High-mobility group box 1 (HMGB1) is a nucleoprotein with proinflammatory functions following cellular release during tissue damage. Moreover, antibody-mediated HMGB1 neutralization alleviates lipopolysaccharide (LPS)-induced shock, suggesting a role for HMGB1 as a superordinate therapeutic target for inflammatory and infectious diseases. Recent genetic studies have indicated cell-intrinsic functions of HMGB1 in phagocytes as critical elements of immune responses to infections, yet the role of extracellular HMGB1 signaling in this context remains elusive. We performed antibody-mediated and genetic HMGB1 deletion studies accompanied by in vitro experiments to discern context-dependent cellular sources and functions of extracellular HMGB1 during murine bloodstream infection with Listeria monocytogenes. Antibody-mediated neutralization of extracellular HMGB1 favors bacterial dissemination and hepatic inflammation in mice. Hepatocyte HMGB1, a key driver of postnecrotic inflammation in the liver, does not affect Listeria-induced inflammation or mortality. While we confirm that leukocyte HMGB1 deficiency effectuates disseminated listeriosis, we observed no evidence of dysfunctional autophagy, xenophagy, intracellular bacterial degradation, or inflammatory gene induction in primary HMGB1-deficient phagocytes or altered immune responses to LPS administration. Instead, we demonstrate that mice devoid of leukocyte HMGB1 exhibit impaired leukocyte recruitment of inflammatory monocytes early during listeriosis, resulting in alterations of the transcriptional hepatic immune response and insufficient control of bacterial dissemination. Bone marrow chimera indicate that HMGB1 from both liver-resident and circulating immune cells contributes to effective pathogen control. Conclusion: Leukocyte-derived extracellular HMGB1 is a critical cofactor in the immunologic control of bloodstream listeriosis. HMGB1 neutralization strategies preclude an efficient host immune response against Listeria. (Hepatology Communications 2021;5:2104-2120).

Inflammation is an integral component of the host response to infectious and sterile injury of vascularized tissues. (1) While the proinflammatory functions of distinct molecular features of pathogens (pathogen-associated molecular patterns [PAMPs]) are well established and their functions increasingly deciphered, signals that stimulate immune responses under sterile conditions remain enigmatic. It is assumed that molecules released from damaged or injured cells can activate immune effectors, often

Abbreviations: BMDM, bone marrow-derived macrophage; Ccl2, chemokine (C-C motif) ligand 2; CD, cluster of differentiation; CFU, colony-forming unit; Cxcl2, C-X-C motif chemokine ligand 2; DAMP, damage-associated molecular pattern; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; fl, floxed; h.p.i., hours postinfection; hep, hepatocyte; HMGB1, high-mobility group box 1; HNF, hepatocyte nuclear factor; IgG, immunoglobulin G; IL, interleukin; L.m., Listeria monocytogenes; LC3, microtubule-associated protein light chain 3; LPS, lipopolysaccharide; Ly6G/C, lymphocyte antigen 6 complex, locus G/C; LysM, lysin motif; MOI, multiplicity of infection; mRNA, messenger RNA; n.s., statistically nonsignificant; NF-kB, nuclear factor kappa B; Nos2, nitric oxide synthase 2; PAMP, pathogen-associated molecular pattern; PMN, polymorphonuclear granulocyte; qPCR, quantitative polymerase chain reaction; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling; WT, wild type.
through shared receptor systems with their PAMP counterparts, to initiate inflammation and wound healing. Very little is known, however, about the specific contributions and mutual interactions of both classes of molecules in the context of infection where pathogen exposure and tissue damage simultaneously affect immune responses.

High-mobility group box 1 (HMGB1) is an abundantly expressed nucleoprotein and considered a prototypical damage-associated molecular pattern (DAMP) with key roles in the initiation of postnecrotic inflammation in various tissues, including the skin, liver, pancreas, skeletal and cardiac muscle, and the central nervous system. Moreover, neutralization of extracellular HMGB1 was shown to alleviate lipopolysaccharide (LPS)-induced septic shock, to reduce lethality in polymicrobial abdominal sepsis, and to attenuate gram-positive bacterial infections in rodents, conclusively indicating that HMGB1 neutralization may be uniformly beneficial during both sterile and infectious inflammatory processes. Consequently, HMGB1 has repeatedly been suggested as a promising therapeutic target for infectious and noninfectious inflammatory diseases.

In a more recent study, however, genetic HMGB1 deficiency in myeloid cells was linked to defective autophagy during abdominal infection, and intracellular effects in HMGB1-depleted peritoneal macrophages were associated with an increased vulnerability of HMGB1-deficient animals to infection. These findings implicate that the main effects of HMGB1 during infection occur intracellularly and generally dispute the prevalent concept of HMGB1 as an extracellular mediator of inflammation (reviewed in Andersson et al. (9)). Even more recently, hepatocyte HMGB1 in particular was identified as a crucial driver of sepsis-induced lethality, further highlighting the complexity of cell- and tissue-specific contributions of HMGB1 to disease pathogenesis. We have previously observed that a highly efficient cell-specific genetic deletion of HMGB1 was not associated with defective autophagy in various cell types during homeostasis or cellular stress and that extracellular HMGB1 derived from parenchymal cells profoundly affects inflammatory and wound healing responses in various tissues, particularly in the liver.

Here, we aimed to assess the role of HMGB1 during bloodstream infection with Listeria monocytogenes, a mouse model of gram-positive bacteremia with high pathogenic relevance for humans and rodents. We performed pharmacologic and genetic HMGB1 deletion strategies followed by multiscale analyses of immune responses to intravenous administration of the pathogen, thus avoiding localized immune responses at the entry site of infection and allowing for a comprehensive analysis of HMGB1 functions during systemic listeriosis. Unlike in LPS-induced shock, antibody-mediated neutralization of extracellular HMGB1 did not alleviate the disease phenotype of systemic listeriosis but effectuated aggravated pathogen burden and exacerbated hepatitis in mice, suggesting a critical role for extracellular HMGB1 during systemic listeriosis. Comparable to intraperitoneal infection, leukocyte HMGB1 was critically required for the immunologic...
control of systemic listeriosis but without altering autophagic responses in HMGB1-deleted tissues or immune cells. Moreover, leukocyte antibacterial functionality, including pathogen uptake and degradation, as well as the induction of proinflammatory genes in response to *Listeria* or LPS were all preserved in the absence of HMGB1. Instead, we demonstrate a key role for leukocyte HMGB1 in the early hepatic recruitment of inflammatory monocytes to mount hepatic inflammatory gene networks in response to disseminated infection. HMGB1 from liver parenchymal cells, on the other hand, did not affect the course of listeriosis over a wide range of inoculation doses. In summary, we identify HMGB1 from myeloid cells as a mediator of host immune responses against circulating *Listeria* by mechanisms differing from localized bacterial infection and conclude that HMGB1 neutralization strategies may not uniformly be beneficial for the host, particularly in the context of disseminated listeriosis.

**Materials and Methods**

**ETHICS STATEMENT**

All animal experiments were approved by the ethics committee of the Behörde für Gesundheit und Verbraucherschutz of the City of Hamburg (permits no. 42/15 and N039/2020) and were conducted under applicable German law governing the care and use of animals for scientific research (Tierschutzgesetz §7 and §8).

**EXPERIMENTAL ANIMALS**

The generation of *Hmgb1*-floxed (f) animals has been described. Hmgb1<sup>fl/fl</sup> mice were crossed with albumin-Cre<sup>(16)</sup> and lysozyme-Cre<sup>(17)</sup> mice (both from Jackson Laboratory). All animals were on a C57BL/6 background and housed under specific pathogen-free conditions in individually ventilated cages with standard food and water *ad libitum* on a 12-hour day/night cycle. Age- and sex-matched control mice from the same colony were used. Mice were injected with LPS or infected with specified doses of wild-type (WT) *Listeria monocytogenes* strain EGD in 200 μL sterile phosphate-buffered saline through the lateral tail vein and analyzed at specified time points post-infection. In some experiments, mice received daily intraperitoneal injections of 100 μg anti-HMGB1 or immunoglobulin G (IgG) control antibodies for 3 consecutive days of the infection. To determine bacterial titers, 200-300 mg of liver tissue was mechanically homogenized in 0.1% Triton X-100 in water, and suspensions were serially diluted, followed by plating on tryptone soya broth agar.

**FLUORESCENCE-ACTIVATED CELL SORTING ANALYSIS**

Cell analysis was performed using a BD LSRFortessa cell analyzer (BD Biosciences). Single-color stainings of each fluorophore (antibodies shown in Supporting Table S1) as well as unstained samples were prepared in order to compensate the fluorescent channels. Cellular events were acquired using BD FACSDiva software (BD Biosciences) and subsequently analyzed using FlowJo software (Tree Star, Inc.). Gating strategies are shown in Supporting Fig. S5.

**WESTERN BLOT, IMMUNOHISTOCHEMISTRY, ENZYME-LINKED IMMUNOSORBENT ASSAY, AND RNA EXPRESSION ANALYSIS**

Interleukin (IL)1β, interferon (IFN)γ (both R&D Systems), and HMGB1 enzyme-linked immunosorbent assays (ELISAs) (IBL International) were performed according to the manufacturers’ instructions. RNA from snap-frozen tissues was column purified using the NucleoSpin RNA kit (Macherey Nagel GmbH and Co. KG, Duren, Germany). Following reverse transcription, quantitative polymerase chain reaction (qPCR) was performed using TaqMan primer-probe pairs (Applied Biosystems) and normalized to 18S by comparative C<sub>T</sub> (ΔΔC<sub>T</sub>, primers shown in Supporting Table S2). Electrophoresis of protein extracts and subsequent blotting were performed as described.<sup>(13)</sup> Blots were incubated with rabbit antibody to HMGB1 (Abcam, ab18256), microtubule-associated protein light chain 3 (LC3; Cell Signaling, 2755), and p62 (Abcam, ab109012) and visualized by the enhanced chemiluminescence light method (Pierce). Blots were reprobed with mouse antibody to -actin (Sigma, A5441) or α-tubulin (Cell Signaling, 3873). Immunohistochemical stainings were performed on paraffin-embedded liver sections after 4% formalin fixation, using primary antibodies against
cluster of differentiation (CD)45 (Abcam, ab10558), F4/80 (Biolegend, 123102), hepatocyte nuclear factor (HNF4)α (Santa Cruz Biotechnology, H-171), and HMGB1 (Abcam, ab18256), followed by horseradish peroxidase-linked anti-rabbit or anti-rat IgG and developed with 3,3’-diaminobenzidine peroxidase substrate (Dako). For immunofluorescence stainings, tissues were fixed using 4% paraformaldehyde, followed by 30% sucrose and frozen. Stainings were performed using antibodies against lymphocyte antigen 6 complex, locus G (Ly6G, phycoerythrin labeled; Biolegend, 127608), p62 (Abcam, ab109012), and L. monocytogenes (Abcam, ab35132), followed by fluorescently labeled secondary antibodies (Thermo Fisher Scientific). Terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining was performed using the In Situ Cell Death Detection Kit (Roche, Germany) according to the manufacturer’s instructions. Microscopy was performed on a Keyence BZ-X710 microscope (Keyence, Japan).

BONE MARROW TRANSPLANTATION

Bone marrow transplantation (BMT) experiments were performed as described. (13) Briefly, 4 × 10⁶ bone marrow cells from donor animals were intravenously injected into lethally irradiated (2 × 6 Gy) recipients. Infection with 2 × 10⁴ colony-forming units (CFU) L. monocytogenes was performed 4 weeks after BMT.

GENERATION AND STIMULATION OF BONE MARROW-DERIVED MACROPHAGES AND PRIMARY POLYMORPHONUCLEAR NEUTROPHILS

Bone marrow was isolated from femurs of naive mice. For the differentiation of bone marrow-derived macrophages (BMDMs), cells were resuspended in growth medium (minimum essential medium alpha + 10% fetal calf serum, 5% antibiotic/antimycotic [Thermo Fisher Scientific]) and 10 ng/μL macrophage colony-stimulating factor (M-CSF; PeproTech). After 7 days of differentiation with M-CSF, BMDMs were left untreated or primed using IFNγ (0.01 μg/mL) for 16 hours. For the phagocytosis and bacterial degradation assays, BMDMs were either exposed to pHrodo-labeled Escherichia coli (Vybrant phagocytosis assay; Thermo Fischer) or infected with L. monocytogenes (multiplicity of infection [MOI], 10) and incubated for 1 hour at 37°C. Listeria-infected cells were washed and suspended in fully supplemented medium containing Gentamicin to neutralize remaining extracellular bacteria. After 30 minutes, cells were again washed and lysed using 0.1% Triton X-100 at the indicated time points, and lysates were plated. In separate experiments, BMDMs were incubated in medium containing LPS (1 μg/mL). For neutrophil isolation, red blood cells were lysed using hypotonic saline, then bone marrow cells were separated using a Histopaque gradient (Thermo Fisher Scientific). BMDMs and neutrophils were incubated with live L. monocytogenes (BMDM MOI, 10; neutrophil-killing MOI, 0.05), as indicated. To analyze autophagic flux, BMDMs were incubated with medium containing L. monocytogenes (MOI, 10) and bafilomycin (30 nM) for 2 hours.

NANOSTRING RNA EXPRESSION AND NCOUNTER DATA ANALYSIS

Analysis was performed on liver samples using the nCounter SPRINTProfiler (NanoString Technologies) and the nCounter mouse myeloid innate immunity panel V2, containing 754 unique gene barcodes in 19 pathways across seven different myeloid cell types. RNA was loaded at 50 ng per sample, and no low-count quality-control flags were observed for any of the samples. Data normalization and differential expression analysis was carried out with nSolver analysis software version 4.0 (NanoString Technologies). Genes with a false discovery rate <0.05 or P < 0.05 for the comparison within control groups/injected groups, respectively, were considered being significantly differentially expressed. Visualization of the normalized counts of differentially expressed genes was performed using the statistical framework R (version 3.5.1), and an overrepresentation enrichment analysis for Gene Ontology terms (biological process) was carried out with WebGestalt (vfcc27621). The complete data set has been deposited at Dryad for reviewing purposes (https://datadryad.org/stash/share/jfT_wz5AB7FiKhPJVcQzLkuwo6CfodmAOM4ehEWx88).
STATISTICS

All data are expressed as mean ± SEM. For comparison of two groups, the Mann-Whitney test was used. For multiple groups, the Kruskal-Wallis test with Dunn's post hoc test was used. Survival curves were compared by the log-rank Mantel-Cox test. *P < 0.05* was considered statistically significant.

Results

ANTIBODY-MEDIATED HMGB1 NEUTRALIZATION FAVORS BACTERIAL DISSEMINATION

In light of the reported beneficial effects of HMGB1-targeted interventions during LPS-induced shock and polymicrobial abdominal sepsis, (4,5) we aimed to assess the consequences of antibody-mediated neutralization of extracellular HMGB1 during murine bloodstream infection with *L. monocytogenes*, a mouse model of gram-positive bacteremia. (18) Daily administrations of well-established HMGB1-neutralizing antibodies (Fig. 1A) did not attenuate the disease phenotype but resulted in higher hepatic bacterial titers 72 hours after infection compared to mice treated with isotype-matched control antibodies (Fig. 1B,C). Fluorescence-activated cell sorting (FACS) analysis demonstrated higher hepatic frequencies of neutrophils but not monocytes or dendritic cells following anti-HMGB1 treatment (Fig. 1D). In summary, antibody-mediated neutralization of extracellular HMGB1 effectuated deficits in bacterial control but not the induction of hepatic inflammation during murine systemic listeriosis.

HEPATOCYTE HMGB1 IS DISPENSABLE FOR THE IMMUNE RESPONSE TO ACUTE LISTERIOsis

During systemic infection with *Listeria*, circulating bacteria are internalized by hepatic and splenic phagocytes and to a lesser extent also enter hepatocytes, triggering protective immune responses. (21) We and others have demonstrated that hepatocyte HMGB1 acts as a key driver of postnecrotic inflammation and maladaptive wound healing in the liver. (13,14,22,23) Intravenous infection with *L. monocytogenes* dose dependently triggered cytosolic HMGB1 translocation in hepatocytes (Fig. 2A). Despite efficient HMGB1 deletion from hepatocytes by Albumin-Cre (*Hmgb1*Δhep; Supporting Fig. S1A-E), (16) HMGB1 serum levels were comparable in *Hmgb1*Δfl and *Hmgb1*Δhep mice (Fig. 2B), suggesting alternative sources of circulating HMGB1 during severe infection. Hepatocyte-specific HMGB1 deficiency also did not affect bacterial dissemination, immune cell recruitment, or microabscess and granuloma formation in the first 72 hours following intravenous injection of *L. monocytogenes*, regardless of the severity of infection (Fig. 2A-D). In terms of inflammatory gene expression, we observed a largely identical induction of key inflammatory cytokines in both genotypes, with an attenuated up-regulation of chemokine (C-C motif) ligand 2 (*Ccl2*) (Fig. 2E). This difference, however, did not affect bacterial titers in the liver 72 hours after infection (Fig. 2C) or infection-induced mortality, as evidenced by comparable survival curves in mice from both groups following administration of a lethal infection dose (2 × 10^5 CFU *L. monocytogenes*) (Fig. 2F). Our findings suggest a subordinate role of hepatocyte HMGB1 in the early immune response to systemic listeriosis and a negligible impact on *Listeria*-induced inflammation in the liver. In conjunction with the effects of antibody-mediated HMGB1 neutralization, the results further indicate that HMGB1 from other cell types may be more relevant to disease pathogenesis. Importantly, while the majority of immune cells constituting hepatic granuloma stained positive for HMGB1, a significant fraction of hepatic immune cells displayed predominantly cytoplasmic, reduced, or even absent immunoreactivity for HMGB1 (Fig. 2A), indicating a potential role for immune cell-derived HMGB1 during listeriosis.

MYELOID CELL HMGB1 DEFICIENCY EFFECTUATES OVERWHELMING HEPATIC INFECTION WITH *L. MONOCYTOGENES*

Kupffer cells are the primary sequestration site of circulating *Listeria*, and phagocytized bacteria were
recently shown to induce necroptosis of liver-resident macrophages, triggering immune responses required for bacterial clearance and the coordinated return to homeostasis.\(^{21,24}\) Myeloid cell-specific HMGB1 ablation by lysin motif (LysM)-Cre effectively deletes HMGB1 from Kupffer cells, monocytes, neutrophils,
and a minority of dendritic cells but not B or T lymphocytes (Hmgb1ΔLysM; Supporting Fig. S1D-G). In contrast to Hmgb1Δhep animals and in line with the aforementioned report, Hmgb1ΔLysM mice displayed approximately 100-fold higher hepatic bacterial titer compared to Hmgb1fl/fl animals 72 hours after intravenous administration of Listeria. This aggravated infection was associated with increased tissue injury and accentuated granuloma formation in the liver but comparable bacterial titers in the spleen (Fig. 3A-C;
FIG. 3. Myeloid-cell HMGB1 deficiency effectuates disseminated listeriosis. (A) H&E stainings of liver sections from Hmgb1<sup>fl/fl</sup> and Hmgb1<sup>ΔLysM</sup> mice at baseline and 72 hours after injection of 2 × 10<sup>4</sup> CFU L. monocytogenes (n = 6–9 animals per group). (B) Hepatic bacterial titers in both groups of mice. (C) FACS analysis of cellular viability of intrahepatic immune cell suspensions. (D) HMGB1 immunostaining of the liver (baseline and after 72 hours) and (E) HMGB1 serum levels assessed by ELISA in Hmgb1<sup>fl/fl</sup> and Hmgb1<sup>ΔLysM</sup> mice over the course of infection. (F) qPCR analysis of key proinflammatory gene expression in the livers of indicated experimental animals. Expression levels are normalized to untreated control mice. (G) FACS analysis of live CD11b<sup>+</sup>Ly6G<sup>+</sup> neutrophils, CD11b<sup>+</sup>Ly6C<sup>+</sup> monocytes, and CD11c<sup>+</sup> dendritic cells at baseline and 72 hours after infection. (H) TUNEL staining and (I) immunostaining for L. monocytogenes (green) and HNF-4α (marking hepatocytes; red) in liver sections of the indicated experimental animals. Single dots indicate individual animals, and vertical bars represent mean ± SEM. Scale bars, (A) 200 μm and (D,H,I) 50 μm. (B,C,E–H) Mann-Whitney test; **P < 0.01. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; H&E, hematoxylin and eosin; IHC, immunohistochemistry.
Supporting Fig. S2A-C). We consistently observed cytoplasmic translocation but not increased expression of Hmgb1 in hepatocytes of Hmgb1ΔLysM mice following intravenous infection with 2 × 10⁴ CFU Listeria, reflecting increased hepatocyte injury and effectuating elevated circulating HMGB1 serum levels (Fig. 3D,E; Supporting Fig S2D,E). Hepatic transcriptional induction of the proinflammatory genes tumor necrosis factor alpha (Tnfa), nitric oxide synthase 2 (Nos2), C-X-C motif chemokine ligand 2 (Cxcl2), and Il1β were all significantly higher in Hmgb1ΔLysM compared to Hmgb1fl/fl animals 72 hours after infection, while adhesion G protein-coupled receptor E1 (Adgre1) (encoding for F4/80) and arginase 1 (Arg1) messenger RNA (mRNA) were significantly reduced (Fig. 3F; Supporting Fig. S2F). Considering only live cells for analysis, we observed comparable hepatic frequencies of inflammatory monocytes and dendritic cells in Hmgb1ΔLysM livers after 72 hours. The frequency of live Cdl11b+Ly6G+ neutrophils was reduced in Hmgb1ΔLysM livers compared to Hmgb1fl/fl mice (Fig. 3G). TUNEL staining revealed that apoptotic cells were mostly located within and in close proximity of hepatic granuloma in Hmgb1ΔLysM animals, whereas very few immune cells were TUNEL positive in Hmgb1fl/fl controls (Fig. 3H). The observed underabundance of neutrophils in Hmgb1ΔLysM may reflect increased leukocyte apoptosis subsequent to overwhelming bacterial burden ²⁵ rather than impaired cellular recruitment in response to infection. Listeria predominantly localized within granuloma (mainly consisting of granulocytes and monocytes), and we did not observe increased numbers of Listeria in surrounding HNF4α+ hepatocytes of Hmgb1ΔLysM mice (Fig. 3I). We thus confirm that leukocyte HMGB1 deficiency effectuates overwhelming infection with Listeria and describe accumulation of apoptotic immune cells within granuloma as a striking hallmark of the disease phenotype in Hmgb1ΔLysM mice.

**PRESERVED AUTOPHAGY INDUCTION AND BACTERICIDAL FUNCTIONALITY IN HMGB1-DEFICIENT LEUKOCYTES FOLLOWING PATHOGEN EXPOSURE**

Increased susceptibility of HMGB1-deficient mice to *Listeria* has been ascribed to impaired autophagy in peritoneal macrophages in an intraperitoneal model of murine listeriosis. ¹⁰ In our *in vivo* infection model, we observed impaired pathogen control in Hmgb1ΔLysM mice following intravenous administration of *L. monocytogenes*, where peritoneal macrophages are not primarily involved. ²¹ We thus re-assessed autophagic responses in the systemic infection model on a cellular and organ level. We did not observe differences in the hepatic conversion of LC3-I to LC3-II or the induction of p62 between Hmgb1fl/fl and Hmgb1ΔLysM mice 24 hours after intravenous infection with *Listeria* (Fig. 4A,B). Both markers are widely used to assess autophagic flux in mammals, ²⁶ and particularly LC3-I conversion to LC3-II has been applied to link HMGB1 to autophagy. ¹⁰ We did, by contrast, observe an increased LC3-II/LC3-I ratio as well as a robust accumulation of p62/sequestosome 1 (SQSTM1), an autophagy receptor with accumulation that is associated with impaired autophagic flux, in whole liver lysates of Hmgb1ΔLysM after 3 days of infection (Fig. 4C,D). Importantly, immunohistochemistry revealed comparable p62 expression levels within hepatic granuloma of both mouse strains but strong p62 accumulation in liver parenchyma outside granuloma (Fig. 4E), indicating that hepatic p62 accumulation is not due to a direct HMGB1-related autophagy defect in myeloid cells but likely a reaction of hepatocytes to excessive bacterial burden in Hmgb1ΔLysM animals. In the same line, we observed near identical p62 accumulation in extracts from Hmgb1fl/fl and Hmgb1ΔLysM BMDMs after infection with *L. monocytogenes* (Fig. 4F) *in vitro* and comparable LC3-II accumulation after additional exposure to bafilomycin to inhibit lysosomal LC3-II degradation ²⁷ (Supporting Fig. S3A), effectively ruling out cell-intrinsic autophagy defects in Hmgb1ΔLysM immune cells in response to infection.

We next tested bactericidal activities of isolated polymorphonuclear granulocytes (PMNs) as well as BMDMs, two main effector cell types of innate antibacterial immunity ²⁴,²⁸ that are targeted by LysM-Cre, ¹⁷ from Hmgb1fl/fl and Hmgb1ΔLysM mice. We observed a similar ~40%-50% reduction of Listeria in the presence of either Hmgb1fl/fl or Hmgb1ΔLysM PMNs *in vitro* after 4 hours (Fig. 4G). Induction of neutrophil cell death in response to Listeria occurred similarly in Hmgb1fl/fl and Hmgb1ΔLysM PMNs (Supporting Fig. S3B). Wild-type but not Hmgb1ΔLysM bone marrow-derived monocytes readily released
substantial amounts of HMGB1 into the medium following exposure to *Listeria* (Fig. 4H). Pathogen uptake into *Hmgb1*-ΔLysM BMDMs was comparable to *Hmgb1*fl/fl BMDMs (Supporting Fig. S3C) and preceded intracellular bacterial degradation independent of the mouse genotype, resulting in >90% degraded
FIG. 4. Autophagy and antibacterial responses in myeloid cells occur independently of their HMGB1 status. (A) Western blotting of LC3-I, LC3-II, p62, and α-tubulin expression in whole liver extracts of Hmgb1Δ/Δ and Hmgb1ΔLysM mice at baseline and 24 hours after intravenous infection with $2 \times 10^4$ L. monocytogenes. (B) Densitometry analysis. (C) Western blotting of aforementioned proteins after 72 hours and (D) corresponding densitometry analysis. (E) Immunofluorescent staining for p62 expression on liver cryosections after 72 hours after infection; scale bars, 50 μm. (F) Western blot analysis of LC3-II, p62, HMGB1, and α-tubulin expression and densitometry of p62 protein in primary isolated BMDMs after in vitro infection with live L. monocytogenes. (G) In vitro bacterial activity of neutrophils from Hmgb1Δ/Δ and Hmgb1ΔLysM mice (MOI, 0.05; n = 6 setups per group). (H) HMGB1 levels in supernatants of untreated and Listeria-stimulated isolated primary BMDMs (MOI, 10). (I) In vitro assessment of intracellular degradation of L. monocytogenes in isolated BMDMs of the indicated genotypes. (J) qPCR analysis of proinflammatory gene expression and (K) ELISA for inflammatory cytokines in the supernatant of primary BMDMs after 4 hours of incubation with L. monocytogenes (MOI, 10). Results are representative of at least three independent experiments. Single dots indicate individual animals, and vertical bars represent mean ± SEM. (H) Kruskal-Wallis test with Dunn’s post hoc test. (I-K) Mann-Whitney test; *P < 0.05. Abbreviations: Gr, granuloma, Med., medium.

### Listeria

8 hours after internalization of bacteria in both groups (Fig. 4I). HMGB1 deletion did not affect inflammatory gene transcription in BMDMs or TNFα or IL1β release at baseline or after bacterial exposure (Fig. 4J,K). To further assess leukocyte functionality in the absence of intracellular HMGB1, we exposed isolated Hmgb1Δ/Δ and Hmgb1ΔLysM splenocytes as well as Hmgb1Δ/Δ and Hmgb1ΔLysM animals to LPS. Both approaches resulted in comparable induction of key inflammatory cytokines in Hmgb1ΔLysM compared to Hmgb1Δ/Δ cells and mice (Supporting Fig. S4A-C), suggesting preserved cellular immune “responsiveness” despite myeloid cell HMGB1 deficiency.

### DIFFERENTIAL MONONUCLEAR CELL RECRUITMENT AND IMMUNE PATHWAY ACTIVATION CONTRIBUTION TO IMPAIRED BACTERIAL CLEARANCE IN Hmgb1ΔLysM

In light of the preserved effector functions of immune cells from Hmgb1Δ/Δ and Hmgb1ΔLysM mice, we aimed to further investigate the impaired immunologic control of systemic listeriosis in Hmgb1ΔLysM animals. We examined immune responses in the early course of infection where bacterial titers diverged between Hmgb1Δ/Δ and Hmgb1ΔLysM animals (Fig. 5A). We observed robust early infiltration of neutrophils into the liver, with a higher frequency in Hmgb1ΔLysM mice after 24 hours, reflecting intact recruitment in both groups proportional to the respective pathogen burden. In contrast, we observed a profound reduction of infiltrating CD11b+Ly6G−Ly6C+ cells and particularly CD11b+Ly6G+Ly6C−high cells into Hmgb1ΔLysM livers (see Supporting Fig. S6 for gating strategies), contrasting the increased bacterial burden (Fig. 5B).

These inflammatory monocytes are rapidly mobilized from the bone marrow and recruited to the liver and spleen where they exert important functions in the orchestration of the immune response to infection. Without the “receptor promiscuity” of HMGB1, which associates with partner molecules, such as LPS, single-stranded DNA, IL1β, and nucleosomes, to enhance activation of their respective receptors, we next studied innate immune activation patterns in livers of Hmgb1Δ/Δ and Hmgb1ΔLysM animals with comparable bacterial titers and thus equivalent exposure to pathogens and associated PAMPs after 24 hours. Heat map analysis demonstrated extensive transcriptional overlap between untreated Hmgb1Δ/Δ and Hmgb1ΔLysM livers, whereas infected mice from both groups clearly clustered according to their genotype (Fig. 5C; Supporting Fig. S5A). Nanostring analysis revealed >log2-fold differential expression of only 48/734 (6.5%) genes involved in innate immune cell regulation between Hmgb1Δ/Δ and Hmgb1ΔLysM livers, with a dominance of proinflammatory gene overexpression in HMGB1-deficient animals. Specifically, we observed a relative underabundance of Cd244, RAS protein-specific guanine nucleotide-releasing factor 2 (Rasgrf2), and linker for activation of T cells family, member 2 (Lat2) expression, which are genes highly enriched in monocytes and macrophages, while Cxcl2, S100 calcium binding protein A8/A9 (S100A8/A9), complement C5a receptor 1 (C5aR1), and Cd14, which are markers predominantly expressed in neutrophils, were all comparably up-regulated in Hmgb1ΔLysM compared to Hmgb1Δ/Δ livers, with only minor alterations of expression levels in the spleen (Fig. 5D; Supporting Fig. S5B). Overrepresentation enrichment analysis of the hepatic data set revealed different patterns of immune pathway activation in Hmgb1Δ/Δ and Hmgb1ΔLysM livers with comparable...
FIG. 5. Impaired monocyte recruitment and differential immune pathway activation in Hmgb1ΔLysM mice early after infection with L. monocytogenes. (A) Hepatic bacterial burden in Hmgb1fl/fl and Hmgb1ΔLysM mice 24 hours after infection (n = 7-8 animals per group) with 2 × 10⁴ CFU L. monocytogenes. (B) FACS analysis of hepatic CD11c+ dendritic cells, CD11b+Ly6G+ neutrophils, and CD11b+Ly6C+ and CD11b+Ly6Chigh monocytes 24 hours after infection. (C) Heat map analysis of hepatic gene expression of Hmgb1fl/fl and Hmgb1ΔLysM mice with identical pathogen burden after 24 hours (marked green in [A]). (D) List of all tested genes with >1 log2 differential expression between infected Hmgb1fl/fl and Hmgb1ΔLysM mice. (E) Overrepresentation enrichment analysis of gene induction in Hmgb1fl/fl and Hmgb1ΔLysM after 24 hours of infection. Single dots indicate individual animals, and vertical bars represent mean ± SEM. (A,B,D) Mann-Whitney test; *P < 0.05, **P < 0.01. Abbreviations: FDR, false discovery rate; h, hours; NIK, NF-κB-inducing kinase; untr., untreated.
bacterial burden. In particular, induction of nuclear factor kappa B (NF-kB)-related pathways was highly enriched in Hmgb1^fl/fl^ livers but largely absent in the livers of Hmgb1^ΔLysM^ animals (Fig. 5E). In contrast, qPCR analysis from severely infected livers revealed timely induction of key proinflammatory genes *Tnf*, *Nos2*, and *Cxc2l2*, which later paralleled the excessive bacterial burden in the hepatic microenvironment, showing that inflammatory gene induction *per se* is not impaired in the absence of leukocyte HMGB1 and eventually reflects the immune response to overwhelming infection and/or increased extracellular HMGB1 concentrations.

**HMGB1 FROM CIRCULATING AND LIVER-RESIDENT IMMUNE CELLS CONTRIBUTES TO MICROBICIDAL ACTIVITY**

Infection with *Listeria* triggers liver-resident phagocyte necroptosis followed by monocyte recruitment and the induction of an antibacterial type 1 inflammatory response. To further elucidate the relative contributions of HMGB1 from liver-resident phagocytes and bone marrow-derived immune cells, we generated Hmgb1^ΔLysM^ bone marrow chimeric mice. Both WT mice replenished with Hmgb1^ΔLysM^ bone marrow and Hmgb1^ΔLysM^ mice with WT bone marrow exhibited impaired clearance of *Listeria*, with an attenuated disease phenotype compared to Hmgb1^ΔLysM^ - Hmgb1^ΔLysM^ chimera (Fig. 6A,B). We observed higher bacterial titers, exacerbated hepatic inflammation, and increased expression of hepatic proinflammatory genes in both Hmgb1^fl/fl^ - Hmgb1^ΔLysM^ and Hmgb1^ΔLysM^ - Hmgb1^fl/fl^ after 3 days (Fig. 6C). Defects in bacterial clearance were marginally more pronounced in WT mice reconstituted with Hmgb1^ΔLysM^ bone marrow; however, HMGB1 from both cell types contributed to immunologic control over *Listeria*, and only HMGB1 deficiency from both cell types effectuated the aforementioned severely adverse phenotype of disseminated infection.

**Discussion**

Inflammation is a critical element of the host response to injury and infection. Whereas molecular signatures of “foreignness” stimulate immune effectors following pathogen exposure, DAMPs are host molecules released from stressed or decaying cells and initiate inflammation and wound healing responses following tissue damage. Intuitively and demonstrably, elevated circulating DAMP levels can also be detected during infections with protozoans, fungi, viruses, and bacteria. The extent of evolutionary conservation and remarkably high expression levels of the prototypical DAMP candidate HMGB1 in virtually all mammalian tissues suggest important functions for the molecule in health and disease, and previous studies have demonstrated that HMGB1 not only triggers postnecrotic inflammation but also mediates lethality in murine LPS-induced shock and promotes tissue injury inferred by gram-positive infections. In light of encouraging results from animal studies using molecular inhibitors or upstream blockade of HMGB1 release even in late phases of septic shock, the molecule has gained significant attention as a potential target for therapeutic interventions. In part due to the early postnatal lethality of global HMGB1 knockout animals, however, our current knowledge about the functions of HMGB1 in *vivo* requires improvement before translation into clinical medicine. Of note, first reports from mice with conditional genetic HMGB1 deletion revealed detrimental effects of myeloid cell HMGB1 deficiency during peritoneal sepsis.

Here, we aimed to address the therapeutic suitability of HMGB1 neutralization during bloodstream infection with *L. monocytogenes*, a widely distributed bacterium causing severe infections predominantly in pregnant women, elderly, and immunocompromised patients. Surprisingly, neutralizing HMGB1 antibodies did not confer host protection but instead resulted in higher bacterial titers, aggravated hepatic inflammation, and exacerbated tissue damage, indicating functions of extracellular HMGB1 that are critical for pathogen defense during incipient infection with *L. monocytogenes*. The reasons for these remarkably divergent consequences of antibody-mediated HMGB1 neutralization between injury models remain elusive but may be attributed to nonspecific effects of employed antibody formulations, highly context-specific functions of HMGB1 in different disease scenarios (i.e., pathogen-specific mechanisms of tissue injury) and in different disease stages, and variations in mouse strain genetics or other confounders (i.e., composition of the intestinal microbiome).
which may all affect experimental outcomes. In our analysis, translocation of HMGB1 into the cytoplasm of hepatocytes and high serum HMGB1 levels were exclusively observed during aggravated infection and may be driven by pathogen-induced signaling, i.e., mediated by interferons. Importantly, however, hepatocyte-specific HMGB1 deletion did not affect bacterial burden, hepatic inflammation, or animal survival over a wide range of pathogen concentrations. Our findings thus constitute a remarkable contrast to the important roles of hepatocyte HMGB1 during sterile hepatic necroinflammation and LPS-induced shock. Similar to antibody-mediated HMGB1 neutralization strategies, it is conceivable that peculiarities of the mode of tissue damage as well as posttranslational HMGB1 modifications, affected by the mode of HMGB1 secretion and physicochemical properties of the extracellular microenvironment, all determine the context-dependent functionality of HMGB1. Distinct differences in inflammatory gene expression observed in Hmgb1Δhep animals at high infection doses may furthermore affect less immediate aspects of infection biology, i.e., induction of wound healing or adaptive immune responses, and require further investigation.

Importantly, our findings from antibody-mediated HMGB1 neutralization were confirmed in mice with myeloid cell-specific HMGB1 deletion during systemic listeriosis. The finding is in general accordance with a report in which the increased sensitivity to the infection was noted.
of mice with myeloid-cell specific HMGB1 deletion toward intraperitoneal infection was ascribed to impaired autophagy in peritoneal phagocytes, arguing against the concept of HMGB1 as an extracellular mediator of tissue-damage signaling. Several other studies have associated HMGB1 with autophagy in different disease scenarios.\textsuperscript{(39–41)} Using our own conditional HMGB1-knockout strategy, however, we have not detected any phenotypic or metabolic alterations typically associated with defective autophagy in HMGB1-deficient cells, including hepatocytes, macrophages, mouse embryonic fibroblasts, and cardiac muscle cells, at baseline or under conditions of cellular stress.\textsuperscript{(12)} Similarly, in the present study, we did not observe autophagy defects in BMDMs or livers of Hmgb1\textsuperscript{ΔLy5M} mice as a potential mechanism of impaired pathogen clearance following intravenous infection. While we did observe hepatic accumulation of p62 in Hmgb1\textsuperscript{ΔLy5M} mice, immunohistochemistry revealed p62 expression almost exclusively in hepatocytes surrounding granuloma, potentially constituting a “stress response” in response to overwhelming bacterial burden and inflammatory excess. In fact, induction of p62 expression (rather than inhibition of its degradation) has been observed in other disease settings\textsuperscript{(42,43)} and may reflect a defense mechanism by directing bacteria toward degradation in hepatocytes.\textsuperscript{(44)} On a more functional level, we observed intact phagocytic activity, intracellular bacterial degradation, and inflammatory cytokine induction in HMGB1-deficient monocytes as well as preserved bactericidal activity of neutrophils in the absence of intracellular HMGB1, suggesting that during listeriosis, these critical immunologic functions are induced by PAMPs and/or other DAMPs. The divergent findings between our own experimental results and those from Yanai et al.\textsuperscript{(10)} regarding autophagy require further exploration but may partly originate from alterations of pathogen and/or host biology inferred by the route of pathogen administration, i.e., local inflammation following intraperitoneal injection compared to intravenous inoculation. Regardless, we conclude that during bloodstream infection with Listeria, the phenotype of uncontrolled hepatic infection in Hmgb1\textsuperscript{ΔLy5M} persists despite intact autophagic responses in involved phagocyte populations. We argue that HMGB1 released from phagocytes regulates hepatic immune responses through modulation of downstream immune effectors. In line with this argument, we report that Hmgb1\textsuperscript{ΔLy5M} mice display an impaired early hepatic recruitment of inflammatory monocytes, a cell population that is mobilized from the bone marrow during bacterial infection in a chemokine (C-C motif) receptor 2 (CCR2)-dependent manner,\textsuperscript{(45)} and subsequently enters the liver and spleen to mediate immune responses.\textsuperscript{(29,30,46)} Consequently, profiling of hepatic transcriptional responses to infection in Hmgb1\textsuperscript{ΔLy5M} and Hmgb1\textsuperscript{ΔLy5M} mice revealed expression profiles specific for the respective mouse genotypes, and overrepresentation enrichment analysis displayed a remarkable scarcity of pathways related to NF-κB activation in Hmgb1\textsuperscript{ΔLy5M} livers despite identical pathogen burden.

These findings likely reflect differential leukocyte recruitment to the liver early during infection (as evidenced by our FACS analysis) rather than a specific inhibition of NF-κB-related pathways because proinflammatory cytokine induction subsequently occurred concomitantly to increased bacterial burden in mice of both genotypes. Thus, impaired recruitment and activation of inflammatory monocytes in the early course of infection likely accounts for an immunologic disadvantage of Hmgb1\textsuperscript{ΔLy5M} mice, effectuating a failure to control Listeria with exacerbated pathogen-induced hepatitis at later stages of infection. Of note, HMGB1 serum levels were comparable or even elevated in mice with hepatocyte or myeloid HMGB1 deficiency, respectively, arguing for alternative sources of HMGB1 that were not affected by our genetic deletion strategies but are involved as critical sources of circulating HMGB1, i.e., vascular endothelial cells and platelets, with functions as HMGB1 donors in the context of listeriosis that remain to be outlined.

Finally, our experiments with mixed bone marrow chimera suggest a framework in which bone marrow-derived cells (i.e., PMNs), which are recruited to the liver in large numbers early after infection, signal to inflammatory monocytes through HMGB1 to attain their recruitment and induction of transcriptional changes required to mount a sufficient innate immune response. The (attenuated) increased susceptibility of Hmgb1\textsuperscript{ΔLy5M} \textsuperscript{ΔLy5M} to infection, however, also argues for HMGB1 signaling from tissue-resident to circulating immune cells during infection, which may affect cellular recruitment and survival, as suggested by high numbers of apoptotic immune cells in granuloma of Hmgb1\textsuperscript{ΔLy5M} mice. It appears that HMGB1 signaling is a dynamic function of time and pathogen...
burden, with parenchymal cells, tissue-resident cells, and circulating immune cells signaling through HMGB1 in context-dependent manners. HMGB1 secretion as well as passive release (i.e., in neutrophil extracellular traps from immune cells\textsuperscript{47}) likely contribute to circulating HMGB1 levels and may exert temporally and locally restricted functions that require further investigation.

In summary, we report an important role for extracellular HMGB1 in the immune response to bloodstream listeriosis. Our results indicate that while immune cell functionality is preserved in the absence of intracellular HMGB1, myeloid cells communicate through HMGB1 during the early phase of infection to direct immune cell migration to the liver and to mount local signaling pathways required for an effective pathogen-directed immune response. Hepatocyte HMGB1 is released when pathogen burden and tissue damage exceed certain thresholds but does not affect hepatic inflammation or early Listeria\textemdash induced lethality. Additional experimental investigations into the context-dependent effects of HMGB1 and its molecular isoforms are needed to precisely identify clinical contexts in which modulation of HMGB1 signaling may confer a benefit for an organism.

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Author names in bold designate shared co-first authorship.

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