HMGB1 as a Therapeutic Target in Disease

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Abstract

High-mobility group box 1 (HMGB1) was initially recognized as a ubiquitous nuclear protein involved in maintaining the nucleosome integrity and facilitating gene transcription. HMGB1 has since been reevaluated to be a prototypical damage-associated molecular pattern (DAMP) protein, and together with its exogenous counterpart, pathogen-associated molecular pattern (PAMP), completes the body's alarmin system against disturbances in homeostasis. HMGB1 can be released into the extracellular matrix (ECM) by either granulocytes or necrotic cells to serve as a chemotaxis/cytokine during infection, endotoxemia, hypoxia, ischemia-reperfusion events, and cancer. Different isoforms of HMGB1 present with distinctive physiological functions in ECM— fully-reduced HMGB1 (all thiol) acts as the initial damage signal to recruit circulating myeloid cells, disulfide HMGB1 behaves as a cytokine to activate macrophages and neutrophils, and both signals are turned off when HMGB1 is terminally oxidized into the final sulfonate form. Targeting HMGB1 constitutes a favorable therapeutic strategy for inflammation and inflammatory diseases. Antagonists such as ethyl pyruvate (EP) inhibit HMGB1 by interfering with its cytoplasmic exportation, while others such as glycyrrhizin directly bind to HMGB1 and render it unavailable for its receptors. The fact that a mixture of different HMGB1 isoforms is present in the ECM poses a challenge in pinpointing the exact role of an individual antagonist. A more discriminative probe for HMGB1 may be necessary to advance our knowledge of HMGB1, HMGB1 antagonists, and inflammatory-related diseases.

1.   Introduction

HMGB1, formally known as high-mobility group protein 1 (HMG-1) or amphoterin, was first discovered in 1973 as a chromosomal non-histone protein and named for its high electrophoretic mobility in polyacrylamide gels (Klune, Dhupar, Cardinal, Billiar, & Tsung, 2008). HMGB1 is a highly conserved and ubiquitously expressed nuclear protein involved in the maintenance of nucleosome structure and regulation of DNA replication, transcription, recombination, and repair (Travers, 2003). HMGB1 has also been found to accumulate in neurite extensions, such as the growth cone of filopodia in the protruding neuron during initial embryonic brain development (Merenmies, Pihlaskari, Laitinen, Wartiovaara, & Rauvala, 1991). In 1999, Wang and colleagues first reported that HMGB1 was released from lipopolysaccharide (LPS)-stimulated macrophages and acted as a pro-inflammatory cytokine of sepsis (H. Wang et al., 1999). This discovery set the stage for later studies on HMGB1's role in the inflammatory process and other chronic inflammatory diseases. Subsequent reports have begun to pour in about the active secretion of HMGB1 in different cells like monocytes, natural killer cells, dendritic cells (DC), platelets, and endothelial cells during infection and passive release from the nuclei of damaged/necrotic cells. Extracellular HMGB1 contributes to several pathologies, including sepsis, ischemia-reperfusion, atherosclerosis, arthritis, neurodegeneration, meningitis, and cancer. Many research groups have explored therapeutic options to regulate HMGB1 in preclinical models and have produced some promising data. Here, we review current progress in the field of HMGB1 biology and the status of HMGB1 as a therapeutic target in HMGB1-related diseases.

2.   HMGB1 structure and Nuclear Function

HMGB1 consists of 215 amino acid residues divided into three domains: two tandem HMG box domains termed A box and B box separated by a short flexible linker and a 30 amino acid glutamate/aspartate-rich C-terminal tail (Bianchi, Falciola, Ferrari, & Lilley, 1992). The A and B boxes are the DNA binding domains of HMGB1, whereas the negatively charged tail can bind histones. The C-terminus also directly interacts with the N-terminal box domains and modulates HMGB1-DNA binding specificity. A box domain can serve as an HMGB1 antagonist to protect against peritonitis-related lethality in mice (Huan Yang et al., 2004). As an evolutionarily conserved molecule, HMGB1 homologies between the human and mouse orthologs differ by less than 2%. HMGB1 has two nuclear localization signal sequences (NLSs) located in A box (aa 28–44) and between B box and the C-terminal tail (aa 179–185). The acetylation of multiple lysine residues in the NLSs promotes HMGB1 translocation from the nucleus to the cytosol and the subsequent release to the extracellular environment. HMGB1 utilizes various membrane receptors during its signaling cascade. The binding to the receptor for advanced glycation end products (RAGE) occurs at residues 150 to 183 (Huttunen, Fages, Kuja-Panula, Ridley, & Rauvala, 2002), while Toll-like receptor 4 (TLR4) binding occurs at residues 89 to 108 within B box of the HMGB1 molecule (H. Yang, Hreggvidsdottir, et al., 2010).

Inside the nucleus, HMGB1 also helps to maintain basal transcription levels by indiscriminately binding/bending duplex DNA, thus exposing binding sites for transcription regulation (Lotze & Tracey, 2005). HMGB1 has been found to increase the binding affinity

of many transcription factors to their cognate DNA sequences, such as p53, p73, the retinoblastoma protein (Rb), NF-κB, and the estrogen receptor. In contrast, the specific binding of HMGB1 to damaged and deformed DNA sequences may contribute to its role in the early stage of DNA repair. Loss of HMGB1 leads to decreased DNA repair efficiency, leading to increased DNA damage and cell death, and attenuating the cell's response to oxidative stress, like UV-irradiation.

3. Mechanism of HMGB1 Release

Besides its nuclear role, HMGB1 is also involved in several extracellular activities as an endogenous danger signal or DAMP. It participates in cell-cell interactions including production of pro-inflammatory cytokines, cell proliferation/differentiation/invasion, and autophagy (Lotze & Tracey, 2005). HMGB1 can be passively released or actively secreted. Damaged or necrotic cells passively release their nuclear HMGB1, which kindles an immediate inflammation response via pro-inflammatory cytokines such as TNFα. HMGB1 knockout cells are unable to activate monocytes during necrosis, thereby delaying the start of the inflammatory response (Scaffidi, Misteli, & Bianchi, 2002). HMGB1 can also be actively secreted from immune cells, endothelial cells, platelets, neurons, astrocytes, and cancer cells during stress or secondary to other DAMP signals as a reinforcement. However, it is still not entirely clear how HMGB1 is secreted to the ECM.

Like other nuclear proteins, HMGB1 is subject to various post-translational modifications including acetylation, N-glycosylation, ADP-ribosylation, phosphorylation, and methylation. The balance between histone acetyltransferases (HATs) and histone deacetylases (HDACs) confers HMGB1 acetylation status, which in turn influences its subcellular location (Bonaldi et al., 2003). This upstream event occurs at the classic nuclear localization signals (NLSs), and hyperacetylation of NLS lysine residues is critical for HMGB1 translocation from the nucleus to the cytoplasm. Activation of the JAK/STAT1 pathway promotes the acetylation of HMGB1 possibly through HDAC. Interferences in the JAK/STAT1 signal cascade through either the JAK-specific inhibitor pyridone 6 or STAT1 gene knockout prohibit LPS-primed HMGB1 hyperacetylation (Lu et al., 2014). The sirtuin 1 (SIRT1) protein represents one of the downstream acetylation signals. In LPS-stimulated murine macrophages, acetylation at lysines 28, 29, and 30 within the NLS breaks the interaction between SIRT1 and HMGB1, which allows HMGB1 translocation out of the nucleus via chromosome region maintenance 1 (CRM1) (Hwang et al., 2015). Additionally, phosphorylation of serine residues within NLSs of HMGB1 also facilitates its translocation from the nucleus to the cytoplasm, an event that has been reported independently by multiple groups in an apparent Ca^{2+}-dependent fashion, through either protein kinase C (PKC) (Oh et al., 2009) or Calcium/calmodulin-dependent protein kinase IV (CalMK-IV) (X. Zhang et al., 2008).

Active secretion of HMGB1 was first observed in LPS-stimulated monocytes by Gardella et al. who hypothesized an unconventional lysosome-mediated pathway for HMGB1 (Gardella et al., 2002). Due to the lack of a leader sequence, HMGB1 is not transported via the classical ER-Golgi secretory system. When tracked via electron microscopy, immunogold-labeled HMGB1 appears to be transported by a subtype of lysosomes—the secretory lysosome. Interestingly, transporting organelles for IL-1β, which also lack the signal peptide

for the Golgi-ER system, share some structural similarities with the HMGB1 lysosome (Gardella et al., 2002). Additional evidence points to a common secretory pathway for both HMGB1 and IL-1β converging in the inflammasome of monocytes during pyroptosis. Later studies of IL-1β$^{-/-}$ and IL-18$^{-/-}$ mice identify HMGB1 as the third pro-inflammatory cytokine downstream of the inflammasome, which mediates LPS-induced toxicity (Lamkanfi et al., 2010). In contrast to IL-1β and IL-18, however, HMGB1 is not a direct proteolytic target of caspase-1. The specific mechanism for caspase-1-dependent HMGB1 release remains elusive.

4. HMGB1 Redox States and function

Post-translational modifications at three redox-sensitive cysteine residues C23, C45, and C106 help to define HMGB1's role in the extracellular matrix as a chemoattractant or cytokine-inducing signal. The fully reduced form of HMGB1, where all three cysteines are in thiol state, behaves as a damage signal to recruit monocytes and other leukocytes. When these three cysteines are replaced with non-oxidizable, polarity-similar serine residues (HMGB1-3s), the resulting HMGB1 loses cytokine-stimulating activity but becomes more effective in recruiting leukocytes (Venereau et al., 2012). A heterocomplex formed by all-thiol HMGB1 and C-X-C motif chemokine 12 (CXCL12) is necessary to interact with C-X-C chemokine receptor type 4 (CXCR4) and initiates the recruitment of leukocytes (Schiraldi et al., 2012). A second semi-oxidized isoform of HMGB1 with a disulfide bridge at C23-S-S-C45 and an intact thiol C106 binds to TLR4 on macrophages and induces production of pro-inflammatory cytokines including TNFα, IL-1, and type-1 interferons (IFNs). Additional modifications at these three cysteine sites by either changing redox statuses, neutral mutations, or site-specific inhibition (targeting thiol C106 with mercury) abrogate HMGB1's cytokine-releasing activity. The unsteady disulfide HMGB1 can easily be reduced to all-thiol HMGB1 via DTT *in vitro* or further oxidized to another isoform, sulfonate HMGB1, by ROS (reactive oxygen species) *in vivo*. The third all-sulfonate form of HMGB1 possesses no known physiological function, and therefore, promotes immune tolerance during apoptosis (Magna & Pisetsky, 2014).

5. HMGB1 receptors and signaling

The function of HMGB1 as an extracellular signal is conferred by an array of receptors including RAGE, TLR-2, TLR-4, TLR-9, CXCR4, CD24, Tim3, IL-1R1, Integrin/Mac1. Various co-factors sometimes assist the binding of HMGB1 to its receptors. As mentioned above, the complex of CXCL12 – HMGB1 is necessary for HMGB1 to bind to CXCR4 (Schiraldi et al., 2012). HMGB1, Siglec-10, and CD24 seem to form a trimer and regulate acetaminophen (AAP)-induced inflammation through the NF-κB pathway (G. Y. Chen, Tang, Zheng, & Liu, 2009). The duplex of HMGB1 and CpG oligodeoxynucleotide, dubbed as the DNA-containing immune complex, exerts its effect through RAGE and TLR-9 to boost cytokine production (J. Tian et al., 2007). Three primary HMGB1 receptors are discussed in detail below.

RAGE

RAGE is a transmembrane receptor belonging to the immunoglobulin gene superfamily. The extracellular module of RAGE consists of three Ig-like domains, among which the distal V-type domain is directly responsible for ligand binding. A single transmembrane domain helps RAGE anchor in the cell membrane, and a short 43-amino acid C-terminal tail mediates cytosolic signaling. As a multiligand receptor, RAGE binds to several classes of molecules including advanced glycation end products (AGEs), HMGB1, S100 family proteins, and β-sheet fibrillar material. RAGE is the first receptor identified to bind to HMGB1 (Hori et al., 1995). Downstream effects of the HMGB1-RAGE axis include cytoskeleton remodeling, cell adhesion, chemotaxis, cell proliferation and differentiation, cell death, and communications with other HMGB1 receptors like TLR4. RAGE-mediated activation plays a vital role in HMGB1-related cancer. Blocking HMGB1-RAGE *in vivo* with the soluble extracellular domain of RAGE (sRAGE) leads to reduced tumor growth and tumor invasion (Taguchi et al., 2000). In addition to the cytoplasmic membrane, RAGE is also found to be present in mitochondria of tumor cells (Kang, Tang, et al., 2014). Interaction between HMGB1 and RAGE regulates cellular metabolism and promotes tumor growth by enhancing ATP production.

TLRs

TLRs are highly conserved proteins that elicit the innate immune response to endogenous or exogenous stimuli. HMGB1's binding to TLRs (TLR2, TLR4, and TLR9) activates NF-κB and interferon regulatory factor (IRF) pathways, stimulating the production of cytokines and chemokines during inflammation. TLR4 has been the focus of study as an HMGB1 receptor during the pre-inflammatory phase. Binding of HMGB1 to TLR4 is critical for a robust TNFα-mediated immune response *in vivo* using three types of knockout (KO) mice: TLR4-KO, TLR2-KO, and RAGE-KO (Venereau et al., 2012). The semi-oxidized form of HMGB1 with a C23-C45 disulfide bond and a reduced C106 has a much higher specificity to TLR4 compared to other forms. A thiol C106 within the B box domain of HMGB1 is key to the stable binding between TLR4 and HMGB1. Using neutralizing HMGB1 monoclonal antibody (mAb) or recombinant HMGB1 with mutant C106 blocks TLR4 binding and abrogates HMGB1-mediated cytokine release (H. Yang, Hreggvidsdottir, et al., 2010). A co-receptor, myeloid differentiation factor 2 (MD-2), is found to be involved in TLR4's binding to disulfide HMGB1, which may explain TLR4's high affinity to this HMGB1 isoform. Decreased expression of MD2 dramatically reduces both LPS- and HMGB1-induced NF-κB activation and subsequent secretion of cytokines (H. Yang et al., 2015).

HMGB1-TLRs binding also displays cell-type specificity. HMGB1 effectively induces IL-8 release only from TLR2-, but not TLR4-, overexpressing HEK293 cells. Consistently, HMGB1-primed HEK293/TLR2-expressing cells treated with TLR2 antagonists have reduced IL-8 production (Yu et al., 2006). Binding of serum DNA-HMGB1 immune complex to TLR9 receptors on dendritic cells and B cells is seen in patients with autoimmune diseases such as systemic lupus erythematosus (SLE). The TLR9–MyD88 pathway mediates HMGB1-DNA-dependent cytokine production, which is synergistically enhanced by coupling with RAGE (J. Tian et al., 2007).

CXCR4

CXCR4 is a member of the G protein-coupled receptors (GPCRs) and widely expressed in hematopoietic cells. CXCR4's main ligand is CXCL12 (also known as stromal cell-derived factor-1 (SDF-1)). CXCL12-CXCR4 pathways are involved in maintaining lymphoid organ homeostasis and lymphocyte trafficking. As discussed above, all thiol-HMGB1 can form a heterocomplex with CXCL12 and together bind to CXCR4 (Schiraldi et al., 2012). CXCR4 seems to function independently from other HMGB1 receptors like RAGE and TLR4 during the initial chemotaxis stage of inflammation. The conformation of CXCR4 activated by HMGB1-CXCL12 is distinctive from that of CXCR4 by CXCL12 alone (Venereau, Schiraldi, Uguccioni, & Bianchi, 2013), but additional information is required to elucidate the exact role of HMGB1 in the CXCL12-CXCR4 interaction. A recent study has reported that mutant HMGB1 (HMGB1-3s), with all three cysteines replaced by non-oxidizable serines, can binds directly to CXCR4 without CXCL12 and induce cardiac fibroblast migration during myocardial infarction (Di Maggio et al., 2017). Subsequent study has shown all thiol (fully reduced) HMGB1 modulates muscle and liver regeneration via CXCR4, and non-oxidizable mutant HMGB1-3s has achieved better tissue repair results than endogenous HMGB1, due to reduced HMGB1-mediated inflammation through the TLR4/MD-2 pathway (Tirone et al., 2018).

6. Current HMGB1 antagonists

As a prototypic DAMP, HMGB1 has been studied for its therapeutic potential in treating sepsis, sterile inflammation, autoimmune disease, and cancer. Many HMGB1 antagonists have been identified including HMGB1-neutralizing antibody, DNA-binding A box (BoxA) protein, platinating agent (like cisplatin), quercetin, ethyl pyruvate (EP), glycyrrhizin, diflunisal, sRAGE, and triptolide (Figure 1). Delayed administration of purified anti-HMGB1 IgG has proven to be effective against LPS-induced lethality in mice (H. Wang et al., 1999). In a dose-dependent manner, the HMGB1 antibody mitigates septic damage and inhibits HMGB1-stimulated release of other pro-inflammatory cytokines like TNFα and IL-6 (Huan Yang et al., 2004). The HMGB1 antibody has also been shown to reduce seizures in kainic acid-induced epilepsy models, probably by blocking the TLR4 receptor. When tested in rats, anti-HMGB1 mAb effectively attenuated secondary brain injuries caused by intracerebral hemorrhage (ICH)-induced stroke (D. Wang et al., 2017). A box domain of HMGB1 can function as a competitive inhibitor to HMGB1 (Huan Yang et al., 2004). Recombinant BoxA protein has been used interchangeably with HMGB1 antibody *in vivo* to protect against HMGB1-induced cell migration, septic lethality, leukocyte recruitment, ischemia/reperfusion injury, inclusion body myositis, acute pancreatitis, and tumor progression (Cottone et al., 2015). Platinating agents, such as cisplatin, are common chemotherapy agents that induce apoptosis in tumor cells by cross-linking DNA. At low nontoxic doses, however, cisplatin is found to limit HMGB1 release from nucleus, possibly via p38 and c-Jun N-terminal kinase (JNK) of mitogen-activated protein kinase (MAPK) signaling and to help reduce secondary damages from LPS-induced inflammation (Pan et al., 2009). Quercetin is a common food supplement derived from fruits and vegetables. It is a natural antioxidant proven to relieve HMGB1-induced endotoxemia lethality possibly

through NF-κB pathway (Tang et al., 2009). Other HMGB1 antagonists are described in detail as follows.

Ethyl Pyruvate

Ethyl pyruvate (EP) is an ester derivative of pyruvic acid, the end-product of glycolysis, with a longer half-life. This structural resemblance allows EP to serve as a protective antioxidant and limits the extent of ROS damage during endotoxemia, sepsis, and inflammatory disease (Ulloa et al., 2002). EP is proven to regulate heme oxygenase-1 (HO-1) and inducible nitric oxide synthase (iNOS) expressions via p38 mitogen-activated protein kinase (MAPK) and NF-E2-related factor 2 (Nrf2) pathways in septic animal models. In addition, EP reduces the lethality of hemorrhage shock in mice, which correlates with a reduction in serum HMGB1 (Ulloa et al., 2002). EP also suppresses the expression of HMGB1's receptor RAGE, which contributes to its ability to attenuate the malignancy of mesothelioma (Pellegrini et al., 2017). Similar studies in hepatocellular carcinoma show that EP acts on HMGB1 through the protein kinase B (Akt) pathway to induce apoptosis and hinder tumor growth (Cheng et al., 2014).

Glycyrrhizin

Glycyrrhizin (GL) is a naturally occurring compound from licorice plant, *Glycyrrhiza glabra,* and can directly bind to and inhibit HMGB1 (Mollica et al., 2007). HMGB1 is released from cells in two ways: passively from nuclei breakdown during necrosis or actively secreted by stimulated myeloid cells during inflammation. As previously discussed, EP blocks HMGB1 release without affecting soluble HMGB1 already present in the ECM. In contrast, GL inhibits HMGB1's release by stimulated macrophages through p38/Nrf2-dependent HO-1 activation as well as blocks extracellular HMGB1 from its receptors by directly binding to A box and B box domains. An NMR-guided docking model demonstrates that both electrostatic force and Van der Waals interactions are important in stabilizing GL's interaction with HMGB1 (Mollica et al., 2007). Additionally, GL inhibits HMGB1-mediated chemotaxis after muscle injury by disrupting HMGB1-CXCL12 heterocomplex (Schiraldi et al., 2012). Research groups focusing on the therapeutic potential of GL continue to generate promising results for its usage in inflammatory diseases including sepsis, ischemia-reperfusion injury, intracerebral hemorrhage, pancreatitis, and cancer.

Diflunisal

Diflunisal (DFL) is a salicylic acid derivative with anti-inflammatory properties, which is approved by the FDA for the treatment of chronic inflammatory diseases including rheumatoid arthritis ("Diflunisal," 2012). Recently, DFL was shown to inhibit HMGB1 by binding to HMGB1-CXCL12 heterocomplex and preventing the recruitment of inflammatory cells (De Leo et al., 2019). Using mutant HMGB1 (HMGB1-3s) with non-oxidizable serines, the authors reported that DFL's inhibition on the HMGB1-CXCL12 axis was mediated through CXCR4 independent of TLR4. DFL inhibited the chemotactic activity of HMGB1 at nanomolar concentrations, further evidence of the anti-inflammatory properties of DFL (De Leo et al., 2019).

Soluble RAGE

Soluble RAGE (sRAGE) is one of the isoforms of the RAGE receptor that is present in the ECM and the bloodstream. It can be produced by either alternative splicing of pre-mRNA or proteolytic cleavage of membrane-bound full-length RAGE. Circulating sRAGE serves as a biomarker for HMGB1-related diseases, especially autoimmune diseases and cancer (Huttunen et al., 2002; Pilzweger & Holdenrieder, 2015). sRAGE can act as a decoy to compete with RAGE in ligand-binding and has been used successfully in blocking the HMGB1–RAGE signaling pathway in animal tumor models. Once bound to HMGB1, RAGE transmits the downstream signals via activation of the ERK/MAP kinase pathway (Sims, Rowe, Rietdijk, Herbst, & Coyle, 2010). sRAGE successfully reduces local HMGB1 concentration in the ECM and weakens HMGB1's stimulatory effects on cancer cell migration and proliferation (R. C. Chen et al., 2014). Administration of sRAGE also helps relieve ischemic damage as seen in the middle cerebral artery occlusion (MCAO) mouse model (Muhammad et al., 2008). The benefits of pre- and post-treatment with sRAGE manifest as a reduction in both infarct volume and activated microglial cells.

Triptolide

One of the most recently discovered antagonists of HMGB1 is triptolide. Triptolide is a natural diterpenoid tri-epoxide extracted from the traditional Chinese herb *Tripterygium wilfordii* Hook F (TwHF) (S. Wang et al., 2012). Studies have shown more than one signaling pathway attributable to the anti-tumor effects of triptolide since its isolation, but only recently triptolide's therapeutic activity has been linked to HMGB1. Triptolide is reported to suppress the expression and release of HMGB1 in breast cancer cells both *in vitro* and *in vivo* (W. Jiang et al., 2019). Moreover, triptolide demonstrates a synergistic effect with EP, another HMGB1 inhibitor, in inhibiting cancer cell proliferation. The anti-growth effect of triptolide may be mediated through the HMGB1/TLR4/NF-κB signaling pathway (W. Jiang et al., 2019). Future studies will hopefully shed light on the value of triptolide as an HMGB1 targeted therapy for anti-cancer treatment.

7. HMGB1 in diseases and HMGB1 targeting strategies in preclinical studies

Due to HMGB1's active role in inflammation and immune response as an endogenous alarmin, many research groups have focused on the therapeutic potential of HMGB1 by suppressing its gene expression, cellular release, or extracellular activities as a cytokine/ chemoattractant. The application of HMGB1 antagonists has proven successful in a wide range of pre-clinical disease models. Here, we discuss the role of HMGB1 in disease and current trends in HMGB1 treatment.

Cancer

HMGB1 overexpression and/or high serum HMGB1 have been found in many types of cancer, including non-Hodgkin lymphoma, liver cancer, melanoma, gastric cancer, cervical cancer, breast cancer, osteosarcoma and mesothelioma (Jube et al., 2012). In a systematic literature review of 18 studies involving 11 types of cancer, HMGB1 overexpression is

associated with poor prognosis (T. Wu et al., 2016). Additional meta-analyses show that high HMGB1 positively correlates with adverse clinical outcomes in non-small cell lung cancer (NSCLC) (Feng, Tu, & Yin, 2016) and cervical cancer (P. Li et al., 2019), further validating HMGB1's role as a prognostic indicator. Elevated HMGB1 has also been associated with malignant phenotypes including tumor invasion and metastasis (M. Chen et al., 2015). In patients of colorectal cancer (CRC), high serum HMGB1 positively correlates with lymph node metastasis. The immunohistochemistry analysis of CRC tissues shows elevated expression of HMGB1, pERK, and c-inhibitor of apoptosis protein (c-IAP2), which may suggest HMGB1's role in inhibiting cancer cell apoptosis (W. Zhang et al., 2019). Another study had similar results in which HMGB1 tested positive in 96 percent of rectal cancer tissues. Knocking down endogenous HMGB1 with shRNA suppresses cancer cell proliferation by decreasing Bcl-2 and activating Bax (Z. Wang et al., 2016). HMGB1-induced chronic inflammation is also the key to explain asbestos-led tumorigenesis and mesothelioma pathogenesis (Carbone & Yang, 2017). Yang, H. et al demonstrated that asbestos fibers cause mesothelial cells to die from programmed necrosis and passively release nuclear HMGB1, which induces nearby macrophages to secrete TNFα creating an inflammatory microenvironment that in turn protects other mesothelial cells from asbestos toxicity (H. Yang et al., 2006; H. Yang, Rivera, et al., 2010). Thus, those cells accumulate DNA damages driven by asbestos fibers and transform into malignant cells.

It is now clear that in addition to passive release during necrosis, HMGB1 is actively secreted by cancer cells. Extracellular HMGB1 can serve as a paracrine/autocrine factor for cancer and a dynamic driver of tumor growth, proliferation, migration, and angiogenesis (van Beijnum et al., 2013). One prominent example is malignant mesothelioma (Carbone & Yang, 2012). Elevated levels of HMGB1 in the microenvironment promote mesothelioma growth and progression through RAGE receptor. *In vivo* studies using either HMGB1 antibody, RAGE antibody, or HMGB1 antagonists like BoxA have effectively reduced tumor burden and increased survival in mice models (Jube et al., 2012).

As an autophagic stimulator, HMGB1 is also a potential solution for chemoresistance. Autophagy is an endogenous cell survival mechanism against starvation and stress. Cancer can upregulate autophagy, which leads to drug resistance thwarting chemotherapy. HMGB1 is a well-recognized autophagy agonist (L. Liu et al., 2011). It has been shown that HMGB1 promotes autophagy by directly binding to Beclin 1 and releasing it from the inhibitory binding of Bcl-2 inside the cytosol (L. Liu et al., 2011). In response to ROS, HMGB1 is oxidized into the pro-inflammatory disulfide form and transported into the cytoplasm where it activates autophagy via the Beclin1-Bcl2 pathway (Tang et al., 2010). HMGB1-p53 interaction serves as a secondary regulator for HMGB1-Beclin 1 binding. A decreased level of p53 correlates with HMGB1's cytoplasmic translocation, which eventually induces autophagy (Livesey et al., 2012). Additionally, extracellular HMGB1 can bind to the RAGE receptor and regulate autophagy through the ERK/MAPK and AMPK/mTOR pathways. Binding of HMGB1 to RAGE leads to ERK phosphorylation and, consequently, autophagy activation, which promotes gastric cancer cell survival. Blocking either HMGB1 or RAGE, on the other hand, suppresses autophagy and enhances the chemosensitivity of colorectal cancer via the ERK/dynamin-related protein 1 (Drp1) pathway (Huang et al., 2018). Similarly, down-regulating HMGB1 via shRNA is shown to improve chemosensitivity and

increase tumor apoptosis via the ERK pathway in colorectal cancer cells (W. Liu et al., 2015). Most recently, autophagy has been implicated in the asbestos-induced carcinogenesis in mesothelioma (Xue, 2020). Asbestos fibers are toxic to primary human mesothelial (HM) cells under tissue culture conditions. It has been recently discovered that autophagy was up-regulated in HM via HMGB1 after asbestos exposure. The autophagy activation serves as a surviving mechanism that protects HM from dying. Surviving HM continue to accumulate DNA-damages due to asbestos fibers and may eventually become malignant cells. Using autophagy inhibitors, like chloroquine (CQ) or desmethylclomipramine (DCMI), the authors can suppress HMGB1-induced autophagy, and consequently, reduce asbestos-caused HM transformation, which may otherwise lead to mesothelioma.

The value of non-coding RNA regulation on HMGB1 and cancer has attracted much attention in recent years. Many microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) have been identified as HMGB1 modulators that can act alone or in combination with chemotherapy drugs to reduce tumor growth. MiRNAs including miR-21, miR-129-2, miR-200a, miR-320a, miR-325, and miR-505 were shown to be involved in the dysregulation of HMGB1 signaling pathways in human hepatocellular carcinoma (HCC) (Yan, Ying, & Cai, 2018). Their expression levels are inversely correlated with that of HMGB1. Further investigations on the miRNA/HMGB1 axis may provide new strategies for HCC therapeutics. In addition, miR-34a, miR-142, and miR-1284 are down-regulated in human cervical cancer, which inversely correlates with HMGB1 overexpression (Chandrasekaran, Sathyanarayanan, & Karunagaran, 2016; J. Chen & Li, 2018; D. Jiang et al., 2017). HMGB1 contains response elements in its 3' untranslated regions (3' UTR), which are often targeted by miRNAs. Upregulation of miR-34a, miR-142, and miR-1284 suppress HMGB1 mRNA levels and, subsequently, reduce cancer cell proliferation and migration/invasion. Additionally, miR-1284 enhances cervical cancer cell response to chemotherapy with cisplatin. Similar tumor suppressor roles for miRNAs are also observed in gastric cancer (miR-129-5p, miR-505, miR-1179) (Y. Li & Qin, 2019; L. Tian, Wang, Hao, & Zhang, 2018; S. Wang et al., 2019) and non-small cell lung cancer (miR-34c, miR-181b, miR-325-3p, and miR-520a-3p) (Y. Liu, Hu, Xia, & Zhang, 2016; Lv, Yao, Nie, & Xu, 2018; Tu et al., 2019; Yao, Zhao, & Jin, 2015), secondary to their HMGB1-inhibitory property. In contrast to the downregulation of miRNAs in cancer, lncRNAs are upregulated, which correlates with HMGB1 overexpression and contributes to tumor initiation and development. Several lncRNAs have been shown to regulate HMGB1 via miRNAs. LncRNA MALAT1 (metastasis-associated lung adenocarcinoma transcript 1) increases HMGB1 expression by directly binding to miR-129-5p and miR-142-3p and, subsequently, promotes development of osteosarcoma (K. Liu et al., 2017) and colon cancer (Q. Wu, Meng, Jie, & Zhao, 2018). Additional lncRNA-miRNA pairs have also been reported in other cancers including lncRNA UCA1-miR-193a (H. Wu & Zhou, 2018), lncRNA ZEB2-AS1-miR-204 (H. Gao et al., 2018), lncRNA NNT-AS1-miR-496 (D. Wu et al., 2019), lncRNA NEAT1-miR-410 (Shan, Tang, Xia, & Qian, 2020), lncRNA TP73-AS1-miR-200a (S. Li et al., 2017) and lncRNA TP73-AS1-miR-142 (R. Zhang, Jin, & Lou, 2018), where they all share a similar mode of cancer inhibition by targeting the HMGB1 signaling pathway.

Sepsis

Sepsis is characterized as a systemic inflammatory response to infectious organisms and is the leading cause of death in ICU patients worldwide. A massive release of cytokines such as TNFα and IL-1β in sepsis drives a largely uncontrolled pro-inflammatory response causing tissue damage and organ dysfunction. TNFα and IL-1β are early mediators in pathogenesis. Antagonists to TNF and IL-1 have shown limited efficacy in clinical trials for suppressing sepsis, in part because the peak mediator activity often has passed before any therapy can be initiated. HMGB1 is identified as a late mediator of endotoxin lethality in mice, which provides a wide therapeutic window for medical intervention. Cultured macrophages release HMGB1 more than 8 hours after stimulation with endotoxin. *In vivo*, increased serum HMGB1 is not seen until 8 to 32 hours after endotoxin exposure, and the administration of HMGB1 antibodies significantly protects against lethal endotoxemia in mice (H. Wang et al., 1999). Several studies have focused on treating sepsis by targeting HMGB1 with molecules such as ethyl pyruvate (Ulloa et al., 2002), nicotine (H. Wang et al., 2004), chloroquine (M. Yang et al., 2013), glycyrrhizin (Y. M. Kim, Kim, & Chang, 2015), haptoglobin (H. Yang et al., 2016), and stearoyl lysophosphatidylcholine (G. Chen et al., 2005) (Figure 1). Both ethyl pyruvate and nicotine attenuate serum HMGB1 elevation and interfere with the activation of the NF-κB pathway, which in turn inhibits cytokine release (H. Wang et al., 2004). Chloroquine, an anti-malaria agent with anti-autophagic properties, also acts through the NF-κB pathway and protects mice from lethal sepsis by suppressing HMGB1-induced IκB degradation (M. Yang et al., 2013). The administration of glycyrrhizin is shown to significantly reduce HMGB1 secretion and protect endotoxic mice from liver injury via p38/Nrf2-dependent HO-1 activation (Y. M. Kim et al., 2015). Similarly, haptoglobin, a serum protein usually involved in hemoglobin degradation, binds to extracellular HMGB1 and haptoglobin-HMGB1 together exerts anti-proinflammatory effects via upholding HO-1 and IL-10 expression in murine macrophages (H. Yang et al., 2016). A different approach using cell-specific siRNA against HMGB1 in macrophages and DCs can reduce human lymphocyte apoptosis, alleviate cytokine storm, and increase survival in a humanized mouse model of sepsis (Ye et al., 2012). The authors successfully delivered the siRNA to the target cells using a short 29-aa peptide derived from the rabies virus glycoprotein fused to 9R residues (RVG-9R), which binds to the acetylcholine receptors on macrophages and DC.

Targeting HMGB1 favors an inflammatory cytokine profile that is associated with better post-septic prognosis. Plasma samples from septic shock patients commonly exhibit pro-inflammatory cytokine compositions that are associated with high HMGB1 and high mortality. Treatment with ovine HMGB1 antibody in a murine septic model leads to altered cytokine responses and resistance to secondary bacterial infection (Stevens et al., 2017). HMGB1 also contributes to neutrophil NADPH oxidase dysfunction in patients surviving from septic shock. Inhibition with HMGB1 antibody improved neutrophil function *in vivo* and reduced post-sepsis immunosuppression (Gregoire et al., 2017). Moreover, anemia is one of the complications of sepsis. Results from a septic mouse model suggest that HMGB1 is the mediator for post-septic anemia (Valdes-Ferrer et al., 2016). Administration of exogenous HMGB1 to healthy mice causes anemia by interfering with extramedullary

erythropoiesis, whereas the application of HMGB1 antibody after sepsis protects against anemia development.

Sterile inflammation

Besides the inflammatory response against invading pathogens, the immune system can also recognize endogenous alarmin released from damaged or dying cells leading to deleterious sterile inflammation. HMGB1, a prototypical DAMP, contributes to the pathogenesis of both infection and sterile inflammation. HMGB1-targeted therapies in several sterile inflammatory diseases including ischemia-reperfusion (I/R) injury, ischemia, pancreatitis, and autoimmune disease, have been extensively studied as described below.

Ischemia-Reperfusion Injury

Ischemia-reperfusion (I/R) injury is a pathophysiologic process causing tissue damage when the blood returns to the tissue after a period of oxygen deprivation or ischemia. In 2005, Watanabe et al. showed elevated HMGB1 levels in serum and affected tissues of three different mouse models following hepatic I/R injury (Watanabe et al., 2005). A subsequent study further reported an increase of HMGB1 as early as one hour after ischemia. This result suggests HMGB1 is an early mediator of the inflammation, contrary to its role in sepsis, where it is thought to be a late mediator (Tsung et al., 2005). Pretreatment with HMGB1 antibody protects against hepatic damage secondary to I/R *in vivo*. Such protection from HMGB1 inhibition is lost in TLR4 knockout mice. Increased serum HMGB1 is first observed as early as 30 minutes after reperfusion. Pretreatment with HMGB1 antibody ameliorates renal dysfunction, lowers the peak of serum creatinine level, and significantly limits the area of renal tubular necrosis. This strict time course efficacy once again supports HMGB1's role as an early mediator for I/R. Ischemia-reperfusion injury is one of the leading concerns for post-surgical patients, especially for patients undergoing liver or kidney transplants. Therefore, prophylactic anti-HMGB1 therapy constitutes a promising new tool to reduce HMGB1-dependent inflammation/damage and improve patient outcomes.

Ischemia

HMGB1 serum level is also elevated in patients with cerebral or myocardial ischemia. Studies show that HMGB1 is translocated from neuronal nuclei to the cytoplasm after ischemia and secreted to the extracellular matrix (J. B. Kim et al., 2006), where it exerts cytokine-like functions by activating microglia, inducing TNFα and iNOS, and suppressing MMP-9 (K. Liu et al., 2007). Pretreatment with HMGB1 shRNA prior to the induction of infarction in mice suppresses microglia activation and provides neuroprotection (J. B. Kim et al., 2006). In addition, EP inhibits HMGB1 phosphorylation and release from activated microglia in the post-ischemic brain, which helps to confine the damage to the original injury (Shin, Lee, Lee, Jin, & Lee, 2014). Treatment with HMGB1 antibodies also reduces the size of brain infarction and improves locomotor function recovery in ischemic mice. In this study, it is reported that targeting HMGB1 suppresses TNFα and iNOS expression and ameliorates microglia-mediated inflammation. Intravenously injected anti-HMGB1 mAb is able to cross the blood-brain barrier and exert its effects on injured areas of the brain (K. Liu et al., 2007). Similarly, the intranasal delivery of siRNA against HMGB1 has also been successful in reducing cerebral infarction volumes. In contrast to HMGB1-related

inflammation, which exacerbates post-stroke brain injury, astrocytic HMGB1 promotes angiogenesis and neurovascular remodeling via endothelial progenitor cells (EPCs) leading to a better prognosis after stroke (Hayakawa, Pham, Katusic, Arai, & Lo, 2012). During stroke recovery, reactive astrocytes release HMGB1 which in turn activates EPC by binding to the RAGE receptor. In an ischemia mouse model, HMGB1 elevation and EPC accumulation are seen in the peri-infarct cortex at 14 days post-stroke. HMGB1 siRNA abrogates such EPC aggregation leading to poor angiogenesis and long clinical recovery (Hayakawa et al., 2012). One possible explanation for the contradictory roles of HMGB1 in stroke is cell specificity or the time-specific function of HMGB1. At the initial stage of stroke, HMGB1 is deleterious and helps microglial cells to aggravate inflammation. However, during the recovery phase of stroke, HMGB1 is beneficial and assists astrocytes in promoting angiogenesis and brain reperfusion.

Pancreatitis

Patients with acute pancreatitis (AP) have significantly increased plasma HMGB1, which is associated with the upregulation of necrosis and autophagy and inversely correlated with plasma sRAGE levels (E. Gao, Jiang, Li, Xue, & Zhang, 2017). Several HMGB1 antagonists such as the HMGB1 antibodies, BoxA protein, glycyrrhizin, and EP have been tested in murine AP models and show some positive results. Blockade of HMGB1 by polyclonal antibodies or BoxA protein ameliorates AP symptoms, decreases the incidence of multiple organ failure, and promotes mouse survival (Yuan et al., 2009). Treatment with glycyrrhizin lowers serum TNFα and IL-6 and limits pancreatic injury in rats with traumatic pancreatitis (Xiang et al., 2014). Administration of EP decreases expression of TNFα and IL-1β via the NF-κB pathway and reduces AP-associated liver injury in rat model (Luan, Zhang, Yin, Ma, & Guo, 2013). In contrast, a study with pancreas-specific HMGB1 knockout (Pdx1-Cre; HMGB1 flox/flox)) mice demonstrates that loss of endogenous HMGB1 leads to more rapid development of AP upon I-Arginine or cerulein challenge. Such an effect seems to be mediated via phosphorylation of NF-κB and mTOR (Kang, Zhang, et al., 2014).

Chronic Inflammatory and Autoimmune Diseases

The discovery of the proinflammatory properties of HMGB1 and the implication of extracellular HMGB1 in autoimmune diseases quickly highlights it as a potential therapeutic target for inflammatory and autoimmune diseases. Rheumatoid Arthritis (RA) is the most studied autoimmune disorder for HMGB1, which is associated with RA development (Hamada et al., 2008). In this study, a collagen-induced arthritis (CIA) mouse model is utilized to show that HMGB1 co-localizes with tissue hypoxia and HMGB1 antibody can relieve the arthritis symptoms. The authors observed that the release or secretion of HMGB1 to the extracellular matrix is induced by hypoxia, alluding to HMGB1's role in inflammation. HMGB1 also promotes angiogenesis in RA via hypoxia-inducible factor (HIF)-1α. HMGB1 binds to the TLR4 receptor on synovial fibroblasts and induces HIF-1α expression through the NF-κB pathway. Vascular endothelial growth factor (VEGF) is increased in HMGB1-stimulated RA fibroblasts, which can be suppressed by HIF-1α inhibition (Park et al., 2015). Several studies have shown other activities of HMGB1 in RA, including promoting proinflammatory cytokine release after LPS, activating synovial fibroblasts (Qin et al., 2014), and ramping up of the production of Th17 cells (Shi et al.,

2012). The value of targeting HMGB1 to treat RA is seen in a spontaneous arthritis mouse model (DNase II$^{-/-}$ x IFNRI$^{-/-}$) with monoclonal HMGB1 antibody (2G7). This mAb therapy prohibits chronic polyarthritis development and partially prevents joint destruction (Schierbeck et al., 2011). Similarly, the administration of BoxA protein, another HMGB1 antagonist, can reduce articular cartilage destruction and ameliorate RA symptoms in both CIA and (DNase II$^{-/-}$ x IFNRI$^{-/-}$) mouse models (Kokkola et al., 2003; Ostberg et al., 2010).

Regarding Multiple Sclerosis (MS), it has been shown that HMGB1 and its receptors RAGE, TLR2, and TLR4 are increased in active lesions in MS and experimental autoimmune encephalomyelitis (EAE, a mouse model of MS). Extracellular HMGB1 level in the spinal cord correlates with EAE progression. *In vitro* study with murine CNS-derived microglia demonstrates that microglial cells behave similarly to regular macrophages in translocating HMGB1 into the cytosol after LPS challenge. Elevated HMGB1 forms a positive feedback loop to promote inflammation in the CNS, which precipitates MS. Intraperitoneal injection of a monoclonal anti-HMGB1 can attenuate serum IL-17 elevation and reduce EAE-associated demyelination (Uzawa et al., 2013). Glycyrrhizin (GL) is shown to inhibit HMGB1 release from primary cortical neurons *in vitro.* Mice treated with GL have decreased TNFα, IFNγ, and IL-17A in both serum and spinal cord homogenate, which are associated with reduced neuronal inflammation/damage, and improved EAE outcomes (Sun et al., 2018).

Systemic sclerosis (SSc), also called scleroderma, is an autoimmune disease affecting the connective tissues of blood vessels, skin, joints, and internal organs. HMGB1 autoantibodies were first reported in 1994 to be elevated in SSc patients (Ayer, Senecal, Martin, Dixon, & Fritzler, 1994). Subsequent study has shown that both HMGB1 and sRAGE are increased in SSc patient serum and in the serum of a scleroderma mouse model (Yoshizaki et al., 2009). The levels of HMGB1 and sRAGE seem to positively correlate with the disease severity as measured by the modified Rodnan total skin thickness score and pulmonary function test. In addition to HMGB1, serum calpain activity is also found to be elevated in SSc patients, particularly SSc patients with interstitial lung disease (Zheng et al., 2020). The combination of HMGB1 and calpain activity may serve as a potential biomarker for SSc. One prevailing theory for high serum HMGB1 in SSc patients is that HMGB1 is released by the activated platelets in the platelet derived microparticles (PDμP) (Maugeri, Rovere-Querini, & Manfredi, 2016). In the serum, SSc patients have a significantly higher fraction of PDμP consistently expressing HMGB1 than healthy control (Maugeri et al., 2012). These HMGB1-expressing PDμPs are shown to promote neutrophil autophagy, neutrophil survival, and the generation of neutrophil extracellular traps (NETs) that may contribute to the microvascular inflammation and vasculopathy of SSc (Maugeri et al., 2018). Targeting HMGB1 with a HMGB1 inhibitor, BoxA, can disrupt the microparticle-neutrophil interaction and potentially reduce the SSc's vascular injury.

Plasma HMGB1 is elevated in SLE patients. High HMGB1 seems to correlate with serum IFNα and the severity of SLE based on SLEDAI (Ma et al., 2012). To confirm its role in SLE, HMGB1 was inhibited with mAb in lupus-prone BXSB mice (C. Zhang et al., 2014). Long-term mAb treatment led to reduced serum levels of proinflammatory cytokines like

IL-1β, IL-6, IL-17, and IL-18, decreased caspase 1 activity in the kidneys, and improved mouse survival.

Although the role of HMGB1 has not been closely examined in other autoimmune disorders, multiple studies point to a shared pattern of HMGB1 function in disease pathogenesis. In Type 1 diabetes mellitus, a study using non-obese diabetic (NOD) mice proposed that the interaction between HMGB1 and TLR4 is critical for selective islet β cell damage during disease development (M. Li, Song, Gao, Chang, & Qin, 2012). In experimental autoimmune myocarditis (EAM) in mice, HMGB1 mediates macrophage reprogramming to an M1-like phenotype via the TLR4-PI3K-ERK pathway and promotes Th17 expansion (Su et al., 2016). Another study on EAM identifies the HMGB1-RAGE axis as a critical component in cardiac troponin I (TnI)-induced myocarditis. RAGE knockout mice are protected from TnI-led cardiac impairment (Bangert et al., 2016). In experimental autoimmune uveitis (EAU), HMGB1 is recognized as an early mediator of intraocular inflammation after interphotoreceptor retinoid-binding protein (IRBP)-specific T cell stimulation. Experiments with neutralizing antibodies indicate that the HMGB1-CXCL12-CXCR4 pathway is responsible for HMGB1-related chemoattraction and infiltration of inflammatory cells in EAU (Yun et al., 2017). Other autoimmune disorders also demonstrated elevated HMGB1 levels, including psoriasis, ankylosing spondylitis, and Sjögren's syndrome (Harris, Andersson, & Pisetsky, 2012).

3. Conclusions

HMGB1, as the most abundant protein second only to histone inside the nucleus, has captured much attention as a prototype DAMP molecule that actively participates in inflammation, inflammatory diseases, and cancer. Building upon the initial knowledge of HMGB1 as a cytokine/chemoattractant, numerous *in vivo* and *in vitro* studies have shown therapeutic potential for targeting HMGB1 and scaling down tissue damage once the inflammation goes awry. A few obstacles need to be resolved before HMGB1 treatment can proceed toward clinical trials. The exact mechanism of HMGB1's translocation from the nucleus to cytoplasm then out into the extracellular matrix is not fully understood. Different redox states of HMGB1 may subject it to *in situ* modulations and make it challenging to pinpoint the specific functions of HMGB1 isoforms. Additionally, multiple HMGB1 receptors with various degrees of preference for a particular HMGB1 isoform or HMGB1-cofactor complex add another layer of complexity in studying the role of HMGB1 and its antagonists under disease settings. Despite the difficulties, targeting HMGB1 has been proven successful in treating inflammation and inflammatory diseases, especially in sepsis, sterile inflammation, autoimmune diseases, and cancer. Continued efforts in the field of HMGB1 can help to fill the gaps in our knowledge and bring HMGB1 antagonists closer to the next step of therapeutics: clinical trials.

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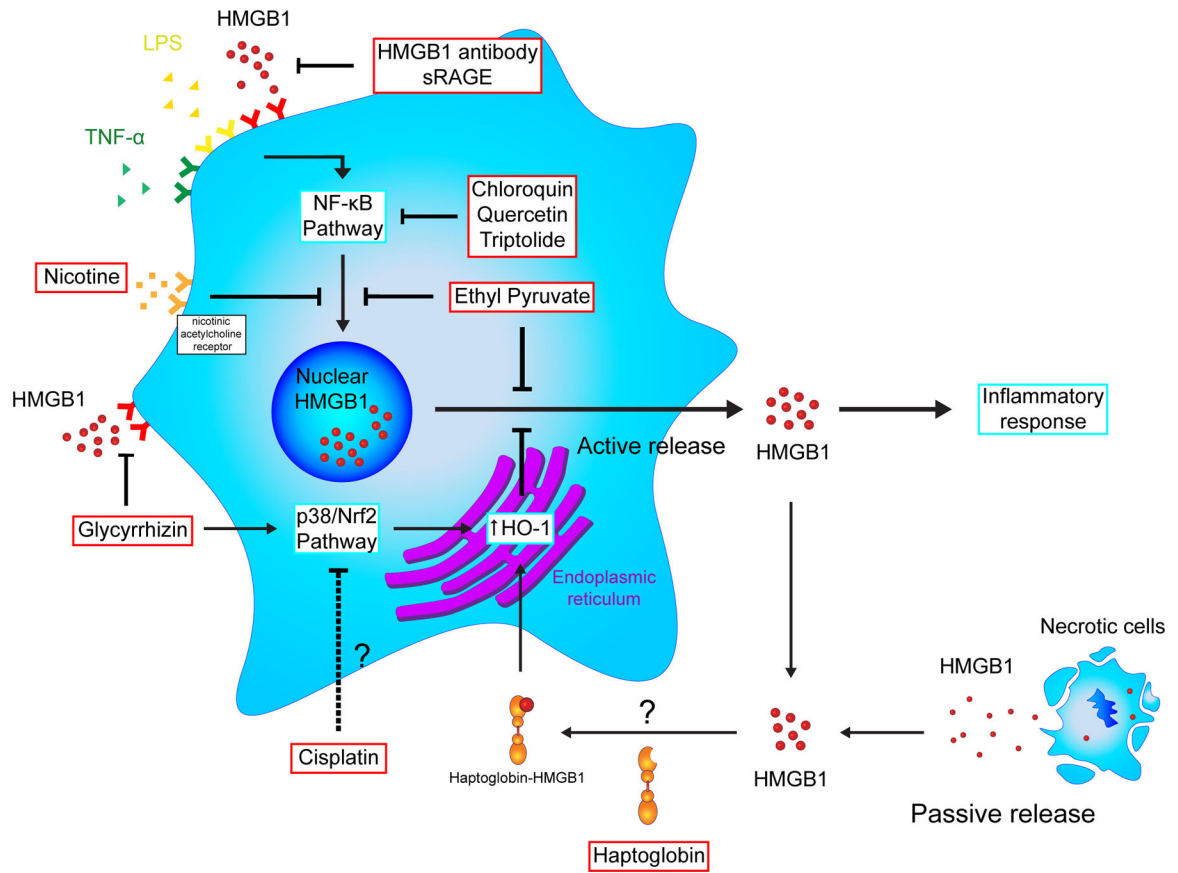
Figure 1.

Actions of high-mobility group box 1 (HMGB1) antagonists. HMGB1 can be released passively by necrotic cells or actively secreted when cells are stimulated. Once outside of the cell, HMGB1 serves as a damage-associated molecular pattern (DAMP) signal to mediate the inflammatory response. Many HMGB1 antagonists are studied for their anti-inflammatory effects. Some, like HMGB1 antibodies and sRAGE, target extracellular HMGB1, while others like ethyl pyruvate and triptolide inhibit intracellular pathways that lead to HMGB1 secretion. HO-1, heme oxygenase-1; LPS, lipopolysaccharide; NF-κB, nuclear factor kappa B; Nrf2, NF-E2-related factor 2; sRAGE, soluble receptor for advanced glycation endproducts; TNF-α, tumor necrosis factor-α