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Molecular Mechanism and Therapeutic Modulation of HMGB1 Release and Action: An Updated Review

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Abstract

High mobility group box 1 (HMGB1) is an evolutionarily conserved protein, and constitutively expressed in virtually all types of cells. Infection and injury converge on common inflammatory responses that are mediated by HMGB1 secreted from immunologically activated immune cells or passively released from pathologically damaged cells. Herein we review the emerging molecular mechanisms underlying the regulation of pathogen-associated molecular patterns (PAMPs)-induced HMGB1 secretion, and summarize many HMGB1-targeting therapeutic strategies for the treatment of infection- and injury-elicited inflammatory diseases. It may well be possible to develop strategies that specifically attenuate damage-associated molecular patterns (DAMPs)-mediated inflammatory responses without compromising the PAMPs-mediated innate immunity for the clinical management of infection- and injury-elicited inflammatory diseases.

Keywords

PAMPs; DAMPs; Infection; Injury; HMGB1; signaling; herbal components

1. INTRODUCTION

High mobility group box 1 (HMGB1), an evolutionarily conserved 30 kDa DNA-binding protein, is constitutively expressed in virtually all types of cells. Bearing two nuclear-localization sequences (NLS), HMGB1 is transported into the nucleus by the nuclear import complexes, thereby maintaining a large nuclear “pool” of pre-formed protein [1]. It carries two internal repeats of positively charged domains (“HMG boxes” known as “A box” and “B box”) in the N-terminus, and a continuous stretch of negatively charged (aspartic and glutamic acid) residues in the C-terminus (**Figure 1**). These HMG boxes enable HMGB1 to

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bind chromosomal DNA, and fulfill nuclear functions in stabilizing nucleosomal structure and regulating gene expression [1]. Disrupted local expression of HMGB1 renders animals susceptible to infectious [2] or injurious insults [3,4], suggesting an overall beneficial role of intracellular HMGB1. In response to infections and injuries, however, HMGB1 is secreted from activated immune cells or passively released from stressed cells. If dysregulated, excessive HMGB1 release adversely contributes to the pathogenesis of both infection- and injury-elicited inflammatory diseases. In this review, we summarize the novel mechanisms underlying the regulation of active HMGB1 secretion by innate immune cells, and highlight potential HMGB1-targeting therapeutic strategies for the treatment of infection- and injury-elicited inflammatory diseases.

2. HMGB1 ACTIVE SECRETION AND PASSIVE RELEASE

2.1. Active HMGB1 Secretion

In response to pathogen-associated molecular patterns (PAMPs, e.g., ds-RNA, CpGDNA and endotoxins) [5,6], macrophages/monocytes sequentially secrete early proinflammatory cytokines (e.g., TNF, IL-1, IFN- β and IFN- γ) and late mediators (e.g., HMGB1, **Figure 1**). Some early cytokines, including IFN- β and IFN- γ , can also stimulate immune cells to secrete HMGB1 in a time- and dose-dependent fashion [7- 9]. Lacking a leader peptide sequence, HMGB1 cannot be actively secreted through classical endoplasmic reticulum - Golgi exocytotic pathways [5]. Instead, macrophages/monocytes activated by exposure to products of infection or injury translocate nuclear HMGB1 into cytoplasmic vesicles which are destined for secreting into the extracellular environment. Recent evidence reveals that the initial HMGB1 nuclear to cytoplasmic translocation is regulated by the JAK/STAT1-mediated acetylation, while the subsequent extracellular release is partly controlled by the double-stranded RNA-activated protein kinase R (PKR)/inflammasome-mediated pyroptosis (**Figure 1**).

2.1.1. Role of JAK/STAT1 in the regulation of nuclear-cytoplasmic

translocation—The nucleus-to-cytoplasm protein shuttle is regulated by posttranslational modifications (e.g., acetylation, phosphorylation, methylation) of the transported protein's nuclear localization or export sequences (NLS or NES), which interact specifically with nuclear import and export complexes on the nuclear membrane [10]. In addition to two NLS sites (**Figure 1**), HMGB1 also contains two non-classical NES, and therefore shuttles continually between the nucleus and the cytoplasm. In quiescent cells, however, the equilibrium is tilted towards nuclear accumulation [11].

In response to exogenous PAMPs or endogenous cytokines (e.g., IFNs), innate immune cells acetylate lysine residues 28, 29, 42, 43, 179, 181, 183 within the NLS sites, leading to sequestration of HMGB1 into cytoplasmic vesicles (**Figure 1**) [7,9,11,12]. The acetylation is controlled both by histone acetylases (HATs) and histone deacetylases (HDACs), and pharmacological inhibition of HDACs also leads to HMGB1 hyperacetylation and nuclear-cytoplasmic translocation [11]. Following hypoxia or ischemia, non-immune cells (e.g., hepatocytes) also acetylate HMGB1 to trigger cytoplasmic translocation [13]. Unlike immune cells which actively secrete HMGB1 to modulate inflammatory responses, hepatocytes mobilize HMGB1 to the cytoplasm for a different purpose: binding to beclin-1

to induce autophagy - a cellular degradation process to remove damaged and reactive oxygen species (ROS)-producing mitochondria [14]. As aforementioned, intracellular HMGB1 is required for the normal physiological cellular response to stress [2,4], and conditional knock-out of liver HMGB1 indeed leads to significant enhancement of hepatic ischemia/reperfusion injury [3].

JAK/STAT1 signaling is critically important for LPS- or IFN-induced HMGB1 hyperacetylation within the NLS sites, thereby functioning as the underlying mechanism that regulates HMGB1 nuclear-cytoplasmic translocation [9] (**Figure 1**). Indeed, pharmacological inhibition or genetic interference with JAK/STAT1 signaling uniformly inhibits HMGB1 secretion induced by IFN- β , IFN- γ or LPS. Given the critical involvement of the JAK/STAT1 signaling in type 1 and type 2 IFNs action, as well as the essential roles of IFNs in the innate immunity against virus and bacteria [15], it is plausible that IFNs-induced cytoplasmic HMGB1 translocation participates in the beneficial host protection against viral or intracellular bacterial invasion. This is likely, as cytoplasmic or extracellular HMGB1 serves a sentinel function that facilitates innate recognition of microbial nucleic acids, an essential step in innate immunity against pathogen infection [6,16].

Phosphorylation of serine residues within the HMGB1 NLS sites may also contribute to the regulation of HMGB1 cytoplasmic translocation [17] (**Figure 1**). Although the upstream signaling pathway remains poorly elucidated, the calcium/calmodulin-dependent protein kinase (CaMK) IV has been implicated in the regulation of LPS-induced HMGB1 phosphorylation and release [18]. Unlike macrophages/monocytes, quiescent neutrophils carry HMGB1 predominantly in the cytoplasm, possibly because the methylation of lysine 42 weakens HMGB1/DNA interaction and forces nuclear HMGB1 to passively diffuse into the cytoplasm [19]. Thus, neutrophils likely provide another important source of extracellular HMGB1 during infection or injury.

2.1.2. Role of PKR in the regulation of HMGB1 secretion—Following cytoplasmic translocation, HMGB1 is secreted into the extracellular space following signal transduction through several pathways, including the Caspase-1/Caspase-11-mediated pyroptosis and the lysosome-mediated exocytosis (**Figure 1**). Indeed, pharmacological inhibition with a broad-spectrum caspase inhibitor (Z-VADFMK), or genetic deletion of Caspase-1/Caspase-11, uniformly reduces HMGB1 secretion from activated macrophages [20,21]. Notably, these caspases function not only as key regulators of the proinflammatory programmed cell death – pyroptosis [21], but also as important modulators of the canonical and non-canonical inflammasomes - key components of innate inflammatory protein complexes. Specifically, Pro-caspase-1 forms a heteromeric protein complex with several other proteins, including an adaptor protein (termed apoptosis-associated speck-like protein containing a CARD, ASC), a NOD-Like Receptor (NLR, e . g . , NLRP1, NLRP3, NLRC4) or a member of the PYHIN family. The assembly of this protein complex, termed the “inflammasome,” cleaves pro-caspase-1 to generate caspase-1, which triggers pyroptosis [22]. Caspase-11, on the other hand, mediates pyroptosis and HMGB1 release in the absence of caspase-1, a process termed the “non-canonical inflammasome pathway” [23]. Although the components of the non-canonical inflammasome remain largely unknown, it has been suggested that an intracellular LPS receptor-like molecule may participate in the mechanism, because

intracellular LPS induces caspase-11 activation even in the absence of TLR4 [24]. In contrast to the ultra-pure LPS that fails to trigger HMGB1 release in the absence of other stimulus (e.g., ATP) [21,25], crude LPS (containing trace amounts of bacterial proteins and nucleic acids) significantly up-regulates PKR expression (> 2-fold) and phosphorylation (> 8-fold), and markedly stimulates HMGB1 secretion [5,26].

Inflammasome activation is the regulatory mechanism of LPS/ATP-induced HMGB1 release [21,25], because genetic disruption of key inflammasome components (e.g., caspase 1 or Nalp3) completely impairs LPS/ATP-induced HMGB1 release. We recently reported a novel role for the double-stranded RNA-activated PKR in the regulation of inflammasome activation and HMGB1 release (**Figure 1**) [25]. In addition to dsRNA, other molecules that activate inflammasome assembly and signaling, ATP and anthrax lethal toxin, also induce PKR autophosphorylation. Genetic disruption of PKR expression or pharmacological inhibition of PKR phosphorylation [with 2-aminopurine (2-AP) or 7-desacetoxy-6,7-dehydrogedunin (7DG)] markedly reduces NLRP3 or NLRP1 agonists-induced inflammasome activation [25,27], pyroptosis [25,27], and HMGB1 release [25]. In agreement with the two-step control of HMGB1 nuclear translocation and extracellular release, pharmacological inhibition of PKR (with 2-AP) abrogates LPS-induced HMGB1 release, but does not prevent HMGB1 cytoplasmic translocation. Thus, the mechanisms of LPS-induced HMGB1 cellular secretion is differentially controlled at two stages: 1) JAK/STAT-mediated nuclear-cytoplasmic translocation; and 2) PKR/inflammasome-dependent pyroptosis and secretion (**Figure 1**).

In addition to pyroptosis, activated innate immune cells may also secrete HMGB1 via other non-classical vesicle-mediated secretory pathways including exocytosis of secretory lysosomes [12] (**Figure 1**). Indeed, a fraction of cytoplasmic HMGB1 has been localized in secretory lysosomes [12], where the key regulator of unconventional protein secretion, caspase-1 [28], co-resides with its enzymatic substrate pro-IL-1 β . It is likely that these secretory lysosomes deliver their cargo (e.g., IL-1 and HMGB1) into the extracellular space via exocytosis, but this hypothesis is as yet unproved, and other mechanisms may be involved.

2.2. Passive HMGB1 Release from Necrotic Cells

HMGB1 can be passively released from damaged cells [29] following sterile tissue injury due to ischemia/reperfusion [30,31], non-penetrating trauma [32,33], or chemical toxemia [34-36]. As a DAMP (damage-associated molecular pattern molecule), extracellular HMGB1 stimulates innate immune cells to respond to sterile injury [1,37], triggering an injury-elicited systemic inflammatory response syndrome (SIRS) that is indistinguishable from microbial infection-induced responses [38]. Necrosis can also be induced by viral infection or proinflammatory cytokines (e.g. TNF, IFNs) (**Figure 1**) [39,40], and HMGB1 is passively released by cells infected by various viruses (e.g., West Nile, Salmon anemia, Dengue, and influenza viruses) [41- 43]. This implicates HMGB1 as a pathogenic mediator of viral infection-elicited inflammatory diseases [44]. Notably, the cytokine-induced necrosis is also a highly regulated programmed process, termed necroptosis or programmed necrosis (**Figure 1**). Several signaling molecules such as the protein kinase receptor-

interacting protein 3 (RIP3) and PKR are involved in the assembly of a “necrosome” protein complex [39,40,45], which contributes to passive HMGB1 release following infection (**Figure 1**). Thus, the innate response mechanisms of infection and injury converge on a common process - inflammation [1], which is orchestrated by HMGB1 and other proinflammatory mediators (e.g., mitochondrial DNA, cold-inducible RNA-binding protein, CIRP) released both by activated immune cells and by damaged tissues [46,47].

2.3. Extracellular HMGB1 as a DAMP Molecule

Once released, extracellular HMGB1 functions as a DAMP to alert, recruit, and activate immune cells. For instance, HMGB1 binds to various PAMPs (e.g., CpGDNA or LPS), thereby facilitating their recognition by respective receptors [48], and consequently augmenting the PAMPs-induced inflammatory responses [48]. Furthermore, HMGB1 can stimulate the migration of monocytes, dendritic cells [49,50] and neutrophils [51], functioning as a chemokine to facilitate leukocyte recruitment to the sites of infection or injury [52] (**Figure 2**). HMGB1 interacts with a family of cell surface receptors and binding proteins including RAGE [48], TLR4 [53], TLR9 [6,48], cluster of differentiation 24 (CD24)/Siglec-10 [54], Mac-1 [51], thrombomodulin [55], as well as single transmembrane domain proteins (e.g., syndecans) [56]. Consequently, it can activate macrophages [57] and endothelial cells [58] to produce proinflammatory cytokines, chemokines, and adhesion molecules (**Figure 2**).

HMGB1 contains three cysteine residues at highly conserved positions: 23, 45 and 106 (C23, C45 and C106, **Figure 1**). They are redox-sensitive and their atomic structure is modified by redox reactions to produce three HMGB1 isoforms (**Figure 2**) [59-61]. The redox status of HMGB1 dictates its chemokine or cytokine-inducing properties. A recent consensus conference proposed nomenclature of the isoforms [62], termed “HMGB1” (which refers to the all thiol form), “disulfide HMGB1” (which is partially oxidized); and oxidized HMGB1. Specifically, the fully reduced (“all-thiol”) HMGB1 binds to other chemokines (e.g., CXCL12) and stimulates leukocyte recruitment via the CXCR4 receptor [63]. On the other hand, the partially oxidized HMGB1 bearing Cys23-Cys45 disulfide can activate immune cells to produce cytokines/chemokines via the TLR4 or other receptors. Once fully oxidized, the HMGB1 is devoid of either chemokine or cytokine activities (**Figure 2**) [60,64]. Thus, the release of redox-modulated HMGB1 from immunologically activated or pathologically injured cells contributes to the sequential leukocyte recruitment, activation, and eventual resolution of inflammation.

The posttranslational modifications of HMGB1 are not limited to the production of redox sensitive isoforms, because HMGB1 secreted via pyroptosis is also hyperacetylated at the NLS sites. This is consistent with the understanding that inflammasome-mediated HMGB1 release is a highly regulated process. Passively released HMGB1, as occurs in necrotic cells, is predominantly the “all-thiol” isoform. Furthermore, different types of inflammasome activation induce distinct HMGB1 post-translational modifications. For instance, the NLRP3 inflammasome stimuli, such as ATP, monosodium uric acids, and adjuvant aluminum, induce the secretion of “disulfide” HMGB1 [25]; whereas the activation of the NLRC4 inflammasome results in the secretion of fully-reduced HMGB1 [65]. One possible

explanation is that activation of the NLRP3 inflammasome, but not the NLRC4 inflammasome, is associated with mitochondrial ROS production, which promotes HMGB1 oxidation and formation of the C23 - C45 disulphide bond [66]. Although both the NLRP3 and the NLRC4 inflammasome mediate maturation of IL-1 β and IL-18, their distinct impact on the redox status of HMGB1 might enable fine-tuning of the immune response against different pathogens. For example, the NLRP3 inflammasome can be activated by several RNA virus [67], and extracellular release of disulfide HMGB1 initiates anti-viral inflammatory responses. On the other hand, as an intracellular sentinel against *Salmonella* infection [68], the NLRC4 inflammasome enables the release of reduced HMGB1, which facilitates leukocyte recruitment to eliminate invading bacteria.

3. HMGB1 AS A MEDIATOR OF INFECTION- AND INJURY-ELICITED INFLAMMATION

In response to infection and injury, the host's innate immune system mounts an immediate inflammatory response to eliminate the invading pathogens and to heal the wounds [69]. To accomplish this, the innate immune cells (e.g., macrophages/monocytes) are equipped with receptors (e.g., CD14, MD-2 and TLR4) that can efficiently recognize both PAMPs (e.g., LPS) [70,71] and DAMPs (e.g., HMGB1 or CIRP) [47,72]. The underlying recognition mechanisms for PAMPs and DAMPs utilize numerous pathways, and extensive evidence reveals an essential role for HMGB1 in both infection- and injury-elicited inflammatory diseases.

3.1. HMGB1 as a Late Mediator of Sepsis

Sepsis refers to the host's deleterious and non-resolving systemic inflammatory response to microbial infection [38], and represents the leading cause of death in the intensive care unit. Substantial evidence has supported the necessity to preserve the early PAMPs-mediated innate immune response to fight against microbial infection. For instance, the impairment of the early inflammatory responses leads to severe immune deficiency during bacterial infection [73]. Although early proinflammatory cytokines (e.g., TNF, IFN- γ) might be protective against infection, the sustained accumulation of late proinflammatory mediators (e.g., HMGB1) contributes to the pathogenesis of lethal infection (**Figure 2**). These scenarios cannot be replicated in the clinic, because by the time patients develop these early cytokine responses, there is no opportunity to intervene. In animal models of lethal infection induced by endotoxemia or cecal ligation and puncture (CLP), HMGB1 is first detected in the circulation eight hours after the disease onset, and subsequently increased to plateau levels from 16 to 32 hours [5,74]. This late appearance of circulating HMGB1 parallels the onset of animal lethality from endotoxemia or sepsis, and distinguishes itself from TNF and other early proinflammatory cytokines [75]. The pathogenic role of HMGB1 in endotoxemia is inferred from studies that HMGB1-neutralizing antibodies confer a dose-dependent protection against endotoxin-induced lethality [5]. In a more clinically relevant animal model of sepsis (induced by CLP), delayed administration of HMGB1-specific neutralizing antibodies, beginning 24 h after CLP, dose-dependently rescue rodents from lethal sepsis [20,74]. Moreover, targeted inhibition of HMGB1 expression in innate immune cells (e.g., macrophages and dendritic cells) reduces systemic HMGB1 accumulation, and similarly

rescues mice from sepsis [76]. Taken together, these experimental data establish extracellular HMGB1 as a critical late mediator of experimental sepsis, which can be therapeutically targeted within wider therapeutic windows than other early cytokines.

3.2. HMGB1 as an Early Mediator of Injury

As a ubiquitous nuclear protein, HMGB1 can also be passively released from necrotic cells [29], and functions as a DAMP to elicit inflammatory responses. Following primary tissue injury, HMGB1 can be passively released from damaged cells, and released into the surrounding periphery, where it accumulates and amplifies inflammatory responses by inducing various cytokines, chemokines, tissue factor and adhesion molecules (**Figure 2**). Indeed, accumulative evidence has suggested a pathogenic role of HMGB1 in injury-elicited inflammatory diseases, as HMGB1-neutralizing antibodies are protective in animal models of ischemia/reperfusion [30,77,78], trauma [79,80], chemical toxemia [34,81,82], atherosclerosis [83], gastric ulcer [84] and hyperoxia [85].

3.3. HMGB1 as a mediator of autoimmune diseases

Extensive evidence has also implicated HMGB1 in the pathogenesis of autoimmune diseases such as the systemic lupus erythematosus (SLE) [86-88] and rheumatoid arthritis [86,89]. The pathogenesis of lupus is partly attributable to the impaired clearance of apoptotic cells, which may gradually enter secondary necrosis to passively release HMGB1 into the extracellular space [86,87]. Extracellular HMGB1, however, may also impair the elimination of apoptotic cells [90], and further exacerbate these vicious inflammatory cascades [91]. In lupus patients with higher photosensitivity, ultraviolet (UV) irradiation induces more pronounced keratinocyte apoptosis and HMGB1 release [92-94], which triggers elevated leukocyte influx to the irradiated skin [95]. Furthermore, emerging evidence suggests that some HMGB1-neutralizing antibodies may reduce systemic accumulation of proinflammatory cytokines and associated glomerulonephritis in a murine model of lupus [96]. As a chronic synovitis, rheumatoid arthritis is characterized by persistent immune cell activation and joint tissue damage [86], which are similarly mediated by extracellular accumulation of HMGB1 and other proinflammatory cytokines [97]. In animal models of rheumatoid arthritis, HMGB1-neutralizing antibodies confer significant protection against joint tissue edema and structural damages [97-99], supporting a pathogenic role for HMGB1 in the pathogenesis of autoimmune diseases.

4. THERAPEUTIC POTENTIAL OF HMGB1-INHIBITING AGENTS

The discovery of HMGB1 as a mediator of lethal infection and injury has prompted to the search of endogenous and exogenous agents that can inhibit HMGB1 release and protect animals against infection or injury.

4.1. Endogenous HMGB1 Inhibitors

Evolution has selected for counter-regulatory or anti-inflammatory mechanisms that suppress the damage to host tissues. For instance, the central nervous system can directly and rapidly respond to PAMPs and DAMPs, and down-regulates production of inflammatory mediators by transmitting efferent vagus nerve signals to tissue-resident T

cells [100] and macrophages [101]. This cytokine suppressing mechanism is dependent on the release of acetylcholine by specific T cells, as well as the presence of the alpha-7 nAChR on targeted immune cells [101-103]. At the sites of infection or injury, another ubiquitous biogenic molecule, spermine, can also be passively released by injured cells, and functions as a local counter-regulatory mechanism for PAMPs- and DAMPs-induced inflammatory responses [104-107]. In response to infection and injury, the liver strategically re-prioritizes the synthesis and systemic release of a group of proteins collectively termed “acute phase proteins” (APPs), which may also function as counter-regulatory mechanisms against infection or injury. For instance, the hepatic expression of fetuin-A is negatively regulated by TNF, IL-1, IL-6 and IFN- γ [108], but positively regulated by HMGB1 [108]. Consistently, fetuin-A functions as a negative APP during infection, but serves as a positive APP in injury. Regardless, supplementation with exogenous fetuin-A confers protection against both injury- [109] and infection-elicited inflammatory responses [108] (**Table 1**). The integral role of fetuin-A in host defense against lethal systemic inflammation was supported by the observations that fetuin-A-deficient C57BL/6J mice were more susceptible to lethal endotoxemic or septic insult than sex- and body-matched (male, 27-29 g) wild-type C57BL/6J mice [109].

In addition, a number of other endogenous molecules (**Table 1**), including the intravenous immunoglobulin (IVIG) [110], anti-coagulant agents (anti-thrombin III, thrombomodulin) [55,111], and endogenous hormones (e.g., vasoactive intestinal peptide and ghrelin) [112,113], have proven protective in animal models of infection through HMGB1-inhibiting mechanisms. Notably, these endogenous molecules are also protective in animal models of ischemia/reperfusion injury [114-118], crush injury [119], burn injury [120], chemical toxemia [121,122], hypoxic injury [123], radiation [124] (**Table 1**). It is thus important to investigate whether the protective effects are associated with similar inhibition of HMGB1 release or activities.

4.2. Exogenous HMGB1-inhibiting Agents

A number of herbal extracts (e.g., Danggui, Mung bean, and *Prunella vulgaris*) confer significant protection against lethal endotoxemia or sepsis (**Table 2**) [125-127]. Similarly, these herbs are also protective in animal models of radiation injury [128] and chemical toxemia [129], although it is not yet known whether the protective mechanism is dependent on the HMGB1 suppression. In addition, an increasing number of herbal components (e.g., nicotine, EGCG, tanshinone, glycyrrhizin, chlorogenic acid, Emodin-6-O- β -D-glucoside, Rosmarinic acid, isorhamnetin-3-O-galactoside, Persicarin, Forsythoside B, chloroquine, acteroside) [103,130-140] have been proven effective in inhibiting endotoxin-induced HMGB1 release (**Figure 3, Table 2**).

Interestingly, different herbal components appear to utilize distinct mechanisms to prevent HMGB1 release by activated macrophages/monocytes. For instance, a major green tea component, EGCG, prevents the LPS-induced HMGB1 release strategically by destroying it in the cytoplasm via a cellular degradation process – autophagy [141]. Autophagy, literally meaning “self-eating”, refers to an evolutionarily conserved process that maintains cellular homeostasis by degrading cytoplasmic macromolecules. The relationship between

autophagy and HMGB1 remains a subject of on-going investigation. In 2009, Thorburn et al proposed a potential role for autophagy in the regulation of HMGB1 release in neoplastic cells, because an agent [i.e., epidermal growth factor receptor (EGFR)-targeted diphtheria toxin] simultaneously induced autophagy and HMGB1 release in glioblastoma tumor cells [142]. Subsequently, Tang et al suggested HMGB1 as an important regulator of autophagy based on the findings that the knock-down of HMGB1 expression led to a reduction of stress-induced autophagy in cancer cells [143,144]. In contrast, Huebener et al recently reported that HMGB1 is dispensable for autophagy regulation in non-transformed cells, because conditional knockout of HMGB1 in hepatocytes or cardiomyocytes completely failed to impair glucagon- or rotenone-induced autophagy [145]. Together with our observations that some agents capable of stimulating autophagy (e.g., EGCG) surprisingly inhibited HMGB1 release in primary macrophage cultures [141], these new findings are calling for further investigation of the seemingly complex relationship between autophagy and HMGB1 in primary and transformed cells.

In contrast, a derivative of tanshinone IIA, TSN-SS selectively inhibits HMGB1 release by facilitating endocytosis of exogenous HMGB1, leading to subsequent degradation via a lysosome-dependent pathway [146]. A pannexin-1 channel blocker, carbenoxolone (CBX), attenuates LPS-induced HMGB1 release by preventing the expression and phosphorylation of PKR [25]. Given the similarity in the chemical structure between CBX and a newly identified PKR inhibitor (7DG, **Figure 3**), it is important to investigate whether CBX directly binds and inhibits PKR activation.

In light of the capacity of herbal ingredients in preventing endotoxin-induced HMGB1 release, we explored the efficacy of several compounds in animal models of CLP-induced sepsis. Considering the late and prolonged kinetics of HMGB1 accumulation in experimental sepsis [74], the first dose of HMGB1 inhibitors was given in a delayed fashion - 24 h after the onset of sepsis. Repetitive intraperitoneal administration of EGCG [130], TSN IIA-SS [131], or CBX [26], at 24, 48, and 72 h post CLP, significantly increased animal survival rates. Even when given orally, EGCG still rescued mice from lethal sepsis, significantly increasing animal survival rates from 16% to 44% [141]. Intriguingly, we found that EGCG facilitated bacterial elimination in selective organs (e.g., the liver and lung) in an animal model of sepsis [147]. It is not yet known whether these antibacterial properties are attributable to the possibilities that EGCG directly kill microbes by altering microbial protein conformations and functions, or indirectly by modulating macrophage-associated innate immune responses. A number of other herbal components have been proven protective against lethal infection or injury by attenuating systemic HMGB1 release or action (**Table 2**), raising further interest in future clinical studies. Importantly, these herbal components have also been proven beneficial in animal models of ischemia [148-155], trauma [156,157], crush injury [158], hemorrhage [159], radiation [160,161], chemical toxemia [162,163]. Nevertheless, it remains unknown whether the protective effects are associated with inhibition of HMGB1 release or chemokine/cytokine activities during injury.

Notably, agents capable of inhibiting HMGB1 release [103,130,131] or action [5,74] confer protection against sepsis, particularly if given in a delay fashion to strategically preserve the

PAMPs-mediated early inflammatory response. At a late stage of infection, the PAMPs-mediated inflammatory response may be accompanied by unintended cell injury and DAMPs release that further amplifies the cytokine storm to precipitate organ dysfunction [164] (**Figure 1 and Figure 2**). This possibility is supported by recent findings that HMGB1 is persistently elevated during a late stage of sepsis despite the cessation of initial infection [165], and contributes to the long-term pathological consequence of sepsis. Although the microbial infection-induced sepsis is similar to the sterile injury-elicited systemic inflammatory response syndrome (SIRS) [38,166], it may be more advantageous to develop strategies that specifically attenuate DAMPs-mediated inflammatory responses without compromising the PAMPs-mediated innate immunity.

5. EXPERT COMMENTARY & FIVE-YEAR VIEW

Therapeutic strategies targeting PAMPs (e.g., endotoxin) [167] or PAMP signaling (e.g., Eritoran) [168] fail to improve survival in clinical trials of human sepsis, raising questions about the feasibility of PAMPs-blocking agents in the treatment of infectious diseases. The investigation of pathogenic cytokines in animal models of diseases has led to the development of successful cytokine-targeting therapeutic strategies (e.g., chimeric anti-TNF monoclonal antibody, infliximab; and a soluble TNF receptors-Fc fusion protein, sTNF-R-Fc, etanercept) for autoimmune diseases such as rheumatoid arthritis (RA) [169]. However, neutralizing antibodies for early cytokines (e.g., TNF) also did not show efficacy in sepsis clinical trials [170], as in the clinic, the early cytokine responses are established or completed prior to the ability to administer these reagents. Thus, it remains highly important to identify feasible therapeutic targets for management of inflammatory diseases.

In contrast to early proinflammatory cytokines, HMGB1 is secreted from immunologically activated innate immune cells and released from pathologically damaged cells, and functions as a critically important mediator in lethal infection and injury. In animal model of sepsis, HMGB1-neutralizing antibodies or inhibitors can rescue mice from the lethality particularly if given in a delayed manner to preserve the potentially beneficial early PAMPs-mediated inflammatory responses. It may be possible to develop novel strategies to specifically modulate DAMP-elicited injurious inflammatory response without impairing the PAMP-mediated beneficial innate immunity against infection. Therefore, it is important to investigate whether HMGB1 can ever be a clinically feasible therapeutic target for human sepsis or other autoimmune diseases.

Future clinical studies are anticipated to test the efficacy of HMGB1-neutralizing antibodies in the clinical management of human inflammatory diseases. Of course, humanized monoclonal antibodies (mAb) are manufactured in low-yield and time-consuming mammalian cells, and are thus tremendously more expensive than small molecule chemical drugs [164]. For example, the recommended dose for frequent injections of Humira (TNF mAb) to treat rheumatoid arthritis is 40 mg every two weeks, totaling > 1 gram (> \$16,000) per year. It is thus essential to develop cost-effective small molecule drugs for the clinical management of human sepsis. One of the most selective HMGB1 inhibitor, TSN-SS, has already been used in China as a medicine for patients with cardiovascular disorders. The dual effects of TSN-SS in attenuating late inflammatory response and improving

cardiovascular function make it a promising therapeutic agent for sepsis. The capacity to facilitate endocytic HMGB1 uptake by professional phagocytes may provide basis for the treatment of both infection- and injury-elicited inflammatory diseases [164]. It is not yet known whether a better protection could be achieved by combinational therapy with several anti-HMGB1 agents, which makes it important to further explore the therapeutic potential of these HMGB1-inhibiting agents in future studies.

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6. KEY ISSUES

- 5.1. PAMPs stimulate immune cells to sequentially release early proinflammatory cytokines and late proinflammatory mediators (e.g., HMGB1).
- 5.2. The secretion of HMGB1 from immunologically activated immune cells is regulated by specific molecular mechanisms: JAK/STAT1 regulates cytoplasmic translocation, and PKR/inflammasomes-dependent pyroptosis regulates cellular secretion.
- 5.2. HMGB1 can be passively released from necrotic cells following ischemiareperfusion, trauma, and injury; in this context it is a DAMP which orchestrates injury-elicited inflammatory responses by interacting with a family of receptors.
- 5.3. Early proinflammatory cytokines (e.g. TNF, IFN- β or IFN- γ) also induce a highly regulated cell death process, termed necroptosis, which may contribute to HMGB1 release.
- 5.4. Extracellular HMGB1 functions as a DAMP molecule to alert, recruit, and activate immune cells, thereby serving as a mediator of lethal infection and injury.
- 5.5. A number of endogenous macromolecules (e.g., intravenous immunoglobulin, anti-coagulants, acute phase proteins, and hormones) have proven effective in inhibiting HMGB1 release, and protecting against lethal infection and injury.
- 5.6. Many herbal extracts and components have been proven effective in inhibiting HMGB1 release and protective against lethal infection and injury.
- 5.7. Different herbal components (e.g., EGCG, TSN-SS, and CBX) inhibit active HMGB1 secretion through divergently distinct mechanisms, ranging from inducing autophagic degradation, stimulating endocytic uptake, to preventing PKR activation.
- 5.8. Many agents capable of inhibiting HMGB1 secretion are also proven protective in various animal models of injury, but the protective mechanisms remains poorly elucidated.

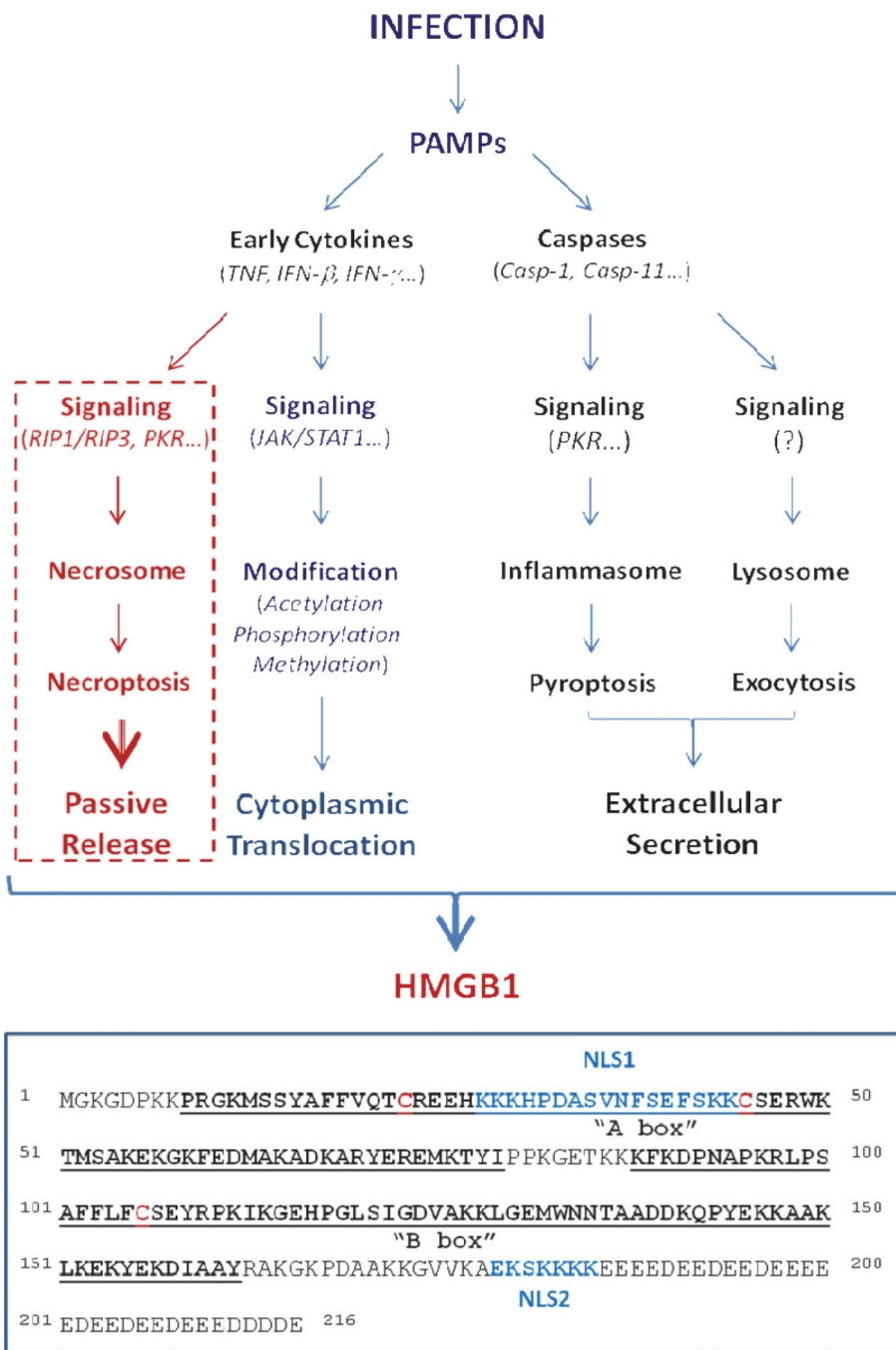


Figure 1. Pathogen-associated molecular patterns (PAMPs) induced active HMGB1 secretion and possibly passive release

The functional domains of human HMGB1 are noted.

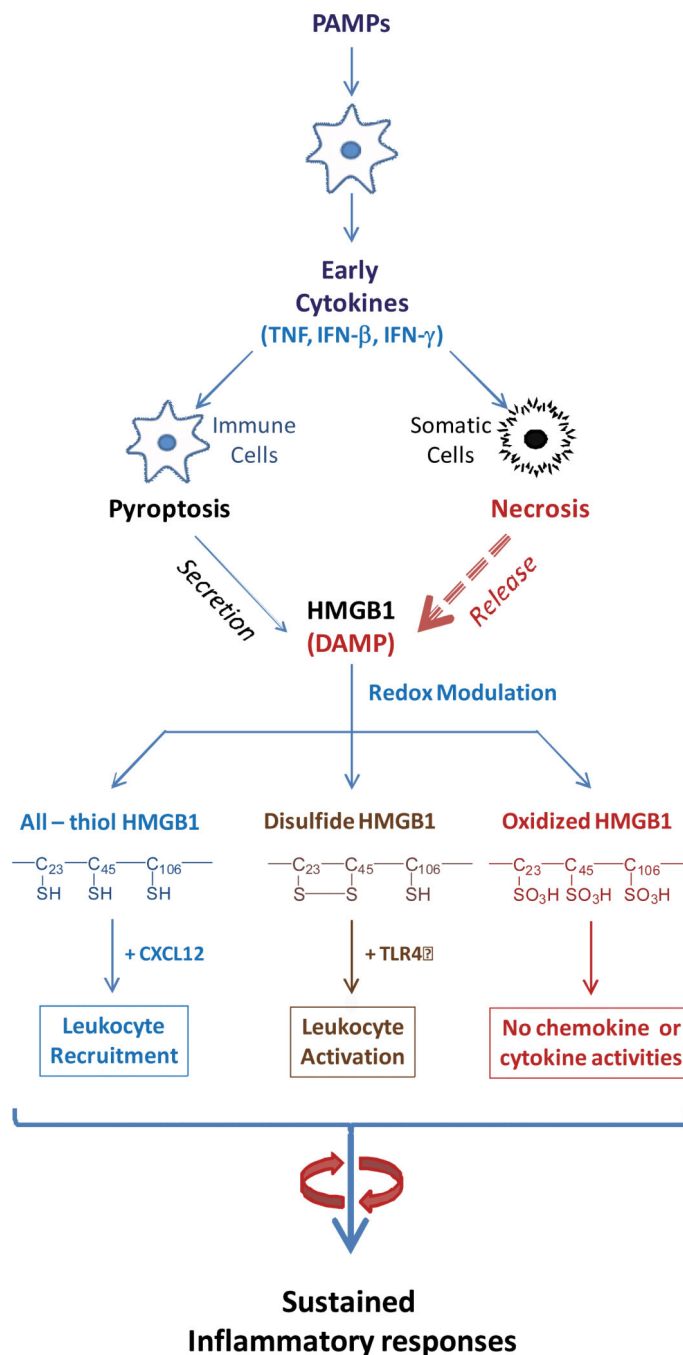


Figure 2. A microbial infection triggers a systemic inflammatory response mediated by HMGB1 secreted by immunologically activated innate immune cells and pathologically damaged cells Microbial invasion leads to the liberation of PAMPs, which trigger active HMGB1 secretion and passive release. Extracellular HMGB1 then amplifies the rigorous inflammatory responses by facilitating leukocyte recruitment and activation, resulting in cytokine storm and organ dysfunction. Note the immunological activities are modulated by the redox status in a divergent fashion.

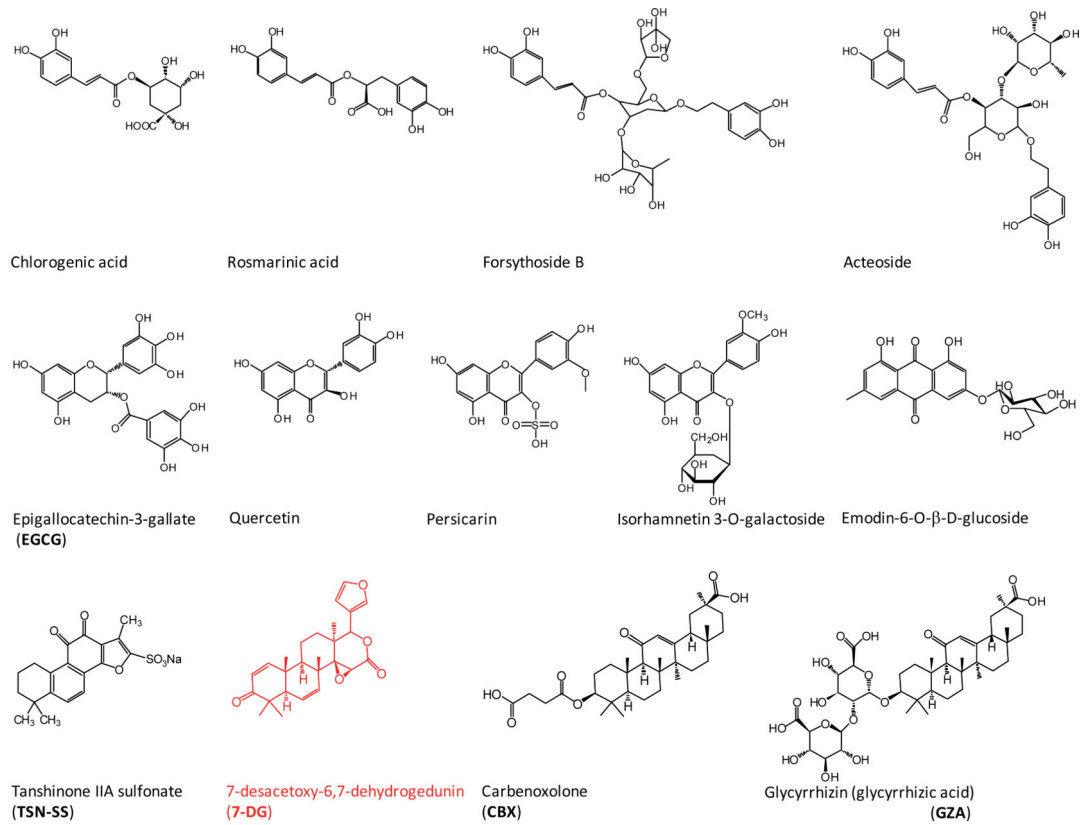


Figure 3. Chemical structures of HMGB1-inhibiting herbal components

Note the chemical structural similarity between two PKR inhibiting agents: CBX and 7DG.

Table 1

Endogenous HMGB1-inhibiting agents.

Agents	Infection Models	Injury Models
Neutralizing antibodies	LPS/CLP	Atherosclerosis Crush Chemical toxemia Liver I/R Brain I/R Heart I/R Hyperoxia Hemorrhagic Trauma Ulcer
Anti-coagulant agents		
Anti-thrombin III	LPS	Ischemia
Thrombomodulin	LPS	I/R Heatstroke
Acute phase proteins		
Fetuin-A	LPS/CLP	Cerebral Ischemia Burn
Endogenous hormones		
Vasoactive intestinal peptide	CLP	Ischemia Hemorrhagic injury
Ghrelin	CLP	Intestinal I/R Hypoxia Radiation
Intravenous immunoglobulin	CLP	Cerebral Ischemia

Note: LPS, lipopolysaccharide; CLP, cecal ligation and puncture; I/R, ischemia/reperfusion

Table 2

Exogenous HMGB1-inhibiting agents.

Agents	Infection Models	Injury
Herbal extract		
Danggui	LPS/CLP	Radiation
Mung bean	LPS/CLP	Chemical toxemia
Prunella vulgaris	CLP	-
Herbal components		
Nicotine	LPS/CLP	-
TSN-SS	LPS/CLP	Cerebral Ischemia
EGCG	LPS/CLP	Pain Crush i I/R
Carbenoxolone	CLP	I/R Trauma
Glycyrrhizin	LPS	Vascular Injury
Chloroquine	LPS/CLP	I/R Radiation
Acteoside	CLP	Chemical toxemia
Chlorogenic acid	LPS/CLP	Pain I/R
Emodin-6-O- β -D-glucoside	CLP	-
Rosmarinic acid (RA)	LPS/CLP	Radiation Oxidative Chemical toxemia
Isorhamnetin-3-O-galactoside	LPS/CLP	-
Persicarin	LPS/CLP	I/R
Forsythoside B	CLP	Hypoxic
Higenamine	-	