

# Convergence and amplification of toll-like receptor (TLR) and receptor for advanced glycation end products (RAGE) signaling pathways via high mobility group B1 (HMGB1)

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**Abstract** Sustained proinflammatory responses in rheumatoid arthritis, atherosclerosis, and diabetic retinopathy, as well as in cancer, are often associated with increased angiogenesis that contributes to tissue disruption and disease progression. High mobility group B1 (HMGB1) has been recognized as a proinflammatory cytokine and more recently, as a proangiogenic factor. HMGB1 can either be passively released from necrotic cells or actively secreted in response to angiogenic and inflammatory signals. HMGB1 itself may signal through the receptor for advanced glycation end products (RAGE), and via toll-like receptors, TLR2 and TLR4. Activation of these receptors results in the activation of NF $\kappa$ B, which induces the upregulation of leukocyte adhesion molecules and the production of proinflammatory cytokines and angiogenic factors in both hematopoietic and endothelial cells, thereby promoting inflammation. Interestingly, HMGB1 seems to be involved in a positive feedback mechanism, that may help to sustain inflammation and angiogenesis in several pathological conditions, thereby contributing to disease progression. Endothelial cells express HMGB1, as well as the receptors RAGE, TLR2, and TLR4, and in diverse pathologies HMGB1 and its receptors are overexpressed. Furthermore, HMGB1-induced signaling can activate

NF $\kappa$ B, which can subsequently induce the expression of HMGB1 receptors. Thus, HMGB1 can mediate amplification of inflammation and angiogenesis through increased secretion of HMGB1 and increased expression of the receptors it can interact with. In this review, we discuss signaling cascades that HMGB1 can induce via TLRs and RAGE, as well as its contribution to pathologies involving endothelial cells.

**Keywords** HMGB1 · Angiogenesis · RAGE · TLR

## Abbreviations

EC	Endothelial cell
HMGB1	High mobility group B1
RAGE	Receptor for advanced glycation end products
TLR	Toll-like receptor
NF $\kappa$ B	Nuclear factor $\kappa$ B
VEGF	Vascular endothelial growth factor
LPS	Lipopolysaccharide
TNF $\alpha$	Tumor necrosis factor $\alpha$
IL-8	Interleukin-8

## Introduction

Angiogenesis contributes to the progression of diverse diseases, including cancer, rheumatoid arthritis, and diabetic retinopathy. Therapies aimed at reducing unwanted angiogenesis will prove beneficial in treating these diseases; however, for maximal efficiency and minimal side effects, therapeutics targeted at key players in these processes are required.

In the past, diverse efforts have been undertaken to identify molecules upregulated during tumor angiogenesis and that can be used as targets for therapy [1]. We have

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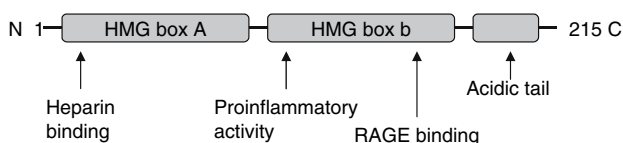
recently identified high mobility group B1 (HMGB1) as a gene overexpressed in endothelial cells in tumors compared to normal endothelial cells [2]. HMGB1 was first identified as a non-histone chromosomal protein involved in DNA binding [3]. A few years later, it was isolated as a heparin-binding protein that promotes neurite outgrowth in rat brain and was called amphoterin [4]. In 1999, HMGB1 was recognized as a proinflammatory cytokine that mediates endotoxin lethality in mice [5]. Subsequently, its cytokine actions on hematopoietic and endothelial cells became subject to investigation [6, 7], but only recently has its involvement in angiogenesis been demonstrated [8, 9].

In this review, we will summarize pathways relating to angiogenesis and inflammation that HMGB1 is involved in, and we will discuss its contribution to angiogenesis in diverse pathologies.

## HMGB1

HMGB1 protein has a dipolar structure and is subdivided in two homologous HMG boxes, A and B, each about 75 amino acids in length, and contains an acidic C-terminal tail. The N-terminus contains heparin-binding motifs whereas the B-box confers proinflammatory activity to the protein and is involved in binding to the receptor for advanced glycation end products (RAGE) [10] (Fig. 1).

HMGB1 is a nuclear protein that is highly conserved during evolution and is present in most eukaryotic cells where it stabilizes nucleosome formation and facilitates transcription [11–13]. However, diverse studies have reported HMGB1 to function also extracellularly [4, 5, 14–16], though it lacks a classical secretion signal. Necrotic cell death can result in passive leakage of HMGB1 from the cell as the protein is then no longer bound to DNA [17]. In addition, HMGB1 can be actively secreted by different cell types, including activated monocytes and macrophages [5, 18], mature dendritic cells [12], NK cells [19], and endothelial cells [15]. Diverse post-translational modifications including methylation [20], acetylation [18], and phosphorylation [21] have been associated with increased cytoplasmic localization and secretion of HMGB1 through interference with the nuclear localization signal of the protein. HMGB1 has been suggested to function as a damage associated molecular pattern molecule (DAMP) or as an alarmin, molecules that when released extracellularly



**Fig. 1** Domain structure of HMGB1 protein

function as a signal of tissue damage [22]. Indeed, during sepsis the serum levels of HMGB1 are markedly elevated [5].

In addition to being secreted, HMGB1 has also been described as a membrane-bound protein that promotes neurite outgrowth [4] and localizes at the advancing plasma membrane and filopodia of various migrating tumor cell lines [14, 23]. At the membrane, HMGB1 can bind plasminogen and tissue plasminogen activator (tPA) to generate active plasmin and matrix metalloproteinases (MMPs) that facilitate migration by degrading extracellular matrix components [24, 25]. Thus, HMGB1 can exert different functions, depending on its cellular localization.

## Cytokine function of HMGB1

In 1999, the cytokine function of the presumed nuclear protein HMGB1 was first appreciated. In an attempt to identify late mediators of endotoxemia and sepsis, HMGB1 was identified as an inflammatory mediator secreted from macrophages. Serum levels of HMGB1 increased markedly several hours after the initial TNF $\alpha$  peak in LPS-challenged mice. Moreover, antibodies against HMGB1 conferred protection against endotoxemia and sepsis [5].

Proinflammatory stimulation of hematopoietic cells, as well as endothelial cells, results in the secretion of HMGB1 which in turn functions to sustain a proinflammatory state [5, 6, 15]. HMGB1 itself acts as a proinflammatory stimulus to activate monocytes and endothelial cells to release proinflammatory cytokines [7, 26]. TNF $\alpha$  and LPS are potent stimulators of HMGB1 release from HUVEC [15]. The release of HMGB1 by endothelial cells may function to attract inflammatory cells to the site of tissue damage. Indeed, HMGB1 was shown to mediate migration of monocytes [18]. Stimulation of endothelial cells with HMGB1 resulted in the secretion of diverse proinflammatory cytokines, including G-CSF, IL-8, TNF $\alpha$  and MCP1 [7, 26], thus creating a positive feedback loop. Furthermore, the expression of adhesion molecules including ICAM-1, VCAM-1, and E-selectin were increased after stimulation of endothelial cells with HMGB1 [7, 15, 26], which contributes to the local invasion of leukocytes and sustains the inflammatory response.

## HMGB1 in angiogenesis

Recently, HMGB1 has been recognized as a putative pro-angiogenic factor [2, 9]. As such, it may act both directly and indirectly. Activation of macrophages by proinflammatory factors, including HMGB1, can result in the production of angiogenic factors such as VEGF. However,

HMGB1 itself was demonstrated to directly induce sprouting of endothelial cell spheroids in a collagen gel [9]. Furthermore, in vitro, HMGB1 stimulated endothelial cell proliferation, chemotaxis, and repair of a wounded monolayer [8]. Thus, in addition to inflammatory activation of endothelial cells, HMGB1 also increases the angiogenic properties of endothelial cells.

Another interesting role of HMGB1 in angiogenesis is its ability to attract endothelial progenitor cells and hematopoietic stem cells to sites of tissue injury and tumors to improve neovascularization [27, 28]. Hence, HMGB1 also directly acts on progenitor cells that contribute to neovascularization.

### RAGE signaling

RAGE is a multiligand receptor of the immunoglobulin superfamily and involved in homeostasis, development and inflammation, and is one of the primary receptors for HMGB1 [29]. RAGE interacts with various ligands, including HMGB1, AGE (advanced glycation end products),  $\beta$ -amyloids, and S100 proteins [10]. RAGE expression is detected on monocytes, macrophages, neurons, and endothelial cells, as well as on a variety of tumor cells [25, 30]. Ligand binding by RAGE can activate two major pathways, one encompassing CDC42/Rac and the other diverse MAPKs that finally lead to NF $\kappa$ B-dependent transcriptional activity (Fig. 2).

HMGB1 has been involved in the migratory phenotype of neurites and cancer cells [25, 31], in which the CDC42/Rac1 pathway is involved. Activation of RAGE by HMGB1 leads to activation of CDC42 and Rac1, giving rise to cytoskeletal changes. Furthermore, stimulation of RAGE induced NF $\kappa$ B activation measured by reporter gene expression. Expression of dominant-negative RAGE eliminated both NF $\kappa$ B activation and neurite outgrowth. Dominant-negative (dn)CDC42 and dnRac1 but not dnRas inhibited neurite outgrowth induced by HMGB1. However, dnRas but not dnCDC42 and dnRac1 inhibited NF $\kappa$ B activation [31]. Thus, HMGB1-induced activation of RAGE was responsible for both NF $\kappa$ B activation and neurite outgrowth, though via separate signaling pathways.

Alternatively, RAGE can signal through the activation of p38 MAPK and Erk1/2 [10, 25], finally leading to the phosphorylation and degradation of I $\kappa$ B, and NF $\kappa$ B-mediated activation of gene expression. Interestingly, the presence of NF $\kappa$ B binding sites in the RAGE promoter creates a positive feedback loop through increasing RAGE expression [32]. As such, HMGB1-mediated stimulation of RAGE may amplify this response by upregulating RAGE expression. Typical NF $\kappa$ B responsive genes are involved in

inflammation, such as adhesion molecules (e.g., ICAM-1, VCAM-1) and cytokines (e.g., TNF $\alpha$ , IL1 $\beta$ , and IL8) (Fig. 2) [7, 33]. Thus, HMGB1 activation of RAGE can both initiate and sustain a proinflammatory phenotype.

### Toll-like receptor signaling

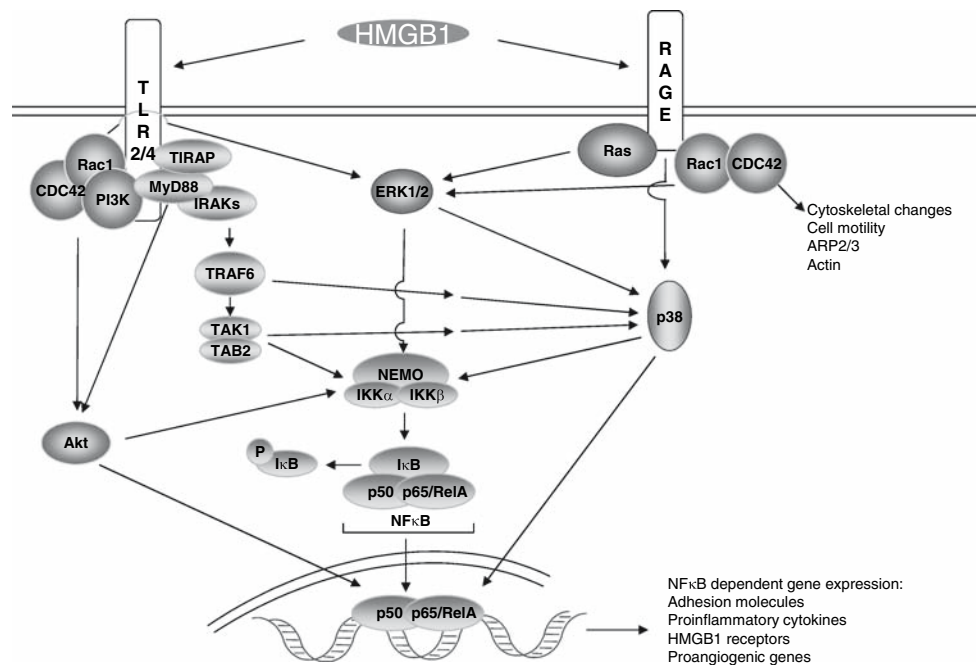
Though RAGE was first identified as the receptor for HMGB1, RAGE alone could not explain all the observed effects of HMGB1, suggesting additional receptors for HMGB1 must exist. Subsequently, toll-like receptors 2 (TLR2) and TLR4 have been identified to be involved in HMGB1 signaling [34–36].

Toll-like receptors are a family of evolutionary conserved proteins that enable cells of the innate immune system to respond to microbial structures and endogenous danger molecules. TLR signaling may require co-receptors and/or oligomerization for proper signal transduction [37]. Endothelial cells express different TLRs, depending on their vascular bed [38]. TLR4 is the main receptor for endotoxin, whereas TLR2 responds to Gram-positive components and fungi [39]. Below we summarize the signaling initiated by ligation of TLR2 and TLR4, and the possible roles for HMGB1 therein.

#### TLR2

The endothelium is not a passive vascular lining; in contrast, it has major functions in the regulation of vascular tone, coagulation, immune function, and inflammatory responses. As such, the endothelial cells are targets for blood-borne pathogens and inflammatory cytokines. The activation of the endothelium in response to microbial agents is mediated by TLRs. TLR2 is present in arterial endothelial cells, as well as in other cells of the hematopoietic lineage. TLR2 signaling can be mediated by different pathways that result in the activation and nuclear translocation of NF $\kappa$ B (Fig. 2).

Involvement of Rac1 and PI3K in TLR2 signaling was demonstrated by stimulation of monocytes with recombinant fimbriin (rFimA) from *Porphyromonas gingivalis*. Upon TLR2 stimulation with rFimA, active Rac1 and PI3K were recruited to the cytoplasmic domain of the TLR for transduction of the signal. This subsequently resulted in an increased CD11b/CD18-dependent adhesion to ICAM-1. Expression of dominant-negative variants of PI3K and Rac1 inhibited NF $\kappa$ B reporter gene expression up to 80% and adhesion to ICAM-1, implicating an important role for PI3K and Rac1 proteins in TLR2 signaling. Subsequently, Akt is activated that leads to NF $\kappa$ B activation, either by directly affecting the p65 transcriptional complex or via



**Fig. 2** Convergence of TLR and RAGE signaling. Schematic representation of signaling events mediated by TLR and RAGE receptor activation. TLRs can signal via MyD88, IRAK, and TRAF to NFκB, as well as via Rac1 and PI3K, and possibly also via ERK1/2 and p38 MAPK. RAGE can also activate the Rac1 and CDC42, as

well as Ras and p38. The common signaling pathway involves activation of NFκB to induce gene expression. The relative contribution of the different receptors and ligands is likely to differ between cell types

IKK [40] (Fig. 2). Thus, TLR2 signaling is mediated by Rac1 and PI3K, though a regulatory role for CDC42, frequently found in complex with Rac1 and PI3K, was not demonstrated [40] (Fig. 2).

As the cytoplasmic domains of TLRs and IL1-receptors are homologous, several signaling mediators play a role in both IL1R and TLR2 activation. Adaptor proteins such as myeloid differentiation primary response protein 88 (MyD88) and TIR-domain containing adaptor protein (TIRAP) are bound to this homologous TIR (Toll/IL-1 receptor) domain of TLRs [41]. An early event after TLR2 activation and receptor oligomerization is the recruitment of IL-1R associated kinase 1 (IRAK1) [41, 42]. Truncated TLR2 failed to recruit IRAK1 and abrogated NFκB activation, suggesting oligomerization of the receptor is necessary for further signal transduction [42]. Furthermore, TLR2-mediated secretion of TNFα in macrophages was inhibited by the expression of dominant-negative (dn)MyD88 and several proteins downstream thereof, pointing to a MyD88 dependent pathway induced by TLR2 that culminates in NFκB activation [41]. In addition to dominant-negative MyD88, dnIRAK1 and dnTRAF6 (TNF-receptor associated factor 6) also attenuated NFκB activation after TLR2 activation, pointing to the involvement of these proteins in TLR2 signaling to NFκB (Fig. 2). Activation of NFκB was not blocked by dnIRAK2, dnTRAF2 and dnRIP [42, 43].

Interestingly, at several levels the TLR-induced MyD88 signaling cascade may cross-talk directly with p38 MAPK signaling (Fig. 2) [44, 45]. Indeed, very recently, this third pathway of TLR2 action was described in neutrophils [46]. Stimulation of neutrophils with the TLR2 agonist P(3)CSK(4) resulted in the activation of p38 MAPK and ERK1/2. Pharmacological inhibition of ERK1/2 but not p38 MAPK inhibited TLR2 induced migration, suggesting ERK1/2 acts upstream of p38 MAPK in TLR2 signaling (Fig. 2) [46].

The above-described studies used TLR2 agonists other than HMGB1. However, a direct interaction of HMGB1 with TLR2 has recently been demonstrated by fluorescence resonance energy transfer (FRET) and immunoprecipitation in RAW264.7 macrophages [34]. Furthermore, HMGB1 stimulation increased TLR2 (and TLR4) mediated NFκB activation in HEK-293 cells [34]. Expression of dominant-negative TLR2 in RAW264.7 cells inhibited HMGB1 induced NFκB driven luciferase activity [35]. In addition, expression of dominant-negative forms of the downstream TLR signaling regulators MyD88, TIRAP, IRAK1, IRAK2, TAK1 (TGF-β activated kinase 1), TAB2 (Tak 1 binding protein 2), TRAF6, p38, and IRAK4 also inhibited HMGB1 induced NFκB activity [35]. These findings were further substantiated by the observation that HMGB1-mediated TNFα release from macrophages of MyD88  $-/-$ , TLR2  $-/-$ , and TLR4  $-/-$  mice was

compromised in comparison to wild-type mice [36]. Interestingly, anti-TLR2 antibodies were capable of inhibiting HMGB1 induced IL-8 and TNF $\alpha$  secretion in several cell lines [36]. Thus, activation of TLR2 by HMGB1 can induce three major signaling pathways, that may cross-talk at several levels, but all culminate in the activation of NF $\kappa$ B (Fig. 2).

#### TLR4

Signaling through TLRs has implicated a major role for MyD88 and IRAK. To investigate the relative contribution of the downstream molecules after TLR4 stimulation, RAW 264.7 macrophages were transduced with a NF $\kappa$ B-dependent luciferase reporter and subsequently with dominant-negative mutants of MyD88, TIRAP, IRAK1, IRAK2, and IRAK4. MyD88 and TIRAP are associated with TLR2 and TLR4 after receptor activation by HMGB1 [35]. MyD88 recruits members of the IRAK family which activate the downstream TRAF6 kinase. Dominant negative MyD88, dnTIRAP, dnIRAK1, and dnIRAK2 inhibited both LPS and HMGB1 induced NF $\kappa$ B reporter gene activity, suggesting these proteins are key players in the TLR4 signaling cascade. IRAK1 activates TRAF6, which results in the formation of a complex with TAK1 and TAB2. However, though expression of dnTRAF6 clearly inhibited the NF $\kappa$ B reporter gene expression after stimulation of both LPS and HMGB1, the expression of dnTAB2 and dnTAK1 was less effective in inhibiting NF $\kappa$ B activation stimulated by HMGB1 than when stimulated by LPS. Thus, TAB2 and TAK1 seem less important in HMGB1 signaling through TLR4 [35].

There is a partial overlap in genes induced after treatment of neutrophils with either HMGB1 or LPS [33], suggesting they may be using the same receptor, TLR4. LPS activates p38 MAPK, PI3K, Akt and Erk1/2. HMGB1 stimulation of neutrophils activated p38 MAPK and to a lesser extent PI3K, Akt and Erk1/2. Inhibition of these signaling components led to a decrease in HMGB1-stimulated cytokine expression through a decrease in NF $\kappa$ B activation [33]. PI3K can also directly interact with TLRs [47]. Furthermore, a putative PI3K binding site is present in MyD88, and phosphorylation of MyD88 upon LPS stimulation results in the formation of a PI3K-MyD88 complex [48]. Moreover, MyD88 can directly interact with the downstream effector Akt [49] (Fig. 2).

The signaling cascades initiated by TLR2 and TLR4 show much overlap. Differentiation of these signaling pathways may occur at the level of adaptor proteins bound to the TIR domain of the TLR [44, 45]. Furthermore, TLR4 signaling may be either MyD88 dependent or independent [44]. Nevertheless, most prominent effect of TLR

activation is the induction of NF $\kappa$ B-dependent gene expression (Fig. 2). The NF $\kappa$ B family of transcription factors contains five family members that function as hetero- or homodimers. The dimers are sequestered in the cytoplasm in an inactive form by I $\kappa$ B. NF $\kappa$ B is activated when I $\kappa$ B is phosphorylated and subsequently degraded, as a result of which NF $\kappa$ B can translocate to the nucleus. The I $\kappa$ B kinase (IKK) complex, consisting of IKK $\alpha$ , IKK $\beta$ , and IKK $\gamma$  (or NF $\kappa$ B essential modulator, NEMO), is the main activator of NF $\kappa$ B and is itself activated by diverse upstream signals (Fig. 2) [50].

Though it is clearly feasible that HMGB1 can signal through either TLR2, TLR4, or both, data substantiating this in endothelial cells is limited. Moreover, most of the data discussed above was generated in rather artificial cell systems for TLR signaling such as HEK293 cells. The extensive use of plasmid constructs (e.g., luciferase reporter gene constructs for NF $\kappa$ B activation, dominant-negative forms of signaling components) may hamper the execution of such experiments in hard to transfect primary cells, including primary endothelial cells. As such, the precise potential of HMGB1-mediated TLR2 and/or TLR4 activation for modulation of endothelial cell activation remains to be fully elucidated. Nevertheless, by inference of the data available regarding the interactions of RAGE, TLR2 and TLR4 with HMGB1, we will discuss below the possible roles of HMGB1 in vasculature-related pathologies.

#### HMGB1 signaling in vasculature-related pathologies

##### Cancer

Overexpression of RAGE and/or HMGB1 has been documented for diverse cancers [23], including colon cancer [51, 52], prostate cancer [53] and glioma [25]. RAGE positively contributes to cancer progression in a number of ways. First, RAGE expression was correlated with VEGF expression and microvessel density in oral squamous cell carcinoma [54]. Moreover, it has been demonstrated that RAGE activation contributes to (tumor) angiogenesis as RAGE activation by AGE-induced angiogenesis in vitro [55]. Second, the homing of EPCs to tumor vasculature in response to HMGB1 is mediated by RAGE as anti-RAGE antibodies inhibited this process; however, anti-TLR antibodies did not [27]. And third, in diverse mouse models, it was demonstrated that blocking HMGB1-RAGE interactions inhibited tumor progression and metastasis. Antibodies against HMGB1 and RAGE were both effective in inhibiting tumor growth, as was soluble RAGE that scavenges HMGB1. Furthermore, expression of dominant-negative RAGE in C6 glioma cells also inhibited tumor

growth [25]. We have recently identified HMGB1 to be overexpressed in tumor endothelial cells [2], suggesting it contributes to tumor angiogenesis and disease progression. Moreover, anti-HMGB1 antibodies inhibited endothelial sprouting in vitro and angiogenesis in the chick chorioallantoic membrane in vivo [2], demonstrating the potential clinical applications of HMGB1 inhibition. Finally, tumor hypoxia may lead to central necrosis and hence infiltration with macrophages [9, 56]. HMGB1 passively released from dead tumor cells can activate macrophages that in turn are activated to secrete TNF $\alpha$  and VEGF [56]. VEGF can directly contribute to increased angiogenesis to overcome tumor hypoxia, and both HMGB1 and TNF $\alpha$  can activate endothelial cells to in an NF $\kappa$ B-dependent response, contributing to tumor progression [9, 15, 26, 56] (Fig. 3a).

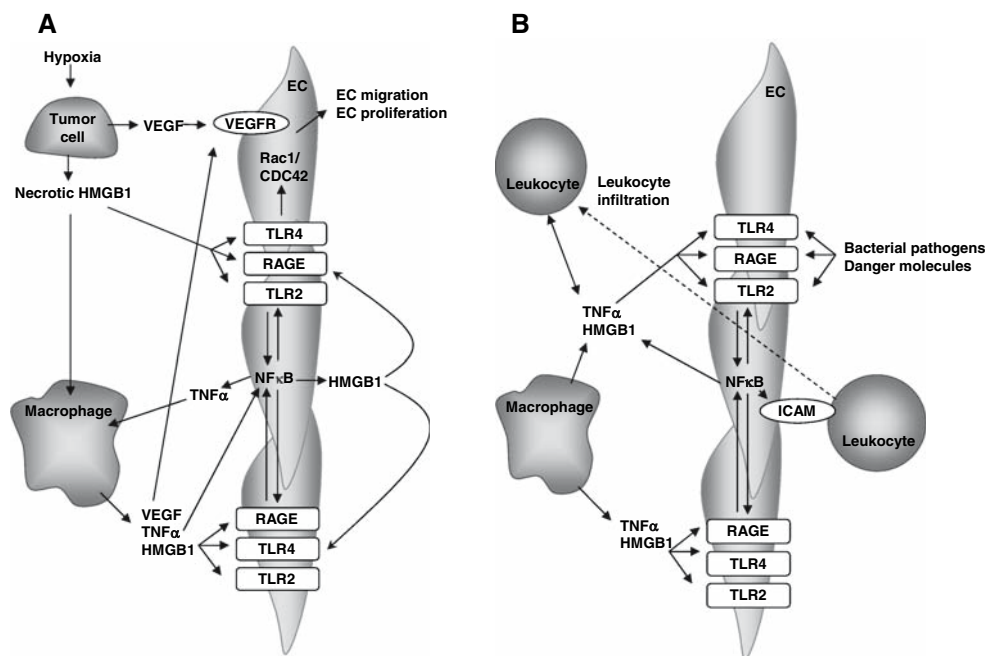
### Diabetes

AGE are non-enzymatical adducts of proteins, lipid and nucleic acids which form in a pro-oxidant environment and the formation of AGE increases with age [57, 58]. In

addition, the hyperglycemic conditions found in diabetes contribute to enhanced generation of AGE. Treatment of endothelial cells with AGE-BSA increased cell proliferation and tube formation and was accompanied by upregulation of VEGF and VEGF receptors through activation of NF $\kappa$ B and AP1 [55, 59], demonstrating the proangiogenic potential of these compounds. Hence, AGE-RAGE interactions can contribute to diabetes-associated retinopathies caused by increased angiogenesis. Indeed, increased expression of AGE, RAGE, and HMGB1 was found in retinas of patients with retinopathy [60].

### Arthritis

In rheumatoid arthritis, new blood vessels invade the joint and destroy articular cartilage, even in the absence of a causative factor, usually Gram-positive bacteria [61]. TLR2, IL-8, and VEGF are increased in arthritic joints, and administration of anti-TLR2 antibodies to chondrocytes inhibited VEGF and IL-8 expression, linking TLR2 to angiogenesis in arthritis [60, 62]. Furthermore, stimulation



**Fig. 3** Signal amplification mediated by HMGB1 in cancer and inflammation. Schematic and simplified visualization of HMGB1 signaling and its contribution to cancer and inflammation. **(a)** Growing tumors can become hypoxic and subsequently start to secrete VEGF that induces angiogenesis. When tumor cells die, HMGB1 is released in the extracellular space, and this can activate macrophages as well as endothelial cells. Activation of macrophages by HMGB1 leads to the secretion of proinflammatory and proangiogenic cytokines. Activation of endothelial cells by HMGB1 leads to NF $\kappa$ B activation that in turn can upregulate inflammatory cytokine production,

HMGB1 secretion, and HMGB1 receptor expression. The Rac1/CDC42 pathway can be activated by both RAGE and TLRs and can contribute to cell migration. **(b)** Bacterial pathogens and danger molecules can initially activate TLRs which results in NF $\kappa$ B-induced gene expression. Adhesion molecules such as ICAM1 are induced, that mediate the infiltration of leukocytes which contribute to inflammation by secreting proinflammatory cytokines. In addition, NF $\kappa$ B mediates the upregulation of HMGB1 receptors and the production of cytokines including HMGB1

of synovial fibroblasts with a TLR2 agonist resulted in the activation of MyD88, IRAK1, IRAK4, and TRAF6. Blocking antibodies to TLR2 inhibited the upregulation of these proteins. Moreover, the expression of IL-8 and VEGF was NF $\kappa$ B activation dependent as NF $\kappa$ B inhibitors decreased TLR2 stimulated expression of these proangiogenic cytokines [62]. Currently, there is no direct evidence on the role of HMGB1-mediated TLR2 stimulation in the progression of arthritis. However, HMGB1 has been detected in synovial macrophages [63, 64], suggesting it may contribute to TLR2-induced VEGF and IL-8 release.

### Atherosclerosis

Endothelial cell injury is central to the initiation of atherosclerosis as this results in the attraction of macrophages, and the progression of atherosclerosis is accompanied by a sustained proinflammatory response [65]. In atherosclerotic lesions, HMGB1 and RAGE are expressed in endothelial cells, smooth muscle cells, and macrophages. Here, different proinflammatory cytokines stimulated the secretion of HMGB1, which was inhibited by inhibitors of ERK1/2, PKC, and PI3K [66]. Thus, upregulation and secretion of HMGB1 may contribute to amplify the inflammatory response in atherosclerosis.

Endothelial cells express both TLR2 and TLR4, though the expression levels depend on the vascular bed they reside in. HUVEC and HMEC express only minimal levels of TLR2, whereas arterial HCAEC expressed higher levels of TLR2; all endothelial cells express TLR4 [38, 67]. TLR2 expression is upregulated under disturbed flow [20, 65]. In these regions of low shear stress, lesion susceptibility is greatest, hence, the ability of TLR2 ligands to activate endothelial cells is greatest in these atherosclerosis-prone arterial branches [65].

TLR2 and TLR4 expression are increased in human atherosclerotic lesions [68]. In combination with increased HMGB1 and RAGE expression, this is likely to contribute to a sustained inflammatory response. A primary event in initiating atherosclerosis may hypothetically be endothelial cell activation through TLRs by bacterial pathogens. This would lead to endothelial cell activation of NF $\kappa$ B and the concomitant increase in leukocyte adhesion molecules and the secretion of cytokines, as well as HMGB1. Subsequently, leukocytes and macrophages invade the vessel wall. Activated infiltrating cells then also secrete cytokines that sustain the inflammatory state of the endothelium. HMGB1 may be released from infiltrated cells or from endothelial cells and in turn stimulate inflammatory molecule expression, thereby amplifying the inflammatory response and leading to progression of the disease (Fig. 3b).

### HMGB1-mediated signal amplification

At several levels HMGB1 signaling may sustain either autocrine or paracrine positive feedback loops. First, HMGB1 stimulation of endothelial cells and macrophages leads to TNF $\alpha$  secretion [6, 7, 26]. In turn, TNF $\alpha$  can induce HMGB1 secretion from macrophages and endothelial cells [5, 15]. Second, activation of RAGE, TLR2, and TLR4 by HMGB1 results in NF $\kappa$ B activation. NF $\kappa$ B activation results in the expression of proinflammatory and proangiogenic genes, as well as in an increased expression of RAGE and TLR2 due to NF $\kappa$ B binding sites in their promoters [10]. Third, TLR2 signaling leads to an NF $\kappa$ B-dependent increase in expression of ICAM-1 on endothelial cells. In leukocytes, TLR2 signaling activates the ICAM-1 receptor CD11b/CD18. Interaction between CD11b/CD18 and ICAM-1 again results in NF $\kappa$ B activation [69]. In addition to TLR2, RAGE also contributes to the activation of CD11b/CD18 on neutrophils [70]. Hence, the interplay between the RAGE and TLR signaling pathways may occur at different levels but all seem to hold a crucial role for NF $\kappa$ B.

### Concluding remarks

All the complex signaling pathways HMGB1 may be involved in seem to affect NF $\kappa$ B activity. Hence, therapeutic modulation of HMGB1 expression will result in altered NF $\kappa$ B signaling and NF $\kappa$ B-induced gene expression. However, because of the complexity of the signaling pathways involved, the effect of such modulation may not be easily predicted. For instance, in tumor angiogenesis and other angiogenesis-related pathologies, at first glance it seems beneficial to reduce sustenance of a proinflammatory loop mediated by active NF $\kappa$ B. However, it has recently been postulated that NF $\kappa$ B activation is required for the activity of diverse anti-angiogenic compounds, and hence should not be inhibited in endothelial cells [71].

In addition to its downstream effector NF $\kappa$ B, HMGB1 itself has been postulated a therapeutic target, not only for cancer [2, 25] but also for the treatment of sepsis [5]. Thus, pathologies characterized by a continuous proinflammatory or proangiogenic state may benefit from therapeutic interference to dampen HMGB1 and the feedback mechanisms it promotes (Fig. 3).

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