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Review Article

HMGB1 as a Therapeutic Target to Alleviate Endothelial Dysfunction in Severe Systemic Inflammation

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Abstract

Excessive endothelial activation causing microvascular dysfunction is key in the pathogenesis of severe systemic inflammation. The ubiquitous nuclear protein high-mobility group box-1 (HMGB1) is extracellularly released as an alarmin and elicits potent proinflammatory effects on endothelial cells after sterile or infectious tissue injury. These effects include increased release of cytokines, chemokines, and factors promoting coagulation and fibrinolytic activities, and luminal endothelial expression of adhesion molecules. HMGB1 is actively discharged from innate immune cells, endothelial cells, and sensory-motor nerves innervating vascular compartments. Discrete HMGB1 redox isoforms signal via toll-like receptor 4 or receptor for advanced glycation end-products (RAGE) to induce inflammation. Furthermore, extracellular HMGB1 avidly complex-binds other extracellular proinflammatory molecules and these complexes are endocytosed via RAGE to the endolysosomal system in many cells, such as endothelial cells. Increased intralysosomal HMGB1 levels disrupt the lysosomal membrane allowing HMGB1-transported co-molecules access to cognate cytoplasmic proinflammatory receptors and inflammasome systems. The subsequent intracellular activation causes endothelial cell pyroptosis, liberating potent procoagulant and proinflammatory molecules. Therapies based on HMGB1-binding antagonists have yielded mixed results in preclinical studies of systemic inflammation. Unsuccessful results were possibly caused by steric hindrance for HMGB1-binding antagonists due to HMGB1 complex formation. An alternative therapeutic strategy is to inhibit HMGB1 release, a process requiring acetylation of nuclear HMGB1 enabling cytoplasmic export. Increased histone deacetylase activity reduces HMGB1 release and is promoted by acetylcholine signaling via alpha 7 nicotinic acetylcholine receptors present on endothelial cells and sensory neurons. Invasive and non-invasive electrical vagus nerve stimulation activates and mobilizes acetylcholine-secreting anti-inflammatory T lymphocytes to the microvascular compartment. This review focuses on determining the role that the innate immune system and sensory nerves have on driving mechanisms of endothelial dysfunction via release of pathogenic extracellular HMGB1.

INTRODUCTION

The endothelium constitutes a physiologically dynamic single cell interface, which lines blood and lymphatic vessels and provides communication between circulating blood and tissues to maintain homeostasis by secreting various vasoactive molecules in response to physical and chemical stimuli and signals [1]. The endothelium promotes homeostasis by (i) tightly controlling vascular tone, permeability and remodeling; (ii) regulating blood fluidity, angiogenesis, and thrombosis; (iii) driving anti-inflammatory, -oxidative and -proliferative mechanisms; and (iv) altering cellular metabolism (Figure 1) [1,2]. Nitric oxide (NO) represents an important and potent endothelium-derived relaxing factor that is generated when the enzyme endothelial nitric oxide synthase (eNOS) catalases the conversion of L-arginine into L-citrulline and NO [1]. NO then diffuses into vascular smooth muscle cells where it activates guanylate cyclase, resulting in cyclic guanosine monophosphate (cGMP)-mediated vasodilation [1]. NO plays a central role in maintaining a quiescent state of the vascular wall by inhibiting mechanisms of inflammation, cell proliferation and thrombosis [1]. Additionally, the endothelium mediates hyperpolarization of vascular smooth muscle cells through NO-independent vasodilation pathway, resulting in augmented potassium conductance and depolarization of vascular smooth muscles, a compensatory mechanism that is favored during loss of NO-mediated vasodilation [1]. A loss of endothelial barrier function, referred to as endothelial dysfunction, has been identified as the first vascular disturbance associated with underlying chronic inflammatory conditions, such as atherosclerosis, hyperhomocysteinemia and sepsis [3-5], and is defined as a systemic pathological state resulting from a plethora of cellular events, including diminished NO availability (partially due to decreased eNOS-derived NO synthesis), increased inflammation and oxidative stress, leukocyte infiltration and adhesion, and dysregulated endothelial cell metabolism [1,2].

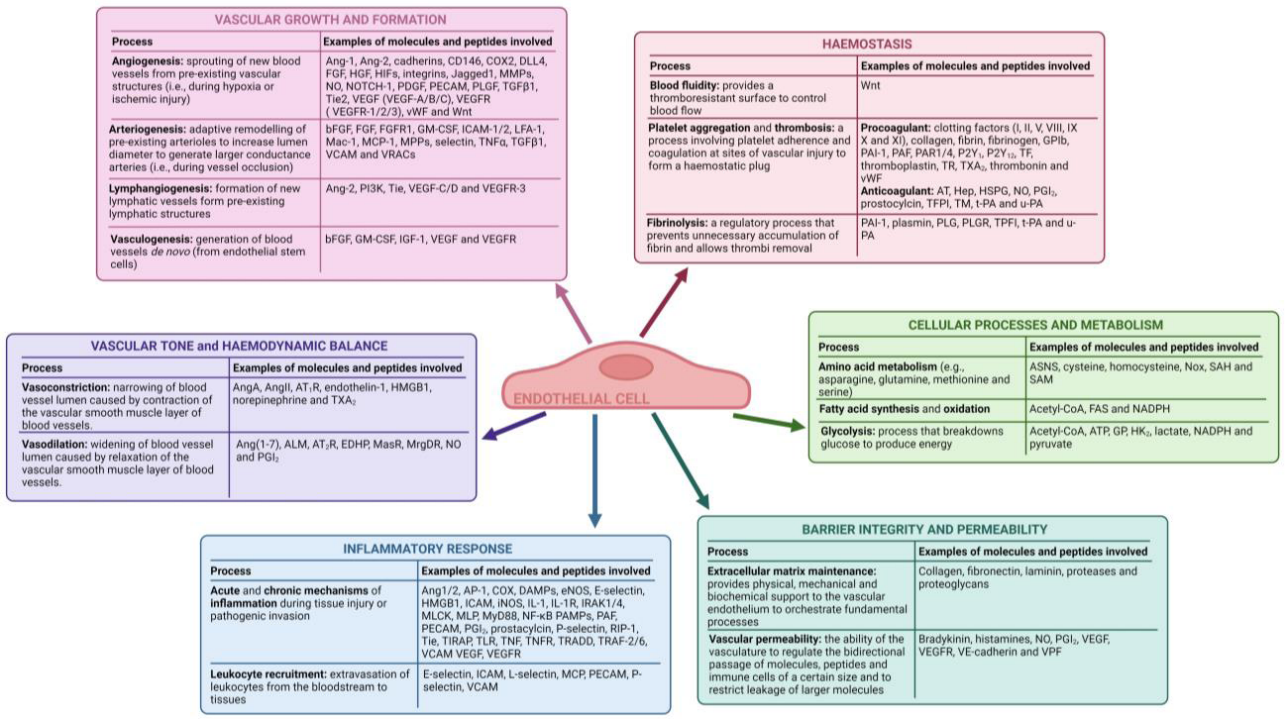

Figure 1 Functions of the endothelium. The endothelium is a dynamic monolayer of cells responsible for (a) vascular growth and formation [10-15], (b) haemostasis and clot formation and degradation [16,17], (c) vital cellular processes [18], (d) providing a physical barrier to control permeability of molecules, peptides and cells [19,20], (e) inflammatory response and leukocyte recruitment [21,22], and (f) haemodynamic balance through regulation of contraction and relaxation responses [23,24].

Abbreviations: Acetyl-CoA: Acetyl Coenzyme A; ALM: Almandine; Ang: Angiopoietin; AngA: Angiotensin A; AngII: Angiotensin II; Ang(1-7): Angiotensin 1-7; AP-1: Activator Protein-1; ASNS: Asparagine Aspartate; AT: Antithrombin; ATP: Adenosine Triphosphate; AT1R: Angiotensin Type 1 Receptor; bFGF: basal Fibroblast Growth Factor; CD146: Cluster Differentiation 146; COX: Cyclooxygenase; DAMPs: Danger-Associated Molecular Patterns; DLL4: Delta-Like Ligand 4; EDHP: Endothelium-Derived Hyperpolarising Factor; eNOS: Endothelial Nitric Oxide Synthase; FGFR: Fibroblast Growth Factor Receptor; FAS: Fatty Acid Synthase; FGF: Fibroblast Growth Factor; GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor; GP: Glycerate 3-Phosphate; GPIb: Glycoprotein Ib; Hep: Heparin-like molecules; HGF: Hepatocyte Growth Factor; HIF: Hypoxia-Inducing Factor; HK: Hexokinase 2; HMGB1: High-Mobility Group Box-1; HSPG: Heparan Sulfate Proteoglycans; ICAM: Intracellular Adhesion Molecule; IGF: Insulin-Like Growth Factor; iNOS: inducible Nitric Oxide Synthase, IL-1: Interluikin-1, IL-1R: Interluikin-1 Receptor; IRAK: IL-1R-Associated Kinase; LFA: Lymphocyte Function-Associated Antigen; MasR: MAS1 Oncogene Receptor; MCP-1: Monocyte Chemoattractant Protein-1; MLCK: Myosin-Light-Chain Kinase; MLP: Myosin Light Chain; MMPs: Matrix Metalloproteinases; MrgDR: MAS1 Oncogene, G-coupled Receptor; MyD88: Myeloid Differentiation Factor 88; NADH: Nicotinamide Adenine Dinucleotide Phosphate; NF-κB: Nuclear Factor Kappa B; NO: Nitric Oxide; Nox: NADPH Oxidase; PAF: Platelet-Activating Factor; PAI-1: Plasminogen Activator Inhibitor 1; PAMPs: Pattern-Associated Molecular Patterns; PAR: Protease Activating Receptor; PDGF: Platelet-Derived Growth Factor; PECAM: Platelet Endothelial Cell Adhesion Molecule; PGI2: Prostaglandin I2; PI3K: Phosphoinositide 3-Kinase; PLGF: Placental Growth Factor; PLG: Plasminogen; PLGR: Plasminogen Receptor; P2Y1: Purinergic Receptor P2Y G protein-coupled 1; RIP-1: Receptor-Interacting Protein 1; SAH: S-Adenosylhomocysteine; SAM: S-adenosylmethionine; TF: Tissue Factor; TFPI: Tissue Factor Pathway Inhibitor; TGF: Transforming Growth Factor; Tie: Angiopoietin Receptor; TIRAP: Toll/IL-1 Receptor Accessory Protein; TLR: Toll-Like Receptor; TM: Thrombomodulin; TNF: Tumor-Necrosis Factor; TNFR: TNF Receptor; TR: Thromboxane Receptor; TRADD: TNFR-Associated Via Death Domain Protein; TRAF: TNFR-Associated Factor; TXA2: Thromboxane; t-PA: tissue Plasminogen Activator; u-PA: urokinase Plasminogen Activator; VCAM: Vascular Cell Adhesion Molecule; VEGF: Vascular Endothelial Growth Factor; VEGFR: Vascular Endothelial Growth Factor Receptor; VRACs: Volume Regulated Anion Channels; vWF: von Willebrand Factor; Wnt: Wingless/integrated

This review focuses on mechanisms exerted by innate immunity and sensory nerves that drive endothelial dysfunction, the central pathophysiological process in life-threatening systemic inflammation. Specific emphasis is directed on the pathogenic features of extracellular high mobility group box protein 1 (HMGB1), a ubiquitous nuclear protein actively released as a proinflammatory mediator from numerous cell types, including sensory neurons during loss of tissue homeostasis [6-9]. Extracellular HMGB1 acts upstream of the classical proinflammatory cytokines and chemokines and all nucleated cells as well as platelets passively release HMGB1 extracellularly during lytic cell death.

Extracellular HMGB1 Biology

HMGB1 is a protein present in the nucleus of all animal cells and expresses 214 amino acid residues arranged in two positively charged DNA-binding domains and a negatively charged C-terminal region [6]. HMGB1 serves vital intranuclear functions required for transcription, nucleosome formation, DNA repair, and appropriate DNA conformation. Lytic cell death mediates passive extracellular HMGB1 release. Active extracellular HMGB1 release, induced by numerous exogenous and endogenous stimuli, occurs in multiple steps [25]. Two nuclear localization sites (NLS) secure a dominant nuclear

HMGB1 localization during cellular homeostasis. Translocation of nuclear HMGB1 to the cytoplasm requires posttranslational molecular modifications. These changes include hyperacetylation of critical lysines in the two NLS sites, preventing the continuous bidirectional shuttle of HMGB1 between the cytoplasm and the nucleus and results in cytoplasmic HMGB1 accumulation [26]. Nuclear hyperacetylation is accomplished via increased histone-acetylase activity and decreased histone-deacetylase (HDAC) activity. Preventing hyperacetylation thus offers a therapeutic strategy to inhibit extracellular HMGB1 release. Cytoplasmic HMGB1 is released extracellularly via several mechanisms. One route proceeds via exocytosis of secretory lysosomes, a pathway also utilized for interleukin (IL)-1β secretion, although HMGB1 and IL-1β are stored in separate vesicles [27]. Another route for HMGB1 to exit cells takes place on the surface of microparticles derived from activated platelets [28]. Vascular injury induces massive extracellular release of HMGB1 from platelets displaying a central role in the pathogenesis of thrombosis formation and neutrophil activation [28]. Programmed, proinflammatory cell death (pyroptosis) is an additional mechanism for regulated HMGB1 release [29,30]. This process is a consequence of inflammasome activation and gasdermin D cleavage inducing pore formation, rupturing the outer cell membrane. This pathway is as an example of the dominant mechanism for HMGB1 release during gram-negative sepsis when caspase-1/caspase-11 double-deficient mice express markedly reduced systemic HMGB1 levels [30-32]. Most importantly, recent research demonstrates that stimulated sensory neurons can actively secrete HMGB1 in an antidromic fashion by mechanisms not fully elucidated [7,8]. Nociceptors harvested from transgenic mice expressing channelrhodopsin-2 in neurons directly release HMGB1 by light exposure causing neuroinflammation in contrast to mice engineered with neuronal HMGB1 gene deficiency. A plausible functional role of HMGB1 released from sensory neurons in the vascular wall during systemic inflammation has not been studied so far but deserves future attention.

The redox state of HMGB1 determines the extracellular HMGB1 receptor usage [33]. The DNA-binding boxes in HMGB1 contain three cysteines and the redox state of each of these residues is key for the extracellular receptor association and activation [33]. All three cysteines reside in a fully reduced state with thiol groups (all-thiol HMGB1) and are present in quiescent cells. This isoform acts extracellularly as a chemotactic factor when operating together with C-X-C motif chemokine ligand (CXCL) 12 via C-X-C chemokine receptor (CXCR) 4 [34]. Mild HMGB1 oxidation generates disulfide HMGB1 characterized by a disulfide bond between Cys^{23} and Cys^{45} while keeping Cys^{106} in the reduced form. Disulfide HMGB1 is a potent proinflammatory molecule operating via toll-like receptor (TLR) 4 [35]. Like lipopolysaccharides (LPS), disulfide HMGB1 binds to myeloid differentiation factor-2 (MD-2) in the TLR4 receptor complex, but at a separate position. Further oxidation of HMGB1 produces sulfonyl groups on any or all cysteine residues creating sulfonyl HMGB1, which is a non-inflammatory isoform.

An additional and important proinflammatory HMGB1 receptor is the receptor for glycated end-products (RAGE) [36]. This is a multi-ligand receptor expressed by many cell types, including endothelial cells, predominantly as a preformed intracellular molecule available for prompt nuclear factor kappa B (NF-kB)-controlled translocation to the cell surface when cellular homeostasis is challenged. Extracellular HMGB1 readily forms heterocomplexes with multiple other extracellular damage-associated molecular pattern molecules (DAMPs) and pathogen-associated molecular pattern molecules (PAMPs) [37], which are endocytosed via RAGE for further transfer into the endolysosomal compartment [30,38] (Figure 2). Most of these DAMPs and PAMPs do not cause inflammation when present extracellularly since their cognate proinflammatory receptors are located intracellularly in target cells but not on cell surfaces. However, HMGB1 provides a transport intracellularly for these danger molecules via RAGE-mediated endocytosis and in addition by performing a unique function inside acidic lysosomes disrupting the lysosomal membrane at low pH allowing its transported molecules to circumvent degradation and leakage into the cytosol. Some of these imported molecules are potent ligands to inflammasomes, retinoic acid-inducible gene I, cGMP-adenosine monophosphate synthase-stimulator of interferon genes, and additional receptors causing inflammation and coagulation.

RAGE expressed on endothelial cells encounters the pathogenic HMGB1 heterocomplexes during the early stage of systemic inflammation. A significant quantitative discrepancy between HMGB1 levels in arterial versus venous plasma samples has been reported in severe trauma patients [39]. Arterial HMGB1 concentration was consistently lower than venous concentration in simultaneously obtained samples (arterial = 0.6 x venous; 95% CI 0.30–0.90) [39]. Significant amounts of HMGB1 are thus hypothesized to be removed in pulmonary circulation, where there is constitutive luminal endothelial RAGE expression in contrast to other organs. The mechanism and reason for HMGB1 elimination remain to be clarified, but one may speculate that it represents a function to degrade PAMP and DAMP molecules carried by HMGB1 via RAGE-mediated endocytosis to the lysosomes in endothelial cells in the pulmonary circulation. Many of these DAMPs and PAMPs share a poor ability to generate antibody responses. Physiological amounts of HMGB1 are insufficient to damage the lysosomal membrane allowing the expected and beneficial degradation of the HMGB1-partner molecule complexes, while pathologically increased intralysosomal HMGB1 levels cause lysosomal damage and leakage to the cytoplasm with subsequent inflammasome activation.

HMGB1 and Endothelial Dysfunction

Endothelial cells both release [40], and respond [41], to HMGB1 and results from *in vivo* and *in vitro* studies indicate that these biological activities have important pathophysiological consequences.

Extracellular HMGB1 Activates Endothelial Cells

Human microvascular endothelium cocultured with HMGB1

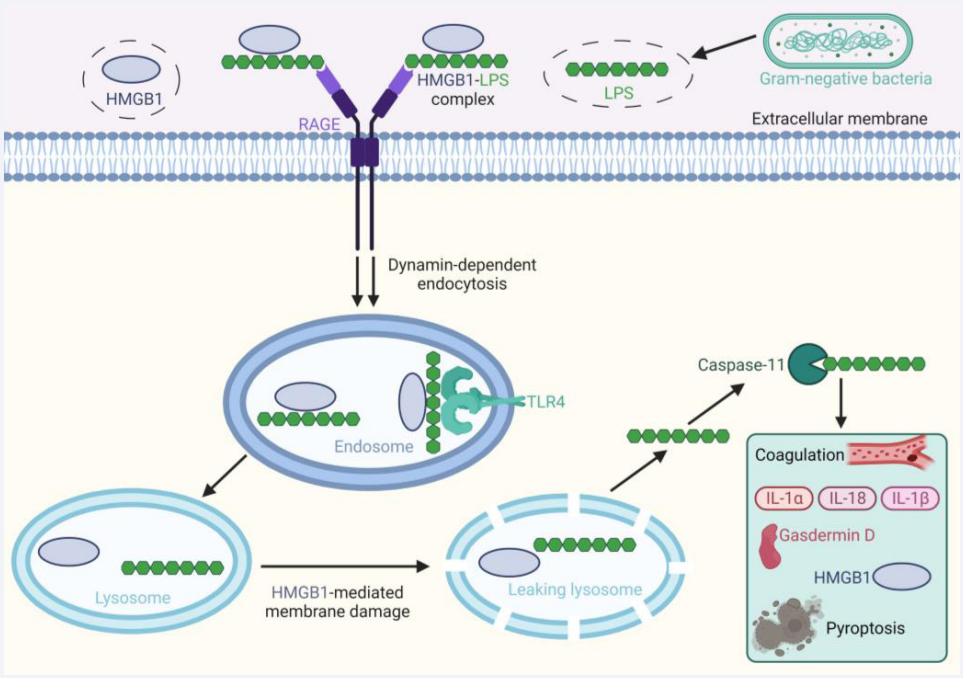

Figure 2 HMGB1 transports multiple extracellular DAMPs and PAMPs, here exemplified by LPS, into the inside of endothelial cells. HMGB1 has two LPS-binding sites and thus forms an extracellular HMGB1–LPS complex that gets endocytosed via RAGE to the endolysosomal system. High intralysosomal levels of HMGB1 present at low pH damages the lysosomal membrane enabling LPS to leak into the cytosol culminating in activation of the intracellular LPS sensor caspase-11 (caspases-4 and −5 in humans) that instigates inflammation and coagulation.
Abbreviations: HMGB1: High-Mobility Group Box-1; IL: Interleukin; LPS: Lipopolysaccharide; RAGE: Receptor For Advanced Glycated End-Products; TLR: Toll-Like Receptor.

becomes activated in a dose- and time-dependent manner to increase the cell surface expression of intercellular adhesion molecule 1, vascular cell adhesion molecule 1, and HMGB1 receptors RAGE and TLR4 [41-45]. Furthermore, HMGB1 induces secretion of the proinflammatory cytokines and chemokines (e.g., tumor necrosis factor (TNF), IL-6, IL-8, and monocyte chemoattractant protein 1) and the critical regulators of fibrinolysis (e.g., plasminogen activator inhibitor 1 and tissue plasminogen activator). HMGB1 stimulation generates a transient phosphorylation of mitogen-activated protein kinases, extracellular signal-related kinase, Jun N-terminal kinase, and nuclear NF-kB translocation in the endothelial cells. Moreover, the pathogenic effect of extracellular HMGB1 is further highlighted by a recent study that reported HMGB1 as an important regulator of various endothelium-dependent activities in genetically modified mice that have an absence of endothelial HMGB1 [46]. Mice in this study displayed elevated mean arterial pressure, increased plasma HMGB1 concentration, reduced endothelium-dependent relaxation and decreased NO and eNOS phosphorylation and peptide expression [46].

Preclinical *in vivo* studies have demonstrated the ability of HMGB1 to induce human endothelial cell cytoskeletal rearrangement and barrier disruption via increasing paracellular gap formation in concert with loss of peripheral organized actin fibers, and dissociation of cell-cell junctional cadherins [47]. Thought-provoking clinical *in vivo* observations have been reported about a possible pathogenic role of HMGB1 in Kawasaki disease, a severe acute vasculitis in infancy and childhood. Kawasaki patients generally express high systemic HMGB1 levels and may develop endothelial damage mediating coronary endothelial dysfunction resulting in coronary artery aneurysms. Results from a recent clinical study indicated that nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3-dependent endothelial cell pyroptosis, activated by HMGB1/RAGE/cathepsin B signaling, represented a central mechanism in the pathophysiology [48]. Human umbilical vein endothelial cells cultured in plasma from Kawasaki patients developed pyroptosis, while the addition of neutralizing anti-HMGB1 antibodies or RAGE-specific antagonists to the cell cultures prevented the pyroptosis and the subsequent release of IL-1a, IL-1b, IL-18, and HMGB1 [48].

Activated Endothelial Cells Release HMGB1

Like many other cell types, stressed endothelial cells release HMGB1 extracellularly via active mechanisms or via passive leakage occurring after lytic modes of cell death [49]. Sterile inflammation and infections are potent stimuli for the generation of active HMGB1 release from endothelial cells. Chemical modifications including hyperacetylation and phosphorylation of HMGB1 in the nucleus are necessary initial steps to allow nuclear HMGB1 export to the cytoplasm for further extracellular

secretion [25]. Antagonizing the nuclear modifications of HMGB1 offers a therapeutic anti-inflammatory strategy that has been successfully used in preclinical models of severe systemic inflammatory disorders, but not yet in patients. Activating the endogenous cholinergic anti-inflammatory pathway is one such therapeutic example that will be outlined later in this review. Administration of histidine-rich glycoprotein (HRG), a 75 kDa plasma protein, has been demonstrated to improve the survival rate of septic mice through the regulation of neutrophils and endothelium barrier function [42]. The role of HRG in endothelial barrier protection was concluded to depend mainly on its effect of inhibiting HMGB1 release from endothelial cells.

HMGB1 Causes Blood Brain Barrier Disruption

The blood-brain barrier (BBB) is composed of vascular endothelial cells with tight junctions, pericytes, extracellular matrix, and endfeet of astrocytic processes [42,44,50-52]. The critical function of the BBB is to protect the central nervous system from the entry of molecules that might harm the brain. High levels of systemic as well as extracellular cerebral HMGB1 have been demonstrated to substantially increase BBB permeability, in association with morphological contractile changes in vascular endothelial cells and pericytes [42,44,50-52]. Nishibori and colleagues have demonstrated that systemic administration of anti-HMGB1 monoclonal antibodies (mAb) protected the BBB integrity and inhibited the translocation of HMGB1 from neurons in experimental models of cerebral ischemia or hemorrhage [53-55]. It seems conceivable that the initial event for the protection of the BBB integrity occurred via antibody mediated HMGB1 neutralization close to the capillary endothelial cells. Using a rat-reconstituted BBB *in vitro* system composed of vascular endothelial cells, pericytes and astrocytes, the same research group confirmed that recombinant HMGB1 incubation caused a contractile response in endothelial cells and pericytes, which was inhibited by anti-HMGB1 mAb [53].

Does HMGB1 Released From Vascular Sensory Nerves Cause Endothelial Dysfunction?

The significance of the role of vascular nerves in the development of endothelial dysfunction in systemic inflammatory diseases remains poorly understood. Vasomotor control is exerted by sympathetic vasoconstrictor nerves in combination with vasodilator influences from transient receptor potential vanilloid subfamily member 1 (TRPV1)-expressing sensory-motor nerve release of substance P and calcitonin gene-related peptide , both of which are neurotransmitters that also possess proinflammatory effects [56]. These nerves run close to each other in the vascular wall and are located between the tunica media and the tunica adventitia, but they do not directly innervate the endothelial cells. However, there is strong evidence from different types of studies that microvascular smooth muscle is electrically coupled to the endothelium and that smooth muscle activation functionally affects endothelial cells [57-60]. This is particularly the case in the microcirculation, where the distance between the media-adventitia border and the tunica intima is minor. Recent discoveries demonstrate that TRPV1-positive sensory neurons also actively release HMGB1 in a retrograde manner and upregulate inflammation in the periphery [7,8]. Optogenetic stimulation of engineered peripheral sensory nerves induces active HMGB1 release and inflammation, while neuronal HMGB1 knock-out or HMGB1 neutralization via antibodies inhibit inflammation in models of arthritis and nerve injury. Analogous studies have not yet been performed in vascular pathology but should be conducted since the results may have important clinical therapeutic implications.

Targeting HMGB1 to Ameliorate Endothelial Dysfunction

Recognition of HMGB1 as a critical molecule in the pathogenesis of dysfunctional microcirculation in systemic inflammatory conditions makes it a valid therapeutic target. There are several successful approaches that have been studied in preclinical models and they will be further discussed.

Activating the Endogenous Cholinergic Anti-Inflammatory Pathway to Inhibit HMGB1 Release and to Reduce RAGE-Mediated Endocytosis of HMGB1-DAMP/PAMP Complexes: Inflammation is regulated by the nervous system, but this insight has so far not led to any clinical therapy for sepsis. Neuronally released HMGB1 accelerates inflammation, while acetylcholine, released via the cholinergic anti-inflammatory pathway, in contrast inhibits excessive inflammation [61,62]. Acetylcholine is released in all vagally innervated, organs including the celiac ganglion, where it activates the catecholaminergic splenic nerve to discharge norepinephrine in the spleen to stimulate a subset of splenic T lymphocytes capable of acetylcholine synthesis [63]. These activated T cells may then leave the spleen and function like mobile neurons to release acetylcholine that will down-regulate inflammation in body compartments that lack vagal innervation. The cholinergic mechanism inhibits proinflammatory HMGB1 release via signals that require acetylcholine and alpha-7 nicotinic acetylcholine receptors (α7nAchR) (Figure 3A) [64, 65].

Acetylation of multiple lysines in HMGB1 is necessary for the nuclear HMGB1 export needed for further active extracellular HMGB1 release [26]. Histone deacetylases, including sirutin 1 (SIRT1), prevent nuclear HMGB1 export. Acetylcholine boosts SIRT1 activity via α7nAChR and promotes nuclear HMGB1 sequestration [66]. HMGB1 is also dependent on NF-kB assistance for the cytoplasmic translocation and SIRT1 inhibits NF-κB activity by deacetylating the RelA/p65 subunit [67]. In addition, acetylcholine inhibits RAGE-mediated endocytosis of HMGB1 complexes, and TLR4/myeloid differentiation factor 88 (MyD88]/NF-κB signaling via a7nAChR interaction [68,69]. Electrical vagus nerve stimulation (VNS) as well as systemic administration of a7nAChR-specific agonists has been demonstrated to improve the outcome in preclinical endotoxemia and sepsis models [64,70-74]. A need for surgical VNS device implantation can be circumvented by transcutaneous auricular VNS (taVNS) using an external pulse generator [75,76]. The auricular branch of the vagus nerve reaches superficial parts in the concha and tragus in

both ears, enabling taVNS as a safer therapeutic alternative to an implanted VNS device. This approach has been successfully tested in murine polymicrobial sepsis resulting in reduced lethality and systemic HMGB1 levels [77].

Activation of α7nAChR can also be achieved via systemic administration of α7nAChR-specific agonists. One such molecule termed GTS-21 dose-dependently suppressed NF-kB activation and inhibited TNF and HMGB1 release from endotoxin-activated mouse macrophages [64]. *In vivo*, GTS-21 significantly reduced serum HMGB1 levels and improved the survival rate in mice with gram-negative polymicrobial severe sepsis. The compound has been therapeutically administered safely and well tolerated to patients with schizophrenia in pilot studies [78,79]. Further studies are warranted to translate the beneficial effects via the cholinergic anti-inflammatory pathway into clinical use for patients with severe systemic inflammation and endothelial dysfunction.

Administration of Low-Dose Heparin or Non-Anticoagulant Heparin: Heparin is a negatively charged polysaccharide that binds to specific, positively charged sites in HMGB1 and prevents HMGB1 from binding to RAGE and thus restricts HMGB1-mediated import in endothelial cells of inflammasome-activating molecules (Figure 3B) [80,81]. Furthermore, non-anticoagulant heparin also binds HMGB1 as well as extracellular histones and prevents histone-mediated cytotoxicity, another dominant pathogenic route in severe sepsis. The effective doses of heparin required to inhibit LPS–HMGB1 toxicity and improve survival in experimental Gram-negative sepsis are much lower than those needed for the anticoagulant effects [80].

The endothelial glycocalyx is a gel-like layer composed of a network of proteoglycans, glycoproteins, and glycolipids at the luminal side of endothelial cells lining the blood vessels throughout the body. Proinflammatory molecules including HMGB1 may disrupt the endothelial glycocalyx, leading to edema, increased macromolecular leakage, and cytosolic LPS delivery. It is thus of therapeutic interest that heparin as well as non-anticoagulant heparin prevent the degradation of glycocalyx [80]. Modified heparin without anticoagulant activity also prevents LPS from binding to HMGB1 and prevents RAGE-mediated endocytosis of HMGB1 and of HMGB1 heterocomplexes. One study reporting successful heparin treatment in experimental Gram-negative sepsis concurrently found that the identical strategy failed in *Staphylococcus aureus*-induced sepsis, indicating that the pathogenic mechanisms operating in Gram-positive versus Gram-negative sepsis are different [80]. Staphylococcal and Streptococcal superantigens induce separate cytokine patterns than LPS with possibly diverse effects on endothelial functions [82,83]. Future clinical studies of heparin treatment in sepsis thus preferably need stratification of patients based on an identification of invading pathogens. There are several clinical studies on the therapeutic efficacy of heparin in treating sepsis with conflicting results, possibly due to the lack of stratification.

Systemic Treatment with Soluble Thrombomodulin: Thrombomodulin is an endothelial cell surface thrombin receptor that converts thrombin into an anticoagulant. During the hypercoagulable state after endothelial injury, thrombomodulin is released into the intravascular space by proteolytic cleavage of the endothelium component [84]. Soluble thrombomodulin binds and neutralizes extracellular histones and HMGB1 and supports the proteolytic thrombin-mediated degradation of HMGB1 (Figure 3C) [85]. Recombinant soluble thrombomodulin has been shown to protect patients with disseminated intravascular coagulation after severe trauma or in severe sepsis [86,87].

DISCUSSION

Based on the presented preclinical results, HMGB1 is a valid and logical therapeutic target molecule to study to further ameliorate the consequences of endothelial dysfunction in severe systemic inflammatory diseases. However, this mission signifies a distinct clinical challenge for several reasons. Extracellular HMGB1 causes endothelial dysfunction via several separate pathways operating in concert. These paths include CXCR4, TLR4, and RAGE and they may possibly need to be counteracted by distinct therapeutic approaches. The general understanding of the relative functional importance of each of these receptor-mediated activities in any given severe systemic inflammatory clinical disease is presently insufficient. Another problem, based on steric hindrance for HMGB1-binding antagonists, is provoked by the exceptional readiness of extracellular HMGB1 to form heterocomplexes *in vivo* with other molecules.

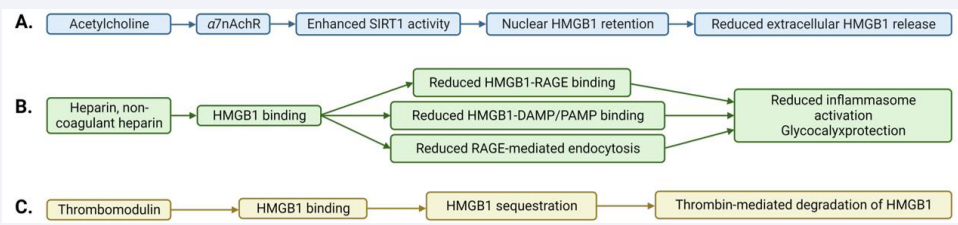

Figure 3 HMGB1 antagonists ameliorate HMGB1-dependent inflammation via separate mechanisms.
Abbreviations: α7nAchR: Alpha-7 Nicotinic Acetylcholine Receptors; DAMP: Danger-Associated Molecular Pattern; HMGB1: High-Mobility Group Box-1; PAMP: Pattern-Associated Molecular Pattern; RAGE: Receptor For Advanced Glycated End-Products; SIRT1: Sirutin 1.

An alternative therapeutic approach, that partially circumvents the discussed limitations, is to reduce the released amounts of HMGB1, occurring passively after lytic cell death or after active cellular secretion. The half-life time of plasma HMGB1 passively released after necrosis in severe trauma patients is 26 min and this early, transient peak of extracellular all-thiol HMGB1 has not been ascertained to generate subsequent severe systemic inflammatory clinical problems [88]. However, trauma patients who go on to develop systemic inflammatory response syndrome (SIRS), produce a second extracellular HMGB1-wave peaking 3-6 hours after the initiating trauma [88]. The cellular source of this secondary HMGB1 release has not been identified but is most likely the result of active HMGB1 secretion. This is a typical example where therapeutic intervention with agents preventing active extracellular HMGB1 release should be important to study. Inhibiting translocation of the nuclear pool of HMGB1 to the cytoplasm in stressed cells is conceivably one way to accomplish this. Promoting histone deacetylase activities inhibit nuclear HMGB1 hyperacetylation and thus prevents HMGB1 from entering the cytoplasm for further extracellular release. Agents that enhance SIRT1- and other HDAC-activities include acetylcholine [66,89,90], metformin [91], resveratrol [92], statins [93], and additional compounds. Strategies aimed to inhibit specific post-translational modifications of HMGB1 needed for nuclear HMGB1 export may conceivably provide novel opportunities to ameliorate endothelial dysfunction in patients with severe inflammation.

AUTHOR CONTRIBUTIONS

Conceptualization, U.A.; writing original draft preparation, H.Y., L.K.G., A.Z. and U.A.; and writing review and editing, H.Y. and U.A. All authors have read and agreed to this submitted version of the manuscript.

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