGB1 receptor complex for cytokine production in macrophages and the interaction requires the disulfide HMGB1 redox isoform, which binds the TLR4 coreceptor MD-2 with nanomolar avidity, just like LPS but at another MD-2 site (3,24). Other redox isoforms of HMGB1 do not bind to MD-2 and hence do not activate the TLR4 system (Table 2) (3). MD-2-deficient macrophages have a markedly reduced HMGB1-mediated nuclear factor (NF)- κ B translocation and TNF release. Taken together, these observations confirm the specificity of the immune-stimulatory activities of HMGB1. There have been concerns

Table 1. HMGB1 receptor families.

Receptors	Complex with	Effects	References
TLR4/MD-2	—	Cytokine release	3,16,39
RAGE	—	Cell migration, pyroptosis, internalization of HMGB1-partner molecules	11,32,37,45
CD24/siglet 10	—	Antiinflammatory	31
Integrin/Mac1	—	Cell recruitment	33
TIM3	—	Reduce tumor immunity	34
TLR4/MD-2	LPS, NMDAR	Synergistic cytokine release, increased neuroinflammation	35,38
TLR2	Pam ₃ CSK ₄ , nucleosomes	Synergistic cytokine release, enhanced autoantibody formation	40,41
TLR3/7/9	Nucleic acids	Synergistic cytokine release	9,10
IL-1R1	IL-1 α/β	Synergistic cytokine release	35,36
CXCR4	CXCL12	Synergistic chemotaxis	24

Extracellular HMGB1, either alone or in conjunction with other molecules, elicits diverse immune responses by activating multiple signaling pathways.

about the specificity of HMGB1 as a ligand for the TLR4 axis because of the possible LPS contamination in preparations of recombinant HMGB1. However, batches of disulfide HMGB1 subjected to cysteine redox changes lose their ability to activate the TLR4/MD-2 receptor complex. These findings prove the capacity of disulfide HMGB1-TLR4/MD-2 signaling without a need for cofactors.

RAGE is the first HMGB1 receptor that was identified, discovered by Rauvala and coworkers in 1999 as a ligand-receptor interaction mediating neurite outgrowth in the fetal mouse brain, without signs of concomitant inflammation (43). The same group later identified the RAGE binding site to be located in the HMGB1 sequence 150–183. Further RAGE-HMGB1 research work on consequences of this signaling pathway per-

formed in many laboratories has focused on cell migration, cell differentiation and upregulation of other HMGB1 receptors (reviewed in [44]). Two recently published HMGB1-RAGE papers bring exciting and essential information to the HMGB1 field. Xu *et al.* (11) uncovered that HMGB1 acting through RAGE binding and dynamin-dependent signaling initiates HMGB1 endocytosis, which in turn induces cell pyroptosis with release of proinflammatory mediators from the studied macrophages (11). They provide evidence that these important cellular events occur both *in vitro* and *in vivo*. As stated above, TLR4 is necessary for HMGB1-dependent cytokine production in macrophages, but at the same time, we and others have previously demonstrated that a quantitatively optimal HMGB1-mediated cytokine response also

requires RAGE (45–47). RAGE knockout macrophages produce substantially lower amounts of cytokines after TLR4 activation (45). It is conceivable that the RAGE-mediated endocytosis of HMGB1, equipped with or without partner molecules, may provide the answer to explain the elusive synergy mechanism occurring when various proinflammatory molecules complexed to HMGB1 are presented to the immune system. Intracellular DAMP and PAMP receptors may thus be exposed to and activated by mediators that would never reach them without the helpful HMGB1 transport and intracellular delivery. The HMGB1-mediated pyroptosis may then enable the proinflammatory molecules synthesized in the endosomes and cytoplasm to be released extracellularly. The second exciting novel HMGB1-RAGE report by LeBlanc *et al.* (48) describes an additional RAGE-binding epitope in HMGB1 located in the box A domain (binding region sequence 23–50) (48). This observation suggests the possibility that HMGB1 may mediate different biological functions when interacting with RAGE depending on which of the two RAGE-binding epitopes is involved. Cysteine redox states in box A need to be sorted out regarding requirements for RAGE interaction, in analogy to the HMGB1-TLR4/MD-2 story. Is the novel RAGE-binding box A epitope important for HMGB1/partner molecule synergy? It has been known since 2002 that recombi-

Table 2. Isoforms of HMGB1 and MD-2 binding and cytokine/chemokine activity.

HMGB1 isoforms	MD-2 binding	Cytokine activity	Chemokine activity
Disulfide	+	+	-
Hg-modified	-	-	-
Fully reduced	-	-	+
H ₂ S-modified	-	-	-
Sulfonyl	-	-	-

Isoforms of HMGB1, generated by a synthetic formation of mercury thiolate on C106 (Hg-modified), by S-sulfhydration (H₂S) to convert cysteine thiol (-SH) group to -SSH (H₂S-modified), by exposure to reducing agent dithiothreitol (fully reduced) or oxidized by hydrogen peroxide (sulfonyl), were tested for their MD-2-binding via Biosensor-based surface plasmon resonance analysis (BIAcore) (3). Only disulfide HMGB1 binds to MD-2 and induced TNF secretion. Fully reduced HMGB1 has chemokine activity.

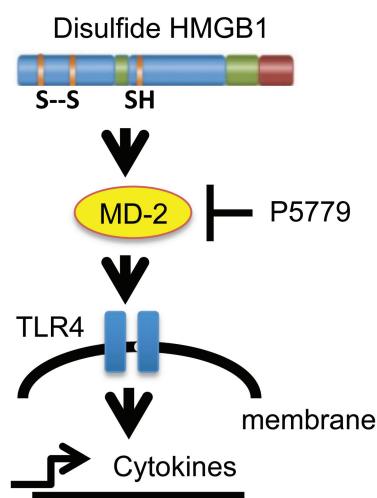


Figure 4. P5779 as an HMGB1 antagonist targeting MD-2. Disulfide HMGB1 binds to MD-2, and this interaction is critically important for HMGB1-TLR4-mediated immune responses. P5779 also binds to MD-2 and thus prevents the HMGB1-MD-2 interaction and subsequent TLR4-dependent signaling and immune response, thereby inhibiting HMGB1-induced cytokine release and toxicity.

nant box A peptide acts as a powerful HMGB1 antagonist in many experimental models of various diseases (18) (reviewed in [4]). However, the effector mechanism is not well understood. It has been suggested that the truncated HMGB1 box A protein may interfere with the TLR4 binding of the box B epitope recognizing MD-2, but the evidence is not convincing. However, the identification of the RAGE-binding box A epitope raises the question of whether recombinant box A protein may work via RAGE blockade, preventing critical full-length HMGB1/RAGE interactions. Future research work is needed to clarify these unresolved HMGB1 issues, which have important therapeutic implications.

P5779 AS AN MD-2 TARGETING HMGB1-SPECIFIC ANTAGONIST

Screening of an HMGB1-derived peptide library revealed that a tetramer peptide (sequence FSSE, P5779) located within the HMGB1 box B domain (aa

105–108) when C106 was replaced by a serine residue, acts as a specific inhibitor of HMGB1. P5779 binds MD-2 and disrupts HMGB1-TLR4/MD-2 ligation and subsequent cytokine induction (Figure 4). While P5779 dose dependently inhibits HMGB1-induced cytokine release from macrophage cultures, it does not alter LPS-TLR4 interaction or other studied ligand-TLR4 collaborations, supporting its role as a specific HMGB1 antagonist. The molecule alleviates HMGB1-induced inflammation in various preclinical models of HMGB1-mediated diseases including CLP-induced sepsis. Administration of P5779 ameliorated liver damage, in sterile injury models induced by liver ischemia/reperfusion or by acetaminophen overdose (3). The results may direct strategies attenuating DAMP-mediated signaling while sparing PAMP signaling through TLR4.

CONCLUSION

Since the discovery of HMGB1 as a proinflammatory mediator 16 years ago, the area of HMGB1 research has grown exponentially. The number of publications reporting advances in HMGB1 biology has increased from 281 during the 5-year period of 1995–2000 to 2,261 reports during 2009–2014 (PubMed). The process of gaining a better understanding of HMGB1 functions has established that once released outside of the cells, HMGB1 acts as a prototypic DAMP and a promiscuous danger sensor for the immune system and other organs. This review has highlighted functions of HMGB1 in its various forms from the whole molecule to individual domains and critical amino acid residues; its immunogenic activity either alone or in complex with cofactors; and the functional importance of different redox states of cysteine residues, to elucidate shifting pathophysiological roles of HMGB1. The worldwide accumulated information regarding HMGB1 biology suggests that the time has come to translate the pathophysiological relevant results

from experimental models into therapeutics for clinical trials.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health (RO1GM098446 to H Yang and RO1AT005076 to H Wang).

DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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Cite this article as: Yang H, Wang H, Chavan SS, Andersson U. (2015) High mobility group box protein 1 (HMGB1): the prototypical endogenous danger molecule. *Mol. Med*. 21 Suppl 1:S6–12.