The HMGB1/RAGE axis triggers neutrophil-mediated injury amplification following necrosis

Peter Huebener, …, John D. Loike, Robert F. Schwabe

*J Clin Invest.* 2015;125(2):539-550. [https://doi.org/10.1172/JCI76887](https://doi.org/10.1172/JCI76887).

In contrast to microbially triggered inflammation, mechanisms promoting sterile inflammation remain poorly understood. Damage-associated molecular patterns (DAMPs) are considered key inducers of sterile inflammation following cell death, but the relative contribution of specific DAMPs, including high–mobility group box 1 (HMGB1), is ill defined. Due to the postnatal lethality of *Hmgb1*-knockout mice, the role of HMGB1 in sterile inflammation and disease processes in vivo remains controversial. Here, using conditional ablation strategies, we have demonstrated that epithelial, but not bone marrow–derived, HMGB1 is required for sterile inflammation following injury. Epithelial HMGB1, through its receptor RAGE, triggered recruitment of neutrophils, but not macrophages, toward necrosis. In clinically relevant models of necrosis, HMGB1/RAGE-induced neutrophil recruitment mediated subsequent amplification of injury, depending on the presence of neutrophil elastase. Notably, hepatocyte-specific HMGB1 ablation resulted in 100% survival following lethal acetaminophen intoxication. In contrast to necrosis, HMGB1 ablation did not alter inflammation or mortality in response to TNF- or FAS-mediated apoptosis. In LPS-induced shock, in which HMGB1 […]
The HMGB1/RAGE axis triggers neutrophil-mediated injury amplification following necrosis

Peter Huebener, Jean-Philippe Pradere, Celine Hernandez, Geum-Youn Gwak, Jorge Matias Caviglia, Xueru Mu, John D. Loike, and Robert F. Schwabe

1Department of Medicine and 2Department of Physiology and Cellular Biophysics, Columbia University, New York, New York, USA.

In contrast to microbially triggered inflammation, mechanisms promoting sterile inflammation remain poorly understood. Damage-associated molecular patterns (DAMPs) are considered key inducers of sterile inflammation following cell death, but the relative contribution of specific DAMPs, including high–mobility group box 1 (HMGB1), is ill defined. Due to the postnatal lethality of Hmgb1-knockout mice, the role of HMGB1 in sterile inflammation and disease processes in vivo remains controversial. Here, using conditional ablation strategies, we have demonstrated that epithelial, but not bone marrow–derived, HMGB1 is required for sterile inflammation following injury. Epithelial HMGB1, through its receptor RAGE, triggered recruitment of neutrophils, but not macrophages, toward necrosis. In clinically relevant models of necrosis, HMGB1/ RAGE–induced neutrophil recruitment mediated subsequent amplification of injury, depending on the presence of neutrophil elastase. Notably, hepatocyte–specific HMGB1 ablation resulted in 100% survival following lethal acetaminophen intoxication. In contrast to necrosis, HMGB1 ablation did not alter inflammation or mortality in response to TNF– or FAS–mediated apoptosis. In LPS–induced shock, in which HMGB1 was considered a key mediator, HMGB1 ablation did not ameliorate inflammation or lethality, despite efficient reduction of HMGB1 serum levels. Our study establishes HMGB1 as a bona fide and targetable DAMP that selectively triggers a neutrophil–mediated injury amplification loop in the setting of necrosis.

Introduction

Inflammation is a key contributor to many disease processes. Whereas ligand-receptor systems regulating microbial-induced inflammation have been characterized in detail in the past 2 decades, the molecular regulation of sterile inflammation remains poorly understood. Sterile inflammation typically occurs in response to cell death (1) and often leads to detrimental exacerbation of the initial insult. It has been proposed that endogenous molecules released from dying cells, termed damage-associated molecular patterns (DAMPs), exert an essential role in this danger response (1–5). However, the role of specific DAMPs in disease processes in vivo and the mechanisms through which they regulate sterile inflammation remain ill-defined. Defining DAMPs that trigger sterile inflammation in response to cell death is of high relevance, as excessive inflammation often results in collateral damage and contributes significantly to morbidity and mortality (6). Accordingly, pharmacologic targeting of DAMPs may be of substantial therapeutic benefit — across organs — in settings that involve significant tissue injury. A large number of candidates including heat-shock proteins (HSPs), mitochondrial DNA, hyaluronan, formyl peptides, ATP, uric acid, S100B, and high–mobility group box 1 (HMGB1) have been suggested to act as DAMPs (5, 7), but only a few of these molecules have a confirmed DAMP function in vivo. As such, HSPs were once considered key proinflammatory DAMPs, but have recently been suggested to dampen immune responses (8). Likewise, HMGB1 has been suggested to amplify inflammation rather than directly triggering it (9–11). Much of the uncertainty about the role of DAMPs in sterile inflammation arises from a lack of clean genetic approaches that allow assessment of the contribution of single DAMPs to relevant disease settings in vivo. As such, there are virtually no mouse models in which candidate DAMPs have been knocked out. At the same time, most DAMP receptors (for which knockouts exist) are promiscuous and recognize multiple endogenous and bacterial ligands, making it difficult to sort out the contribution of specific DAMPs to relevant disease processes.

HMGB1 is a key DAMP candidate with proposed functions in the regulation of inflammation following tissue injury, LPS–induced shock, and UV irradiation (3, 4, 12, 13). In the healthy organism, HMGB1 functions as a DNA-binding protein that, without apparent sequence specificity, induces bends in the DNA helix, allowing interactions between DNA and proteins such as p53, NF-κB, homeobox-containing proteins, recombinases, and steroid hormone receptors (14). Although mice with global Hmgb1 deletion die shortly after birth (15), several recent studies have shown that intracellular HMGB1 is not required for cell homeostasis and organ function in the healthy adult organism (16–18). During tissue injury or sepsis, HMGB1 is passively released from necrotic cells and actively secreted by inflammatory cells, with signature posttranslational modifications that are characteristic of the respective release mechanism (19). In addition, the oxidation status of critical cysteine residues, which is largely determined by...
Together, these data implicate HMGB1 as a potential therapeutic approach. However, HMGB1 has been shown to act as an amplifier of TLR-dependent inflammation rather than a primary trigger of inflammation. In particular, contaminating DAMPs have been suggested to play a role in proinflammatory effects of recombinant HMGB1, thus challenging its function as a true DAMP. To date, virtually all functions of HMGB1 have been assessed using pharmacologic HMGB1 inhibition, recombinant HMGB1, or cell lines. Of note, previous septic shock studies, in which HMGB1 was shown to mediate lethality, have demonstrated major differences between experimental approaches using TNF- and IL-1-neutralizing antibodies, which prevented LPS-induced lethality, and genetic approaches. In a subsequent study, TNF- and TNFR-, and IL-1R-deficient mice displayed normal sensitivity to LPS-induced septic shock. Subsequent clinical studies that spanned more than a decade did not find clear benefits of TNF neutralization, and this approach has subsequently been abandoned as a therapeutic strategy in septic shock. These findings emphasize the importance of genetic approaches in understanding the contribution of inflammatory mediators to disease processes such as septic shock and tissue injury.

Here, we studied the role of HMGB1 in sterile and LPS-induced inflammation using conditional HMGB1 ablation in a recently generated transgenic mouse. We demonstrate that HMGB1 exerts an essential role in recruiting neutrophils to sites of necrotic tissue injury, resulting in sterile inflammation, injury amplification, and decreased survival. Surprisingly, HMGB1 did not promote inflammation or death in LPS-induced shock. Together, these data implicate HMGB1 as a potential therapeutic target in the setting of tissue necrosis to interrupt the HMGB1-triggered amplification loop that follows organ injury.

Results

**HMGB1 does not mediate LPS-induced lethal shock.** One of the most striking functions of extracellular HMGB1 in the regulation of inflammation has been demonstrated in LPS-mediated septic shock, an often lethal syndrome that affects 750,000 patients annually in the US alone and accounts for 10% of all ICU admissions. A previous study demonstrated that HMGB1 acts as a late mediator of LPS-mediated shock, with a neutralizing HMGB1 antibody increasing survival rates in LPS-treated mice from 0% to 70% (13). To study the contribution of HMGB1 to LPS-mediated shock, we used conditional ablation of HMGB1 using a recently generated transgenic mouse. This well-characterized mouse model does not display altered gene transcription, cellular homeostasis, or organ function after HMGB1 deletion in adulthood, making it ideally suited to investigate HMGB1 contributions to inflammatory disease processes in vivo. Using Mx1-Cre as an inducible and highly efficient strategy to delete Hmgb1 from IFN-responsive cells, we achieved complete inhibition of the LPS-induced increase in serum HMGB1 at early and late time points (Figure 1A) as well as almost complete loss of HMGB1 expression in the liver, bone marrow (BM), and macrophages (Supplemental Figure 1, A–C; full uncut gels are shown in the supplemental material). Despite this efficient reduction of LPS-induced serum HMGB1, we observed no influence on LPS-induced inflammatory gene expression or cytokine secretion and even observed a trend toward slightly increased mortality (Figure 1, B–D), in contrast therefore with results from previous antibody-based studies demonstrating that HMGB1 neutralization was protective against LPS-induced death (13, 31). These data were further confirmed by crossing Vav1-Cre-transgenic mice, which display Cre-mediated recombination in hematopoietic cells (32), with Hmgb1 flox/flox (Hmgb1Δ/Δ) mice, resulting in almost complete loss of HMGB1 expression levels in BM, spleen, and F4/80-positive hepatic macrophages, but no significant reduction in overall HMGB1 levels in the liver (Supplemental Figure 1, D and E). Deletion in endothelial cells, as previously reported for a different Vav1Cre-transgenic mouse (33), occurred only in a minority of endothelial cells in several tested organs including the liver (20.9% ± 0.9%), kidneys (21.1% ± 9.2%), and heart (23.0% ± 3.0%) (Supplemental Figure 1, E and F). As with Mx1-Cre-mediated deletion, Vav1-Cre-mediated Hmgb1 deletion resulted in significantly reduced levels of circulating HMGB1 (Figure 1E), but did not significantly affect LPS-induced lethality at 2 different LPS doses (Figure 1, F and G). Taken together, these data suggest that HMGB1 does not constitute a key mediator of LPS-induced inflammation or lethal shock. Based on these findings, we investigated the hypothesis that HMGB1 operates as a regulator of inflammation in settings other than LPS-induced shock.

**HMGB1 promotes neutrophil but not macrophage migration toward necrotic tissue.** In addition to active secretion from inflammatory cells, e.g., after stimulation with LPS, HMGB1 is also passively released from necrotic cells, consistent with the concept of a proinflammatory DAMP. We therefore tested the role of HMGB1 in host responses to injury using necrotic tissue from Hmgb1-deleted or Hmgb1Δ/Δ control mice. Neutrophils, the first responders to tissue injury, displayed a greater than 7-fold increase in migration toward Hmgb1Δ/Δ tissue lysates. Of note, Hmgb1 deletion almost completely mitigated the ability of tissue lysates to induce neutrophil migration (Figure 2A). In contrast to neutrophils, macrophage migration was not affected by Hmgb1 deletion of tissue lysates (Figure 2A). We made similar observations in vivo, where inflammatory cell infiltration was reduced after i.p. injection of Hmgb1-deleted liver lysates compared with injection of Hmgb1Δ/Δ lysates (Figure 2A). In summary, these data indicate that HMGB1 may be a key DAMP that triggers the migration of neutrophils but not macrophages toward necrotic tissue.

**HMGB1 promotes neutrophil migration and injury amplification after necrosis in vivo.** To test the DAMP function of epithelial HMGB1 in vivo and its role in linking necrosis to neutrophil-mediated inflammatory responses in clinically relevant disease models, we subjected mice with an albumin-Cre-driven hepatocyte-specific Hmgb1 knockout (Hmgb1 ΔCre) to ischemia and reperfusion (I/R) injury. Of note, this hepatic epithelial-knockout mouse shows a virtually complete absence of HMGB1 in HNF4α-
tive hepatocytes and cytotkerin-positive biliary epithelial cells, but preserves HMGB1 expression in other hepatic cell populations including F4/80-positive macrophages, CD31-positive endothelial cells, and desmin-positive hepatic stellate cells, as well as HMGB1 expression in other organs (Supplemental Figure 2, A–F). I/R injury is not only involved in the pathophysiology of a wide range of diseases and in complications of organ transplantation (34), but also represents a prototypical setting in which DAMPs elicit detrimental effects (6). Hmgb1Δhep mice displayed a profound reduction of infiltrating neutrophils and inflammatory gene expression following hepatic I/R injury, whereas the initial injury, as demonstrated by similar serum ALT levels and necrosis area, was similar to that seen in Hmgb1Δhep mice (Figure 2, B–D). Similar to our in vitro data, Hmgb1 ablation in hepatocytes selectively affected neutrophils without impacting the number of F4/80-positive macrophages in the liver (Figure 2E). At later time points, the reduced inflammation translated into amelioration of liver injury (Figure 2, F and G), consistent with the notion that recruited neutrophils promote collateral damage and that HMGB1 is part of this neutrophil-mediated injury amplification loop. To confirm the relevance of HMGB1 as a disease-promoting DAMP in a second clinically relevant disease setting, we subjected Hmgb1fl/fl mice to acetaminophen-induced death of primary hepatocytes (Figure 3B) were similar in Hmgb1Δhep and Hmgb1Δhep mice. Since neutrophil infiltration preceded differences in chemokine expression between Hmgb1Δhep and Hmgb1Δhep mice (Supplemental Figure 4A), the effects of HMGB1 on neutrophils appeared not to be directly caused by HMGB1-mediated modulation of chemokine expression. Accordingly, we found that neutrophil recruitment toward necrotic liver tissue was not altered by pharmacologic inhibition of CXCL2 or its receptor CXCR4 by neutralizing antibodies or AMD3100, respectively (Supplemental Figure 4B). Hence, the differences in chemokine levels between Hmgb1Δhep and Hmgb1Δhep mice we observed at later time points were likely a consequence of different levels of neutrophil-mediated inflammation and liver injury. It is conceivable that these differences in chemokine expression additionally contribute to differences in neutrophil accumulation between Hmgb1Δhep and Hmgb1Δhep mice at later stages. At later time points, we found that the decreased neutrophil infiltration in Hmgb1Δhep mice translated into attenuated liver injury, with striking reductions in serum ALT and AST (Figure 3G) and liver necrosis (Figure 3H). Of note, 100%
not alter inflammation, injury, or survival in FAS- or TNF-mediated apoptotic liver injury (Figure 4, A–J), consistent with the notion that apoptosis is considered a low-inflammatory form of cell death (3). The unaltered hepatocyte injury in apoptotic injury models further corroborates our finding that HMGB1 status does not affect the intrinsic sensitivity of hepatocytes to cell death, thus emphasizing the cell-extrinsic nature of HMGB1-mediated inflammation in necrotic injury models.

HMGB1 from different cellular compartments differentially affects the inflammatory response after necrosis. The contribution of Hmgb1Δhep mice survived a lethal dose of acetaminophen versus only 22% of Hmgb1fl/fl mice (Figure 3I). Despite slight differences in kinetics, these data strikingly resemble our findings in the I/R model, confirming HMGB1 as a bona fide DAMP and a key trigger of an inflammation-mediated injury amplification loop.

HMGB1 does not promote sterile inflammation or injury amplification after apoptosis. To determine whether HMGB1 mediates inflammatory responses to all forms of cell death, we next tested its role in TNF- and FAS-mediated apoptosis. In striking contrast to our findings in necrotic injury, hepatic HMGB1 deficiency did not alter inflammation, injury, or survival in FAS- or TNF-mediated apoptotic liver injury (Figure 4, A–J), consistent with the notion that apoptosis is considered a low-inflammatory form of cell death (3). The unaltered hepatocyte injury in apoptotic injury models further corroborates our finding that HMGB1 status does not affect the intrinsic sensitivity of hepatocytes to cell death, thus emphasizing the cell-extrinsic nature of HMGB1-mediated inflammation in necrotic injury models.

HMGB1 from different cellular compartments differentially affects the inflammatory response after necrosis. The contribution

Figure 2. HMGB1 promotes neutrophil recruitment in vitro and in vivo. (A) Neutrophil and macrophage migration toward Hmgb1-floxed and Hmgb1-deleted (Hmgb1Δhep) liver extracts (induced by Mx1-Cre), determined in Boyden chambers (left 2 panels, n = 3 per group, with representative results from 3 separate isolations). Insert shows immunoblot confirming Hmgb1 deletion. Peritoneal inflammatory cell accumulation after i.p. injection of lysates from Hmgb1fl/fl (n = 7) and Hmgb1Δhep (n = 8) livers. (B–E) Hmgb1fl/fl (n = 8) and Hmgb1Δhep (n = 9) mice were subjected to warm hepatic I/R and sacrificed 6 hours later. H&E staining (B, left panel) and serum ALT (B, right panel) demonstrate similar initial injury, whereas hepatic neutrophil infiltration (C) and hepatic expression of inflammatory genes (D) differed. Numbers of hepatic macrophages determined by F4/80 staining and staining with pan-macrophage antibody in Hmgb1fl/fl (n = 8) and Hmgb1Δhep (n = 9) mice (E). (F and G) Hepatic injury 24 hours after I/R injury in Hmgb1fl/fl (n = 11) and Hmgb1Δhep (n = 10) mice, determined by H&E staining (F) and serum ALT (G). *P < 0.05 and **P < 0.01 by 1-way ANOVA followed by Tukey’s multiple comparisons test (A) and unpaired 2-tailed t test (B–G). Scale bars: 200 μm.
Of HMGB1 from different cellular sources, such as dying epithelial cells and HMGB1-secreting inflammatory cells, remains only poorly understood (4, 38). To investigate whether HMGB1 from BM-derived cells affects injury responses in addition to the observed role of epithelial HMGB1, we used the above-described Vav1-Cre-deleted Hmgb1<sup>ΔBM</sup> mice. In contrast to Hmgb1<sup>Δhep</sup> mice, Hmgb1<sup>ΔBM</sup> mice did not display altered hepatic neutrophil infiltration, inflammatory gene expression, or liver injury following necrosis (Supplemental Figure 5, A–D), thus emphasizing the key role of epithelial HMGB1 in triggering injury responses following necrotic cell death. There are distinct isoforms of HMGB1, including fully reduced HMGB1 and disulfide HMGB1, each in nonacetylated or acetylated form, which are likely to differ in their inflammatory functions (39, 40). Further studies are required to determine the specific functions of these isoforms and how deletion of HMGB1 in epithelial cells shifts the balance.

HMGB1 promotes necrosis-induced sterile inflammation through RAGE. Having established epithelial HMGB1 as a disease-promoting DAMP in clinically relevant settings, we next aimed to identify the receptor through which HMGB1 recruits neutrophils and amplifies injury in vivo, focusing on Toll-like receptor 4 (TLR4) and the receptor for advanced glycation end-products (RAGE) as the best-established HMGB1 receptors (41). Whereas TLR4-deficient neutrophils displayed normal migration toward liver lysates, RAGE-deficient neutrophils showed reduced migration toward liver lysates (42, 43) and did not recruit neutrophils to liver lysates in vivo (Figure 5A). To confirm these results in vivo and to test whether RAGE deficiency affects injury responses in a manner similar to that of HMGB1 ablation, we subjected WT, TLR4-deficient, and RAGE-deficient mice to a sublethal dose of acetaminophen. In line with the above in vitro findings, we did not detect differences in neutrophil infiltration, inflammation,
or liver injury between WT and TLR4-deficient mice (Figure 5, B–E), but noted a profound reduction of these parameters in RAGE-deficient mice, similar to that observed in Hmgb1Δhep mice (Figure 5, F–I). Like Hmgb1Δhep hepatocytes, RAGE-deficient hepatocytes displayed normal acetaminophen metabolization and preserved sensitivity to acetaminophen-induced cell death (Supplemental Figure 6). To substantiate the hypothesis that RAGE expressed on BM-derived inflammatory cells and not other RAGE-expressing cell types is responsible for neutrophil infiltration, inflammation, and injury in response to necrosis, we generated RAGE BM–chimeric mice (Supplemental Figure 7). Following a sublethal dose of acetaminophen, chimeric mice with RAGE-deficient BM displayed strongly reduced neutrophil infiltration, inflammation, and late liver injury (Figure 6, A–D), similar to that seen in mice with global RAGE deficiency. Of note, macrophage depletion did not affect hepatic neutrophil recruitment after acetaminophen treatment (Supplemental Figure 8), confirming that the HMGB1-triggered neutrophil infiltration into the liver was not an indirect effect through RAGE-expressing macrophages. These findings are consistent with our observation that necrotic tissue could directly stimulate the migration of RAGE-expressing, but not RAGE-deficient, neutrophils in vitro (Figure 5A).

HMGB1-recruited neutrophils amplify injury following necrosis. To directly link neutrophil infiltration to amplification of liver injury, we investigated whether inhibition of neutrophil function ameliorates acetaminophen-induced injury. We tested the role of neutrophils in injury amplification using chimeric mice with BM-specific deficiency of neutrophil elastase (ElaneΔ/Δ), a protease that is essential for neutrophil effector functions but not for neutrophil migration (42). Mice with ElaneΔ/Δ BM displayed profoundly reduced necrosis and ALT levels after acetaminophen challenge despite intact hepatic neutrophil recruitment (Figure 6, E–I), thus confirming that neutrophils in injured tissue amplify already existing injury. To further determine whether this HMGB1-dependent neutrophil-mediated injury amplification pathway could be pharmacologically exploited, we tested the effects of the HMGB1 inhibitor glycyrrhizin (12, 43). This inhibitor is a natural component of liquorice and has been widely used for the treatment of liver disease in Asia and Europe (44), but its mechanisms of action...
are not understood. Glycyrrhizin markedly attenuated acetaminophen-induced hepatic inflammation and injury (Supplemental Figure 9) to almost the same degree as that of genetic HMGB1 ablation. Moreover, treatment with glycyrrhizin 2 hours after acetaminophen intoxication also reduced liver injury, albeit to a lesser extent than did pretreatment (Supplemental Figure 9), suggesting the possibility of pharmacologic prevention of HMGB1-mediated injury amplification.

**Discussion**

Using conditional HMGB1 ablation, our study could for the first time to our knowledge reliably characterize the contributory role of HMGB1 to inflammatory processes driven by LPS and necrosis, the 2 proposed key functions of HMGB1. Surprisingly, our results revealed no role of HMGB1 in the promotion of LPS-induced lethal shock. Although we cannot fully explain differences in a previous study by Tracey et al., which demonstrated a 70% reduction of lethality by HMGB1-neutralizing antibodies (13), our approach virtually eliminated LPS-induced serum HMGB1 by 2 genetic approaches and therefore effectively rules out a key role of HMGB1 in promoting LPS-induced lethality. Similar differences between pharmacologic and genetic approaches have been observed in studies of TNF in septic shock in mice (21-27), and subsequent studies in humans have failed to reproduce therapeutic effects of TNF neutralization seen in mice (28). A recent study by Yanai et al., using conditional ablation of HMGB1 exons 2-4 in myeloid cells also reported no protection from LPS-induced lethality by Hmgb1 deletion (18). However, in contrast to our study, Yanai et al. reported only marginally lowered levels of serum HMGB1 levels, thus precluding conclusions about the functional contribution of extracellular HMGB1 to LPS-induced shock (18). In our study, there was even a trend toward slightly increased LPS-induced lethality in mice with Mx1-Cre-mediated Hmgb1 deletion, but not in mice with Vav1-Cre-mediated deletion (at 2 dif-

**Figure 5. RAGE, but not TLR4, mediates neutrophil recruitment following necrosis.** (A) Migration of WT, Tlr4−/−, and RAGE-deficient (RAGE encoded by Ager) neutrophils (Ager−/− neutrophils, n ≥ 2 separate isolations per experiment) toward WT liver lysates. GM-CSF served as a positive control. (B-E) Analysis of hepatic neutrophil infiltration (B), inflammatory gene expression (C), H&E staining and necrosis quantification (D), and serum ALT concentrations (E) in WT (n = 9) and Tlr4−/− (n = 7) mice treated with acetaminophen (300 mg/kg). (F-I) Analysis of hepatic neutrophil infiltration (F), inflammatory gene expression (G), H&E staining and necrosis quantification (H), and serum ALT concentrations (I) in WT (n = 10) and Ager−/− (n = 9) mice treated with acetaminophen (300 mg/kg). *P < 0.05 and **P < 0.01 by unpaired 2-tailed t test. Scale bars: 200 μm.
in response to necrosis but not apoptosis — as HMGB1 ablation did not alter TNF- or FAS-induced injury responses or lethality. Our study further highlights the role of RAGE as the receptor target for HMGB1, promoting neutrophil migration to necrotic tissue. We observed the requirement of RAGE not only in neutrophil migration toward necrotic tissue in vitro, but also in the acetaminophen model in vivo. The contribution of RAGE on non–BM-derived cell populations to acetaminophen-induced neutrophil migration was excluded using BM-chimeric mice. Likewise, we did not observe a role for TLR4, another receptor target of HMGB1, in inflammatory responses to necrotic injury, nor in the migration of neutrophils toward necrotic tissue in vitro. Although liver-resident macrophages typically do not increase following liver injury, BM-derived macrophages often migrate to sites of injury (45). However, we did not observe effects of epithelial Hmgb1 deletion on macrophage migration in vitro, nor on the number of hepatic macrophages in vivo, suggesting that the HMGB1/RAGE axis selectively modulates neutrophil migration toward injured tissue. Moreover, our in vitro migration assays and in vivo data, including experiments in which mice were depleted of macrophages, demonstrate that the HMGB1/RAGE axis provides a direct link between necrotic cells and neutrophils without requirement for macrophage-mediated signals. Using Elane−/− BM-chimeric mice,

Figure 6. RAGE-expressing neutrophils promote injury amplification after necrotic injury. (A–D) Neutrophil infiltration (A), inflammatory gene expression (B), H&E staining and necrosis (C), and serum ALT concentrations (D) in chimeric mice with WT BM (n = 8) and Ager−/− BM (n = 10) treated with acetaminophen (300 mg/kg). (E–I) Chimeric mice with WT BM and Elane−/− BM (n = 12 per group) were treated with acetaminophen (300 mg/kg). Hepatic neutrophil infiltration was determined by staining for Ly6-B (E). BM reconstitution was confirmed by qPCR for Elane in spleens of BM-chimeric mice (F). Hepatic inflammatory gene expression was determined by qPCR (G). Liver injury was assessed by hepatic H&E staining and necrosis quantification (H), and serum ALT concentrations (I). *P < 0.05 and **P < 0.01 by unpaired 2-tailed t test. Scale bars: 200 μm.
our study firmly established injury amplification by HMGB1-recruited neutrophils. Our study focused primarily on pathways through which necrotic tissue triggers neutrophil recruitment and sterile inflammation and did not determine in detail effector pathways through which neutrophils amplify tissue injury. The incomplete inhibition of liver injury in mice lacking Elane is consistent with previous studies that demonstrate the essential role of additional pathways such as proteinase 3 in neutrophil activation (46). Moreover, recent studies have also highlighted the role of additional DAMP receptors such as CLEC12A in the negative regulation of inflammation in response to cell death responses (47), suggesting that these may counteract the proinflammatory effects of HMGB1. Future studies need to further investigate the functional interactions between pro- and antiinflammatory DAMP receptors on neutrophils following necrosis and determine the effectors through which HMGB1-recruited neutrophils exacerbate tissue injury. These are likely to not only include neutrophil proteases and ROS, but possibly also neutrophil extracellular traps, a key effector of neutrophils in infection (48).

Before being known as a DAMP, HMGB1 was described as a nuclear protein with intracellular functions, such as binding of DNA, to facilitate DNA-protein interactions (14). We previously did not find evidence for a requirement of intracellular HMGB1 in the regulation of gene expression, organ function, and viability after tissue-specific ablation of exons 2–4 of Hmgb1 (16), consistent with the lack of a spontaneous phenotype in mice with conditional HMGB1 ablation from other groups (17, 18). Our results indicate that intracellular functions of HMGB1 are likely restricted to development and/or specific cell types and that the main function of HMGB1 in epithelial cells lies in its role as a danger signal in the extracellular space in the context of tissue injury. Consistent with these findings, we also do not observe a role of intracellular HMGB1 in epithelial injury responses: (a) in our 2 necrosis models, initial injury of epithelial cells is similar and only diverges at later time points after differences in inflammation and neutrophil infiltration have occurred; (b) in the acethanophen models, we found APAP metabolism and similar APAP-induced death of primary hepatocytes in floxed and Hmgb1-deleted mice; (c) the sensitivity to FAS- and TNF-induced injury was not altered by HMGB1 status. Consistent with these findings, Yanai et al. found no effect of Hmgb1 deletion on early liver injury after hepatic I/R (18). However, their study did not further investigate DAMP functions of HMGB1, such as neutrophil infiltration, inflammation, and late liver injury (18). In contrast to our study and that of Yanai et al., conditional ablation of HMGB1 exons 2–3 resulted in worsened hepatic injury, even at early time points after I/R, as well as in worsened pancreatitis (17, 49). Of note, the targeting strategy in these studies appears to affect mitochondrial quality and function, which are likely to affect the injury response (17, 49), whereas our approach avoids this potential confounder (16). Our data indicate that HMGB1 derived from different cellular compartments may serve highly context-specific roles, as evidenced by our finding that HMGB1 absence in epithelial cells is correlated with reduced neutrophil recruitment and injury. Importantly, disulfide HMGB1 has recently been shown to exert cytokine-like properties, whereas all-thiol HMGB1 exerts chemotactic activity via CXCL12 heterodimerization and CXCR4 activation (20). Since the chemotactic activity of HMGB1-containing liver extracts toward neutrophils was not mediated via CXCR4, further mechanistic studies are required to determine which isofrom of HMGB1 promotes neutrophil chemotaxis after acute liver injury.

While our study characterized the role of the HMGB1/RAGE axis using the liver as a model system, in which necrotic injury is accompanied by massive neutrophil infiltration and often results in life-threatening acute liver failure, we did not study its contribution to disease processes in other organs or in other contexts. Our data showing reduced neutrophil migration toward HMGB1-deleted cardiac tissue lysates (Supplemental Figure II) suggest, in principle, that the neutrophil-recruiting HMGB1/RAGE axis may be operational in other tissues. Since other DAMPs such as formyl-peptides and SAP130 and their corresponding receptors (FPR-1 and Mincle, respectively) may mediate neutrophil recruitment in settings such as heat-induced liver injury (50) or irradiation-induced thymocyte death (51), the relative contribution of specific DAMPs needs to be further studied and compared between different injury types and organs. Moreover, the role of HMGB1 released from immune cells, e.g., undergoing pyroptosis or NETosis during infection, is likely to differ substantially from HMGB1 released during necrosis (52). In this regard, the benefits of HMGB1-mediated neutrophil recruitment in sterile inflammation and LPS-induced shock need to be better understood. It could be speculated that HMGB1 recruits neutrophils not only to promote phagocytosis of cellular debris, but also to constitute a preemptive strike against secondary infection of necrotic tissue — at the price of increased tissue injury. Likewise, the increase in HMGB1 secretion after LPS — rather than mediating lethal complications of LPS — may be involved in recruiting or activating effector cells in order to fight bacterial infections, as was recently suggested by Yanai et al. (18). Finally, cell death is a key driver of many disease processes including cancer, suggesting that DAMPs such as HMGB1 provide a link between epithelial injury and cancer in the setting of chronic injury.

Methods

Animals. Mice were maintained on a 12-hour dark/12-hour light cycle with free access to food and water unless otherwise indicated. Six- to 8-week-old mice were used for all experiments. Hmgb1cre mice and MHC-Cre mice have been previously described (16). C57BL6 mice, albumin-Cre mice, Mxl1-Cre mice, Varl-Cre mice, and TLR4-knockout mice were obtained from The Jackson Laboratory. RAGE-knockout mice were a gift of Ann-Marie Schmidt (New York University, New York, New York, USA). Mxl1-Cre activation for deletion of Hmgb1 was induced by 3 i.p. injections of poly(I:C) (10 mg/kg; GE Healthcare), given every other day (30).

Models of LPS-induced inflammation and mortality. Four weeks after administration of poly(I:C), Hmgb10/0 and Hmgb1c0/0 mice were injected i.v. with LPS (80 mg/kg) dissolved in saline to induce systemic inflammation and lethal shock. Serum was collected after 3 and 18 hours to determine HMGB1 serum levels by ELISA (IBL International). For some experiments, mice were sacrificed after 18 hours to collect tissue. For survival analysis, animals were injected with 80 mg/kg LPS and monitored without further manipulation. To determine the contribution of HMGB1 from BM-derived cells, Varl-Cre-negative Hmgb1cre and Varl-Cre-positive Hmgb10/0 mice (Hmgb10/0+0/0) were treated accordingly, without prior exposure to poly(I:C).
Liver injury models. Mice were injected i.v. with hamster anti-mouse anti-CD95 antibody (10 μg/mouse, clone Jo2, catalog 554254; BD Biosciences) or with 700 μg/kg D-galactosamine i.p. followed by 100 μg/kg LPS i.v. (both from Sigma-Aldrich) 2 hours later. Mice were sacrificed 4 hours after the LPS injection or monitored for survival. Acetaminophen intoxication was induced in overnight-starved male mice by i.p. injection of 300 mg/kg (sublethal dose) or 500 mg/kg (lethal dose) acetaminophen (Sigma-Aldrich), dissolved in sterile warm saline. For some experiments, mice were pretreated with either clodronate liposomes or PBS liposomes (200 μl/mouse, obtained from Nico van Rooijen, Vrije Universiteit, Amsterdam, The Netherlands), followed by treatment with acetaminophen 96 hours later. For some experiments, mice were pretreated with 50 mg/kg glycyrrhizic acid dissolved in saline (catalog 50531; Sigma-Aldrich). For ischemia/reperfusion experiments, male mice were starved overnight and subjected to warm partial hepatic ischemia as previously described (53). Briefly, after midline abdominal incision, the liver hilum was exposed, and the hepatic artery, portal vein, and bile duct were occluded using a microvascular clamp (Fine Science Tools) proximally to caudate hepatic branches to avoid portal venous congestion. Rapid blanching of the respective liver lobes indicated ischemia. Body temperature was monitored with a UV thermometer (Fisher Scientific) and maintained at 37°C ± 1°C with heat pads and warm lamps. After 60 minutes of ischemia, the clamp was removed, and reperfusion of all segments was visually confirmed. In the event of incomplete reperfusion, the animal was excluded from further analysis.

BM transplantation. BM transplantation (BMT) experiments were performed as previously described (54). Briefly, 5 × 10⁶ BM cells from donor animals were injected i.v. into lethally irradiated (2 × 6 Gy) recipients. Successful BMT in RAGE chimeric mice was confirmed via GFP fluorescence as described (55), taking advantage of GFP expression in Ager⁻/⁻ mice. Successful BMT in Elane chimeric mice was confirmed by quantitative PCR (qPCR) in spleens. APAP treatment was performed 8 weeks after BMT.

Serum transaminase, chemokine, and HMGB1 measurements. Serum ALT and AST measurements were performed using ALT/GPT and AST/GOT kits (both from Thermo Scientific) according to the manufacturer’s instructions and calculated using a standard curve. IL-6 and MCP-1 ELISA (both from R&D Systems) and HMGB1 ELISA (IBL International) were performed according to the manufacturer’s instructions.

Chemotaxis assays. To assess the effects of HMGB1 on neutrophil migration toward necrotic tissue, HMGB1-containing and HMGB1-deficient liver extracts were prepared according the following procedure: Hmgbr3/–/ Mx1-Cre-positive and Mx1-Cre-negative mice were injected 3 times with poly-IC (100 μg/mouse) and were harvested 10 days later. Livers were lysed in sterile PMN buffer (PBS containing glucose and BSA) and subjected to 5 freeze-thaw cycles, followed by centrifugation. For in vitro chemotaxis assays, neutrophils were isolated from the peripheral blood of healthy donors using a histopaque gradient as previously described (56). For additional experiments involving WT and Ager⁻/⁻ neutrophils, neutrophils were isolated from the BM of WT or Ager⁻/⁻ mice as previously described (57). For some experiments, neutrophils and liver extracts were pretreated with either the CXCR4 inhibitor AMD3100 (Sigma-Aldrich) or anti-mouse CXCL2 antibody (AF-452-NA; R&D Systems). Neutrophil chemotaxis through 3-μm inserts (BD) coated with laminin, fibronectin, or fibrinogen was determined in Boyden chambers, with 1 × 10⁴ neutrophils placed in the upper chamber and 30 mg liver extract in the lower chamber, both in sterile PMN buffer. Migration was quantified after 3 hours. Chemotaxis of macrophages was performed in an analogous manner using BM-derived macrophages, isolated as previously described (58), using 8-μm-pore-size filters. For in vivo chemotaxis, 30 mg lyed liver tissue was injected i.p. into recipient mice. After 20 hours, the peritoneum of the recipient mice was lavaged with 4 ml sterile PBS, and cells were counted using a hemocytometer.

RNA extraction, qPCR, Western blotting, and immunohistochemistry. RNA from snap-frozen tissues was column purified (Roche Diagnostics). Following reverse transcription, qPCR was performed as previously described using primer-probe pairs (Applied Biosystems), with normalization to 18S and relative quantification by the standard curve method (59). Electrophoresis of protein extracts and subsequent blotting were performed as previously described (54). Blots were incubated with mouse antibodies against β-actin (A5441; Sigma-Aldrich) or GADPH (G9225; Sigma-Aldrich). Immunohistochemical staining was performed on paraffin-embedded liver sections after 10% formalin fixation using a primary antibody against Ly-6B.2 (clone MCA771G; AbD Serotec) and anti-F4/80 (clone CI:A3-1, catalog MCA497R; AbD Serotec), followed by biotinylated anti–rabbit or anti–rat IgG, respectively, and developed with DAB peroxidase substrate (Vector Laboratories). HMGB1 immunohistochemistry was performed using rabbit anti–HMGB1 (ab18256; Abcam) and anti–rat IgG, respectively, and developed with DAB peroxidase substrate. Neutrophils and liver extracts were isolated from the BM of WT or Elane–/– mice as previously described (56). For additional experiments, neutrophils and liver extracts were isolated from the BM of WT or Ager⁻/⁻ mice as previously described (57).

Hepatocyte isolation. Mouse hepatocytes were isolated as previously described via 2-step collagenase perfusion (59). Hepatocytes were allowed to attach for 90 minutes on collagen-coated plates in RPMI containing 10% FBS (Gibco), followed by overnight starvation in serum-free medium before experiments. Cells were harvested for protein analysis 3 hours after APAP exposure, and cell death was quantified by assessing the percentage of PI-positive nuclei 24 hours after APAP exposure.

Glutathione measurements. Glutathione measurements were performed using a glutathione assay kit according to the manufacturer’s instructions (Cayman Chemicals).

Although analysis of HMGB1 by electrospray ionization liquid chromatography MS/MS was performed, an investigative committee at the University of Liverpool recently identified evidence of data fabrication relating to the MS data contributed by Daniel J. Antoine.
Statistics. All data are expressed as the mean ± SEM. For comparison of 2 groups, an unpaired 2-tailed t or Mann-Whitney U test was used. Differences in survival were compared using a log-rank (Mantel-Cox) test. A P value of less than 0.05 was considered statistically significant.

Study approval. All animal procedures were performed with Columbia University IACUC approval and were in accordance with the Guide for the Care and Use of Laboratory Animals (National Academies Press. 8th edition. Revised 2011). Isolation of neutrophils from the blood of volunteers was performed with Columbia University IRB approval.

Acknowledgments

This study was supported by NIH grants 5R01DK76920 and 1U01AA021912 (to R.F. Schwabe). P. Huebener was supported by a fellowship from the German Research Foundation (Hu 1953/1-1). J.M. Caviglia was supported by NIH grant 1K22CA178098.

Address correspondence to: Robert F. Schwabe, 1150 St. Nicholas Ave., Russ Berrie Pavilion Room 415, New York, New York 10032, USA. Phone: 212.851.5462; E-mail: rfs2102@cumc.columbia.edu.


