HMGB1 and RAGE in Inflammation and Cancer

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Abstract

The immune system has evolved to respond not only to pathogens, but also to signals released from dying cells. Cell death through necrosis induces inflammation, whereas apoptotic cell death provides an important signal for tolerance induction. High mobility group box 1 (HMGB1) is a DNA-binding nuclear protein, released actively following cytokine stimulation as well as passively during cell death; it is the prototypic damage-associated molecular pattern (DAMP) molecule and has been implicated in several inflammatory disorders. HMGB1 can associate with other molecules, including TLR ligands and cytokines, and activates cells through the differential engagement of multiple surface receptors including TLR2, TLR4, and RAGE. RAGE is a multiligand receptor that binds structurally diverse molecules, including not only HMGB1, but also S100 family members and amyloid-β. RAGE activation has been implicated in sterile inflammation as well as in cancer, diabetes, and Alzheimer's disease. While HMGB1 through interactions with TLRs may also be important, this review focuses on the role of the HMGB1-RAGE axis in inflammation and cancer.

PAMP: pathogen-associated molecular pattern

DAMP: damage-associated molecular pattern

HMGB1: high mobility group box 1

RAGE: receptor for advanced glycation end products

AGEs: advanced glycation end products

INTRODUCTION

Sensing the presence of a pathogen is the first step for the immune system to mount an effective response to eliminate an invading organism and establish protective immunity. Over the last decade, the molecular and cellular mechanisms required for efficient microbial detection have begun to be appreciated following the identification of a number of pattern-recognition receptors that detect components of microbes though pathogen-associated molecular patterns (PAMPs). The best studied of these pattern detectors are the Toll-like receptors (TLRs). TLRs are, however, not alone in this function; other molecules, including the cytoplasmic sensing molecules, the helicases, and the nucleotide-binding oligomerization domain (NOD)- or caspase recruitment domain (CARD)-containing proteins, also play critical roles as pathogen sensors. Furthermore, we now know that, in addition to recognizing PAMPs, the immune system has evolved to recognize endogenous danger signals or, by analogy, damage-associated molecular patterns (DAMPs), many of which are released by dying or necrotic cells and contribute to inflammation in a noninfectious sterile setting. In this regard, liberation of the nuclear DNA-binding molecule high mobility group box 1 (HMGB1) may play a critical role in mediating the immune responses to damage.

DANGER SIGNALS, DAMPs, AND STERILE INFLAMMATION

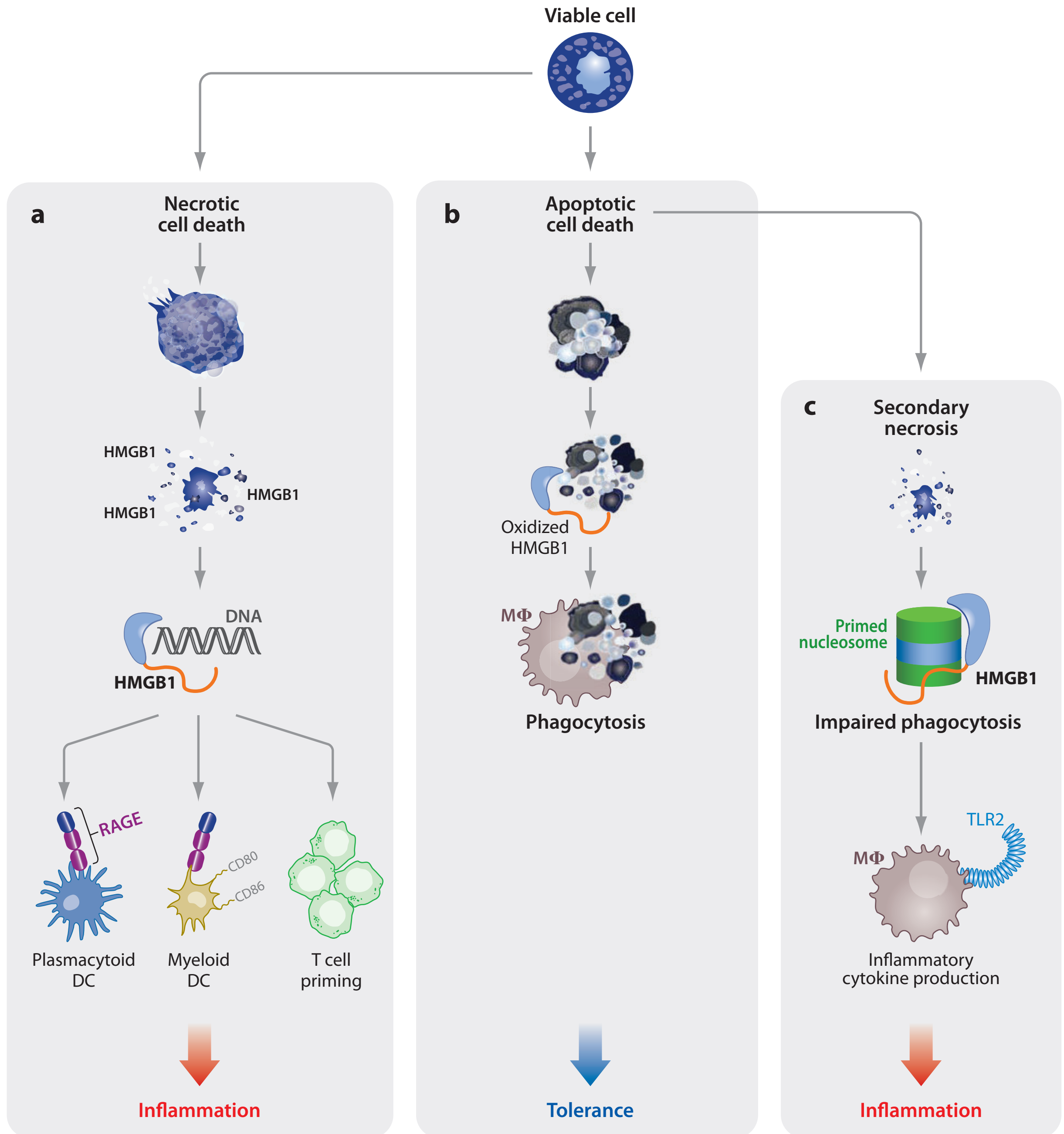
How cells die has profound effects on the immune system. Our current understanding is that when cells undergo necrosis, necrotic debris leads to inflammation and priming for adaptive T cell responses, whereas cell death by apoptosis leads primarily to tolerogenic responses (**Figure 1**). The recognition of necrotic cell death is mediated by DAMPs, a structurally and sequence-diverse family of endogenous molecules, generally intracellular, often released from necrotic cells, that activate the innate immune system (1, 2). Our understanding of the DAMP superfamily is growing, such that known members now include breakdown products of the extracellular matrix such as hyaluronan fragments (3), heat shock proteins (HSPs), S100 family of proteins (4), fibrils of amyloid-β (5), uric acid (6, 7), cytokines including IL-1α and IL-33 (8, 9), and nuclear-associated proteins such as HMGB1.

Unlike the PAMPs, the molecules that detect DAMPs are less fully understood, although recently an important role for the NALP3 inflammasome has been implicated in mediating the response to uric acid and fibrils of amyloid-β (5, 10). Some of the responses to DAMPs are also through TLRs; for example, TLR4 not only responds to LPS, but also is activated by hyaluronan (11) and S100A8/9 (4). There is emerging interest in the role of the multiligand receptor RAGE, or receptor for advanced glycation end products. As the name suggests, RAGE binds advanced glycation end products (AGEs), but more recently it has been described to bind to a diverse array of DAMPs, including HMGB1. RAGE activation plays a role in various diseases, including sepsis, rheumatoid arthritis, diabetic nephropathy, atherosclerosis,

Figure 1

HMGB1 and immune homeostasis during cell death. How cells die has important consequences for the host and immune homeostasis. Cell death through necrosis is highly proinflammatory (*a*); cell death through apoptosis is tolerogenic (*b*); and cell death in situations of impaired recognition of apoptotic cells can lead to secondary necrosis (*c*). All these mechanisms can contribute to disease pathology, but in different ways. In necrotic cell death, molecules that include HMGB1 are released, associate with other molecules (including DNA or immune complexes), and activate plasmacytoid DCs, myeloid DCs, and macrophages to induce IFN-α and TNF-α, upregulate costimulatory molecules such as CD80 and CD86, and expand effector T cells (*a*). In contrast, during apoptosis, through the generation of reactive oxygen species, molecules including HMGB1 are oxidized and deliver tolerogenic signals to dampen immune activation (*b*). If, however, professional phagocytes have an impaired ability to remove apoptotic cells, cells undergo secondary necrosis and release nucleosomes associated with HMGB1 that can induce inflammatory cytokine production from macrophages and potentially represent an alternative pathway to immune complex–mediated diseases (*c*).

and neurological diseases. The focus of this review is the role of HMGB1 and the multiligand receptor RAGE in inflammation and cancer, although HMGB1-TLR4 may also be important. Recent reviews on the importance of RAGE in diabetes (12), cardiovascular disease (13), and Alzheimer's disease (14) are highlighted elsewhere.



DANGER SIGNALS AND THE INFLAMMASOME

Recently, interest has focused on mechanisms that lead to caspase-1-dependent activation of IL-1β, and the term inflammasome is now used to describe the assembly of the high-molecular-weight complex that integrates several extracellular and intracellular signals required for IL-1β processing. Although the diversity of the inflammasome has yet to be fully appreciated, the NALP3 inflammasome is the best characterized. This system is activated by danger signals that include monosodium urate crystals, alum, and silica, as well as ATP and UV exposure, bacterial toxins such as *Staphylococcus aureus*, viral DNA, and NOD2 ligands. The Pyrin domain of NALP3 interacts with the adaptor molecule ASC (apoptosis-associated speck-like protein), which contains a caspase recruitment domain and in turn recruits caspase-1 to the complex. Manipulation of the inflammasome has implications in vaccine therapy, given that NALP3 deficiency impairs primary antibody responses. In addition, in diseases such as gout, therapeutic approaches to inhibit the inflammasome may be of benefit.

HMGB1 STRUCTURE AND CONFORMATION

HMGB1 is a relatively small protein of 215 amino acid residues. Structurally, the protein is organized into three distinct domains: two tandem HMG box domains (A box and B box), which are spaced by a short flexible linker, and a 30 amino acid–long acidic C-terminal tail. As structural units, HMG boxes are well conserved throughout evolution and are characterized by three α-helices, which are arranged in an L-shaped configuration (15). In the nucleus, HMGB1 is a nonhistone DNA-binding protein and serves as a structural component to facilitate the assembly of nucleoprotein complexes (16). The unique conformation of the A and B box domains likely plays an important role in the way HMG box proteins interact with chromatin (17). The HMGB proteins bind DNA in a conformation-dependent but sequence-independent manner. The A and B box domains of HMGB1 bind the minor groove of DNA. HMGB1 also binds four-way Holliday junctions as well as platinum-modified DNA. The ability of nuclear, chromatin-associated HMGB1 to bend DNA is likely related to the function of HMGB1 as a transcriptional regulator (17). The nuclear localization of HMGB1 is facilitated by two nuclear localization signal sequences, one embedded within the A box and the other located just before the acidic tail sequence.

Whereas the HMG box domains of HMGB1 have been studied in detail, much less is known about the function of the unusual acidic C-terminal tail. Early studies suggested that the C-terminal tail regulates the interaction of HMGB1 with DNA (18). Further analysis demonstrated that the acidic tail region can bind both HMG boxes of HMGB1, although the affinity for the A box appears to be higher than the affinity for the B box (19). This intramolecular interaction of the acidic tail provides the basis for the regulation of HMGB1 binding to DNA. The intra- as well as extracellular function of HMGB1 is further regulated by post-translational modification of the protein. More recently, HMGB1 has been shown to undergo oxidation (20) that may have the potential to modulate various aspects of HMGB1 function, including subcellular localization, interaction with DNA, cytokine activity, and proinflammatory activity.

HMGB1 Release from Cells

For HMGB1 to function as a cytokine, it must be released into the extracellular milieu. This can be accomplished either by active secretion or by passive release from damaged or necrotic cells. In 1999, Wang and colleagues first reported that HMGB1 was liberated from cells stimulated with cytokines and that HMGB1 played an important role in mediating experimental sepsis (21). Subsequently, it was demonstrated by Scaffidi and colleagues (22) that HMGB1 is released from cells undergoing necrosis, but not from apoptotic cells, because HMGB1 is tightly bound to chromatin. Importantly, the form of HMGB1 released from necrotic mouse embryonic fibroblasts has

cytokine activity and can stimulate monocytes to produce tumor necrosis factor (TNF)-α (22). Recent reports demonstrate that HMGB1 can also be released by apoptotic cells (23, 24). HMGB1 released from such cells appears to be tolerogenic rather than proinflammatory (25) (**Figure 2**). During apoptosis, HMGB1 is oxidized on Cys106 in a process that requires

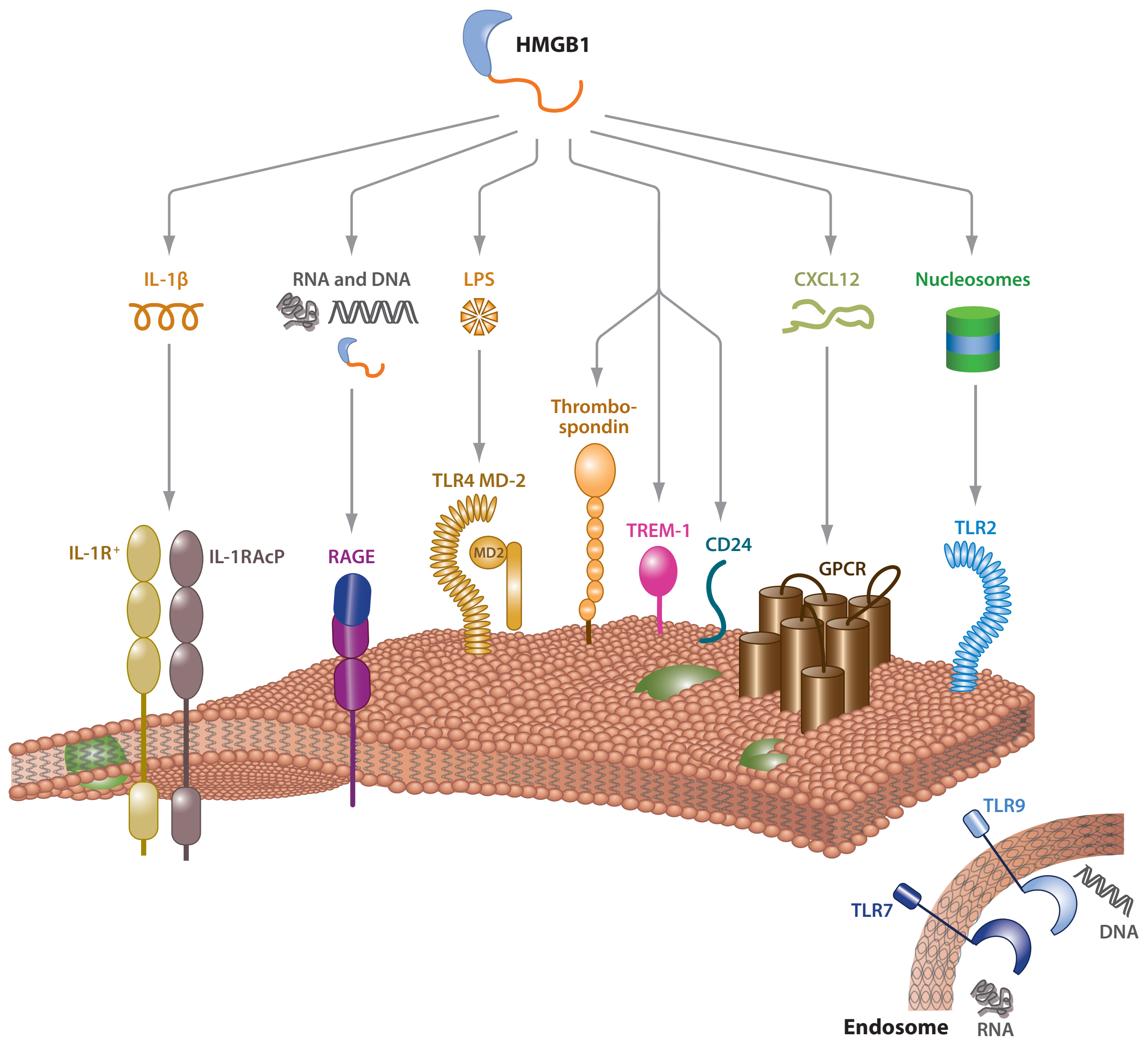


Figure 2

HMGB1-associated molecules and differential interaction with cell-surface molecules. Whether HMGB1 is released from either necrotic cells or apoptotic cells or is actively induced by cytokines, HMGB1 can potentially bind to and associate with other molecules. HMGB1 can bind to DNA and RNA and signal through RAGE. HMGB1 can also bind to IL-1β and signal through the IL-1R/IL-1RAcP complex or associate with lipopolysaccharide and activate TLR4. When HMGB1 is associated with the nucleosome, released during secondary necrosis, TLR2 is preferentially engaged. HMGB1 has also been reported to bind to TREM-1 (triggering receptor expressed on myeloid cells-1). Thrombospondin and CD24 also bind to HMGB1 and may provide important negative regulatory signals to inhibit HMGB1-mediated coagulation and inflammation.

iDC: immature dendritic cell

pDC: plasmacytoid dendritic cell

caspase activity and mitochondrial reactive oxygen species (ROS). Interestingly, Cys106 lies within the B box, which could explain the lack of inflammatory activity of oxidized HMGB1 from apoptotic cells (25), although whether Cys106 HMGB1 interacts with different binding partners and/or receptors remains to be determined.

Active secretion of HMGB1 requires shuttling of the protein from the nucleus into the cytosol or prevention of nuclear import of newly synthesized HMGB1. Several forms of post-translational modifications, such as phosphorylation, methylation, and acetylation, result in the accumulation of HMGB1 in the cytosol (26–28). Because HMGB1 does not contain a leader sequence, the protein is released via a nonclassical secretory pathway that may involve specialized vesicles of the endolysosomal compartment (29). The secretion of HMGB1 can be triggered by different stimuli. For example, IFN-γ can induce HMGB1 release from macrophages that, at least in part, requires induction and signaling through TNF-α (30). HMGB1 secretion can also be triggered by endotoxin [lipopolysaccharide (LPS)] and IL-1β (21). HMGB1 release in response to LPS and TNF-α appears to depend on the NF-κB pathway and can be blocked by compounds that prevent NF-κB activation (21, 31). In addition, molecules such as ethyl pyruvate, a lipophilic pyruvate derivative, and cholinergic agonists prevent HMGB1 release and improve survival in experimental mouse models of sepsis (31–33). In contrast, LPS-induced release of HMGB1 from macrophages may require IFN-β signaling via JAK kinases and Stat-1, but not via NF-κB (34). Differences between this and previous studies may be, at least in part, due to differences in the experimental systems and the pharmacological inhibitors used to interrogate signaling pathways. Given the different mechanisms of HMGB1 release and the variety of post-translational modifications (and combinations thereof) involved, caution must be exercised when comparing soluble forms of HMGB1 generated under different conditions.

In Vitro Functions of HMGB1

HMGB1 can promote inflammatory responses by numerous mechanisms. Comparisons of the necrotic cell debris from HMGB1-deficient and wild-type cells demonstrate that HMGB1-deficient cells have a profoundly reduced capacity to induce cytokines (22). Recombinant HMGB1 also induces cytokine production from human monocytes but not from lymphocytes (35). However, several groups have more recently shown that highly purified HMGB1 does not induce significant amounts of proinflammatory cytokines (36–39). Indeed, the capacity of HMGB1 to bind other molecules (see below) may be the underlying basis for these observations. However, it should also be noted that recombinant HMGB1 may be different from the native protein, with changes in the oxidative status having a profound impact on its biological activity (25). HMGB1 induces the phenotypic maturation of immature DCs (iDCs) with upregulation of costimulatory molecules and MHC class II and the secretion of proinflammatory cytokines, including IL-12 and IFN-γ, that together potently stimulate allogeneic T cells, polarizing them toward a Th1 phenotype (40, 41). HMGB1 is also implicated in the maturation of plasmacytoid DCs (pDCs) (42, 43). As DCs mature, they switch their function from antigen uptake to antigen presentation. During this process, they reorganize their cytoskeleton and become more responsive to chemokines in draining lymph nodes. HMGB1 induces the migration of iDCs but not of mature DCs; furthermore, RAGE expressed on iDCs is necessary for homing to lymph nodes (44, 45).

Stimulation of neutrophils and monocytes with HMGB1 induces cytokine release and increases the interaction between Mac-1 and RAGE that enables these cells to adhere to activated vascular endothelium and migrate into inflamed tissue (46, 47). HMGB1 also primes the vascular endothelium by upregulating TNF-α and monocyte chemotactic protein (MCP)-1, as well as upregulating intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 to facilitate

the adhesion of inflammatory cells (48). An additional mechanism by which HMGB1 may promote inflammation is by interfering with phagocytosis. If apoptotic cells are not efficiently removed by phagocytosis, cells become necrotic, and their contents fuel an inflammatory response. Interestingly, HMGB1 binds to phosphatidylserine and blocks phosphatidylserine-mediated phagocytosis of apoptotic neutrophils (49).

HMGB1 Interactions and Receptors

Although the role of HMGB1 inside the nucleus is beyond the scope of this review (for a recent review, see Reference 50), it is important to highlight the vital contribution HMGB1 makes to nuclear function. DNA was the first molecule to be described that interacts with HMGB1. Subsequent work revealed that HMGB1 preferentially binds to altered DNA structures and can bend DNA upon binding to confer transcriptional stability (51). These traits are central to the notion that HMGB1 acts as a molecular linchpin facilitating the arrangement of complex nucleoprotein structures (52).

One emerging concept in HMGB1 biology is its ability to interact with other molecules, including DNA, RNA, IL-1β, and LPS, and also with nucleosomes that augment or modify the function of HMGB1 itself (**Figure 2**). Recognition of self-DNA is normally kept in check by extracellular nucleases and by the endosomal localization of TLR9. Breakdown of this mechanism can occur either when extracellular DNA is protected from nucleases and/or when DNA has a means to enter the endosomal pathway where it can encounter TLR9. A well-studied example of this mechanism is with pathogenic DNA-containing immune complexes. These complexes contain DNA bound to a protein component (nucleosomes, HMGB1) and can enter the endosomal pathway of pDCs or B cells via CD32-mediated uptake (53). Because HMGB1 can directly bind DNA, we hypothesize that extracellular HMGB1-DNA complexes, alone or bound to autoantibodies, may activate the immune system via RAGE and TLR9. Indeed, HMGB1-CpG-A complexes stimulate greater IFN-α production from mouse bone marrow–derived pDCs than does either component alone. In addition, not only was HMGB1 present in DNA-containing immune complexes, it was also essential for inducing cytokine production in a TLR9-mediated manner (37). Ivanov et al. (39) similarly reported that HMGB1-deficient immortalized fetal liver DCs (iFLDCs) produce less IFN-α/β in response to CpG-DNA than do wild-type iFLDCs. Interestingly, HMGB1 can also interact with TLR9 independently of DNA (37, 39).

iFLDC: immortalized fetal liver dendritic cell

Autoantibodies, particularly autoantibodies to nuclear antigens, are a common feature in many systemic autoimmune diseases, especially systemic lupus erythematosus (SLE), and impairment of apoptotic cell clearance likely contributes to disease pathogenesis (55–57). Recently, Urbonaviciute et al. (58) implicated HMGB1-containing nucleosomes derived from secondary necrotic cells as potent proinflammatory activators of macrophages and DCs (**Figure 1**). To elucidate the mechanism of action, a series of activation experiments were performed on macrophages defective in RAGE and several TLR pathways. Surprisingly, the deletion of *MyD88* or *TLR2* inhibited activation (58). Thus, if HMGB1 is associated with DNA, then the complex signals through RAGE/TLR9, whereas if HMGB1 is associated with the nucleosome, signaling is strictly TLR2 dependent (**Figure 2**).

HMGB1 also has the potential to bind to other PAMPs, including LPS (59). HMGB1, like LPS-binding protein, can actively destabilize LPS aggregates and present LPS monomers to CD14, thus increasing the overall sensitivity of the TLR4/MD-2 receptor complex. As with HMGB1-DNA complexes, HMGB1 and LPS synergize for optimal cytokine production (59). Whether this is due to the LPS transfer properties of HMGB1 or is a direct result of HMGB1 signaling remains to be determined.

HMGB1 administration to LPS-resistant C3H/HeJ mice can induce lethality, suggesting a TLR4-independent pathway (21). A

clue to this outcome came in 2007 with the identification of TREM-1 (triggering receptor expressed on myeloid cells-1) as a potential detector of HMGB1-mediated cytokine induction (60). In this case, investigators employed neutralizing antibodies to identify HMGB1 and HSP70 as key danger signals present in necrotic cell lysates and to implicate TREM-1 as their receptor on exposed cells. However, no direct binding of HMGB1 to TREM-1 has been demonstrated thus far. More recently, investigators have explored the interaction of HMGB1 with various cytokines and chemokines. First, purified HMGB1 from cells treated with doxycycline and either TNF-α, IFN-γ, or IL-1β acquired enhanced proinflammatory activity (38). Biochemical studies uncovered the ability of HMGB1 and IL-1β to form a reversible complex. Furthermore, studies with blocking antibodies to IL-1β or using IL-1RA completely abrogated this newly acquired activity of HMGB1. Interestingly, a common theme emerged in this study as well: the capacity of HMGB1 to synergize with normally inert amounts of a compound and, by doing so, exert proinflammatory effects (**Figure 2**).

Yet another binding partner of HMGB1 is CXCL12 (also known as stromal cell-derived factor-1). As described by Campana et al. (61), HMGB1 secretion is required for CXCL12-dependent migration of DCs and to a lesser extent migration of macrophages. In addition, HMGB1 protects the conformation of CXCL12 in a reducing environment, a state existing in the draining lymph node. However, experiments using the HMGB1 specific inhibitor HMGB1 A box and a CXCL12 inhibitor only partially inhibited DC migration, suggesting that multiple receptors are responsible for mediating the response to the HMGB1/CXCL12 complex.

Several negative regulators of HMGB1 have also been identified. CD24-deficient mice exhibit increased responsiveness to acetaminophen-mediated liver necrosis. Using a series of biochemical approaches, investigators demonstrated that CD24 is associated with HMGB1 (62). Anti-HMGB1 antibody reversed the increased lethality seen in CD24-deficient mice, leading to the hypothesis that CD24 recognition of HMGB1 suppresses its proinflammatory effects. Further experiments identified Siglec-10 as the signal transducer of the CD24-HMGB1 interaction, and similar results were obtained for endogenous danger signals HSP70 and HSP90. Thus, the CD24 and Siglec-10 receptor complex appears to provide a molecular break for endogenous danger signals without compromising responses to infectious agents (62). This mechanism would provide the host a unique way of sensing damage to inflamed tissues during sterile inflammation, but it would also allow an appropriate response to invading organisms.

Lastly, thrombospondin also inhibits HMGB1. Thrombospondin is a cell-surface glycoprotein, predominately expressed on vascular endothelial cells, that mediates thrombin-dependent activation of protein C, which has critical anticoagulant activity (63). The N-terminal lectin-binding domain of thrombospondin binds HMGB1 and blocks its interaction with RAGE (64). Binding promotes the cleavage of the N-terminal domain of HMGB1, thereby reducing its proinflammatory potential (65). Thrombospondin may therefore act as a natural negative regulator of HMGB1 and modulate vascular inflammatory responses (**Figure 2**).

HMGB1 AND DISEASE

HMGB1 and Sepsis

Sepsis is induced by infectious organisms that enter the blood stream and disseminate throughout the body. Despite advances in antibiotics and supportive care, the incidence of sepsis syndromes is increasing and is responsible for over 200,000 deaths in the United States annually (66). Sepsis syndromes result from an exaggerated systemic inflammatory response characterized by a massive release of mediators such as TNF-α and IL-1β that drives a largely uncontrolled proinflammatory response.

In a model of murine sepsis, systemic administration of LPS induces a lethal proinflammatory response. Within the first few hours, LPS triggers TNF-α and IL-1β production followed by the delayed release of HMGB1, all of which induces cellular damage. HMGB1 antibodies inhibit endotoxin lethality in mice (67, 68) and also inhibit lung inflammation following airway LPS exposure (69). RAGE-deficient mice are also protected from endotoxemia, suggesting that HMGB1-induced lethality is mediated through RAGE signaling (64). RAGE expression on the endothelium appears to be essential and sufficient to mediate lethality (70). The important roles of HMGB1 and RAGE in mediating pathological innate responses in sepsis have been confirmed in the cecal ligation and puncture (CLP) model, a lethal polymicrobial insult (70–72). The C5a-HMGB1 axis is important in the development of sepsis, and C5L2, rather than C5AR, appears to play a key role in HMGB1 release (73). TLR9-deficient mice are also protected in the CLP model, which is associated with increased numbers of peritoneal DCs and neutrophils, reduced bacterial dissemination, and reduced circulating proinflammatory cytokines. Transfer of TLR9-deficient DCs to wild-type mice conveys protection (74) and suggests that HMGB1-DNA complexes released from dying cells may reduce phagocytic activity and cellular recruitment in a TLR9-dependent manner. HMGB1 also mediates the secretion of plasminogen activator inhibitor-1 and tissue plasminogen activator from endothelial cells (48). This finding, in conjunction with the release of tissue factor from monocytes, suggests that HMGB1 plays a key role in the microvasculature and in the initiation of the coagulation cascade in the pathophysiology of sepsis.

HMGB1, Trauma, and Sterile Inflammation

In the absence of pathogens, severe trauma and hemorrhagic shock can also induce systemic autoinflammatory reactions with clinical manifestations similar to those in sepsis syndrome, indicating commonality in the pathophysiology. HMGB1 levels are elevated in patients with mechanical trauma, strokes, acute myocardial infarction, acute respiratory distress, and liver transplantation (75–79). In a model of hepatic ischemia-reperfusion injury, HMGB1 levels are increased as early as 1 h after reperfusion and are sustained for at least 24 h (79). Administration of soluble RAGE or neutralizing anti-HMGB1 antibody decreased liver damage (79, 80). TLR4-deficient mice are also protected during hepatic ischemia-reperfusion injury. Compelling evidence using TLR4-defective mice also supports a role for a HMGB1-TLR4 axis in hemorrhagic shock/resuscitation-induced injury, systemic inflammation, and end-organ damage induced by bilateral femur fractures (81, 82).

Improved survival and liver regeneration in RAGE-deficient mice following partial hepectomy is associated with reduced apoptosis and IL-6 production (83). RAGE-deficient mice also exhibit less damage following ischemia-reperfusion injury in the heart (84). Interestingly, in addition to being one of the potential receptors for triggering HMGB1-mediated inflammation, TLR4 is also responsible for the secretion of HMGB1. Early after ischemia-reperfusion, hypoxia induces the active release of HMGB1 from hepatocytes, a process regulated by TLR4-dependent ROS production and downstream calcium-dependent kinase signaling (80). In marked contrast to these studies, investigators have also shown that preconditioning with HMGB1 prior to hepatic insult protects mice from injury in a TLR4-dependent manner (85). Which cells are responsible for mediating this effect and whether this phenomenon occurs in other ischemia-reperfusion models are still unclear. Nevertheless, outcome of HMGB1-TLR signaling likely depends on other signaling events and may be context dependent.

Accommodating all these findings into a simple unifying hypothesis is challenging. Most probably, multiple receptors mediate HMGB1 responses, and their relative importance may depend on the levels of HMGB1, the expression of the receptors in the target organ, and

IPF: idiopathic pulmonary fibrosis

the ability of HMGB1 to interact with other molecules, as shown in **Figure 2**. Potentially, there is significant cross-talk between receptors, and the deficient or defective receptor may influence the function of other receptors. The use of specific ligand-blocking antibodies may help clarify the roles of the individual receptors.

HMGB1 in Chronic Inflammatory and Autoimmune Diseases

HMGB1 may also play a role in chronic autoimmune disorders (86). In this regard, increased levels of HMGB1 and increased numbers of HMGB1-secreting cells have been identified at specific sites of inflammation, including in the inflamed synovium of patients with rheumatoid arthritis. Anti-HMGB1 antibodies inhibit the development of synovial inflammation and joint swelling in experimental models of arthritis (87, 88), and this appears to be independent of the TNF-α pathway (88). Moreover, anti-TNF-α monoclonal antibody therapy in patients with rheumatoid arthritis had no notable effect on HMGB1 expression (89). Thus, TNF-α may not be the main inducer of extranuclear HMGB1. Rather, HMGB1 may represent a TNF-α-independent pathway for possible future therapeutic intervention in arthritis.

Elevated levels of HMGB1 have also been reported in patients with SLE. The autoantibodies that form against double-stranded DNA and nucleosomes are characteristic of SLE. Evidence suggests that impaired phagocytosis of apoptotic cells and subsequent secondary necrosis may contribute to disease pathogenesis (90). As discussed above, HMGB1, when released from apoptotic cells, remains bound to nucleosomes and can be detected in the plasma of SLE patients (58, 91). Notably, HMGB1 is also part of immunostimulatory complexes that bind to and activate autoreactive B cells (37). Finally, HMGB1-DNA complexes induce cytokine secretion from mesangial cells in vitro (92). Serum HMGB1 levels are higher in patients with scleroderma and correlate with skin fibrosis and impaired lung function (93). The association with fibrosis is not restricted to autoimmune disorders, given that elevated levels of HMGB1 occur in patients with idiopathic pulmonary fibrosis (IPF) (94). The potential pathogenic role of HMGB1 in IPF is supported by experimental animal model studies demonstrating that anti-HMGB1 antibodies prevent bleomycin-induced lung fibrosis (94).

HMGB1 and Cancer

The involvement of HMGB1 in cancer is complex, and intracellular/nuclear and extracellular forms of HMGB1 have been implicated in tumor formation, progression, and metastasis and in the response to chemotherapeutics. Elevated expression of HMGB1 occurs in several solid tumors, including melanoma, colon cancer, prostate cancer, pancreatic cancer, and breast cancer (20, 95, 96). In gastrointestinal stromal tumors, the overexpression of HMGB1 is closely associated with gain-of-function mutations in c-kit (97). Importantly, HMGB1 in cancer is associated with invasion and metastasis (95, 98). HMGB1 may be directly involved in tumor cell metastasis through its ability to promote cell migration, to modulate the adhesive properties of cells, and to modify components of the extracellular matrix. In certain cases, however, the effect may be indirect, and, by enhancing the activity of NF-κB p65, HMGB1 leads to the induction of melanoma inhibitory activity (MIA) (99, 100), an 11-kDa secreted protein, which binds to fibronectin as well as to certain integrins and enhances migration and invasion of tumor cells (99). Thus, HMGB1 does not necessarily have to be secreted by tumor cells to enhance their invasive and metastatic potential.

One rate-limiting step in tumor growth and progression is neoangiogenesis. As the growing tumor exceeds the capacity of the existing vasculature, hypoxia results in necrotic cell death within the tumor. As a cytokine, HMGB1 not only stimulates inflammatory cells, but also can activate vascular endothelial cells. HMGB1

can stimulate endothelial cell proliferation and sprouting in vitro and neovascularization in vivo (101). The importance of this pathway in vivo is suggested by the observation that blockade of HMGB1 and RAGE suppresses tumor growth and metastasis in a murine model of lung cancer (102). Van Beijnum and colleagues (103) analyzed the gene expression profile of tumor-associated endothelium compared with normal tissue endothelium. HMGB1 was one of several genes specifically overexpressed in tumor endothelium. Furthermore, an anti-HMGB1 antibody inhibited angiogenesis in the chick chorioallantoic membrane assay (103). Together, these data suggest that HMGB1 is a proangiogenic cytokine that may contribute to tumor growth and progression by promoting neoangiogenesis. Whether HMGB1 needs to be associated with other cellular components to mediate the proangiogenic effect is currently unknown.

Dying tumor cells can, in some circumstances, induce an effective antitumor response if cells die in such a manner as to induce maturation of DCs that can promote a cytotoxic T lymphocyte response through cross-presentation of tumor antigens. HMGB1 has been shown to be released from both irradiated and doxorubicin treated tumor cells (104). In this context, HMGB1 plays an important role in activation of DCs through TLR4 for the efficient presentation of tumor antigens liberated by dying cells (104). Notably, the role of HMGB1 in this process appears to be independent of the contribution of HMGB1 to DCs' maturation and migration, which primarily involves RAGE activation (41), but appears to modulate processing and cross-presentation of tumors through TLR4 (104).

THE RECEPTOR FOR ADVANCED GLYCATION END PRODUCTS (RAGE)

The loci (*AGER*) encoding RAGE is located on chromosome 6 near MHC class III in humans and mice, close to the genes encoding TNF-α, lymphotoxin, and HOX12 (105). RAGE is a type I transmembrane protein composed of three extracellular immunoglobulin-like domains (V, C1, and C2), a single transmembrane domain, and a short cytoplasmic tail (106). A combination of biochemical approaches revealed that the V and C1 domains form an integrated structural unit, separated from a fully independent C2 domain by a flexible linker (107). Interactions between RAGE and its ligands are mapped to the V/C1 domain, with the amino-terminal V domain providing the major contribution (107, 108).

RAGE has two potential N-glycosylation sites, both located in the V domain. Carboxylation of these N-glycans enhances the binding of HMGB1 and AGEs and subsequent signal transduction (109). Because the carbohydrate groups are easily removed from the native protein by PNGase F (109), they are not predicted to alter the conformation of the protein. The surface exposure of the carboxylated glycan groups may influence ligand binding directly.

Numerous human RAGE mRNA species have been isolated from different cell types (110). Most mRNA species, including the N-truncated isoform, are likely to be targeted to the nonsense-mediated mRNA decay pathway or to otherwise fail to generate the protein. Some rare mRNA species with alterations in the V/C1 domain could be interesting because they could differentially bind ligands; however, their roles remain speculative in the absence of extensive functional characterization. Only two functionally relevant spliced isoforms are currently known: the abundant full-length transmembrane form, which can initiate signaling through its intracellular tail, and a soluble isoform (known as esRAGE, C-truncated RAGE, and RAGE_v1), which can act as a decoy receptor. The transmembrane form of RAGE may also be proteolytically cleaved proximal to the membrane to generate a soluble form of RAGE that will have the same decoy capacity as esRAGE (111).

RAGE is constitutively expressed at high levels in the lung. Expression is localized to

alveolar type II cells (112) and on the basolateral membranes of the alveolar type I epithelial cells (113). The physiological relevance of the high expression in these cells is unknown. Elsewhere, there is widespread but relatively low expression of RAGE on vascular endothelial cells, neutrophils, monocytes/macrophages, lymphocytes, DCs, cardiomyocytes, and neurons (114, 115). RAGE expression increases in circumstances when ligands, such as AGEs, and inflammatory mediators accumulate (106, 116). Because the RAGE promoter contains multiple functional NF-κB and SP-1 transcription factor–binding sites (117, 118), ligands and proinflammatory cytokines can promote the expression of RAGE, potentially triggering a receptor-dependent autoinflammatory loop.

RAGE AS A MULTILIGAND RECEPTOR

As its name suggests, the receptor for advanced glycation end products was initially identified as a receptor for AGEs (106). Since then, we have learned that this receptor has various binding partners. Rather than binding to a single specific ligand or even a group of closely related ligands, RAGE binds to several classes of molecules that lack sequence similarities (**Figure 3**). These ligands include HMGB1, several members of the calcium-binding S100 family of proteins, some species of AGEs, and β-sheet fibrillar material such as amyloid-β, serum amyloid A, immunoglobulin light chains, transthyretin, and prions, among others (**Figure 3**). Consequently, RAGE can be considered a pattern-recognition receptor that

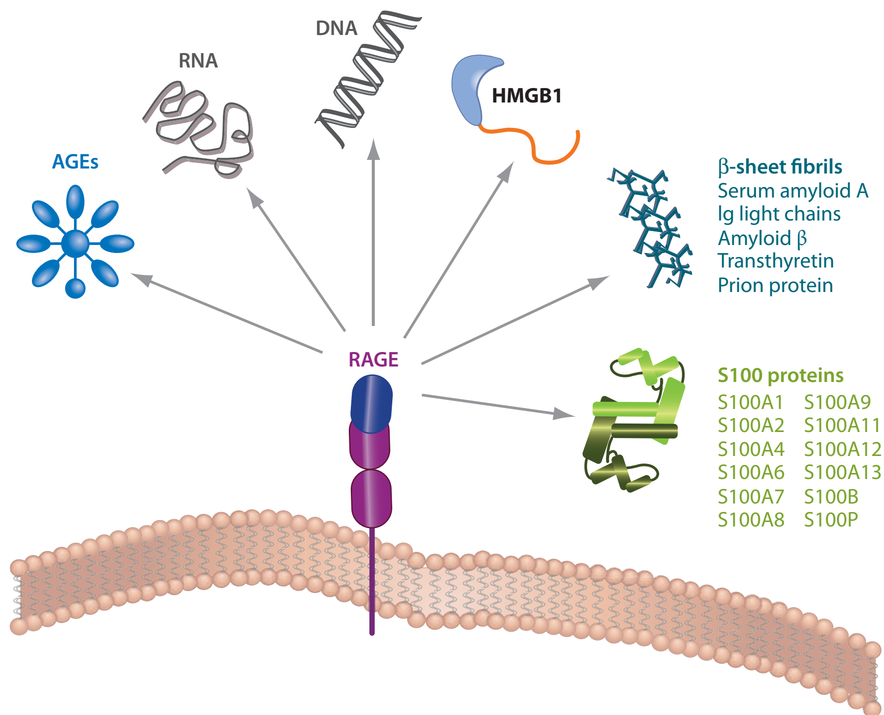


Figure 3

RAGE is a multiligand receptor and the prototypic DAMP receptor. RAGE can bind to structurally diverse molecules that include not only AGEs, but also DNA and RNA that bind in a sequence-independent manner, HMGB1, an array of S100 family member proteins, and fibrillar proteins that include amyloid-β. The association of ligands with RAGE results in a complex signal transduction cascade leading to cell activation.

binds predominately endogenous molecules that are either generated or released during cellular or physiological stresses.

RAGE SIGNALING

Although there are compelling data to support the hypothesis that RAGE is central to many inflammatory disorders, the structural features (or lack thereof) at the C terminus of RAGE raise questions as to whether RAGE is a signal transducer. Insight into this question was gleaned through the use of a RAGE construct lacking the intracellular tail. In these studies, this RAGE construct behaved like a dominant negative, supporting the notion that RAGE can indeed function as a bona fide signaling molecule (102, 116, 119). RAGE engagement by a myriad ligands is linked to an array of signaling pathways. These include the activation of NF-κB (120–122), MAPKs (122–124), PI3K/Akt (125), Rho GTPases (126), Jak/STAT (127), and Src family kinases (128), among others. This variety of reported RAGE signal transduction pathways is quite extraordinary. However, the diverse nature of the RAGE ligands, and possibly their contaminating elements, coupled with the broad expression pattern of RAGE may account for such an assortment of signals.

The predicted cytoplasmic domain of RAGE consists of a short 43 amino acid tail with no obvious signaling domains or motifs. Hence, a major question in the field was how RAGE transduces signals from the cell surface to the nucleus. Several papers have tried to address this question by searching for direct binding partners using the cytoplasmic tail of RAGE as bait. Ishihara et al. (129) identified both extracellular signal-related kinase-1 and -2 (ERK1/2) as direct RAGE-binding partners. Further truncation of the cytoplasmic domain unveiled a putative ERK docking site at the membrane-proximal region. HMGB1 stimulation of RAGE-transfected HT1080 cells induced the interaction of RAGE and ERK1/2. Hudson et al. (126) employed a yeast two-hybrid system to explore possible cytoplasmic-binding partners of RAGE. Results from this study indicate that the FH1 domain of mammalian Diaphanous-1 (mDia-1) interacts with RAGE. A functional link between the relationship of mDia-1 and RAGE was illustrated in mDia-1 siRNA knockdown assays that impaired the GTPase activity of Rac-1 and Cdc42, two Rho GTPases involved in cellular migration (130, 131). Interestingly, mDia-1 had previously been implicated in cytokinesis and Src signal transduction (132).

OTHER RAGE LIGANDS

Advanced Glycation End Products (AGEs)

AGEs result from the nonenzymatic reaction between reducing sugars and cellular components, including proteins, lipids, and nucleic acids. Glycation of proteins usually occurs at the N terminus and at particular amino acid side chains (133). The reaction, also known as the Maillard reaction, is initiated by the interaction between the sugar carbonyl moiety and the primary amino group of the protein (in the case of N-terminally modified proteins). The resulting Schiff base is transformed into a more stable Amadori product. Next, a series of lesser understood oxidation and dehydration reactions lead to the formation of AGEs. Furthermore, AGEs can cause extensive cross-linking of proteins, leading to their deposition. Recently, alternative pathways leading to AGE formation have been uncovered (134, 135).

Overall, the accumulation of AGEs in the body increases with time and in certain disease states such as diabetes and Alzheimer's, with disease progression linked to higher AGE levels (136, 137). AGEs can also bind to other receptors, including AGE-R1 and lysozyme, that facilitate the degradation and clearance of AGEs (138). The binding of AGEs to RAGE appears to be proinflammatory, however, and upregulates TNF-α, IL-6, and nitric oxide (124). Thus, the stimulation of RAGE through AGEs may contribute to several pathological conditions and diseases.

S100 Family Members

The S100s are a family of over 20 related calcium-binding proteins that are exclusively expressed in vertebrates, where their expression patterns are tissue- and cell-type specific (139). These small, acidic proteins (10–12 kDa) are characterized by two calcium EF-hand motifs: a C-terminal canonical EF-hand, common to all EF-hand proteins, and an N-terminal pseudo EF-hand, characteristic of S100 proteins, connected by a central hinge (140). Calcium binding to the EF-hand motif occurs in response to increased intracellular calcium concentrations and triggers conformational changes that expose a wide hydrophobic cleft that interacts with target proteins. The structural homology of the S100s permits the formation of active heterodimers, such as S100A1/A4 and S100A8/A9 (141). S100A4, S100A8/9, S100A12, and S100B can generate tetramers, hexamers, and higher-order multimeric structures that alter binding properties and physiological responses (108, 142). S100s modulate an array of intracellular functions, including calcium homeostasis, cytoskeletal organization, cell cycle progression, and cell growth and differentiation (139, 140). Besides their intracellular functions, S100s released from different cell types during inflammation serve as useful markers of disease activity (143, 144).

S100A12 [also known as extracellular newly identified RAGE-binding protein (EN-RAGE) or Calgranulin C] and S100B were the first identified S100 RAGE ligands (120, 121). Subsequently, S100A1, S100A2, S100A4, S100A5, S100A6, S100A7, S100A8 (MRP8, Calgranulin A), S100A9 (MRP14, Calgranulin B), S100A11, S100A13, and S100P have also been shown to bind to RAGE (145). The extracellular functions of all the S100 proteins have not been completely delineated. Some S100s, such as S100A1, S100A7, and S100B, are primarily associated with inflammation in the heart, skin, and brain, respectively, whereas a subset of the S100s known as the calgranulins, S100A8 (Calgranulin A, MRP8), S100A9 (Calgranulin B, MRP14), and S100A12 (Calgranulin C, EN-RAGE), are predominately expressed by granulocytes and monocytes and have a broad role in inflammatory responses (4). The S100A8/A9 heterodimer and S100A12, released from activated phagocytes, mediate the recruitment of leukocytes (146, 147). The importance of S100 proteins in inflammation is also demonstrated by the fact that migration of neutrophils and monocytes during monosodium crystal–mediated gouty arthritis is inhibited by anti-S100 antibodies (147).

S100A12 and S100A8/A9 also increase the expression of the proinflammatory cytokines IL-1β and TNF-α (120, 148). Using anti-RAGE antibodies and a DN-RAGE signaling–deficient construct, investigators observed that the S100A12-induced response is mediated by RAGE. However, despite the sequence similarity of S100A12 with S100A8 and S100A9, S100A8-mediated cytokine induction was mediated by TLR4, not by RAGE (148). Interestingly, S100A9 and the S100A8/A9 heterodimer failed to activate macrophages in the absence of LPS, suggesting that S100A9 modulates the activity of its partner (148).

Much remains to be understood regarding the role of S100s in inflammation. These proinflammatory S100 proteins are almost always up-regulated at inflammatory lesions and in the blood of patients with a range of inflammatory and autoimmune disorders (4, 144). Whether they are molecular patterns that may be useful diagnostic markers or whether they actively contribute to disease pathogenesis remains to be determined. Recently, investigators reported that a quinoline-3-carboxamide derivative that inhibits S100A9 interaction with RAGE and TLR4 suppresses inflammation in an experimental model of autoimmune encephalomyelitis. This finding provides a proof of concept in early clinical evaluation in multiple sclerosis and type 1 diabetes, suggesting that S100A9 may indeed be an important mediator of inflammatory disorders (149).

CONCLUSIONS AND FUTURE PERSPECTIVES

HMGB1 is undoubtedly an important effector molecule and plays an important role in many

pathological settings from acute sepsis to sterile inflammation during trauma, as well as in chronic inflammatory diseases from rheumatoid arthritis to SLE. In addition, HMGB1 is implicated in other diseases characterized by cell death and damage including diabetes and Alzheimer's disease. Despite this key role, many questions remain to be addressed. Given that highly purified recombinant HMGB1 possesses little if any inflammatory properties, what is the true nature of the pathological form of HMGB1 in disease? Do post-translational modifications of HMGB1 influence biological activity? Indeed, given the growing number of HMGB1-binding partners, are there different types of HMGB1 complexes that are formed in the context of different pathological situations, as has been reported in SLE, where HMGB1 nucleosomes are believed to be pathogenic? Oxidative stress of the cell also has important implications for the role of HMGB1 and presumably switches the ability of HMGB1 to activate DCs and prime T cells to a molecule that promotes tolerance. Whether the oxidized form of HMGB1 mediates tolerance through RAGE is unknown. Recently, HMGB2 has also been shown to be liberated from necrotic cells and to exert proinflammatory effects (150), raising the possibility that effective therapies will require inhibition of both HMGB1 and HMGB2. In this respect, not only HMGB1 (151), but also HMGB2 and HMGB3 have been reported to function as universal sentinels for nucleic acid–mediated activation of innate immune responses (151). The relative contribution of intracellular and extracellular HMGBs in this response remains to be determined, however. The role of RAGE as the principal receptor for HMGB1 is also unresolved, as studies also support a role for TLR2 and TLR4. The molecular basis for differential receptor engagement may be related to the nature of the HMGB1 complex. In this respect, most studies performed to date have relied on inhibition of HMGB1 with polyclonal antibodies. To target this complex protein successfully with monoclonal antibodies, careful characterization of the biological activities specifically associated with particular diseases will be necessary.

DISCLOSURE STATEMENT

A.J.C., R.H., G.S., S.T.R., and D.R. are all employees of Medimmune LLC. The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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