

Review Article

Life after death: targeting high mobility group box 1 in emergent cancer therapies

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Abstract: High mobility group box 1 (HMGB1), an evolutionarily highly conserved and abundant nuclear protein also has roles within the cytoplasm and as an extracellular damage-associated molecular pattern (DAMP) molecule. Extracellular HMGB1 is the prototypic endogenous 'danger signal' that triggers inflammation and immunity. Recent findings suggest that posttranslational modifications dictate the cellular localization and secretion of HMGB1. HMGB1 is actively secreted from immune cells and stressed cancer cells, or passively released from necrotic cells. During cancer development or administration of therapeutic agents including chemotherapy, radiation, epigenetic drugs, oncolytic viruses, or immunotherapy, the released HMGB1 may either promote or limit cancer growth, depending on the state of progression and vascularization of the tumor. Extracellular HMGB1 enhances autophagy and promotes persistence of surviving cancer cells following initial activation. When oxidized, it chronically suppresses the immune system to promote cancer growth and progression, thereby enhancing resistance to cancer therapeutics. In its reduced form, it can facilitate and elicit innate and adaptive anti-tumor immunity, recruiting and activating immune cells, in conjunction with cytotoxic agents, particularly in early transplantable tumor models. We hypothesize that HMGB1 also functions as an epigenetic modifier, mainly through regulation of NF- κ B-dependent signaling pathways, to modulate the behavior of surviving cancer cells as well as the immune cells found within the tumor microenvironment. This has significant implications for developing novel cancer therapeutics.

Keywords: Cancer, HMGB1, NF- κ B signaling, activation, innate immunity, dendritic cells, CD8⁺ T cells, epigenetic pathways

Introduction

The high-mobility group box-1 (HMGB1) protein, also known as high-mobility group 1 (HMG-1) and amphoterin, is a highly conserved, abundant non-histone nuclear protein expressed in almost all eukaryotic cells [1]. Within the nucleus, HMGB1 modulates and 'fluidizes' nucleosomes and bends DNA and binds bent DNA in chromatin structure, facilitating transcription of many genes (for a review, see ref# [2]). As a component of the innate immune system, the HMGB1 protein functions as a common signal that alerts the host to stress, unscheduled cell death, and to microbial invasion [3, 4]. As a result, HMGB1 is an endogenous molecule that facilitates host inflammation and the resultant immune response [5]. In response to inflammatory stimuli following infection or injury, HMGB1

is secreted by immune cells such as macrophages [6, 7], natural killer (NK) cells [8], neutrophils [9] and mature dendritic cells (DC) [10], functioning as a cytokine-like molecule. Cancer cells may actively secrete HMGB1 [11-13], or passively release HMGB1 in the process of dying. Through binding to individual surface receptors, including the receptor for advanced glycation end products (RAGE) and Toll-like receptors (TLR) 2/4 on cancer and other cells, HMGB1 can activate signalling pathways, including mitogen-activated protein kinases (MAPKs) and the serine/threonine kinase AKT, which play important roles in tumor growth and inflammation [5, 14]. Increased expression of HMGB1 occurs in many cancers and has functional implications. Here we will focus on the posttranslational modification, localization, and release of the HMGB1 molecule in immune

cells and cancer cells, its dynamics and function during cancer treatment, and as a potential target for improved combination therapy. Interestingly, we propose that HMGB1 functions as an epigenetic modifier via epigenetic pathways to regulate surviving cancer cells and immune cells within the tumor microenvironment, thus regulating their function. This is an important factor to consider in developing combination strategies for patients with cancer.

Molecular biology of HMGB1

HMGB1 is a protein encoded by the *HMGB1* gene, a member of the HMG superfamily in humans. The human *HMGB1* gene contains 14 individual introns (12 gt-ag, 2 gc-ag) and five exons. In addition 16 different splice variant mRNAs and 2 unspliced forms are found. There are 6 probable alternative promoters, 2 non overlapping alternative terminal exons and 9 validated alternative polyadenylation sites. Interestingly, 799-base pairs of this gene are antisense to the ubiquitin-specific peptidase-like protein 1 (USPL1), suggesting regulated alternate expression. Both *USPL1* and *HMGB1* are located on chromosome 13q12 [15] (**Figure 1**). The gene is driven by a strong TATA box-less promoter, flanked with additional regulatory elements, including a silencer located upstream and an enhancer located in the first intron of the gene [16]. Even though expression is ubiquitous, the levels of expression can vary significantly in individual cell types presumably due to regulation of its transcription [17]. HMGB1 is often overexpressed not only in cancer cells, such as carcinomas of the breast [18], cervical [19], gastrointestinal tract [20], head and neck [21], nasopharynx [22], and lymphomas [23], but also in activated endothelial cells found within the cancer tissue [24].

At the protein level, members of the HMG superfamily are typically 25 to 30 kDa in size with homologous basic domains that bind DNA, mediated by individual HMG boxes [25]. HMGB1 is composed of three domains: two positively charged domains (A and B boxes) and a negatively charged carboxyl terminus [26, 27] (**Figure 1**). Boxes A and B are DNA-binding domains. Intriguingly, the box B contains the cytokine-like activity that can induce macrophage secretion of additional proinflammatory cytokines [28], dependent on the cysteine 106 being nonoxidized and the more proximal cyste-

ines at positions 23 and 45 being oxidized as the dithiol. The protein structure involved in the binding of HMGB1 with RAGE is located between amino acid residues 150 and 183 [29]. The acidic tail at the C-terminus interacts with HMG boxes and regulates HMGB1 DNA binding specificity via a unique mechanism [30, 31].

HMGB1 exerts its key functions within the nucleus by binding (without sequence specificity) to the minor groove of DNA. This enables it to facilitate the assembly of nucleosomes on the chromatin and interact physically with DNA and a variety of molecules, including p53 [32], NF- κ B [33], and steroid hormone receptors [34], thus regulating transcription of a number of important genes (for a review, see ref# [35]). HMGB1 is also found outside of the nucleus, within the cytoplasm following any type of cellular stress [17]. Some cells express HMGB1 on the plasma membrane as well. Membrane-associated HMGB1 controls neurite outgrowth, smooth muscle cell chemotaxis, and tumor cell metastasis [36-39]. In addition, HMGB1 can be found within mitochondria [40-42] (**Figure 1**).

HMGB1 is released into the extracellular space during both sterile inflammation and infection [43, 44]. Immunocompetent cells also secrete HMGB1 following stimulation with other danger-associated molecular pattern (DAMP) and pathogen-associated molecular pattern (PAMP) molecules [45, 46]. There is a novel role for double-stranded RNA-dependent protein kinase (PKR) in inflammasome activation and HMGB1 secretion in macrophages, offering a potential new target for intervention [47]. In addition to innate immune cells, at least three studies have demonstrated that HMGB1 can be actively secreted from cancer cells including human colon cancer [11, 13] and mesothelioma [12]. HMGB1 can also be released passively from dying/stressed cancer and normal cells. Originally, it was thought that HMGB1, ATP and other inflammatory molecules were released only from necrotic, but not apoptotic cells [48]. Later studies indicated that cells dying via apoptosis could also release HMGB1 late following dissolution of an undigested cell. In a number of cancer cell lines, HMGB1 is released following treatment with chemical inducers of cell death (staurosporine, etoposide, or camptothecin), and this release can be diminished by application of the apoptosis-

inhibitor Z-VAD-fmk [49]. Thus release of HMGB1 from both apoptotic and necrotic cells can be observed [50]. Murine macrophages stimulated with lipopolysaccharide (LPS) or polyinosinic-polycytidylic acid release HMGB1 and this is correlated with the occurrence of apoptosis in these cells [51]. This observation is in part confounded by the fact that HMGB1 is actively secreted from macrophages which have taken up apoptotic cells. Autophagy also regulates selective release of HMGB1. Stressed cancer cells, in which autophagy is induced, selectively release HMGB1 without associated lysis of the cell membrane or classical necrosis [52].

Signaling pathways of HMGB1

HMGB1 can interact with other TLR ligands as well as cytokines, and activate cells through multiple cell surface receptors including TLR2, TLR4, and RAGE, functioning as a major *in vivo* sensor of tissue damage by eliciting inflammatory reactions, functioning much like a cytokine. HMGB1 binding to RAGE triggers Ras, PI3K and Rho activation, one of the major downstream signaling molecules being NF- κ B, the latter itself inducing a number of other inflammatory molecules. HMGB1 binding to TLR2/4 triggers signaling cascades with substantial overlap, activating three signaling pathways, eventually leading to NF- κ B activation. There are crosstalk and convergence between these various signaling pathways (for a review, see ref # [5, 14, 53]). These signaling cascades lead to activation of NF- κ B, and then other pro-inflammatory cytokines, leukocyte adhesion molecules and angiogenic factors in both hematopoietic and endothelial cells, thus promoting inflammation in cancer and other disease states. Negative signaling pathways can also be identified. For example, inflammatory responses to DAMPs, but not to microbial PAMPs, are repressed by the interaction of CD24 and Siglect-10 in humans or Siglec-G in mice [54, 55]. Interestingly, tumor-associated dendritic cells (DC) express on the cell surface high levels of another molecule, T cell immunoglobulin and mucin domain containing protein-3 (TIM-3) that binds HMGB1 and also inhibits the antitumor immunity induced by DNA vaccines and chemotherapy [56].

HMGB1 may form complexes with other proteins, bind to other receptors in synergy with endogenous and exogenous danger signals to

promote inflammation, and contribute to pathogenesis or effective therapy [57, 58]. In activation of human PBMC or synovial fibroblast cultures, HMGB1 on its own does not induce detectable IL-6 production. However, when the protein associates with one of several individual proinflammatory molecules (IL-1 β , TLR4 ligand LPS, the TLR9 ligand CpG-ODN, or the TLR1-TLR2 ligand Pam3CSK4) it potently enhances proinflammatory cytokine production. TLR9-dependent activation by DNA-containing immune complexes is mediated by HMGB1 and RAGE [59]. The complexes bind to TLR9 as well as to RAGE and activate pDCs and B cells in response to DNA and contribute to autoimmune pathogenesis [59]. One could take advantage of this interaction in therapeutic settings where a potent immune response is desired. Microbial CpG-DNA or its analog CpG-ODNs activate macrophages, monocytes, and DC to secrete proinflammatory cytokines, driving the Th1 response. HMGB1 can function as a CpG-ODN-binding protein, pre-associates with TLR9 in the endoplasmic reticulum-Golgi intermediate compartment, and facilitates the redistribution of TLR9 to early endosomes in response to CpG-ODN. CpG-ODN stimulates macrophages and dendritic cells to secrete HMGB1; extracellular HMGB1, in turn, accelerates the delivery of CpG-ODNs to its receptor, leading to a TLR9-dependent augmentation of IL-6, IL-12, and TNF α secretion [60], following effective recruitment of NF- κ B. Interestingly, HMGB1-nucleosome complexes activate antigen presenting cells via binding to TLR2 and may crucially contribute to the pathogenesis of systemic lupus erythematosus via breaking the immunological tolerance to against nucleosomes/dsDNA [61]. HMGB1/DNA complexes could thus also serve as attractive adjuvants in vaccine strategies for patients with cancer or other diseases.

The mechanism by which HMGB1 promotes cytokine release subsequent to its interaction with TLR4 thereby promoting cell migration is still not completely understood. Inhibitors of NF- κ B kinases α (IKK α) and β (IKK β) are important for HMGB1-elicited chemotaxis of fibroblast, innate immune cells *in vitro* and neutrophils *in vivo* [62], and may play critical roles.

DAMPs are critically regulated by oxidation with terminal oxidation by myeloperoxidases as means for extinguishing the 'danger signals'

[63-66]. Functioning as either chemoattractant or proinflammatory cytokine, HMGB1 orchestrates both processes by switching among mutually exclusive redox states [67]. Reduced cysteines make the molecule a chemoattractant, whereas a disulfide bond between the vicinal C23 and C45 cysteines makes it a proinflammatory cytokine. Further cysteine oxidation to sulfonates by reactive oxygen species abrogates both activities. In other words, HMGB1 orchestrates both key events in sterile inflammation, leukocyte recruitment and their induction to secrete inflammatory cytokines, by adopting mutually exclusive redox states. CXCL12 plays an important role in HMGB1-induced recruitment of inflammatory cells. HMGB1 and CXCL12 form a heterocomplex that acts exclusively through CXCR4 and not through other HMGB1 receptors. This heterocomplex promotes conformational rearrangements of CXCR4 from that of CXCL12 alone. Mononuclear cell recruitment *in vivo* into air pouches and injured muscles depends on the heterocomplex and is inhibited by AMD3100, an inhibitor of CXCR4 signaling, and glycyrrhizin, which directly binds HMGB1. Thus, both inflammatory cell recruitment and activation depend on HMGB1 [68].

Post-translational modifications dictate intracellular distribution and key functions of HMGB1

HMGB redistribution is controlled, in part, by one of several posttranslational modifications. These include acetylation [6], phosphorylation [69], methylation [9] and oxidation [70]. These changes modulate its structure, its localization and subsequent biological functions.

HMGB1 contains two nuclear localization signals (NLSs) for controlled nuclear transport [6] (**Figure 1**). Both acetylation and phosphorylation of the two NLSs of HMGB1 is involved in nuclear transport toward secretion. In 2003, Bianchi, Agresti and associates showed that, in all cells, HMGB1 shuttles actively between the nucleus and the cytosol. HMGB1 is acetylated extensively upon activation with LPS in monocytes and macrophages. In resting macrophages, forced hyperacetylation of the molecule leads to its translocation to the cytosol. Cytosolic HMGB1 is then concentrated by default into secretory lysosomes, and secreted

when monocytic cells receive an appropriate second signal [6]. The postsynthetic acetylation of HMGB1 protein and its truncated form significantly affects its properties as an “architectural” factor - recognition of bent DNA and binding of short DNA fragments. In a study of liver ischemia/reperfusion (I/R) injury, we found that serum HMGB1 released following liver I/R *in vivo* is acetylated, and that hepatocytes exposed to oxidative stress *in vitro* also released acetylated HMGB1. They identified the histone deacetylases-1 (HDAC1) and -4 (HDAC4) as critical in regulating acetylated HMGB1 release [71]. We subsequently found that interferon regulatory factor 1 (IRF-1) mediates acetylation and release of HMGB1 via histone acetyltransferases [72]. Phosphorylation of HMGB1 dictates the dynamic shuttling between cytoplasmic and nuclear compartments [69]. Hyperphosphorylated HMGB1 relocates to the cytoplasm. In a nuclear import assay, phosphorylated HMGB1 in the cytoplasm did not enter the nucleus. The authors mutated serine residues of either or both NLSs of HMGB1 to glutamic acid to mimic a phosphorylated state and examined the binding of HMGB1 to karyopherin- α 1, a nuclear import protein for HMGB1. Substitution of serine with glutamic acid in either NLS decreased the binding with karyopherin- α 1 by about 50%. Substitution, however, of both NLSs showed no binding, and HMGB1 was relocated to the cytoplasm and subsequently secreted. PKC- ζ phosphorylates HMGB1, and the phosphorylation of specific serine residues is related to enhanced HMGB1 secretion in colon cancer cells [13]. These studies establish the notion that phosphorylation and acetylation of the two NLS regions of the protein regulate the direction of transport of HMGB1.

HMGB1 can be modified by methylation as well. In neutrophils, HMGB1 is post-translationally mono-methylated at Lys42. The methylation alters the conformation of HMGB1 and weakens its DNA binding activity, causing it to become largely distributed in the cytoplasm by passive diffusion out of the nucleus [9]. This novel pathway may help explain the distribution of nuclear HMGB1 to the cytoplasm and is important for understanding how neutrophils release HMGB1 into the extracellular milieu [9].

HMGB1 is also regulated by reduction-oxidation (redox) reactions. Oxidative stress can induce a

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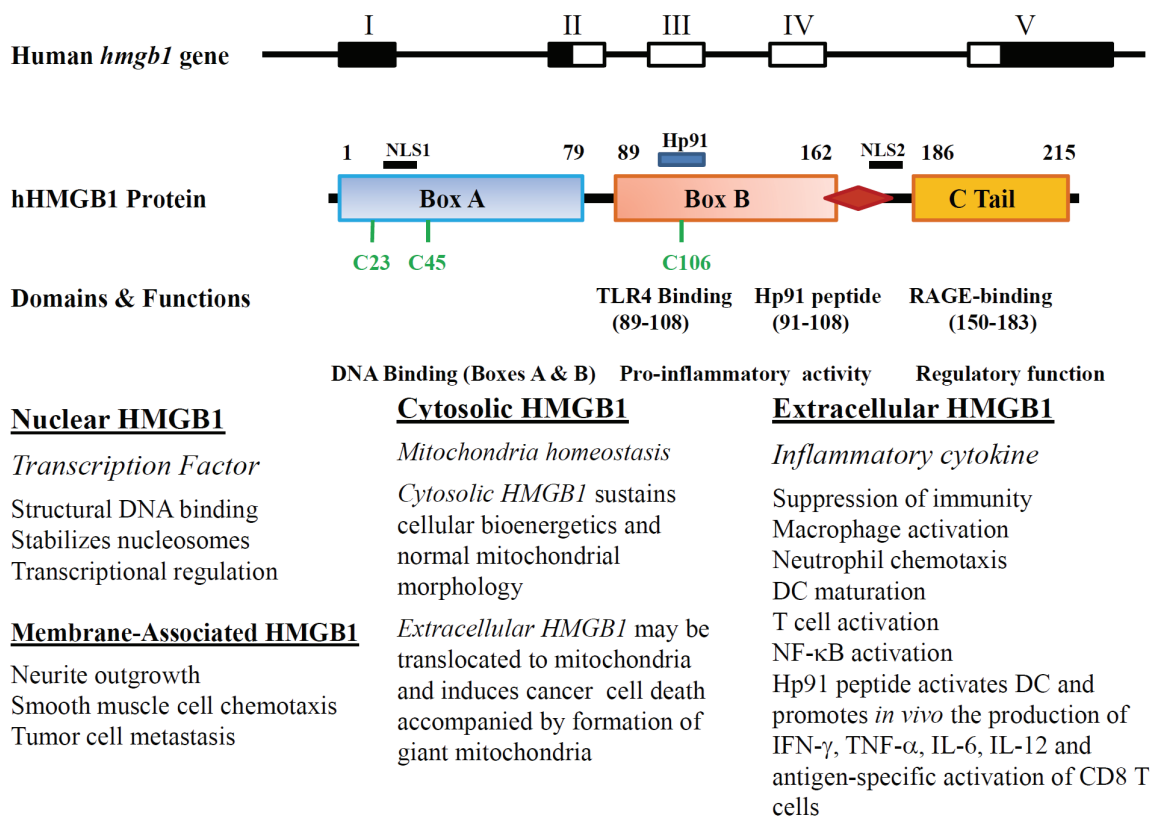


Figure 1. A schematic representation of gene structure, protein domains and their associated functions of human HMGB1. Upper Panel: The 5 exons of the gene are indicated by boxes (open for translated regions and solid for untranslated regions). Middle Panel: The human HMGB1 has 215 amino acid residues and can be divided into three domains. Box A domain at the N-terminal is required for DNA binding, Box B is also required for DNA binding, and a fragment peptide within (#80 to 123) is required for cytokine activity. Peptide #89-108 is required for TLR4 binding and peptide #150-183 for RAGE-binding. Peptide Hp91 (#91 to 108) is an immune adjuvant peptide [113]. The C tail is acidic and codes for regulatory functions. There are two nuclear localization signals (NLS1, #27-43, and NLS2, #178-184) [6]. Three cysteine residues, C23, C45 and C106 are labeled. C23 and C45 can form an intramolecular disulfide bridge, while C106 is essential for binding to TLR4. Lower Panel: Listed are main functions of four different localized forms of HMGB1. The interplay between mitochondria and two forms of HMGB1 are based on two recent studies [41, 42].

covalent disulfide bond between protein and peptide thiols that is reversible through enzymatic catalysis. HMGB1 contains three conserved cysteine (C) residues: C23 and C45 can form an intramolecular disulfide bridge, whereas C106 is unpaired and is essential for binding to TLR4 (Figure 1). The disulfide bond between C23 and C45 is a target of glutathione-dependent reduction by glutaredoxin. Cysteine targeted mutational analysis suggests that C23 and 45 in response to oxidative stress, undergo conformational changes whereas C106 appears to be critical for the nucleocytoplasmic shuttling of HMGB1 [70].

In cancer therapy with cisplatin, it is believed that HMGB1 enhances the anticancer efficacy

of cisplatin by shielding platinated DNA lesions from repair. A recent study showed that C23 and C45 in the domain A form a reversible disulfide bond under mildly oxidizing conditions. The reduced domain A protein binds to a 25-bp DNA probe containing a central 1,2-d(GpG) intrastrand cross-link, the major platinum-DNA adduct, with a 10-fold greater binding affinity than the oxidized box A domain. Thus, the cellular redox environment can influence the interaction of HMGB1 with platinated DNA and suggests that the redox state of the box A domain modulates the activity of cisplatin as an anticancer drug [73]. The C106 thiol and C23-C45 disulfide bond are required for HMGB1 to induce NF-κB nuclear translocation and TNF production in macrophages [74].

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Table 1. Redox regulation of HMGB1 and biological effects

Redox states	Biological models	Biological effects	References
Native and exclusive redox states	<ol style="list-style-type: none"> 1. Examine its function as a cytokine or chemoattractant in different redox states; 2. "Native state" of the molecule (with C106 thiol and C23-C45 disulfite bond) is required for HMGB1 to induce nuclear NF-κB translocation and TNF production in macrophages. 	<ol style="list-style-type: none"> 1. Reduced cysteines make it a chemoattractant; 2. A disulfite bond (C23-C45) makes a proinflammatory cytokine. 3. Full cysteine oxidation to sulfonates by ROS abrogates both activities. 4. Both irreversible oxidation and complete reduction of these cysteines inhibit TNF production. 	[67] [74]
Oxidation	Apoptosis: HMGB1 is predominately oxidized in apoptotic cells and from primary and secondary necrotic cells. The oxidation is caspase-dependent.	<ol style="list-style-type: none"> 1. This usually leads to immunological tolerance. 2. However, it may stimulate immune responses by scavenging or by mutating a mitochondrial caspase target protein when ROS activity was prohibited 	[50] [153]
Oxidation and reduction	Autophagy and apoptosis: Stimuli that enhance ROS promote cytosolic translocation of HMGB1 and thereby enhance autophagic flux. HMGB1 directly interacts with the autophagy protein Beclin1 displacing Bcl-2.	<ol style="list-style-type: none"> 1. Reduced HMGB1 binds RAGEs, but not to TLR4, induces Beclin1-dependent autophagy and promotes tumor resistance; 2. Oxidized HMGB1 increases the cytotoxicity of the agents and induces apoptosis mediated by the caspase-9/-3 intrinsic pathway. 	[82, 84]
Mild reduction	Regeneration and remodeling of skeletal muscle: HMGB1, weakly expressed in healthy muscles, increases during regeneration in parallel with the antioxidant response in both fibers and leukocytes.	The early antioxidant response in regenerating muscle may limit HMGB1 oxidation, thus allowing successful muscle regeneration.	[154]
Oxidation	Prolonged ischemia and upon reperfusion: Oxidation of HMGB1 takes place and may attenuate its pro-inflammatory activity.	The results confirm that post-translational oxidation of HMGB1 attenuates its pro-inflammatory activity	[75]

Oxidation of HMGB1 occurs during liver I/R injury, and leads to attenuation of its pro-inflammatory activity after its release from cells [75]. Reduced and oxidized HMGB1 have selective roles in extracellular signaling and regulation of immune responses that are mediated by signaling through individual receptors [76]. Together, these studies demonstrate that HMGB1 post-translational modifications are critical for the localization, DNA binding and pro-inflammatory activity of the protein (**Table 1**).

Relationship between autophagy, apoptosis and release of HMGB1 and immune responses

Most PAMPs and DAMPs serve as so-called 'Signal Os' that bind specific receptors to promote autophagy [77]. We and others have studied how HMGB1 and other DAMPs affects autophagy and apoptosis, and conversely how

they regulate HMGB1 release and function. Autophagy, or autophagocytosis, is the basic catabolic mechanism that involves degradation of unnecessary or dysfunctional cellular components through either extracellular secretion or degradation within lysosome [78]. In the context of disease, autophagy has been seen as an adaptive response to survival, however when excessive, can promote cell death and morbidity [79]. The balance between apoptosis and autophagy is important in tumor development and response to therapy. Often, tumor suppressors positively regulate autophagy, whereas oncoproteins inhibit autophagy. Autophagy in cancer can either suppress or promote tumor growth depending on the status of the cells [80, 81].

Endogenous HMGB1 regulates autophagy [82]. HMGB1 and p53 form a complex that regulates

the balance between tumor cell death and survival [83]. Stimuli that enhance reactive oxygen species promote cytosolic translocation of HMGB1 and thereby enhance autophagic flux. HMGB1 directly interacts with the autophagy protein Beclin1 displacing Bcl-2. Pharmacological inhibition of HMGB1 cytoplasmic translocation by agents such as ethyl pyruvate limits starvation-induced autophagy. HMGB1 is a redox-sensitive regulator of the balance between autophagy and apoptosis [84]. In cancer cells, anticancer agents enhance autophagy and apoptosis, as well as HMGB1 release. HMGB1 release may be a prosurvival signal for residual cells following various cytotoxic cancer treatments. Diminished HMGB1 leads predominantly to apoptosis and decreased autophagy in stressed cancer cells. Under these conditions, reducible HMGB1 binds to RAGE, but not to TLR4, inducing Beclin1-dependent autophagy and thereby promoting tumor resistance to a number of chemotherapeutic agents [84, 85]. In contrast, oxidized HMGB1 increases the cytotoxicity of these agents and induces apoptosis mediated by the caspase-9/-3 intrinsic pathway. HMGB1 release, as well as its redox state, thus links autophagy and apoptosis, representing a suitable target when coupled with conventional tumor treatments [84].

Autophagy also regulates release of HMGB1 [52, 86]. Autophagy modulates cell death by epidermal growth factor receptor-targeted diphtheria toxin (DT-EGF) lysis of cells. DT-EGF kills epithelial and glioblastoma tumor cells with similar efficiency but by different mechanisms that depend on whether the cells activate autophagy when treated with the drug. Dying cells in which autophagy is induced selectively release the immune modulator HMGB1 without causing disruption of the cell membrane or necrosis. Conversely, cells that are killed by DT-EGF where autophagy is blocked, activate caspases but retain HMGB1. These data suggest that it may be feasible to manipulate the immunogenicity of dying cells by increasing or decreasing autophagy [52].

Antineoplastic chemotherapies are particularly efficient when they elicit immunogenic cell death, thus provoking an anticancer immune response. Autophagy, which is often disabled in cancer, is dispensable for chemotherapy-induced cell death but required for its immunogenicity [87]. The key feature is that, when

responding to chemotherapy, autophagy-competent, but not autophagy-deficient, cancers attracted dendritic cells and T lymphocytes into the tumor bed. Suppression of autophagy inhibits the release of ATP from dying tumor cells. Conversely, inhibition of extracellular ATP-degrading enzymes increases pericellular ATP in autophagy-deficient tumors, reestablishing recruitment of immune cells, and restoring chemotherapeutic responses but only in immunocompetent hosts. Thus, autophagy is essential for the immunogenic release of ATP from dying cells, and increased extracellular ATP concentrations improve the efficacy of antineoplastic chemotherapies in short term tumor-bearing hosts when autophagy is disabled [87]. It is important to point out that this study deals with a vaccine instead of a therapeutic tumor model and that more chronic release of HMGB1 and ATP, as occurs presumably in human tumors, more likely promotes immunosuppression.

HMGB1 promotes cancer growth and development

Six biological capacities acquired during the multistep development of cancer make up the hallmarks of cancer [88]. Unfortunately, overexpression of HMGB1 is associated with each of the six hallmarks of cancer [89] (**Figure 2**). HMGB1 protein serves predominantly to promote wound healing, but when excessive can cause disease [90, 91]. In cancer progression and during treatment of cancer, HMGB1 signaling has also paradoxical dual effects. On the one hand, chronic release of HGMB1 promotes tumor growth and progression, enhances tumor neoangiogenesis, and promotes resistance to drug therapy (**Figure 2** and **3A**). On the other hand, under ideal settings such as acute release of large quantity of HMGB1 and other inflammatory molecules, it can also promote anti-tumor immunity that can promote tumor regression (**Figure 3A**). These yin-yang properties and functions make it an ideal therapeutic target in cancer therapy [92].

HMGB1 promotes tumor growth. HMGB1 is over-expressed in many types of cancer [18-23]. HMGB1 is critical for survival of cancer cells and knockdown of HMGB1 results in apoptosis in prostate and gastric cancer cells [93, 94]. Diminished HMGB1 promotes apoptosis and decreased autophagy in stressed colon and pancreatic cancer cells [84]. In hypoxic

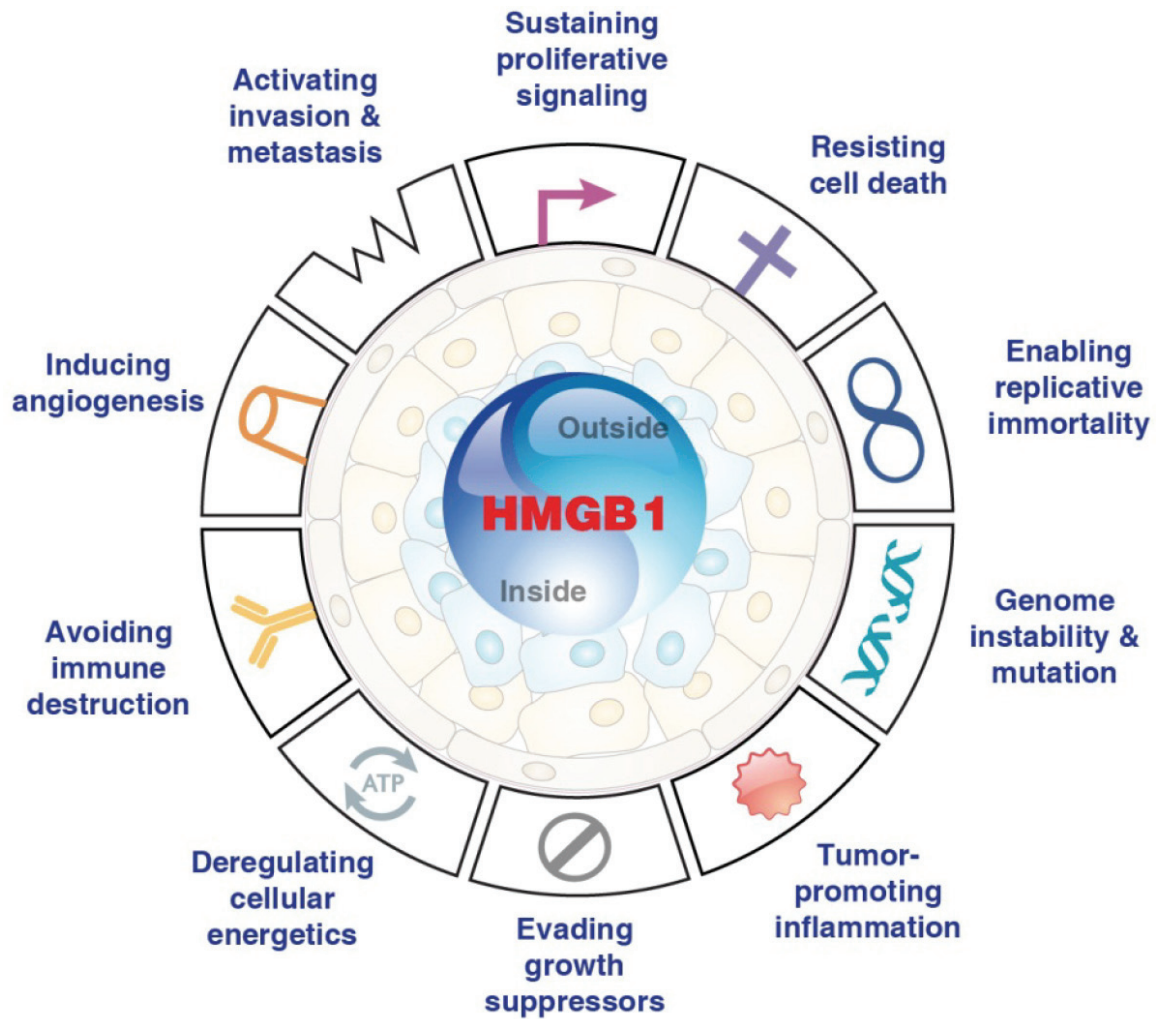


Figure 2. Cancer promoting properties of HMGB1. In cancer, HMGB1 dysfunction is associated with each of the six hallmarks of cancer the latter of which were summarized by Hanahan and Weinberg [88]. We have presented an earlier version of this association in our previous review [89].

hepatocellular carcinoma, HMGB1 activates TLR4 and RAGE-signaling pathways to induce caspase-1 activation with subsequent production of multiple inflammatory mediators. As a result, these inflammatory signals promote invasiveness and metastases [95]. In gastric cancer, HMGB1 promotes invasiveness through enhanced MMP-9 expression [94]. In a colon cancer model, HMGB1 released from necrotic cancer cells enhances regrowth and metastasis of the remaining cancer cells via RAGE activation [96]. In malignant mesothelioma, active secretion (and passive release) of HMGB1 from cancer cells supports progression of the disease [12]. The motility, survival, and anchorage-independent growth of HMGB1-secreting malignant mesothelioma cells are inhibited *in*

in vitro by treatment with monoclonal antibodies directed against HMGB1 or RAGE. Significantly, HMGB1 inhibition diminishes the growth of mesothelioma xenografts in immunodeficient mice and extends host survival. HMGB1 also promotes drug resistance in osteosarcoma [97], and leukemia [85], and this could be attributed to HMGB1-mediated autophagy [85]. In ovarian cancer, nucleus accumbens-1 (NAC1) contributes to drug resistance. Treatment with cisplatin caused an activation of autophagy in ovarian cancer cells. Regulation of autophagy by NAC1 was mediated by HMGB1, as the functional status of NAC1 was associated with the expression, translocation and release of HMGB1 [98]. Therefore, one mechanism of HMGB1-mediated drug resistance to cisplatin

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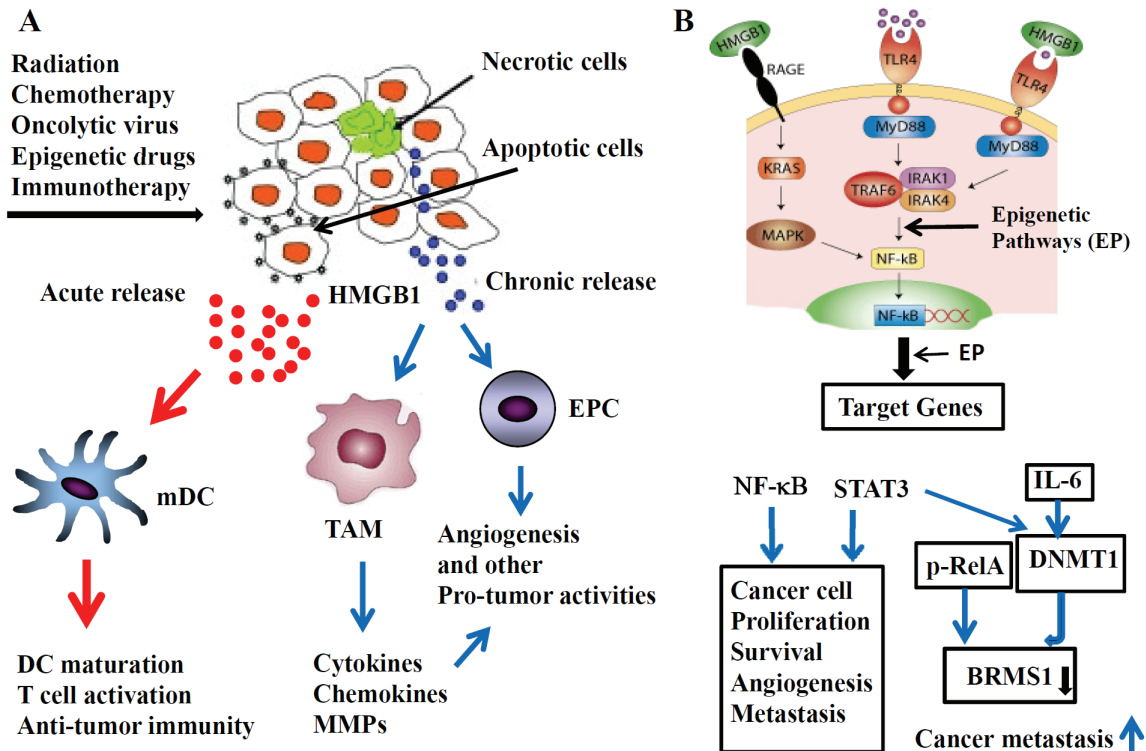


Figure 3. HMGB1 in the tumor microenvironment and hypothesis of HMGB1 functioning via NF- κ B and epigenetic pathways. A. The release of HMGB1 in the tumor microenvironment. HMGB1 has yin-yang effects on cancer. Acute release of HMGB1 after cancer therapies promotes maturation of DCs through interaction with TLR4 and clonal expansion of tumor antigen-specific T cells and thus elicits antitumor responses. In contrast, persistent hypoxia in growing tumors leads to necrosis, causing chronic release of HMGB1, which promotes angiogenesis and tumor growth through the recruitment of macrophages (TAM) and endothelial precursor cells (EPC) and activation of local endothelial cells through RAGE signaling. The acutely and chronically released HMGB1 molecules are represented in red and black, indicating that they may be differently modified by redox or other mechanisms. This figure is modified from a figure by Srikrishna and Freeze [58]. B. The hypothesis of HMGB1 acting via NF- κ B and epigenetic pathways to exert its long lasting effects on surviving tumor cells, immune cells and stromal cells in the tumor microenvironment. The NF- κ B pathway is regulated partly via epigenetic pathways (EP) both upstream and downstream. STAT3 and NF- κ B, both inflammation-regulated genes, regulate genes to promote cancer cell proliferation, survival, cancer angiogenesis and metastasis. As an example to illustrate the point of role of epigenetic mechanisms, we showed that IL-6, produced and released from macrophage stimulated by HMGB1 and modulated via epigenetic mechanisms, regulates transcription of DNMT1, one of the key enzymes for DNA methylation in cancer cells. The TNF-stimulated and phosphorylated p65 binds and recruits DNMT1 to the promoter of BRMS1 gene, thus inhibits the production of BRMS1, an inhibitor of tumor metastasis.

is via enhanced autophagy stimulated by NAC1 in ovarian cancer.

Redox regulates interaction of HMGB1 with individual receptors and its functions. Reducible HMGB1 binds to RAGE, but not to TLR4, induces Beclin1-dependent autophagy and promotes tumor resistance to various chemotherapeutic agents. In contrast, oxidized HMGB1 increases the cytotoxicity of these agents and induces apoptosis mediated by the caspase-9/-3 intrinsic pathway [84], but may have lost its pro-inflammatory activity [75].

HMGB1 can also be highly immunosuppressive, particularly in the setting of absence of

other TLR ligands. It actively affects the immune functions of many types of cells including DC, macrophages, NK cells, T lymphocytes, and regulatory T cells (Tregs) [3, 10, 99]. Recent findings suggest that the release of HMGB1 as an endogenous danger signal is important for priming an adaptive immune response that promotes malignant progression [100]. Tumor-associated DCs express on the cell surface high levels of TIM-3 that binds HMGB1, thereby inhibiting the antitumor immunity elicited by DNA vaccines and chemotherapy [56]. HMGB1 suppresses cytokine secretion and maturation of plasmacytoid DCs in response to TLR9 agonists including the hypomethylated oligodeoxy-

nucleotide CpG- and DNA-containing viruses. HMGB1 inhibits secretion of several proinflammatory cytokines including IFN- α , IL-6, TNF- α , inducible protein-10, and IL-12 from pDC [101]. Knockdown of HMGB1 in tumor cells attenuates their ability to induce Tregs and leads to naturally acquired CD8 T cell- or IFN- γ -dependent tumor rejection [99]. HMGB1 directly enhances immune inhibitory functions of Treg via RAGE-mediated mechanisms and limits the number and activity of Teff. HMGB1 effects on Treg may alter immune reactivity in the setting of chronic inflammatory states such as cancer [21, 102].

HMGB1 during cancer therapy

Essentially all therapeutic modalities inducing necrosis and/or apoptosis of cancer cells would lead to release of HMGB1 from cancer cells, and subsequent secretion of HMGB1 from activated immune cells. These would include, but not limit to, most types of chemotherapy, radiation, and immunotherapy. The release of HMGB1 has been demonstrated with killing of cancer cells using cytolytic cells such as NK and T cells *in vitro* [103], in prodrug-suicide gene therapy [104], oncolytic virotherapy [105], and epigenetic therapy [49].

The beneficial effects of HMGB1 in cytotoxic therapeutic regimens

HMGB1 sensitizes cells to cisplatin-mediated cytotoxicity by shielding its major DNA adducts from nucleotide excision repair. Treatment of steroid hormone receptors-expressing human cancer cells with the either estrogen and/or progesterone, significantly increases the potency of cisplatin and its analogue carboplatin by causing overexpression of HMGB1 [106]. Binding of HMGB1 to DNA (chromatin), could be modulated by the redox state of HMGB1. Two cysteine residues in the HMGB1 A box domain form a reversible disulfide bond under mildly oxidizing conditions. The reduced domain A protein binds to a 25-bp DNA probe containing a central 1,2-d(GpG) intrastrand cross-link, the major platinum DNA adduct, with a 10-fold greater binding affinity than the oxidized domain A. Thus, the cellular redox environment can influence the interaction of HMGB1 with the platinated DNA and suggest that the redox state of the box A domain of HMGB1 could modulate the potency of cisplatin as an anti-

cancer drug [73]. In summary, these findings suggest that the proper combination of drugs and hormones could have potential benefit in treating hormone receptor-expressing ovarian, prostate, or breast cancers [107]. Similarly a role for HMGB1 in the deeper understanding of Vitamin D biology and therapy is in order, not just for microbial infections but also in cancer as another steroid hormone important in its biology [108].

HMGB1 plays an important role in eliciting innate and adaptive anti-tumor immunity [3, 44]. The emerging concept that cancer is not just a disease of a tissue or an organ but also a host disease relies on evidence of tumor-induced immunosuppression and polymorphisms in genes involved in host protection against tumors. Many therapeutic effects require the immunoadjuvant effect of tumor cell death induced by cytotoxic anticancer agents. Zitvogel and colleagues were among the first to explore the role of immunity in conjunction with anticancer chemotherapy and radiotherapy [107]. Release of HMGB1 by dying tumor cells is mandatory to license host DCs to process and present tumor antigens. HMGB1 interacts with TLR4 on DCs, which are selectively involved in the cross-priming of anti-tumor T lymphocytes *in vivo*. A TLR4 polymorphism that limits the binding of HMGB1 to TLR4 predicts early relapse following anthracycline-based chemotherapy in breast cancer patients [107]. In a recent study, the significance of HMGB1 release and induction anti-tumor immunity was analyzed in patients with esophageal squamous cell carcinoma (ESCC) receiving chemoradiation. The authors found that chemoradiation induces tumor antigen-specific T-cell responses, and HMGB1 production is related to clinical outcome after chemoradiation [109].

DNA alkylating agents induce sporadic cell necrosis and regression of apoptosis-deficient tumors [93]. Sporadic tumor cell necrosis is associated with extracellular release of intercellular contents including HMGB1 and subsequent recruitment of innate immune cells into tumor tissues. Although DNA alkylating therapy led to a complete tumor regression in an athymic mouse tumor xenograft model, it failed to do so in tumors deficient in HMGB1. The HMGB1-deficient tumors have an impaired ability to recruit innate immune cells including

macrophages, neutrophils, and NK cells into the treated tumor tissue [93]. Suppression of innate immunity and HMGB1 using depleting Abs limits tumor regression in response to these therapies.

The epigenetic modulator apicidin has been applied in combination with the cytotoxic agent docetaxel in tumor breast cell lines characterized by different grades of invasiveness [110]. Combined treatment with apicidin and docetaxel, at minimally toxic doses, stimulates in metastatic breast cancer cells the expression of CTCF-like protein and other cancer antigens, thus favoring an antitumor immune response. Importantly, following combined exposure to these agents, metastatic cells express calreticulin on the cell surface and release considerable amounts of HMGB1, thus promoting the translation of induced cell death into effective antitumor immune responses [110].

HMGB1 mediates endogenous TLR2 activation and leads to brain tumor regression in a glioblastoma multiforme model [111]. Combined immunotherapy/conditional cytotoxic approaches that utilize adenoviral vectors (Ad) expressing Fms-like tyrosine kinase 3 ligand (Flt3L) and thymidine kinase delivered into the tumor mass lead to secretion of HMGB1. This in turn is specifically recognized by its receptor TLR2 on myeloid dendritic cells (MDC). The presence of HMGB1 attracts and activates the MDC within the brain which, in turn, activates T lymphocytes. The HMGB1-TLR2 axis signaling pathway thus activates anti-tumor T cell immunity and leads to brain tumor regression. Another important function of HMGB1 in immune response is that it is an endogenous immune adjuvant [112-115]. Necrotic HMGB1(-/-) cells have a reduced ability to activate APCs, and HMGB1 blockade reduces the activation induced by necrotic wild-type cell supernatants. *In vivo*, HMGB1 enhances the primary antibody responses to soluble antigens and transforms poorly immunogenic apoptotic lymphoma cells into efficient vaccines [112]. A short peptide derived from HMGB1 potentiates cellular immune responses to peptide antigen and cellular and humoral immune responses to protein antigen *in vivo*. The short peptide promotes *in vivo* production of the immunomodulatory cytokines, IFN- γ , TNF- α , IL-6, and IL-12 (p70), as well as antigen-specific activation of CD8⁺ T cells [113, 114]. HMGB1 adjuvant properties enhance the adaptive effector and memory

immune responses against influenza virus infection [115]. Together, these results suggest that HMGB1 could function as an adjuvant in the context of cancer vaccine to promote potent anti-cancer immunity.

HMGB1 in treatment with epigenetic drugs

Epigenetic therapy of cancer has shown very promising results in both preclinical and clinical studies [116, 117]. So far four epigenetic drugs, including inhibitors of DNA methyltransferases (DNMT) and histone deacetylases (HDAC), have been approved by the FDA. They are used to treat myelodysplastic syndromes, a set of bone marrow conditions that often progress into terminal leukemias, or cutaneous T cell lymphomas. The use of epigenetic drugs to treat solid cancers has been actively pursued with limited results. Extracellular release of HMGB1 during nominal 'apoptotic' death has been found [49]. The HDAC inhibitor trichostatin A causes biochemical changes that promote enhanced release of HMGB1 from apoptotic cells [118]. We have applied DNMT and HDAC inhibitors to study induction of cancer/testis antigens and a transcription factor *Rhox5* in cancer cells as a novel form of immunotherapy [119-121]. Epigenetic drugs such as HDAC inhibitors induce apoptosis and autophagy in cancer cells [120, 122], which likely leads to the release of HMGB1. HMGB1 is released into the extracellular milieu from cancer cells treated with decitabine (Guo ZS et al., unpublished data).

HMGB1 is involved in epigenetic silencing of tumor necrosis factor alpha (TNF- α) and interleukin 1 beta (IL-1 β) transcription in blood leukocytes of animals and humans after the initiation of severe systemic inflammation (SSI). In the case of endotoxin tolerance, HMGB1 and linker histone H1 couple as a component of the epigenetic complex that silences acute proinflammatory TNF- α during the assembly of heterochromatin in the severe systemic inflammation (SSI) phenotype. Depletion of HMGB1 results in dissociation of RelB, one of the five NF- κ B proteins, from the promoter and partially restores TNF- α transcription [123].

HMGB1 in treatments with oncolytic viruses

Oncolytic virotherapy for cancer has shown great promise in both preclinical and clinical studies [124, 125]. The death of cancer cells infected with oncolytic virus (OV) results from

direct oncolysis (a mixture of apoptosis and necroptosis), and necrotic death induced indirectly by the effects of anti-angiogenesis and hypoxia induced by the virus, or by anti-cancer immunity elicited by the viruses and the virus-infected cells and cytokines. As a result, the biologically active HMGB1 is released from dying cancer and endothelial cells or actively secreted from activated immune cells into the cancer tissue milieu.

Our group first reported that cancer cells infected by an oncolytic virus, in this case vaccinia virus, were induced into apoptotic/necrotic death pathways and released HMGB1 from the dying cells into the extracellular milieu [105]. Subsequently, other investigators have also observed the release of HMGB1 as well as other inflammatory signals from cancer cells infected with other OVs, such as an oncolytic adenovirus (Ad5/3-hTERT-E1A-hCD40L) [118], an oncolytic measles virus from infected melanoma cells [126], a herpes simplex virus type -2 (HSV-2) from human endometrial cancer cells [127], and with a coxsackievirus B3 [128]. Together, these studies strengthen the notion that OV exert antitumor effects through multiple mechanisms including oncolysis (necrosis), apoptosis, release of HMGB1 and other inflammatory cytokines, and induction of innate immunity and anti-tumor T cell response.

HMGB1 interacts physically and functionally with a number of viruses including OVs: 1) exogenous HMGB1 inhibits replication of human immunodeficiency virus in monocyte-derived macrophages [129]; 2) HMGB1 binds to the influenza virus nucleoprotein and promotes replication of this virus [130]; and 3) HMGB1 also regulates dengue virus (DENV) infection in human DCs [131]. DENV infection causes translocation of nuclear HMGB1 into the cytosol and secretion into the extracellular milieu. When DENV-infected DCs are co-cultured with autologous T cells, there is increased production of HMGB1 by both cell types. HMGB1 regulated TNF- α , IL-6, IL-8 and IFN- α secretion in DENV-infected DCs. As would be expected, elevated level of HMGB1 lowers the rate of DENV replication in DCs [131]. Thus, the released HMGB1 may modulate the replication and the efficacy of various OVs depending on the specific virus.

Oncolytic virotherapy itself is a form of immunotherapy [132, 133]. In most cases, the positive

immune effects of OV are mediated by the pro-immune environment created in part by the release of immunogenic molecules, such as HMGB1, heat shock protein and IFNs. Thus, it is logical to combine OV with other forms of immunotherapy in order to further enhance its efficacy. For example, one recent study demonstrated improved therapeutic outcomes when combining an OV with a potent agonist antibody specific for the costimulatory molecule 4-1BB [134]. It is interesting to note that some most promising OVs are Ad, HSV or VV armed with GM-CSF, another immunostimulatory molecule. The HSV version, called OncoVEX GM-CSF, has shown good efficacy in cancer patients with an approximately 30% response rate against systemic disease, following local injection into accessible tumors. OncoVEX GM-CSF is currently completing a pivotal phase III trial in melanoma, and a phase III trial in head and neck cancer is also underway [135]. HMGB1 and other pro-inflammatory molecules may work in concert with GM-CSF to elicit potent anti-cancer immunity which may account for significant better clinical outcomes. Another combination strategy has been to combine OV with chemotherapeutic agents. Synergistic anti-tumor effects between oncolytic vaccinia virus and paclitaxel are mediated by IFN and HMGB1 released from the infected cells [136].

Hypothesis: HMGB1 functions via epigenetic pathways

We hypothesize that HMGB1 plays a significant role via epigenetic pathways in shaping the surviving cancer cells and immune cells within the emergent tumor microenvironment arising in the setting of chronic inflammation (**Figure 3B**). During cancer progression and treatment, the interplay between environment and epigenetics directs distinct inflammatory responses [137]. Cancer evolution at all stages is driven by both epigenetic abnormalities as well as genetic alterations. Alterations in DNA methylation have been observed during inflammation and inflammation-associated carcinogenesis [138]. Enzymes involved in both acetylation and methylation of histones are involved in inflammatory responses and in immunity [139, 140].

One of the key molecules activated by HMGB1 signaling cascades, and a number of other TLR-ligand-initiated signaling, is the activation and nuclear localization of NF- κ B [14, 53]. The

NF- κ B/Rel family includes NF- κ B1 (p50/p105), NF- κ B2 (p52/p100), RelA (p65), RelB, and c-Rel. NF- κ B is well connected both upstream and downstream with a number of key factors of the epigenetic pathways in cancer and immune responses [141-143]. Some recent progress further strengthens this critical connection. Constitutive NF- κ B activation has causative roles in adult T cell leukemia (ATL) caused by HTLV-1 and other cancers. An epigenetic pathway is involved in Polycomb-mediated miRNA silencing and NF- κ B activation [144]. MiR-31 negatively regulates the noncanonical NF- κ B pathway by targeting NF- κ B inducing kinase. In ATL cells, miR-31 level is epigenetically regulated, and aberrant upregulation of Polycomb proteins contribute to miR-31 downregulation in an epigenetic fashion, leading to activation of NF- κ B and apoptosis resistance. Inflammation may promote metastatic disease via NF- κ B and epigenetic pathways. In response to TNF in the tumor milieu, NF- κ B member p65 is phosphorylated at S276 and the phosphorylated p65 recruits DNA methyltransferase 1 (DNMT-1) to the promoter and represses transcription of the tumor metastasis suppressor gene BRMS1 [145]. p65 directly recruits DNMT-1 to chromatin, resulting in promoter-specific DNA methylation and transcriptional repression of BRMS1. This highlights a new mechanism through which the inflammation-induced NF- κ B can regulate metastatic disease. A number of TLR4-regulated proinflammatory genes in macrophages, also targets of HMGB1, are controlled by regulated trimethylation and demethylation of histone H4K20 located in the promoters. Signal-dependent erasure of H4K20me3 is required for effective gene activation and is achieved by NF- κ B-dependent delivery of the histone demethylase PHF2 [146]. NF- κ B modulates IL-6 via epigenetic pathways, which in turn affects other genes [142, 147]. IL-6 and STAT3, another key inflammation-triggered signaling molecule, modulate transcription of DNMT1 in leukemia and cancer cells [148, 149]. These studies clearly link epigenetic pathways with inflammation-activated NF- κ B-mediated gene regulation and directly contribute to disturbed cancer biology. As for immune cells, epigenetic processes and NF- κ B contribute to the development and activation of a variety of immune cells [150, 151]. In addition, a number of cytokine genes in immune cells are regulated through epigenetic pathways, and accompany

the activation or suppression of immune cells. The findings of these and other studies suggest that epigenetic programs from HMGB1 to NF- κ B and further downstream oncogenic signaling play important roles in cancer initiation and progression. Thus, both HMGB1 and NF- κ B offer ideal targets for interventions in cancer therapeutics. In this regard, it is interesting to note that both HMGB1 and NF- κ B play 'yin-yang' roles in cancer and immunity.

Conclusions and perspectives

The majority of therapeutic regimens for cancer lead to release of HMGB1. Therefore, how to best utilize the released HMGB1 in combination therapy is one of the major questions we have addressed. In the setting of cancer arising over several years, the major role of HMGB1 is to promote a wound healing response and limit the effectiveness of immune effectors. Still, immune reactivity is a dynamic process and means to limit myeloid derived suppressor cells and Tregs in the setting of oncolytic therapy and enhancement of immune reactivity, perhaps by targeting HMGB1 cofactors or receptors is in order.

Following acute cytotoxic treatments including chemotherapy, radiation therapy, epigenetic drugs, oncolytic viruses, and immunotherapy, stressed/dying cancer cells often release HMGB1, ATP and other inflammatory molecules [152]. At the same time, activated macrophages/monocytes and other innate immune cells actively secrete HMGB1 and other inflammatory cytokines into the tumor milieu. These events could be potent signals to prime for potent anti-tumor immune responses. Any rational combinatorial therapeutic regimen should take advantage of the potential synergy of HMGB1 signaling. (1) acute release of HMGB1 to stimulate innate and adaptive immunity against cancer; and/or (2) the effect of HMGB1 to sensitize the cancer cells to certain type of chemotherapy (such as cisplatin) or other therapeutic regimens. This is a promising new direction for treatment of patients with cancer.

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Conflict of interest statement

None.

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