THE ROLE OF HMGB1 IN IMMUNITY

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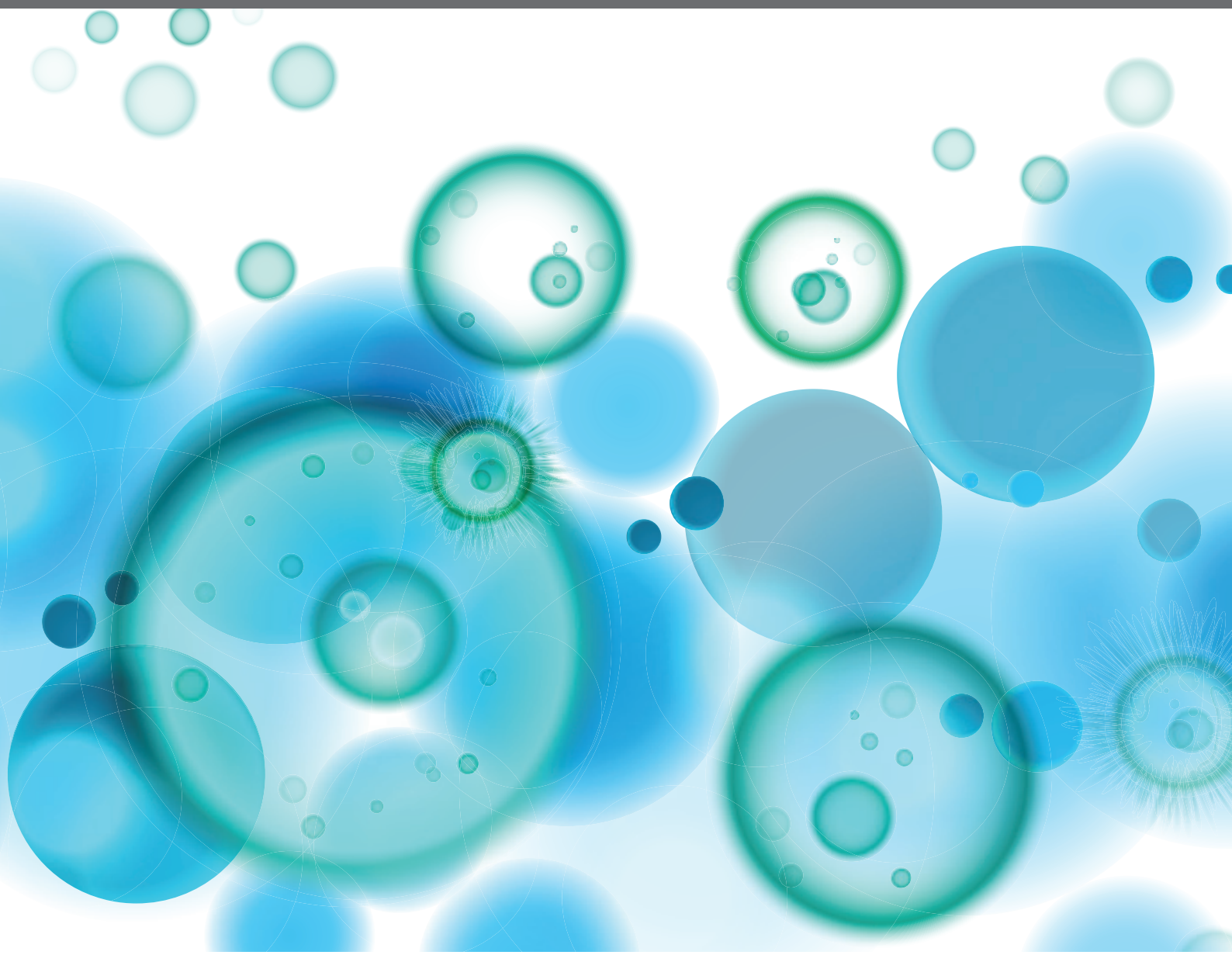

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THE ROLE OF HMGB1 IN IMMUNITY

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EDITORIAL
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Editorial: The Role of HMGB1 in Immunity

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Keywords: HMGB1, secretion, inflammation, sepsis, cancer, immune function, potential therapeutics

Editorial on the Research Topic

The Role of HMGB1 in Immunity

High mobility group box 1 (HMGB1) is an evolutionarily conserved nuclear protein that can be released by almost all cell types. Scientists have uncovered a variety of molecular mechanisms by which HMGB1 in both immune and non-immune cells modulates the nature and magnitude of immune responses (1–3). In recent years, HMGB1-targeted therapies have been exploited in multiple preclinical studies of inflammatory conditions and there is robust clinical evidence for HMGB1 levels as a potential biomarker for early prediction or progression of various diseases. However, it is not presently possible to specifically target HMGB1 in any clinical setting. A significant obstacle to developing therapeutics lies in gaps in knowledge of the post-translational modification of HMGB1 as well as the timing and type of microenvironments to which HMGB1 is exposed.

This Research Topic provides a comprehensive overview of current understanding of the contribution of HMGB1 to various diseases and HMGB1 specific therapeutics. Nine articles are included: five original articles, three review articles, and one mini-review. The authors invited the scientific contributors to this collection based on their unique and pioneering discoveries on the role of HMGB1 in physiological and pathological conditions including: (i) HMGB1-related immune functions (ii) Post-translational modification and secretion mechanisms of HMGB1 (iii) Molecular pathways activated by HMGB1 in acute lung injury, lupus, cancers, and other diseases (iv) Agents to modulate HMGB1 function.

HMGB1-RELATED IMMUNE FUNCTIONS

While many researchers have focused on HMGB1 as an inflammatory mediator that prolongs various inflammatory diseases, another aspect of HMGB1, which is related to its role in tissue healing and regeneration, is being highlighted (4, 5). Yamashiro et al. describe the potential tolerogenic role of HMGB1 in periodontal disease progress, including the cause of inflammation and, conversely, regeneration of periodontal tissue. Further studies are needed regarding HMGB1 isoforms and their receptors that play major roles in the oral cavity to open up opportunities for therapeutics.

Serum HMGB1 is elevated in systemic lupus erythematosus (SLE) patients, and it correlates with disease activity (6). There are several preclinical studies of HMGB1-specific antagonists in experimental lupus models showing inconsistent results. Liu et al. provide a mini-review about the role of HMGB1 in SLE disease phenotypes and a novel agent forcing anti-inflammatory macrophages polarization.

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In the tumor microenvironment, HMGB1 has a protective role in cancer immunity during the early stage of disease. In contrast, sustained HMGB1 recruits immunosuppressive myeloid-derived suppressor cells and regulatory T cells during tumor progression (7). Soloff et al. provide insight into how HMGB1 impacts the microenvironment of malignant pleural effusions (MPEs). The level of HMGB1 was inversely correlated to the diversity of γδ T cells in MPE. The authors suggest some novel therapeutic strategies for targeted HMGB1-neutralization and its usage in pleural effusions.

POST-TRANSLATIONAL MODIFICATION AND SECRETION MECHANISMS OF HMGB1

The dynamics of HMGB1 oxidation in health and disease are unknown. Ferrara et al. confirmed our understanding of functions of HMGB1 redox isoforms using novel applications of *in vivo*-based assay. They demonstrate that the redox state of HMGB1 is controlled at both tissue and cell levels, suggesting that HMGB1 oxidation is a spatially regulated process. Kwak et al. provide an overview of the protein secretion mechanisms. The authors highlight the importance of multiple post-translational modifications and the redox biology of HMGB1, focusing on the vital role of HMGB1 oxidation in its secretion.

MOLECULAR PATHWAYS BY HMGB1 IN HUMAN DISEASES

Sepsis is a life-threatening inflammatory condition with no known cure. HMGB1 is a critical mediator of acute and chronic inflammation in sepsis caused by endotoxin (8). Li W. et al. assess a novel mechanism through which hepatocytes secrete HMGB1 following LPS stimulation that is relevant to sepsis pathogenesis and inflammatory diseases of the liver. The cytoplasmic translocation and later release of HMGB1 from hepatocytes are mediated by a TLR4, Caspase-11, and Gasdermin D-dependent mechanism. HMGB1 is secreted in exosomes. Kim et al. demonstrate the anti-inflammatory effect of sulfatide in suppressing the secretion of HMGB1 and disrupting lipid rafts following LPS stimulation. They suggest that sulfatide is a potential therapeutic agent against sepsis. Li R. et al. explore how HMGB1/PI3K/Akt/mTOR signaling participates in acute lung injury and acute respiratory distress syndrome which are characterized by persistent hypoxemia, disruption of the alveolar-capillary barrier, and widespread inflammation in the lung.

AGENTS TO MODULATE HMGB1 FUNCTION

As mentioned above, HMGB1 antagonists have achieved therapeutic success in a broad set of preclinical inflammatory disease animal models. Yang et al. summarize recent advances in the understanding of HMGB1 as a pro-inflammatory molecule.

Collectively, these articles provide information for other researchers in the field that will eventually help develop novel therapeutic approaches to regulate the function of HMGB1 for the benefit of patients. The next step should be to translate these preclinical studies into clinical settings. Many inflammatory diseases, including the current pandemic COVID-19, are characterized by increased circulating HMGB1 levels (9). HMGB1 possibly plays a role in the increased risk for severe outcomes in COVID-19 patients with inflammatory comorbidities. Overall, HMGB1 is relevant in many diseases and research on HMGB1 can benefit all fields of medicine.

AUTHOR CONTRIBUTIONS

MS wrote the manuscript. BD and J-SS contributed to the elaboration of the manuscript. All authors have approved it for publication.

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REVIEW
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Targeting Inflammation Driven by HMGB1

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High mobility group box 1 (HMGB1) is a highly conserved, nuclear protein present in all cell types. It is a multi-facet protein exerting functions both inside and outside of cells. Extracellular HMGB1 has been extensively studied for its prototypical alarmin functions activating innate immunity, after being actively released from cells or passively released upon cell death. TLR4 and RAGE operate as the main HMGB1 receptors. Disulfide HMGB1 activates the TLR4 complex by binding to MD-2. The binding site is separate from that of LPS and it is now feasible to specifically interrupt HMGB1/TLR4 activation without compromising protective LPS/TLR4-dependent functions. Another important therapeutic strategy is established on the administration of HMGB1 antagonists precluding RAGE-mediated endocytosis of HMGB1 and HMGB1-bound molecules capable of activating intracellular cognate receptors. Here we summarize the role of HMGB1 in inflammation, with a focus on recent findings on its mission as a damage-associated molecular pattern molecule and as a therapeutic target in inflammatory diseases. Recently generated HMGB1-specific inhibitors for treatment of inflammatory conditions are discussed.

Keywords: HMGB1, inflammation, danger signal, RAGE, TLR4, drug target

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INTRODUCTION

Cells are constantly challenged by sterile or infectious stimuli that may cause injury or death. The alarm system sensing danger-induced cellular stress makes use of preformed endogenous molecules termed alarmins or damage-associated molecular pattern molecules (DAMPs) for this interaction (1). They have specified intracellular missions during homeostasis, but promotes inflammation when released in response to danger signals. HMGB1 is a chromatin-binding protein that among several undertakings regulates gene transcription, but operates as a critical DAMP after being released. Excessive amounts of extracellular HMGB1 may cause tissue injury and organ dysfunction in the pathogenesis of many different diseases of both sterile and infectious origin (2–5). Important questions in studies of HMGB1 biology concern how the molecule senses and mediates danger signals during infectious and sterile inflammation. What are the most effective approaches to specifically block HMGB1-driven inflammation? Here we focus on reviewing recent findings addressing these important issues.

HOW DOES EXTRACELLULAR HMGB1 INITIATE INFLAMMATION?

Excessive quantities of extracellular HMGB1, released after cell death or via active secretion, produce inflammation. Receptor usage causing inflammation is totally dependent on whether HMGB1 acts on its own or in complex with partner molecules. HMGB1 is prone to bind other proinflammatory molecules including DNA, RNA, histones, nucleosomes, lipopolysaccharide (LPS), SDF-1, IL-1α, IL-1β, and additional factors. These complexes act in synergy via cognate receptors to the HMGB1-partner molecules. The HMGB1 redox isoform is key when HMGB1 acts on its own as a pro-inflammatory mediator. The redox state of the 3 cysteines present in an HMGB1 molecule determines subsequent bioactivities. Nuclear HMGB1 in a quiescent cell is always in the fully reduced form with all three cysteines expressing thiol groups. The fully reduced HMGB1 released extracellularly forms a complex with the chemokine CXCL12 (SDF-1) and initiates enhanced chemotaxis via CXCR4 compared to CXCL12 acting alone (6). Gentle HMGB1 oxidation generates a disulfide bond between Cys23 and Cys45, but keeping Cys106 in the reduced form. This modification converts extracellular HMGB1 to a potent activator of pro-inflammatory cytokine production via TLR4 receptor stimulation (7). Disulfide HMGB1 loses its capacity to activate TLR4 when it is either reduced or further oxidized. The ability to bind to MD-2 is also abolished by substituting Cys 45 or Cys 106 by an alanine residue (8). Additional oxidation of HMGB1 generates a sulfonyl groups on one or several cysteines resulting in molecules without any proinflammatory capacity on its own (7). The interchange between the reduced and disulfide isoforms is reversible, while sulfonyl HMGB1 is irreversibly converted.

Even if the list of reported HMGB1 receptors is quite extensive, only two receptor systems, RAGE and TLR4, are fully confirmed to act as established HMGB1 receptors. Many of the receptor systems claimed to perform as HMGB1 receptors are actually receptors for molecules complex-bound to HMGB1. When disulfide HMGB1 activates the TLR4 complex, it binds to MD-2 which forces two TLR4 chains together to form a complex that can bind intracellular signal transduction molecules (9). The binding site for HMGB1 on the MD-2 molecule is distinct from that for LPS. The biology created by HMGB1-RAGE interactions is a fascinating story that has recently been delineated (10, 11). There are approximately 700 publications on PubMed examining HMGB1-RAGE activation. The great majority concludes that HMGB1 binding to RAGE leads to a direct NF-kB activation and subsequent cytokine formation. However, macrophages expressing both TLR4 and RAGE, do not produce cytokines when stimulated by any HMGB1 isoform if TLR4 is functionally inactivated or absent. That would not be the expected result if HMGB1-RAGE activated cytokines directly. The novel discoveries by Lu and Billiar revealed that RAGE provides a transport route for HMGB1, and above all, for HMGB1-partner molecule complexes by endocytosis to the endolysosomal compartment (11). Under the acidic conditions in the lysosome system, HMGB1 has the unique ability to act as a detergent in the lysosomal membrane. The HMGB1-transported partner molecules will thus not be degraded in the lysosomes as expected, but leak out from the permeabilized lysosomes into the cytosol to reach cognate cytoplasmic receptors that will be activated to cause inflammation (11).

HMGB1 holds two defined LPS-binding sites enabling HMGB1 to bring LPS from the extracellular space via RAGE and the lysosomal compartment to cytosolic caspase 11. TLR4 deficient mice have been shown to succumb to endotoxemia in the presence of increased levels of HMGB1, while caspase 11 gene deficient mice survived (12, 13). These results emphasize the functional importance of caspase-11 as a pathogenic LPS receptor. HMGB1 operates as an LPS-carrier necessary to enable caspase-11-mediated pyroptosis. Caspase-11 oligomerization and activation are caused by LPS lipid A binding to the CARD domain of caspase-11 (14). This activated oligomerized form of caspase-11 cuts gasdermin D and the truncated gasdermin D will subsequently generate pores in the plasma membrane resulting in secretion of IL-1α, IL-1β, and HMGB1 and may terminally cause cellular pyroptosis (15). Another fundamentally important function exerted by cleaved gasdermin D is to promote coagulation running the risk to escalate into disseminated intravascular coagulation (DIC), a life-threatening event during systemic inflammation. It has recently been demonstrated that gasdermin D-induced pores can generate enhanced cell membrane expression of rotated phosphatidylserine enabled via a calcium-dependent phospholipid scramblase (15). This process markedly promotes the pro-coagulant activity of tissue factor, a central initiator of coagulation. The HMGB1-mediated transfer of LPS to caspase-11 thus represents the initial step in the cascade culminating in DIC generation.

An analogous strategy used by extracellular LPS to reach cognate intracellular receptors has also been identified for extracellular nucleic acids (16). Extracellular DNA bound to HMGB1 can be endocytosed by cells via RAGE to reach cognate DNA receptors like endosomal TLR9 or cytoplasmic cGAS or the AIM2 inflammasome complex (16–19). This biology may have detrimental effects in flares of lupus or in response to major trauma. The HMGB1/RAGE-assisted cellular import system thus performs an important task by alerting cells about a dangerous environment. HMGB1 is an alarmin with dual functions-warning the extracellular environment about cells in distress and informing cells about a hazardous extracellular surrounding.

PATHOGENIC ROLE OF HMGB1 IN IMMUNOSUPPRESSION IN SEPSIS

Sepsis is attributable to both exaggerated inflammatory responses and subsequent immunosuppression (20–22). When initially secreted by innate immune cells at relatively low amounts, HMGB1 might still be pro-inflammatory during the early stages of sepsis (23). However, when it is released by the liver (11) and other somatic cells at overwhelmingly higher quantities, HMGB1 could also induce immune tolerance (24, 25), macrophage pyroptosis (10, 11), and immunosuppression (26), thereby impairing the host's ability to eradicate microbial infections (27, 28).

This notion is supported by the relative higher affinity of HMGB1 to receptors (e.g., TLR4/MD-2 complex, with a disassociation equilibrium constant of 12 nM) that are involved in the activation of innate immune cells (9), whereas HMGB1 has a relative lower binding affinity to other receptors (e.g., RAGE with a disassociation equilibrium constant of 97–710 nM) (29, 30) that are required for HMGB1 endocytosis and the resultant macrophage pyroptosis. We thus propose that upon active secretion by innate immune cells or passive release by somatic cells, extracellular HMGB1 binds TLR4 (31) to induce the expression and production of various cytokines and chemokines, but triggers macrophage pyroptosis if it binds to RAGE and is internalized via receptor-mediated endocytosis (10, 11).

As aforementioned, HMGB1 can also bind many negatively charged pathogen-associated molecular pattern molecules (PAMPs, e.g., CpG-DNA, endogenous extracellular DNA or LPS) to facilitate their cellular uptake via similar RAGE-receptor-mediated endocytosis. Consequently, HMGB1 not only augments the PAMP/DAMP-induced inflammation (16), but also promotes the PAMP/DAMP-induced pyroptosis (11), leading to dysregulated inflammatory responses as well as macrophage depletion and possible immunosuppression during sepsis. In light of our recent finding that an HMGB1-neutralizing mAb (e.g., m2G7), capable of rescuing animals from lethal sepsis and acute liver injury could also inhibit HMGB1 endocytosis (32), we propose that therapeutic strategies capable of modulating HMGB1-mediated immune over-activation and/or associated immunosuppression could be developed in the clinical management of inflammatory diseases.

HMGB1 ANTAGONISTS OF POTENTIAL CLINICAL INTEREST

Several different strategies have been shown successful in inhibiting HMGB1-dependent inflammatory processes, especially aiming at blocking TLR4-HMGB1 or RAGE-HMGB1 pathways. Anti-HMGB1 antibodies and recombinant HMGB1 box A protein have each demonstrated beneficial effects in a wide range of preclinical models of inflammatory diseases (5, 33). Here we report on selected HMGB1 antagonists with a potential of being brought to clinical trials in HMGB1-driven inflammatory diseases.

MOLECULES INHIBITING RAGE-MEDIATED ENDOCYTOSIS OF HMGB1 OR LPS-HMGB1 COMPLEXES

Previous studies established that RAGE mediates HMGB1 endocytosis via dynamin-dependent signaling (10). The concept that extracellular HMGB1-LPS complexes are imported via cell surface-expressed RAGE to the endolysosomal system from where LPS leaks out into the cytosol to activate caspase 11 has been discussed in this review (11). The study by Deng et al. also confirmed one previous report that treatment with anti-HMGB1 mAb m2G7 improves survival in experimental gram-negative sepsis (34). The observation that RAGE-mediated endocytosis of HMGB1 complexes is a pivotal event in gram-negative sepsis prompted us to study therapeutic candidate molecules with a capacity to prevent the cellular internalization of HMGB1/LPS and subsequent inflammation. We thus generated an *in vitro* assay to identify agents that inhibited RAGE-dependent import in macrophages of fluorochrome-labeled HMGB1 or fluorochrome-labeled complexes of HMGB1 and LPS (32). Our main discoveries were that m2G7, recombinant HMGB1 box A protein, acetylcholine, the nicotinic acetylcholine receptor subtype alpha 7 agonist GTS-21, and a dynamin inhibitor, all prevented cell activation and endocytosis of HMGB1, as well as of HMGB1/LPS complexes in cultured macrophages (**Figure 1**). The intriguing clinical therapeutic correlate to each one of these identified HMGB1 antagonists is that they can be delivered with exceptional delay (up to 24 h after sepsis initiation) with beneficial effects (35–38). This unique, and clinically important, wide therapeutic window is most likely mechanistically enabled by obstructing the HMGB1/RAGE transport route.

HMGB1 Box A Protein

Recombinant HMGB1 box A protein has been successfully used to treat a number of experimental inflammatory models, but its mode of action has, until now, been an unresolved issue. The identification of box A-blockade of RAGE-mediated cellular import of HMGB1 and HMGB1-partner molecule complexes thus represents considerable progress, not the least because this knowledge enables an opportunity to evaluate the biological activity of individual box A batches *in vitro*. A lack of such technology has so far prevented a clinical development of box A protein. Beneficial preclinical effects by box A therapy was first reported in experimental arthritis (39), followed by CLP sepsis (35), transplantation (40), stroke (41), ischemia-reperfusion injury (42), pancreatitis (43), and acute lung injury (44).

MOLECULES INHIBITING HMGB1/TLR4-MEDIATED INFLAMMATION

Peptide P5779

Macrophages that do not express TLR4 do not display nuclear NF–κB translocation or cytokine production when activated by any HMGB1 redox isoform (7). Disulfide HMGB1 subjected to cysteine mutations or redox changes loses the ability to activate TLR4. MD-2 gene-deficient macrophages do not release TNF in response to disulfide HMGB1 or any other HMGB1 isoform. Disulfide HMGB1, like LPS, binds to MD-2 with low nanomolar avidity, but to distinct MD-2 epitopes. We generated a tetramer peptide (FSSE, designated P5779) as an HMGB1 inhibitor specifically targeting the HMGB1-TLR4/MD-2 pathway (9). P5779 binds exclusively to MD-2 at the HMGB1 binding site, which preserves the responsiveness to endotoxin. The P5779 peptide does not inhibit RAGE-mediated endocytosis of HMGB1 or HMGB1/LPS complexes. P5779 (but not the scrambled control peptide) dose-dependently inhibited HMGB1-induced TNF release without affecting LPS-induced cytokine and chemokine release in primary human macrophage cultures. Therapeutic administration of P5779

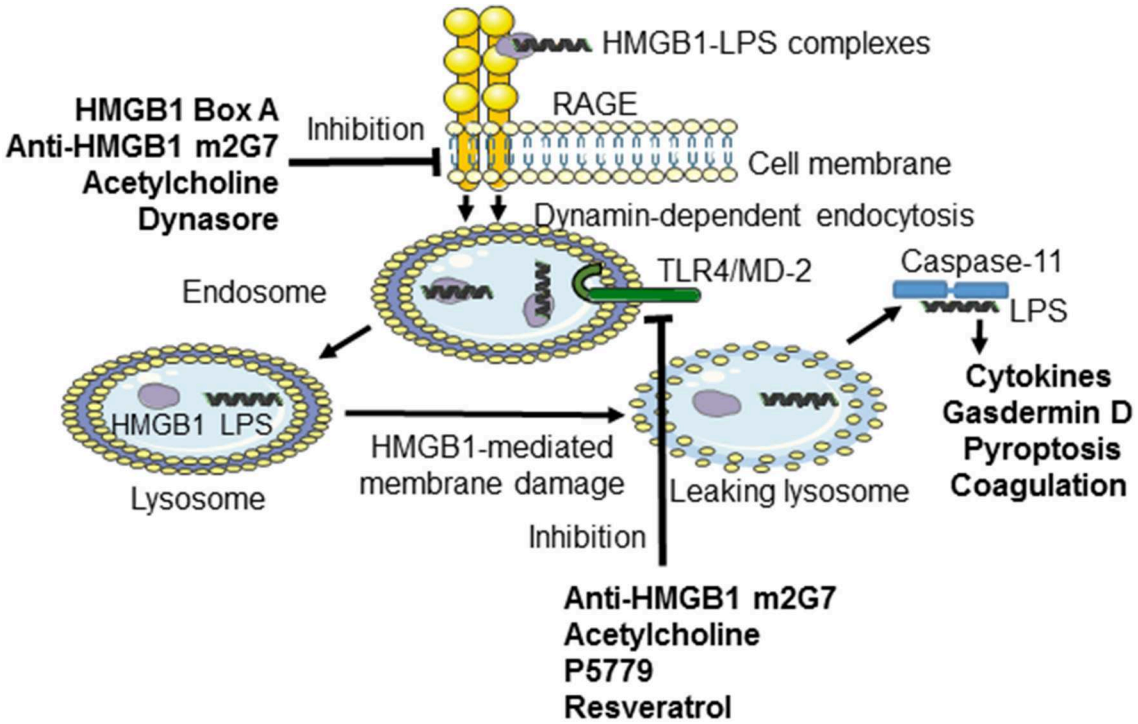

FIGURE 1 | Inhibiting TLR4- or RAGE-mediated effects induced by HMGB1 or LPS-HMGB1 complexes. During endotoxemia, LPS and extracellular HMGB1 forms complexes that are endocytosed via the RAGE-dependent pathway. LPS and HMGB1 activate TLR4 system. The unique contribution by HMGB1 is disruption of the lysosomal membrane enabling LPS to reach and activate its cytosolic receptor caspase-11, which cleaves gasdermin D to form an active oligomer. Activated gasdermin D will subsequently start coagulation and cause cellular pyroptosis in murine macrophages. The HMGB1-specific inhibitors recombinant HMGB1 box A, anti-HMGB1 m2G7, and acetylcholine each inhibits the cellular internalization of LPS-HMGB1 complexes and resultant immune activation. Anti-HMGB1 m2G7 and acetylcholine also inhibit HMGB1/TLR4-mediated inflammation, whereas P5779 and resveratrol selectively block the HMGB1/TLR4 pathway only.

protected against experimental hepatic ischemia/reperfusion-induced injury, acetaminophen-induced liver toxicity and CLP sepsis lethality (9). Furthermore, the clinical outcome of murine influenza infection was significantly improved by treatment with P5779 (45). Therapy based on P5779 administration also alleviated experimental endoluminal arterial injury-induced intimal hyperplasia and up-regulation of TLR4, HMGB1, and IL-6 expression in the affected carotid vessels. Global TLR4 gene-deficient mice demonstrated reduced inflammation and diminished HMGB1 expression after arterial injury, further supporting that HMGB1 and TLR4 are essential for vascular inflammatory responses (46). P5779 treatment conferred a striking survival advantage in an experimental pulmonary arterial hypertension model (47). Using a molecular dynamic simulation approach and surface plasmon resonance analysis, Sun et al. (48) identified that several folic acid peptides mimic the binding interaction of P5779 at the TLR4/MD-2 interaction. Addition of these P5779 mimetic peptides inhibited HMGB1-induced TNF release in cultured human macrophages. Taken together, P5779 acts as an HMGB1-inhibitor specifically targeting HMGB1-TLR4 interaction and efficiently ameliorates HMGB1/TLR4-driven inflammatory diseases (**Table 1**; **Figure 1**).

TABLE 1 | Summary of efficacy of P5779 in HMGB1-driven inflammatory diseases.

Models	Findings	References
Acetaminophen liver toxicity in mice	Improved survival, reduced serum liver enzymes, reduced liver necrosis	(9)
Liver ischemia/reperfusion in mice	Reduced serum liver enzymes and liver inflammation	(9)
CLP-sepsis in mice	Improved survival	(9)
Arterial injury model in mice	Reduced carotid artery injury-induced intimal hyperplasia and TLR4, HMGB1, and IL-6 expression in injured vessels	(46)
Influenza in nuce	Improved survival and reduced lung edema in influenza infection	(45)
Puhnonary hypertension in rats	Improved survival in monocrotaline-induced severe pulmonar y hypertension	(47)
In vitro	Reduced HMGB1-induced TNF release from cultured human macrophages	(48)

Anti-HMGB1 mAb (m2G7)

Since the development of our anti-HMGB1 m2G7 (34), many laboratories have independently confirmed the efficacy of other

anti-HMGB1 mAb in many different models of sterile or infectious inflammation. The m2G7 binds to an epitope in the box A (located in HMGB1 sequence amino acids 53–63) and this binding functionally affects both HMGB1 interactions with RAGE and TLR4. Other published anti-HMGB1 mAbs have not been studied from the perspective of HMGB1 receptor inhibition and thus will not be further discussed in this section.

The m2G7 has been demonstrated to inhibit TNF production in macrophages activated by recombinant disulfide HMGB1, by HMGB1 from cultured HMGB1-transfected mammalian cells, and by HMGB1 derived from necrotic fibroblasts (7, 9). This is proof of m2G7-caused antagonistic effects on HMGB1-TLR4-mediated processes. There are many examples of preclinical HMGB1-dependent models which respond favorably to therapeutic administration of the m2G7 (**Table 2**). However, the inflammation is generally caused by HMGB1 activation of both TLR4 and RAGE and it is most often not possible to discriminate between the specific contributions by each receptor system. The first evidence of successful performance by the m2G7 *in vivo* came from CLP sepsis studies (34), when m2G7 therapy improved survival, a result which was confirmed in the recent report by Deng et al. (11). Systemic HMGB1 levels are increased during the acute stage of sepsis, but persistently elevated for weeks or months in both mice and patients for unknown reasons (50, 56–58). The increased HMGB1 levels post-sepsis exert a causative role for post-sepsis complications including cognitive dysfunction and anemia in the mouse CLP model. Both complications also occur after clinical sepsis, but the molecular background for this is unresolved. It is tempting to suggest HMGB1 as a cause also in the clinical situation, since HMGB1 is 99% identical in all mammals. Mice surviving CLP sepsis developed significant and persistent impairment in learning and memory, and anatomic changes in the hippocampus. Administration of the m2G7 10 days from the onset of CLP-sepsis to the survivors significantly ameliorated memory and learning disabilities, and hippocampal pathology. Systemic administration of disulfide HMGB1 reproduced the neuropathology seen after CLP sepsis (49). Systemic HMGB1 administration also caused anemia with extramedullary erythropoiesis just like CLP surviving mice. Treatment with the m2G7, provided post the acute CLP-sepsis stage, prevented the development of anemia in sepsis survivors in mice (50).

Multiple preclinical inflammatory sterile injury models likewise respond positively to m2G7 therapy. Improved islet viability and reduced inflammation after syngeneic islet graft transplantation in diabetic mice were observed in response to systemic m2G7 therapy (51). Collagen-induced arthritis and a spontaneous arthritis model were both ameliorated by m2G7 treatment. Joint destruction was prevented and clinical arthritis scores improved (52). Intrathecal m2G7 injection reversed collagen antibody-induced arthritis-induced chronic pain reactions (53). HMGB1 is an important down-stream mediator in the pathogenesis of acetaminophen intoxication and causes serious liver damage. Treatment with m2G7 significantly inhibited acetaminophen-induced release of hepatic enzymes, pro-inflammatory cytokines, and improved survival in mouse studies (9). Lundback et al. (54) confirmed these experimental results and demonstrated that administration of a humanized version of the m2G7 significantly attenuated acetaminophen-induced elevation of microRNA-122, a liver-specific microRNA, and serum levels of TNF, MCP-1, and CXCL1. Likewise, survival in experimental pulmonary hypertension in rats was significantly enhanced after m2G7 treatment (47). Systemic, as well as cardiac, HMGB1 levels are increased in mice with troponin-induced experimental autoimmune myocarditis and m2G7-based therapy reduced the cardiac inflammation and HMGB1 expression (55) (**Table 2**).

TABLE 2 | Summary of efficacy of anti-HMGB1 m2G7 in HMGB1-driven inflammatory diseases.

Models/species	Findings	References
Infectious diseases		
CLP sepsis or endotoxemia in mice	Reduced lethalit y in CLP-induced sepsis and in endotoxemia	(11, 34)
CLP sepsis-survivors in mice	Reduced sepsis-induced memory impairments and brain pathology in survivors	(49)
CLP sepsis-survivors in mice	Ameliorated sepsis-induced development of anemia and stress erythropoiesis	(49, 50)
Sterile injury		
Islet transplantation in diabetic mice	Improved islet viability and reduced transplantation-induced inflammation	(51)
Chronic arthritis in mice	Ameliorated clinical arthritis scores, partially prevented joint destruction	(52)
Arthritis pain in mice	Ameliorated pain-like behavior in collagen antibody induced arthritis	(53)
Acetaminophen (APAP)-induced liver toxicity in mice	Attenuated APAP-induced release of ALT, microRNA-122, and abrogated inflammation	(9, 54)
Autoimmune myocarditis in mice	Reduced cardiac inflammation	(55)
Puhnonary hypertension in rats	Improved survival in monocrotaline-induced severe puhnonary hypertension	(47)

There are also reported therapeutic failures with the m2G7 in preclinical trials. Administration of m2G7 in a mouse model of amyotrophic lateral sclerosis showed overall very limited efficacy (59).

Treatment with m2G7 did not affect lupus nephritis in MRL/lpr mice, despite the fact that systemic levels of HMGB1 are increased in lupus (60). Successful therapeutic outcome has in contrast been reported in another mouse lupus model using a different anti-HMGB1 mAb (61).

Resveratrol

Resveratrol is a phytoalexin phenol molecule acting as a protective endogenous antibiotic when produced in plants under

stress. Resveratrol also reduces LPS-induced levels of HMGB1, IL-6, NO, and TNF in RAW 264.7 cell cultures. This TLR4-dependent process was downregulated by resveratrol-mediated inhibition of TLR4 expression (62). Resveratrol, markedly inhibited microglia activation and display of TLR4, HMGB1, MyD88, and NF-κB in the brain cortex in an experimental subarachnoid hemorrhage model (63). Furthermore, resveratrol demonstrated similar neuroprotective and anti-inflammatory effects in a neonatal hypoxic-ischemic brain injury model. Mechanistic *in vitro* and *in vivo* studies indicated that resveratrol activated SIRT1 to reduce HMGB1/TLR4/MyD88/NF-κB signaling and subsequent neuroinflammatory responses (64). The compound also demonstrated beneficial effects in an asthma model by decreasing the expression of HMGB1, TLR4, MyD88, and NF-κB mRNA levels in the lung tissue and significantly decreased the thicknesses of the airway walls (65). Together, these results indicate that resveratrol ameliorates inflammation in part via inhibition of HMGB1/TLR4-mediated inflammation (**Figure 1**).

Dexmedetomidine

Dexmedetomidine is a α2-adrenoceptor agonist with anti-inflammatory effects mediated via activation of the cholinergic anti-inflammatory pathway (66). Dexmedetomidine treatment in experimental endotoxemia attenuated inflammation through downregulated TLR4 expression via a α7 nicotinic acetylcholine receptor-dependent pathway (67). It is thus of great interest that acetylcholine has the capacity to functionally inhibit both the TLR4 and RAGE pathways, the major receptor HMGB1 systems (32, 67, 68).

ADDITIONAL HMGB1 ANTAGONISTS OF CLINICAL INTEREST

Anti-HMGB1 mAb #10–22

Another extensively studied anti-HMGB1 mAb has been developed by a Japanese research group (69). The antibody, termed #10–22, recognizes an epitope in the repetitive C-terminal sequence. Successful therapeutic interventions are reported in a number of experimental neuro-inflammatory conditions, including stroke (70), traumatic brain injury (71), cognitive dysfunction after traumatic brain injury (72), spinal cord injury (73), epilepsy (74, 75), blood brain barrier dysfunction after CNS ischemia (76), hemorrhage-induced brain injury (77), neuropathic pain (78–83), and neuropathic pain-related depressive behavior (84). The antibody has also demonstrated beneficial effects in severe mouse influenza models (85, 86). Taken together, these findings demonstrated impressive treatment results in severe preclinical disease models.

Thrombomodulin

Thrombomodulin is an endothelial cell thrombin receptor that converts thrombin into an anticoagulant. Soluble thrombomodulin also binds to HMGB1 and aids the proteolytic cleavage of HMGB1 by thrombin (87). Recombinant thrombomodulin is successfully used in Japan to treat patients with disseminated intravascular coagulation in sepsis (88).

Haptoglobin

The major task of the acute phase protein haptoglobin is to bind and eliminate extracellular hemoglobin. Haptoglobin is in addition capable of capturing extracellular HMGB1. The haptoglobin-HMGB1 binds to CD163 on macrophages activating an anti-inflammatory response mediated via IL-10 and heme-oxygenase 1 production (89). Therapeutic administration of haptoglobin improved septic shock, lung injury, and survival in a canine pneumonia model (90). Haptoglobin is approved as an adjuvant therapy for patients in Japan with trauma, burns, and transfusion-related hemolysis.

Metformin

Metformin occupies an important role in type 2 diabetes treatment. Metformin also has an anti-inflammatory effects, although these effects are not mechanistically fully understood. Metformin inhibits nuclear HMGB1 translocation to the cytosol and thus retains HMGB1 in the nucleus after cell activation (91). Metformin also binds directly to the C-terminal domain of HMGB1 and down-regulates inflammation by counteracting the extracellular activity of HMGB1 (92). Furthermore, the compound inhibits HMGB1 release and increases survival rate of endotoxemic mice (93).

DNA-Conjugated Beads

HMGB1 is released and present at high levels in intestinal tissue and feces in patients with chronic inflammatory bowel diseases (IBD). Several experimental IBD models have responded very well to systemic treatment with neutralizing anti-HMGB1 antibodies. HMGB1 is a well-known DNA-binding protein, which offers an opportunity to sequester HMGB1 via DNA-conjugated beads that has been studied in experimental colitis. Oral treatment with DNA-conjugated beads significantly improved outcome in two different preclinical colitis models (94).

CONCLUSION

HMGB1 antagonists have been highly successful in a broad set of preclinical inflammatory disease models**,** corroborating HMGB1 as an appealing therapeutic target in both infectious and sterile inflammatory conditions that currently lack efficient therapy. The next step should be to translate these preclinical studies to a clinical setting. Most preclinical treatment trials have targeted extracellular HMGB1. We suggest that this strategy should also be the preferred approach in initial future clinical studies, since we need to learn more about critical intracellular functions of HMGB1 before starting therapy studies with intracellular HMGB1 antagonists. Regardless of the indication, the success of future therapy with HMGB1 antagonists will depend on an ability to accurately measure HMGB1 on standard hospital-based instruments

in order to target patients expressing excessive quantities of HMGB1.

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LPS Induces Active HMGB1 Release From Hepatocytes Into Exosomes Through the Coordinated Activities of TLR4 and Caspase-11/GSDMD Signaling

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High-mobility group box-1 (HMGB1), a ubiquitous nuclear protein, acts as a late mediator of lethality when released extracellularly during sepsis. The major source of circulating HMGB1 in sepsis is hepatocytes. However, the mechanism of HMGB1 release of hepatocytes during sepsis is not very clear. We have previously shown that bacterial endotoxin [lipopolysaccharide (LPS)] sensing pathways, including Toll-like receptor (TLR)4 and caspase-11, regulate hepatocyte HMGB1 release in response to LPS. Here, we report the novel function of caspase-11 and gasdermin D (GsdmD) in LPS-induced active HMGB1 released from hepatocytes. HMGB1 release during endotoxemia was caspase-11/GsdmD dependent via an active way *in vivo* and *in vitro*. Caspase-11/GsdmD was responsible for HMGB1 translocation from nucleus to the cytoplasm via calcium changing-induced phosphorylation of calcium-calmodulin kinase kinase (camkk)β during endotoxemia. Cleaved GsdmD accumulated on the endoplasmic reticulum, suggesting this may lead to calcium leak and intracellular calcium increase. Furthermore, we investigated that exosome was an important pathway for HMGB1 release from hepatocytes; this process was dependent on TLR4, independent of caspase-11 and GsdmD *in vivo* and *in vitro*. These findings provide a novel mechanism that TLR4 signaling results in an increase in caspase-11 expression, as well as increased exosome release, while caspase-11/GsdmD activation/cleavage leads to accumulation of HMGB1 in the cytoplasm through a process associated with the release of calcium from the endoplasmic reticulum and camkkβ activation.

Keywords: gasdermin D (GsdmD), endotoxemia, caspase-11, extracellular vesicles, calcium, innate immunity

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INTRODUCTION

Sepsis is a dysregulated inflammatory and metabolic state associated with infection. This dysregulated state is associated with multi-organ dysfunction and high mortality (1). Endotoxin [lipopolysaccharide (LPS)], a constituent of Gram-negative bacteria, stimulates immune and non-immune cells to release excessive levels of inflammatory mediators (e.g., cytokines), which can precipitate tissue injury and lethal shock. However, blocking single cytokines early in the course of sepsis has not improved outcomes during clinical trials (2). This led to the search for late mediators of lethality in sepsis, and this search yielded high-mobility group box-1 (HMGB1), a nuclear protein that is released by the liver during sepsis that can drive pyroptosis, immune dysfunction, and lethality in sepsis models (3, 4).

Our findings (5) and the findings of others (6) established that active release of HMGB1 by hepatocytes is the dominant source of systemic levels of HMGB1 during endotoxemia and sepsis. HMGB1 contributes to lethality in sepsis by delivering extracellular LPS to cytosolic caspase-11 in macrophages and endothelial cells (5). This, in turn, leads to macrophage and endothelial cell pyroptosis that then propagates the systemic inflammatory response and immune dysfunction (7–9). Caspase-11 (caspase 4 and 5 in humans) belongs to the family of inflammatory caspases and is also referred to as the non-canonical inflammasome. The binding of cytosolic LPS to the caspase activation and recruitment domain (CARD) of caspase-11 leads to its oligomerization/activation (8). Active caspase-11 promotes caspase-1 activation, and both caspases cleave gasdermin D (GsdmD) (10, 11). The N-terminal fragment of GsdmD forms 10- to 14-nm pores in artificial or natural phospholipid mixtures (12, 13).

The intracellular steps that lead to the active release of HMGB1 by hepatocytes in response to LPS are unknown. Interestingly, this release is known to involve Toll-like receptor (TLR)4-mediated LPS uptake by hepatocytes and is caspase-11 dependent (5, 14). While TLR4 is required for the upregulation of caspase-11 in hepatocytes exposed to LPS, how these two LPS sensing pathways then regulate the release of HMGB1 is not known. Here, we show that hepatocytes mobilize HMGB1 from the nucleus to the cytosol through a process that requires caspase-11-dependent GsdmD cleavage, increases in intracellular calcium, and calcium-calmodulin kinase kinase (camkk)β activation. We provide evidence that a cleavage fragment of GsdmD inserts into the endoplasmic reticulum (ER) membrane and may initiate calcium-dependent signaling. Extracellular release of HMGB1 takes place via exosomes, and this requires receptor-specific roles for TLR4 and caspase-11/GsdmD. These findings illuminate a novel pathway for the active release of HMGB1 from hepatocytes that is relevant to sepsis lethality.

EXPERIMENTAL PROCEDURES

Exosome Isolation and Quantification

Exosome isolation from hepatocyte culture media was performed as described previously (15). Briefly, cell culture media was centrifuged at 500 × g for 10 min, 16,500 × g for 20 min, followed by filtration through a 0.2-μm filter (Life Sciences). Exosomes were pelleted at 120,000 g for 70 min with Type 70.1 Ti rotor (Beckman). The exosomes were further washed once with phosphate buffered saline (PBS) and centrifuged at 120,000 g for 70 min, then resuspended in a small volume of PBS for NanoSight™ assessment or in lysis buffer (1:10 dilution, Cell Signaling Technology, #9803) for Western blot. Plasma exosomes were isolated using the total exosome isolation kit (Invitrogen) according to manufacturer's instructions. The pellet was resuspended in sample dilution buffer for ELISA or lysis buffer for Western blot.

Animal Model

Male C57BL/6J wild-type (WT) mice were purchased from Jackson Laboratory. GsdmD knockout (KO) mice were obtained from Dr. Vishva Dixit (Genetech). TLR-4 KO (16), caspase-11 KO (5), and GsdmD KO (17) mice on C57BL/6 background were bred in Dr. Billiar's lab. We also generated mice with selective Hmgb1 deletion (5) in either myeloid cells ($HMGB1^{f/f}$ Lyz2-cre^{+}) or hepatocytes ($HMGB1^{f/f}$ Alb-cre^{+}). All animals were housed or bred in the specific pathogen-free animal facility at the University of Pittsburgh School of Medicine and were kept under a 12-h dark/light cycle, fed standard chow *ad libitum*.

Mice were intraperitoneally (i.p.) injected with 5 mg/kg LPS for the time indicated in the experiments. Knockdown of Rab27a *in vivo* was performed as previously (18). Briefly, 1×10^9 plaque forming unit (PFU) adenoviruses Rab27a shRNA (Vector Biolabs, Malvern, PA) were injected into the penile vein of mice anesthetized by isoflurane. Two days after virus injection, mice were injected i.p. with LPS or saline. For exosome release inhibition *in vivo*, GW4869 dissolved in dimethyl sulfoxide (DMSO) (0.005%) was pre-injected into the penile vein at one dose of 2.5 mg/kg 1 h before LPS treatment.

Isolation and Culture of Hepatocytes

Cells were isolated from mice by an *in situ* collagenase (type VI; Sigma) perfusion technique, modified as described previously (19). Cell viability was typical >95% by trypan blue exclusion. Hepatocytes (4×10^5 cells/plate for six-well plates, 5×10^6 cells/plate for 10-cm plates) were plated on gelatin-coated culture plates in Williams medium E with 10% calf serum, 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10^{-6} M insulin, 2 mM L-glutamine, 100 U/ml penicillin, and streptomycin. Cells were allowed to attach to plates for at least 4 h before treatment.

Cell Treatment

Primary hepatocytes were treated with or without 1 μg/ml LPS in serum-free liver media (15 mM HEPES, 10^{-6} M insulin, 2 mM L-glutamine, 100 U/ml penicillin, and streptomycin) for 24 h. Culture media from two 10-cm plates for each group were harvested for exosome isolation. Proteins in the supernatant were extracted using methanol/chloroform. Total lysates were prepared using lysis buffer (1:10, Cell Signaling Technology). GW4869 and spiroepoxide were prepared as previously described (20). For exosome inhibition *in vitro*, GW4869 or spiroepoxide

was added 2 h before LPS treatment (1 μg/ml LPS for 4 or 8 h). For knockdown of caspase-11 or GsdmD, 300 ng siRNA was diluted in 400 μl serum-free medium with 12 μl HiperFect™ transfection reagent (Qiagen) and mixed by vortexing. The mixture was incubated for 10 min at room temperature, added dropwise to the cells in 2-ml medium with 10% fetal bovine serum (FBS) (final siRNA concentration was 10 nM), swirled, and cultured the cells for 48 h.

Isolation of Nuclear/Cytoplasmic Protein and Endoplasmic Reticulum

Nuclear and cytoplasmic proteins were prepared using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher) according to the manufacturer's instructions. ER was prepared using an ER isolation kit (Sigma) according to the manufacturer's instructions. Nuclear/cytoplasmic or ER proteins were quantified using Pierce™ BCA protein assay kit (Thermo Fisher).

Immunofluorescent Staining on Primary Hepatocytes and Tissue Sections

Primary hepatocytes cultured on coverslips were treated as described and then fixed with 2% (w/v) paraformaldehyde in PBS for 15 min. Residual paraformaldehyde was removed by multiple washes with PBS, and cells were permeabilized with 0.1% Triton X-100 in PBS for 15 min at room temperature, washed with PBS and PBB (0.5% BSA in PBS) and blocked for 1 h with 20% normal goat serum (NGS, Sigma) in PBS. Then samples were incubated with the specific primary antibody for HMGB1 (Abcam, 1:200) in 1% BSA for 1 h, washed, and incubated with secondary antibody (goat anti-rabbit-Cy3, Jackson ImmunoResearch, 1:1,000). Additional *in vitro* experiments were performed with primary hepatocytes cultured on coverslips that were treated with Zombie Red™ viability dye (1:1,000, Biolegend) at room temperature in the dark for 30 min. All immunofluroescent staining sets involved staining the nuclei with Hoechst (1 mg/100 ml; Sigma) was applied at room temperature for 30 s followed by a single rinse of PBS to remove excess dye. *In vitro* samples cultured to collagen coated coverslips were adhered on the cell surface side of the coverslip to slides using Gelvatol [23 g of poly(vinyl alcohol 2000), 50 ml of glycerol, 0.1% sodium azide to 100 ml of PBS].

Liver tissue removed after perfusion with cold PBS and 2% paraformaldehyde was incubated for an additional 2 h to complete tissue fixation and then incubated for 24 h in 30% sucrose, followed by cryopreservation in liquid nitrogen cooled 2-methylbutane. Tissue sections of 6 μm were permeabilized with 0.3% Triton X-100 for 20 min, followed by staining according to the manufacturer's protocol of the *in-situ* Cell Death Detection Kit-TMR red (Roche). Samples were washed with PBS prior to being coverslipped using Gelvatol.

Regardless of the source of samples, all imaging conditions were maintained at identical settings with original gating performed using the primary delete control (no primary antibody). Large area images in X and Y were taken at a magnification of 20× with a two-fold digital zoom for the equivalent of nine fields/section with a Nikon A1 confocal microscope (purchased with 1S10OD019973-01 awarded to Dr. Simon C. Watkins). Quantification was performed in a blinded fashion using NIS Elements Software (Nikon). In brief, the Nikon NIS elements quantification software measure amount of cell death (either TMR or Zombie) fluorophore colocalized with the nuclear Hoechst fluorescences. The amount of HMGB1 content was measured for total HMGB1 fluorescences, as well as the amount of HMGB1 that colocalized with the nuclear content to enable the reporting of nuclear HMGB1, and cytosolic HMGB1 was analyzed as the amount of HMGB1 that did not colocalize with the nuclear HMGB1 content.

Liver Damage Assessment

Mouse plasma was used for alanine aminotransferase (ALT) test. ALT levels were measured using the DRI-CHEM 4000 Chemistry Analyzer System (Heska). The ALT values were expressed as international units per liter.

Intracellular Ca^{2+} Measurement

Cells were plated on a 96-well black clear bottom plate. After LPS treatment, cells were washed and loaded with the ratiometric Ca^{2+} indicator Fura-2/AM in calcium-free Hank's balanced salt solution (HBSS) [at 37°C, 5% carbon dioxide (CO_2)] for 30 min, washed, and incubated for an additional 30 min prior to testing. Excitation was carried out at 340 and 380 nm, and emissions were collected at 510 ± 10 nm using BioTek SynergyMx multi-format microplate readers.

Exosome NanoSight™ Analysis (Nano Tracking Analysis)

Exosome samples were analyzed as previously described (21). Briefly, exosomes isolated from 100 μl plasma were resuspended in 100 μl PBS and diluted 1:10,000 in particle-free water (W4502, Sigma). Exosomes isolated from 10^7 cells were resuspended in 50 μl PBS and diluted 1:10,000 in particle-free water. After vortexing, the diluted samples were injected into the NTA LM-10 system continuously using a syringe pump. Particles were acquired by the machine, and data were analyzed with NTA particle analysis software.

ELISA Assay

HMGB1 ELISA Kit (IBL, Hamburg, Germany) was used to detect plasma HMGB1 levels according to the manufacturer's instructions. CD81 ELISA Kit (Cusabio, Wuhan, China) was used to detect plasma exosome samples according to the manufacturer's instructions.

Western Blot

Antibodies for Western blot analysis were as follows: anti-HMGB1 (1:1,000, Abcam), anti-caspase-11 (1:500, Sigma), anti-TSG101 (1:500, Novus), anti-CD81 (1:500, Novus), anti-Rab27a (1:1,000, Abcam), anti-Rab27b (1:1,000, Abcam), anti-beta actin (1:5,000, Abcam), anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:5,000, Bio-Rad), anti-tubulin (1:5,000, Bio-Rad), anti-specificity protein 1 (SP1) (1:500, Santa Cruz), anti-phospho-camkk2 (1:1,000, Cell

Signaling), anti-camkk2 (1:1,000, Novus), anti-calnexin (1:1,000, Novus), anti-ERp72 antibody (1:1,000, Cell Signaling). Secondary antibodies (1:10,000) were from Thermo Fisher Scientific. The procedure of Western blot analysis was as previously described (22). For *in vitro* experiments, hepatocytes were washed with PBS at the endpoint of experiments, collected in lysis buffer (Cell Signaling Technology) with phenylmethylsulfonyl fluoride (PMSF) and protease inhibitors, and centrifuged at 16,000*g* for 10 min, and supernatant was collected for Western blotting. For *in vivo* experiments, frozen liver (ischemic lobe) was homogenized in lysis buffer and centrifuged at 16,000*g* for 10 min, and supernatant was collected. Protein concentrations of the supernatants were determined with the bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific). Sodium dodecyl sulfate (SDS) loading buffer was then added to the samples. Denatured protein samples were analyzed by 10% or 15% SDS–polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene difluoride membrane at 250 mA for 2 h. The membrane was blocked in 5% milk (Bio-Rad) in Tris-buffered saline (TBS) for 1 h and then incubated overnight with primary antibody in 1% milk in TBS overnight. Membranes were washed three times in TBS-Tween (TBS-T) for 10 min, incubated with horseradish peroxidase-conjugated secondary antibody for 1 h, and then washed three times for 10 min in TBS-T before being developed for chemiluminescence (Bio-Rad). Densitometry analysis was performed using the ImageJ Gel Analysis tool. GAPDH, β-actin, and tubulin are used as loading controls.

Statistical Analysis

All data were analyzed using GraphPad Prism software (version 6.0). For *in vivo* and *in vitro* experiments, numerical measures will be compared using Student's *t*-test and were used for comparison between two groups or one-way ANOVA followed by *post hoc* Bonferroni test for multiple comparisons. If the data do not satisfy the assumptions necessary for this analysis, variables will be transformed, or a non-parametric alternative will be used. For the test above, there will be enough cultures/mice to attain a power of at least 80% at a significance level of 0.05. The required effect sizes for each analysis were estimated using the results from the preliminary studies. Based on these numbers, it was confirmed that the planned sample sizes are sufficient in attaining the desired power. Further, for statistical analysis of our *in vitro* studies, data will be expressed as mean ± SEM of three independent experiments performed in triplicate. A $p < 0.05$ was considered statistically significant for all experiments. All values are presented as the mean ± SEM.

RESULTS

Lipopolysaccharide-Induced High-Mobility Group Box-1 Release From Hepatocytes Is Caspase-11 and Gasdermin D Dependent

We have previously demonstrated that hepatocytes release HMGB1 in sepsis and that this requires caspase-11 and GsdmD (5). Here, we confirmed that hepatocytes are the dominant source of the increases in circulating HMGB1 during endotoxemia.

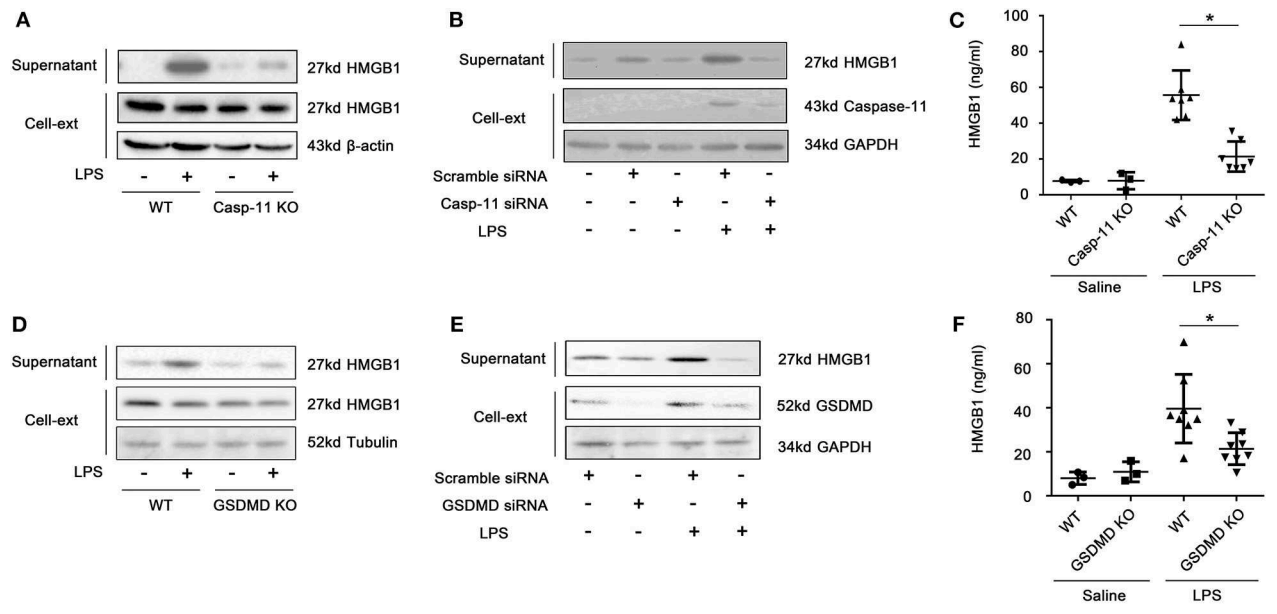

FIGURE 1 | Lipopolysaccharide (LPS)-induced high-mobility group box-1 (HMGB1) release from hepatocytes is caspase-11 and gasdermin D (GsdmD) dependent. Immunoblots for HMGB1, β-actin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), or tubulin in the supernatant and cell lysates (Cell-Ext) in **(A)** wild-type (WT) and caspase-11$^{-/-}$ [caspase-11 knockout (KO)] and **(D)** WT and GsdmD$^{-/-}$ (GsdmD KO) hepatocytes at 24 h after LPS (1 μg/ml). **(B,E)** Hepatocytes pretreated with siRNA to knock down caspase-11 or GsdmD prior to LPS treatment for 24 h as above. **(C,F)** Plasma HMGB1 levels in WT, caspase-11$^{-/-}$, or GsdmD$^{-/-}$ mice at 4 h after intraperitoneal injection with LPS (5 mg/kg). Each point represents one mouse. *$P < 0.05$. $n = 3$.

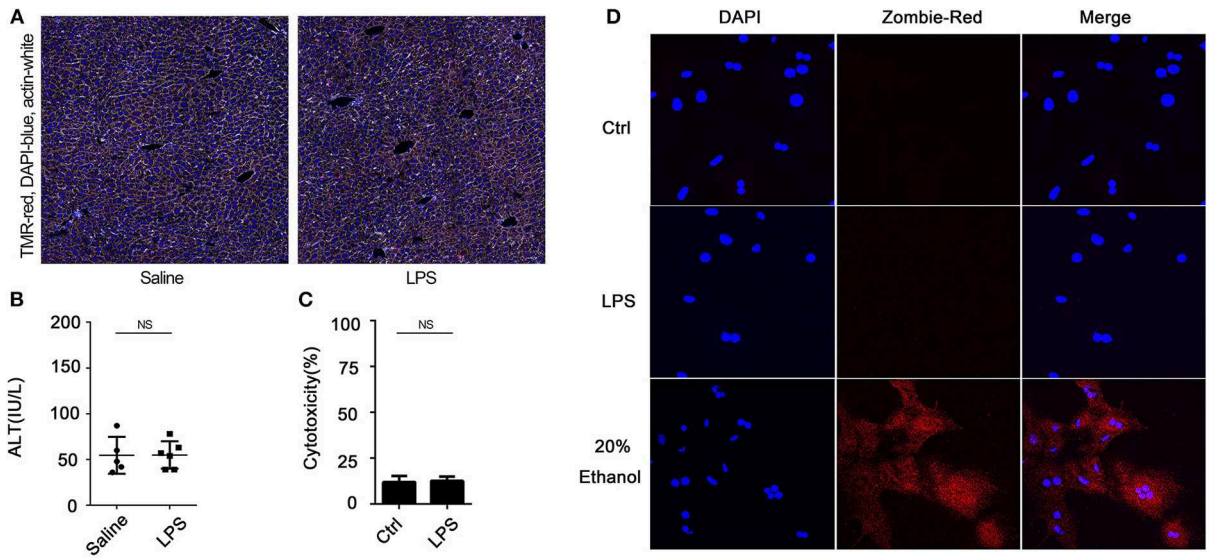

FIGURE 2 | Lipopolysaccharide (LPS) does not induce hepatocyte death *in vitro* or *in vivo*. **(A)** Immunofluorescence of liver from wild-type (WT) mice at 4 h after intraperitoneal injection with LPS (5 mg/kg). TMR = red; 4′,6-diamidino-2-phenylindole (DAPI) = blue; actin = white. **(B)** Plasma alanine aminotransferase (ALT) level in WT mice at 4 h after intraperitoneally injection with LPS (5 mg/kg). Each point represents one mouse. **(C)** WT hepatocytes were treated with LPS (1 μg/ml) for 24 h. Cytotoxicity was measured by using lactate dehydrogenase (LDH) release in the culture media. Data are expressed as mean ± SEM. **(D)** Immunofluorescence of Zombie-red staining (cell death) of WT hepatocytes 24 h after treatment with LPS (1 μg/ml). DAPI = blue. NS, no significant difference. $n = 3$.

As shown in **Supplemental Figure 1**, cell-specific deletion of HMGB1 in hepatocytes, but not myeloid cells, prevented the rise in plasma HMGB1 observed in mice after LPS injection. As expected, LPS treatment *in vivo* led to an increase in liver levels of caspase-11 by 4 h that further increased at 8 h. The levels of caspase-11 at 24 h decreased to a similar level as at 4 h (**Supplemental Figure 2**). We confirmed that LPS treatment of cultured hepatocytes led to a caspase-11-dependent cleavage of GsdmD (**Supplemental Figure 3**). Deletion or knockdown of caspase-11 or GsdmD prevented LPS-induced HMGB1 release by cultured hepatocytes (**Figures 1A,B,D,E**; **Supplemental Figure 6**), while deletion of caspase-11 or GsdmD prevented the circulating rise in HMGB1 following LPS treatment *in vivo* (**Figures 1C,F**). The increase in extracellular HMGB1 induced by LPS treatment *in vitro* and *in vivo* was not due to cell death (**Figure 2**). LPS treatment *in vivo* failed to increase terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells in the liver or ALT levels in the plasma. Furthermore, LPS, at a concentration shown to induce HMGB1 release from hepatocytes *in vitro*, did not increase lactate dehydrogenase (LDH) release or Zombie-red uptake in cultured WT hepatocytes. Thus, hepatocytes actively release HMGB1 after exposure to LPS via a process that requires caspase-11 and GsdmD.

Caspase-11 and Gasdermin D Are Required for High-Mobility Group Box-1 Translocation From the Nucleus to the Cytoplasm

The active secretion of HMGB1 from cells requires at least two steps. First, HMGB1 accumulates in the cytoplasm, instead of the nucleus, and second, HMGB1, a leaderless protein, is released into the extracellular space (23). Therefore, we next assessed whether the nucleo-cytoplasmic translocation of HMGB1 in hepatocytes following LPS treatment required caspase-11 and/or GsdmD. As shown in **Figure 3** and **Supplemental Figure 7**, LPS treatment led to an increase in cytosolic HMGB1 levels by 8 h. Whereas, deletion of caspase-11 or GsdmD had no impact on baseline levels of nuclear HMGB1 in hepatocytes, deletion of either gene prevented the accumulation of HMGB1 in the cytoplasm induced by LPS exposure. Specificity protein 1 (SP1) and GAPDH/tubulin were used to verify the compartment specificity of the proteins isolated from the nucleus and cytoplasm (**Figure 5B**; **Supplemental Figure 9**). These data establish that caspase-11 and GsdmD are involved in the nucleo-cytoplasmic translocation of HMGB1 that occurs after exposure of hepatocytes to extracellular LPS.

Caspase-11 and Gasdermin D Are Required for Lipopolysaccharide-Induced Phosphorylation of Camkkβ

We have previously shown that hypoxia-induced HMGB1 release by hepatocytes requires camkkβ (24). Camkkβ belongs to the serine/threonine-specific protein kinase family and to the Ca^{2+}/calmodulin-dependent protein kinase subfamily. Camkkβ catalyzes the phosphorylation of threonine residues located in the activation loop of the CaMKI and CaMKIV, enhancing their kinase activity. Therefore, we tested whether inhibition of camkkβ would reduce HMGB1 release from hepatocytes exposed to LPS. STO-609, a specific camkk inhibitor, blocked

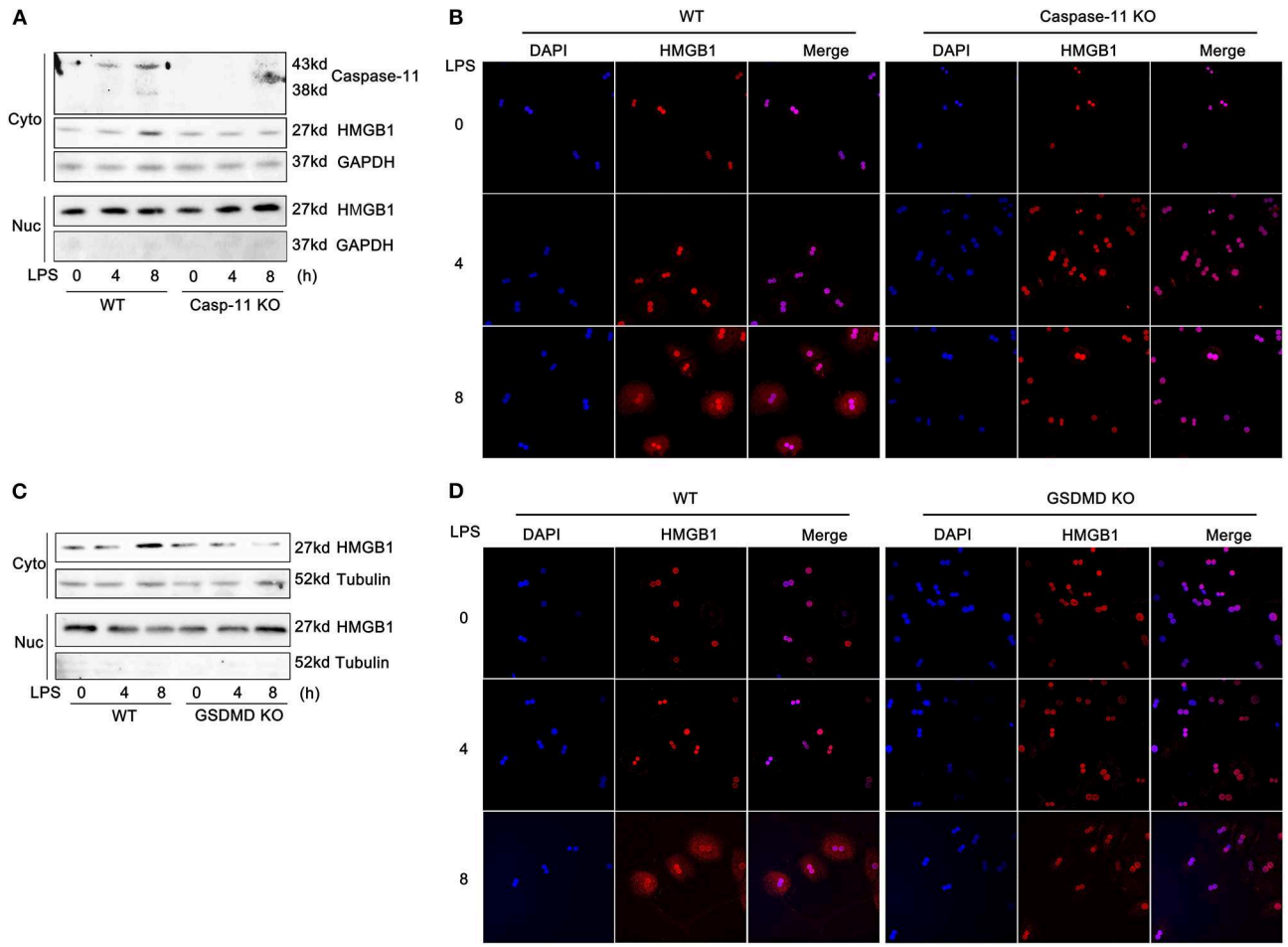

FIGURE 3 | Caspase-11 and gasdermin D (GsdmD) are required for high-mobility group box-1 (HMGB1) translocation to the cytosol in response to lipopolysaccharide (LPS). **(A,C)** Immunoblots for HMGB1, caspase-11, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), or tubulin in the cytoplasmic (Cyto) and nuclear (Nuc) lysates from wild-type (WT), caspase-11$^{-/-}$ [Casp-11 knockout (KO)], or GsdmD$^{-/-}$ (GsdmD KO) hepatocytes treated with LPS (1 μg/ml) for the time indicated. **(B,D)** Immunofluorescence of WT, caspase-11$^{-/-}$, or GsdmD$^{-/-}$ hepatocytes treated with LPS (1 μg/ml) for the indicated time. HMGB1 = red; 4′,6-diamidino-2-phenylindole (DAPI) = blue; colocalization = magenta. $n = 3$.

LPS-induced HMGB1 release (**Supplemental Figure 4A**). Interestingly, previous reports suggest that camkkβ (25) and its downstream targets, CaMKI (26, 27) and CaMKIV (28), may regulate HMGB1 nucleo-cytoplasmic translocation. Therefore, we investigated whether caspase-11 or GsdmD were required for camkkβ pathway activation. As shown in **Figure 4** and **Supplemental Figure 8**, deletion of either caspase-11 or GsdmD reduced the phosphorylation of camkkβ in hepatocytes exposed to LPS (**Figures 4A,B**). Consistent with this result, we also found that intracellular free calcium was increased following LPS treatment in hepatocytes, and this required caspase-11 and GsdmD (**Figures 4C,D**). Furthermore, A23187, an ionophore that increases intracellular calcium levels, promoted HMGB1 release in response to LPS in caspase-11$^{-/-}$ and GsdmD$^{-/-}$ hepatocytes to a level similar to that seen in WT hepatocytes (**Supplemental Figure 4B**). Taken together, these data show that the Ca^{2+} signaling regulates LPS-induced HMGB1 release in hepatocytes, and this signaling pathway requires both caspase-11 and GsdmD.

Lipopolysaccharide Triggers Gasdermin D Association With the Endoplasmic Reticulum

Next, we investigated how GsdmD regulates intracellular calcium transients. Calcium storage is one of the functions commonly attributed to the ER in non-muscle cells (29). The N-terminal cleavage fragment of GsdmD can form pores in phospholipid membranes (13). Therefore, we sought evidence for GsdmD association with the ER in LPS-treated hepatocytes. The purity of the isolated ER was confirmed using electron microscopy and the ER protein markers, calnexin and ERp72 (**Figures 5A,B**). Western blot analysis demonstrated the presence of a GsdmD cleavage fragment in the ER lysate from LPS-treated WT

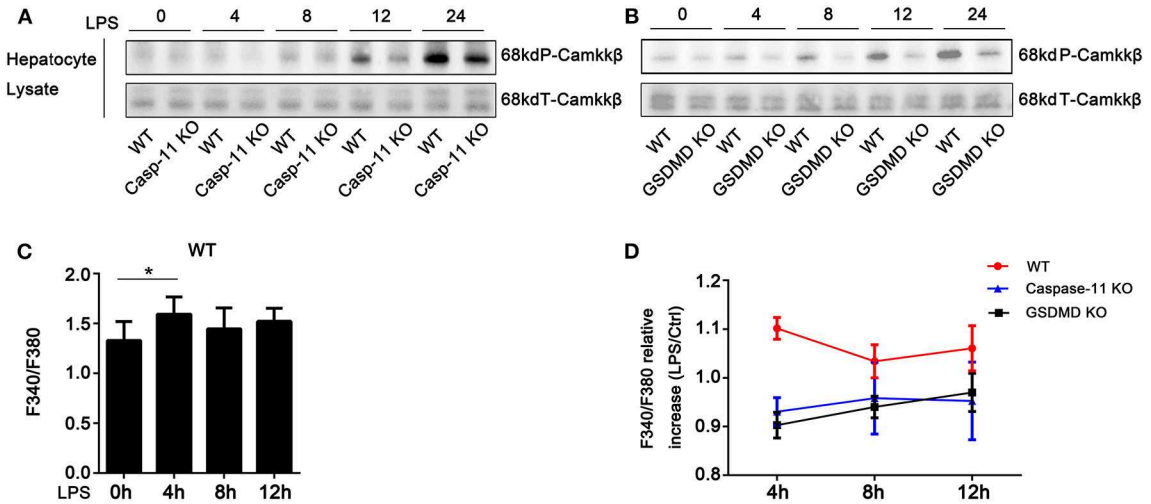

FIGURE 4 | Caspase-11 and gasdermin D (GsdmD) inhibited the phosphorylation of calcium-calmodulin kinase kinase (camkk)β. **(A,B)** Immunoblots for phospho-camkkβ (P-camkkβ) and total-camkkβ (T-camkkβ) in whole cell lysates from wild-type (WT), caspase-11$^{-/-}$ [Casp-11 knockout (KO)], or GsdmD$^{-/-}$ (GsdmD KO) hepatocytes were treated with lipopolysaccharide (LPS) (1 μg/ml) for the indicated times. **(C)** Intracellular Ca^{2+} measured by fluorescence intensity of Fura-2AM (F340/F380) in WT hepatocytes treated with or without LPS (1 μg/ml) for the indicated time. Data are expressed as mean ± SEM. **(D)** Intracellular Ca^{2+} measured by fluorescence intensity of Fura-2AM (F340/F380) in WT, caspase-11$^{-/-}$, or GsdmD$^{-/-}$ hepatocytes treated with or without LPS (1 μg/ml) at indicated times. Data are expressed as relative levels compared with baseline controls and as mean ± SEM. $^{*}P < 0.05$. $n = 3$.

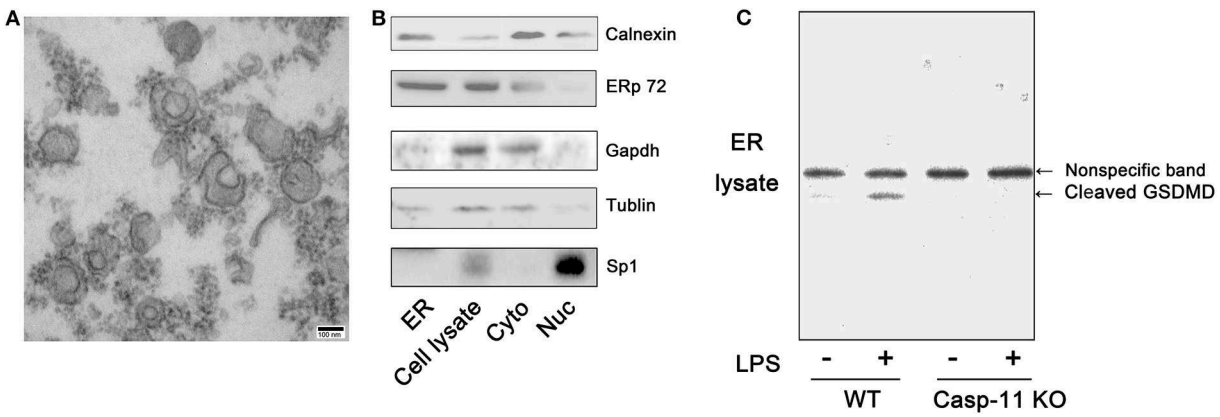

FIGURE 5 | Gasdermin D (GsdmD) is recruited to the endoplasmic reticulum (ER). **(A)** Morphological structure of isolated mouse ER as seen on standard transmission electron microscopy (TEM) (100,000 × magnification; scale bar, 100 nm). **(B)** Immunoblots for calnexin, ERp72, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), tubulin, and specificity protein 1 (SP1) in isolated ER, whole cell (Cell lysate), cytoplasm (Cyto), and nucleus (Nuc) of wild-type (WT) hepatocytes. **(C)** Immunoblots for GsdmD in ER (ER lysate) isolated from WT and caspase-11$^{-/-}$ [Casp-11 knockout (KO)] hepatocytes after treatment with lipopolysaccharide (LPS) (1 μg/ml) for 8 h. $n = 3$.

hepatocytes but not caspase-11$^{-/-}$ hepatocytes (**Figure 5C**). These findings raise the possibility that caspase-11 cleaves GsdmD, and the cleavage fragment then inserts in the ER membrane to release calcium into the cytoplasm.

High-Mobility Group Box-1 Is Released From Hepatocytes in Exosomes

We have recently provided evidence that HMGB1 is released into the extracellular space inside vesicles (5). To determine if these HMGB1-containing vesicles were exosomes, we confirmed that HMGB1 found in cell supernatant and plasma after LPS challenge was within CD81- and TSG101-positive vesicles (**Figures 6A,B**; **Supplemental Figure 10**). To further establish that HMGB1 is released via exosomes, NanoSight™ nanoparticle tracking analysis was used to show that mean size of particles isolated from hepatocyte supernatant *in vitro* and plasma were both in the range consistent for exosomes (40–100 nm) (**Figures 6C,D**). GW4869 and spiroepoxide, inhibitors of neutral sphingomyelinase associated with exosome release (20), reduced HMGB1 release from hepatocytes in a dose-dependent manner (**Figures 6E,F**). *In vivo*, GW4869 treatment before LPS challenge significantly suppressed HMGB1 levels in plasma (**Figure 6G**).

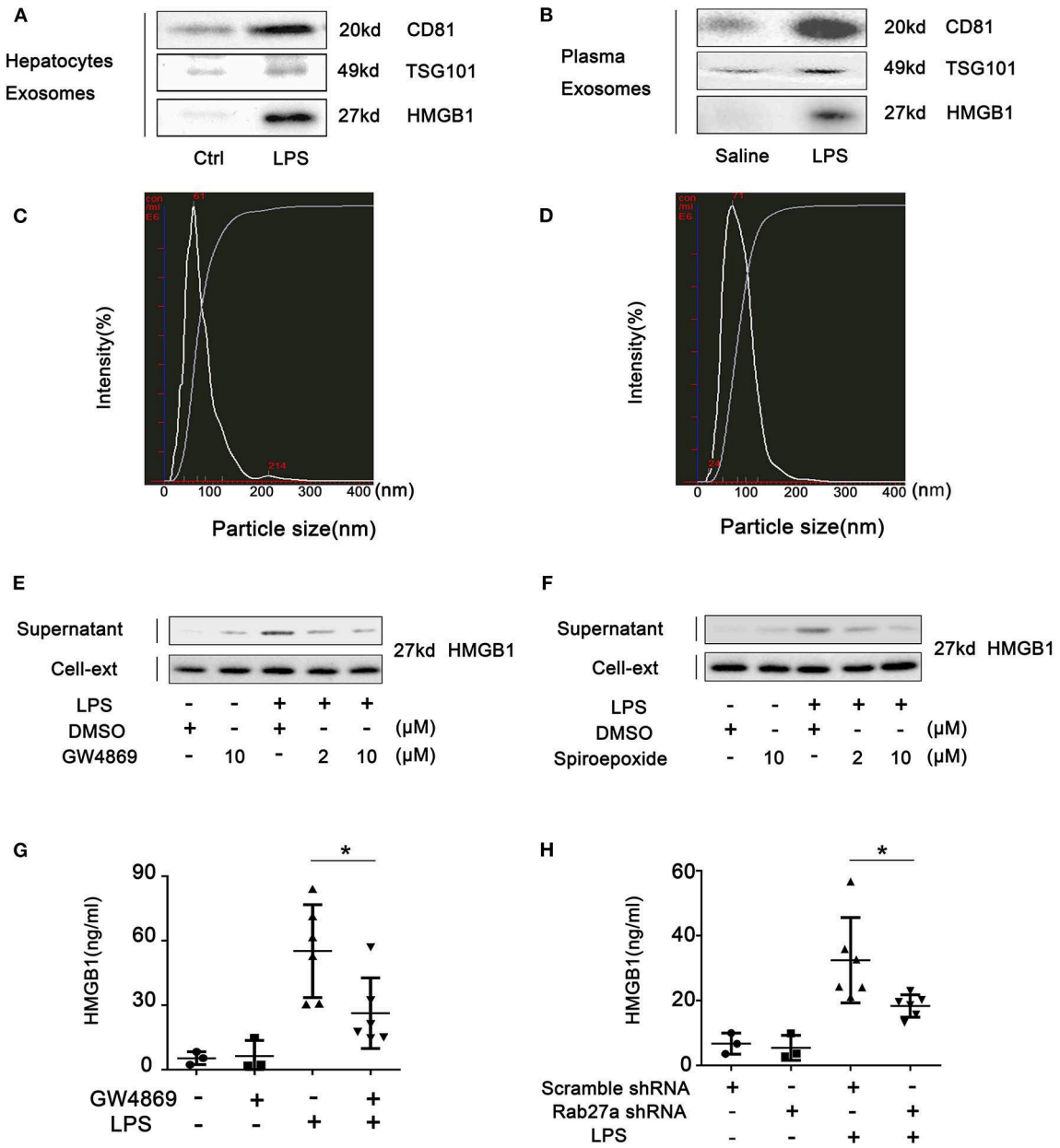

FIGURE 6 | Hepatocytes release high-mobility group box-1 (HMGB1) in exosomes. Immunoblots for CD81, TSG101, and HMGB1 in exosomes isolated from **(A)** cell culture media from wild-type (WT) hepatocytes treated with lipopolysaccharide (LPS) (1 μg/ml) for 24 h and **(B)** plasma from WT mice intraperitoneally injected with LPS (5 mg/kg) for 4 h. NanoSight™ analysis of exosomes isolated from **(C)** cell culture media and **(D)** mouse plasma. **(E,F)** Immunoblots for HMGB1 in the supernatant and cell lysates of hepatocytes treated with GW4869 or spiroepoxide for 2 h, then challenged with LPS (1 μg/ml) for 24 h. **(G)** Plasma HMGB1 level in WT mice injected intravenously with GW4869 (2.5 mg/kg) for 1 h prior to intraperitoneal injection of LPS (5 mg/kg) for 4 h. **(H)** Plasma HMGB1 level in WT mice pretreated for 48 h with scrambled (control) or Rab27a-targeted shRNA via intravenous injection followed by intraperitoneal injection with LPS (5 mg/kg) for 4 h. Each point represents one mouse. *$P < 0.05$. $n = 3$.

Rab27a is required for exosome release (30). An adenovirus-expressing shRNA targeting Rab27a was used to suppress liver Rab27a (**Supplemental Figure 5**). Knockdown of Rab27a also significantly prevented HMGB1 increases in the plasma of LPS-treated mice (**Figure 6H**). Combined, these observations support the conclusion that HMGB1 is released into the extracellular space in exosomes.

High-Mobility Group Box-1 Release in Exosomes Is Toll-Like Receptor 4, Caspase-11, and Gasdermin D Dependent During Endotoxemia

We show above that the nucleo-cytoplasmic translocation of HMGB1 and the active extracellular release of HMGB1 in

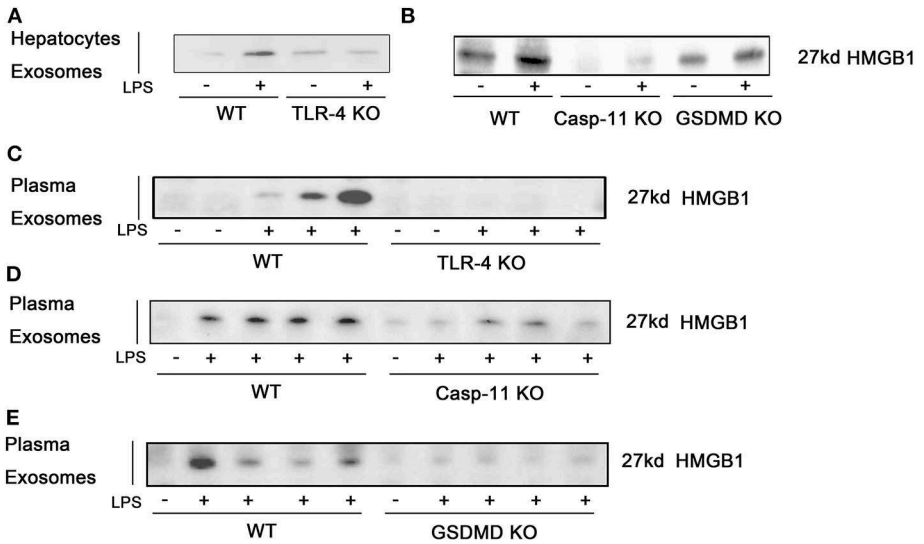

FIGURE 7 | High-mobility group box-1 (HMGB1) release in exosomes is dependent on Toll-like receptor (TLR)4, caspase-11, and gasdermin D (GsdmD). **(A,B)** Immunoblots for HMGB1 in exosomes isolated from cell culture media from wild-type (WT), TLR4$^{-/-}$ [TLR4 knockout (KO)], caspase-11$^{-/-}$ (Casp-11 KO), or GsdmD$^{-/-}$ (GsdmD KO) hepatocytes were treated with lipopolysaccharide (LPS) (1 μg/ml) for 24 h. **(C–E)** Immunoblots for HMGB1 in exosomes isolated from plasma of WT, TLR4$^{-/-}$, caspase-11$^{-/-}$, or GsdmD$^{-/-}$ mice treated with LPS (5 mg/kg) for 4 h. Each lane represents one mouse. $n = 3$.

response to LPS exposure both require TLR4 and caspase-11/GsdmD. We hypothesized that the release of HMGB1 into exosomes would depend on the same pathways. Using KO mice, we confirmed that LPS-induced HMGB1 release in exosomes was TLR4, caspase-11, and GsdmD dependent both *in vitro* (**Figures 7A,B**; **Supplemental Figure 11**) and *in vivo* (**Figures 7C–E**). We next asked whether the TLR4 and caspase-11 pathways regulated total exosome release in response to LPS. LPS treatment markedly increased exosome numbers based on the Western blots for CD81 and TSG101. Only the deletion of TLR4 and not the deletion of caspase-11 or GsdmD prevented exosome release into the cell supernatant of cultured hepatocytes or into the plasma of mice after the LPS challenge (**Figures 8A–E**; **Supplemental Figure 8**). To further confirm these findings, we used ELISA for quantification of CD81, and the results were consistent with the Western blot analysis (**Figure 8F**). Thus, TLR4 regulates exosome formation, while caspase-11 and GsdmD are required only for HMGB1 delivery into exosomes.

DISCUSSION

In this study, we sought to establish the mechanisms involved in the LPS-induced release of HMGB1 into the extracellular space by hepatocytes. This area of investigation is important because active HMGB1 release by hepatocytes has been shown to be critical in the pathogenesis of not only sepsis lethality but also many liver-based diseases (5, 31). Hepatocytes are known to sense the presence of pathogen-associated molecular patterns (PAMPs) in the circulation, and the detection of PAMPs triggers the release of immune regulators such as HMGB1 or chemokines (5, 14, 32). Taken together, our previously published findings (5, 14) combined with our current work demonstrate that hepatocytes utilize surface TLR4 to detect and take up LPS, which occurs concurrently with TLR4-dependent upregulation of caspase-11. Intracellular LPS leads to caspase-11-dependent cleavage of GsdmD, and this promotes increases in free calcium in the cell, camkkβ activation, and relocation of nuclear HMGB1 to the cytoplasm. This is followed by a TLR4- and caspase-11/GsdmD-dependent release of HMGB1 in exosomes. The localization of a cleavage fragment of GsdmD in the ER in LPS-treated cells suggests that the source of free calcium in this signaling cascade may be the ER. These findings introduce a novel mechanism involving the coordinated interaction between the two canonical LPS sensing pathways, surface TLR4 and cytoplasmic caspase-11, for the secretion of HMGB1 into exosomes by hepatocytes. Our observations also raise the possibility that targeting caspase-11 in sepsis could improve outcomes not only by directly blocking pyroptosis in macrophages and endothelial cells but also by suppressing HMGB1 release from the liver.

Our understanding of LPS sensing by immune cells evolved with the discovery that LPS is recognized not only by the cell surface TLR4 receptor complex (33) but also by cytosolic caspase-11 in mice and caspases 4/5 in humans (8). In macrophages and endothelial cells, LPS triggers an inflammatory program aimed at initiating antimicrobial defenses by interacting with cell surface TLR4 (34–36). This inflammatory signaling promotes the upregulation of caspase-11 in the cytosolic compartment of macrophages (37). The delivery of LPS to the cytosol in these cells requires the endocytic uptake of LPS-containing outer membrane vesicles from live Gram-negative bacteria (38), the uptake of live bacteria, and the release of LPS from phagolysosomes, or the uptake of LPS–HMGB1 complexes via cell surface RAGE followed by the release of LPS from

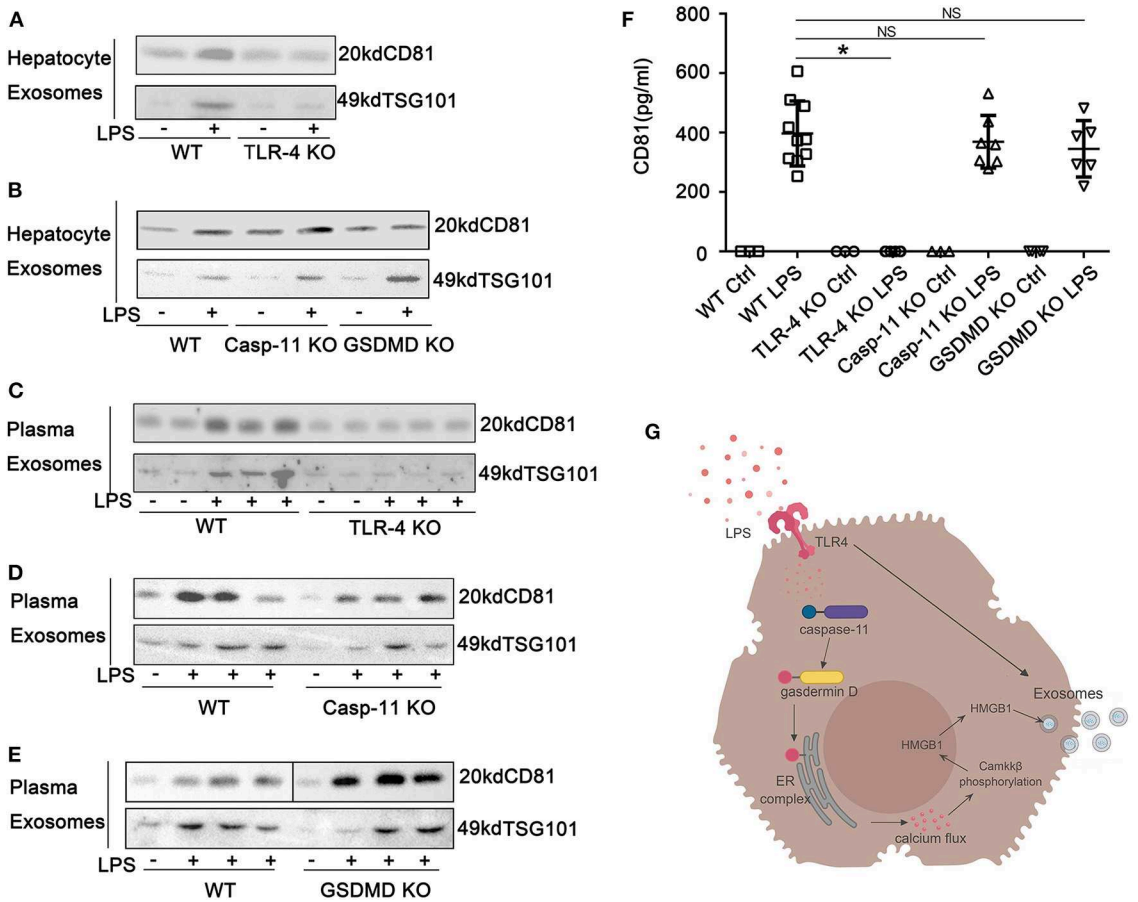

FIGURE 8 | Exosome release from hepatocytes is dependent on Toll-like receptor (TLR)4 but independent of caspase-11 and gasdermin D (GsdmD). **(A,B)** Immunoblots of CD81 and TSG101 in exosomes isolated from cell culture media of wild-type (WT), TLR4$^{-/-}$, caspase-11$^{-/-}$, or GsdmD$^{-/-}$ hepatocytes treated with lipopolysaccharide (LPS) (1 μg/ml) for 24 h. **(C–E)** Immunoblots for CD81 and TSG101 in exosomes isolated from plasma of WT, TLR4$^{-/-}$, caspase-11$^{-/-}$, or GsdmD$^{-/-}$ mice treated with LPS (5 mg/kg) for 4 h. Each lane represents one mouse. **(F)** CD81 levels in exosomes isolated from plasma of WT, TLR4$^{-/-}$, caspase-11$^{-/-}$, or GsdmD$^{-/-}$ mice treated with LPS (5 mg/kg) for 4 h. Each point represents one mouse. **(G)** A proposed model describing TLR4 signaling results in an increase in caspase-11 expression, as well as increased exosome release, while caspase-11/GsdmD activation/cleavage leads to accumulation of high-mobility group box-1 (HMGB1) in the cytoplasm through a process associated with the release of calcium from the endoplasmic reticulum and calcium-calmodulin kinase kinase (camkk)β activation. *$P < 0.05$. NS, no significant difference. $n = 3$.

lysosomes when HMGB1 destabilizes lysosomal membranes (5). The release of LPS into the cytosol following uptake of bacteria occurs through the actions of interferon-induced GTP-ases (39). The only known consequence of LPS-induced activation of caspase-11 in macrophages or endothelial cells is pyroptosis, which is thought to be a mechanism for the destruction of intracellular microbial niches and the release of local pro-inflammatory molecules [e.g., interleukin (IL)-1α and HMGB1]. Our previous and current work shows that LPS sensing by hepatocytes has a number of characteristics that are unique from macrophages and endothelial cells. Hepatocytes utilize a cell surface TLR4 receptor complex that incorporates CD14 and CD11b/CD18 to uptake LPS into the cell (14). We show here that this response to TLR4 stimulation includes an increase in caspase-11 expression and an increase in exosome numbers released by hepatocytes. In contrast to the cell death seen in macrophages and endothelial cells, caspase-11 activation and GsdmD cleavage in hepatocytes do not lead to cell injury or death but instead mobilize HMGB1 from the nucleus to be released into the exosomes. This coordinated TLR4 and caspase-11/GsdmD interaction represents a novel pathway activated by LPS sensing by hepatocytes. This pathway leads to the massive systemic release of the alarmin/damage-associated molecular pattern (DAMP) and LPS binding protein, HMGB1, from the liver.

Mechanisms for cellular release of HMGB1 fall under two broad categories: passive and active (40, 41). Passive release follows necrosis or programmed cell death, while active release follows a posttranslational modification of nuclear HMGB1. Critical in this process is the acetylation of lysines in the two nuclear localization domains present in HMGB1 (42–44). Although we cannot rule out the possibility that low levels of cell death contribute to the systemic release of HMGB1 during endotoxemia, our results indicate that most of the

HMGB1 released by hepatocytes in response to LPS is through an active process that requires both TLR4- and caspase-11-dependent signaling steps. Deletion of TLR4, caspase-11, or the downstream cleavage target of caspase-11, GsdmD, prevents the nucleo-cytoplasmic translocation of HMGB1 induced by LPS. These findings parallel our previous findings where hypoxia-induced HMGB1 release by hepatocytes involves the transfer of acetylated HMGB1 from the nucleus to cytoplasm following the inhibition of nuclear histone deacetylase-1 (HDAC1) and the transfer of histone deacetylase-4 (HDAC4) from the nucleus to the cytoplasm (42). HMGB1 acetylation may also involve an upregulation of histone acetyltransferase in response to Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling downstream of TLR4 (45, 46). Others have linked JAK/STAT signaling to HMGB1 hyperacetylation (27) and HDAC4 degradation in macrophages (47). We have also shown that hypoxia-induced hepatocyte HMGB1 release requires intracellular calcium signaling through camkkβ and CaMKIV downstream of TLR4 (24), and that CaMK signaling is upstream of HDAC inhibition. Our findings that caspase-11/GsdmD are required for calcium increases in LPS-treated hepatocytes and that the inhibitor of camkkβ blocks LPS-induced HMGB1 release from hepatocytes suggest that caspase-11/GsdmD regulate HMGB1 through calcium signaling, while TLR4 signaling upregulates caspase-11 expression and may regulate acetylation of HMGB1 through JAK/STAT. We speculate that the insertion of the N-terminal fragment of GsdmD into the ER membrane may explain the caspase-11/GsdmD-dependent increase in cytosolic calcium observed in our experiments. This would represent a novel function for cleaved GsdmD but will require further proof that the source of cytosolic calcium is indeed the ER in hepatocytes.

Our data suggest that caspase-11 or GsdmD transiently regulates the calcium flux in hepatocytes at an early time point after LPS treatment, which is prior to the upregulation of caspase-11 levels. Unlike macrophage, there is a measurable amount of caspase-11 in hepatocytes at baseline. Therefore, caspase-11 may not be needed to upregulate in hepatocytes. However, caspase-11 at baseline may be activated at early time points to cause the early (within 4 h) Ca^{2+} increase after GsdmD cleavage and localization to ER. This then activates camkk and HMGB1 translocation. The increase of caspase-11 by later time points may be responsible for other functions, such as packaging and release of exosomes. However, the mechanisms remain elusive.

If the transfer of HMGB1 from the nucleus to the cytoplasm is the first step in the active release of HMGB1 in response to LPS, our findings also support the notion that delivery of cytosolic HMGB1 into exosomes is the second step. Exosomes form in the cytoplasm when cytosolic contents are packaged into multivesicular bodies (48). By blocking factors critical to exosome release including neutral sphingomyelinase or the GTPase Rab27a, we prevented extracellular HMGB1 release in response to LPS, suggesting that HMGB1 is one of the cytosolic proteins that are incorporated as cargo in the exosomes released in response to LPS. Part of the mechanism for exosome release appears to be an increase in overall exosome formation in response to LPS-induced TLR4 signaling, while both TLR4 and caspase-11/GsdmD are required for HMGB1 to accumulate in the cytoplasm. How HMGB1 in the cytoplasm is selected for transfer into exosomes is not known. Furthermore, it is unclear whether the secreted HMGB1 could further act as an internalization signal, thereby mediating the switch-off of the pathway.

In summary, we provide evidence that hepatocytes sense LPS through both TLR4 and caspase-11, and this leads to the active release of HMGB1 in exosomes. As depicted in **Figure 8G**, each of the LPS-sensing pathways plays unique but interconnected roles in this signaling cascade. An important conceptual advance of this work is that hepatocytes utilize TLR4 and caspase-11 in ways that are distinct from macrophages. The two most striking differences are that TLR4 signaling is critical to the delivery of LPS into hepatocytes, and that the caspase-11/GsdmD pathway does not lead to pyroptosis but instead promotes calcium-dependent signaling for the active release of HMGB1. The disease implications relate to the recently discovered central roles of hepatocyte-derived HMGB1 to sepsis pathogenesis and inflammatory diseases of the liver.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

This animal study was reviewed and approved by University of Pittsburgh Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

TB, MS, and BL conceived the project, designed the experiments, analyzed the data, and wrote the paper. WL and MD designed the experiments, performed the experiments, analyzed the data, and wrote the paper. MY, ML, CY, WG, SL, SJ, and JC helped to perform to the experiments. PL performed imaging experiments and edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00229/full#supplementary-material

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HMGB1 in Systemic Lupus Erythematosus

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The high-mobility group box 1 (HMGB1) has been shown to exert proinflammatory effects on many cells of the innate immune system. Originally identified as a nuclear protein, HMGB1 has been found to play an important role in mediating inflammation when released from apoptotic or necrotic cells as a damage-associated molecular pattern (DAMP). Systemic lupus erythematosus (SLE) is a disease of non-resolving inflammation, characterized by the presence of autoantibodies and systemic inflammation involving multiple organ systems. SLE patients have impaired clearance of apoptotic debris, which releases HMGB1 and other DAMPs extracellularly. HMGB1 activity is implicated in multiple disease phenotypes in SLE, including lupus nephritis and neuropsychiatric lupus. Elucidating the various properties of HMGB1 in SLE provides a better understanding of the disease and opens up new opportunities for designing potential therapeutics.

Keywords: HMGB1, SLE, neuropsychiatric SLE, lupus nephritis, innate immunity, adaptive immunity

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INTRODUCTION

Systemic Lupus Erythematosus (SLE)

SLE is an autoimmune disease characterized by the production of autoantibodies and multi-organ system involvement with a wide array of clinical manifestations. The dominant clinical features include fever, arthritis, serositis, cutaneous lesions, neuropsychiatric and renal involvements (1). SLE is caused by aberrant activation of autoreactive B cells and subsequent production of autoantibodies against nucleic acid and nucleic acid binding proteins. These bind to tissue, often through cross-reactivity to a tissue antigen, and cause organ damage (2). Immune complexes containing nucleic acid can be internalized through Fc receptor engagement and activate cells of the innate immune system. Thus, neutrophils, monocytes, macrophages, and dendritic cells (DCs) contribute to SLE pathologies (3), in part following, cytosolic sensing of DNA or RNA in part through an impairment in the usual non-immunogenic clearance of apoptotic debris, and in part due to cell intrinsic genetic alterations.

Genetic factors in the context of environmental triggers are thought to play important roles (4). Some identified risk gene loci for SLE include BLIMP1, IRF5 and C1q (5–8). C1q binds to opsonized cellular debris to mediate the clearance of dead and dying cells (9–11). Genetic deficiency of C1q predisposes strongly to SLE (7, 12, 13). SLE occurs in approximately 90% of C1q-deficient individuals in many studies. These patients have severe central nervous system and renal autoimmune disease.

High-Mobility Group Box 1 Protein (HMGB1) in SLE

HMGB1 is a member of the family of high-mobility group (HMG) proteins which were identified as important non-histone nuclear proteins (14, 15). Also known as amphoterin, HMGB1 has

a molecular weight of 25 kDa and two positively charged nucleic acid binding motifs, A box and B box, and a negatively charged C-terminal tail (16, 17). The function of HMGB1 in the cell is context-dependent. In the nucleus, HMGB1 plays the essential role of bending DNA and facilitating its interaction with transcription factors. HMGB1 can also function as a DAMP outside the cell, activating the immune system and promoting inflammation. It is released from damaged cells or activated cells to exert its inflammatory effects (18). It binds both receptor for advanced glycation endproducts (RAGE), toll-like receptor 2 (TLR2) and TLR4 (19–21). There are three cysteine residues (C23, C45, C106) in HMGB1 and their redox states dictate the function of HMGB1. Histone H1 is most effective at inhibiting the DNA bending activities of oxidized HMGB1 (22). Additionally, HMGB1 oxidation is known to alter its extracellular receptor binding and subsequent functions. As reviewed by Janko et al., fully reduced HMGB1 can induce autophagy through binding to RAGE or together with CXCL12 can promote cell migration through binding to CXCR4. When C23 and C45 are oxidized to form a disulfide bond, HMGB1 can signal through TLR4 and cause pro-inflammatory cytokine release (23). Oxidative stress is known to be increased in SLE and contributes to immune system dysregulation (24) and it is likely that partially oxidized, disulfide HMGB1 contributes to this process. Serum HMGB1 is elevated in SLE patients and levels of serum HMGB1 correlate with disease activity (25). The present review discusses the role of HMGB1 as a DAMP in both the innate and the adaptive aspects of SLE pathogenesis (**Figure 1**).

HMGB1'S ROLE IN SLE PATHOGENESIS

Adaptive Immunity

Antibodies to nuclear antigens are the hallmark of SLE (26). These autoantibodies to ubiquitous self-antigens lead to immune complex formation, to deposition in tissue and ensuing tissue damage. Apoptotic defects are an important aspect of SLE pathogenesis (27). When apoptotic cells are not efficiently cleared, they can undergo secondary necrosis, releasing their intracellular contents (28). The HMGB1 released in this process can play a role as an autoadjuvant in the breakdown of B

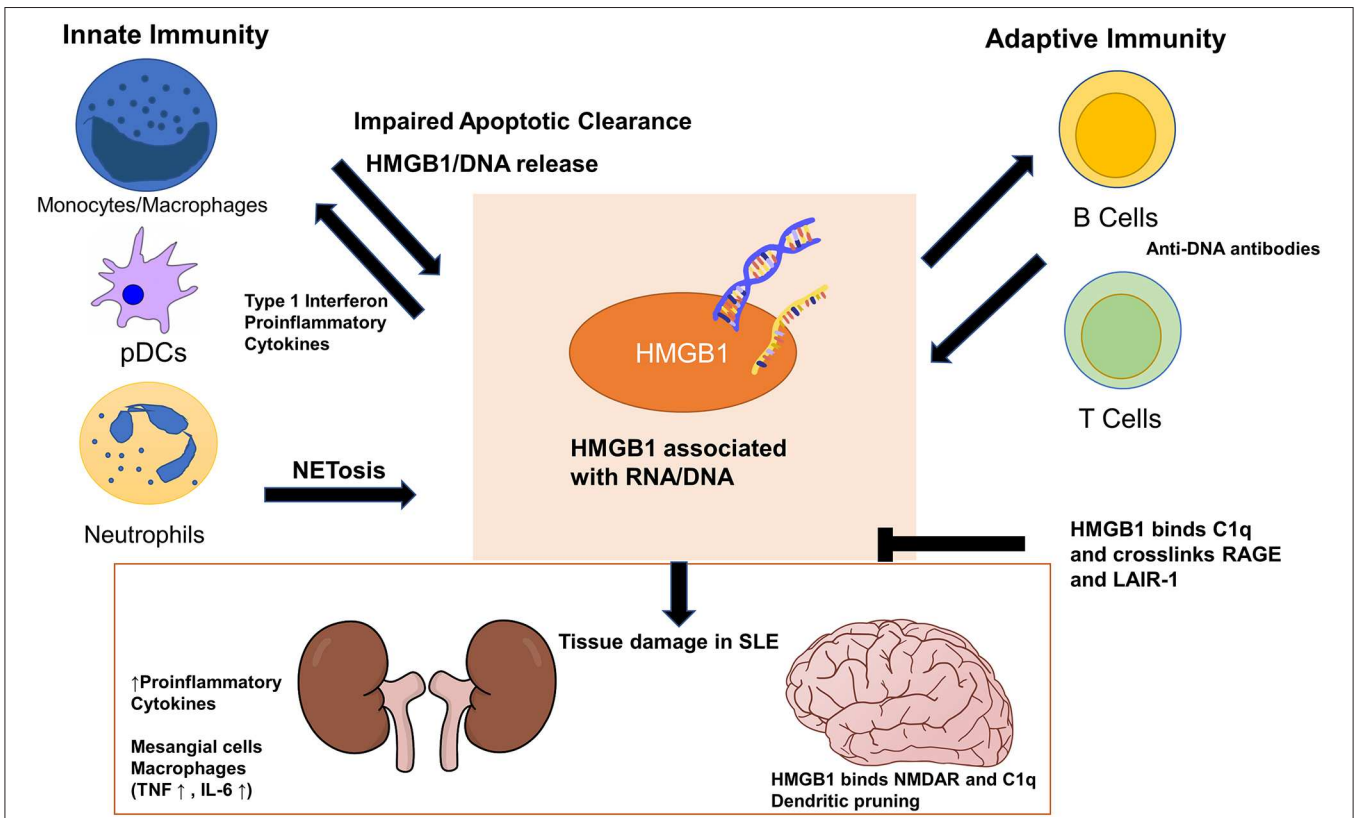

FIGURE 1 | High-mobility group box 1 (HMGB1) exerts its pathogenic effects in systemic lupus erythematosus (SLE) through cells in both the innate and adaptive immune systems. Impaired apoptotic clearance by macrophages prolongs exposure of HMGB1/nucleic acid-containing debris to the adaptive immune system as autoantigens. HMGB1 enhances adaptive immune response in generating autoantibodies against DNA/RNA/HMGB1, which cross react with tissues and cause organ damage. HMGB1 also locally increases proinflammatory cytokines and stimulate mesangial cells and macrophages in the kidneys. In the brain, HMGB1 bridges binding of C1q to N-methyl-D-aspartate receptor (NMDAR) to promote dendritic pruning and spatial memory deficit. HMGB1 can stimulate monocytes and plasmacytoid dendritic cells to sustain the production of type 1 interferon seen in SLE. HMGB1 can perpetuate its extracellular presence both by inducing more HMGB1/DNA release from neutrophil NETosis and by deviating macrophage polarization away from M2, which further impairs apoptotic clearance. HMGB1 and C1q together crosslink RAGE and LAIR-1 to exert anti-inflammatory and pro-resolving effects on monocytes.

cell tolerance and the generation of autoantibodies in SLE. As HMGB1 can bind both RNA and DNA it can activate cytosolic nucleic acid receptors after entering cells in a RAGE dependent fashion (29). It has been shown *in vivo* that HMGB1-nucleosome complexes activate antigen presenting cells and elicit an anti-dsDNA and anti-histone IgG response in a TLR2-dependent manner, whereas HMGB1-free nucleosome do not (30). Although anti-nuclear antibodies (ANA) in SLE most commonly bind to DNA and histones in nucleosomes, they are also reported to bind to HMGB1 itself (31, 32), although this may represent binding to DNA associated with HMGB1. Elevated anti-HMGB1 antibodies are observed in SLE and correlate to disease severity (33, 34). Coupled with elevated circulating HMGB1 seen in SLE patients, this can be a mechanism for immune complex formation that includes nucleic acid which is bound to the HMGB1.

Innate Immunity

Although the adaptive immune system has been studied extensively for its roles in producing autoreactive antibodies in SLE, the innate immune system is increasingly appreciated as playing an important role in the pathogenesis of SLE (35). Activating Fcγ receptors are highly expressed on monocyte-derived dendritic cells (mo-DC) and macrophages. Immune complexes formed by DNA or RNA/HMGB1 and IgG can activate these innate immune cells through their Fcγ receptors to elicit their inflammatory functions (36), which include secretion of type 1 interferon (IFN), TNFα, IL-6 and more. The IFN pathway is a crucial contributor to the disease in some models of SLE. Type I IFN can cause the loss of peripheral tolerance by maturing dendritic cells, which activates T cells that eventually help expand autoreactive B cells (37).

While plasmacytoid DCs (pDCs) make the most type 1 IFN on a per cell basis, monocytes are important IFN producers in SLE because of their abundance compared to pDCs (38). Nucleic acids need to be internalized into monocytes and delivered to TLRs 7 and 9 to trigger the production of IFNs. HMGB1chaperones nucleic acid to endosomal TLRs through a RAGE dependent pathway (39). Porat et al. described two pathways by which SLE serum can activate monocytes, one of which involves HMGB1 delivering its nucleic acid cargo by binding and internalization with RAGE (40). The induction of the IFN signature genes by HMGB1 was shown to be inhibited by a DNA mimetope binding to HMGB1, preventing its interaction with RAGE (40).

PDCs, mentioned above, are specialized to produce high amounts of type I interferons (41). Upon TLR 7 or 9 activation, HMGB1 leaves the nuclei of pDCs and pDCs increase their expression of RAGE as a part of their maturation (42). This creates an autocrine loop which sustains type I IFN production. The pathogenic role of pDCs in SLE is often considered to be a consequence of their production of type I IFNs. Patients with SLE have reduced numbers of pDCs in the blood and an accumulation of pDCs in tissues (43). Reciprocally, IFN regulates HMGB1 secretion by driving its translocation from the nucleus to the cytoplasm prior to release into the extracellular space (44). The activation of the JAK/STAT1 signaling pathway by type 1 IFN stimulation induces this process (45). Additionally, IFN-γ has also been shown to dose-dependently induce HMGB1 release through a TNF-dependent mechanism (46). Taken together, these processes highlight the important role HMGB1 plays in initiating nucleotide-induced IFN signature in SLE.

Neutrophils in SLE can mediate tissue damage and produce IFNs (47). Neutrophils can undergo a specialized form of cell death known as NETosis, releasing neutrophil extracellular traps (NETs), primarily composed of DNA and nuclear proteins. Normally, this process functions to prevent the dissemination of pathogens. In SLE, uncleared NETs can become a source of nuclear self-antigens and immune complexes and complement activation, thereby perpetuating the inflammatory response (48). HMGB1 is both released from neutrophils as a part of NETs and itself can induce the release of NETs. It has been shown that HMGB1 promotes the formation of NETs in mice in a TLR4 dependent manner (49). NETs are confirmed as a source of HMGB1 in SLE patients and are positively correlated with disease progression in lupus nephritis (50).

It is important to note, however, that macrophages, especially those expressing SLE risk alleles, also contribute to SLE (51). Macrophages from SLE patients are defective at clearing apoptotic debris and this delayed clearance can lead to prolonged exposure of autoantigens to the adaptive immune system (52, 53). Monocytes can differentiate into classically activated macrophages (M1) responsible for inflammation and tissue destruction, or alternatively activated macrophages (M2) involved in phagocytosis, inflammation resolution and tissue repair (54). Gene expression profiles have revealed that SLE patients have a biased activation toward M1 macrophages (55). Part of this activation pattern may be explained by the elevated HMGB1 in SLE patients. HMGB1 is known to polarize monocytes into M1-like macrophage phenotypes, skewing macrophage phenotype away from M2-like differentiation and thus decreasing phagocytosis of apoptotic cells (56), leaving patients susceptible to the breakdown of peripheral B cell tolerance and the generation of autoantibodies.

HMGB1 has also been shown to bind to C1q, a component of the classical complement pathway. Leukocyte-associated immunoglobulin-like receptor 1 (LAIR-1) is an inhibitory receptor for C1q on the membrane of many immune cells (57, 58). Son el al showed that HMGB1 and C1q can form a tetramolecular complex on the lipid raft with RAGE and LAIR-1 on monocytes, causing M2-like pro-resolving macrophage polarization (59). In the absence of C1q, or when C1q is low due to immune complex-mediated complement consumption, the high level of HMGB1 in SLE patients may skew to M1 macrophage polarization unchecked, promoting inflammation and further reducing clearance of apoptotic cells, exposing autoantigens and thus creating a favorable environment for the adaptive immune system to generate autoantibodies. It is also reported that in pediatric SLE, elevation of serum HMGB1 and type 1 IFN occur with together decreased expression of LAIR-1 on pDCs, suggesting a potential mechanism for a loss of the inhibitory function of LAIR-1 in SLE (60).

ROLE OF HMGB1 IN SPECIFIC SLE DISEASE PHENOTYPES

HGMB1 in Lupus Nephritis

The role of HMGB1 in lupus nephritis (LN) illustrates its central role in linking the innate and adaptive aspects to cause the disease phenotype. Lupus nephritis is an example of immune complex-mediated end organ damage in SLE. It is a frequent complication and an important cause of long-term disability and death in the disease (61). Its etiology, as with that of SLE as a whole, involves the loss of immune tolerance resulting in the production of autoantibodies against nuclear autoantigens, potentially through increased exposure to specific antigens and also through polyclonal B cell activation. These immune complexes activate intrarenal TLRs and IFN signaling, resulting in the local production of proinflammatory cytokines by glomerular endothelium, mesangial cells and macrophages. Damage to renal parenchyma triggers tissue repair mechanisms that lead to glomerulosclerosis and chronic kidney failure (61).

Putterman et al. have shown that in the MRL/lpr mouse model of SLE, anti-DNA antibodies can alter the gene expression in mesangial cells of the kidney, upregulating proinflammatory genes and facilitating kidney damage (62). They further demonstrated that HMGB1 has a synergistic effect with anti-DNA antibodies on this process in a RAGE/TLR2 dependent manner (63). It has also been shown that, through TLR2, HMGB1 can induce proliferation of glomerular mesangial cells. Inhibition of either HMGB1 or TLR2 resulted in the decrease in fibronectin and collagen IV, accompanied by improved glomerular histological changes and sclerosis levels (64).

Renal macrophages from the SLE mice were found to be strong producers of the proinflammatory cytokines TNFα and IL-6, which have been suggested as important pathogenic cytokines in mediating kidney inflammation and damage in SLE (65). HMGB1 overexpression in mice resulted in an increased macrophage proinflammatory cytokine response and increased severity of lupus nephritis, whereas administration of glycyrrhizin, a blocker of HMGB1 had an opposite effect (66). Both *in vivo* and *in vitro* experiments confirmed that HMGB1's enhancement of macrophage response is through receptor RAGE (66). These results demonstrate that HMGB1 has kidney-specific effects in addition to its global contribution to SLE's etiology. Finally, urinary HMGB1 has been shown to differentiate SLE patients with active LN from inactive and from healthy individuals (67), again suggesting high local concentrations of HMGB1 in LN.

HGMB1 in Neuropsychiatric SLE

Neuropsychiatric systemic lupus erythematosus (NPSLE) is a manifestation of SLE reported in up to 80% of patients. It can affect both the central and the peripheral nervous systems and is mostly characterized by cognitive impairment (68). Damage to the blood-brain-barrier can be seen in NPSLE, which allows anti-DNA antibodies access to the central nervous system (69). A subset of anti-DNA antibodies termed DNRAbs cross reacts with the N-methyl-D-aspartate receptor (NMDAR). In mouse models of SLE, enhanced NMDAR signaling by DNRAbs result in spatial memory impairments (70–73) and in patients, elevated titers of DNRAb correlate with memory impairment. Transient exposure to DNRAb leads to long-term neuronal dysfunction through a 2-stage process with stage 1 involving excitotoxic neuronal death and stage 2 involving microglia activation and neuronal pruning (74). HMGB1 can be secreted by stressed or activated cells, including neurons activated through the NMDAR. Interestingly HMGB1 binds to NMDARs. Nestor et al. showed that dendrites bound to C1q are targeted for destruction, resulting in the deficits in spatial memory seen in SLE. C1q uses HMGB1 as a bridge that connects it to the NMDAR. Both *in vivo* and *in vitro* data showed that NMDAR-HMGB1-C1q complexes formed on dendrites target them for destruction by microglia, which itself is activated by HMGB1 through RAGE/TLR4 (74).

HMGB1-BASED THERAPEUTICS

The standard treatments options for SLE are currently centered around corticosteroids and immunosuppressive drugs with numerous unwanted side effects (75). Therapeutics have shifted toward targeting specific pathways (76). Small molecule inhibitors of HMGB1 such as tashinone IIA derivatives and glycyrrhizin are being investigated with some clinical success (77). The possible therapeutic effects of HMGB1-specific antagonists have also been explored in several preclinical studies. The A box domain of HMGB1 alone can bind to HMGB1 receptors such as TLR2/4 and RAGE without eliciting proinflammatory responses and can, therefore, serve as a potent competitive inhibitor of HMGB1 (18). Administration of HMGB1 A box as an HMGB1 antagonist has been shown to reverse lethality in a model of sepsis (78). The effects of monoclonal HMGB1-neutralizing antibodies have also been investigated in various diseases. In SLE specifically, studies on monoclonal HMGB1 antibodies showed conflicting results, with some experiments demonstrating amelioration of SLE disease phenotypes in MRL/lpr mice and BXSB mice (79, 80) while another finding no effects in disease progression in MRL/lpr mice (81). Contrasting clinical outcomes have led to efforts in inhibiting HMGB1 by other means. In addition to direct inhibition of HMGB1, pathways involving HMGB1 can be harnessed for their anti-inflammatory effects. HMGB1 is known to regulate macrophage polarization through its interaction with RAGE, LAIR-1 and C1q (59). Further studies showed that HMGB1 through a positive feedback loop involving IRF5 increases leukotriene B4 production in activated monocytes while HMGB1 plus C1q increase the production of specialized pro-resolving lipid mediators (82). In the same study, a fusion protein that contains the RAGE-binding fragment of HMGB1 and the LAIR-1-binding fragment of C1q were shown to crosslink the two receptors the same way HMGB1 and C1q do and to exert the same pro-resolving effects both *in vivo* and *in vitro* (82). Recognizing that HMGB1 can be harnessed to enhance tolerogenic properties of the immune system opens up novel opportunities for potential therapeutics.

CONCLUDING REMARKS

HMGB1 has been shown to affect a wide array of disease processes in SLE. Functioning both as a DAMP, HMGB1 is able to exerts its pathogenic effects on both the innate and the adaptive immune systems. HMGB1 also interacts with local cells in the diseased organs in SLE, exacerbating disease progression. Investigating the various effects of HMGB1 on the immune system can be extremely valuable in enhancing our understanding of SLE, and in the development of new therapeutics.

AUTHOR CONTRIBUTIONS

TL wrote the manuscript and prepared the figure. MS contributed to the conception and scheme of the manuscript. BD contributed to the conception, supervision, and critical revision of the manuscript.

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REVIEW
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Immunological Significance of HMGB1 Post-Translational Modification and Redox Biology

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Most extracellular proteins are secreted via the classical endoplasmic reticulum (ER)/Golgi-dependent secretion pathway; however, some proteins, including a few danger-associated molecular patterns (DAMPs), are secreted via non-classical ER/Golgi-independent secretion pathways. The evolutionarily conserved high mobility group box1 (HMGB1) is a ubiquitous nuclear protein that can be released by almost all cell types. HMGB1 lacks signal peptide and utilizes diverse non-canonical secretion mechanisms for its extracellular export. Although the post-translational modifications of HMGB1 were demonstrated, the oxidation of HMGB1 and secretion mechanisms are not highlighted yet. We currently investigated that peroxiredoxins I and II (PrxI/II) induce the intramolecular disulfide bond formation of HMGB1 in the nucleus. Disulfide HMGB1 is preferentially transported out of the nucleus by binding to the nuclear exportin chromosome-region maintenance 1 (CRM1). We determined the kinetics of HMGB1 oxidation in bone marrow-derived macrophage as early as a few minutes after lipopolysaccharide treatment, peaking at 4 h while disulfide HMGB1 accumulation was observed within the cells, starting to secrete in the late time point. We have shown that HMGB1 oxidation status, which is known to determine the biological activity in extracellular HMGB1, is crucial for the secretion of HMGB1 from the nucleus. This review summarizes selected aspects of HMGB1 redox biology relevant to the induction and propagation of inflammatory diseases. We implicate the immunological significance and the need for novel HMGB1 inhibitors through mechanism-based studies.

Keywords: high mobility group box1 (HMGB1), oxidation, inflammation, therapeutic target, danger-associated molecular pattern (DAMP)

INTRODUCTION

High mobility group box 1 (HMGB1) is an abundant non-histone nuclear protein that was discovered over four decades ago. The protein was isolated from calf thymus chromatin by 0.35 M NaCl extraction (1) and was then biochemically characterized (2). Based on its mobility during polyacrylamide gel electrophoresis, Goodwin et al. termed the proteins "high mobility group," or HMG proteins; however, the group of proteins that migrated more slowly during polyacrylamide

gel electrophoresis were termed "low-mobility group" proteins. HMG proteins were therefore divided into two groups based on their molecular weight: higher—HMG-1 and HMG-2 (now HMGB1 and HMGB2), lower—HMG-14 and HMG-17 (now HMGN1 and HMGN2), and HMG-I, -Y (now HMGA1a and HMGA1b) (3–9).

HMG proteins are categorized into three superfamilies based on the specific functional domains or motifs via which they recognize individual DNA structures on chromatin: HMGA, HMGB, and HMGN. Proteins in the HMGA family contain an AT-hook, which is a DNA-binding motif with a preference for A/T rich regions. In contrast, those in the HMGB family contain A-box and B-box functional motifs and those in the HMGN family contain a nucleosomal binding domain (NBD). HMGB proteins are ubiquitous and abundant in most cells and can bind to DNA without sequence specificity (10–12).

The human HMGB1 protein has 215 amino acid (aa) residues (MW: 25–30 kDa) that form two homologous DNA-binding domains (A-box, 1–79 aa; B-box, 89–162 aa) and a negatively charged C-terminal acidic tail (186–215 aa; **Figure 1A**) (9, 13). HMGB1 is located in the nucleus as a result of bipartite nuclear localization signals (NLS; NLS1, 28–44 aa; NLS2, 179–185 aa) mediated by the nuclear importin karyopherin (KAP)-α1; however, the affinity between the two molecules is decreased by HMGB1 phosphorylation (14, 15). Conversely, the DNA-binding domain of HMGB1 contains a nuclear-export signal (NES), and its cytoplasmic localization is mediated by the nuclear exportin chromosome-region maintenance 1 (CRM1) (16). The acidic C-terminal of HMGB1 regulates DNA binding and bending by interacting with its DNA-binding domains (8, 17) or histones H1/H3 (18); thus, HMGB1 lacking the C-terminal domain displays improved DNA looping and binding abilities (19).

The C-terminal acidic domain of HMGB1 also functions as a transcriptional activator (20, 21), while HMGB1 B-box has been reported to induce pro-inflammatory signals upon extracellular stimulation, and the A-box induces antagonistic effects (22). In particular, the 201–205 aa residues in the C-terminal acidic tail play a crucial role in the antibacterial activity of HMGB1 (23). Moreover, interactions with diverse receptors, extracellular partners, and intracellular partners play important roles in the activity and biological functions of HMGB1. HMGB1 residues 89–108 bind to Toll-like receptor (TLR) 4 and increase pro-inflammatory signaling (22), whereas residues 150–183 interact with the receptor for advanced glycation end products (RAGE) to regulate cell migration (24) and stimulate inflammation (25). HMGB1 has also been shown to bind to dendritic cell (DC)-derived TIM-3 and suppress nucleic acid-mediated innate immune responses (26). In addition, residues 1–15 and 80–96 have been found to inhibit lipopolysaccharide (LPS)-induced cytokine production in a subclinical endotoxemia mouse model (27). HMGB1 binds to lipoteichoic acid (LTA) and enhances proinflammatory responses by mediating the transfer of LTA to CD14 and TLR2 (28). Furthermore, HMGB1 residues 6–12 are responsible for binding heparin and compete with binding between RAGE and HMGB1 (29). A- and B-box of HMGB1 bind to C1q, but only B-box of HMGB1 can induce the complement activation leading to sterile inflammation (30). In addition, complex formation between HMGB1 and IL-1β enhances inflammation and destruction mechanisms in arthritic joints (31), whereas the HMGB1 and C-X-C motif chemokine ligand 12 (CXCL12) complex binds to C-X-C chemokine receptor 4 (CXCR4) and promotes the recruitment of inflammatory cells (32). Extracellular HMGB1 binds to single-stranded oligonucleotides, forming HMGB1-5'-C-phosphate-G (CpG)-DNA complex, interacting with TLR9 to augments cytokine production (33, 34). Also, HMGB1 released from apoptosis binds to the nucleosomes and induces cytokine production or dendritic cells (DCs) activation through interaction with TLR2 (35). In contrary, HMGB1-CD24 complex selectively represses the tissue damage-induced inflammation via interaction with Siglec-10 protein (36). Cytoplasmic HMGB1 binds to Beclin 1 using intramolecular disulfide bridge (Cys23 and Cys45) to affect autophagosome formation (37). Cys23 or Cys45 in HMGB1 can also bind to reactive cysteine residues in peroxiredoxins I and II (PrxI/II) to form intramolecular disulfide bonds that promote its secretion in response to inflammatory stimuli (38). Residues 7–74 are responsible for binding the p53 transactivation domain and thus increasing gene transcription (39) (**Figure 1A**).

Studies have modified HMGB1 A-box and B-box structures using the PyMol program based on 2YRQ [Protein Data Bank (PBD) ID: 2yrq]. These two DNA-binding domains consist of three alpha helices (helix-I, -II, and -III) and two loops (loop-I and -II) that form an L-shaped structure (40). HMGB1 binds to the minor groove of pre-bent or linear DNA with little sequence specificity (41, 42); however, both A- and B-box have the remarkable ability to unwind and bend DNA with different properties. For instance, the A-box domain recognizes pre-bent or linear DNA, whereas the B-box domain binds to mini-circles and bends linear DNA (43–45). In the crystal structure of HMGB1 showing A-box domains and an AT-rich DNA fragment, the two HMGB1 A-box domains were found to collaborate in order to interact with pre-bent or kinked DNA. The Phe37 (Phe38 in HMGB1 described here) residues from both domains were shown to play important roles in initiating intercalation with CG base pairs and thus generating highly kinked DNA (**Figure 1B**) (46). The B-box domain is structurally similar to the A-box in its DNA-binding characteristics and its Ile34 (Ile35 in B-box or Ile122 in the full HMGB1 sequence described here) residue is sterically comparable to the Phe37 residue in the A-box domain (**Figure 1B**).

HMGB1 senses and coordinates the cellular stress response. As mentioned earlier, HMGB1 contains three conserved cysteines: Cys23, Cys45, and Cys106 (**Figure 1A**). Cys23 and Cys45 can form an intramolecular disulfide bond depending on the reactive oxygen species (ROS) concentration and environmental conditions under which HMGB1 binds to its ligands (**Figure 1C**) (47). Indeed, the half-life of all-thiol-HMGB1 ranges from ∼17 min in human serum and saliva to 3 h in cell culture medium (47). The oxidation state of HMGB1 determines its interactions with diverse receptors (32, 48) and its DNA-binding affinity (49). Depending on its redox status,

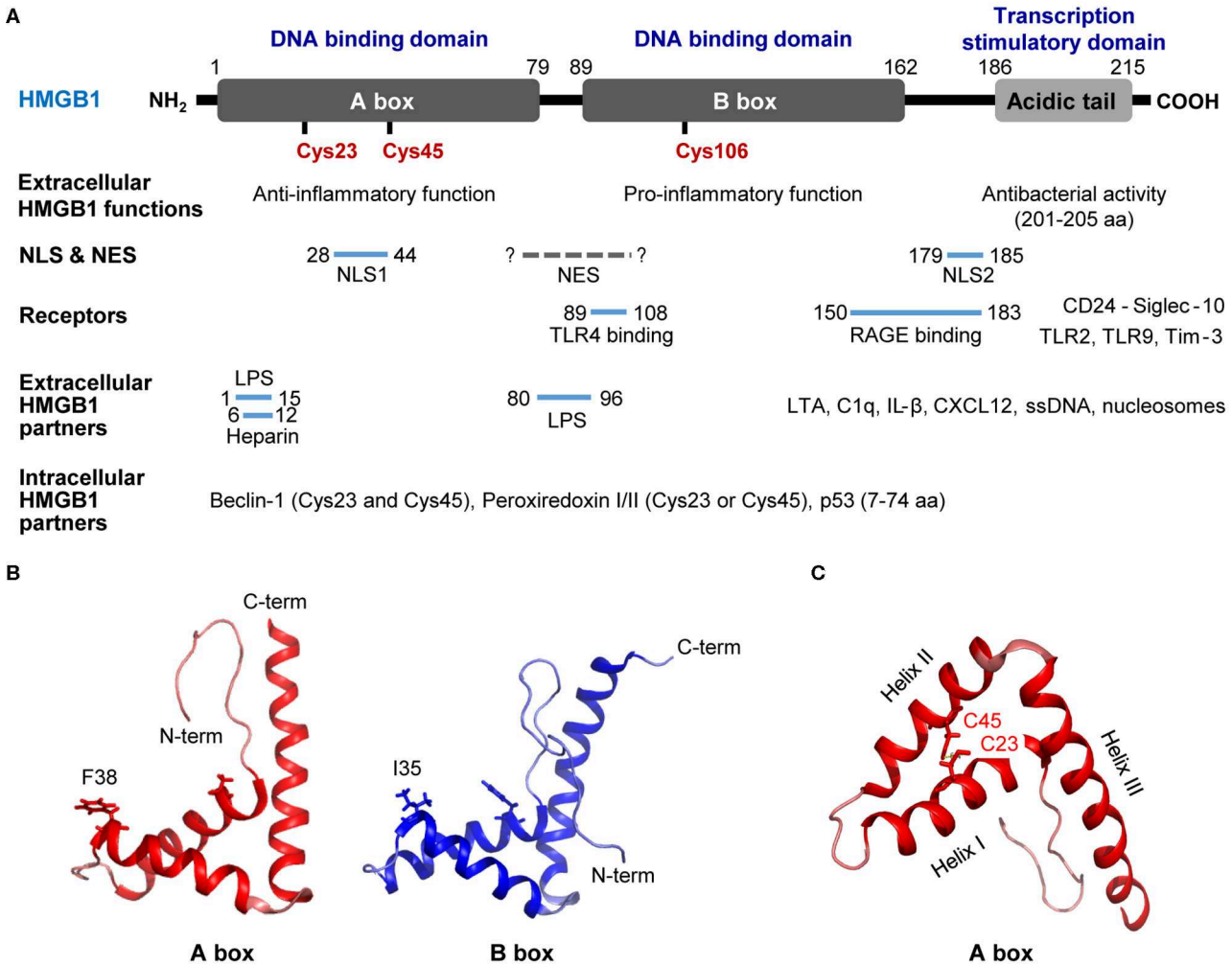

FIGURE 1 | Structure of HMGB1. **(A)** Detailed summary of various domains and motifs in HMGB1. **(B)** Signature L-loop structure of the A-box and B-box structures of HMGB1. **(C)** Overall structure of HMGB1 showing the functional A-box cysteines (C23 and C45) and their geometric distribution in HMGB1. A-box and B-box structures modified based on 2YRQ [Protein Data Bank (PDB) IP: 2yrq] using PyMol. NLS, nuclear localization sequence; NES, nuclear export signal; TLR4, toll-like receptor 4; RAGE, receptor for advanced glycation endproducts; Tim-3, T-cell immunoglobulin and mucin domain-containing protein-3; LPS, lipopolysaccharide; LTA, lipoteichoic acid; C1q, complement component 1q; IL-1β, interleukin 1β; CXCL12, C-X-C motif chemokine 12; F, phenylalanine; I, isoleucine; C, cysteine; C-term, C-terminal; N-term, N-terminal. Solid line; known binding domain or sequence of HMGB1, Dotted grey line; unknown sequence of HMGB1.

extracellular HMGB1 can trigger numerous effects: (1) all-thiol-HMGB1 can exert chemoattractive effects by binding to CXCR4; (2) all-thiol-HMGB1 can prompt autophagy by binding to RAGE (50); (3) disulfide-HMGB1 can exert pro-inflammatory effects by binding to TLR4; and (4) fully oxidized-HMGB1 is inert. Cys106 plays a crucial role in the translocation of HMGB1 from the nucleus to the cytosol (51). Moreover, C23-C45 oxidation induces a shift between helix I and helix II in the A-box domain that reduces DNA binding affinity by altering the orientation of Phe37 (52), resulting in cytoplasmic translocation. HMGB1 can also affect transcription in the nucleus, requiring rapid transition between the all-thiol-and disulfide forms of HMGB1 (53).

Purification of HMGB1 under its native conditions yields both homodimers and oligomeric forms of the protein; however, these forms are dissociated when acid-extracted (54). Our group has also described the Cys106-mediated formation of HMGB1 dimers under conditions of excessive ROS generation at the cellular level (unpublished data). As previously discussed, HMGB1 is a versatile molecule because of intra- and inter-molecular interactions in its different domains; moreover, the protein can be either actively secreted by activated immune cells or passively released due to necrotic cell death where it acts as a damage-associated molecular pattern (DAMP). Because of its versatile and variable nature, it is important to understand the mechanism underlying the secretion of HMGB1 to fully appreciate its therapeutic and pathological potential. In this review, we briefly summarize the conventional and non-conventional mechanisms of cytokine secretion, and describe in detail the mechanisms of HMGB1 oxidation and

secretion that have been determined so far, with a focus on immunological function.

CONVENTIONAL AND NON-CONVENTIONAL CYTOKINE SECRETION MECHANISMS

Most soluble secretory proteins utilize a well-known conventional secretion system involving the endoplasmic reticulum (ER) and Golgi network, and contain a signal peptide to target them to the ER (55). When such proteins are synthesized in the ribosome, the signal peptide is recognized by a signal recognition particle (SRP) complex. The protein-ribosome-SRP complex then moves to the Sec61 translocon complex in the ER outer membrane and proteins translocate into the ER lumen via the translocon complex (56, 57). Within the ER lumen, the protein meets chaperone proteins such as Bip and undergoes modification (glycosylation) followed by protein folding (58, 59). These proteins are then translocated to the Golgi and plasma membrane via several processes, concluding the conventional protein secretion pathway. A recent study found that when the ER/Golgi pathway is blocked, some proteins are secreted via an independent mechanism. These proteins lack a signal peptide targeting them to the ER and are secreted under specific conditions, such as ROS accumulation, inflammation, and cell growth factors. These forms of protein secretion are considered unconventional, and the secreted proteins are generally involved in immune surveillance, cell survival, and cellular stress (60).

Cytokines regulate immunological functions via their secretion in immune environments; therefore, controlling cytokine secretion is crucial for regulating immune function. Many cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-2, and IL-12 are secreted via the conventional secretion mechanism; however, some, including HMGB1, IL-1β, fibroblast growth factor (FGF)-1/2, and galectins, utilize non-conventional secretion mechanisms. Here, we briefly explore different cytokines that use various secretion mechanisms, and summarize them in **Table 1**.

Tumor Necrosis Factor (TNF)-α

TNF-α was first identified for its anti-tumor activity but is now also known to act as a multifunctional cytokine in host defense mechanisms during inflammatory responses (77). Since it contains an ER signal peptide, TNF-α is translocated across the ER and through the Golgi apparatus to the plasma membrane (61). Newly synthesized TNF-α is localized in the trans-Golgi network (TGN) with golgin and p230/golgin-245 for intracellular trafficking and cell surface delivery (78). TNF-α is then released from the granule via fusion with the plasma membrane (62) and is cleaved at the cell surface by the tumor necrosis factor-α converting enzyme (TACE) between Ala^{76} and Val^{77} (79).

Interleukin (IL)-1β

IL-1β plays important roles in the cytokine response to inflammation and immunity during bacterial or viral infection and is mainly secreted by monocytes, macrophages, and CD in response to inflammasome activation under conditions such as LPS and adenosine triphosphate (ATP) stimulation (80). Although IL-1β is passively released during pyroptotic cell death (63), secretion of cleaved-IL-1β requires cytosolic compartments such as the secretory lysosome (64), microvesicles shed from the plasma membrane (81) or exosomal release (65). Previous studies have reported that caspase-1/11 cleave gasdermin D (GSDMD), whose N-terminal then forms pores in the plasma membrane that are crucial for the passive release of IL-1β during pyroptosis (66, 67). Although the pathway is unknown, IL-1β secretion is known to require ABC transporters whose knockdown or inhibition is reported to ameliorate IL-1β secretion (82, 83).

Fibroblast Growth Factor (FGF) -1 and -2

FGF-1 and -2 belong to a family of heparin-binding growth factors (84) and control mitogenic activity (85) and tumor-induced angiogenesis (86). FGF-1/2 are not only secreted via the ER/Golgi-dependent secretion pathway, but also the non-conventional secretion system (68–70); however, the secretion pathways of FGF-1 and FGF-2 are distinct (87). Unlike FGF-1, FGF-2 secretion is sensitive to Na^+/K^+-ATPase inhibition and is dependent on forming higher-order complexes with Na^+/K^+-ATPase ion transporters, with its export occurring in a membrane

TABLE 1 | Conventional and non-conventional secretion of cytokines.

Cytokine	Conventional or Non-conventional	Secretion mechanism	References
TNF-α	Conventional	- Translocated across the ER and through the Golgi apparatus to the plasma membrane	(61, 62)
IL-1β	Non-conventional	- Secretion by secretory lysosome, microvesicles shed, or exosome - Gasdermin D (GSDMD)-dependent	(63–67)
FGF-1/2	Conventional/ Non-conventional	- FGF-1/2 are not only secreted via the conventional secretion pathway, but also the non-conventional secretion system FGF-1 - Secretion is increased by cellular stresses such as heat shock, hypoxia, and serum starvation FGF-2 - Dependent on forming complexes with Na^+/K^+-ATPase	(68–73)
Galectins	Non-conventional	- Accumulate at the plasma membrane and induce the formation of exosomes pinched off and released into extracellular space	(74–76)

potential-independent manner (71). Conversely, FGF-1 secretion is increased by cellular stresses such as heat shock (88), hypoxia (72), and serum starvation (73), while copper also can induce FGF-1 secretion by forming multiprotein aggregates in response to stress (89); however, FGF1 folding does not prevent its export (90).

Galectins

Galectins are a family of abundant β-galactoside-specific lectins that reside in the extracellular matrix and are implicated in many cellular processes, such as proliferation, differentiation, and apoptosis (91, 92). Since galectins lack the signal peptides found in IL-1β and FGF-1/2 for ER/Golgi-mediated secretion, their secretion is not blocked by the ER/Golgi-dependent inhibitors brefeldin A and monensin (74, 93). Moreover, galectin-1/3 are not packaged into vesicles during extracellular export (74, 75, 94, 95) but accumulate at the plasma membrane and induce the formation of exosomes that are pinched off and released into extracellular space (74, 75, 94, 95). Secreted galectins bind to the extracellular surface of the plasma membrane or extracellular matrix (75, 76) via the *N*- and *O*-glycosylated β-galactose-terminated oligosaccharide side chains of glycoproteins (74, 92).

HMGB1 SECRETION

Generally, cytokines containing a leader sequence undergo secretion via the ER/Golgi secretion pathway; however, the non-histone nuclear protein HMGB1 lacks this signal peptide, and studies have suggested that HMGB1 secretion involves diverse unconventional secretion pathways. For instance, infection or cellular stress has been shown to increase the cytoplasmic accumulation of HMGB1, which is then passively released into the extracellular space or actively secreted via secretory lysosomes (9) (**Figure 2**). Here, we describe in detail the mechanisms that participate in HMGB1 secretion.

Passive Release of HMGB1

HMGB1 can be passively released during various forms of cell death, including pyroptosis, apoptosis, autophagy, necroptosis, and necrosis (**Figure 2A**). Pyroptosis refers to inflammatory programmed cell death that occurs after inflammasome formation caused by bacterial or viral infection. During pyroptosis, double-stranded RNA-dependent protein kinase (PKR) induces inflammasome formation, caspase-1 activation, and HMGB1 release upon exposure to diverse inflammasome-activating agents (96). Apoptosis is another form of programmed cell death that occurs when cells die due to injury and involves caspase-3/7, which belong to a family of protease enzymes. Apoptotic cells induce HMGB1 release, and it has been reported that Z-VAD, a pan-caspase inhibitor, can reduce the levels of HMGB1 released (97). Autophagy is an intracellular degradation system that balances energy sources in response to nutrient stress by regulating the degradation of cellular material using lysosomes or vacuoles; however, excessive autophagy can lead to cell death. Indeed, studies have shown that epithelial and glioblastoma tumor cells release HMGB1 when treated with the autophagy-inducing agent epidermal growth factor receptor-targeted diphtheria toxin (DT-EGF) (98), also ATG5 knock-out bone marrow-derived macrophages (BMDMs) reduced HMGB1 secretion under EBSS starvation conditions (99). In addition, autophagosome-mediated HMGB1 secretion has been identified, with ATG5 deficient cells or those treated with an early autophagy inhibitor displaying all-thiol-HMGB1 secretion (data not published). Necrosis is a form of premature cell death caused by the loss of membrane integrity, intracellular organelle swelling, and ATP depletion, and it has been shown that HMGB1 is passively released by necrotic or damaged cells (100). Necroptosis is a form of programmed necrosis mediated by death signals that cause the phosphorylated mixed lineage kinase domain-like protein (MLKL) to be inserted into and permeabilize the plasma membrane (101). Moreover, TNF-α/Z-VAD-induced necroptosis has been shown to phosphorylate MLKL proteins and increase HMGB1 secretion levels (102).

Post-Translational Modifications (PTMs) and Active Secretion of HMGB1

HMGB1 can undergo several extensive PTMs that increase its cytoplasmic accumulation and extracellular secretion during infection or cell stress, including acetylation (16), phosphorylation (14, 15), ADP-ribosylation (103), methylation (104), glycosylation (105), and oxidation (38, 51) (**Figure 2B**). Various PTMs increase the interaction between HMGB1 and the nuclear transport receptor CRM1, thus favoring its translocation from the nucleus to the cytoplasm. PTM-mediated HMGB1 secretion is caused by lysosomal exocytosis wherein cytoplasmic HMGB1 co-localizes with the lysosomal marker LAMP1 for secretion (106). The PTMs and subsequent events that HMGB1 undergoes are summarized below and visualized in **Figure 2B**.

1) Acetylation is a major PTM that can affect protein function by altering properties such as hydrophobicity, solubility, and surface properties. Protein acetylation refers to the reaction during which the acetyl group of acetyl coenzyme A (Ac-CoA) is transferred to the lysine (Lys) residue of the target protein. HMGB1 has two acetylation clusters at Lys27–29 and Lys181–183, and it has been shown that nuclear localization is unaffected by mutating either Lys cluster (16). The poly(ADP-ribose) polymerase-1 (PARP1) induces the cytoplasmic translocation and extracellular secretion of HMGB1 by catalyzing its acetylation (107). Compare to HMGB1, mimicking acetylated HMGB1 (six lysine residues for glutamines) increases the TNF-α production in RAW264.7 cells and reduces DC maturation (108). Various triggers which induces HMGB1 acetylation includes inflammatory signal such as LPS or TNF-α (109), and cell stress triggered by chemotherapeutic reagent such as cisplatin (110). Such conditions can be experimentally mimicked using trichostatin A (TSA), an inhibitor of histone deacetylase complex (HDAC) (111).
2) Phosphorylation is a molecular mechanism via which amino acid residues are phosphorylated by a protein kinase to regulate the functional response of proteins to various extra- or intracellular stimuli. HMGB1 phosphorylation is mediated by classical protein kinase C (cPKC) in a calcium-dependent

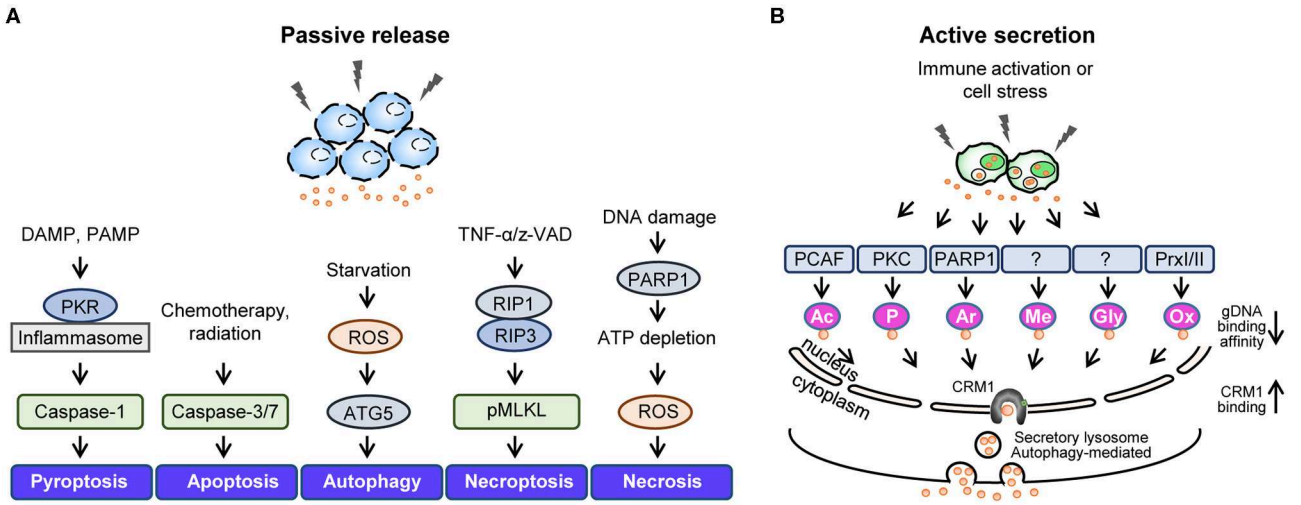

FIGURE 2 | Active secretion and passive release of HMGB1. Summary of stimuli leading to passive HMGB1 release **(A)** and active HMGB1 secretion **(B)**. **(A)** Passive release mechanisms involve the disruption of the plasma membrane via various cell death mechanisms. **(B)** Active secretion involves various HMGB1 post-translational modifications that reduce its genomic DNA binding activity and increase its CRM1 binding affinity. DAMP, danger-associated molecular pattern; PAMP, pathogen-associated molecular pattern; PKR, double-stranded RNA-dependent protein kinase; ROS, reactive oxygen species; ATG5, autophagy related 5; z-VAD, carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]; RIP1, receptor-interacting serine/threonine-protein kinase 1; RIP3, receptor-interacting protein kinase 3; pMLKL, Phosphorylated mixed linage kinase domain like; PARP1, poly [ADP-ribose] polymerase 1; PCAF, P300/CBP-associated factor.

manner via the PI3K-PKC signaling pathway (14). In HMGB1, Ser35, 39, 42, and 46 in NLS1, 181 in NLS2, and 53 close to NLS1 have been shown to be phosphorylated in macrophages after TNF-α and okadaic acid treatment (15), while Ser39, 53, and 182 of HMGB1 are phosphorylated by PKC-ζ in colon cancer cells (112). Moreover, HMGB1 phosphorylation has been found to reduce its binding affinity with the nuclear import protein KAP-α1 and promote its cytoplasmic translocation and extracellular secretion (15).

3) ADP-ribosylation refers to the process wherein one or more ADP-ribose moieties are added to target proteins, and includes mono-ADP-ribosylation, poly-ADP-ribosylation, ADP-ribose cyclization, and *O*-acetyl-ADP-ribose formation. PARP activation regulates the translocation of HMGB1 from the nucleus to the cytoplasm during DNA-alkylating damage (103), while hyper poly(ADP)-ribosylated HMGB1 has been shown to inhibit efferocytosis by binding to phosphatidylserine (PS) on apoptotic cells and RAGE on macrophages (113). Such activation was reported in cell death related stimuli such as activation of tumor necrosis factor [ligand] superfamily member 10 (TNFSF10)—TNF-related apoptosis-inducing ligand (TRAIL) pathway (114) or daunorubicin treatment (115), and inflammatory assault with LPS (103).
4) Methylation is a PTM in which a methyl group is added to proteins, usually on the side-chain nitrogens of arginine and lysine or carboxyl groups of glutamate and leucine. During the process of neutrophilic differentiation, Lys42 in HMGB1 can be mono-methylated which significantly reduces its DNA binding activity, causing its translocation from the nucleus to the cytoplasm (104). Lys112 has also been found to be mono-methylated in HMGB1 and contribute toward its cytoplasmic localization (116). However, it is still unclear which stimuli exclusively leads to the methylation of HMGB1 during active secretion.
5) Glycosylation is a common PTM characterized by the attachment of sugar moieties to proteins. HMGB1 derived from calf thymus and chicken erythrocytes undergoes *O*-linked GlcNac glycosylation with sugars such as Fuc, Man, $GalNH_2$, $GlcNH_2$, and Gal monosaccharides (117), while it has recently been reported that HMGB1 can also undergo *N*-linked glycosylation at two consensus (Asn37 and Asn137) residues and one non-consensus (Asn135) residue. *N*-glycosylation of HMGB1, induced by PMA, TSA and LPS, can persuade its secretion into the extracellular space by reducing its DNA binding affinity and increasing the association with CRM1 (105).
6) Oxidation is a covalent modification that proteins undergo during redox reactions involving the transfer of oxygen, hydrogen, and electrons. HMGB1 contains three redox-sensitive cysteines: Cys23, Cys45, and Cys106. Under mild oxidative stress, Cys23 and Cys45 rapidly form an intramolecular disulfide bond that increases the cytoplasmic localization and extracellular secretion of HMGB1 (51). In response to inflammatory stimuli, PrxI and PrxII induce HMGB1 oxidation to its disulfide form and lead to its nucleocytoplasmic translocation and secretion (38). Such oxidative stresses may come from external sources such as H_2O_2 or glucose oxidase, or by internal activation of molecules by LPS, TNF-α, or interferon-γ which in turn causes HMGB1 release through TNF-dependent manner (118). Since the oxidation status and immunological properties of HMGB1

crucially influence its biological function, we dedicated a separate section to discussing HMGB1 redox biology in detail.

HMGB1 Oxidation Mechanisms

The function and secretion of HMGB1 are dependent on its redox status, which is controlled by three redox-sensitive cysteines: Cys23 and Cys45 in A-box and Cys106 in B-box. Thus, HMGB1 can take three different oxidation forms: an all-thiol form, disulfide form (Cys23-Cys45 intramolecular disulfide bond with Cys106 thiol form), and a fully oxidized form. During the active secretion of HMGB1, the Cys23-Cys45 intramolecular disulfide bond induces HMGB1 cytoplasmic translocation. This process also requires Cys106, as demonstrated by the nuclear localization of Cys106-mutated even with Cys23 and Cys45 mutations (51). Hydrogen peroxide is a ROS that induces the release of HMGB1 from macrophages and monocytes, reportedly by increasing its interaction with CRM1 and thus increasing HMGB1 secretion (119). Under elevated ROS conditions, PrxI and PrxII cause disulfide-HMGB1 formation (38) (**Figure 3**). The diversity of HMGB1 redox status also affects its passive release from necrotic and apoptotic cells, with the majority of HMGB1 released from necrotic cells being in an all-thiol state but that released from apoptotic cells being in a fully oxidized form. Moreover, HMGB1 oxidation status plays an important role in receptor binding and subsequent cytokine-like activities.

Hoppe et al. previously described the mechanism of HMGB1 oxidation (51), identifying that HMGB1 interacts with the de-glutathionylation enzyme glutaredoxin (Grx) during the nuclear extraction of Chinese hamster ovary (CHO) cells after diamide treatment. Electrophoretic mobility assays revealed that HMGB1 oxidation increases in a diamide concentration-dependent manner, while disulfide-HMGB1 could be reversed by incubating diamide-treated retinal pigment epithelium (RPE) cells with thioredoxin (Trx) or Grx/glutathione (**Figure 3**). Conversely, we found that HMGB1 can be oxidized by PrxI/II in the nucleus after exposure to inflammatory stimuli (**Figure 3**) (38). PrxI/II can interact with all-thiol-HMGB1 generated by mutagenesis (Cys^{23}-to-Ser or Cys^{45}-to-Ser) after hydrogen peroxide stimulation; however, such HMGB1 oxidation is suppressed in PrxI/II-deficient mouse embryonic fibroblast

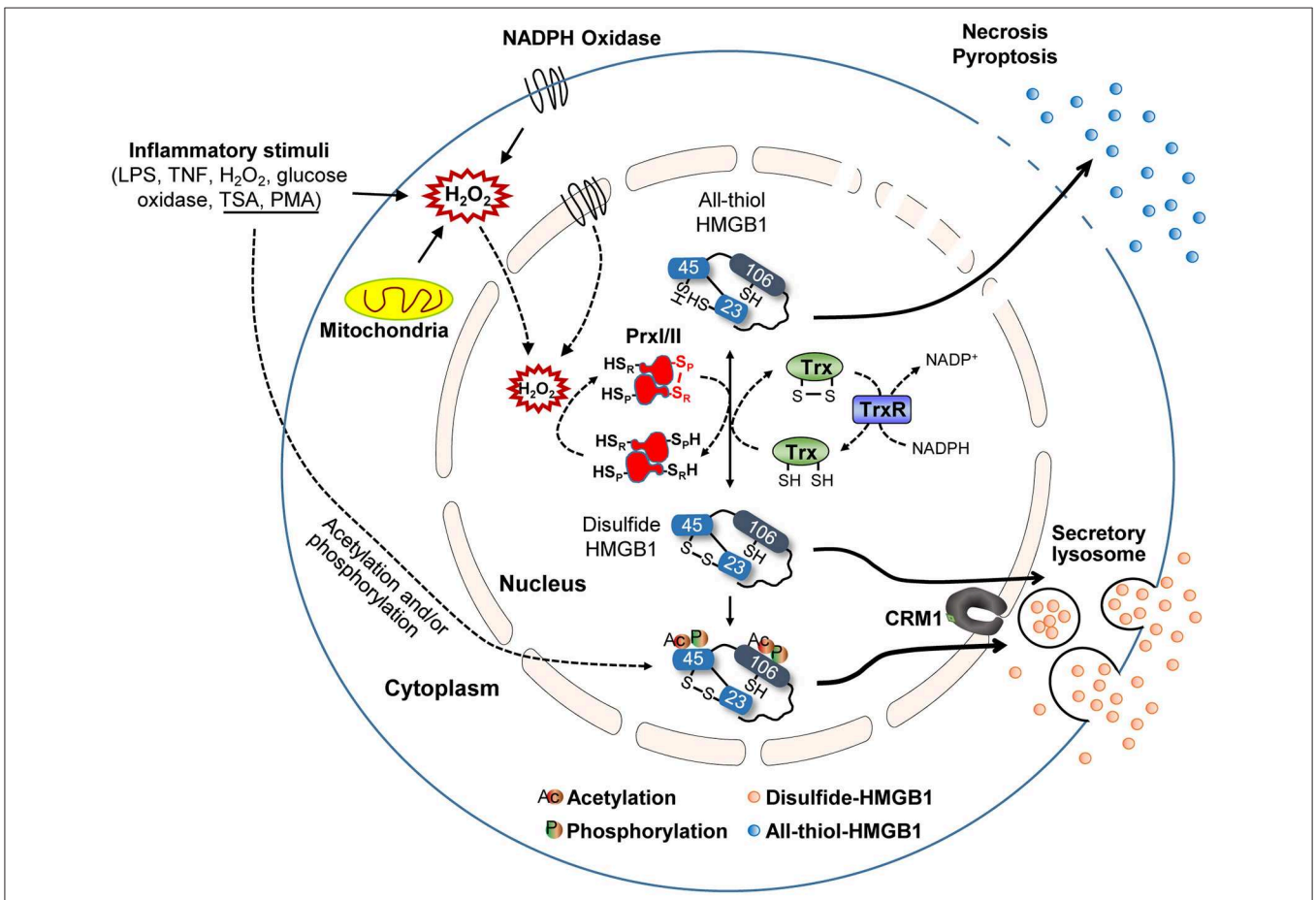

FIGURE 3 | HMGB1 redox biology. Summary of HMGB1 redox biology and the crucial role of peroxiredoxin and thioredoxin. Various stimuli cause oxidative stress that promotes HMGB1 oxidation via the peroxiredoxin-dependent pathway. NADPH, nicotinamide adenine dinucleotide phosphate; S_P, peroxidatic cysteine; S_R, resolving cysteine; H, hydrogen; Trx, thioredoxin; TrxR, thioredoxin reductases; NADP, nicotinamide adenine dinucleodebtide phosphate.

(MEF) cells, even when exposed to inflammatory stimuli. All-thiol-HMGB1 cannot translocate into the cytoplasm or extracellular space, while PrxI/II-deficient BMDMs lack the ability to secrete HMGB1 despite treatment with diverse inflammatory stimuli, such as LPS, phorbol-12-myristate-13-acetate (PMA), trichostatin A (TSA), or TNF-α (38). HMGB1 phosphorylation by PMA and/or acetylation by TSA promotes its nuclear transport and extracellular secretion (15, 16). Although cells treated with PMA or TSA display increased HMGB1 secretion, this secretion is inhibited by treatment with the antioxidant *N*-acetylcysteine (NAC). Moreover, HMGB1 secretion induced by PMA can be inhibited by the NADPH oxidase inhibitor diphenyleneiodonium (DPI), indicating that PMA-induced HMGB1 secretion requires H_2O_2 production by the mitochondria and/or Nox. Intracellular disulfide bond formation in HMGB1 (Cys^{23}-Cys^{45}) has been shown to be important for its nucleocytoplasmic translocation and extracellular secretion. Indeed, HMGB1 mutants with defective phosphorylation or acetylation sites undergo less translocation into the extracellular space than WT HMGB1 (38). In summary, these findings indicate that the mechanisms of HMGB1 oxidation and reduction are induced by PrxI/II and Trx or Grx/glutathione, respectively (**Figure 3**). It is possible that, like acetylated-HMGB1, oxidized-HMGB1 may be less favored for nuclear import and thus accumulates in the cytosol. Oxidized-HMGB1 in the cytosol is packed into lysosomes through an as yet unknown mechanism and then secreted. Nevertheless, extracellular HMGB1 can induce an immune response and HMGB1 oxidation decides its immune function. Also, as mentioned previously, HMGB1 oxidation has a more substantial influence on its secretion compared to acetylation and phosphorylation. Thus, control of HMGB1 oxidation both in intracellular and extracellular is important for the therapeutic approach based on blockade of HMGB1 secretion and immune response.

HMGB1 Secretion Kinetics

The PTMs of HMGB1 in the nucleus occurs rapidly after exposure to diverse stimuli; however, after binding to CRM1 in the nucleus, the export of modified HMGB1 into the cytoplasm is known to take around 6–8 h, whereas it can take up to 18 h for HMGB1 secretion into the extracellular space to peak. The degree of HMGB1 oxidation has been reported to be crucial for its immune function (9, 51, 120) and is also very important for its secretion. For instance, HMGB1 secretion induced by PTMs such as phosphorylation or acetylation also requires oxidation, with anti-oxidant treatment reducing HMGB1 secretion even when treated with PMA or TSA (38). Therefore, we examined instances when HMGB1 PTMs and extracellular secretion occur under oxidative conditions by treating mouse BMDMs with 100 ng/mL of LPS and separating their nuclei at a series of time points to determine the HMGB1 oxidation ratio. HMGB1 oxidation increased with time, with oxidation first detectable after just 30 min, and disulfide-HMGB1 was maintained for up to 4 h and then gradually decreased after 8 h. Despite rapid HMGB1 oxidation in the nucleus, its secretion began only after 4 h and increased up to 16 h (**Figure 4**). Further studies investigating the mechanism underlying the delay between oxidation and secretion would improve our understanding of HMGB1 secretion kinetics. It is possible that secretion-ready cytosolic HMGB1 is packed into a secretory lysosome or autophagosome and secreted via non-conventional secretion mechanisms, requiring a very complex, as yet unknown, packaging mechanism. Conversely, a recent article by Wang *et al.* observed HMGB1 localization in mitochondria and peroxisome in neuron cells via electron microscopy and immunofluorescence, but not in lysosomes (121). In macrophages or macrophage lineages, the release of HMGB1 occurred through a lysosomal pathway after acetylation of the HMGB1 (16). It has also been demonstrated that LPS-induced HMGB1 secretion by monocytes is mediated by lysosomal exocytosis (106). Various explanations may be

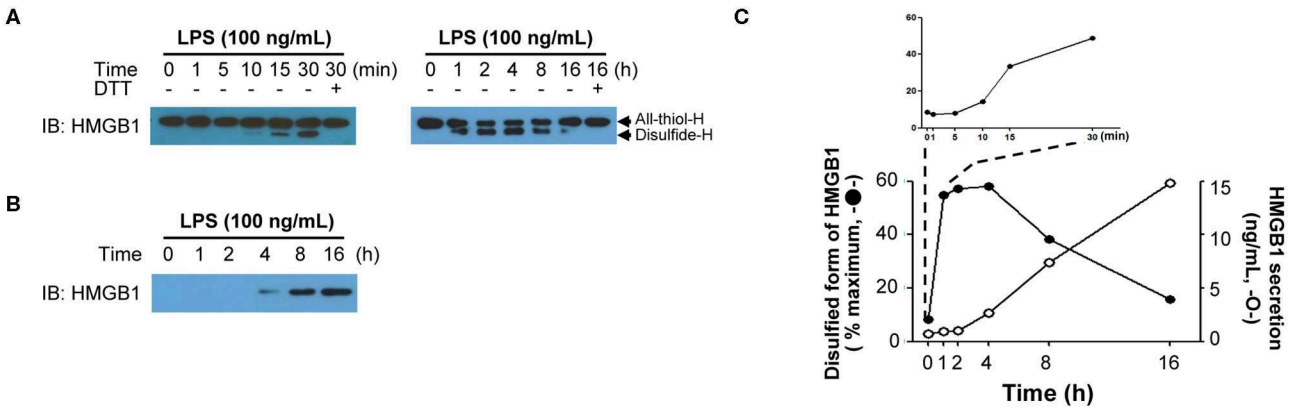

FIGURE 4 | HMGB1 redox kinetics. **(A)** Short-term (left) and long-term (right) changes of HMGB1 redox status upon LPS stimulation. Western blot showing the All-thiol-H (all-thiol-HMGB1) or Disulfide-H (disulfide-HMGB1) expression in BMDM whole-cell lysates, which was treated with LPS (100 ng/mL) for indicated times. Methods were used as our previous study (38). **(B)** HMGB1 secretion timeframe. Disulfide-H from culture supernatant was measured by Western blot. **(C)** Graphical representation of the relationship between HMGB1 oxidation (left *y*-axis, closed circle) quantified as % maximum from **(A)** and HMGB1 secretion (right *y*-axis, opened circle). The level of secreted HMGB1 was determined by ELISA (38).

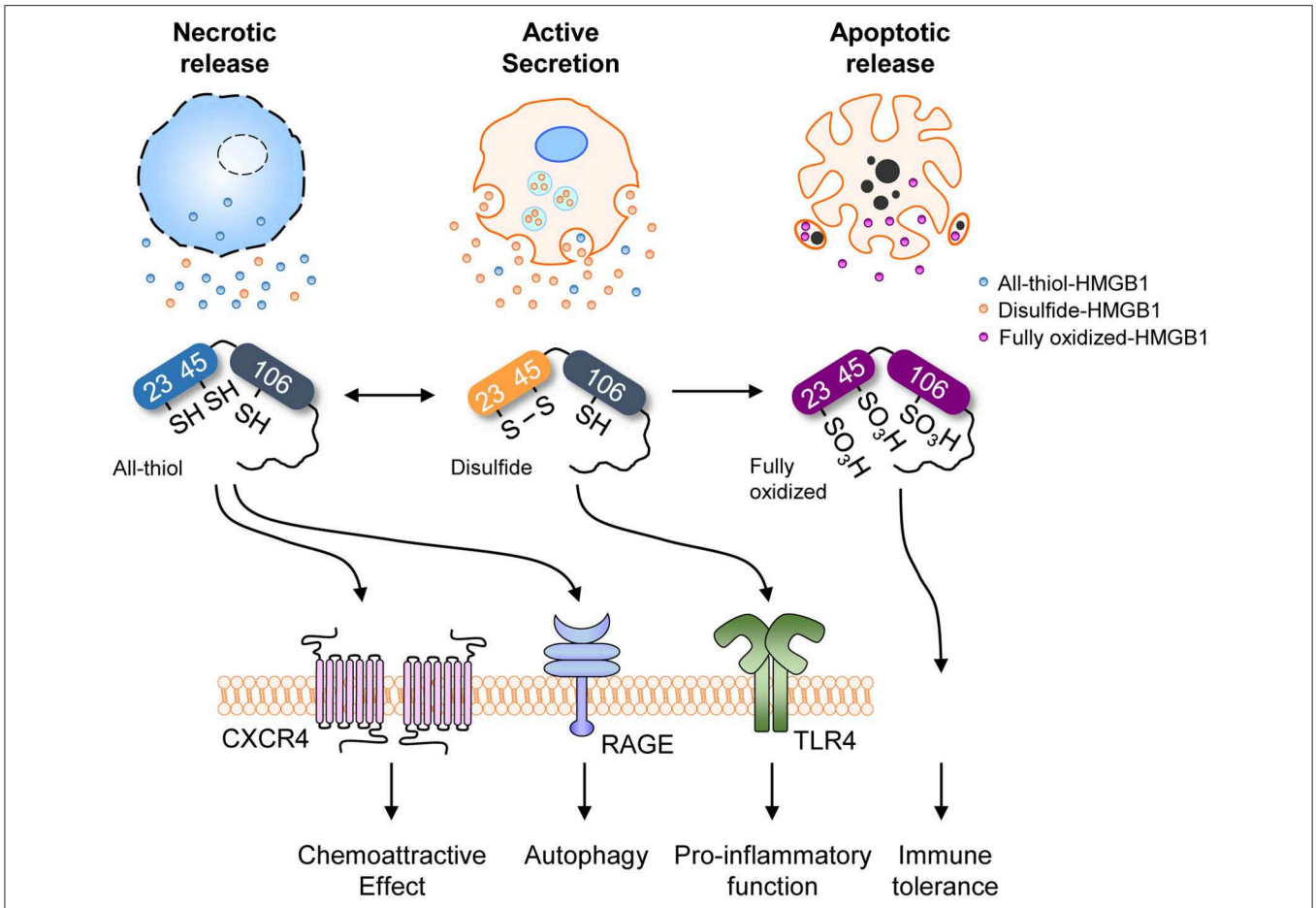

FIGURE 5 | HMGB1 redox status and receptors. Redox status of HMGB1 from various sources with different receptors and their representative functions. Different redox states are associated with different release mechanisms, each linked to various immunological and cell biological functions.

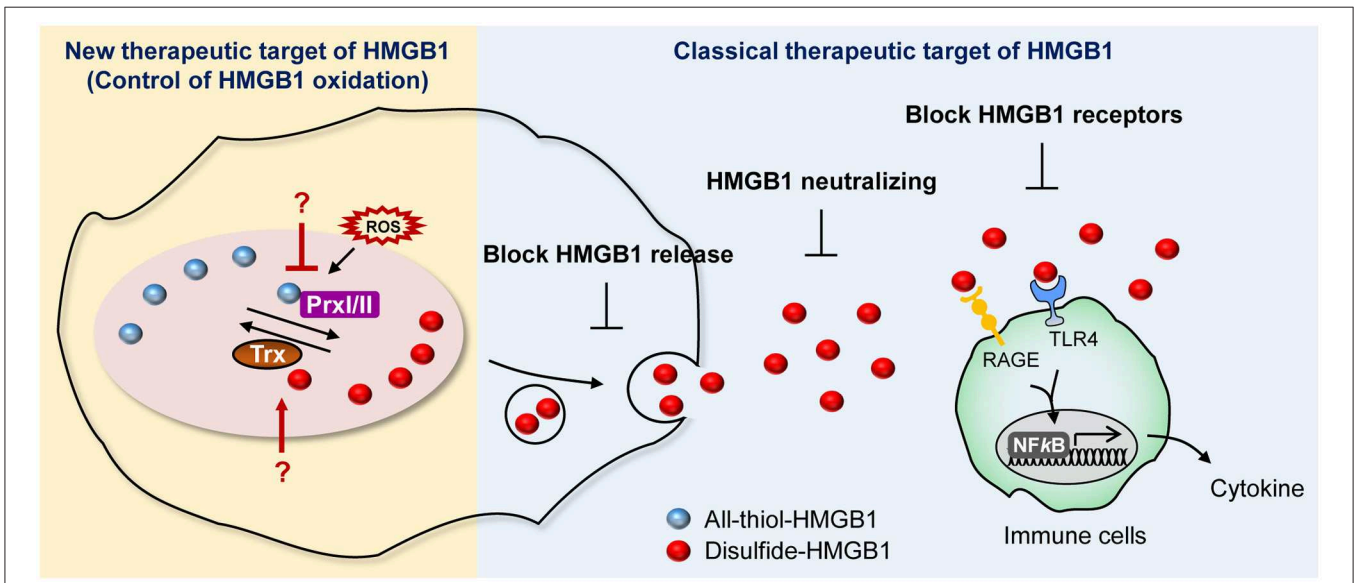

FIGURE 6 | Current and potential strategies for HMGB1 inhibition. Summary of newly proposed inhibition targets (left), targets utilized by conventional inhibitors (right).

available for such discrepancy; however, the diverse origin of cells (macrophage, epithelial, neuronal, etc.) may have different mechanisms that translocated HMGB1 takes to be secreted to the extracellular milieu. A complete understanding of the oxidation kinetics mechanism of HMGB1 is necessary to predict the fate of HMGB1-mediated inflammation.

HMGB1 Receptors and Immune Functions

As mentioned in the Introduction, extracellular HMGB1 via active secretion or passive release binds to diverse partners (**Figure 1A**). HMGB1 associates and shows interactions with several molecules, such as Heparin, LPS, LTA, IL-1β, RNA, and DNA, CXCL12, nucleosomes and C1q (9, 27–30). HMGB1-partner complex (or HMGB1 alone) interacts with immune receptors or surface molecules such as TLRs and RAGE, which then activate the downstream signaling pathways such as MyD88, IFN regulatory factors (IRFs), nuclear factor κB (NF-κB), MAPKs and phosphatidylinositol 3-kinase (PI3K) to enhance the inflammation and immune response (9, 22, 25, 26, 28, 34, 36). Besides, HMGB1 oxidation status alters its receptor bindings and subsequent cytokine-like activities (9, 51). As mentioned in section Post-Translational Modifications (PTMs) and Active Secretion of HMGB1, multiple PTMs are involved in extracellular secretion of HMGB1. Effect of PTM in receptor-ligand interaction kinetics between extracellular HMGB1 and its receptors, however, are understood to a lesser degree. One example of PTMs positively affecting the HMGB1-receptor interaction is portrayed using acetylation-mimicking mutant HMGB1 (six lysine residues for glutamines), increasing TNF-α production in RAW264.7 cells (108). Thus, although specific effects for each PTM are yet to be reported, diversely modified HMGB1 may have an important role in the receptor binding and downstream signaling pathway. Of all PTMs associated with HMGB1, only the effects of oxidation are studied in detail, hence we will focus our scope to the redox status of extracellular HMGB1 in regards to its activity. HMGB1 binds to different receptors depending on the redox state of its three cysteines (C23, C45, C106), which subsequently determines its functions (120). HMGB1 containing three thiol-form cysteines exerts chemoattractive effects by forming a heterocomplex with CXCL12, which then binds to CXCR4 and induces cell migration. The formation of a complex between HMGB1 and CXCR4 induces conformational changes in aa residues 3–12 of CXCL12 alongside specific conformational changes in the CXCR4 homodimer, which promotes better chemotactic abilities than CXCL12 alone (32). All-thiol-HMGB1 can also bind to RAGE and promote autophagy (122) by inhibiting mTOR and promoting Beclin 1-Ptdlns3KC3 complex formation (123). ROS induces HMGB1 oxidation and cytosolic translocation from the nucleus. Cytoplasmic HMGB1 binds to Beclin 1 using an intramolecular disulfide bridge (Cys23 and Cys45) to enhance autophagic flux (37, 124, 125). Moreover, the interaction between HMGB1 and RAGE activates NF-κB, the MAP kinase pathways and affects cell migration by inducing the expression of adhesion molecules (126, 127). Extracellular HMGB1 can also stimulate RAGE expression (128). Conversely, disulfide-HMGB1 stimulates cytokine production and inflammation by forming a complex with CD14 and MD-2 via TLR4. The disulfide bond between C23-C45 and the thiol form of C106 residues are not only required for binding TLR4 but also inducing the translocation of NF-κB and release of TNF-α (48). These findings were confirmed in apoptotic cells where fully oxidized-HMGB1 produced by excessive ROS contributed toward immunological inertness and apoptotic cell death (32). Although its specific functions remain unclear, fully oxidized-HMGB1 is known to prevent the cytokine or chemokine activities of other HMGB1 forms and ultimately induce immune tolerance (32) (**Figure 5**). In contrast, CD24-Siglec-10 and TIM-3 are negative receptors that inhibit HMGB1 immune activity in macrophages, DCs and tumor cells. HMGB1 can bind to CD24, first identified as a B cell differentiation marker, and selectively represses the tissue damage-induce inflammation through induction of CD24-Siglec-10 complex formation, negatively regulating NF-κB (36). Also, HMGB1 can bind to TIM-3, a member of the T-cell immunoglobulin domain and mucin domain family, and its binding suppresses the nucleic acid-mediated antitumor immunity via A-box competing with nucleic acid (26). The aforementioned reports indicate extracellular HMGB1 have not only pro-inflammatory effects but also anti-inflammatory effects according to the microenvironment.

Extracellular HMGB1 not only differ in functions by its PTM derivations, but also by the types of cells responding to HMGB1. Monocytes exposed to HMGB1 polarized toward pro-inflammatory (M1) macrophages, upregulating the production of inflammatory cytokines both *in vitro* and *in vivo* (129, 130). Silencing of HMGB1, on the other hand, prevents macrophage polarization to the M1 phenotype following LPS stimulation (131). Similarly, neutrophils react to extracellular HMGB1 by promoting its neutrophil extracellular trap formation (132) and heighten its immune reactions (133). DCs consider HMGB1 as an endogenous adjuvant to boost its effectiveness in antigen-presenting to its adaptive counterparts (134). Extracellular HMGB1, as discussed above, has exhibited significance in various immunological and physiological contexts, sparking an interest in suppressing its functions. Controlling HMGB1 as a potential therapeutic target in the immune diseases must be exquisitely controlled depending on its purpose.

HMGB1 Inhibition

The involvement of HMGB1 in various pathologies ranging from inflammatory diseases to cancer has been discussed thoroughly and has resulted in the development of HMGB1 secretion inhibitors. Currently, several companies and research centers sought to control the effects of HMGB1 by modulating its expression, translocation, secretion, and receptor binding ability using diverse chemicals as an approach to develop therapeutic agents. These strategies for suppressing HMGB1 secretion can be divided into three categories: (1) small molecules inhibiting HMGB1 release; (2) neutralizing HMGB1 itself; and (3) blocking HMGB1 receptors (**Figure 6**).

Numerous studies have reported small molecules capable of inhibiting HMGB1 secretion, from newly synthesized molecules to those isolated from natural sources. For instance, naturally isolated small molecules such as glycyrrhizin have been reported

to be effective in treating numerous pathological conditions, such as septic shock, neuroinflammation, atopic dermatitis, and *Pseudomonas aeruginosa* keratitis (135–138). Synthetic molecules such as ethyl pyruvate, atorvastatin, and simvastatin have also demonstrated promising therapeutic activity by targeting HMGB1. In addition, the natural flavonoid kaempferol was found to alleviate neuroinflammation by suppressing HMGB1 release and down regulating the TLR4/MyD88 pathway (139), and the rare ginsenosides Rk1 and Rg5 have shown promise by reducing HMGB1 release and thereby improving survival in cecum ligation- and puncture-induced murine sepsis models (140). Inflachromene, a novel small molecule developed as a potential anti-inflammatory drug, was also found to inhibit HMGB1 secretion via directly binding to HMGB1 and inhibiting autophagy (141, 142). Despite most of the candidates are yet to be approved by the Food and Drug Administration (FDA), Metformin, clinically approved drug for metabolic disease and type 2 diabetes, has investigated as an inhibitor for HMGB1 through direct binding each other, inhibiting the cytosolic translocation within the cells and receptor binding in the extracellular space (143, 144). Other candidates of HMGB1 secretion inhibitors are also being discovered through drug repositioning efforts, such as salicylic acid, methotrexate, and (-)-epigallocatechin-3-gallate (145–147).

Neutralizing antibodies against HMGB1 have been used to confirm its involvement in mouse models of various pathologies, such as arthritis, suggesting that HMGB1-neutralizing antibodies could be used therapeutically (148, 149). Indeed, neutralizing the effects of HMGB1 by competitively inhibiting its activity with soluble receptors or neutralizing antibodies could be a straightforward and approach. A soluble form of RAGE was reported to effectively reduce neutrophilic asthma attacks and angiotensin II-induced cardiomyocyte hypertrophy by inhibiting the HMGB1-RAGE axis (150, 151). Similarly, studies have reported the inhibition of the HMGB1-receptor signaling pathway using neutralizing antibodies against its receptors. For instance, neutralizing monoclonal antibodies recognizing TLR4 were used to reduce IL-8 secretion upon LPS stimulation in human primary monocytes, and efforts to use HMGB1 neutralizing antibodies in stroke patients are being continually made (152, 153). Moreover, the continual administration of neutralizing antibodies against RAGE in murine models of neurological pain were reported to reverse mechanical hyperalgesia (154, 155) Further information about HMGB1 inhibitors could be found in this Frontiers Research Topics of "The Role of HMGB1 in Immunity" by Yang et al. (156).

We suggest expanding on the importance of modulating the HMGB1 oxidation mechanism. HMGB1 oxidation is the major PTM that drives secretion and its oxidized form of extracellular HMGB1 induce inflammatory signaling, which leads to many diseases, including neuroinflammation, hyperalgesia, drug-induced liver injury, and sepsis (157–159). Of particular note is the fact that it is important to develop specific inhibitors targeting the enzymes involved in altering HMGB1 redox status. Prx, which is a major direct modulator of HMGB1 redox status, could possibly be a plausible candidate for inhibition (38), whereas well-established redox enzymes with a strong connection to Prx, such as Trx and sulforedoxins, could also be potential targets for inhibition (160) (**Figure 6**).

CONCLUDING REMARKS

Although several strategies have been shown successfully in inhibiting HMGB1-dependent inflammatory processes (156), there is still a lack of specificity originating from HMGB1's involvement in pathologies. This review aims to overcome the aforementioned weak points by suggesting various plausible aspects of inhibition, increasing the specificity of inhibition therapies. We provide an overview of the protein secretion mechanisms and discuss the HMGB1 secretion mechanisms and pathways in depth. Besides, we highlight the importance of multiple PTMs and the redox biology of HMGB1, with a particular focus on the important role of HMGB1 oxidation in its secretion. Finally, we discuss multiple immunological and non-immunological diseases involving HMGB1, as well as attempts to inhibit its secretion, extracellular activity, or the receptors that bind to HMGB1. The next step should to unveil fine-tuned process of HMGB1 PTMs in physiological and pathological conditions. Future researches would benefit from extensive quantitative analysis of extracellular HMGB1 and its PTM patterns in various cell types and different pathological conditions to further develop disease-specific inhibition strategies.

AUTHOR CONTRIBUTIONS

MK and HK wrote the manuscript, contributed to the conception, design, and analysis of the study. BL and YK contributed to interpretation of the study and drafted the manuscript. MK assisted in manuscript editing and figure preparation. MS and J-SS contributed to the conception and design of the study and the discussion, writing, supervision, and critical revision of the manuscript.

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HMGB1/PI3K/Akt/mTOR Signaling Participates in the Pathological Process of Acute Lung Injury by Regulating the Maturation and Function of Dendritic Cells

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Background: High-mobility group box 1 protein (HMGB1) was identified as a highly conserved DNA binding nuclear protein, which participates in the processes of acute lung injury (ALI). HMGB1 binds to its specific receptors not only to activate the nuclear factor (NF)-κB and mitogen-activated protein kinase (MAPK) pathways but also to regulate the activation of the phosphatidylinositol 3′-kinase/protein kinase B/mammalian target of the rapamycin (PI3K/AKT/mTOR) pathway. Mature dendritic cells (DCs) regulate acute lung inflammation and pathological injury in ALI. In addition, studies have shown that the activation of the PI3K/AKT/mTOR signaling pathway may regulate the function and maturation of DCs.

Objective: Therefore, we speculate that HMGB1/PI3K/Akt/mTOR signaling participates in regulating the pathological process of ALI by regulating the maturation and function of DCs.

Methods: Anti-HMGB1 antibody, rHMGB1, or LY294002 (PI3K inhibitor) was administered in a murine model of lipopolysaccharide (LPS)-induced ALI. For *in vitro* studies, generated bone marrow-derived dendritic cells (BMDCs) primed by LPS were stimulated with the same reagents. The effects of these different treatments were observed on the expression of PI3K, AKT, and mTOR and on the function of DCs.

Results: HMGB1 upregulated the expression of PI3K, Akt, and mTOR mRNA and phosphorylated proteins in BMDCs. The HMGB1/PI3K/Akt/mTOR signaling pathway induced the maturation and antigen-presenting ability of lung DCs, mediated the percentage of myeloid DCs (mDCs), and enhanced the adhesion and chemotactic ability of lung DCs.

Conclusions: HMGB1/PI3K/Akt/mTOR signaling participates in the pathological process of ALI by regulating the maturation and functions of DCs.

Keywords: HMGB1, PI3K/Akt/mTOR signaling, acute lung injury, lipopolysaccharide, dendritic cell

INTRODUCTION

Acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) is a complex clinical syndrome characterized by persistent hypoxemia due to pulmonary interstitial edema, damage and disruption of the alveolar–capillary barrier, and widespread inflammation in the lung (1, 2). Although the clinical treatment and management of ALI/ARDS have improved, it still has high morbidity and mortality in the intensive care unit (3). Previous studies have shown that an excessive inflammatory response storm plays a vital role in the pathological process of ALI/ARDS (1). Therefore, elucidation of molecular and cellular mechanisms associated with inflammation in ALI may be helpful in identifying new therapeutic targets.

High-mobility group box 1 protein (HMGB1) was identified as a highly conserved DNA binding nuclear protein and participates in the processes of replication, recombination, transcription, and DNA repair. HMGB1 is reported to contribute to inflammatory dysfunction in sepsis and ALI, and the levels of HMGB1 in the plasma and tissue were significantly increased in a mouse model of lipopolysaccharide (LPS)-induced mouse model of ALI (4, 5). Increasing evidence supports the role of HMGB1 as a regulator of ALI. The downstream pathways of HMGB1 may lead to neutrophil infiltration, injury of lung tissue, inflammatory cytokine release, and the development of ALI. HMGB1 binds to its specific receptors, including the receptor for advanced glycation end products, toll-like receptor (TLR)2 and TLR4, in turn activating the nuclear factor (NF)-κB and mitogen-activated protein kinase (MAPK) pathways, which mediate inflammatory molecules such as TNF-α, IL-1β, IL-18, and IL-6 (6–8). HMGB1 also induces an inflammatory response through the phosphatidylinositol 3′-kinase/protein kinase B/mammalian target of the rapamycin (PI3K/AKT/mTOR) pathway (9). Previous studies have shown that HMGB1 inhibitors or HMGB1 siRNA effectively inhibited the activation of the TLR4/NF-κB and PI3K/AKT/mTOR pathways (9, 10). Previous research has shown that the PI3K/Akt/mTOR pathway regulated multiple physiological activities such as cell proliferation, autophagy, and apoptosis (11, 12). In addition, the PI3K/Akt/mTOR pathway has an important role in pulmonary inflammation and pathological progression of ALI (13, 14). Thus, HMGB1 induces an inflammatory response in ALI through the PI3K/AKT/mTOR pathway.

Dendritic cells (DCs), the most prominent antigen-presenting cells, play a key role in initial and adaptive immune responses and are ideally positioned to serve a priming and central role in the immune response during inflammation (15, 16). Previous studies have shown that the number of mature conventional DCs (cDCs) in lung tissue during ALI is significantly increased, and the maturation of pulmonary DCs regulate acute lung inflammation and pathological injury (17, 18). Therefore, DCs play an important role in the pathological progress of ALI, and regulating the function and maturation of DCs may have great clinical significance for the treatment of ALI.

Studies have shown that promoting the activation of PI3K/AKT/mTOR signaling regulates the function of immune cells including DCs (19, 20). Moreover, the activation of the PI3K/Akt/mTOR pathway participates in sepsis-induced ALI (11, 13, 21). Thus, we speculated that HMGB1/PI3K/Akt/mTOR signaling participates in regulating the pathological process of ALI by regulating the maturation and function of DCs.

Abbreviations: ALI, acute lung injury; LPS, lipopolysaccharide; ARDS, acute respiratory distress syndrome; HMGB1, high mobility group box1; BALF, bronchoalveolar lavage fluid; DCs, dendritic cells; MNCs, lung mononuclear cells; NF-κB, nuclear factor kappa B; TLR, toll-like receptor; BMDCs, bone marrow-derived dendritic cells; MAPK, mitogen-activated protein kinase; cDCs, conventional DCs; RT-qPCR, real-time reverse-transcriptase polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; TNF, tumor necrosis factor; MCP, monocyte chemotactic protein; cDNA, complementary DNA; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; ICAM, intercellular adhesion molecule; mDCs, myeloid dendritic cells; pDCs, plasmacytoid dendritic cells.

MATERIALS AND METHODS

Mice

Male C57BL/6 mice (6–8 weeks old, 20–22 g) were obtained from the Experimental Animal Centre of Hubei province (Wuhan, China). The mice were maintained in the animal laboratory of the Experimental Animal Center of Hubei province under standard laboratory conditions for 1 week prior to the experiments. The mice were kept under specific pathogen-free conditions at 25°C in a 12-h light/dark cycle, with a humidity of 45–55% in a ventilated cage. All experimental procedures were performed in accordance with the requirements of the Institutional Animal Care and Use Committee at Huazhong University of Science and Technology (Wuhan, China).

Experimental Protocol for the ALI Murine Model

All mice were divided into the following groups using the randomized grouping method (n = 4–6 per group): control group, LPS group, LPS+anti-HMGB1 group, LPS+rHMGB1 group, LY294002 (PI3K inhibitor) positive control group, and LPS+LY294002 group. The ALI murine model was induced by intraperitoneal (i.p.) injection of LPS as described previously (22). Anti-HMGB1 or rHMGB1 was administered as previously described (22). The LY294002 intervention group was injected with 1 mg/25 g LY294002 via the tail vein 2 h after LPS injection (23). All experimental mice were sacrificed using cervical dislocation 24 h after receiving the LPS challenge, and lung tissues and bronchoalveolar lavage fluid (BALF) were extracted for further analysis.

Collection of BALF

We collected BALF as described in detail in a previous study (24).

TABLE 1 | Primer sequences and product sizes.

Gene name	Primer sequences (5′-3′)	Product size (bp)
PI3K Forward:	5′- ACACCACGGTTTGGACTATGG -3′	140
Reversed:	5′- GGCTACAGTAGTGGGCTTGG -3′	
Akt Forward:	5′- TGGGTCAAGGAACAGAAGCA -3′	111
Reversed:	5′- TCACACTGACCACTGACACA -3′	
mTOR Forward:	5′-CGGGACTCTTTACACTGCG-3′	82
Reversed:	5′-CCTTCAGGCTCAACCAACA-3′	
GAPDH Forward:	5′-TGTGTCCGTCGTGGATCTGA-3′	150
Reversed:	5′-TTGCTGTTGAAGTCGCAGGAG-3′	

(a) PI3K, Phosphatidylinositol 3′-kinase; (b) Akt, Protein kinase B; (c) mTOR, Mammalian target of rapamycin; (d) GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

Generation of Bone Marrow-Derived Dendritic Cells (BMDCs)

BMDCs were generated as previously described (24). The obtained BMDCs were stimulated with or without 1 μg/ml LPS (Sigma Aldrich, St. Louis, MO, USA), anti-HMGB1 (10 μg/ml) (22), rHMGB1 (50 μg/ml) (22), or LY294002 (25 μM) (25) for 24 h. BMDCs and cell supernatants were collected for subsequent real-time reverse-transcriptase polymerase chain reaction (RT-qPCR) and enzyme-linked immunosorbent assay (ELISA) analyses.

Cytokine Analysis

Concentrations of interleukin (IL)-12p40, tumor necrosis factor (TNF)-α, IL-6, IL-18, IL-1β, and monocyte chemotactic protein (MCP)-1 secreted by DCs from each sample were determined by ELISA according to the manufacturer's instructions (eBioscience, San Diego, CA, USA). Cytokine concentrations are expressed as pg/ml.

RNA Extraction and RT-qPCR

Total RNA was extracted from the right lung tissue and BMDCs by a Trizol reagent (Invitrogen/Thermo Fisher Scientific Inc., Carlsbad, CA, USA), and the RNA was reverse-transcribed into complementary DNA (cDNA) using a ReverTra Ace qPCR RT kit (Toyobo CO., LTD., Tokyo, Japan) according to the manufacturer's protocol.

RT-qPCR assay on the samples was carried out using the SYBR Premix Ex Taq™ (Takara Bio Inc., Otsu, Japan) as previously described (24, 26). RT-qPCR data were analyzed by QuantStudio 6 Flex (ABI Life Technology, USA). The $2^{\Delta\Delta Ct}$ method was used to evaluate the relative expression of each target gene after normalization by glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primer sequences for each target gene are listed in **Table 1**.

Western Blot Analysis

BMDCs were harvested at 24 h after stimulation with LPS and frozen at −80°C. Total protein was extracted using a lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). Protein concentrations were determined by BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China). Protein (40 μg/well) was separated via 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Corp., Billerica, MA, USA). Membranes were blocked with 5% non-fat milk in Tris-buffered saline containing Tween 20 (TBST) for 2 h at room temperature, then incubated with primary antibodies (p-PI3K p85, p-Akt T308, p-mTOR, Abcam, Cambridge, UK) diluted in a blocking solution (1:1,000) overnight at 4°C, and finally treated with diluted horseradish peroxidase (HRP)-conjugated secondary antibody (1:50,000, BOSTER Biological Technology Co., Wuhan, Chine) at 37°C for 2 h. The immunoreactive bands were analyzed using the BandScan 5.0 (Glyko, Novato, CA, USA) gel imaging software.

Flow Cytometry

To determine the phenotype of DCs in lung tissue and BMDCs, prepared lung mononuclear cells (MNCs) and BMDCs were suspended in a FACS buffer at 2×10^6 cells/ml. DCs express the marker CD11c but not the marker F4/80 on the cell surface; therefore, DCs were gated as $CD11c^+/F4/80^-$ cells using the PE-CD11c antibody (eBioscience, San Diego, CA, USA) and the APC-Cy7-F4/80 antibody (BioLegend Inc., San Diego, CA, USA). Next, the cells were stained with the following antibodies: FITC-MHCII, FITC-CD80, FITC-CD40, FITC-B220, FITCICAM-1 (eBioscience, San Diego, CA, USA), APC-CD86, APC-CCR7, APC-CD11b (Biolegend Inc., San Diego, CA, USA). All cells were analyzed by flow cytometry (FACSAria™ III, BD Biosciences, USA).

Statistical Analysis

The data are presented as the mean ± standard deviation (SD). All experiments were repeated at least three times. One-way analysis of variance was used to compare differences. Statistical analysis was carried out using the GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA) and the SPSS 22.0 software (IBM SPSS, Chicago, IL, USA). Differences were considered significant at $p < 0.05$. * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$.

RESULTS

HMGB1 Activated PI3K/Akt/mTOR Signal Pathway in BMDCs

Previous studies have shown that HMGB1 regulates the PI3K/Akt/mTOR pathway in myocardial ischemia reperfusion injury and the ALI mouse model (9, 27). We examined the effect of HMGB1 on PI3K/Akt/mTOR signaling in BMDCs following administration with rHMGB1 or anti-HMGB1 by Western blot and RT-qPCR analysis. HMGB1-mediated PI3K/Akt/mTOR pathway activation was assessed by detecting p-PI3K, p-Akt, and p-mTOR protein and PI3K, Akt, and mTOR mRNA expression levels. Western blot and RT-qPCR analysis revealed a remarkable increase in the expression of p-PI3K, p-Akt, and p-mTOR protein

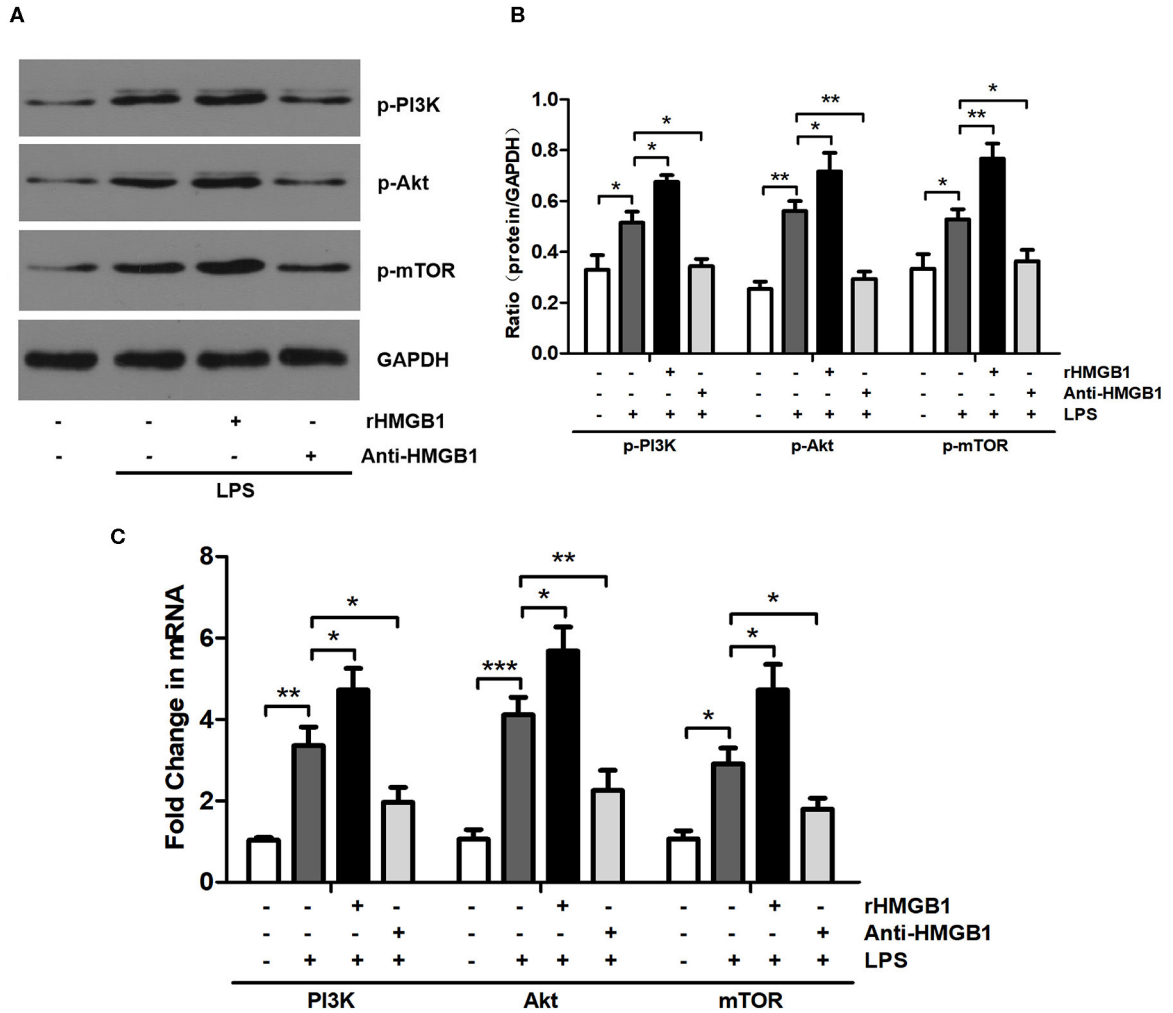

FIGURE 1 | Anti-HMGB1 and rHMGB1 regulated the expression of the PI3K/Akt/mTOR signaling pathway in BMDCs. **(A,B)** Expression of p-PI3K, p-Akt, and p-mTOR in BMDCs of different groups was measured by Western blot analysis. GAPDH served as a loading control. **(C)** Expression of PI3K, Akt, and mTOR mRNA in BMDCs of different groups was measured by RT-PCR. GAPDH served as the housekeeping gene. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

(**Figures 1A,B**) and PI3K, Akt, and mTOR mRNA (**Figure 1C**) in LPS-primed BMDCs. Moreover, rHMGB1 treatment further increased the expression of these proteins and transcripts, but this increase was attenuated by anti-HMGB1 (**Figures 1A–C**). These results suggest that HMGB1 is an activator of the PI3K/Akt/mTOR pathway in DCs.

HMGB1 Induced the Maturation of DCs *in vivo* and *in vitro*

The activation and maturation of DCs are characterized by the expression of MHCII and various costimulatory molecules on their surface and the secretion of related inflammatory cytokines (28, 29). To determine whether HMGB1 affects the maturation and function of DCs, we observed the effect of HMGB1 on MHCII, CD80, CD86, and CD40 of lung DCs in the LPS-induced ALI model. The left lungs from mice of different groups were collected 24 h after LPS intervention, and the prepared lung MNCs were stained for CD11c and F4/80 and analyzed by flow cytometry. DCs were marked as $CD11c^{+}F4/80^{-}$ (**Figure 2A**). The analysis results showed that the positive expression percentage of MHCII, CD80, CD86, and CD40 was significantly increased in the ALI group in contrast to those in the control, and the increase in MHCII, CD86, and CD40 was further augmented by rHMGB1 treatment. By contrast, the increase in MHCII, CD80, CD86, and CD40 was obviously lowered in the ALI group treated with anti-HMGB1 (**Figures 2B,C**). Subsequently, we observed the effect of HMGB1 on proinflammatory cytokines released by LPS-primed BMDCs *in vitro*. The levels of TNF-α, IL-6, IL-18, IL-1β, MCP-1, and IL-12 cytokines released by DCs, which reflect the maturation of DCs, were significantly upregulated by LPS stimulation in the BMDC culture supernatant compared with those in the control group. On the other hand, rHMGB1 stimulation also significantly increased the production of these proinflammatory

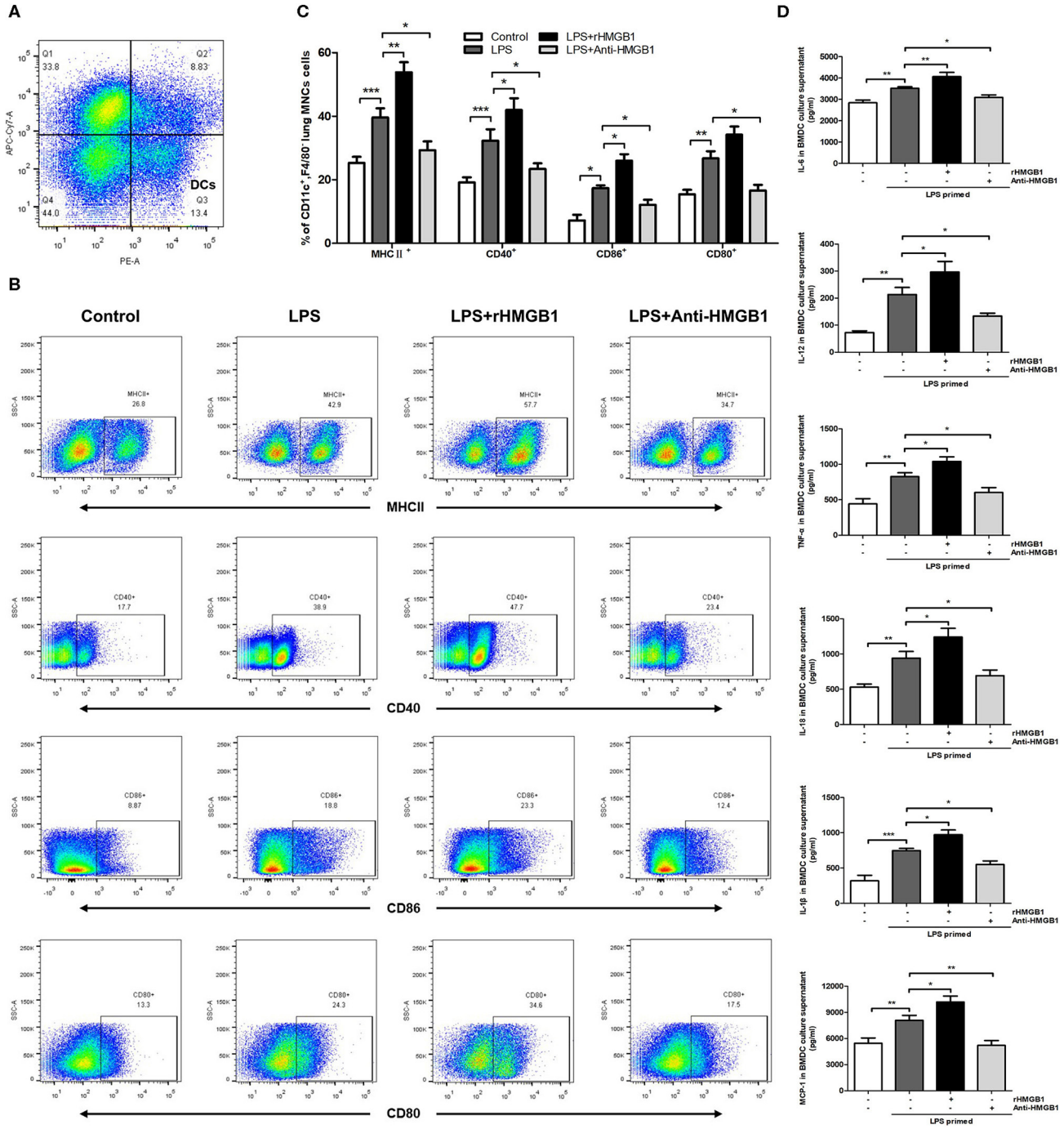

FIGURE 2 | Anti-HMGB1 and rHMGB1 regulated the maturation of DCs *in vivo* and *in vitro*. **(A)** Lung DCs were stained as $CD11c^{+}F4/80^{-}$. **(B,C)** Positive expression percentage of MHCII, CD80, CD86, and CD40 was measured in DCs ($CD11c^{+}F4/80^{-}$) by flow cytometric analysis. **(D)** The levels of cytokines TNF-α, IL-6, IL-18, IL-1β, MCP-1, and IL-12 secretion in BMDC culture supernatants were measured by ELISA. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

cytokines. Opposite results were observed in the groups receiving anti-HMGB1 stimulation (**Figure 2D**). These data suggested that HMGB1 induces the maturation and enhances the antigen-presenting ability of DCs in the ALI mice model and in LPS-primed BMDCs.

HMGB1 Affected the Phenotypic Changes of DCs in ALI Mice Model

Subsequently, we observed the effect of anti-HMGB1 and rHMGB1 on phenotypic and functional changes in the DCs of lung tissue from the LPS-induced ALI mice model. While

detecting intercellular adhesion molecule 1 (ICAM-1), CD11b (a marker for myeloid dendritic cells [mDCs]), B220 (a marker of plasmacytoid dendritic cells [pDCs]), and chemotactic factor CCR7 in the surface of lung DCs by flow cytometric analysis, we found that the percentage of ICAM-1, CD11b, or CCR7-positive expression was significantly elevated in the LPS-administrated ALI group in contrast to the control. The increase in ICAM-1, CD11b, and CCR7 percentage was significantly higher in the rHMGB1-treated ALI group relative to those of the ALI group. In addition, the increase in ICAM-1, CD11b, and CCR7 was reduced after anti-HMGB1 treatment (**Figures 3A,B**). However, the percentage of B220-positive expression among different groups was not significantly different (**Figures 3A,B**). These data demonstrated that HMGB1 upregulated the percentage of mDCs and enhanced the adhesion and chemotaxis of DCs.

Inhibition of PI3K/Akt/mTOR Signaling Pathway Weakens the Maturation of DCs *in vivo* and *in vitro*

We next verified whether the PI3K/Akt/mTOR signaling pathway affects the maturation and function of DCs in the presence of a PI3K inhibitor (LY294002) *in vivo* and *in vitro*. Flow cytometric analysis *in vivo* showed that the percentage of $MHCII^+$, $CD80^+$, $CD86^+$, or $CD40^+$ DCs was significantly higher in ALI mice compared with control mice. A significant decrease was also observed in the percentage of $MHCII^+$, $CD80^+$, $CD86^+$, and $CD40^+$ DCs in the LY294002-treated mice compared with that in the LPS-induced ALI mice (**Figures 4A,B**). *In vitro* experiments with BMDCs revealed that stimulation with LPS significantly augmented the levels of TNF-α, IL-6, IL-18, IL-1β, MCP-1, and IL-12 in the culture supernatant. The levels of these proinflammatory cytokines produced by BMDCs were reduced with the inhibition of PI3K (in the LPS+LY294002 group) (**Figure 4C**). Thus, inhibiting the PI3K/Akt/mTOR signaling pathway attenuated the mature differentiation of DCs in ALI.

PI3K/Akt/mTOR Signaling Pathway Affected the Phenotypic Changes of DCs in ALI Mice Model

To demonstrate the role of the PI3K/Akt/mTOR signaling pathway in phenotypic and functional changes of DCs in the ALI mice model, we also treated LPS-induced mice with LY294002 and detected the percentage of ICAM-1, CD11b, B220, and CCR7 in lung DCs by flow cytometric analysis. In comparison with the control group, LPS-induced ALI groups showed a significant increase in the percentage of DCs ($CD11c^+F4/80^-$MNCs) expressing ICAM-1, CD11b, and CCR7. These changes were weakened with LY294002 treatment. In addition, no significant difference arose in the percentage of B220 between different groups (**Figures 5A,B**). These results suggested that, similarly to HMGB1, the PI3K/Akt/mTOR signaling pathway also influenced the percentage of mDCs and mediated the adhesion and chemotactic ability of DCs to T cells.

DISCUSSION

In the current study, we show that HMGB1 activates the PI3K/Akt/mTOR signaling pathway in BMDCs and upregulates the expression of PI3K, Akt, and mTOR mRNA and corresponding phosphorylated proteins. HMGB1 and the PI3K/Akt/mTOR pathway form the HMGB1-PI3K/Akt/mTOR signaling pathway in lung DCs, and this pathway then induces the maturation and antigen-presenting ability of lung DCs while also mediating the percentage of mDCs and enhancing the adhesion and chemotactic ability of lung DCs.

HMGB1 functions to regulate the innate immune system. Recent studies have shown that as a late proinflammatory cytokine, HMGB1 has a key role in the pathological progress of ALI and regulates the lung inflammatory response (4, 5). HMGB1 is an upstream mediator of TLR2 and TLR4, the main receptors of HMGB1. Together these form the HMGB1-TLR2, TLR4 pathway, which contributes to inflammatory response via multiple mechanisms (6–8). Besides activating the NF-κB and MAPK pathways, recent studies also have demonstrated that HMGB1 regulates the PI3K/Akt/mTOR signaling pathway in myocardial ischemia/reperfusion injury and LPS-induced pulmonary inflammation in ALI models (9, 10, 13, 14). The PI3K/AKT/mTOR signaling pathway has been shown to contribute to the regulation of cell survival during oxidative stress and to participate in the pulmonary inflammatory progression of ALI (11–14). These studies agree with our results for the relationship between HMGB1 and the PI3K/Akt/mTOR pathway in DCs during ALI.

As specialized antigen-presenting cells pivotal for the initial and adaptive immune response, DCs are outpost cells of immune defense in the respiratory system. Under the stimulation of pathogens, DCs are activated and then mature and migrate to lymph nodes (30). These mature and activated DCs upregulate the surface expression of MHCII and diverse costimulatory molecules (CD80, CD86, and CD40) and release inflammatory cytokines, which train and stimulate Th cells to differentiate into various subtypes (28–30). DCs regulate acute lung inflammation and injury in LPS-induced ALI, and mature DCs participate in aggravating acute lung tissue injury and inflammatory response (17, 18).

In humans and mice, DCs have two important subsets: mDCs ($CD11c^+F4/80^-CD11b^+$) and pDCs ($CD11c^+F4/80^-B220^+$). Compared with pDCs, the mDC subset displays high expression levels of costimulatory molecules CD80, CD86, CD40, and MHCII and also better stimulate the proliferation and differentiation of T cells (31). This indicates that the maturity of pDCs is inferior to that of mDCs. Moreover, pDCs do not express LPS-specific receptors TLR2 and TLR4 and therefore cannot be stimulated to maturation by LPS (31). In addition, DCs also express an important adhesive molecule on their cell surface, ICAM-1, which mediates adhesion reaction, and release an important chemokine, CCR7, which has the ability to induce directional chemotaxis (32–34). In our present study, we detected the expression of surface markers (CD80, CD86, CD40, MHC II, B220, CD11b, ICAM-1, CCR7) to reflect the

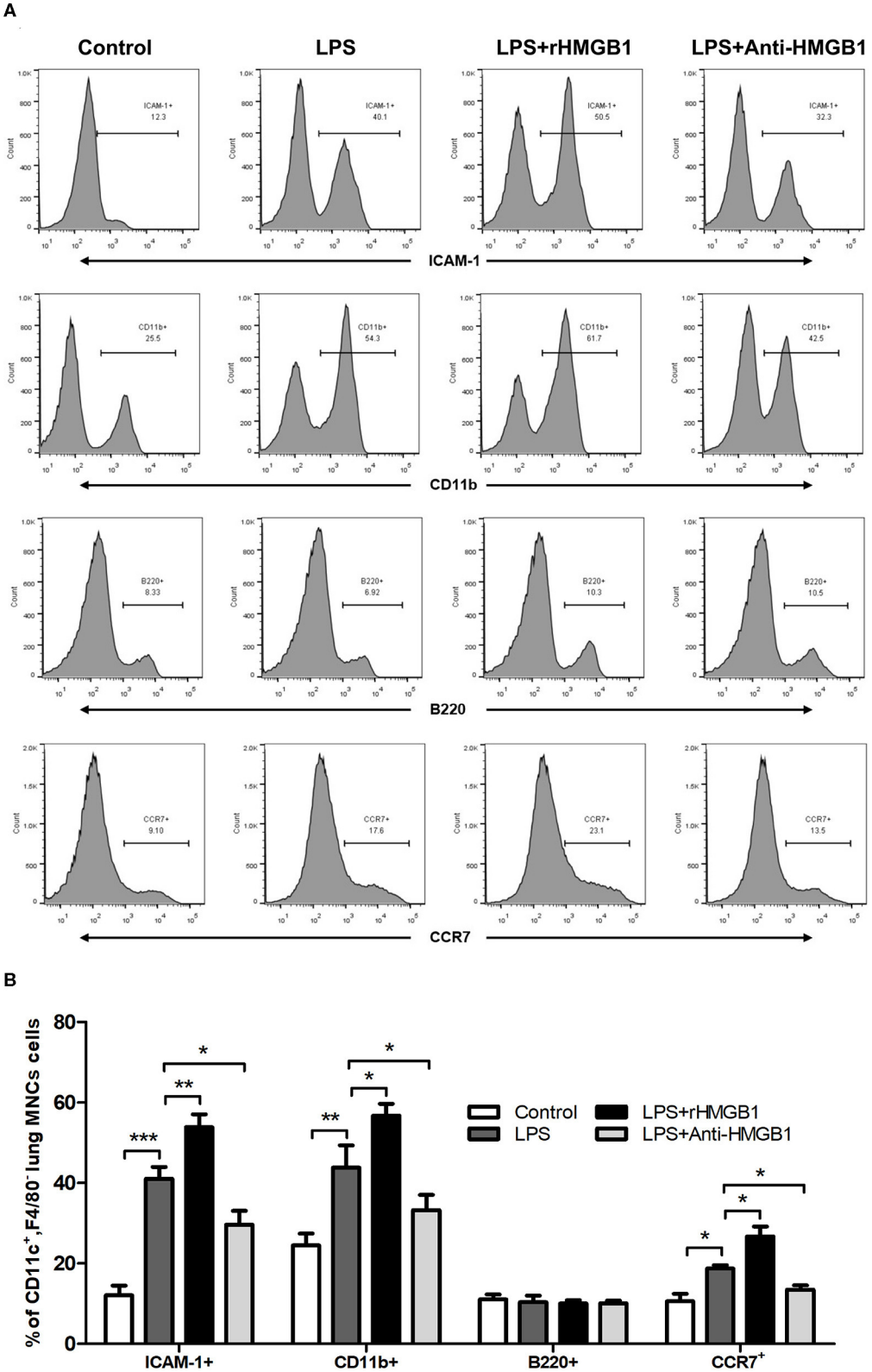

FIGURE 3 | Anti-HMGB1 and rHMGB1 affected the phenotype and function of DCs. **(A,B)** Positive expression percentage of ICAM-1, CD11b, B220, and CCR7 was measured in DCs ($CD11c^+F4/80^-$) by flow cytometric analysis. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

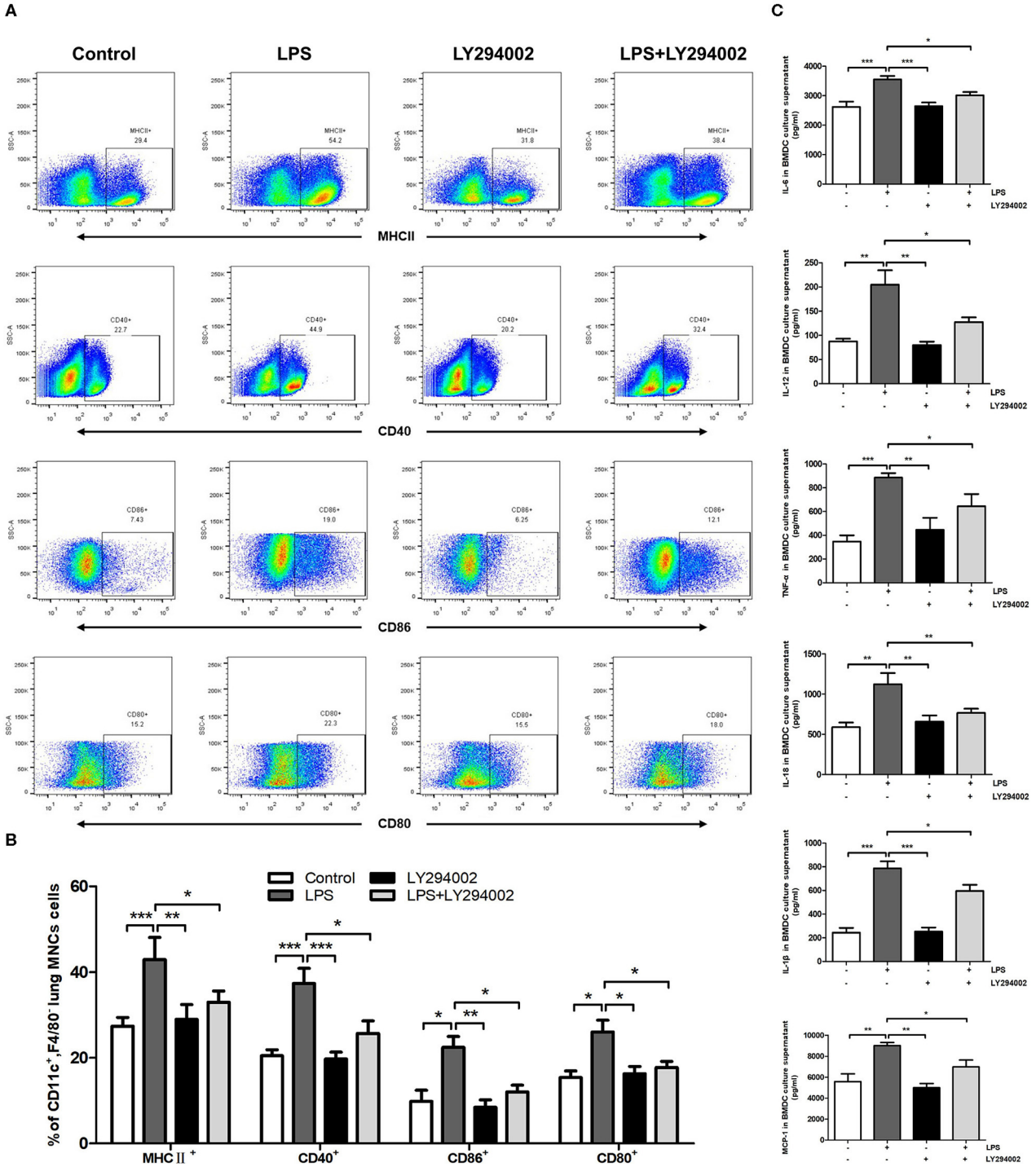

FIGURE 4 | Inhibition of PI3K by LY294002 regulated the maturation of DCs *in vivo* and *in vitro*. **(A,B)** Positive expression percentage of MHCII, CD80, CD86, and CD40 was measured in DCs ($CD11c^{+}F4/80^{-}$) by flow cytometric analysis. **(C)** Levels of secreted cytokines TNF-α, IL-6, IL-18, IL-1β, MCP-1, and IL-12 in BMDCs culture supernatant were measured by ELISA. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

effect of the HMGB1/PI3K/Akt/mTOR signaling pathway on the phenotype and function of DCs in ALI. Our results showed that the HMGB1/PI3K/Akt/mTOR signaling pathway induced upregulation of markers CD80, CD86, CD40, MHC II, CD11b, ICAM-1, and CCR7, suggesting that the proportion of mature DCs increased; the ratio of mDCs also increased, accompanied by the augmentation of antigen presentation, adhesion, and chemotactic ability.

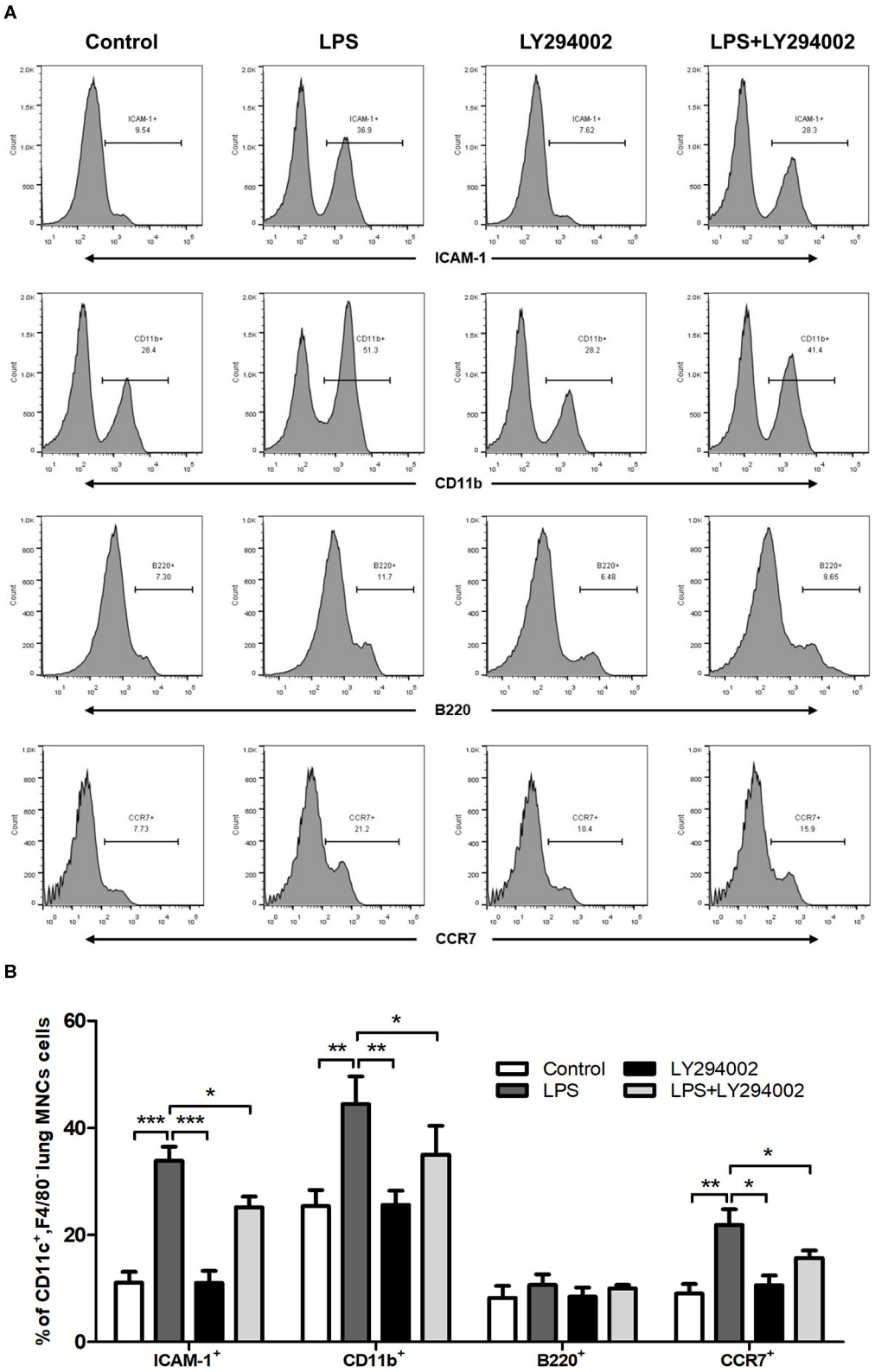

FIGURE 5 | Inhibition of PI3K by LY294002 affected the phenotype and function of DCs. **(A,B)** Positive expression percentage of ICAM-1, CD11b, B220, and CCR7 was measured in DCs ($CD11c^{+}F4/80^{-}$) by flow cytometric analysis. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

In conclusion, our present study provides evidence of the role of the HMGB1/PI3K/Akt/mTOR signaling pathway at the level of DCs in ALI and further confirms that HMGB1/PI3K/Akt/mTOR signaling participates in the pathological process of ALI by regulating the maturation and functions of DCs.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because access to this dataset is restricted. Requests to access the datasets should be directed to 498676772@qq.com.

ETHICS STATEMENT

The animal study was reviewed and approved by Huazhong University of Science and Technology.

AUTHOR CONTRIBUTIONS

RL and YS designed the research. RL, XZ, YY, HZ, PL, SP, and YO performed the experiments. RL analyzed the data and produced the figures. RL, HH, and YS wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Sulfatide Inhibits HMGB1 Secretion by Hindering Toll-Like Receptor 4 Localization Within Lipid Rafts

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The high mobility group box 1 (HMGB1) is a well-known late mediator of sepsis, secreted by multiple stimuli, involving pathways, such as the mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-κB) pathways, and reactive oxygen species (ROS) under inflammation. Sulfatide, in contrast, is a sphingolipid commonly found in myelin sheets with a disputed immunological role. We sought to determine the immunological characteristics of sulfatide in the periphery by analyzing the secretion of HMGB1 triggered by lipopolysaccharide (LPS) stimulation in Raw 264.7 cells. Suppression of HMGB1 secretion by inhibiting its cytosolic translocation was observed after pre-treatment with sulfatide before LPS stimulation. Further analysis of the downstream molecules of toll-like receptor (TLR) signaling revealed suppression of c-Jun N-terminal kinase (JNK) phosphorylation and p65 translocation. LPS-mediated ROS production was also decreased when sulfatide pre-treatment was provided, caused by the down-regulation of the phosphorylation of activators, such as IRAK4 and TBK1. Investigation of the upstream mechanism that encompasses all the aforementioned inhibitory characteristics unveiled the involvement of lipid rafts. In addition to the co-localization of biotinylated sulfatide and monosialotetrahexosylganglioside, a decrease in LPS-induced co-localization of TLR4 and lipid raft markers was observed when sulfatide treatment was given before LPS stimulation. Overall, sulfatide was found to exert its anti-inflammatory properties by hindering the co-localization of TLR4 and lipid rafts, nullifying the effect of LPS on TLR4 signaling. Similar effects of sulfatide were also confirmed in the LPS-mediated murine experimental sepsis model, showing decreased levels of serum HMGB1, increased survivability, and reduced pathological severity.

Keywords: sulfatide, HMGB1, TLR4, lipid raft, sepsis, NF-κB, ROS

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INTRODUCTION

Approximately 45 years have passed since the HMGB1 protein, an abundant nuclear protein and a well-defined danger-associated molecular pattern (DAMP) molecule, was first purified (1). Since its discovery, HMGB1 has been discussed in various contexts. Nuclear HMGB1 is well-known for its chaperone-like functions, playing a role in deoxyribonucleic acid (DNA)

unwinding (2) and DNA synthesis (3) by binding to DNA in a sequence-independent manner (4) and in the structuring of chromatin (5). In contrast, research regarding cytosolic HMGB1 is still in its relatively early stages, revealing its role in autophagy regulation (6) and unconventional protein secretion (7).

HMGB1 can be either passively released through non-apoptotic cell death, such as in necrotic cells (8), or actively secreted through multiple pathways, such as in inflammasome-mediated release (9). In this paper, we intend to limit the scope to active secretion of HMGB1, triggered by inflammatory signals transduced by toll-like receptor (TLR)-related signaling. When TLRs are stimulated by their ligands, NF-κB (10) and MAPK (10–12) are responsible for the translocation and secretion of these receptors to the extracellular space. Our research concentrates on TLR4, a member of the TLR family, which recognizes lipopolysaccharides (LPS), and its mechanism of action regarding the active secretion of HMGB1. Under physiological conditions, bacterial LPS, which normally forms a micelle, is recognized by the LPS-binding protein (LBP), which facilitates its monomerization by CD14 (13). The LPS–LBP complex, now bound to CD14, is then transferred to the myeloid differentiation protein-2 (MD-2)–TLR4 complex (14). This complex then forms a dimer, completing its activation process. TLR4 dimers, however, require the formation of a lipid raft, a special nano-scale membrane structure consisting of various lipids (15). TLR4, which contains lipid-binding motifs, is attracted and can readily form a dimer within the lipid rafts, providing a platform on which the TLR4s can be within closer proximity (16).

Most studies addressing the immunological role of HMGB1 have focused on the role of extracellular HMGB1 as a DAMP molecule and its chemokine-like behavior. Depending on its redox status, HMGB1 exerts different characteristics: 1) as a thiol isoform, in which all of the three active cysteine residues (Cys23, 45, and 106) are in free-thiol(-SH) form, HMGB1 binds to C-X-C motif ligand 12 (CXCL12) and shows chemokine-like activity, recruiting immune cells to the site of inflammation (17); 2) the disulfide isoform of HMGB1, which possesses one intra-molecular disulfide bond between the two cysteine molecules Cys23 and Cys45, exerts cytokine-like activity, activating macrophages and lymphocytes (18–20); 3) the oxidized isoform of HMGB1, containing fully oxidized cysteine residues (-SOOOH) is considered immunologically inert (21, 22). In sepsis, extracellular HMGB1 is known to be released in its reduced form (23); it is considered a potent pro-inflammatory cytokine (24) and a promising therapeutic target in clinical studies (25, 26).

Sulfatide, also known as 3-O-sulfogalactosylceramide, is a lipid commonly found in the myelin sheath in both the central and peripheral nervous system (27). First isolated and partially characterized over 40 years ago (28), sulfatide was suggested to play a varying role in physiological functions, ranging from myelination of nerves (29, 30) to insulin secretion (31–34). Similar to HMGB1, intracellular (or membrane-bound) sulfatide and extracellular sulfatide play different roles. While the intracellular (or membrane-bound) form performs the abovementioned functions, extracellular sulfatide can bind to selectins to cause hemostasis (35) or metastasis of tumors (36) or bind to CD1d activating natural killer T (NKT) cells with various anti-inflammatory abilities (37–41). Although most papers discussing the anti-inflammatory functions of sulfatide emphasize on NKT cells, a report suggested that sulfatide may have a direct effect on brain-resident immune cells, causing inflammation (42). This discrepancy between immune cells residing in the central or peripheral nervous system led us to investigate the direct effect of sulfatide in peripheral immune cells, namely the macrophages.

In this study, we aimed to elucidate the effect of sulfatide in the context of innate immunity by investigating its effect on HMGB1 secretion under LPS stimulus and discuss the specific molecules involved in the process.

MATERIALS AND METHODS

Cell Culture and Treatment Reagents

Raw 264.7 cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Waltham, MA, USA), 100 μg/mL of penicillin, and 100 μg/mL of streptomycin (Sigma, Saint Louis, MO, USA). Treatment was performed after allowing the cells to adapt to Opti-MEM (Gibco) for 2 h, after which the media was replaced.

LPS (*Escherichia coli* O111:B4; > 3 x 10^6 EU/mL; Sigma), sulfatide (Bovine; brain; Matreya, State College, PA, USA), 18:0(2R-OH) sulfogalactosylceramide (synthetic; Avanti, Alabaster, AL, USA), C24:0 mono-sulfogalactosylceramide (synthetic; Avanti), C24:0 mono-sulfogalactosylceramide (synthetic; Avanti), galactosylceramide (Bovine; Matreya), and ceramide (Bovine; Matreya) were used as indicated in the figures. All experiments were performed using vehicle as a negative control.

Bone Marrow-Derived Macrophage (BMDM) Preparation

Wild-type C57BL/6 mice obtained from Orient Bio (Seongnam, Gyeonggi-do, South Korea) were housed in a SPF-grade facility with controlled temperature, humidity, and light. For all experiments, 8-week old female mice with approximate body weight of 20 g were used. The animals were ethically sacrificed, and the femur and tibia were extracted. Bone marrow was collected via warm, serum-free DMEM lavage until no remaining bone marrow was visible. Bone marrow was collected and filtered through cell strainer with 40 μm pore (SPL, Pocheon-si, Gyeonggi-do, South Korea) to remove any undesirable debris and washed with excessive media to further remove unfiltered debris. The resulting cells were plated to 100 mm cell culture-treated dish (Corning, Oneonta, NY, USA), and then differentiated using 20 ng/mL GM-CSF in complete medium for 7 days to yield BMDMs.

Sample Preparation (Culture Media)

Culture media after treatment were collected after 24 h to compare HMGB1 secretion between groups. Culture media were then centrifuged at 3500 × g for 5 min to remove any cell debris. The supernatant was collected for trichloroacetic

acid (TCA)/acetone precipitation. Then, 10% by volume of ice-cold TCA was added to the samples and mixed by inverting. After incubating overnight at−20°C, the samples were thawed and centrifuged at 20000 × g for 90 min. Supernatants were then discarded. The remaining pellets were washed with−20°C acetone by vortexing vigorously and left overnight at−20°C. Samples were centrifuged at 20000 × g for 90 min, and the resulting supernatants were removed. The remaining pellets were then dried and boiled with 2X sample buffer.

Sample Preparation (Whole Cell Lysate)

Cells were harvested by scraping using cold Dulbecco's phosphate buffered saline (PBS) after the indicated time periods; they were then collected by centrifuging at 3000 × g for 5 min. Supernatants were discarded, and radioimmunoprecipitation (RIPA) buffer was added before sonication. Lysed cells were centrifuged at 20000 × g for 10 min to remove any debris. The resulting whole cell lysates were collected, and protein concentration was quantified using the bicinchoninic acid (BCA) assay. The cell lysates were then prepared by heating to 65°C for 10 min after adding sample buffer to minimize the loss of phosphorylated protein to beta-elimination.

Western Blot

SDS-PAGE was performed on samples prepared via the abovementioned methods, and proteins were transferred to a polyvinylidene difluoride (PVDF) membrane for western blotting. Transferred membranes were blocked using 5% skimmed milk. Primary antibodies for HMGB1 (Abcam; Cambridge, UK), JNK (phospho- and whole; Cell Signaling Technology; Danvers, MA, USA), ERK1/2 (phospho- and whole; Cell Signaling Technology), p38 (phospho- and whole; Cell Signaling Technology), phospho-IκBα (Cell Signaling Technology), phospho-IRAK4, phospho-TBK1 (Cell Signaling Technology), caveolin 1 (Merck; Darmstadt, Germany), TLR4 (Santa Cruz; Dallas, TX, USA), and β-actin (Santa Cruz) were diluted in 5% skimmed milk solution and incubated overnight at 4°C. After extensive washing, the corresponding secondary antibody solutions were incubated for 1 h at room temperature (20∼25°C). The membranes were then washed, and signals were detected using enhanced chemiluminescence substrate solution (Gendepot; Katy, TX, USA) and X-ray film (AGFA; Mortsel, Belgium). Membranes were stripped using stripping solution (BioMax, Seoul, South Korea) for re-blotting, as necessary. Densitometry analysis was performed using Image J.

Immunofluorescence

Raw 264.7 cells were seeded in 4-well chambered glass slides coated with poly-L-lysine (Sigma). Treatment dosage for LPS was increased to 200 ng/mL to facilitate visualization via immunofluorescence, and sulfatide dosage was adjusted accordingly to maintain molar ratio. Treatment was performed for the duration indicated in Figure Legends. After treatment, cells were then fixed with 4% paraformaldehyde overnight in 4°C. On the subsequent day, the cells were washed with PBS and permeabilized with 1% Triton X-100 and blocked with bovine serum albumin (BSA). Primary antibodies anti-p65 (Santa Cruz) or anti-HMGB1 (Abcam) were diluted in BSA solution and left to incubate overnight at 4°C. After thorough washing, the respective secondary antibodies conjugated with Alexa Fluor 488 (Invitrogen; Waltham, MA, USA) were diluted in BSA solution and incubated at 37°C for 45 min. Slides were then washed, dried, and mounted using mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI; Vector). Sealed slides were observed via FV1000 confocal microscopy (Olympus). Localization of sulfatide was determined by treating Raw 264.7 cells with biotin-sulfatide and staining them with streptavidin-Alexa Fluor 488 (Invitrogen). Localization of TLR4 was detected using mouse anti-TLR4 antibodies (Invitrogen).

ROS Detection

Raw 264.7 cells were pre-treated with either vehicle control or 20 μM of sulfatide and with vehicle control or 100 ng/mL of LPS for 1 h. The treatment medium was removed, and culture dishes were washed twice with warm culture medium. H2-DCFDA (Thermo Fisher; Waltham, MA, USA) was treated as per the manufacturer's instructions. Cells were viewed under a fluorescence microscope. For flow cytometric analysis of ROS levels, the cells were detached before H2-DCFDA treatment.

Lipid Raft Staining

Raw 264.7 cells were seeded in 4-well chambered glass slides coated with poly-L-lysine (Sigma). Treatment dosage for LPS was increased to 1 μg/mL to maximize lipid raft formation and facilitate visualization via immunofluorescence, and sulfatide dosage was adjusted accordingly to maintain molar ratio. Treatment was performed for the duration indicated in Figure Legends. After treatment, the cells were then washed once with 4°C complete growth medium. Washed cells were incubated in cholera toxin B-Alexa Fluor 549 (Invitrogen) staining solution, prepared in 4°C complete growth medium. Cells were washed with ice-cold PBS three times and fixed with ice-cold 4% paraformaldehyde for 15 min.

Lipid Raft Isolation

Raw 264.7 cells were treated with reagents for 8 min as indicated in the legends, and cells were briefly washed three times with ice-cold PBS to halt the internalization of lipid rafts. Cells were then lysed using the ice-cold buffer provided by Caveolea/Rafts Isolation Kit (Merck) supplemented with Triton X-100. Lysates then underwent ultracentrifugation with OptiPrep™ density gradient, provided by the aforementioned kit, and nine fractions were collected. Collected fractions were then supplemented with 1% SDS to assist complete dissociation of the protein from the lipids. Treated samples were concentrated using TCA/Acetone and analyzed by immunoblotting.

Animal Experiments

Wild-type C57BL/6 mice obtained from Orient Bio (Seongnam, South Korea) were housed in a SPF-grade facility with controlled temperature, humidity, and light. For all experiments, 8-week old female mice were used. For serum collection, mice were anesthetized using an isoflurane–oxygen mixture, and combinations of PBS, LPS (3 mg/kg), or sulfatide (25 nmol)

were injected with a total of 100 μL injection volume, intraperitoneally. The animals were allowed 60 min between injections to fully recover from the effects of anesthesia. Serum samples were collected after 18 h. Survival rate was measured by following the same procedure as mentioned above, with increased doses of LPS and sulfatide injection (to 20 mg/kg and 175 nmol, respectively). Mice were checked twice every day and observed until completion. Survival data were then analyzed through Kaplan-Meier survival analysis. Pathological scores were obtained using the scoring regimen described by Shrum et al. (43), and the obtained scores were then analyzed through ANOVA and Dunnett's multiple comparison

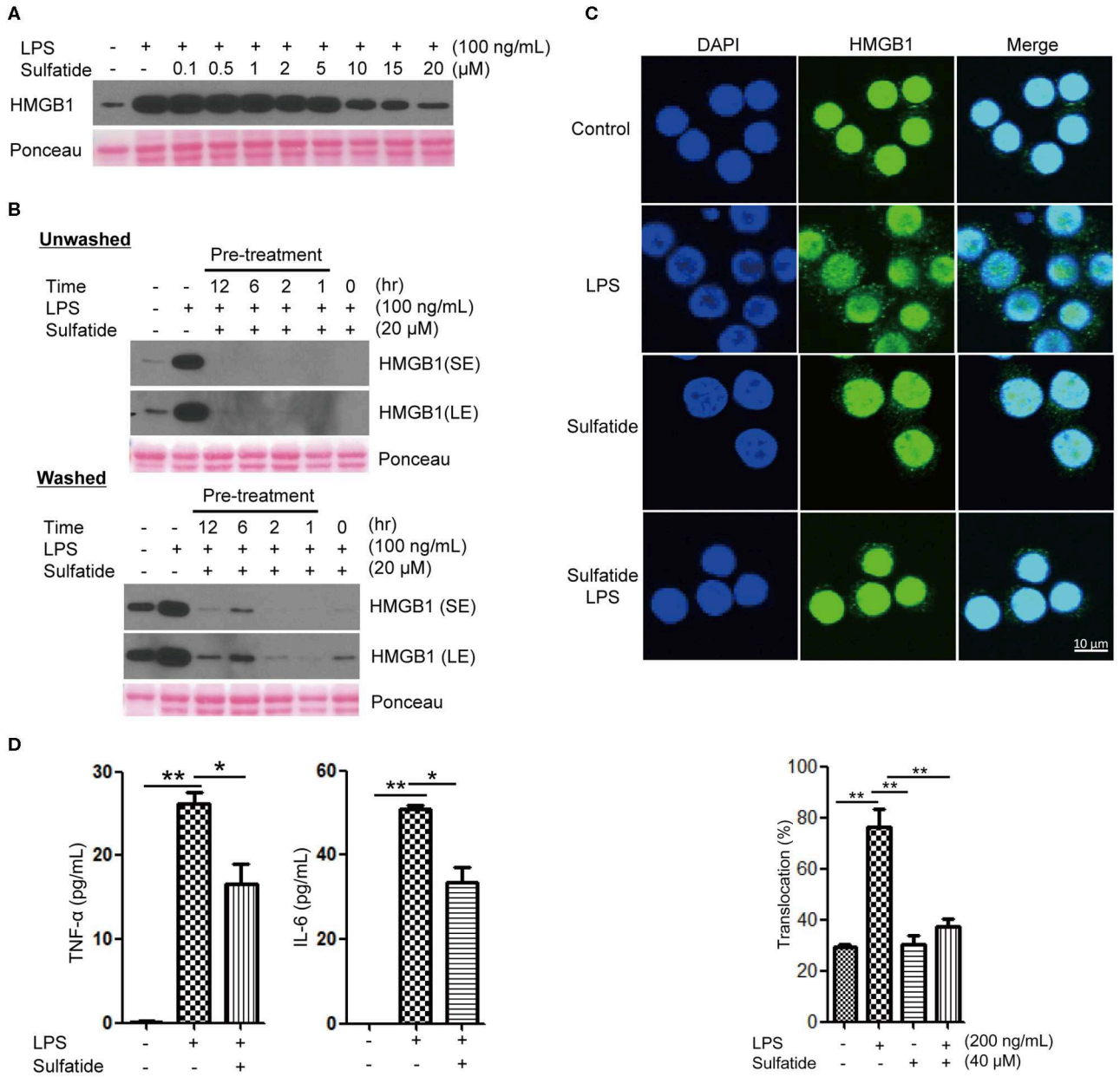

FIGURE 1 | Sulfatide inhibits HMGB1 translocation and release in Raw 264.7 cells. **(A)** Dose-dependency of sulfatide regarding HMGB1 secretion was accessed 24 h after LPS treatment. Varying dosage of sulfatide was treated 10 min prior to LPS treatment. **(B)** Efficacy of sulfatide pre-treatment for indicated time on HMGB1 secretion and its effect after removal of sulfatide was observed. Washed cells received two 36°C PBS wash to remove the residual sulfatide prior to LPS treatment, whereas unwashed cells were left unperturbed. (SE, Short Exposure; LE, Long Exposure) **(C)** Raw 264.7 cells received vehicle control, LPS 200 ng/mL, sulfatide 40 μM only, or 10 min of sulfatide 40 μM pre-treatment, followed by LPS 200 ng/mL for 6 h. Cells were fixed for analysis by immunofluorescence, as described in the Methods section. In total, 100 cells were counted, and those containing HMGB1 signals in the cytoplasm were counted as positive. **(D)** Culture media were analyzed by ELISA for TNF-α and IL-6 titer. Cells were treated with vehicle control (PBS with DMSO), LPS 100 ng/mL, or 10 min of sulfatide 20 μM pre-treatment, followed by LPS 100 ng/mL. Graphs show the mean value and error bars of three independent experiments performed. $^{*}p < 0.01$, $^{**}p < 0.001$.

test. All experiments were conducted according to procedures approved by the Institutional Animal Care and Use Committee of the Yonsei Laboratory Animal Research Center (YLARC, 2015-0275).

Enzyme-Linked Immunosorbent Assay (ELISA)

Tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6) ELISA were performed using Raw 264.7 cell culture medium. Cells were treated with vehicle control (negative control), 100 ng/mL LPS, and sulfatide 20 μM, followed by LPS 100 ng/mL. Culture media were collected after 12 h of treatment and centrifuged to remove any cell debris. ELISA was performed with the resulting supernatant following the manufacturer's instructions (Invitrogen). Serum obtained from murine experimental sepsis models was analyzed for HMGB1 levels with a HMGB1 ELISA kit (IBL International), following the manufacturer's instructions.

Statistical Analysis

Unless specified otherwise, statistical analysis of experimental data present in this paper were performed with Student's t test and ANOVA, with Tukey's multiple comparison test as *post-hoc* test, using GraphPad Prism 5. The data represent the mean value and SD. The difference was considered statistically significant at $p < 0.05$.

RESULTS

Sulfatide Inhibits HMGB1 and Pro-inflammatory Cytokines Release

To study whether sulfatide treatment shows pro-inflammatory or anti-inflammatory characteristics, we analyzed the secretion level of a well-known DAMP molecule, HMGB1. When treated simultaneously, sulfatide exhibited an inhibitory effect in HMGB1 secretion without toxicity in a dose-dependent manner, as shown (**Figure 1A**, **Supplementary Figure 1A**). This phenotype was unique to sulfatide, and was not seen in its precursors, galactosylceramide, and ceramide (**Supplementary Figure 1B**). Further analysis using ligands of other extracellular TLRs shows complete inhibition of HMGB1 secretion (**Supplementary Figure 1C**). This indirectly suggests that the anti-inflammatory effect does not come from inhibiting the ligand-receptor interaction by acting as a competitive inhibitor or aggregating reagent against TLR ligands, since it is unlikely that a molecule can act as broad-range inhibitor or aggregating reagent against multiple TLR ligands with different characteristics. Next, the time point-dependent effect of sulfatide was studied to further investigate the mechanism of action (**Figure 1B**). Interestingly, sulfatide not only exhibited dose- and time-dependent manner in HMGB1 release suppression, but also removal of sulfatide only induced a slight increase—lower than the secretion level of negative control, nevertheless—in HMGB1 secretion in 6 and 12 h-pretreatment samples. These results, combined with the results collected above,

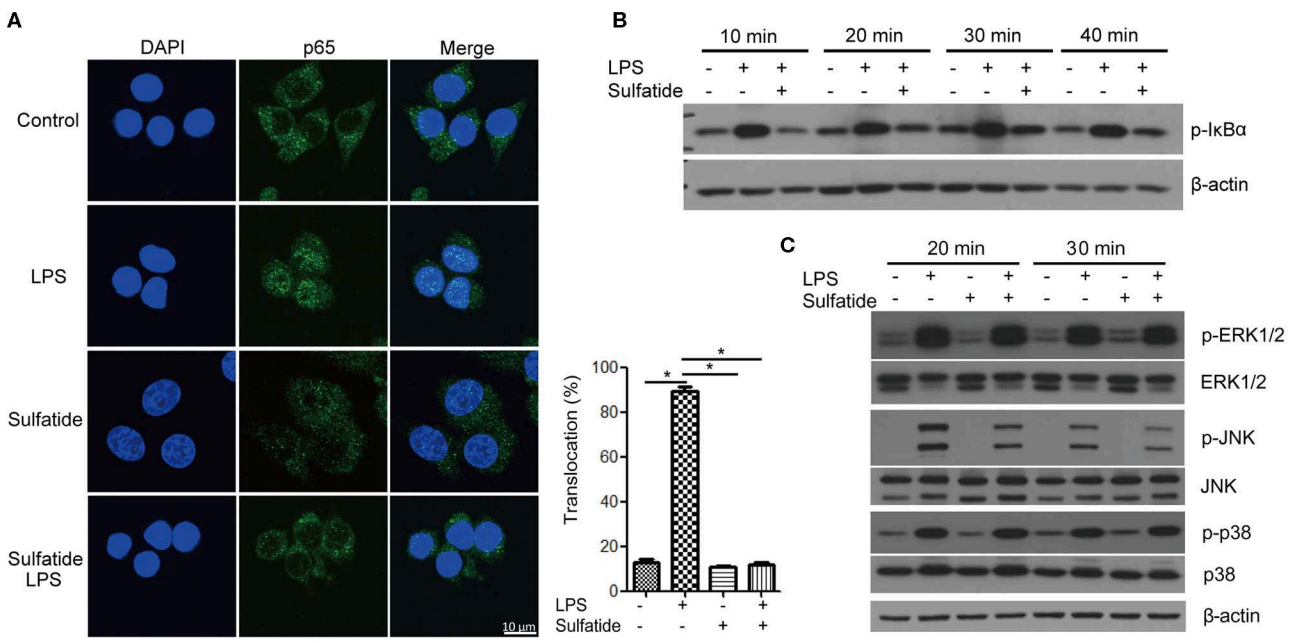

FIGURE 2 | Sulfatide suppresses NF-κB activation and JNK phosphorylation. **(A)** Raw 264.7 cells received vehicle control, LPS 200 ng/mL, sulfatide 40 μM, or 10 min of 40 μM sulfatide pre-treatment, followed by LPS 200 ng/mL for 40 min. Cells were then fixed for analysis by immunofluorescence as described in the Methods section. In total, 100 cells were counted, and those with p65 signals co-localizing with DAPI were counted as positive. *$p < 0.001$. **(B,C)** Raw 264.7 cells received vehicle control, LPS 100 ng/mL, sulfatide 20 μM, or 10 min of 20 μM sulfatide pre-treatment followed by LPS stimuli, as shown in the figure. Cells were harvested after the indicated times and analyzed for the phosphorylation level of IκBα **(B)**, p-ERK, p-JNK, and p-p38 **(C)** by immunoblotting.

suggest that sulfatide is neither an aggregating reagent nor competitive inhibitor, nor a reversible non-competitive inhibitor of TLR ligands.

Although multiple points of inhibition are potentially available throughout the HMGB1 secretion pathway, they can be categorized into two large categories: initial signal transduction, and the release step. In order to clarify whether sulfatide affects the former or the latter, we treated Raw 264.7 cells with LPS or sulfatide and investigated HMGB1 localization via immunofluorescence microscopy (**Figure 1C**). Confocal microscopy images show sulfatide inhibits nuclear HMGB1 translocation to the cytoplasm caused by LPS stimulation. This indicates that the inhibition mechanism of sulfatide does not target the release of HMGB1 to the extracellular space itself, but the pathway that precedes HMGB1 translocation.

Previous reports state sulfatide to play a pro-inflammatory role in brain-resident immune cells (42). In order to confirm its anti-inflammatory characteristics shown within our experimental setup, we treated Raw 264.7 cells with vehicle control, LPS alone, or LPS stimuli after sulfatide pre-treatment (**Figure 1D**). Contrary to previous reports made with brain-resident immune cells, sulfatide did not induce any significant secretion of pro-inflammatory cytokines, namely TNF-α and IL-6. Interestingly, a combination of LPS and sulfatide, however, did result in a significant decrease in the secretion levels of both TNF-α and IL-6, indicating that sulfatide indeed has an anti-inflammatory role in the peripheral immune system.

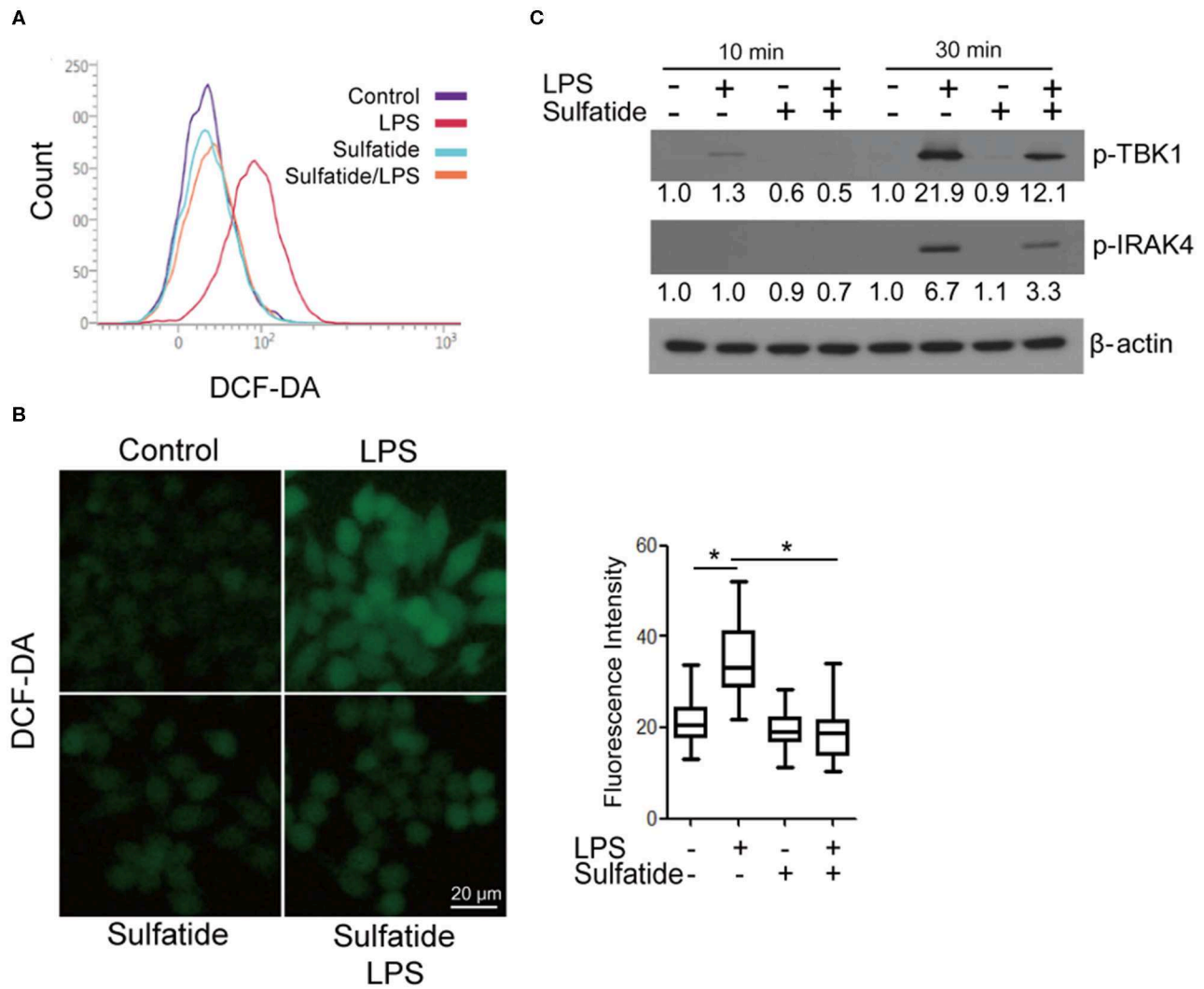

FIGURE 3 | Sulfatide down-regulates LPS-induced ROS production. Raw 264.7 cells were treated with 20 μM sulfatide or vehicle control for 10 min prior to receiving LPS 100 ng/mL, and were analyzed through **(A)** flow cytometry and **(B)** fluorescence microscopy. More than 150 cells were counted. *$p < 0.001$. **(C)** Cells were pre-treated with vehicle control or 20 μM sulfatide before treatment with 100 ng/mL LPS. Cells were lysed, and the samples were immunoblotted for p-TBK1 and p-IRAK4. Numbers below the immunoblots represent the relative band intensity, obtained by densitometry analysis. Vehicle controls of each groups were considered as standards.

Sulfatide Down-Regulates NF-κB Signaling Pathway and JNK Phosphorylation

The pathway most frequently associated with TLR signaling, NF-κB signaling pathway, is a cascade of signaling molecules that results in the degradation of NF-κB inhibitory molecules and the translocation of NF-κB to the nucleus, acting as a transcription factor. Concerning this pathway, we performed immunofluorescence microscopy, tracking the location of the p65 molecule, and immunoblotting of the IκBα molecule (**Figures 2A,B**). Our immunofluorescence data shows that NF-κB activation, signified by the translocation of p65, decreased when cells were pre-treated with sulfatide. Immunoblotting also indicated that phosphorylation of IκBα, a crucial step that precedes its ubiquitination and degradation, significantly decreases when pre-treated with sulfatide.

Further analysis of the MAPKs within the TLR signaling pathway revealed specific kinases affected by sulfatide treatment. The phosphorylation levels of ERK, JNK, and p38 MAPK were analyzed via immunoblotting (**Figure 2C**). Immunoblots revealed that only the phosphorylation level of JNK, but not of ERK or p38, was decreased by pre-treatment with sulfatide. Overall, sulfatide blocks the NF-κB signaling pathway and JNK-mediated HMGB1 translocation.

LPS-Mediated ROS Production Is Decreased by Sulfatide

Since an alternate mechanism exists, where HMGB1 release can be triggered via LPS-TLR4 signaling through ROS production, we sought to measure the changes in the level of intracellular ROS in the presence/absence of sulfatide pre-treatment (**Figures 3A,B**). Flow cytometric analysis and measurement of relative fluorescence intensity both show a significant decrease in intracellular ROS levels in sulfatide pre-treated groups. Such a decrease in ROS levels can be accredited to the decreased phosphorylation of both TBK1 and IRAK4, molecules that play crucial roles in the regulation of NOX activity (**Figure 3C**). These results, paired with those presented in earlier experiments, propose that the point of inhibition, which sulfatide utilizes to suppress HMGB1 release is positioned higher in the signaling hierarchy.

Sulfatide Hinders the Translocation of TLR4 Into Lipid Rafts

We hypothesized that sulfatide, a well-known component of the cell membrane, may interfere with the lipid composition of the cell membrane, inhibiting its signaling pathways. Since

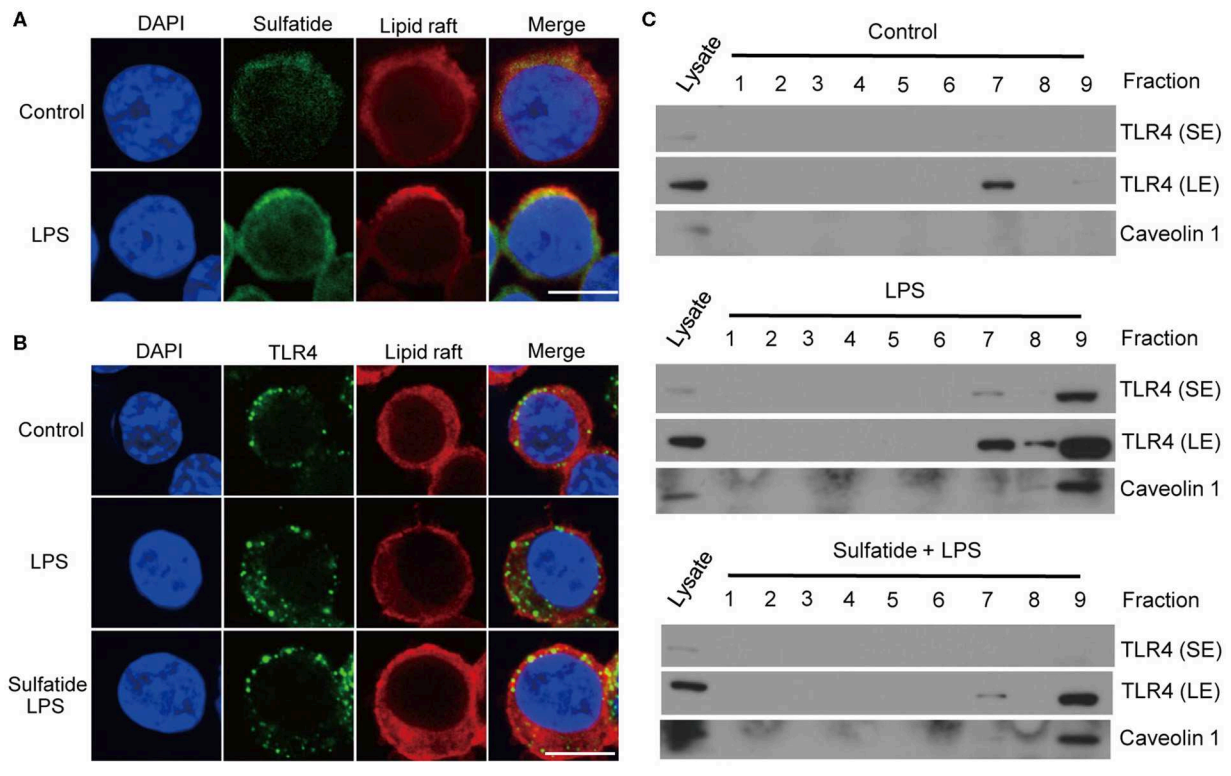

FIGURE 4 | TLR4–lipid raft complex formation is reduced by sulfatide. **(A)** Raw264.7 cells were treated with 200 μM of biotinylated sulfatide, with or without 1 μg/mL of LPS for 8 min. Biotinylated sulfatide was stained with streptavidin-Alexa Fluor 488 and TLR4 with anti-rabbit-Alexa Fluor 549, and the cells were prepared for confocal microscopy as described in the Methods section. (Scale bar : 10 μm) **(B)** Raw 264.7 cells were treated with vehicle control or 40 μM of sulfatide for 10 min, before 8 min of vehicle control or 200 ng/mL of LPS treatment. **(C)** Raw 264.7 cells were identically treated as those in **(B)**, and obtained samples were immunoblotted for TLR4 and caveolin 1. All membranes were immunoblotted under identical medical X-ray film for accurate comparison. (SE, Short Exposure; LE, Long Exposure).

TLR4 requires its monomers to be localized within the lipid raft microdomains to form dimers, we sought to assess (1) whether sulfatide localizes to the lipid raft microdomains, and (2) whether sulfatide treatment curbs the localization of TLR4 to lipid rafts. Utilization of biotinylated sulfatide revealed the co-localization of sulfatide and lipid rafts (**Figure 4A**), indicating the possibility of direct involvement of sulfatide in the lipid raft machinery. Next, to observe the co-localization of TLR4 and lipid rafts, we treated cells with appropriate stimuli and were prepared for immunofluorescence. Results insinuated that sulfatide plays a role in significantly decreasing the localization of TLR4 into the lipid microdomains. Such findings were reinforced by subjecting the cells to identical conditions and fractionating the cell lysate for lipid rafts. Results showed significantly decreased co-localization of TLR4 within the lipid raft fractions, signified by caveolin-1, in sulfatide-treated groups. In summary, sulfatide was found to interfere with the localization of TLR4 within lipid rafts, decreasing the efficacy of TLR4 signaling (**Figures 4B,C**).

Relase of HMGB1 Is Suppressed by Sulfatide in BMDM and the Murine Experimental Sepsis Model

The effects of sulfatide in primary cells and *in vivo* murine models were measured. BMDMs of 8 weeks old female C57BL/6 mice were harvested and were subjected to the same stimuli used above (**Figure 5A**). BMDMs pre-treated with sulfatide showed significantly decreased HMGB1 secretion, compared to cells treated with LPS alone, congruent with data obtained with Raw 263.7 cells. Such conformity led us to induce an experimental septic shock by the means of a sub-lethal dose injection of LPS into the peritoneum of C57BL/6 mice. Measurement of serum HMGB1 level was taken from sera obtained from a total of 18 mice (**Figure 5B**). Serum HMGB1 level was significantly decreased in the groups pre-treated with sulfatide, compared to groups treated only with LPS (**Figure 5B**). These results show that sulfatide regulates the release of HMGB1, a late time point cytokine of sepsis, in the murine experimental sepsis model. Such decrease in the serum HMGB1 level is also reflected in the murine

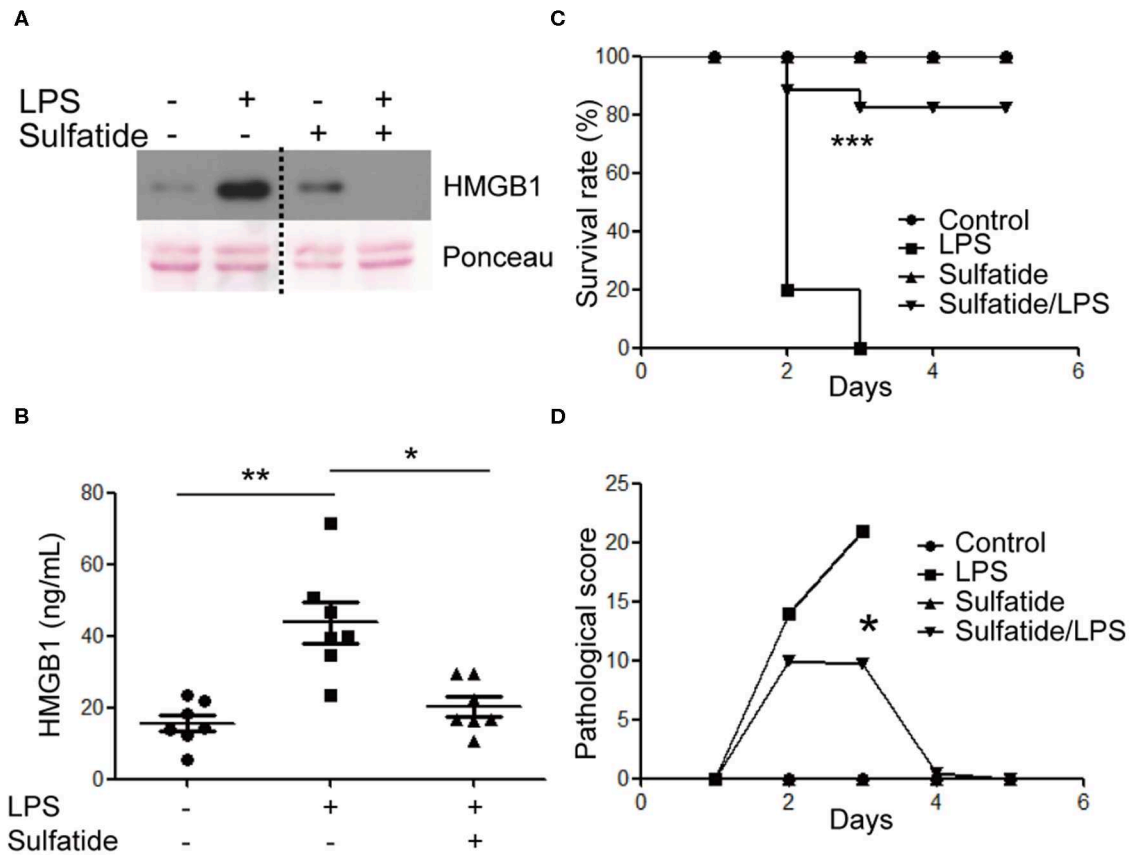

FIGURE 5 | Sulfatide decreases HMGB1 release in mouse BMDMs and the murine experimental sepsis model. **(A)** BMDMs were subjected to vehicle control, LPS 100 ng/mL, and 10 min of 20 μM sulfatide pre-treatment, followed by LPS 100 ng/mL for 24 h. The dotted line indicates where different portion of the identical membrane have been presented together. **(B)** C57BL/6 mice (7 mice per group) were intraperitoneally injected with PBS, LPS, or sulfatide pre-treatment, followed by LPS injection, as discussed in the Methods section. Sera were harvested and prepared for ELISA to measure serum HMGB1 level. **(C)** C57BL/6 mice (5 mice per group) were subjected to a survival test against LPS-induced lethal septic shock. Two independent trials were completed, and the results were pooled for statistical analysis. **(D)** A pathological score was obtained from the mice described in **(C)**. Mice from the second trial were used. $*p < 0.01$, $**p < 0.001$, $***p < 0.0001$.

model injected with a lethal dosage of LPS, mimicking acute septic shock. Although showing the telltale signs of septic shock (decreased physical activity, shivering etc.), mice pre-injected with sulfatide before LPS injection experienced no death in the population, in contrary to those that received saline pre-injection (**Figure 5C**). Additionally, to accurately compare the severity of the septic shock and the effect of sulfatide in decreasing its severity, pathological scores were measured every 24 h. Sulfatide pre-injected mice showed similar increase in pathological scores as the mice injected only with LPS for the first 24 h; however, groups that only received LPS injection showed a continuous increase in pathological scores, whereas the scores of the sulfatide pre-injected group plateaued, followed by a decrease in the pathological score (**Figure 5D**). Generally, sulfatide successfully blocked the LPS-mediated HMGB1 release in sepsis, decreasing the level of serum HMGB1 and preventing severe symptoms and death caused by sepsis.

DISCUSSION

Our experiments showed sulfatide reducing HMGB1 secretion and cytosolic translocation upon LPS stimulation. Sulfatide decreased the activation of NF-κB translocation into the nucleus, and inhibition of multiple kinases, such as JNK, IRAK4, and TBK1, was also seen throughout the experiment. JNK is a well-known signaling molecule playing a crucial role in cellular stress conditions, and when activated, phosphorylated JNK can also alter the mitochondria to increase its ROS production significantly, creating a positive feedback loop (44). Mice expressing inactive mutant form of IRAK4 were found to be more susceptible to *Listeria monocytogenes* and *Mycobacterium smegmatis* systemic infections due to impaired induction of inducible nitric oxide synthase (iNOS) mRNA (45). Since TBK1 was also involved in mitophagic regulation of mitochondrial physiology and expression of iNOS mRNA during inflammatory assault, paired with the reduction of ROS production, we hypothesized that the inhibitory characteristics of sulfatide may come from the upper hierarchy (46, 47). Further experiments showed sulfatide was hindering the lipid raft–TLR4 interaction, thereby diminishing the TLR4 signaling pathway (**Figure 6**).

Based on our research, the possibility of exogenous sulfatide as regulator of lipid raft—receptor complex formation may be suggested in clinical scenarios, in addition to the experimental sepsis model provided within. Pathological action of angiotensin II, a potent vasoconstrictor which binds to the AT_1 receptor, are ascribed to multiple vascular diseases, such as hypertension and secondary cardiac hypertrophy (48). AT_1 receptors are reported to be associated with lipid rafts (49); thus, sulfatide can be used to alter the lipid composition of the microdomains to deter the pathology in angiotensin II-mediated hypertension patients. Moreover, the immunological synapse, crucial for B/T cell activation, also depends on lipid raft formation (50–53), proposing a potential treatment strategy against autoimmune diseases such as rheumatoid arthritis, Type I diabetes, and multiple sclerosis (54–56) by blocking abnormal B/T cell activation.

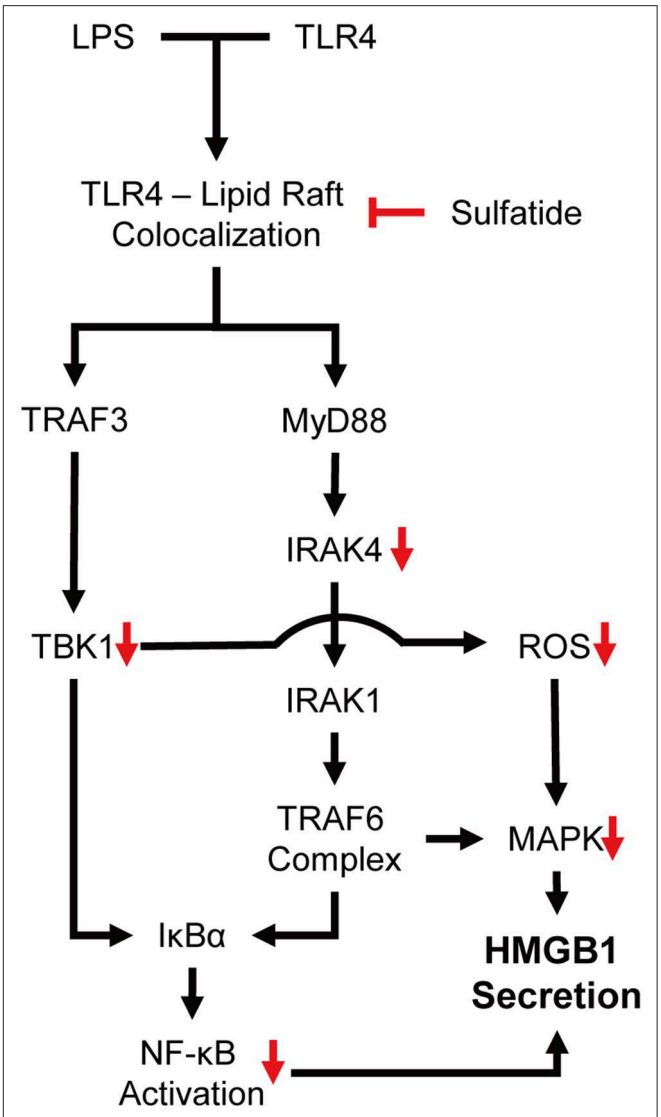

FIGURE 6 | Schematic summary of the proposed mechanism of action.

Sulfatide, however, has been reported as a possible auto-antigen in multiple sclerosis and experimental autoimmune encephalomyelitis (EAE). Lipid microarrays showed specific antibodies against various lipids in the cerebrospinal fluid, including ones against sulfatide in the murine EAE model and in multiple sclerosis (57, 58). Kanter et al. also reported the increase in disease severity as the mice were immunized with sulfatide and myelin membrane proteins. A further role of sulfatide as a pro-inflammatory molecule in pathogenesis was discovered in autoimmune hepatitis (59). In contrast, the anti-inflammatory roles of sulfatide were also revealed in autoimmune neuritis and asthma, mediated by sulfatide-activated type II NKT cells (37, 60). These reports suggest that sulfatide can be a double-edged sword, depending on the organ and pathological context, and that caution must be taken when attempting to adapt the "natural" form of sulfatide as a potential therapeutic agent.

Further studies regarding the mechanism of action of sulfatide in the abovementioned pathologies should be pursued to minimize or ameliorate side effects, possibly by utilizing small molecules mimicking the action of sulfatide.

Our research was able to report the anti-inflammatory effect of sulfatide in the periphery, specify the kinases within the NF-κB and MAPK pathway affected by sulfatide, and elucidate its mechanism of action. Sulfatide, nevertheless, is naturally a mixture of varying lengths of carbon chain backbone; therefore, the sulfatide used in this experiment is close to its natural form but far from being homogenous. Such properties could control the accessibility of sulfatide isoforms to various molecules via steric hindrance and variation in affinity. The composition of sulfatide isoforms has been connected to MS prognosis, enabling physicians to differentiate remitting MS from progressive MS by studying the composition of sulfatide isoforms (61). Although we were able to discover sulfatide hampering the localization of TLR4 and lipid rafts in our research, the specific roles of each component of sulfatide are yet to be discovered. According to the composition sheet provided by the supplier, C24-related isoforms were dominant in the making of sulfatide. This may explain the difference in phenotype between our experiment and others, as we carefully suggest the difference stems from the variability of sulfatide composition, depending on the provider. Isaac et al. has reported the importance of C18 sulfatide in astrocyte functionality (62), whereas many researchers including Buschard et al. and Blomqvist et al. have reported the crucial role of the C16:0 isoform in diabetes mellitus (63, 64). Such reports describing distinct role of various sulfatide components could be used to aid in indirectly understanding the phenotype difference between our group and the others. We sought to specify the isoform solely responsible for the phenotype shown within C24-related isoforms and C18 sulfatide, but to no avail (data not shown). Although we were not able to establish the isoform of sulfatide that is responsible for its properties, we were able to suggest that the mixture of C24-related isoforms and the C18 isoform that mimics the natural composition of sulfatide could also mimic its suppressive phenotype (**Supplementary Figure 2**). Fine-tuning the composition of specific isoforms by the means of supplementing the patients with sulfatide isoforms as needed may prove to be useful to alter the overall phenotype of sulfatide, further opening its therapeutic potential.

In conclusion, our study showed the effect of sulfatide in suppressing the secretion of HMGB1 under LPS stimulation, and its potential as anti-sepsis treatment. We have also firstly described the mechanism of inhibition where sulfatide inhibits the localization of TLR4 within the lipid microdomains, nullifying LPS-TLR4 signaling cascade. Further investigations regarding the interaction of exogenous sulfatide with lipid microdomains, importance of sulfatide isoform composition in various inflammatory diseases, and in-depth studying of isoform lipid biology are necessary to pursue future therapeutic applications of sulfatide.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of the Yonsei Laboratory Animal Research Center.

AUTHOR CONTRIBUTIONS

HK performed the immunoblotting and ELISA analysis with regard to HMGB1 secretion and its mechanisms. MH and IP helped in the planning and execution of the animal experiments. CP aided with the overall experiments using BMDMs. MK and IP contributed to the detailed experimental planning. J-SS gave the final approval of the manuscript version and has overseen the project and provided overall guidance for the experimental design. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01305/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Oxidation of HMGB1 Is a Dynamically Regulated Process in Physiological and Pathological Conditions

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Acute inflammation is a complex biological response of tissues to harmful stimuli, such as pathogens or cell damage, and is essential for immune defense and proper healing. However, unresolved inflammation can lead to chronic disorders, including cancer and fibrosis. The High Mobility Group Box 1 (HMGB1) protein is a Damage-Associated Molecular Pattern (DAMP) molecule that orchestrates key events in inflammation by switching among mutually exclusive redox states. Fully reduced HMGB1 (frHMGB1) supports immune cell recruitment and tissue regeneration, while the isoform containing a disulphide bond (dsHMGB1) promotes secretion of inflammatory mediators by immune cells. Although it has been suggested that the tissue itself determines the redox state of the extracellular space and of released HMGB1, the dynamics of HMGB1 oxidation in health and disease are unknown. In the present work, we analyzed the expression of HMGB1 redox isoforms in different inflammatory conditions in skeletal muscle, from acute injury to muscle wasting, in tumor microenvironment, in spleen, and in liver after drug intoxication. Our results reveal that the redox modulation of HMGB1 is tissue-specific, with high expression of dsHMGB1 in normal spleen and liver and very low in muscle, where it appears after acute damage. Similarly, dsHMGB1 is highly expressed in the tumor microenvironment while it is absent in cachectic muscles from the same tumor-bearing mice. These findings emphasize the accurate and dynamic regulation of HMGB1 redox state, with the presence of dsHMGB1 tightly associated with leukocyte infiltration. Accordingly, we identified circulating, infiltrating, and resident leukocytes as reservoirs and transporters of dsHMGB1 in tissue and tumor microenvironment, demonstrating that the redox state of HMGB1 is controlled at both tissue and cell levels. Overall, our data point out that HMGB1 oxidation is a timely and spatially regulated process in physiological and pathological conditions. This precise modulation might play key roles to finetune inflammatory and regenerative processes.

Keywords: inflammation, regeneration, injury, leukocyte, tumor, cancer cachexia, muscle, liver

INTRODUCTION

Inflammation is commonly perceived as a detrimental process and people often react to its five signs (pain, heat, redness, swelling, and loss of function) by taking anti-inflammatory drugs. Indeed, many chronic and degenerative diseases are associated to inflammatory processes, but inflammation is also important for the elimination of infections, the clearance of damaged cells and the regeneration of tissue (1). Many of the mechanisms that link inflammation to damage repair and regeneration in mammals are conserved during evolution, underlying the importance of this physiological process. Hence, chronic unresolved inflammation can lead to tissue damage and chronic disorders, including cancer and fibrosis, but self-limiting acute inflammation is essential for a proper healing process.

The Damage-Associated Molecular patterns (DAMPs) have been identified as key mediators of inflammation in response to infection or tissue damage (2). These sophisticated molecules have physiological roles inside the cell and, without damage, they are hidden to the immune system. Upon injury, DAMPs are exposed to the extracellular environment where they acquire additional functions: they alert the body about danger and contribute to inflammatory response and tissue repair (3). The High Mobility Group Box 1 protein fits all the criteria of DAMPs: it leads a double life having both intracellular and extracellular functions. HMGB1 has been first identified as a nuclear non-histone protein that regulates many processes in the nucleus from DNA repair to nucleosome dynamics (4, 5). However, HMGB1 is a very motile protein that can translocate to the cytoplasm and be passively released following traumatic death or actively secreted during severe stress to alert other cells of danger (6). This DAMP has been characterized as an inflammatory mediator, inducing both leukocytes recruitment and production of inflammatory cytokines and chemokines (7–9). Interestingly, the activities of HMGB1 in the extracellular microenvironment are tightly regulated by its redox state (7, 10, 11).

The HMGB1 protein is composed of two DNA-binding domains, called A box and B box, and of an acidic tail. This redox-sensitive protein contains 3 cysteines: C23 and C45 in the A box, which can form a disulphide bond, and the unpaired C106 in the B box. Notably, the redox state of these cysteines modulates the extracellular activities of HMGB1 and dictates its binding to different receptors. Fully reduced HMGB1 (frHMGB1) associates with the chemokine CXCL12 and activates the CXCR4 receptor, which recruits circulating leukocytes and stem cells to the site of damage, promoting tissue regeneration (7, 12, 13). Conversely, HMGB1 containing a disulphide bond (dsHMGB1) induces the expression of pro-inflammatory cytokines and chemokines by macrophages through its binding to MD-2, the TLR4 adaptor, or to the Receptor for Advanced Glycation End products (RAGE) (13, 14). Further cysteine oxidation to sulfonates by reactive oxygen species (ROS) abrogates both activities (7). It has been reported that HMGB1 inside the nucleus is fully reduced in normal conditions (15). It has also been suggested that the tissue itself determines the redox state of the extracellular space and most probably of released HMGB1, although it has not been demonstrated yet.

In the present work, we analyzed the expression of HMGB1 redox isoforms in different inflammatory conditions in skeletal muscle, from acute injury to chronic conditions of muscle wasting such as cancer cachexia, in tumor microenvironment, in spleen, and in liver after drug intoxication. Interestingly, we found that the presence of dsHMGB1 was tightly associated with an inflammatory state and we identified leukocytes as a main source of dsHMGB1. Overall, our data point at dsHMGB1 as a biomarker of inflammation and as a therapeutic target to dampen the inflammatory response.

MATERIALS AND METHODS

Mice and Models

Eight-wk-old C57BL/6 and Balb/c WT mice were purchased from Charles River Laboratories. C57BL/6 HMGB1 fl/fl and HMGB1 fl/fl:MyoD-Cre (thereafter termed mKO) mice were bred in the animal facility at San Raffaele Scientific Institute. In mKO mice, Cre recombinase is under the control of MyoD promoter to knock out HMGB1 in all myogenic cells. All mice were housed under standard or specific pathogen–free conditions and allowed access to food and water *ad libitum* with the exception of the 16 h fasting prior to acetaminophen (APAP) injection, as described below. All experimental protocols were approved by the San Raffaele Institutional Animal Care and Use Committee (IACUC 838, 972, and 1,111) in accordance with Italian law. All efforts were made to minimize suffering.

In the acute muscle injury model, animals were anesthetized by intraperitoneal injection of Avertin (T48402, 2,2,2-Tribromoethanol 97%; Sigma-Aldrich), and sterile injury was induced by injection of 50 μl of 15-μM cardiotoxin (CTX, C9759 Sigma-Aldrich) in tibialis anterior or triceps muscles. WT mice were euthanized at 1 h, 6 h, 1 d, 3 d, 5 d or 7 d after CTX injection, while HMGB1 mKO mice were euthanized 1, 2, 5, and 7 d after CTX injection. Muscles were collected and either sectioned for histological analyses or subjected to protein quantification for Western blot analyses.

For the liver acute injury, 8-wk-old C57BL/6 males were fasted 16 h before intraperitoneal injection of 300 mg/Kg (body weight) APAP (Sigma-Aldrich) dissolved in sterile warm saline. Mice were i.p. injected with APAP or control saline, and after 1, 2, 3 and 7 days, were i.v. injected with 5 mg/Kg (body weight) of Evans Blue (Sigma-Aldrich) followed by euthanasia 30 min later. Spleen and liver were collected either for histological analyses or subjected to protein quantification by Western blot analyses. At the indicated time points, blood was collected for serum Alanine Aminotransferase (sALT) and HMGB1 quantifications.

For the cancer cachexia models, Lewis lung carcinoma (LLC) cells and C26 colon adenocarcinoma cells were maintained in DMEM (ThermoFisher) with 10% Fetal Bovine Serum (FBS). C57BL/6 and Balb/c WT mice were subcutaneously injected on the right flank with 5×10^6 LLC cells or 1×10^6 C26 cells, respectively, in 100 μl of Phosphate Buffered Saline (PBS). Blood was collected for HMGB1 quantification just before euthanasia. LLC- and C26-bearing mice were sacrificed 3- and 2-weeks post-injection of cancer cells, respectively. Skeletal muscles (tibialis anterior, quadriceps, gastrocnemius) and tumors were collected

and either sectioned for histological analyses or subjected to protein quantification by Western blot analyses.

Histology and Immunohistochemistry

Tibialis anterior muscles were fixed with 4% buffered paraformaldehyde solution for 3 h, then dehydrated in 15 and 30% sucrose and subsequently frozen in liquid nitrogen–cooled isopentane. Serial muscle sections, 8-μm thick, were then stained with anti-HMGB1 (1:800, ab18256 Abcam) and anti-CD45 (1:1000, ab10558 Abcam) antibodies.

Livers were collected and pieces of liver were either fixed with 4% buffered paraformaldehyde solution or zinc-formalin. The livers fixed over-night with 4% buffered paraformaldehyde solution were then equilibrated in 10, 20, and 30% sucrose, embedded in OCT for quick freezing at −80°C and cryosectioned (20 μm thickness) for subsequent fluorescent detection of Evans Blue damaged areas. The liver samples fixed in zinc-formalin were then embedded in paraffin, cut and stained with hematoxylin/eosin, anti-CD45 (1:1000, ab10558 Abcam), or anti-HMGB1 (1:800, ab18256 Abcam) antibodies.

Tumors were collected and fixed 24 h in formalin and then transferred in 70% ethanol solution. Fixed tumors were then embedded in paraffin, cut, and stained with anti-HMGB1 (1:800, ab18256 Abcam) and anti-CD45 (1:1000, ab10558 Abcam) antibodies.

Image Acquisition and Analyses

Bright-field images were taken with a Leica DM750 microscope equipped with Leica ICC50 HD camera or with a Zeiss AxioImager M2m with AxioCam MRc5. Representative images were acquired at 20x magnification and analyzed by using ImageJ software (http://rsbweb.nih.gov/ij/).

Confocal images were acquired using a Leica TCS SP5 confocal system (Leica Microsystems) available at the SRSI Advanced Light and Electron Microscopy BioImaging Center (ALEMBIC). Twenty-micrometer z-stacks were projected in 2D and processed using Imaris image processing software.

Leukocyte Isolation and Lysates

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of human healthy donors by Ficoll-Paque density centrifugation as previously described (7). PBMCs were then plated in RPMI 10% FBS and lysed after 30 min or treated with either combination of 1 μg/ml anti-CD3 (16-0037-85, Life Technologies) and 1 μg/ml anti-CD28 (16-0289-85, Life Technologies) or 1 μg/ml LPS (L4641, Sigma-Aldrich), and lysed after 24 or 72 h.

Intrahepatic leukocytes (IHLs) isolation was performed as previously described (16). Both PBMCs and IHLs ($2–10 \times 10^6$ cells) were lysed in 100 μl of RIPA buffer (50 mM Tris-HCl-pH 7.4, 1% IGEPAL, 0.5% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 50 mM NaF).

Supernatant Collection and Tissue/Cell Lysates

Isolated muscles were incubated overnight at 4°C in 200 μl of Phosphate Buffered Saline with protease inhibitors cocktail (P8340, Sigma-Aldrich). Supernatants were collected and centrifuged at 12,000 rpm for 15 min at 4°C. Pellet was discarded and supernatants were analyzed by Western blot assays.

Tissues (muscles, tumor masses, livers, spleens) and cells (PBMCs, IHLs, tumor cells) were lysed in RIPA buffer (50 mM Tris-HCl-pH 7.4, 1% IGEPAL, 0.5% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 50 mM NaF) with protease inhibitors cocktail. Tissues were disrupted in RIPA buffer with TissueLyser LT (Qiagen). Lysates were then centrifuged at 12,000 rpm for 15 min at 4°C and the supernatants were collected.

Western Blot Assays

Total protein content in muscle, tumor, liver, spleen, and cells lysates was determined using the BCA protein Assay Kit (ThermoFisher). Laemmli buffer to 1X final concentration (45 mM Tris-HCl-pH 6.8, 1.5% SDS, 3.5% β-mercaptoethanol, 3.5% Glycerol, 0.01% Bromophenol Blue) was added to equivalent protein amounts of cell lysates (5 μg for PBMCs lysates), tissue lysates (20 μg for muscle, spleen, liver, or tumor lysates) or lysate volumes (5 or 25 μl for IHLs lysates or muscle supernatants, respectively). To detect fully reduced and disulphide-HMGB1 isoforms, Western blot assays were performed in non-reducing conditions by diluting samples in Laemmli buffer without reducing agent (β-mercaptoethanol or DTT). Protein samples were separated on 14% SDS-PAGE (in reducing or non-reducing conditions) and transferred onto nitrocellulose membranes, which were blocked with 5% milk in Tris-buffered saline, pH 7.0, containing 0.1% Tween 20 (TBS-T). Membranes were probed with monoclonal rabbit anti-HMGB1 (1:10,000, EPR3507 Abcam) or rabbit anti-CD45 antibodies (1:500, ab10558 Abcam) in TBS-T plus 5% milk overnight at 4°C, washed several times with TBS-T, and incubated for 1 h with anti–rabbit peroxidase-conjugated antibody. For loading control, membranes were incubated with Ponceau Red (P7170 Sigma Aldrich) for a couple of minutes and then washed several times with TBS-T, or with monoclonal anti-GAPDH antibody (1:10,000, G9545 Sigma-Aldrich) in TBS-T plus 5% milk overnight at 4°C, washed several times with TBS-T, and incubated for 1 h with anti–rabbit Cy5-conjugated antibody. Western blots assays were visualized using a chemiluminescence kit or a Typhoon instrument according to the manufacturer's instructions (GE Healthcare).

ELISA and Blood Analysis

Blood samples were collected, and serum was obtained by centrifugation for 10 min at 3,500 rpm at 4°C. The levels of HMGB1 protein were measured by ELISA (Tecan) according to manufacturer's instructions.

sALT levels were quantified in serum after APAP-induced intoxication with an International Federation of Clinical Chemistry and Laboratory Medicine-optimized kinetic UV method in an ILab Aries chemical analyzer (Instrumentation Laboratory).

Statistical Analysis

Every experiment was replicated at least twice and was performed at least in biological triplicates. Sex-matched animals were assigned randomly to experimental groups and no animals were

excluded from the study. According to the 3R rules, a power calculation analysis was previously performed. The evaluator was blinded to the identity of the specific sample as far as the nature of the experiment allowed it. Bars represent the mean ± SEM. Statistical significance was assessed by using the tests indicated in the figure legends (Prism 8; GraphPad Software). $P < 0.05$ were considered statistically significant.

RESULTS

Redox Modulation of HMGB1 in Skeletal Muscle Upon Acute Injury

Several reports highlighted a role of HMGB1 in skeletal muscle regeneration (17–19) and we previously demonstrated that HMGB1 redox isoforms orchestrate regeneration in muscle and liver after acute injury (13). HMGB1 is highly expressed in the nuclei of regenerating myofibers (**Figure 1A**) and both fr- and dsHMGB1 isoforms are abundant in the medium bathing injured muscles (7). We speculated that dsHMGB1 might derive from leukocytes infiltrating the injured muscle. To address this issue, we concomitantly analyzed the presence of leukocytes (CD45-positive cells) and the expression of HMGB1 redox isoforms in injured muscles at different time points after cardiotoxin (CTX)-induced acute injury. As evidenced by CD45 immunostaining on muscle sections and lysates, leukocytes were nearly undetectable in healthy muscle, and their infiltration started at 6 h post-injury and persisted until day 7 post-injury (**Figures 1A,B**). While the expression of total HMGB1 was increased from days 3–7 post-injury, both CD45-positive cells and dsHMGB1 appeared between 6 h and day 1 post-injury and persisted until day 7 post-injury (**Figures 1B–D**). The proportion of frHMGB1 on the total amount of HMGB1 decreased in muscle lysate from 6 h to day 7 post-injury and conversely, the proportion of dsHMGB1 was increased at these time points (**Supplementary Figure 1A**). Specifically, frHMGB1 was the isoform predominantly expressed at time points characterized by the absence of CD45-positive cells (control and 1 h post-injury), while dsHMGB1 represented about 30% of the total HMGB1 protein in muscle lysate in the presence of CD45-positive cells (from 6 h to day 7 post-injury) (**Figure 1E**).

HMGB1 is a marker of tissue damage as it is released by dead or stressed cells (6). In addition, leukocytes have been identified as professional cells for HMGB1 release upon injury and infection (9, 20). Hence, we analyzed the expression of released HMGB1 in serum and supernatant of injured muscles. Circulating HMGB1 was increased at early timepoints, from 1 to 6 h post-injury (**Supplementary Figure 1B**). This first peak of HMGB1 in the serum is most probably due to the release of the protein by necrotic cells from the injured muscle. To a lesser extent, we observed a second peak of HMGB1 from days 3–7 post-injury, which might be attributed to HMGB1 release by infiltrating leukocytes. The amount of HMGB1 was increased in supernatant of muscles excised from days 3–7 post-injury (**Figures 1F,G**). Notably, the expression of dsHMGB1 in the supernatant was much higher compared to those in muscle lysate and perfectly overlaps the expression of CD45 over time (**Figures 1F–H**, **Supplementary Figure 1C**). In the presence of CD45-positive cells, the percentage of dsHMGB1 on total HMGB1 protein level in the supernatant raised about 50% (**Figure 1I**).

Overall, our data demonstrate that the redox state of HMGB1 is highly modulated in skeletal muscle following acute injury, with a very low amount of dsHMGB1 in normal condition that strongly increases upon damage and is tightly associated with the presence of infiltrating leukocytes.

Infiltrating and Circulating Leukocytes Represent Major Sources of dsHMGB1

To demonstrate that dsHMGB1 derives from infiltrating leukocytes and not from resident muscle cells, we took advantage of muscle cells-specific HMGB1 knockout mice (hereafter mKO mice). These mice were generated by crossing C57BL/6 HMGB1 fl/fl mice with MyoD-Cre mice. In the latter, the Cre enzyme is under the control of the promoter of MyoD, a transcription factor expressed during myogenesis, which enables the deletion of lox-flanked sequences in all myogenic cells (muscle stem cells, myoblasts, myofibers). In this model, HMGB1 is deleted in myogenic cells but it is still expressed in skeletal muscle by non-muscle cell types, such as endothelial and nervous cells. As expected, the level of total HMGB1 was strongly decreased in uninjured muscle lysates from mKO mice compared to controls, and CD45-positive cells were nearly absent (**Figures 2A–C**). The level of HMGB1 and the number of CD45-positive cells strongly increased in both wildtype and mKO mice from day 1 to day 7 post-injury (**Figures 2A–C**), indicating that the absence of HMGB1 in myogenic cells does not dramatically affect leukocyte recruitment in injured muscle and that HMGB1 in injured muscle mainly derives from non-muscle cells. Similarly, we observed no difference in the distribution of HMGB1 redox isoforms between wildtype and HMGB1 mKO mice with detection of CD45 and dsHMGB1 only in injured muscles (**Figures 2D,E**). These data demonstrate that dsHMGB1 does not derive from myogenic cells in the injured muscle and strongly suggest that it originates from non-muscle cells such as infiltrating leukocytes.

Although the release of HMGB1 by leukocytes has been widely studied, very little is known on the redox modulation of the protein in these cells. Hence, we analyzed the expression and redox state of HMGB1 in peripheral blood mononuclear cells (PBMCs) from human healthy donors. We observed a high expression of HMGB1 in lysate of freshly isolated PBMCs with around the 50% of the total corresponds to dsHMGB1 (**Figures 3A,B**), demonstrating that these cells represent a reservoir of dsHMGB1. To determine whether the expression and redox state of HMGB1 might be modulated during cell activation, we treated PBMCs with anti-CD3/anti-CD28 or lipopolysaccharide (LPS) to stimulate T cells, dendritic cells, and monocytes/macrophages. We observed a high amount of dsHMGB1 in all conditions with no major difference upon the various stimuli, although

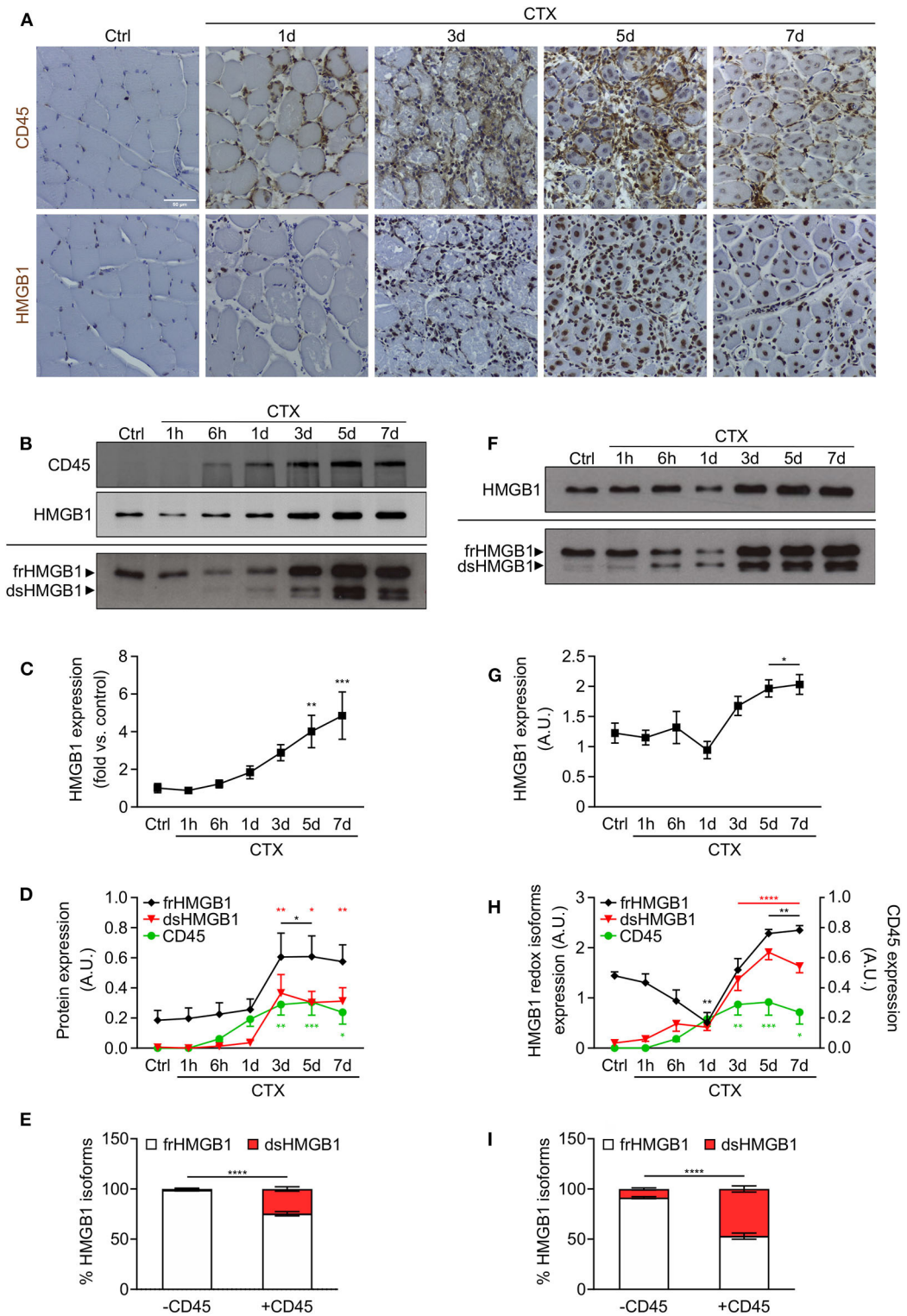

FIGURE 1 | HMGB1 redox isoforms expression and leukocyte infiltration during acute muscle injury. **(A)** Representative images of immunohistochemical staining for CD45 (upper panel) and HMGB1 (lower panel) on tibialis anterior (TA) muscle sections at indicated time points after cardiotoxin (CTX) injection. Scale bars, 50 μm. Ctrl, uninjured control muscles. **(B–E)** Western blot probed with anti-CD45 (upper panel) and anti-HMGB1 (middle panel) antibodies in reducing conditions or with anti-HMGB1 antibody in non-reducing conditions (lower panel) on muscle lysates at indicated time points after CTX injection. The upper and lower bands in non-reducing conditions correspond to the fully reduced-HMGB1 (frHMGB1) and the disulphide-HMGB1 (dsHMGB1) isoforms, respectively. **(C)** Quantification of

(Continued)

FIGURE 1 | total HMGB1 protein expression levels, relative to control (Ctrl) and normalized on Ponceau staining, at indicated time points after CTX injection. A.U. = arbitrary unit ($n \geq 10$ muscles, 3 mice/time point). **(D)** Quantification of CD45 and HMGB1 redox isoforms expression (frHMGB1 and dsHMGB1), normalized on Ponceau staining, at indicated time points. **(E)** Distribution of HMGB1 redox isoforms expression in muscle lysates in absence (controls and at 1 h post-injury) or presence of CD45-positive cells (from 6 h to day 7 post-injury). **(F–I)** Western blot probed with anti-HMGB1 antibody in reducing (upper panel) and non-reducing conditions (lower panel) on supernatant of muscles isolated at indicated time points after CTX injection **(F)**. Total HMGB1 protein expression at indicated time points after CTX injection **(G)**. A.U. = arbitrary unit ($n = 6$ muscle supernatants, 3 mice/time point). **(H)** Quantification of HMGB1 redox isoforms expression (frHMGB1 and dsHMGB1) in muscle supernatants, from Western blot assays in non-reducing conditions, at indicated time points after CTX injection and compared with CD45 expression as in **(D)**. **(I)** Distribution of HMGB1 redox isoforms expression in supernatants of muscle in absence (controls and at 1 h post-injury) or presence of CD45-positive cells (from 6 h to day 7 post-injury). Data represent the means ± SEM and statistical significance was calculated by One-way **(C,D,G,H)** and Two-way ANOVA **(E,I)**. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

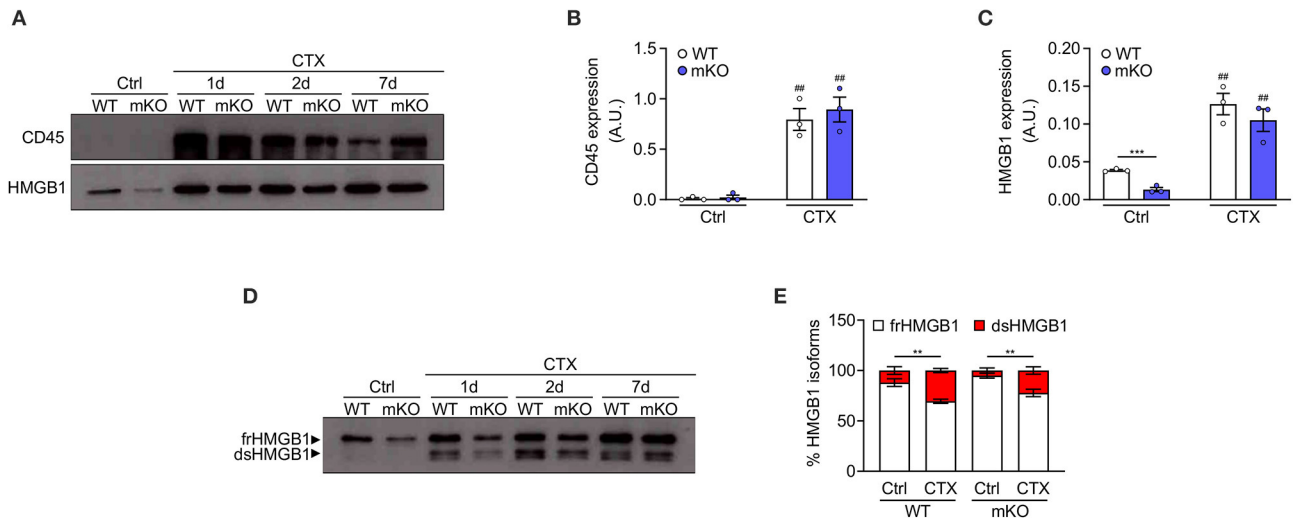

FIGURE 2 | Disulphide-HMGB1 derives from non-myogenic cells in injured muscle. **(A)** Western blot probed with anti-CD45 (upper panel) and anti-HMGB1 (lower panel) antibodies in reducing conditions on tibialis anterior (TA) muscle lysates from WT or HMGB1 mKO mice at indicated time points after cardiotoxin (CTX) injection. Ctrl, control uninjured muscles. **(B,C)** Quantification of CD45 **(B)** and HMGB1 **(C)** protein expression, normalized on Ponceau staining, before (Ctrl) and after CTX injection (CTX at 1, 2, and 7 d) in TA and triceps muscle lysates ($n \geq 4$ muscles/time point, $n = 3$ mice/genotype). A.U. = arbitrary unit. **(D,E)** Western blot probed with anti-HMGB1 antibody in non-reducing conditions **(D)** on TA muscle lysates from WT or HMGB1 mKO mice at indicated time points after CTX injection. The upper band corresponds to the fully reduced-HMGB1 (frHMGB1) and the lower band to the disulphide-HMGB1 (dsHMGB1). **(E)** Percentage of HMGB1 redox isoforms expression from WT or HMGB1 mKO mice before (Ctrl) and after CTX injection (CTX at 1, 2, 5, and 7 d) in TA and triceps muscle lysates ($n \geq 3$ muscles/time point; $n \geq 4$ mice/genotype). Data represent the means ± SEM and statistical significance was calculated by Student *T*-test **(B,C)** and Two-way ANOVA **(E)**. $^{**}P < 0.01$; $^{***}P < 0.001$; $^{\#\#}P < 0.01$ (Ctrl vs. CTX).

LPS appeared to slightly increase the level of dsHMGB1 at 72 h (**Figures 3C,D**).

Overall, our findings demonstrate that both circulating and infiltrating leukocytes contain a high amount of dsHMGB1 that could be released into the injured muscle.

Leukocytes Operate as Transporters of dsHMGB1 in the Tumor Microenvironment

To further investigate the ability of leukocytes to transport dsHMGB1, we extended our results to cancer and to cachexia, a severe muscle wasting syndrome associated to tumor progression (21). Cancer-related inflammation has emerged as a hallmark of cancer and evidences from animal models indicate a compelling link between cachexia and inflammation (22, 23). Beside leukocyte invasion in the tumor microenvironment, cancer cachexia is associated to systemic inflammation, but no leukocyte infiltration in cachectic muscle (22, 24). To study the expression of HMGB1 redox isoforms in tumors and cachectic muscles, we employed two well-established mouse models of cancer cachexia: C57BL/6 and BalB/C mice injected subcutaneously with Lewis Lung Carcinoma (LLC) cells and colon adenocarcinoma C26 cells, respectively. In these models, mice undergo body weight loss and muscle wasting, mainly through increased levels of circulating Tumor Necrosis Factor-α and Interleukin-6, respectively (25, 26). We observed a loss of body weight and muscle mass in these two models, but the level of circulating HMGB1 was decreased in C26-bearing mice while it was increased in LLC-bearing mice (**Supplementary Figures 2A–C**), further underlining that tumor growth and cachexia progression are regulated by different mechanisms in these two models.

As expected, CD45-positive cells were nearly absent in cachectic muscles (**Figures 4A–C**, **Supplementary Figures 2D,E**). We observed a slight increase of total HMGB1 level in cachectic muscles from both mouse models (**Figures 4B,C**, **Supplementary Figures 2D,E**). Interestingly, frHMGB1 was the predominant isoform with no increase

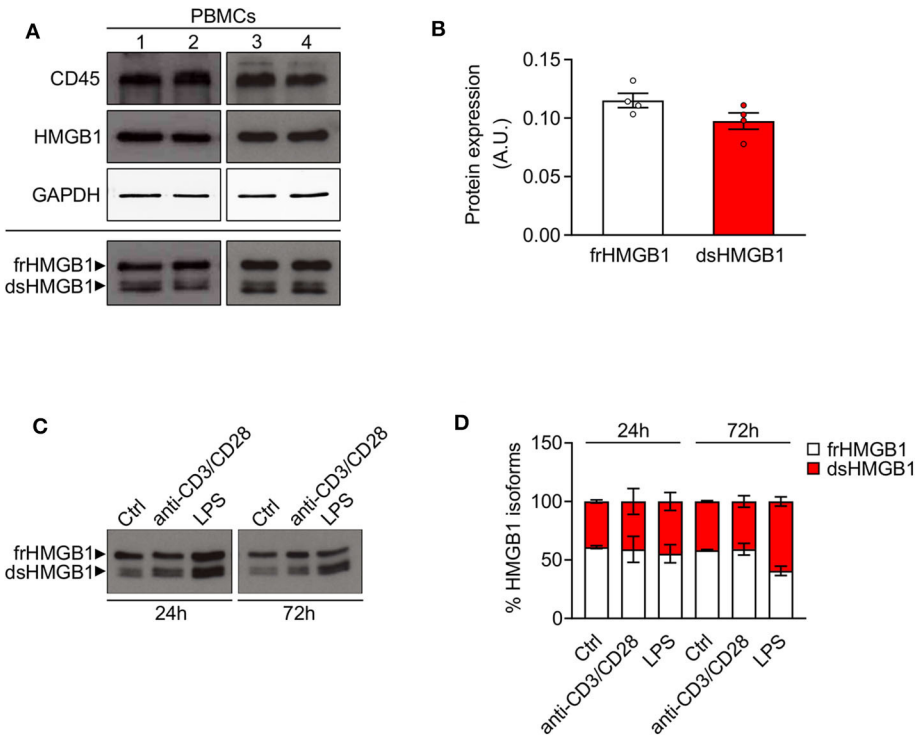

FIGURE 3 | High expression of disulphide-HMGB1 in human leukocytes. **(A)** Western blot probed with anti-CD45, anti-HMGB1, and anti-GAPDH antibodies in reducing conditions (upper panels) or probed with anti-HMGB1 antibody in non-reducing conditions (lower panel) on peripheral blood mononuclear cells (PBMCs) isolated from four healthy human donors. The upper band corresponds to the fully reduced-HMGB1 (frHMGB1) and the lower band to the disulphide-HMGB1 (dsHMGB1) in the lower panel. **(B)** Quantification of HMGB1 redox isoforms expression normalized on Ponceau staining. A.U. = arbitrary unit ($n = 4$ healthy donors). **(C,D)** Western blot probed with anti-HMGB1 antibody in non-reducing conditions on PBMCs stimulated with anti-CD3/anti-CD28 antibodies or lipopolysaccharide (LPS) for 24 or 72 h **(C)**. Percentage of HMGB1 redox isoforms expression **(D)**. Ctrl, control unstimulated cells ($n = 2$ healthy donors). Data represent the means ± SEM and statistical significance was calculated by Two-way ANOVA **(D)**.

of dsHMGB1 in cachectic muscles compared to controls (**Figures 4D,E**, **Supplementary Figures 2F,G**). These data demonstrate that leukocytes are not recruited and that the redox state of HMGB1 is not shifted toward dsHMGB1 in cachectic muscles.

We next analyzed the expression of CD45 and HMGB1 in tumors isolated from cachectic mice. Both CD45 and HMGB1 were highly expressed in LLC- and C26-derived tumors (**Figures 4F–H**, **Supplementary Figures 2H,I**). While the expression of total HMGB1 was comparable in isolated tumors and cultured tumor cell lines, CD45 and dsHMGB1 were highly expressed only in isolated tumors (**Figures 4G–J**, **Supplementary Figures 2H–K**).

Overall, these results demonstrate that the redox state of HMGB1 is modulated locally during cancer cachexia progression and indicate that leukocytes act as transporters of dsHMGB1 isoform in the tumor microenvironment.

Redox Modulation of HMGB1 in Spleen and Liver

To determine whether resident leukocytes, as opposed to infiltrating/circulating leukocytes, also produce dsHMGB1, we analyzed the expression of HMGB1 redox isoforms in spleen and liver, two organs characterized by a high number of resident leukocytes. We observed a high expression of both CD45 and dsHMGB1 in spleen whereas comparable percentage of dsHMGB1 expression was associated to much lower CD45 expression in liver (**Figures 5A–C**), suggesting additional cell population(s) expressing dsHMGB1 in liver. Beside differences in leukocytes number, these findings indicate that these two organs represent important sources of dsHMGB1.

Previous studies have demonstrated that endogenous HMGB1 is a mediator of drug-induced hepatoxicity by promoting inflammation via its interaction with TLR4/MD-2 (14). We decided to analyse the recruitment of leukocytes and the expression of HMGB1 redox isoforms in liver upon acetaminophen (APAP) intoxication. We hypothesized that no difference in dsHMGB1 percentage would be observed in liver upon acute injury as we already observed a comparable percentage of dsHMGB1 in circulating leukocytes and uninjured liver (around 50% of total HMGB1). As expected, we observed hepatocyte necrosis, CD45-positive cell recruitment and HMGB1 release in injured areas of the liver after APAP injection (**Figure 5D**). Notably, the temporal dynamics of circulating HMGB1 levels perfectly overlaps that of sALT, a marker of liver damage (**Figure 5E**), which peaks at day 1 post-APAP. As

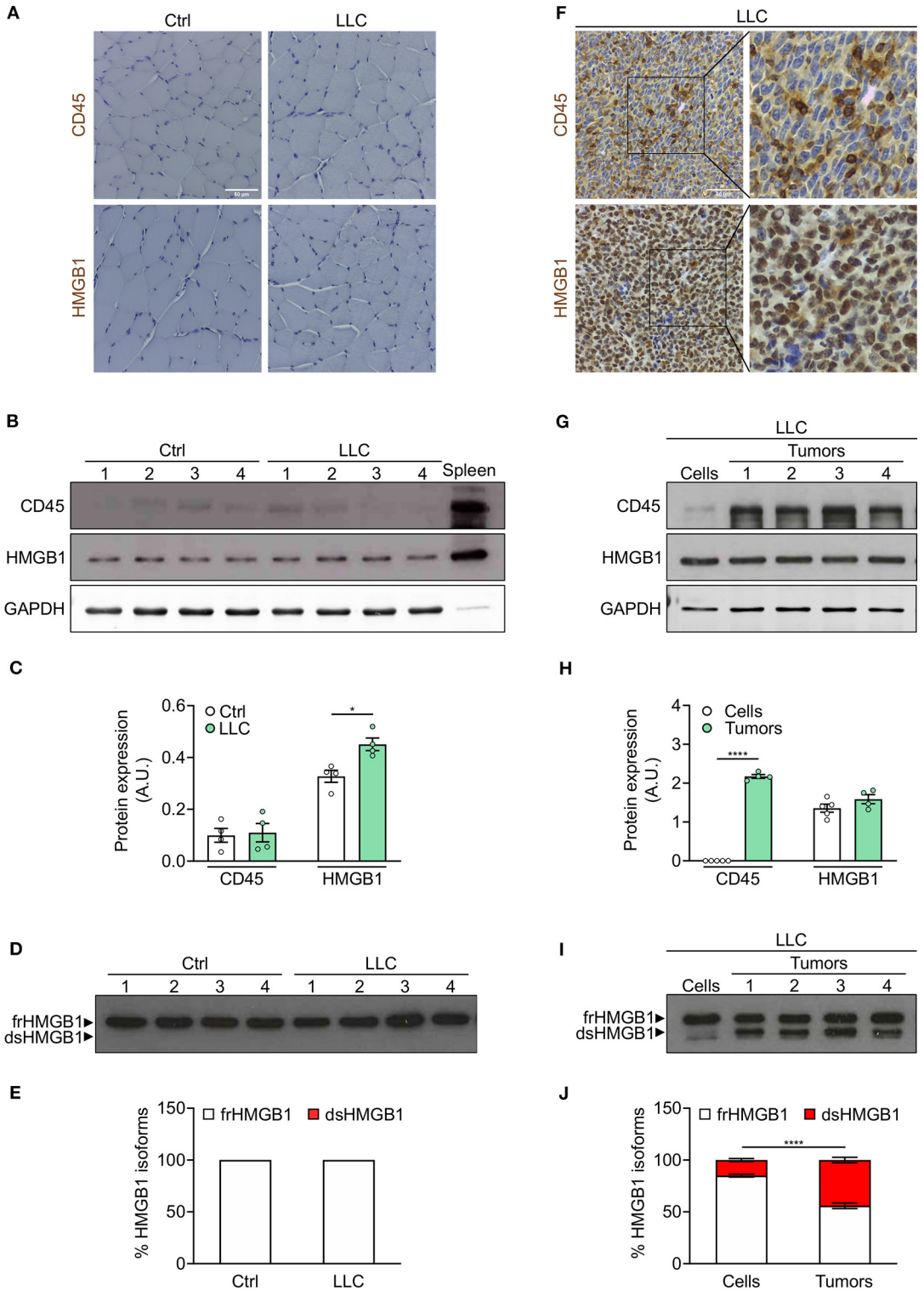

FIGURE 4 | Leukocytes operate as transporter of dsHMGB1 in tumor microenvironment. **(A)** Representative images of immunohistochemical staining for CD45 (upper panel) and HMGB1 (lower panel) on tibialis anterior (TA) muscle sections from control (Ctrl) vs. Lewis lung carcinoma (LLC)-bearing mice. Scale bars, 50 μm. **(B,C)** Western blot probed with anti-CD45, anti-HMGB1, and anti-GAPDH antibodies in reducing conditions **(B)**, and quantification of total CD45 and HMGB1 protein levels normalized on GAPDH **(C)** ($n = 4$ mice). In **(B)**, spleen lysate (5 μg) was added as positive control for CD45 expression. **(D)** Western blot probed with anti-HMGB1 antibody in non-reducing conditions on tibialis anterior (TA) lysates from control or LLC-bearing mice. The upper and lower bands in non-reducing conditions

(Continued)

FIGURE 4 | correspond to the fully reduced-HMGB1 (frHMGB1) and the disulphide-HMGB1 (dsHMGB1) isoforms, respectively. **(E)** Percentage of HMGB1 redox isoforms expression. A.U. = arbitrary unit ($n = 4$ mice/group). **(F)** Immunohistochemical staining for CD45 (upper panel) and HMGB1 (lower panel) on tumoral sections from LLC-bearing mice. Scale bars, 50 μm. **(G–J)** Western blot probed with anti-CD45, anti-HMGB1, and anti-GAPDH antibodies in reducing conditions on LLC cells and tumoral masses isolated from mice injected with LLC cells **(G)**, and quantification of total CD45 and HMGB1 protein levels normalized on GAPDH **(H)**. **(I)** Western blot probed with anti-HMGB1 antibody in non-reducing conditions on LLC cells and tumoral masses isolated from mice injected with LLC cells. **(J)** Percentage of HMGB1 redox isoforms expression in LLC cultured cells and tumoral masses from LLC-injected mice **(J)**. A.U. = arbitrary unit ($n = 5$ cell replicates and $n = 4$ mice for tumoral masses). Data represent the means ± SEM and statistical significance was calculated by Student *T*-test **(C,H)** and Two-way ANOVA **(E,J)**. $^{*}P < 0.05$; $^{****}P < 0.0001$.

previously described (13), the peak of intrahepatic leukocytes (IHLs) occurs at day 2 post-APAP (**Figures 5D,F**). We performed Western blot analyses on IHLs isolated from liver, and we observed that both resident and infiltrating leukocytes upon APAP intoxication express high level of dsHMGB1 (**Figure 5G**, **Supplementary Figure 3A**). As expected, although the number of CD45 positive cells was higher in APAP-treated mice, the percentage of dsHMGB1 in total liver lysate was similar in control and intoxicated mice (**Supplementary Figures 3B–D**), indicating that cell population(s) distinct from leukocytes might also produce dsHMGB1 in liver.

Overall, these findings demonstrate that spleen and liver express high level of dsHMGB1 in physiological conditions, and that both resident/infiltrating leukocytes and additional uncharacterized cell population(s) are sources of dsHMGB1 in normal and intoxicated livers.

DISCUSSION

Although it is well-established that HMGB1 is a critical mediator of inflammation and is involved in numerous inflammatory disorders, clinical trials to specifically target the protein are still to come. A deeper understanding of both intracellular and extracellular functions of HMGB1 is essential to develop efficient therapeutic interventions targeting this alarmin. In this context, the discovery of HMGB1 redox modulation represented a breakthrough in the field, and our findings now reveal a highly dynamic regulation of HMGB1 oxidation *in vivo*, both upon tissue injury and in the tumor microenvironment, which is tightly associated to inflammatory processes. In addition, we identified the leukocyte cell population as a reservoir and transporter of dsHMGB1.

A growing body of evidence indicates that frHMGB1 orchestrates cell recruitment and tissue regeneration while dsHMGB1 contributes to inflammation by activating immune cells (11–13). However, most studies were performed using recombinant HMGB1 redox isoforms. Here, we analyzed the dynamics of expression of endogenous HMGB1 isoforms, demonstrating that the redox state of HMGB1 is highly modulated *in vivo* in different tissues, both in physiological and pathological conditions. Indeed, our results indicate that the redox modulation of HMGB1 is tissue-specific, with a high expression of dsHMGB1 in normal conditions in spleen or liver while it is almost absent in skeletal muscle. Similarly, dsHMGB1 is highly expressed in the tumor microenvironment while it is absent in cachectic muscles from the same tumor-bearing mice. It is well-established that cancer cachexia is characterized by systemic inflammation and leukocytes infiltration in the tumor, but not in the cachectic muscles (24). Accordingly, we observed a high expression of both CD45 and dsHMGB1 in tumors, but not in cachectic muscles. Hence, these data clearly establish that the redox state of HMGB1 is locally controlled and demonstrate that the presence of dsHMGB1 is tightly associated with leukocytes infiltration.

Besides being spatially restricted, HMGB1 oxidation is regulated in time. In skeletal muscle, dsHMGB1 appears a couple of hours after an acute injury. In liver, dsHMGB1 is highly expressed both at basal level and upon drug intoxication, indicating that cell populations other than leukocytes might contribute to the production of dsHMGB1 in liver. Overall, our findings point out to an accurate and dynamic regulation of HMGB1 redox state in physiological and pathological conditions, most probably to finetune the inflammatory and regenerative processes.

Extracellular HMGB1 has been identified as a drug-target protein in multiple diseases, in particular in inflammation-associated disorders, and as a target of aspirin (27), the most widely used drug worldwide, and of the salicylate diflunisal (28), demonstrating the importance of HMGB1 in clinic. A high level of serum HMGB1 appears to be a sensitive biomarker in diverse disorders, such as mesothelioma, but the different HMGB1 isoforms represent novel biomarker candidates that provide additional mechanistic information (29). Indeed, total HMGB1 is indicative of both cell death and immune cell activation while the characterization of the oxidation state can provide pivotal information on the type of injury and on inflammation degree. So far, it is not possible to detect the different isoforms by ELISA assay due to the difficulty to generate antibodies specific for each isoform. Mass spectrometry analyses have been widely employed to analyse the posttranslational modifications of HMGB1 such as acetylation and oxidation. However, this methodology is costly and time-consuming, considerably limiting its potential application in the clinic. Iwahara et al., proposed an NMR-based approach to study the kinetics of HMGB1 oxidation in extracellular fluids (30). Although this technique has multiple advantages, it can be applied only to extracellular fluids. In our study, we showed that it is possible to perform Western blot assays to analyse the expression of HMGB1 redox isoforms both at cell and tissue levels. Other studies reported the detection of HMGB1 redox isoforms in serum and plasma by Western blot assay (31, 32), showing that this method can also be applied to extracellular fluids. Western blot has a wide range of applications in the clinic, such as the application of medical diagnosis for

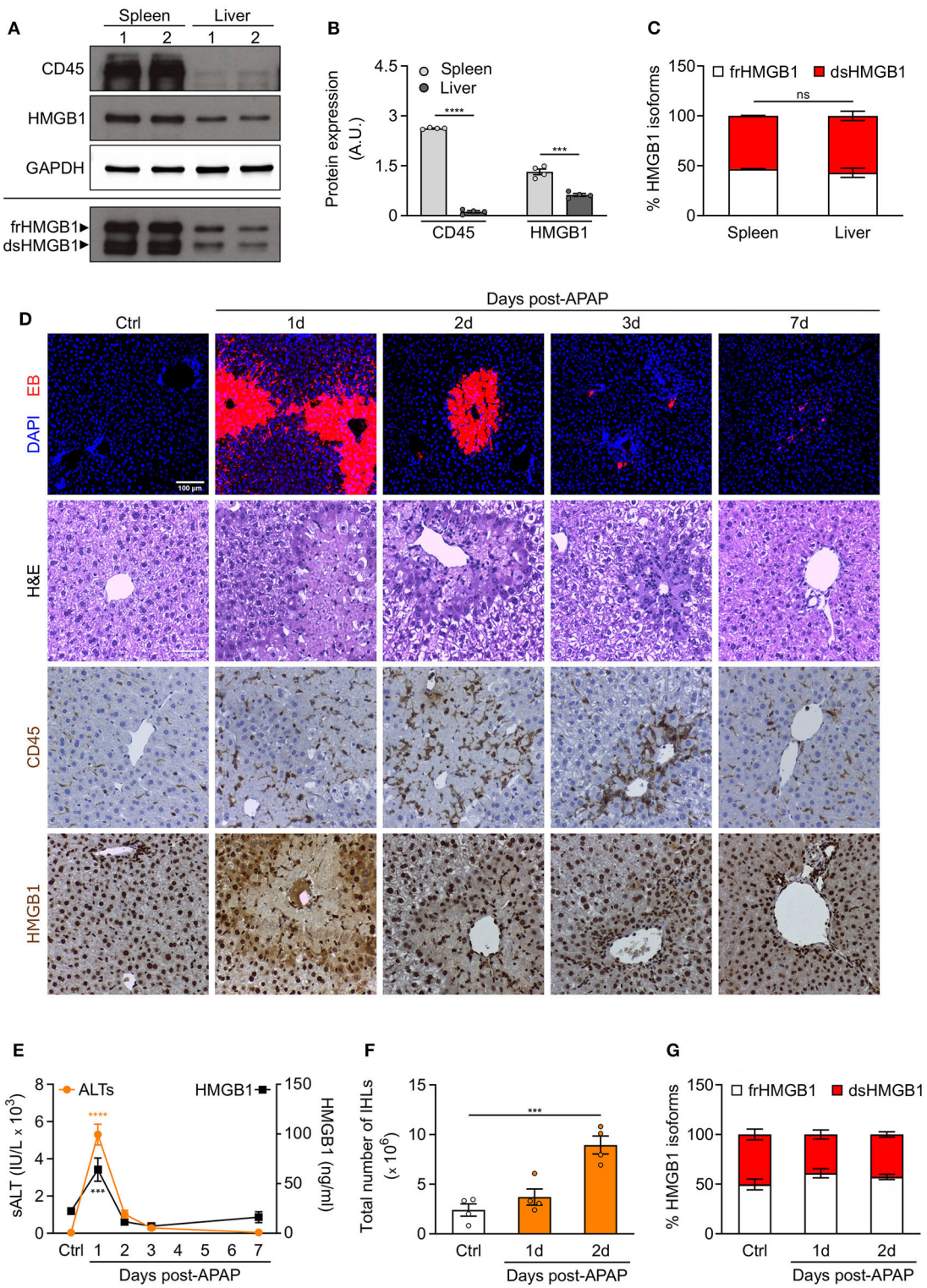

FIGURE 5 | Redox modulation of HMGB1 in spleen and in drug-intoxicated liver. **(A)** Western blot probed with anti-CD45, anti-HMGB1, and anti-GAPDH antibodies in reducing conditions (upper panels) or probed with anti-HMGB1 antibody in non-reducing conditions (lower panel) on lysates of spleen and liver isolated from control WT mice. In the lower panel, the upper band corresponds to the fully reduced-HMGB1 (frHMGB1) and the lower band to the disulphide-HMGB1 (dsHMGB1). **(B,C)** Quantification of total CD45 and HMGB1 protein levels normalized on GAPDH **(B)**, and HMGB1 redox isoforms percentage **(C)** in spleen and liver lysates. A.U. = arbitrary unit (n = 4 mice/group). **(D–G)** Drug-induced liver injury (DILI) was induced by i.p. injection of acetaminophen (APAP), 300 mg/kg (body weight). Serum

(Continued)

FIGURE 5 | collection and necroscopy were performed at the indicated time points. **(D)** Representative images of DAPI and Evans Blue (EB) staining, Haematoxylin & Eosin (H&E) staining, and CD45 and HMGB1 immunostaining in liver sections from control mice (Ctrl) and at days 1, 2, 3, and 7 after DILI. Scale bars, 50 μm. **(E)** Alanine aminotransferase (sALT) and HMGB1 levels in serum before and after APAP injection in mice ($n \geq 5$ mice/group). **(F)** Quantification of total number of intrahepatic leukocytes (IHLs) in control mice and at days 1 and 2 post-APAP injection ($n = 4$ mice/group). **(G)** Quantification of HMGB1 redox isoforms percentage, from Western blot assays performed in non-reducing conditions with anti-HMGB1 antibody, in IHLs isolated from control mice and at days 1 and 2 post-APAP injection ($n = 4$ mice/group). Data represent the means ± SEM and statistical significance was calculated by Student *T*-test **(B)**, One-way **(E,F)** and Two-way ANOVA **(C,G)**. $^{***}P < 0.001$; $^{****}P < 0.0001$; ns, not significant.

infectious diseases including hepatitis C (HCV), HIV, Lyme disease, and syphilis, as well as autoimmune disorders such as paraneoplastic disease and myositis (33). In conclusion, the analysis of HMGB1 redox isoforms expression by Western blot assay might be useful not only for research but also for clinical applications.

An important issue to address is to determine in which conditions HMGB1 gets oxidized and if its oxidation occurs outside and/or inside the cells. HMGB1 is secreted through a non-classical vesicle-mediated secretory pathway, bypassing the endoplasmic reticulum (ER) (20). The redox potential of the ER is continually preserved as an oxidizing environment to facilitate the oxidative process of disulphide bond formation during protein folding. Hence, the avoidance of the ER limits HMGB1 oxidation. Conversely, a recent study indicates that HMGB1 oxidation can occur in the nucleus of mouse bone marrow-derived macrophages, mouse embryonic fibroblasts and HEK293T cells (34). The authors demonstrate that disulphide bond formation is required for HMGB1 nucleocytoplasmic translocation and secretion, and is mediated by peroxiredoxins (Prxs), a ubiquitous family of antioxidant enzymes highly expressed in cells. We observed high levels of dsHMGB1 in lysates of leukocytes from mice and healthy donors, demonstrating that HMGB1 was already oxidized inside the cells. Indeed, it is well-known that leukocytes produce Reactive Oxygen Species (ROS) as part of the killing response against microbial invasion and as intra- and intercellular messengers. Conversely, a recent study showed that HMGB1 is maintained in a reduced state, owing to the activity of the thioredoxin antioxidant system, in monocytes from patients with active rheumatoid arthritis (35). Future investigation should characterize the molecular mechanisms driving HMGB1 oxidation in extracellular and intracellular spaces, in particular in leukocytes.

Inflammatory conditions are associated with the release of ROS in the microenvironment, in particular by leukocytes. Hence, HMGB1 is most probably oxidized also when it is present in the extracellular space, especially in inflammatory conditions. Accordingly, it has been reported that HMGB1 is rapidly oxidized in the extracellular space and that the half-life for frHMGB1 is 17 min *in vitro* in serum before it gets converted to dsHMGB1 (30). The authors showed large variations in the kinetics for HMGB1 oxidation and clearance in different extracellular fluids, clearly demonstrating that the balance between fr- and dsHMGB1 depends on the extracellular environment. Similarly, we observed higher level of dsHMGB1 in supernatants than in lysates of injured muscles, suggesting that HMGB1 was partially oxidized outside the cells. Most importantly, our results identify leukocytes as a source of dsHMGB1 in muscle, spleen, liver, and tumor. These findings are relevant because they demonstrate that leukocytes can also operate as vehicle of dsHMGB1 in the tissue. However, the relative contribution of non-muscle cell types (e.g., endothelial cells) resident in skeletal muscle to HMGB1 release and redox regulation is still unknown. Similarly, the high expression of dsHMGB1 in healthy liver suggests that it originates from a cell population different from leukocytes. Hence, further investigation is required to decipher the regulation of HMGB1 redox state at both tissue and cell levels.

Overall, our study underlines a close association of dsHMGB1 expression with an inflammatory state characterized by immune cells presence, and identifies leukocytes as reservoirs and transporters of dsHMGB1. These findings emphasize that HMGB1 oxidation is a timely and spatially regulated process in physiological and pathological conditions, most likely to finetune inflammatory and regenerative processes.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the San Raffaele Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

MF and GCh designed, carried out most of the experiments and analyzed data. LF and GS designed and carried out experiments of acetaminophen intoxication in mice, and analyzed data. GCa and ER contributed to design, carry out experiments of acute muscle injury in mice, and analyze data. AP performed ELISA for HMGB1. RP provided advice for design and technical details of experiments of cancer cachexia. MB discussed results and provided advice on experimental design. EV designed experiments, directed the project and wrote the manuscript with comments from all authors. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01122/full#supplementary-material

Supplementary Figure 1 | HMGB1 redox isoforms expression in muscle and HMGB1 level in serum upon acute muscle injury. **(A)** Percentage of HMGB1 redox isoforms expression, quantified from Western blot assays performed in non-reducing conditions with anti-HMGB1 antibody, on tibialis anterior (TA) and triceps muscle lysates at indicated time points after cardiotoxin (CTX) injection. A.U. = arbitrary unit ($n = 11$ muscles, 3 mice/time point). **(B)** Quantification of HMGB1 protein level (ng/ml) by ELISA in the serum of control (Ctrl) and CTX-treated mice at indicated time points ($n \geq 3$ mice/time points). **(C)** Percentage of HMGB1 redox isoforms expression, quantified from Western blot assays performed in non-reducing conditions with anti-HMGB1 antibody, on muscle supernatants at indicated time points after CTX injection. A.U. = arbitrary unit ($n = 6$ muscle supernatants, 3 mice/time point). Data represent the means ± SEM and statistical significance was calculated by One-way ANOVA **(A–C)**. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

Supplementary Figure 2 | Redox modulation of HMGB1 during cancer cachexia. **(A)** Body weight (g) of mice injected with LLC or C26 cells at day 0 or at the endpoint of the experiment. **(B)** Weight loss percentage of gastrocnemius (GAS), tibialis anterior (TA), and quadriceps (QUAD) muscles from LLC- or C26-bearing mice ($n \geq 4$ mice/group). **(C)** Quantification of HMGB1 protein level (ng/ml) by ELISA in the serum of control (Ctrl) or tumor-bearing mice (LLC or C26) ($n \geq 4$ mice/group). **(D,E)** Western blot probed with anti-CD45, anti-HMGB1, and anti-GAPDH antibodies in reducing conditions **(D)** on tibialis anterior (TA) muscle lysates from control (Ctrl) or C26-bearing mice. In **(D)**, spleen lysate (5 μg) was added as positive control for CD45 expression. **(E)** Quantification of total CD45 and HMGB1 protein levels normalized on GAPDH. A.U. = arbitrary unit ($n \geq 4$ mice/group). **(F,G)** Western blot probed with anti-HMGB1 antibody in non-reducing conditions on TA muscles isolated from control or C26-bearing mice **(F)**. The upper and lower bands correspond to the fully-reduced HMGB1 (frHMGB1) and the disulphide-HMGB1 (dsHMGB1) isoforms, respectively. **(G)** Quantification of HMGB1 redox isoforms percentage. A.U. = arbitrary unit ($n \geq 4$ mice/group). **(H,I)** Western blot probed with anti-CD45, anti-HMGB1, and anti-GAPDH antibodies in reducing conditions on C26 cultured cells (Cells) and on tumoral masses (Tumors) isolated from mice injected with C26 cells **(H)**. Quantification of total CD45 and HMGB1 protein expression normalized on GAPDH **(I)**. A.U. = arbitrary unit ($n = 4$ cell replicates and $n = 5$ mice for tumoral masses). **(J,K)** Western blot probed with anti-HMGB1 antibody in non-reducing conditions on cultured C26 cells (Cells) and on tumoral masses (Tumors) isolated from C26-bearing mice **(J)**. Quantification of HMGB1 redox isoforms percentage in cultured C26 cells (Cells) and in tumoral masses (Tumors) isolated from C26-injected mice (**K**; $n = 4$ cell replicates and $n = 5$ mice for tumoral masses). Data represent the means ± SEM and statistical significance was calculated by Student *T*-test **(A,C,E,I)** and Two-way ANOVA **(G,K)**. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$; ns, not significant.

Supplementary Figure 3 | CD45 and HMGB1 redox isoforms expression in liver after drug intoxication. Drug-induced liver injury (DILI) was induced by i.p. injection of acetaminophen (APAP), 300 mg/kg (body weight). Liver and intrahepatic leukocytes (IHLs) isolations were performed at indicated time points after APAP treatment. **(A)** Western blot probed with anti-CD45, anti-HMGB1, and anti-GAPDH antibodies in reducing conditions (upper panels) or probed with anti-HMGB1 antibody in non-reducing conditions (lower panel) on IHLs isolated from control (Ctrl) and APAP-treated mice at indicated time points. In the lower panel, the upper band corresponds to the fully-reduced HMGB1 (frHMGB1) and the lower band to the disulphide-HMGB1 (dsHMGB1). **(B)** Western blot probed with anti-CD45, anti-HMGB1, and anti-GAPDH antibodies in reducing conditions (upper panels) or probed with anti-HMGB1 antibody in non-reducing conditions (lower panel) on liver lysates of control (Ctrl) and APAP-injected mice at indicated time points. **(C)** Quantification of total CD45 and HMGB1 protein expression in control (Ctrl) and APAP-treated mice at indicated time points. A.U. = arbitrary unit ($n = 4$ mice/group). **(D)** Quantification of HMGB1 redox isoforms percentage in liver lysates from control (Ctrl) and APAP-treated mice ($n = 4$ mice/group). Data represent the means ± SEM and statistical significance was calculated by One-way **(C)** and Two-way ANOVA **(D)**. $^{**}P < 0.01$; $^{****}P < 0.0001$.

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Conflict of Interest: MB is founder and part owner of HMGBiotech, a company that provides goods and services related to HMGB proteins, and AP is partially supported by HMGBiotech.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer UA declared a past collaboration with one of the authors MB to the handling Editor.

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REVIEW
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High Mobility Group Box 1 Expression in Oral Inflammation and Regeneration

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High mobility group box 1 (HMGB1) is a non-histone DNA-binding protein of about 30 kDa. It is released from a variety of cells into the extracellular milieu in response to inflammatory stimuli and acts on specific cell-surface receptors, such as receptors for advanced glycation end-products (RAGE), Toll-like receptor (TLR)2, TLR4, with or without forming a complex with other molecules. HMGB1 mediates various mechanisms such as inflammation, cell migration, proliferation, and differentiation. On the other hand, HMGB1 enhances chemotaxis acting through the C-X-C motif chemokine ligand (CXCL)12/C-X-C chemokine receptor (CXCR)4 axis and is involved in regeneration. In the oral cavity, high levels of HMGB1 have been detected in the gingival tissue from periodontitis and peri-implantitis patients, and it has been shown that secreted HMGB1 induces pro-inflammatory cytokine expression, such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α, which prolong inflammation. In contrast, wound healing after tooth extraction or titanium dental implant osseointegration requires an initial acute inflammation, which is regulated by secreted HMGB1. This indicates that secreted HMGB1 regulates angiogenesis and bone remodeling by osteoclast and osteoblast activation and promotes bone healing in oral tissue repair. Therefore, HMGB1 can prolong inflammation in the periodontal tissue and, conversely, can regenerate or repair damaged tissues in the oral cavity. In this review, we highlight the role of HMGB1 in the oral cavity by comparing its function and regulation with its function in other diseases. We also discuss the necessity for further studies in this field to provide more specific scientific evidence for dentistry.

Keywords: high mobility group box 1, inflammation, periodontal regeneration, periodontitis, osseointegration, tooth movement, wound healing

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INTRODUCTION

An inflammatory or immune response in oral tissues occurs when biological reactions such as microbial infection, physical trauma, neoplastic processes, and autoimmune conditions occur in the oral environment. The oral cavity contains complex microbial flora, and the immune system promotes pro-inflammatory cytokine production (1). Sterile injuries, such as tooth extraction

and orthodontic tooth movement also cause an immune reaction and subsequent tissue repair. Immediately after a dental extraction is performed, blood platelets are recruited into the collagen connective tissue, a clot starts to form, and growth factors and angiogenesis mediators are being produced (2). In the context of orthodontic tooth movement, an initial inflammatory reaction is generated at the compression sites caused by constriction of the periodontal ligament. The periodontal ligament releases pro-inflammatory cytokines and promotes tissue resorption (3). It has been mentioned that chronic inflammation and oxidative stress promote carcinogenesis (4). In the oral cavity, it has been suggested that both cyclooxygenase (COX) 2 and chronic inflammation are involved in the initiation of carcinogenesis process of oral squamous cell carcinoma (5). Becht's disease, a well-known autoimmune condition, exhibits symptoms such as aphthous ulcers in the oral cavity, and it is suggested that there is a relationship between this disease and periodontitis, which is a major chronic oral inflammatory pathology (6). It is thought that the inflammatory and immune response are closely related to both the progress of the diseases and tissue repair.

HMGB1 is a nuclear protein that regulates transcription and is one of the damage-associated molecular patterns (DAMPs), which act as major mediators in immune reactions. HMGB1 has several isoforms, which have distinct biological implications. These isoforms are: "fully reduced HMGB1," "disulfide HMGB1," and "sulfonyl HMGB1" named after the different redox reactions that occur in the three cysteines at positions 23, 45, and 106 of HMGB1 (7). Necrotic cell death or cell stress promotes fully reduced HMGB1, which forms a heterocomplex with CXCL12. The heterocomplex binds to the CXCR4 receptor with increased affinity and enhances chemotaxis (8). Fully reduced HMGB1 can be oxidized to disulfide HMGB1, which forms a disulfide bond between C23 and C45, and exerts a pro-inflammatory effect by promoting cytokines production via the TLR4/myeloid differentiation factor 2 (MD-2) complex. Fully oxidized HMGB1 and sulfonyl HMGB1 are thought to be inert (9, 10). The difference in the isoforms is thought to be one of the reasons why HMGB1 is involved in two opposing functions: progression of inflammation and tissue repair.

Initially, HMGB1 was shown to cause a danger signal in acute inflammatory diseases such as sepsis. Wang et al. (11) reported that HMGB1 was liberated from cells stimulated with cytokines and that HMGB1 plays an important role in mediating experimental sepsis. Yamamoto et al. (12) reported that lipopolysaccharide (LPS) increased pro-inflammatory cytokine secretion from peritoneal macrophages and initiated intracellular signaling to activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) by binding RAGE and TLR2. In the report, they mentioned that LPS-mediated RAGE signaling accelerates acute inflammation and vascular dysregulation, leading to tissue damage, which then mediates HMGB1 release in the late phase, resulting in a pernicious cycle of RAGE-dependent lethality in septic shock. Yang et al. (9) reported that disulfide HMGB1 binds MD-2/TLR4, and MD-2 antagonists inhibited hepatic ischemia/reperfusion injury, chemical toxicity, and sepsis in mice. HMGB1 is not only involved in acute inflammation but also in chronic inflammation. Gasiorowski et al. (13) reported that RAGE activation should be perceived as a primary mechanism that determines self-perpetuated chronic inflammation in Alzheimer's disease, and the crosstalk; RAGE cooperation with TLRs amplifies inflammatory signaling via extracellular signal-regulated kinase (ERK)1/2, mitogen-activated protein kinase (MAPK), p38, c-Jun N-terminal kinase (JNK), and NF-κB signaling. In a recent report, it was shown that the HMGB1/CXCL12 heterocomplex can be maintained in rheumatoid arthritis (RA) by the activity of the prostaglandin E2 (PGE2)/COX2 pathway, the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, and the thioredoxin system, all of which are associated with the activation of the disease (14). In addition, in juvenile idiopathic arthritis patients (JIA), the presence of three functional HMGB1 redox isoforms confirms the complexity of their pathogenic role during chronic inflammation (15).

Until now, many researchers have focused on HMGB1 as an inflammatory mediator that prolongs various inflammatory diseases, and because of this research has focused on inhibiting the function of HMGB1 to treat such diseases (16, 17). However, in recent reports, another aspect of HMGB1, which is related to its role in tissue healing and regeneration, is being highlighted (18, 19). Originally, inflammation is believed to be not only a chronic and degenerative disease, but also part of the physiological process that initiates tissue repair and regeneration. Infection or injury of epithelium leads to the generation of DAMPs and pathogen-associated molecular patterns (PAMPs), which then activate immune cells for regeneration by stimulating cell proliferation and differentiation (20). Vénéreau et al. (18) created the mutant HMGB1 (3S HMGB1), in which the cysteines are replaced with serines, which are resistant to oxidation, and behave as reduced HMGB1. Tirone et al. (21) reported that 3S HMGB1 orchestrates muscle and liver regeneration via CXCR4. A recent study also reported that fully reduced HMGB1 forms a heterocomplex with CXCL12, which binds to CXCR4 and then accelerates skeletal, hematopoietic, and muscle regeneration (22).

In addition, an explanation of the HMGB1 function is that HMGB1-C1q complexes regulate macrophage polarization by inducing the differentiation to anti-inflammatory M2-like macrophages (23). The activation of complements is strongly involved in immune cell migration. There are three pathways of complement activation: the classical pathway, the Mannan-binding lectin pathway, and the alternative pathway, all of which promote immune cell migration by producing the cleaved complement component 3 (C3a) and cleaved complement component 5 (C5a) (24). On the other hand, the C1 complex (C1q), which normally triggers the classical pathway, is thought to regulate both inflammation and regeneration by the coexistence of DAMPs. Liu et al. (25) reported that the HMGB1-C1q complex induces production of proresolving mediators such as resolvin (Rv)D1 and RvD2. The resolution of inflammation and macrophage polarization may result in tissue regeneration.

The oral cavity has a complex environment having a variety of different tissues such as epithelium, connective tissue, and hard tissue such as teeth and bone, along with various bacterial species. Thus, in this complex environment HMGB1 may play

its dual role in prolonged inflammation and tissue regeneration. However, we still do not know the detailed mechanism by which the isoform of HMGB1, or how the HMGB1 forming complex is involved in oral inflammation and regeneration in the oral cavity. In this review, we specifically focus on the role of HMGB1 in oral inflammation and regeneration. We introduce past reports and suggest future directions.

HMGB1 IN ORAL INFLAMMATORY CONDITIONS

Periodontal Inflammation

Periodontal diseases are dysbiotic conditions in the gingival margin, which are characterized by an imbalance between subgingival microbial communities and the host immune response (26, 27). Clinical studies have demonstrated that the levels of TLR2 and TLR4 in periodontitis patients were significantly higher than those in control groups (28, 29). Li et al. (29) also demonstrated the presence of TLR4, CD14, and MD-2 expression in both cultured human gingival keratinocytes and fibroblasts. *Porphyromonas gingivalis* (*P. gingivalis*) is considered a keystone pathogen for periodontitis (30). *P. gingivalis* fimbriae can activate the TLR2 and TLR4 pathways, leading to excessive production of pro-inflammatory cytokines and chemokines in monocytic cells (31). PAMPs are conservative molecules associated with groups of pathogens or their products, and are involved in the development of periodontitis. LPS of *P. gingivalis* is an effective ligand for TLR4, and Li et al. (32) discovered that the ability of human periodontal ligament stem cells (hPDLSCs) to differentiate into osteoblasts was impaired by LPS, through a TLR4-mediated NF-κB pathway. Lipoprotein derived from *P. gingivalis* can serve as a ligand for TLR2 and activate the NF-κB pathway (33). Okugawa et al. (34) reported that soluble peptidoglycans (PGNs) of *P. gingivalis* and *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans)* induced IL-8 production in cultured oral epithelial cells. TLRs on gingival tissues such as gingival epithelium, gingival fibroblasts, periodontal ligaments, and immune cells recognize PAMPs, and the TLR signals induce significant amounts of inflammatory mediators. An exaggerated reaction response by the immune response promotes the production of receptor activator of nuclear factor kappa-B ligand (RANKL), activates osteoclasts, and then cause tissue destruction and bone resorption.

The Cells in Periodontal Tissue Produce HMGB1

Not only PAMPs but also alarmins such as HMGB1 are considered as a significant factor during osteoclastogenic. Infection promotes HMGB1 secretion from periodontal tissue, and the secreted HMGB1 is involved in the lingering or aggravation of periodontitis. HMGB1 was detected at high levels in gingival crevicular fluid (GCF) in periodontitis patients (35, 36). There were significant positive correlations between the levels of HMGB1 in GCF and all periodontal parameters, including plaque index, bleeding index, probing depth, and clinical attachment level. The abundance of HMGB1 in GCF in chronic periodontal patients suggests that human gingival epithelial cells secrete HMGB1 up on stimulation by bacterial infection (37). That study also confirmed that TNF-α promotes HMGB1 production *in vitro*, using rat gingival epithelial cells and Ca9-22 cells, which is an oral epithelial cell line. Ito et al. (38) reported that IL-1β promoted the secretion of HMGB1 in human gingival epithelial and fibroblast cells. They also confirmed that gene expression of RAGE was highly upregulated by IL-1β stimuli in cultured human gingival epithelial cells, and that HMGB1 and RAGE were highly expressed in gingival epithelial cells in patients with oral inflammation. Another study reported that HMGB1 was dislocated from the nucleus of the cells in the pocket epithelium, which faces the infected root surface, but it was mainly localized in the nucleus in the gingival epithelium of periodontitis patients (39). They also confirmed that butyric acid, which is a metabolite of periodontal pathogens and a virulence factor of *P. gingivalis,* induced HMGB1 production in Ca9-22 cells *in vitro*. Our previous report suggested that HMGB1 translocated from the nucleus into the cytoplasm in the gingival epithelium *in vivo*, in a periodontal mouse model with *P. gingivalis*-soaked ligatures (40). *In vitro* analysis using cultured progenitor human gingival epithelial cells (HGECs) and THP-1 cells, which is a macrophage-like cell line, showed that TNF-α induced HMGB1 production. Interestingly, the amount of HMGB1 production was lower in HGECs (<20 ng/mL) than in THP-1 cells (more than 60 ng/mL) (40). Gingival connective tissue located between the epithelium and the root surface contains gingival fibroblasts. It has been reported that cultured human gingival fibroblasts (HGF) produce HMGB1 upon stimulation by LPS of *A. actinomycetemcomitans*, *P. gingivalis*, and *Escherichia coli,* and upon apoptotic and necrotic initiation (41). The periodontal ligament, which is also a connective tissue lying between the alveolar bone and tooth root, contains periodontal ligament fibroblasts (PDLF). Nogueira et al. (42) reported that HMGB1 was produced in cultured human PDLF upon treatment LPS and IL-1β. They also confirmed the expression of HMGB1 in the periodontal ligament in an experimental periodontitis rat model (42). However, we still do not know which tissue is the main source of HMGB1 production in periodontium.

Secretion of HMGB1 around periodontal tissue is considered to promote pro-inflammatory cytokine production and prolong periodontitis. Kim et al. (43) reported that recombinant HMGB1 induced the expression of TNF-α, IL-1β, IL-6, IL-11, and IL-17 mRNA in immortalized human PDL cells (hPDLCs). They also showed that TLR4 and TLR2 expression was increased in hPDLCs exposed to HMGB1 and that neutralizing anti-TLR2 and anti-TLR4 antibodies specifically inhibited HMGB1-induced expression and secretion of osteoclastogenic cytokines and expression of RANKL (43). Parks et al. (44) reported that RAGE plays only a minor role in macrophage activation by HMGB1, whereas signaling through TLR 2 and TLR4 prompted the release of TNF-α, IL-1β, and IL-6 from cultured mouse neutrophils and macrophages. In addition to other inflammatory diseases, secreted HMGB1 is considered to promote inflammation; however, there is little evidence to have examined the difference in HMGB1 isoforms in this

research area. One of the reasons for these limitations was the difficulty in using recombinant HMGB1 proteins because they easily form complexes with other molecules such as LPS and IL-1β.

Periapical lesions are osteolytic bone defects that are inflammatory immune defensive reactions that originate as a consequence of microbial and toxin invasion into the root canal. It has been established that a variety of cytokines and chemokines participate in the innate immune-inflammatory response and later in the adaptive immune response (45). These cytokines, including matrix metalloproteinases (MMPs), IL-1β, and IL-6 play a critical role in osteoclast formation and alveolar resorption (46). Liu et al. (47) reported the presence of HMGB1 and TLR4-positive cells around periapical lesions surrounding the apical foramen via osteoclast activation.

In addition to teeth, dental implants also suffer from bacterial infection. Peri-implantitis is a destructive inflammatory process caused by bacteria surrounding dental implants (48). Previously, it was reported that RAGE levels were elevated in patients susceptible to periodontitis compared to healthy patients, but TLR2 and TLR4 levels did not change before implant therapy. After implant therapy, RAGE and TLR4 levels were upregulated but TLR2 levels were downregulated (49). In addition, a higher concentration of HMGB1 has been found in the GCF from inflammatory gingival tissue in comparison to the healthy site around the dental implant (35, 50). The expression levels of other pro-inflammatory factors, such as IL-1β, Il-6, IL-8, and TNF-α, were also higher in the GCF from peri-implantitis sites than in the GCF from healthy sites. They reported that HMGB1 expression level in GCF is indicative of the progression of peri-implantitis and may be a useful diagnostic biomarker.

HMGB1 Blockade Inhibits Periodontitis Progression

It was revealed that HMGB1 is involved in the aggravation of periodontitis by HMGB1 blockade analysis. Glycyrrhizin is the chief sweet-tasting constituent of *Glycyrrhiza glabra* root and is contained in various oral hygiene products such as toothpaste and mouth wash to exert an anti-inflammatory effect (51, 52). Mollica et al. (52) reported that glycyrrhizin binds to HMGB1 specifically and inhibits cytokine activity. In our previous report, the progression of periodontitis was inhibited in a mouse periodontitis model with glycyrrhizin (53). A recent study reported that glycyrrhizic acid suppressed inflammation and reduced the increased glucose levels induced by the combination of *Porphyromonas gulae* and ligature placement in mouse model of diabetes (54). In this study, glycyrrhizic acid also suppressed ligature/*P. gulae*-induced increases in HMGB1 and RAGE both at the mRNA and serum levels in the gingiva of diabetic mice. The anti-HMGB1 antibody is one of the most powerful HMGB1 inhibitors and has been used in many inflammatory disease models such as sepsis and brain infarction (11, 55). In our study, administration of anti-HMGB1 antibody in a murine periodontitis model inhibited myeloperoxidase (MPO) activity, neutrophil migration, and bone resorption in a dose-dependent manner. This result suggested that a faster resolution of periodontal inflammation can be achieved by blocking HMGB1. The antibody inhibited the expression of IL-1β and C-X-C motif chemokine ligand 1 (CXCL1) in cultured cells. The antibody also inhibited TNF-α-induced IL-1β production in HGECs and TNF-α-induced GM-CSF production in THP-1 cells *in vitro* (40). In early inflammation, gingival epithelial cells release various cytokines and chemokines, and HMGB1 is then translocated from the nucleus to the cytoplasm upon stimulation by TNF-α. The released HMGB1 induces the translocation in an autocrine-related manner; the released HMGB1 also induces GM-CSF secretion from gingival epithelial cells, resulting in the differentiation and activation of immune cells. As inflammation proceeds via the continuous secretion of HMGB1, macrophages release more cytokines, chemokines, and HMGB1. Moreover, the released IL-1β promotes osteoclastogenesis and bone resorption. Therefore, periodontal inflammation is initiated, exacerbated, and prolonged by the HMGB1 secretion cycle. The present study demonstrated that anti-HMGB1 antibody succeeded in preventing prolonged immunostimulation and bone-resorbing activity of osteoclasts by inhibiting the release of cytokines in periodontal tissue. However, HMGB1 blockade with anti-HMGB1 antibody partially inhibited periodontal progression, thus indicating that there might be another HMGB1-independent pathway.

Periodontitis has been associated with many other systemic diseases; for instance, there is a two-way relationship between diabetes and periodontitis (56). In 2012, a hypothesis was reported that secreted HMGB1 acting through RAGE, on monocytes, macrophages, and vascular endothelial cells, and might play an important role in the development of diabetes-associated periodontitis (57), and many reports regarding this relationship are currently being conducted. RAGE is one of the receptors for HMGB1, and its expression is higher in gingival tissue of patients with type 2 diabetes than in healthy patients (58). Blockade of HMGB1 by soluble RAGE (sRAGE) suppressed periodontitis-associated bone loss in diabetic mice (59), and serum levels of sRAGE and cleaved RAGE were significantly lower in periodontitis patients (60). However, soluble RAGE to neutralize RAGE receptors does not specifically block HMGB1, as there are multiple other RAGE ligands, such as AGEs, S100As, and lysophosphatidic acid (LPA), which may bind to this receptor as well as to HMGB1 (61, 62). Metformin, the first-line medication for the treatment of type 2 diabetes, is also considered an HMGB1 inhibitor because it directly binds HMGB1 and inhibits the pro-inflammatory activity (63). Metformin and metformin hydrochloride-loaded poly lactic-co-glycolic acid nanoparticles decreased the inflammatory response and bone loss in a rat periodontitis model (64, 65). These findings indicate that metformin does not only have a hypoglycemic action but also has an anti-inflammatory effect to block HMGB1, and it might be effective in diabetes-associated periodontitis. In summary, HMGB1, and RAGE are involved in the two-way relationship between diabetes and periodontitis, and metformin has the potential to resolve them.

ORAL REGENERATION ASSOCIATED WITH HMGB1

Wound Healing Around the Gingival Tissue and After Tooth Extraction

Tancharoen et al. (66) reported that HMGB1 promotes intraoral palatal wound healing through RAGE. *In vivo* analysis showed that the wound closure of palatal gingival tissue was attenuated in heterozygous HMGB1 (*Hmgb1*$^{+/-}$) mice compared to wild type (WT) mice. In the *Hmgb1*$^{+/-}$ mice, the number of proliferating cell nuclear antigen (PCNA), NF-κB p50, and vascular endothelial growth factor (VEGF) were lower than those in WT mice. *In vitro* analysis using cultured HGF revealed that keratinocyte proliferation and migration during re-epithelialization was delayed in RAGE knockdown cells compared to that of the control, as determined by the wound scratch assay and the gene expression level of PCNA. Bone healing after tooth extraction is representative of intramembranous ossification. Healing after tooth extraction requires an initial acute inflammation, which is regulated by secreted HMGB1. Anti-HMGB1 antibody inhibits MPO activity, IL-1β and VEGF-A expression, and migration of CD31-, CD68-, TRAP-, and osteocalcin-positive cells. This indicates that the secreted HMGB1 regulates angiogenesis and bone remodeling by osteoclast and osteoblast activation, thus promoting bone healing in the tooth extraction socket (67). This indicates that anti-HMGB1 antibody inhibits not only aggravation of inflammatory diseases such as periodontitis, but also inhibits initial acute inflammation, which is essential for tissue repair.

Periodontal Regeneration

Periodontal regeneration is defined histologically as regeneration of the tooth's supporting tissues, including alveolar bone, periodontal ligament, and cementum on a previously diseased root surface (68). Particularly, PDLFs are thought to play an important role in periodontal wound healing and regeneration because they contain stem cells that can be used to regenerate periodontal tissues (69). HMGB1 protein induced PDLF proliferation and migration in *in vitro* studies. It also promoted osteogenic differentiation parameters such as alkaline phosphatase (ALP), osteopontin, osteocalcin, RUNX2, and bone morphogenetic protein (BMP) (70). However, the authors considered that HMGB1 might support the reestablishment of the structural and functional integrity of the periodontium, following periodontal trauma such as orthodontic tooth movement described later, and might not support periodontal regeneration. Indeed, there is no evidence that HMGB1 is involved in periodontal regeneration. For further study, it must be explored whether the different isoforms of HMGB1 are involved in periodontal regeneration.

Orthodontic Tooth Movement

In addition to the *in vitro* analysis with PDLF (70), Wolf et al. (71) also indicated that HMGB1, initially produced in PDLFs by mechanical loading during orthodontic tooth movement, decreased gradually. Initial HMGB1 production enhances the activity of monocytes and macrophages by clearing cellular debris and activating RANKL to initiate bone remodeling (71). Cui et al. (72) also reported that mechanical stress during orthodontic tooth movement induces PDLFs to secrete HMGB1. Mechanical stress also induced pro-inflammatory cytokine expression, such as TNF-α and IL-6, from macrophages, which activates the innate immune response. HMGB1 and these pro-inflammatory cytokines are reduced in a time-dependent manner (72). HMGB1 is thought to react in an acute innate immune response, and the gradual reduction of HMGB1 in PDLF is necessary for the achievement of orthodontic tooth movement; however, more evidence is still needed.

Dental Pulp Regeneration

Many studies regarding tissue regenerative procedures have found that dental pulp cells (DPCs) are one of the stem cell sources (73, 74). Zhang et al. (75) showed that in healthy dental pulps, HMGB1 remains in the nuclei (confirming its nuclear localization), but in inflamed pulps, the presence of HMGB1 in the cytoplasm of infiltrated inflammatory cells, fibroblasts, and endothelial cells increases. Moreover, HMGB1 mRNA levels in these cells have been demonstrated to increase, which means that the pulp infection also stimulates the synthesis of this molecule. Through *in vitro* studies, these authors have also demonstrated that elevated cytoplasmic presence of HMGB1 mRNA levels after *E. coli* LPS stimulation in cultured DPCs. It has also been demonstrated that high levels of cytokines in pulpitis such as IL-6, IL-1, and TNF-α are also released by HMGB1 secretion (76). Some studies have concluded that HMGB1 and its receptor, RAGE, are involved in stem/progenitor cell differentiation in order to repair damaged tissues (77, 78). In a study by Zhang et al. (75) it was demonstrated that HMGB1 promoted DPCs migration in a dose-dependent manner, and that HMGB1 also activated Rho signaling and cytoskeletal reorganization. Thus, the formation of new dentin could be established, confirming the findings from Qi et al. (79) who found that HMGB1 promotes odontoblast differentiation from DPCs. However, excessive quantities of this molecule may amplify inflammation and may cause tissue damage. Based on these findings, it can be concluded that HMGB1 plays crucial roles not only in dental pulp inflammation, but also in dentine regeneration, enhancing DPC recruitment into the pulp injury, stimulating their differentiation into odontoblasts, and new dentin formation for healing of damaged tissues. Furthermore, we recently reported that RvD2 induces active resolution of inflammation through pulp-like tissue regeneration after root canal infection (80). It is possible that the HMGB1-C1q complex induces the production of RvD2 for dental pulp regeneration, as suggested by Liu et al. (25).

Ti Osseointegration

Interestingly, in the latest report, HMGB1 is involved not only in peri-implantitis but also in osseointegration. Osseointegration is defined as the direct structural and functional connection between the living bone and the surface of a load-bearing artificial dental implant. Biguetti et al. (81) reported that the released HMGB1 binding to RAGE contributes to titanium (Ti)-mediated osseointegration in dental implants. In this report, HMGB1 was detected at high levels at bone Ti implantation sites immediately after implantation, followed by a gradual decrease in later time points. Inhibition of HMGB1 with glycyrrhizic acid and RAGE

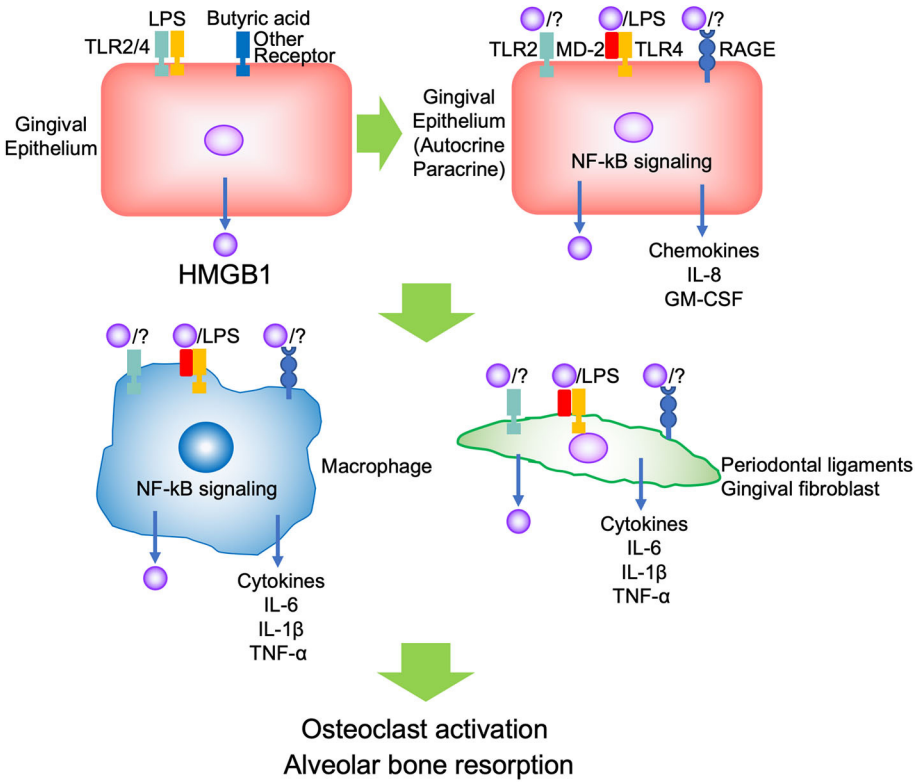

FIGURE 1 | Scheme of the role of HMGB1 in periodontitis progression.

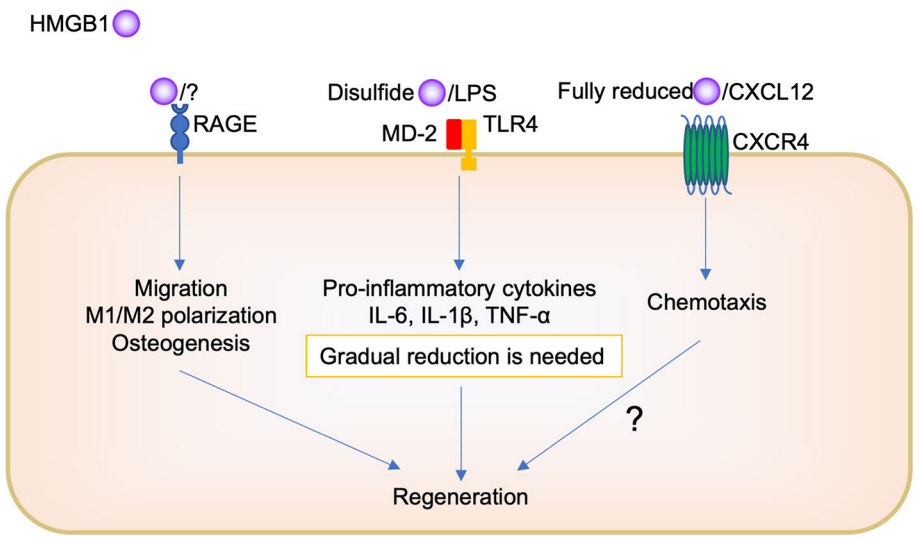

FIGURE 2 | Scheme of the role of HMGB1 in oral regeneration.

antagonistic peptide decreased bone matrix formation, blood vessel formation, and migration of osteoblasts and osteoclasts around the Ti surface. The growth factors and mesenchymal stem cell markers were upregulated in the oral osteointegration model, but these were reduced in the HMGB1 inhibition models.

CONCLUSION AND FUTURE DIRECTIONS

Evidence indicates that HMGB1 is associated with inflammation or immune response in both pathogenic and repair processes in the oral cavity. The reason may depend on the amount

and duration of HMGB1, and the kind of stimulus, such as whether the conditions were sterile or infectious, not its dual role. In the context of periodontal inflammation, PAMPs such as LPS initially bind TLR2/4 and butyric acid binds to other receptors on the gingival epithelium, and promotes HMGB1 secretion. The secreted HMGB1 forms a complex with other molecules such as LPS, binds TLR2/4 and RAGE on the adjacent epithelium, thus promoting autocrine/paracrine signaling. The inflamed epithelium produced pro-inflammatory cytokines and chemokines such as IL-8 and GM-CSF to migrate and differentiate immune cells. Migrated immune cells, such as neutrophils and macrophages, are activated by secreted HMGB1, and produce pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α, and HMGB1 via TLR2/4 and RAGE. These cytokines induce further HMGB1 production from other tissues, such as gingival fibroblasts and periodontal ligaments. Then, the aggravated inflammation promotes osteoclastogenesis and causes alveolar bone resorption (**Figure 1**). There was no evidence regarding which isoform of HMGB1 was mostly involved in the progression of periodontal disease. In JIA, which is characterized by chronic inflammation and periodontitis, it was indicated that the presence of various functional HMGB1 redox isoforms confirms the complexity of their pathogenic role during chronic inflammation (15). Thus, subsequent studies should focus on improving the understanding of the biological effects of different isoforms of HMGB1 and different receptors in periodontitis.

On the other hand, regarding tissue repair, it is believed that because of the complexity of HMGB1, its different pathways depending on the redox forms, and its complex formation with other molecules, it is difficult to know how it orchestrates its biological function (**Figure 2**). The administration of HMGB1 antibody inhibited chemotaxis, such as neutrophil and macrophage migration, during socket repair. Tirone et al. (21) reported that fully reduced HMGB1 induced muscle and liver regeneration via CXCR4, whereas "disulfide HMGB1" and its receptors TLR/MD-2 and RAGE are not involved. However, we believe that HMGB1/RAGE signaling is also important in oral tissue repair. Keratinocyte proliferation and migration during oral palate healing are regulated by HMGB1/RAGE signaling (66). Biguettiet al. (81) reported that HMGB1/RAGE signaling is involved in stem cell migration, macrophage M1/M2 polarization, and osteogenesis during Ti-implant osseointegration. In addition, further studies are needed to examine whether fully reduced HMGB1, CXCL12, and CXCR4 participate in the biological process.

Furthermore, there is need to produce a certain amount of HMGB1; disulfides HMGB1 binding TLR2 or TL4, to produce pro-inflammatory cytokines for initial inflammation during tissue repair. The presence of oral bacteria may be important to determine whether HMGB1 plays a role in inflammation or regeneration in the oral cavity. Pathogen removal by physical approach or immune cell activity such as phagocytosis decreases HMGB1 secretion following pro-inflammatory cytokine reduction, M1/M2 polarization change, and then promotes tissue repair. However, the remaining pathogen induces further HMGB1 secretion, continued pro-inflammatory cytokine production, and impaired healing or chronic inflammation. To understand the detailed mechanism of this complexity of HMGB1, further studies are required. For example, the use of different isoforms of recombinant HMGB1 or knock down analysis of HMGB1 receptors are needed in this research field. In addition, mostly, *in vivo* and *in vitro* studies have been included in this review; thus, further clinical studies, such as a translational study using anti-HMGB1 antibody or HMGB1 protein as a therapeutic agent, are needed to examine the biological effects of HMGB1 in the human body.

AUTHOR CONTRIBUTIONS

KY contributed to the conception, design, analysis, and interpretation of the study and wrote the manuscript. HI and HA contributed to the analysis and interpretation of the study and drafted the manuscript. HA, CY-H, AH, RS-K, and YZ contributed to *in vivo* experiments. HW and TY contributed to the interpretation of the study. MN and ST contributed to the conception, design, analysis, and interpretation of the study and drafted and critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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HMGB1 Promotes Myeloid Egress and Limits Lymphatic Clearance of Malignant Pleural Effusions

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Pleural effusions, when benign, are attributed to cardiac events and suffusion of fluid within the pleural space. When malignant, lymphatic obstruction by tumor and failure to absorb constitutively produced fluid is the predominant formulation. The prevailing view has been challenged recently, namely that the lymphatics are only passive vessels, carrying antigenic fluid to secondary lymphoid sites. Rather, lymphatic vessels can be a selective barrier, efficiently coordinating egress of immune cells and factors within tissues, limiting tumor spread and immune pathology. An alternative explanation, offered here, is that damage associated molecular pattern molecules, released in excess, maintain a local milieu associated with recruitment and retention of immune cells associated with failed lymphatic clearance and functional lymphatic obstruction. We found that levels of high mobility group box 1 (HMGB1) were equally elevated in both benign and malignant pleural effusions (MPEs) and that limited diversity of T cell receptor expressing gamma and delta chain were inversely associated with these levels in MPEs. Acellular fluid from MPEs enhanced γδ T cell proliferation *in vitro*, while inhibiting cytokine production from γδ T cells and monocytes as well as restricting monocyte chemotaxis. Novel therapeutic strategies, targeting HMGB1 and its neutralization in such effusions as well as direct delivery of immune cells into the pleural space to reconstitute normal physiology should be considered.

Keywords: HMGB1, malignant pleural effusions, benign pleural effusions, immune repertoire, tumor immunology, γδ T cells, adaptome, monocytes

INTRODUCTION

At advanced stages, many types of cancer can infiltrate the pleural cavity, disrupting the normal mechanism of fluid secretion and absorption, resulting in an unopposed collection of cancer containing fluid termed malignant pleural effusion (MPE). MPEs pose a major detriment to the quality of life for a cancer patient, are marked by persistent inflammation, associated with reduced

life expectancy, and often heralding the terminal stages of cancer (1–4). While we are able to palliate some of the symptoms from MPEs, systemic treatments most often fail to reverse course and localized strategies are not often successful. The inflammatory milieu of MPEs may provide insight regarding tumor biology related to immune evasion and immune dysfunction (5). MPEs possess an abundance of immune cells, often exceeding 10^8 leukocytes per effusion, that may harbor dormant effector cells which could be expanded and utilized for therapy. Unfortunately, our understanding of the phenotypes of immune cells, their tumor specificity, and how they are impacted by the MPE environment are only beginning to be understood. The lymphatics themselves, regulating pleural fluid dynamics, are not just passive conduits. Evolutionarily they emerged after the appearance of cartilaginous fish, with organized lymph nodes found later, primarily in mammals and in some birds. Pleural fluid is responsible for lubrication between the visceral and parietal layers. Water, and associated solute less than 4 nm, pass freely through mesothelial cells. Materials >1000 nm, are phagocytosed. Pleural lymphatics cycle pleural fluid at a rate of 0.4 mL/kg/h (6–10). In the pleural space of sheep, where it has been measured, effusions can be completely removed by the lymphatics in a linear manner at a rate of 0.28 mL/kg/h, ∼28 times greater than the rate of pleural fluid formation.

High mobility group box 1 (HMGB1) is a multifaceted nuclear protein that has diverse biological roles, regulating inflammation and orchestrating cellular immune responses (11–13). Outside the cell, HMGB1 functions as a prototypic damage-associated molecular pattern (DAMP) molecule with established roles in pathobiology such as cancer initiation and progression, toxic shock, and trauma (11–13). In the setting of cancer, HMGB1 is released from lysed and stressed cells into the extracellular space, causing chronic inflammation, attracting immune cells to the tumor site and engaging RAGE and Toll-like receptors to initiate and propagate inflammatory responses (12). HMGB1 can also be released by activated immune cells, which possibly contribute to the local pathophysiology. In a MPE, it is unknown whether HMGB1 has a role in anti-tumor immune activation or tumor progression (14). It may indeed serve as a vehicle to regulate lymphatic egress, limiting pathology to sites of tissue damage and preventing propagation of tumor and microbes.

During tumor development and therapy, pleiotropic HMGB1 mediates diverse biologic functions, promoting both cell survival and death by regulating unique signaling pathways (14). HMGB1 provides a protective role in cancer immunity by initially inducing immunogenic tumor cell death, contributing to immune-mediated eradication of tumors during their early development (15–17). Release of HMGB1 into the extracellular space contributes to the maturation of dendritic cells (DCs) and prompts cytotoxic T lymphocyte responses (18–20). In contrast, HMGB1 also plays an adverse role in tumor immunity. HMGB1 recruits and sustains immunosuppressive myeloid-derived suppressor cell and regulatory T cell populations during chronic inflammation. Sustained HMGB1 signaling limits chemotherapeutic responses in tumor cells, promoting resistance via enhanced autophagy, inhibiting both intrinsic and extrinsic-mediated apoptotic pathways in cancer cells (18–21). The presence of heightened levels of HMGB1 in human tumor tissues and in the circulation is frequently associated with disease severity and progression.

Inflammatory signaling among tumor cells, vasculature, and immune cells contributes to the development of pleural effusions (22). The pleural space is a sterile, secluded location in the thoracic cavity that is a frequent metastatic site for various histologic subtypes (2). The development of a MPE is the product of three associated processes; inflammation, lymphangiogenesis, and vascular leakage. More than 80% of MPEs feature elevated lymphocyte populations that play an important role in MPE pathogenesis (2, 3, 22). Lymphatic vessels respond to tumor and pathogen-induced changes in fluid transport, helping to regulate host immunity (23, 24). Based on these studies in melanoma and viral infection, we therefore hypothesized that, in the context of persistent tumor and other inflammatory mediators within the pleural space, that released DAMPs serve as cues to influence regional lymphatic vessel function, downstream immune induction, and host antitumor defense. Given the established role of HMGB1 and HMGB1-induced inflammation in the pathology of malignant disease, and the unique interface between immunity and tumor cells within the microenvironment established within MPEs, we examined the potential influence of HMGB1 on immune composition of MPEs.

MATERIALS AND METHODS

Collection of Specimens

Informed consent for participation was obtained prior to effusion drainage from all patients, and no subjects were under the age of 18. The use of human tissue samples and the experiments were approved by the Institutional Review Board at the University of Pittsburgh (IRB#PRO16110093). Samples were collected as excess pathologic specimens and experiments were not performed on humans. Effusions were collected for clinically indicated drainage of symptomatic effusions, either by thoracentesis, or from a temporary or indwelling tunneled catheter. These specimens would otherwise be medical waste. All methods were carried out in accordance with relevant guidelines and regulations. Seventy pleural effusions resulting from malignant disease (N = 46) or benign etiology (N = 24) were included. Ten patients (N = 7 malignant and 3 benign), underwent repeated collection of samples between 6 and 301 days apart. Quantities of 350–1000 cc were processed immediately upon collection wherein red blood cells were lysed, and cell pellets and acellular fluid were isolated and preserved. All effusions were examined by a cytopathologist. For normal serum controls, subjects (N = 404) were drawn from two population-based cohorts, the Shanghai Cohort Study and the Singapore Chinese Health Study (25). Serum from a cohort of patients with metastatic clear cell renal cell carcinoma (ccRCC) (N = 30) were obtained in the context of an IRB approved protocol, 11-080 conducted within the Cytokine Working Group. These three cohort studies have been approved by the Institutional Review Boards of the Shanghai Cancer Institute, the National University of Singapore, and the University of Pittsburgh.

HMGB1 ELISA

High mobility group box 1 levels in the acellular fractions of pleural effusions and sera were measured using a specific ELISA according to the manufacturer's protocol (IBL International-Shino Test Corporation, Kandajimbocho Chiyoda-ku, Japan). All measurements were performed in duplicate.

Cell Isolation and Culture

Peripheral blood mononuclear cells (PBMC) were isolated from consenting healthy volunteers using lymphocyte separation media (Corning). Cryopreserved PBMC were thawed, and γδ T cells were negatively selected (STEMCELL Technologies). γδ T cells were cultured for 10 days in complete media containing RPMI-1640, 5% human AB serum (GemCell), 1% Pen-Strep, and recombinant cytokines IL-2 (3000 IU/ml, aldesleukin, Clinigan), IL-15 (70 ng/ml, Miltenyi Biotech), and IL-21 (30 ng/ml, Miltenyi Biotech). γδ T cell phenotype was confirmed by flow cytometry and viable cultures with >80% γδ TCR^+, <0.5% αβ TCR^+, and <5% $CD3^-$ $CD56^+$ were utilized for subsequent studies. $CD14^+$ monocytes were isolated via MojoSort positive magnetic bead isolation kit (BioLegend) per manufacturers protocol and cultured as described below in complete RPMI-1640 without additional cytokines. Acellular MPE fluid used for *in vitro* assays was generated by pooling three individual donors with the final solution containing 54.38 ng/ml HMGB1 as determined by ELISA. When used at 50% in our assays (1:1 with media), this yielded a final concentration of 27.19 ng/ml HMGB1 replicating median levels identified in our cohort.

γδ T Cell Expansion

For expansion studies, 2×10^5 γδ T cells were seeded in 24-well plates and cultured in complete media containing IL-2, IL-15, and IL-21 with or without addition of rHMGB1 (200 ng/ml, R&D Systems), acellular MPE fluid (50%), neutralizing anti-HMGB1 polyclonal chicken Ab (10 μg/ml, Tecan), or humanized CD3/CD28 agonist (20 μl/1:100, T cell TransActTM Miltenyi Biotech) as indicated. γδ T cells were cultured for up to 11 days incubated at 37°C, 5% CO_2 and maintained at 0.5–1.5 $\times$ 10^6 cells/well with rHMGB1, acellular MPE fluid, and anti-HMGB1 Ab added upon well splitting to maintain initial culture conditions. Live cell counts were determined with acridine orange and propidium iodide staining on day 6 and day 11 with an automated cell counter (Cellometer K2, Nexcelom Biosciences).

Cytokine Analysis

To determine cytokine production, 2×10^5 γδ T cells were washed in PBS and plated in 200 μl in a 96-well plate and cultured in cytokine free complete media with addition of rHMGB1, pooled acellular MPE fluid, anti-HMGB1 antibody, or CD3/CD28 agonist as indicated. Following 24-h incubation, 50 μl culture supernatant was assayed with the Th1/Th2/Th17 cytometric bead array kit (BD Biosciences) measuring IL-2, IL-4, IL-6, IL-10, TNFα, IFNγ, and IL-17A per the manufacturer's instructions. Similarly, 5×10^4 $CD14^+$ monocytes were cultured in 100 μl in a 96-well plate using complete media in the presence or absence of acellular MPE fluid, rHMGB1, anti-HMGB1 Ab, or LPS (10 μg) (BioLegend) for 4-h. Subsequently, 50 μl culture supernatant was assayed using the human inflammation cytometric bead array kit (BD Biosciences) measuring IL-1β, IL-6, IL-8, IL-10, IL-12p70, and TNFα per the manufacturer's instructions. Data was collected on a 5-laser Aurora (Cytek Biosciences) or 4-laser BD LSR Fortessa flow cytometers and analyzed with FlowJo V10.7 (BD Biosciences).

Monocyte Migration

Chemotaxis of $CD14^+$ monocytes was performed using a 96-well ChemoTX system (Neuroprobe) with membranes containing 8 μm pores per manufacturer's protocol. Briefly, monocytes were rested overnight in serum-free RPMI-1640 then 10^5 cells in 80 μl were plated onto top chamber of the membrane. Lower chambers contained 325 μl of complete RPMI-1640 with or without addition of rHMGB1 (200 ng/ml), anti-HMGB1 Ab (10 μg/ml), LPS (10 μg/ml), and pooled acellular MPE fluid (50%). ChemoTX plates were incubated at 37°C, 5% CO_2, for 12 h. Transwell membranes were wiped with cotton and washed in PBS to remove unbound cells, then stained with 0.2% crystal violet. Transwell membranes were imaged using a Leica DMI 3000B digital microscope and cell quantitation performed by ImageJ (United States National Institutes of Health) to extrapolate cell counts of three randomly selected fields to represent the total area of the membrane well.

Flow Cytometry

Immunophenotyping of pleural effusions was performed following cryopreservation of samples. All reagents were purchased from BioLegend unless otherwise specified. 1–5 $\times$ 10^6 cells per sample were stained in Cell Staining Buffer using combinations of mAbs specific followed by labeling with amine-reactive viability dye (LiveDead, Molecular Probes). To determine leukocyte composition in pleural effusions, cells were labeled with mAbs specific for: EpCAM (9C4), CD45 (HI30; BD Biosciences), CD3 (UCHT1), CD4 (RPA-T4; Invitrogen), CD8a (RPA-R8), HLA-DR (L243), CD11b (ICRF-44), CD14 (HCD-14), CD16 (3G8), CD15 (W6D3), CD66b (G10F5; BD Biosciences), CD123 (6H6), CD11c (3.9), CD56 (HCD56; BD Biosciences), and CD19 (SJ25C1). Lineage gating for DCs includes CD3, CD19, and CD56. Samples were fixed in 1% paraformaldehyde and data was collected on a five laser LSR Fortessa (BD Bioscience). FlowJo (BD) software was used for conventionally gated data analysis.

TCR Repertoire Analysis

The cellular component of MPEs was isolated, cryopreserved, and transported to iRepertoire for T cell receptor (TCR) analysis. RNA extraction from MPE cells using a RNeasy Micro Kit (Qiagen, Valencia, CA, United States) according to the manufacturer's instruction. RNA concentrations were measured by spectrophotometry. iRepertoire multiplex primer sets (iRepertoire, Inc. Huntsville, AL, United States) were used to amplify the CDR3 region of TCR α, β, γ, and δ chains by using RNA as template as described by Wang et al. (26). The whole amplification process and library preparation process

for next generation sequencing were fully automated in the iR-Procecessor and iR-Cassette (iRepertoire, Inc.). Then, paired-end sequencing was performed on purified PCR products using an Illumina MiSeq v2 300-cycle Reagent Kit (Illumina Inc.), for an average read depth of 30,000 reads per sample. Raw cDNA sequences were first analyzed to identify V and J genes by using iR-map and visualized in iRweb (iRepertoire, Inc.). Multiple alignments and hierarchical clustering of conserved amino acid sequences were analyzed as described (26). RNA samples were split into two reactions and processed as technical replicates. The coefficient of determination (R^2) was calculated by linear regression to show the correlation between the replicates of CDR3 frequencies prior to data analysis to exclude PCR and sequence errors. The diversity of the TCR repertoire was calculated based on the diversity index (DI) defined mathematically by Wu et al. (27). Tree maps were used to reveal the diversity and characteristics of TCR repertoire. In a tree map, each rounded rectangle represents a unique V–J combination of uCDR3, where the size of a spot denotes the relative frequency.

Statistical Analysis

All results were expressed as means ± standard error of the mean (SEM) unless otherwise stated. Data were analyzed using non-parametric Mann–Whitney U tests for comparisons of patient groups, paired Student's *t*-test for analysis of change in HMGB1 levels and cell densities within patients over time, unpaired Students *t*-test for analysis of healthy donor cell expansion, cytokine production, or migration *in vitro*, or Spearman rank-order correlation tests performed using GraphPad Prism8 (GraphPad Software). For all hypothesis tests, a $p < 0.05$ was considered statistically significant.

RESULTS

HMGB1 Levels Are Elevated in Pleural Effusions

To characterize levels of soluble HMGB1 in pleural effusions from malignant and benign etiologies, an HMGB1-specific ELISA was performed on the acellular fraction of pleural effusions from patients with metastatic MPE ($N = 46$) or benign pleural effusion (BPE) ($N = 24$). MPEs were secondary to various primary cancers including breast ($N = 19$), lung ($N = 11$), ovarian ($N = 8$), sarcoma ($N = 3$), salivary gland ($N = 1$), and colon ($N = 1$) (**Table 1**). As a reference control, we measured HMGB1 levels in the serum of healthy donors ($N = 404$) and patients with metastatic ccRCC ($N = 30$). Levels of HMGB1 in the sera of ccRCC patients (16.16 ng/ml ± 4.708) were significantly higher than those of healthy controls (2.64 ng/ml ± 0.229) ($p \leq 0.0001$) (**Figure 1**). Notably, levels of HMGB1 in pleural effusions were significantly higher than serum HMGB1 in both healthy and ccRCC cohorts ($p = \leqq 0.0011$), irrespective of effusion etiology (**Figure 1**). Soluble HMGB1 was comparable between pleural effusions ($p = 0.872$), with BPEs containing 53.07 ng/ml ± 11.21 and MPEs containing 48.89 ng/ml ± 12.13, respectively (**Figure 1**). HMGB1 levels in sera from ccRCC patients, BPEs, and MPEs were all significantly greater than those detected in healthy control sera ($p \leq 0.0001$). Additionally, in our cohort, intrapleural HMGB1 from lung cancer patients (70.05 ng/ml ± 24.70) was significantly elevated compared to levels observed in ovarian cancer patients (21.01 ng/ml ± 3.54; $p = 0.025$) and raised compared to breast cancer patients (34.50 ng/ml ± 7.38; $p = 0.057$) (**Supplementary Figure 1**). No differences were observed between HMGB1 levels in MPEs secondary to breast or ovarian cancers.

Association of Intrapleural HMGB1 and Cell Density of Pleural Effusions

We next examined associations between HMGB1 levels detected in MPEs and BPEs and gross cellularity of the effusion. As total cell number and volume of each effusion varies dramatically, values were represented as total live cells per liter of effusion. Although there was no association between HMGB1 and cell density within MPEs, a minor correlation between BPE cell density and HMGB1 was identified within our cohort ($p = 0.061$) (**Figure 2A**). To examine the temporal dynamics of this interaction, serial effusions were collected up to four times from patients with MPE ($N = 7$) or BPE ($N = 3$). HMGB1 levels and intrapleural cell densities in MPEs were highly variable over time, whereas we observed a continual decrease in both HMGB1 levels and gross cellularity in BPEs upon serial drainage (**Figures 2B,C**). To evaluate if the state of the effusion microenvironment, i.e., HMGB1 levels, was associated with a systemic phenotype, neutrophil, lymphocyte, and monocyte levels were measured in the peripheral blood by complete blood count (**Table 1**). In patients with MPE, intrapleural HMGB1 positively correlated with increased lymphocytes, percentage ($p = 0.019$) and number ($p = 0.005$), and percentage of neutrophils ($p = 0.021$) in the circulation. By contrast, HMGB1 levels within BPEs were not associated with the percentage or numbers of these cellular subsets in the peripheral blood.

HMGB1 Expression Is Associated With Unique Leukocyte Profile Within MPEs

Malignant pleural effusions contain a highly heterogeneous population of innate and adaptive immune cells, representing diverse states of activation. As HMGB1 is a potent inflammatory mediator capable of inducing chemotaxis, we performed comprehensive immunophenotyping on a subset of MPEs. Polychromatic flow cytometry was used to simultaneously identify $CD4^+$ and $CD8^+$ T cells ($CD3^+$), NK cells ($CD3^-CD56^+$), monocytes ($CD14^+$), macrophages ($CD11b^+CD66b^-$), myeloid DCs ($Lin^-HLA\text{-}DR^+CD11c^+$), plasmacytoid DCs ($Lin^-HLA\text{-}DR^+CD123^+$), B cells ($CD19^+$), and neutrophils ($CD66b^+$ $CD16^+$). Correlative analysis was performed to identify associations between intrapleural HMGB1 and unique cell populations within the MPE. HMGB1 levels in MPEs were associated with increased presence, as both proportion of all cells as well as total numbers of $CD45^+$ leukocytes within effusions (**Figures 3A,B**). Additionally, we observed a strong inverse correlation between the concentration

TABLE 1 | Characteristics of patients with pleural effusion.

	Number of Patients	MPE	BPE	*p*-Value (MPE/BPE)
Total number of patients	70	46 (65.7)	24 (34.2)	
Age	65.89 (±1.46)	64.6 (±2.03)	68.3 (±1.70)	0.176
Gender				
Male	21 (30.0)	6 (13.0)	15 (62.5)	
Female	42 (70.0)	40 (87.0)	9 (37.5)	
HMGB1				
Mean ± SEM	50.33 (±8.90)	48.89 (±12.31)	53.07 (±11.21)	0.872
Median	26.25	22.88	32.78	
Effusion cells				
Tumor (% total)	25.74 (±6.07)	33.10 (±7.03)	0 (±0.0)	0.000
Leukocytes (% total)	63.70 (±5.96)	56.90 (±6.83)	87.50 (±5.59)	**0.007**
Peripheral blood				
Neutrophil (10^9/L)	7.897 (±1.19)	6.778 (±1.48)	10.76 (±1.76)	**0.006**
Neutrophil (%)	73.61 (±1.93)	70.43 (±2.38)	81.73 (±2.38)	**0.002**
Lymphocyte (10^9/L)	1.008 (±0.10)	1.065 (±0.13)	0.861 (±0.10)	0.861
Lymphocytes (%)	13.68 (±1.54)	15.42 (±2.08)	9.211 (±1.40)	0.123
Monocytes (10^9/L)	0.674 (±0.05)	0.645 (±0.07)	0.748 (±0.06)	0.129
Monocytes (%)	8.934 (±0.83)	9.649 (±1.10)	7.117 (±0.78)	0.062
Effusion etiology				
Breast cancer		19 (41.3)		
Lung cancer		11 (23.9)		
Ovarian cancer		8 (17.4)		
Sarcoma		3 (6.52)		
Colon cancer		1 (2.17)		
Salivary gland cancer		1 (2.17)		

Values are represented as n (%) and mean (±SEM) unless noted. p-values are derived using a Mann–Whitney U test. Bold values are statistically significant.

of intrapleural HMGB1 and the proportion and absolute number of $CD14^+$ monocytes (**Figures 3A,B**). When these subjects were stratified into HMGB1 high (41.28 ng/ml ± 4.03) and HMGB1 low (16.88 ng/ml ± 4.03) groups (N = 3 patients each) based on the median HMGB1 level in our MPE cohort (22.88 ng/ml) (**Table 1**), we observed pronounced differences in MPE immune composition (**Figures 3C,D**). Notably, patients with high levels of intrapleural HMGB1 were found to have greatly increased proportion of neutrophils (19.9% vs 5.4%), T cells (30.6% vs 15.5%), and B cells (6.8% vs 1.4%) compared to HMGB1 low subjects. By contrast, MPEs containing low HMGB1 were composed predominantly of monocytes (18.6% in low vs 3.0% in HMGB1 high) and a major population of undefined myeloid cells (36.6% vs 2.0% in HMGB1 high), likely to represent myeloid-derived suppressor cell populations.

MPE Fluid Inhibits Monocyte Migration and Cytokine Production

We observed that intrapleural HMGB1 levels were associated with unique immune composition within MPEs, including decreased percentage and absolute numbers of monocytes. Consequently, we evaluated the effect of HMGB1 and acellular MPE fluid on chemotaxis and cytokine production from $CD14^+$ monocytes isolated from the peripheral blood of healthy donors. Herein, monocyte migration was measured via ChemoTX Transwell membrane system following a 12 h culture in the presence or absence of 50% acellular MPE fluid, exogenous rHMGB1 (200 ng/ml), high dose LPS (10 μg/ml), or combinations of these factors (**Figure 4A**). LPS was evaluated as a known surrogate for other TLR4 ligands such as HMGB1 and taxanes and potent inducer of inflammatory responses in monocytes. In the absence of acellular MPE fluid, both rHMGB1 and LPS induced robust monocyte migration (**Figure 4A**). Notably, monocyte migration was dramatically inhibited in the presence of acellular MPE fluid with migration induced by rHMGB1 and LPS reduced by 90.3 and 60.1%, respectively (**Figure 4A**). Culture with rHMGB1 and LPS combined was insufficient to regain migratory ability (**Figure 4A**). As expected, blocking HMGB1 in MPE culture using HMGB1-specific antibody failed to enhance migration (data not shown). We next determined the capacity of monocytes to produce inflammatory TNFα following 4 h culture in the above conditions. Treatment of monocytes with LPS induced robust TNFα production measured in culture supernatants by cytometric bead array. Notably, addition of acellular MPE fluid reduced the levels of TNFα generated by monocytes following LPS stimulation by 37.1%, an effect that was independent of further addition of rHMGB1 (36.6% reduction) or anti-HMGB1 blocking antibody (43.6% reduction) (**Figure 4B**, and data not shown). Collectively, these findings suggest that the local microenvironment of MPEs exerts profound inhibitory effects upon monocyte functionality that are independent upon the presence of HMGB1.

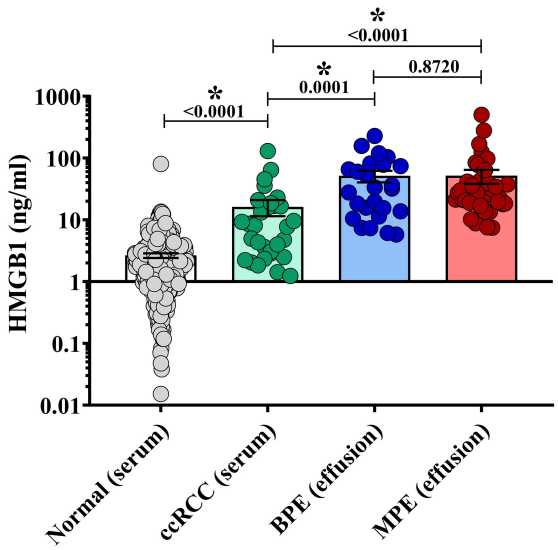

FIGURE 1 | HMGB1 levels are elevated in pleural effusions. HMGB1 levels were measured in pleural effusions from patients with metastatic malignant disease (MPE) (N = 46), in effusions from patients presenting with congenital heart failure or effusion of benign origin (BPE) (N = 24), within the sera of healthy donors (N = 404) and sera from patients with metastatic clear cell renal cell carcinoma (ccRCC) (N = 30) by specific ELISA. Levels of HMGB1 in the sera of ccRCC patients (16.16 ng/ml ± 4.708) were significantly higher than those of healthy controls (2.64 ng/ml ± 0.229) (p = 0.001) HMGB1 was comparable between pleural effusions (p = 0.872), BPE containing 53.07 ng/ml ± 11.21 and MPE possessing 48.89 ng/ml ± 12.31. HMGB1 levels in serum from ccRCC patients, BPE, and MPE were all significantly elevated compared to healthy controls ($p \leq 0.0001$). Data represent means ± SEM and Mann–Whitney U tests were used for comparisons. *denotes statistical significance.

High HMGB1 Levels Are Associated With Reduced Diversity of γδ TCRs Within MPEs and γδ T Cell Proliferation *in vitro*

The clonal repertoire of T cells establishes diversity responding to pathogenic and endogenous insults. In the context of malignant disease, a greater breadth of TCR diversity may afford superior protection from tumor cells expressing neoantigens (28). To examine the effects of intrapleural HMGB1 on TCR diversity within pleural effusions, cells isolated from MPEs from 14 patients underwent multiplexed TCR amplification followed by next generation sequencing providing comprehensive detection of unique TCR clones. Between patients, we observed a wide range of TCR diversity, as illustrated in representative tree plots of the TCRδ chain from individuals with high, moderate, or low DIs, respectively (**Figure 5A**). Patients were then separated into HMGB1 high (N = 5; 161.5 ng/ml ± 86.6) and HMGB1 low (N = 9; 18.75 ng/ml ± 2.68) groups based on the MPE cohort median level of HMGB1 as before (**Figure 5B**). Heterogeneity of the TCR α and β chains was unaffected by levels of intrapleural HMGB1 in MPEs (**Figure 5C**). Interestingly, patients with high intrapleural HMGB1 levels had significantly diminished diversity indices for both TCRδ and γ chains present on T cells within MPEs (**Figure 5C**). Given that a decrease in TCR diversity may be associated with emergence of dominant clonotypes, we subsequently examined the 10 most highly expressed TCRδ clones from the five patients with high HMGB1 levels and corresponding low TCRδ diversity. Notably, the 10 dominant TCRδ clones per MPE were unique to each patient, with no individual clone being shared amongst individuals.

To examine the effect of HMGB1 on γδ T cell function, γδ T cells were isolated from the peripheral blood of healthy donors and assessed for their proliferative capacity and cytokine production in the presence or absence of rHMGB1 (200 ng/ml), anti-HMGB1 blocking antibody (10 μg/ml), acellular MPE fluid (50% of culture media), and polyclonal activation with a CD3/CD28 agonist. In media containing IL-2, IL-15, and IL-21, γδ T cells underwent a ∼7.5-fold expansion by 11 days in culture (**Figure 5D**). Notably, addition of rHMGB1 reduced γδ T cell expansion by ∼41% at day 11 (p = 0.005) compared to media control, whereas provision of acellular MPE fluid increased γδ T cell numbers by ∼35% (p = 0.005). Depletion of HMGB1 from acellular MPE fluid in culture further enhanced γδ T cell yield to ∼60% (p = 0.0005) by day 11. When γδ T cell culture was performed in the presence of an activating CD3/CD28 agonist, MPE fluid again augmented γδ T cell proliferation, with the highest γδ T cell numbers obtained following culture in HMGB1-depleted acellular MPE fluid with a 92% increase over CD3/CD28 agonist alone (p = 0.0006) (**Figure 5D**). Culture of activated γδ T cell with rHMGB1 decreased proliferation by 21% compared to CD3/CD28 agonist treated cells but did not attain statistical significance. Collectively, these findings suggest that HMGB1 inhibits γδ T cell growth which is conversely augmented by unidentified factors present within the acellular MPE environment.

We next asked whether HMGB1 present in culture media or acellular MPE fluid could effect cytokine production from γδ T cells. We determined cytokine levels by cytometric bead array in supernatants following 24 h culture of 2×10^5 γδ T cells in the above conditions. Culture of γδ T cells in IL-2, IL-15, IL-21 containing media resulted in robust TNFα production that was further enhanced upon stimulation with CD3/CD28 agonist (**Figure 5E**). Notably, addition of acellular MPE fluid, with or without anti-HMGB1 antibody, significantly decreased TNFα production from both unstimulated and stimulated γδ T cells ($p \leq 0.002$ for both conditions; data not shown) (**Figure 5E**). Provision of exogenous rHMGB1 did not effect TNFα production. Similarly, production of IL-10 by γδ T cells was enhanced by CD3/CD28 agonist stimulation, and again, substantially inhibited by the presence of acellular MPE fluid with or without anti-HMGB1 ($p < 0.05$) (**Figure 5E**). Culture with rHMGB1 did not effect IL-10 production by γδ T cells in this setting. Although provision of acellular MPE fluid profoundly inhibits cytokine production form γδ T cells, this effect seems to be independent of HMGB1.

DISCUSSION

Various pathologic processes regulate the development of pleural effusions. To assess the presence of the prototypic DAMP,

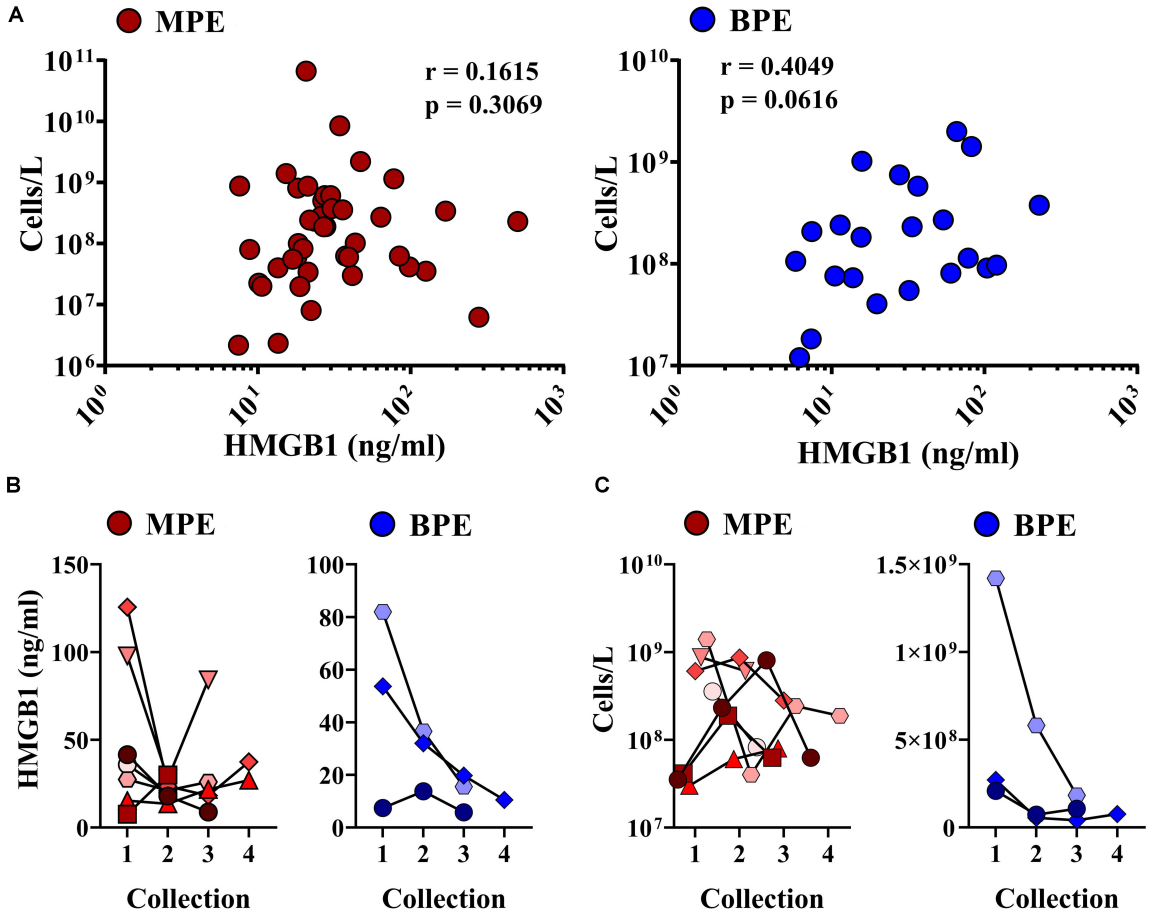

FIGURE 2 | Correlations between intra-pleural HMGB1 and gross cellularity of pleural effusions. **(A)** Levels of intra-pleural HMGB1 did not predict cellularity as cells/liter of MPEs (left, red), but were significantly correlated with cell density in BPEs (right, blue). **(B)** When measured serially following multiple drainage events of pleural effusions, HMGB1 decreased in BPE (right, blue). No consistent trend was observed in HMGB1 levels longitudinally in MPE (left, red). **(C)** Over time, cell density decreased in both MPE (left, red) and BPE (right, blue) when serially sampled. Correlations were performed using Spearman rank-order tests.

associated with high levels of unscheduled cell death and/or cellular stress, within the unique tumor microenvironment of MPEs, we quantified intrapleural HMGB1 using BPEs as a comparator. Previous studies have shown that transudative effusions resulting from congestive heart failure or liver cirrhosis had significantly lower levels of HMGB1 when compared with exudative effusions arising from infection or malignancy (29, 30). Although levels of intrapleural HMGB1 varied between studies (15.0–36.62 ng/ml transudate), (35.1–118.0 ng/ml infectious), and (29.6–111.45 ng/ml malignancy), quantities were similar to those identified in our local cohort with expected variability due to limited sample sizes (29, 30). We found, compared to reference cohorts from sera of both healthy controls and metastatic ccRCC patients, that intrapleural HMGB1 levels in both MPEs and BPEs were significantly elevated. Notably, intrapleural HMGB1 was comparable in effusions resulting from malignant and benign processes. Because both malignant and benign effusions can have a component of associated inflammation, it is of interest that MPEs and BPEs demonstrate increased levels of HMGB1, whereas HMGB1 is rapidly cleared from serum in the setting of trauma. As such, the presence of elevated HMGB1 may serve as a novel therapeutic target if further studies implicate DAMPs in the pathologic subversion of fluid reabsorption in the pleural cavity.

High mobility group box 1 has both immune stimulating and suppressing properties (31). HMGB1 promotes maturation and subsequent cell death in macrophage−derived DCs (32). HMGB1 also enhances the function of regulatory T cells via enhanced IL-10 production, while inhibiting the effector function of conventional T cells including IFNγ production and proliferation (21, 33). Activated macrophages, natural killer cells, and mature DCs can release HMGB1, which may promote a positive feedback loop to propagate subclinical inflammation, tumor initiation and progression (34–38). Collectively, the immunosuppressive effects of HMGB1 serve to inhibit the generation of *de novo* tumor-specific immunity, as well as suppress the maintenance of pre-existing anti-tumor responses. In MPEs, HMGB1 levels are associated with an increase in leukocyte infiltration with reduced monocyte

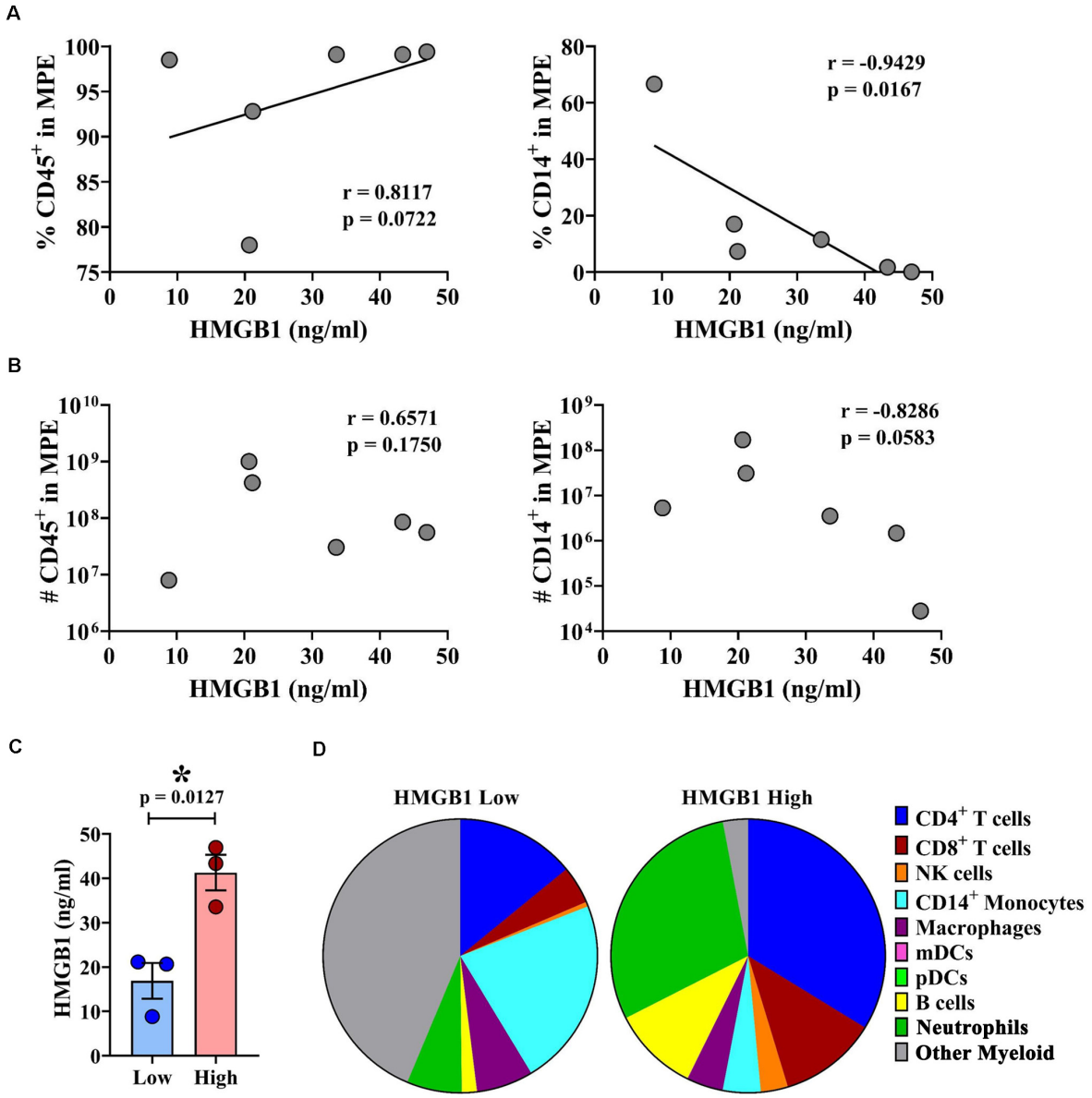

FIGURE 3 | HMGB1 levels are associated with leukocyte infiltration and unique immune composition within MPEs. Multiparametric flow cytometry was used to immunophenotype cells isolated from MPEs. **(A)** The proportion and **(B)** absolute number of CD45+ leukocytes (left) was found to increase with greater levels of intra-pleural HMGB1. By contrast, **(A)** the proportion and **(B)** absolute numbers of CD14+ monocytes within MPEs declined with increased HMGB1. **(C)** Patients were segregated into HMGB1 low (16.88 ng/ml ± 4.03) and HMGB1 high (41.28 ng/ml ± 4.00) groups for comparative analysis. **(D)** Immune composition as proportion of CD45+ cells determined by select markers by flow cytometry of MPE-resident leukocytes in patients with low or high intra-pleural HMGB1 (*N* = 3 each), respectively. Group means were compared using a Mann–Whitney U test and correlations performed using Spearman rank-order tests. * denotes statistical significance.

numbers. Notably, the presence of acellular MPE fluid alone restricted monocyte chemotaxis and reduced inflammatory cytokine release *in vitro*, and may, in the setting of chronic HMGB1 exposure *in vivo*, induce apoptosis in effusion-resident myeloid cells. In our cohort, high intrapleural HMGB1 was associated with increased T cell, B cell, and neutrophil recruitment into the MPEs, mirrored by increased number of lymphocytes and neutrophils in the systemic circulation, suggesting that the chemotactic properties of HMGB1 propagated an active inflammatory environment with substantial involvement of adaptive immune cells. In contrast, low intrapleural HMGB1 was associated with a significant proportion of undefined myeloid cells, likely representing various myeloid-derived suppressor cell populations. Future mechanistic studies will be required to define the causal effect of intrapleural HMGB1 on leukocyte recruitment,

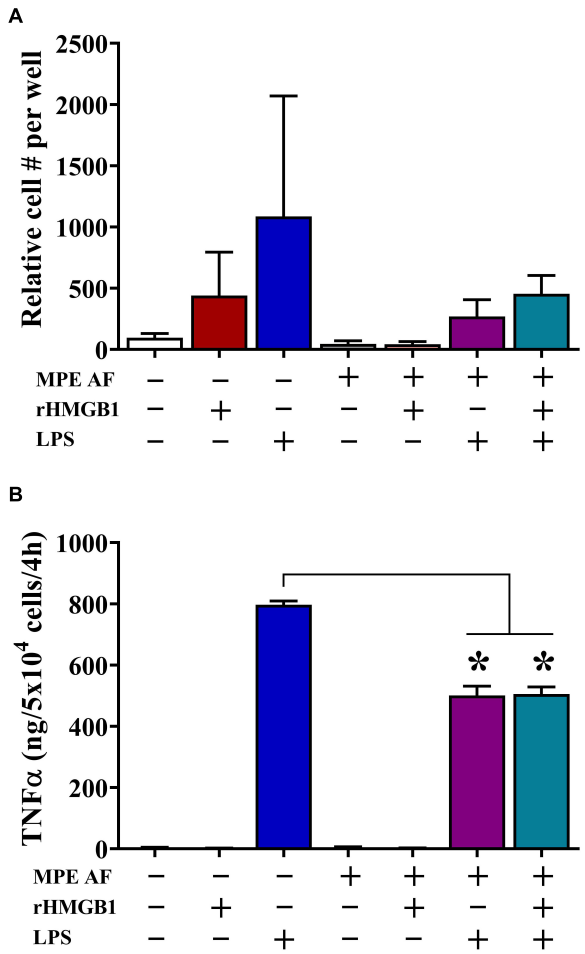

FIGURE 4 | Acellular MPE Fluid Inhibits Monocyte Migration and TNFα Production Independent of HMGB1. The effect of HMGB1 and acellular MPE fluid (MPE AF) was assessed on healthy donor peripheral blood $CD14^+$ monocyte function. **(A)** Twelve-hour monocyte migration (10^5 cells/well) was evaluated in the presence of rHMGB1 (200 ng/ml), LPS (10 μg/ml), and 50% acellular MPE fluid (*N* = 5 repeats). While rHMGB1 and LPS enhanced migration, addition of acellular MPE fluid dramatically inhibited monocyte chemotaxis. **(B)** Soluble TNFα was measured via cytometric bead array following 4-h *in vitro* stimulation of 5×10^4 monocytes (*N* = 3 repeats). In the presence of acellular MPE fluid, TNFα production following LPS stimulation (10 μg/ml) was significantly decreased irrespective of additional treatment with rHMGB1. $p < 0.05$. Data represent means ± SEM and Mann–Whitney U tests were used for comparisons. * denotes statistical significance.

retention, lymphatic clearance, and ultimately, their impact on malignant disease.

The ability of adaptive cellular immunity to account for the myriad of pathogenic and endogenous threats is afforded through the diversity of the T cell and B cell receptors, collectively referred to as the adaptome (28). Using PCR-multiplexed amplification and next-generation sequencing of TCR sequences from bulk T cell populations isolated from MPEs, we have found that high intrapleural HMGB1 levels resulted in substantial reduction in TCRδ and TCRγ diversity specifically. Such a decrease in breadth of diversity is likely accompanied by the expansion of one or several dominant clones that have been actively recruited to the MPE space (e.g., clonality). These findings suggest that HMGB1 release may be associated with induction of an antigen-specific γδ T cell response, or alternatively, that aberrant release of DAMPs liberates yet unidentified molecular antigens driving the recruitment of specific γδ T cell populations. The early recruitment of such cells to tissues such as the skin, lung, and gut is in part for them to regulate lymphatic fluid flux and clearance of DAMPs, pathogens and cancer (39–43). Notably, decreased T cell diversity, primarily within the αβ T cell population, has been identified in the setting of cancer (44–46). Furthermore, TCRβ diversity has been associated with better patient responses to immunotherapy during checkpoint treatment for lung cancer (47). Herein, we have observed that cultured healthy donor γδ T cell proliferation was inhibited by rHMGB1, enhanced in the presence of acellular MPE fluid, and further augmented with addition of neutralizing HMGB1 antibody. Given the limited inflammatory cell migration in the setting of MPE, enhanced intrapleural HMGB1 concentrations could inhibit (1) infiltration of circulating γγδ T cells and (2) subsequent proliferation of all but the most reactive clonotypes, limiting repertoire diversity. Alternatively, recognition of non-peptide stress antigens (MICA/B; ULBP1-6) in the pleural environment could result in clonal expansion and reduction in repertoire diversity (48). Increased understanding of the biologic role of TCR diversity in health and disease has broad implications for cellular immunity and identification of specific and effective clonotypes, potentially useful for adoptive cell transfer therapy.

The effects of HMGB1 on T cell proliferation and phenotype are dependent on the source of HMGB1, resulting from tumor or myeloid cells, and the T cell activation status (35). Secreted HMGB1 from activated DCs results in CD4 Th1 polarization and expansion that is limited in the presence of a HMGB1 blocking antibody. HMGB1 mediated clonal expansion was dependent on CD3 and CD28 crosslinking and T cells are more sensitive to HMGB1 secreted by mature DCs than recombinant HMGB1 (49). rHMGB1 enhances CD4 proliferation at suboptimal doses of plate-bound OKT-3, but has limited effects on CD8 T cells (50). rHMGB1 induces CD4 Th17 polarization and apoptosis of regulatory cells with diminished IL-10 production (51). Although our findings suggest that increased HMGB1 levels within MPEs are capable of promoting T cell recruitment, further studies are necessary to determine the phenotype of recently recruited lymphocytes and if such cells are endowed with tumor-specific reactivity.

γδ T cells are MHC unrestricted effector cells that recognize non-peptide antigens, with an underappreciated role in tumor immune surveillance. Similar to their αβ T cell cousins, γδ T cells are highly susceptible to the composition of the tumor microenvironment, which may impart either antitumor or immunoregulatory capabilities depending upon the local signaling context (52). γδ T cells have been previously identified in the MPE of patients with NSCLC. Compared to circulating patient lymphocytes, γδ T cells in the MPE were found to have a predominant Vδ1/Vδ1-Vδ2- subtype and decreased expression of CD27 and CD28, with a suggested impaired activation and

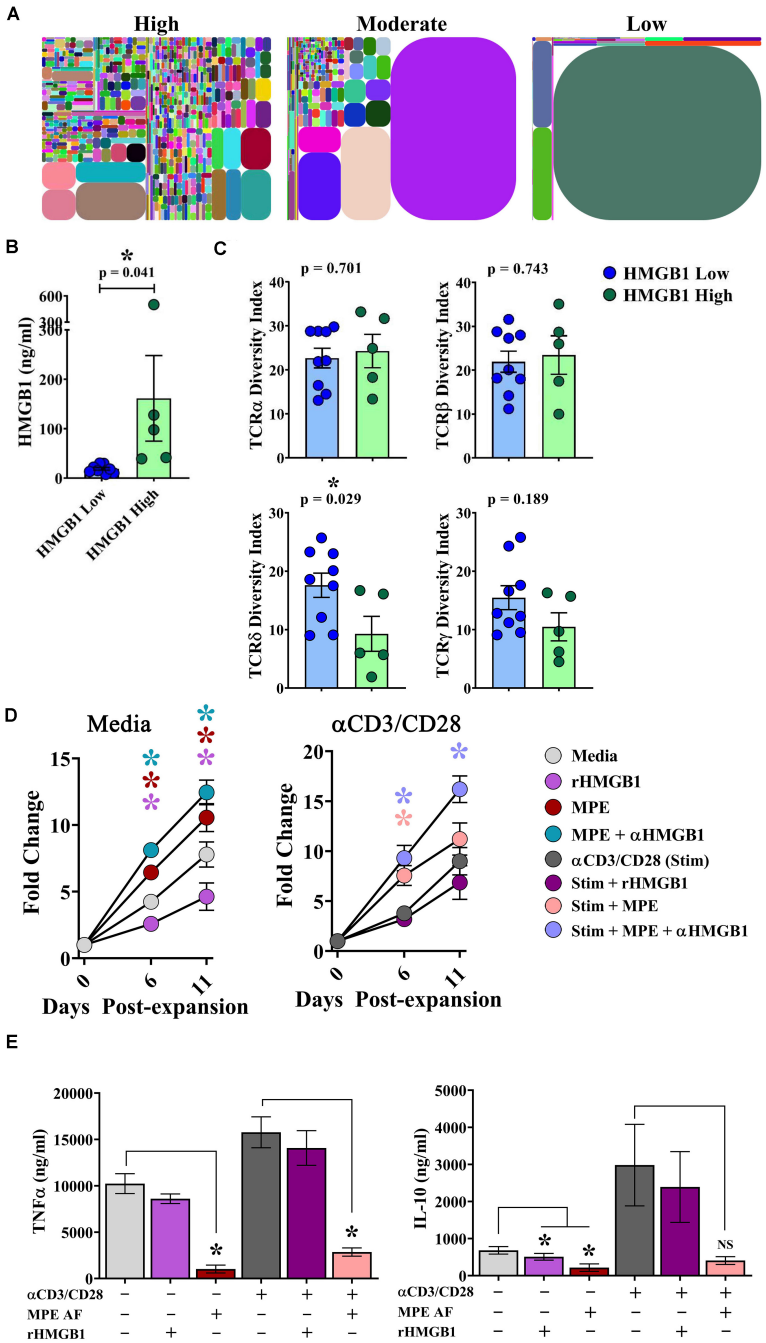

FIGURE 5 | HMGB1 is associated with reduced γδ TCR diversity within MPEs and inhibition of γδ T cell proliferation *in vitro*. Amplification and next generation sequencing of the α, β, δ, and γ TCR chains was performed from bulk cells isolated from 14 MPEs. **(A)** Clonal diversity represented via tree maps illustrating the relative frequency of unique CDR3s as geometric shapes in patients with high, moderate, and low diversity of TCRδ chains within MPEs. **(B)** For comparative analysis, patients were segregated into HMGB1 low (N = 9) (18.75 ng/ml ± 2.68) and HMGB1 high (N = 5) (161.5 ng/ml ± 86.6) groups. **(C)** Diversity of TCRα and TCRβ chains, as calculated by diversity index, within cells of the MPE was not different between patients with high or low intrapleural HMGB1 (p = 0.743) (top). Diversity indices for TCRδ and TCRγ chains were significantly decreased in patients with high compared to low intrapleural HMGB1 (p = 0.029 and 0.189, respectively). **(D)** Expansion of cultured γδ T cells isolated from peripheral blood of healthy donors (N = 5) was observed over 11 days in the presence of rHMGB1 (200 ng/ml), anti-HMGB1 Ab (10 μg/ml), CD3/CD28 agonist (1:100 transact), and acellular MPE fluid (50%). rHMGB1 decreased unstimulated γδ T cell growth, while addition of acellular MPE fluid enhanced proliferation that was further increased with addition of anti-HMGB1 blocking antibody. Similar trends were observed in CD3/CD28 agonist stimulated γδ T cells. **(E)** Soluble TNFα and IL-10 in culture supernatants was determined by cytometric bead array following 24-h *in vitro* culture of 2 × 10^5 γδ T cells under the above conditions. rHMGB1 had no effect on cytokine production; however, in unstimulated and stimulated γδ cells, acellular MPE fluid decreased both TNFα and IL-10 production. Cytokine production studies were completed in n = 3 different donors. Group means were compared using a Mann–Whitney U test with significance of $p \leqq 0.05$. * denotes statistical significance.

cytokine releasing state (53). In a murine model of Lewis lung carcinoma-derived MPE, IL-10 deficiency led to increased γδ T cell intrapleural proliferation and IL-17a production, reduced MPE volume, and longer survival that was dependent on γδ T cells. However, IL-$10^{-/-}$ KO γδ T cells expressed lower levels of NKG2D and FasL, typically associated with activated γδ T cells (54, 55). Adding further complexity, γδ T cell-derived IL-17 mediates both anti-tumor and pro-tumor effects that are temporally regulated (56–58). Our findings suggests that in the presence of acellular MPE fluid γδ T cells are simultaneously driven to proliferate, while restricted in their ability to mount a cytokine response. Such processes may drive terminal exhaustion of this effector population leading to immune evasion and tumor escape.

DATA AVAILABILITY STATEMENT

The data will be available on request without restriction through the corresponding author.

ETHICS STATEMENT

Studies have been approved by the Institutional Review Boards of the Shanghai Cancer Institute, the National University of Singapore, and the University of Pittsburgh. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AS, AP, PM, RD, and ML wrote the main body of the text. KJ, AP, YW, PM, KR, MB-S, J-MY, SM, and AS assisted in data collection, data analysis, and figure preparation. SM performed cytologic analysis and figure preparation. AS, KJ, AP, PM, YW, RD, AL, J-MY, JH, and ML assisted in experimental design and manuscript review and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.02027/full#supplementary-material

FIGURE S1 | HMGB1 levels are elevated in MPEs secondary to lung cancer. HMGB1 levels were measured by specific ELISA in MPEs from lung (N = 11), breast (N = 19), and ovarian (N = 8) cancer patients. HMGB1 levels were significantly higher in lung cancer MPEs when compared to levels within ovarian MPEs (p = 0.025) and elevated in comparison to levels in breast cancer MPEs (p = 0.057). Data represent means ± SEM and Mann–Whitney U tests were used for comparisons.

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