Leukocyte-Derived High-Mobility Group Box 1 Governs Hepatic Immune Responses to *Listeria monocytogenes*

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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 hepatic 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.

Received February 24, 2021; accepted June 13, 2021.

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep4.1777/suppinfo.

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Supported by the German Research Foundation (grant HU1953/2-1 to P.H.) and the Clinician-Scientist Program of the University Medical Center Hamburg-Eppendorf (to P.H.).

through shared receptor systems with their PAMP counterparts, to initiate inflammation and wound healing.[2] 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.[3] 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,[4–7] 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.[8,9] 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.[10] 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,[11] 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[12] and that extracellular HMGB1 derived from parenchymal cells profoundly affects inflammatory and wound healing responses in various tissues, particularly in the liver.[13,14]

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

View this article online at wileyonlinelibrary.com.
DOI 10.1002/hep4.1777
Potential conflict of interest: Dr. Schwabe has received grants from Takeda. The other authors have nothing to report.

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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 Behoerde 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.[12,15] *Hmgb1*$^{fl/fl}$ mice were crossed with albumin-Cre[16] and lysozyme-Cre[17] 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 immosorbent 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_t ($\Delta\Delta C_t$, primers shown in Supporting Table S2). Electrophoresis of protein extracts and subsequent blotting were performed as described.[13] 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 (HNF)4α (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, 4×10^6 bone marrow cells from donor animals were intravenously injected into lethally irradiated (2 × 6 Gy) recipients. Infection with 2×10^4 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 stimulated 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 SPRINT Profiler (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_wz5AB7FrKhPjVcQzLkuwo6CfodmAOM4eehEWx88).

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)[19,20] 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), and hepatic inflammatory cytokine induction was comparable or higher following HMGB1 neutralization when compared to controls (Fig. 1E). 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*$^{\Delta hep}$; Supporting Fig. S1A-E),[16] HMGB1 serum levels were comparable in *Hmgb1*$^{fl/fl}$ and *Hmgb1*$^{\Delta hep}$ 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*) in *Hmgb1*$^{\Delta hep}$ mice 72 hours after injection of 1×10^5 CFU *L. monocytogenes* (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

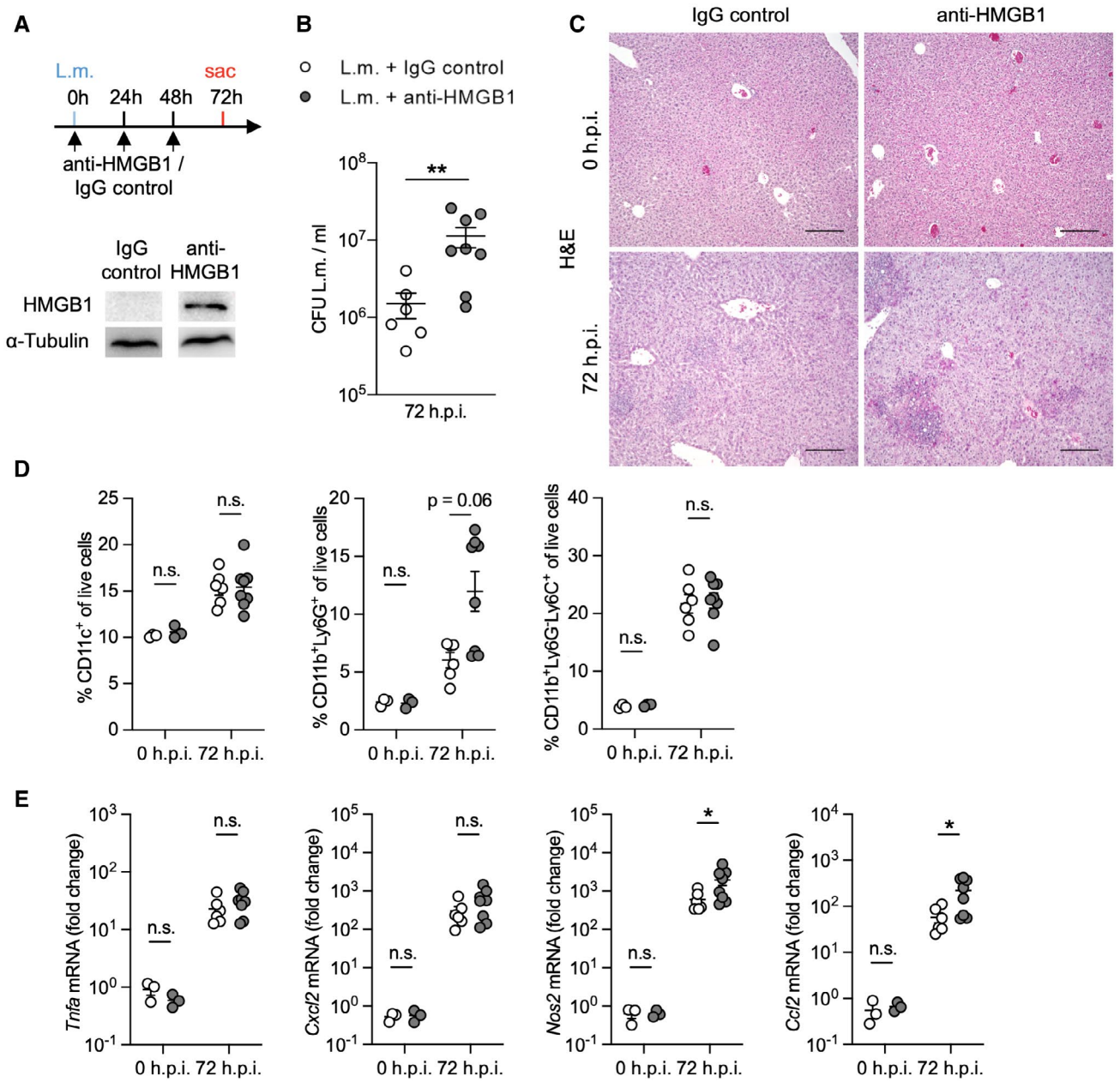

FIG. 1. Antibody-mediated HMGB1 neutralization impairs clearance of *L. monocytogenes*. (A) Experimental setup and assessment of the respective antibody affinities toward HMGB1 by western blot analysis, using WT whole liver lysates. (B) Hepatic titers of *L. monocytogenes* in anti-HMGB1-treated and IgG control-treated mice 72 hours after intravenous injection of 2×10^4 *L. monocytogenes* (n = 6 and n = 8 animals per group, respectively). (C) Photomicrographs of H&E-stained liver sections; scale bars, 200 μm. (D) FACS analysis of $CD11c^+$ dendritic cells, $CD11b^+Ly6G^+$ neutrophils, and $CD11b^+Ly6C^+$ monocytes at baseline and 72 hours after infection. (E) qPCR analysis of hepatic expression of key proinflammatory genes at baseline and 72 hours after infection with *Listeria*. Expression levels are normalized to untreated control mice. Single dots indicate individual animals, and vertical bars represent mean ± SEM. (B,D,E) Mann-Whitney test; $^*P < 0.05$, $^{**}P < 0.01$. Abbreviations: h, hours; H&E, hematoxylin and eosin; sac, animal sacrification.

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,

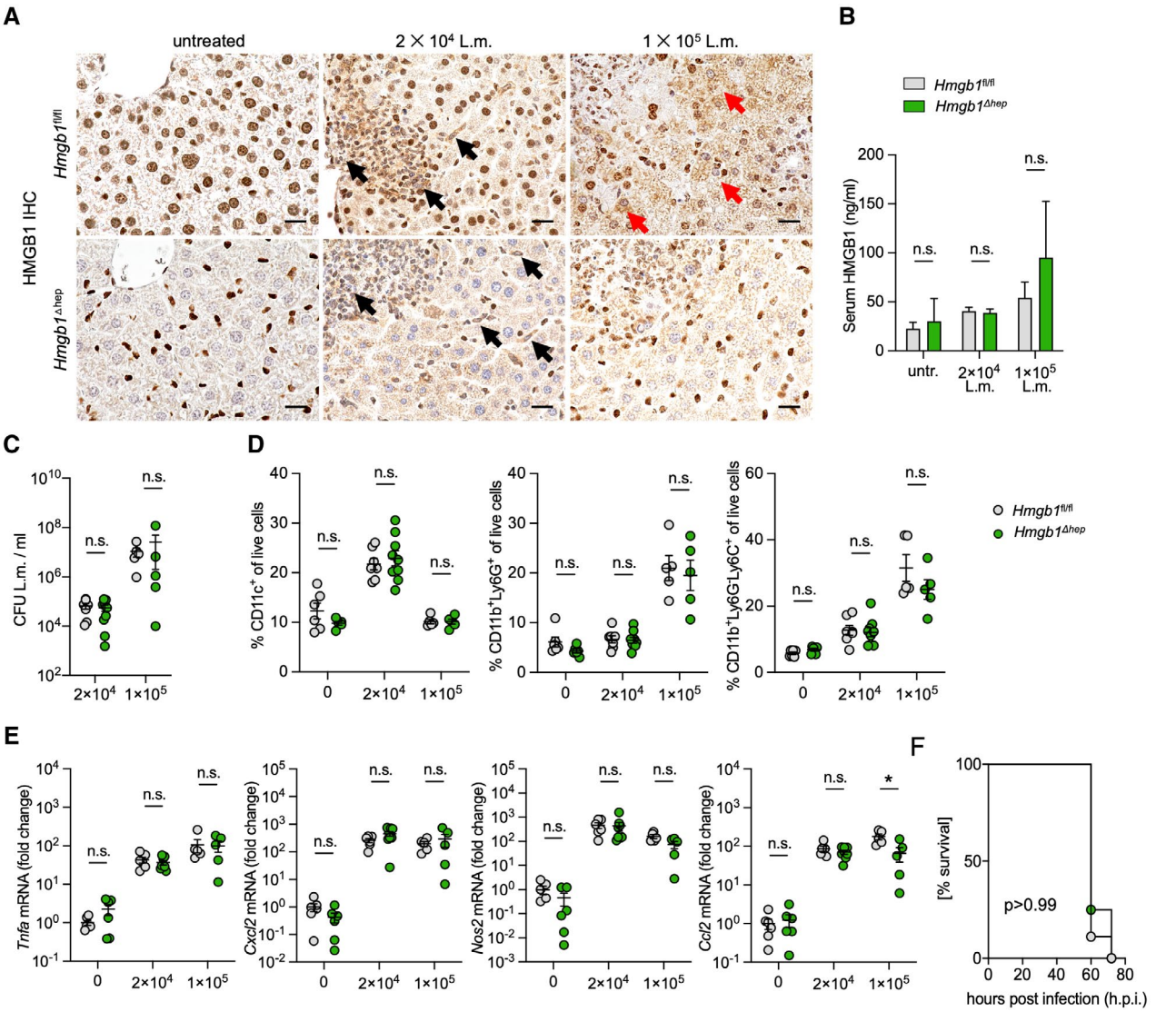

FIG. 2. Hepatocyte HMGB1 is dispensable for antibacterial immunity. (A) HMGB1 immunohistochemistry of liver sections from *Hmgb1*$^{fl/fl}$ and *Hmgb1*$^{\Delta hep}$ mice at baseline and 72 hours after the specified infection doses. Black and red arrows indicate HMGB1 translocation in infiltrating immune cells and in hepatocytes, respectively; scale bars, 50 μm. (B) Serum HMGB1 levels in *Hmgb1*$^{fl/fl}$ and *Hmgb1*$^{\Delta hep}$ mice at baseline and 72 hours after the specified injection doses. (C) Hepatic titers of *L. monocytogenes* in *Hmgb1*$^{fl/fl}$ and *Hmgb1*$^{\Delta hep}$ animals 72 hours after injection of 2 × 10^4 and 1 × 10^5 CFU *L. monocytogenes*. (D) FACS analysis of live hepatic CD11c$^+$ dendritic cells, CD11b$^+$Ly6G$^+$ neutrophils, and CD11b$^+$Ly6C$^+$ monocytes in *Hmgb1*$^{fl/fl}$ and *Hmgb1*$^{\Delta hep}$ mice at baseline and 72 hours after infection with the specified doses of *L. monocytogenes*. (E) qPCR analysis of hepatic proinflammatory *Tnfα*, *Cxcl2*, *Nos2*, and *Ccl2* in *Hmgb1*$^{fl/fl}$ and *Hmgb1*$^{\Delta hep}$ mice at baseline and 72 hours after injection of the specified doses of *L. monocytogenes*. Expression levels are normalized to *18S* and shown as fold induction from untreated control mice. Single dots indicate individual animals, and vertical bars represent mean ± SEM. (F) Survival curves of experimental mice following injection of 2 × 10^5 *Listeria* (n = 8 mice per group). (B-E) Mann-Whitney test. (F) Log-rank (Mantel-Cox) test; $^*P < 0.05$. Abbreviations: IHC, immunohistochemistry; untr., untreated.

and a minority of dendritic cells but not B or T lymphocytes (*Hmgb1*$^{\Delta LysM}$; Supporting Fig. S1D-G).[17] In contrast to *Hmgb1*$^{\Delta hep}$ animals and in line with the aforementioned report,[10] *Hmgb1*$^{\Delta LysM}$ mice displayed approximately 100-fold higher hepatic bacterial titer compared to *Hmgb1*$^{fl/fl}$ 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;

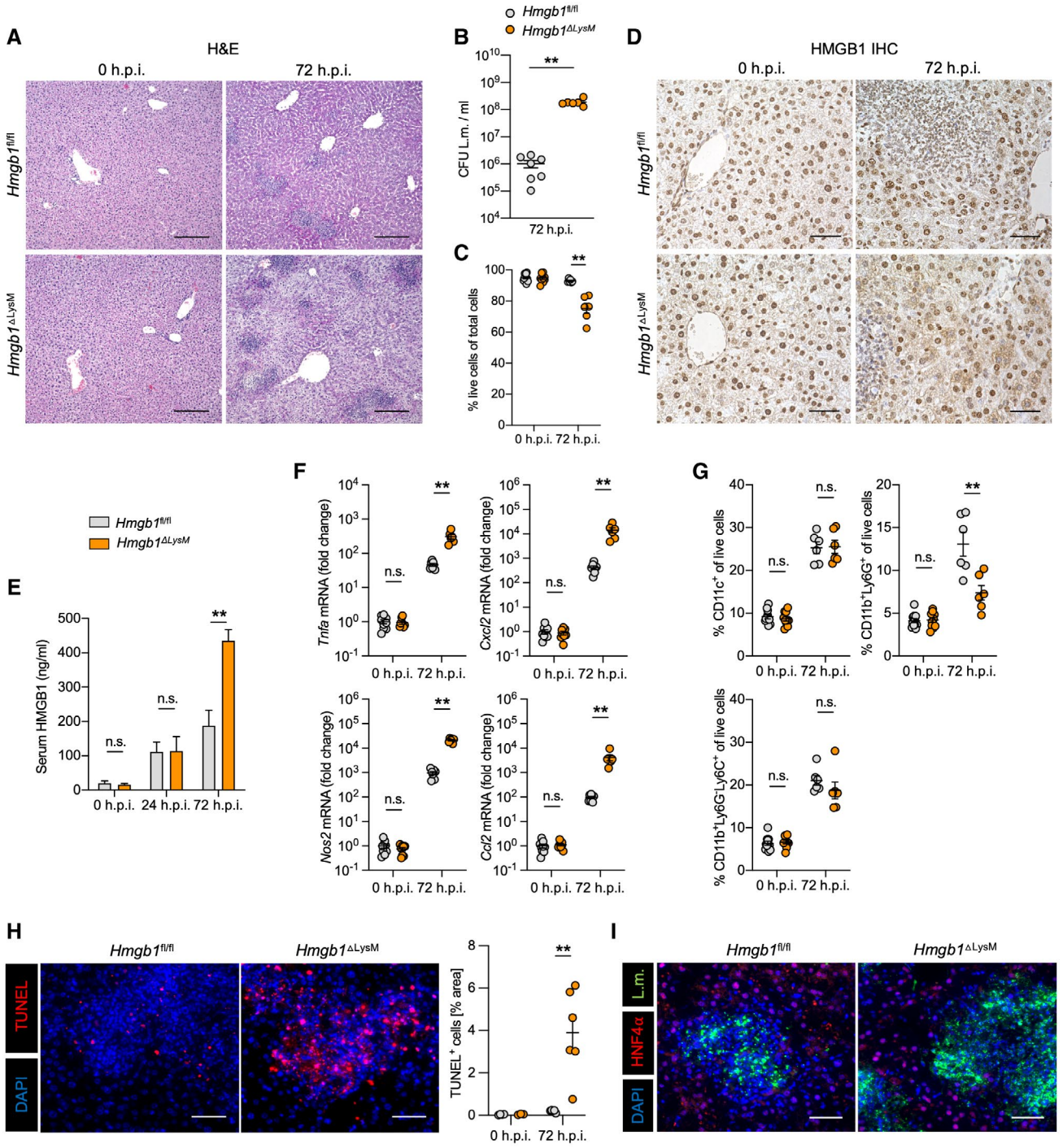

FIG. 3. Myeloid-cell HMGB1 deficiency effectuates disseminated listeriosis. (A) H&E stainings of liver sections from *Hmgb1*$^{fl/fl}$and *Hmgb1*$^{\Delta LysM}$ mice at baseline and 72 hours after injection of 2 × 10^4 CFU *L. monocytogenes* (n = 6-9 animals per group). (B) Hepatic bacterial titers 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*$^{fl/fl}$ and *Hmgb1*$^{\Delta LysM}$ 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 $CD11b^+Ly6G^+$ neutrophils, $CD11b^+Ly6C^+$ monocytes, and $CD11c^+$ 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 *Hmgb1*$^{\Delta LysM}$ mice following intravenous infection with 2×10^4 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 (*Tnfα*), nitric oxide synthase 2 (*Nos2*), C-X-C motif chemokine ligand 2 (*Cxcl2*), and *Il1β* were all significantly higher in *Hmgb1*$^{\Delta LysM}$ compared to *Hmgb1*$^{fl/fl}$ 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 *Hmgb1*$^{\Delta LysM}$ livers after 72 hours. The frequency of live Cd11b$^+$Ly6G$^+$ neutrophils was reduced in *Hmgb1*$^{\Delta LysM}$ livers compared to *Hmgb1*$^{fl/fl}$ mice (Fig. 3G). TUNEL staining revealed that apoptotic cells were mostly located within and in close proximity of hepatic granuloma in *Hmgb1*$^{\Delta LysM}$ animals, whereas very few immune cells were TUNEL positive in *Hmgb1*$^{fl/fl}$ controls (Fig. 3H). The observed underabundance of neutrophils in *Hmgb1*$^{\Delta LysM}$ 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 *Hmgb1*$^{\Delta LysM}$ 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 *Hmgb1*$^{\Delta LysM}$ 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 *Hmgb1*$^{\Delta LysM}$ 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 *Hmgb1*$^{fl/fl}$ and *Hmgb1*$^{\Delta LysM}$ 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 *Hmgb1*$^{\Delta LysM}$ after 3 days of infection (Fig. 4C,D). Importantly, immunohistochemistry revealed comparable low 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 *Hmgb1*$^{\Delta LysM}$ animals. In the same line, we observed near identical p62 accumulation in extracts from *Hmgb1*$^{fl/fl}$ and *Hmgb1*$^{\Delta LysM}$ 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 *Hmgb1*$^{\Delta LysM}$ 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 *Hmgb1*$^{fl/fl}$ and *Hmgb1*$^{\Delta LysM}$ mice. We observed a similar ~40%-50% reduction of *Listeria* in the presence of either *Hmgb1*$^{fl/fl}$ or *Hmgb1*$^{\Delta LysM}$ PMNs *in vitro* after 4 hours (Fig. 4G). Induction of neutrophil cell death in response to *Listeria* occurred similarly in *Hmgb1*$^{fl/fl}$ and *Hmgb1*$^{\Delta LysM}$ PMNs (Supporting Fig. S3B). Wild-type but not *Hmgb1*$^{\Delta LysM}$ bone marrow-derived monocytes readily released

substantial amounts of HMGB1 into the medium following exposure to *Listeria* (Fig. 4H). Pathogen uptake into *Hmgb1*$^{\Delta LysM}$ 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

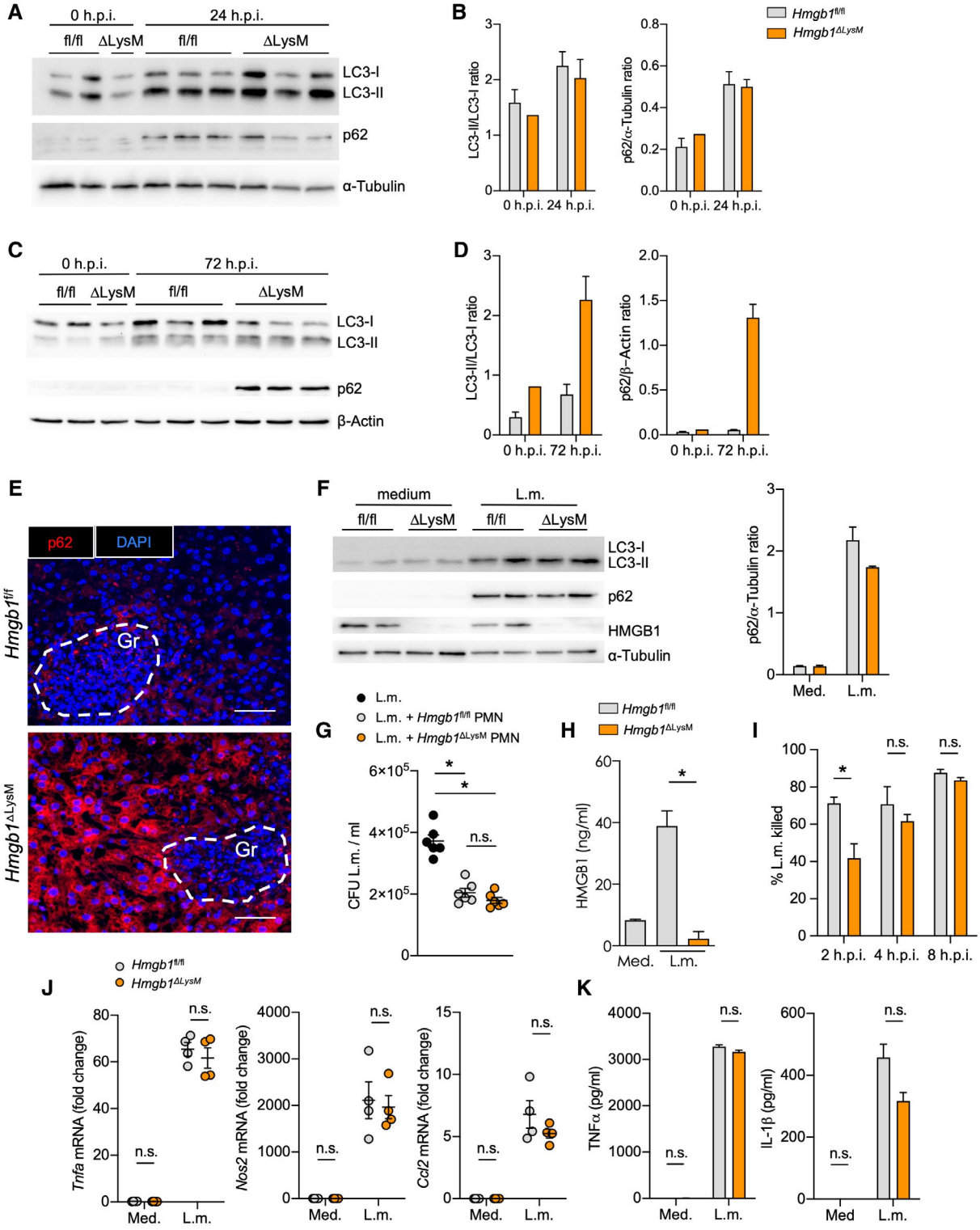

FIG. 4. Autophagy and antibacterial responses in myeloid cells occur independently of their HMGB1 status. (A) Western blotting of LC3-I, LC3-II, p62, and α-tubulin expression in whole liver extracts of *Hmgb1*$^{fl/fl}$ and *Hmgb1*$^{\Delta LysM}$ mice at baseline and 24 hours after intravenous infection with 2 × 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 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* bactericidal activity of neutrophils from *Hmgb1*$^{fl/fl}$ and *Hmgb1*$^{\Delta LysM}$ 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 *Hmgb1*$^{fl/fl}$ and *Hmgb1*$^{\Delta LysM}$ splenocytes as well as *Hmgb1*$^{fl/fl}$ and *Hmgb1*$^{\Delta LysM}$ animals to LPS. Both approaches resulted in comparable induction of key inflammatory cytokines in *Hmgb1*$^{\Delta LysM}$ compared to *Hmgb1*$^{fl/fl}$ 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 CONTRIBUTE TO IMPAIRED BACTERIAL CLEARANCE IN *Hmgb1*$^{\Delta LysM}$

In light of the preserved effector functions of immune cells from *Hmgb1*$^{fl/fl}$ and *Hmgb1*$^{\Delta LysM}$ mice, we aimed to further investigate the impaired immunologic control of systemic listeriosis in *Hmgb1*$^{\Delta LysM}$ animals. We examined immune responses in the early course of infection where bacterial titers diverged between *Hmgb1*$^{fl/fl}$ and *Hmgb1*$^{\Delta LysM}$ animals (Fig. 5A). We observed robust early infiltration of neutrophils into the liver, with a higher frequency in *Hmgb1*$^{\Delta LysM}$ 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 *Hmgb1*$^{\Delta LysM}$ 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.[29,30] Given 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,[31] we next studied innate immune activation patterns in livers of *Hmgb1*$^{fl/fl}$ and *Hmgb1*$^{\Delta LysM}$ 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 *Hmgb1*$^{fl/fl}$ and *Hmgb1*$^{\Delta LysM}$ 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 *Hmgb1*$^{fl/fl}$ and *Hmgb1*$^{\Delta LysM}$ 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,[32] were all comparably up-regulated in *Hmgb1*$^{\Delta LysM}$ compared to *Hmgb1*$^{fl/fl}$ 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 *Hmgb1*$^{fl/fl}$ and *Hmgb1*$^{\Delta LysM}$ livers with comparable

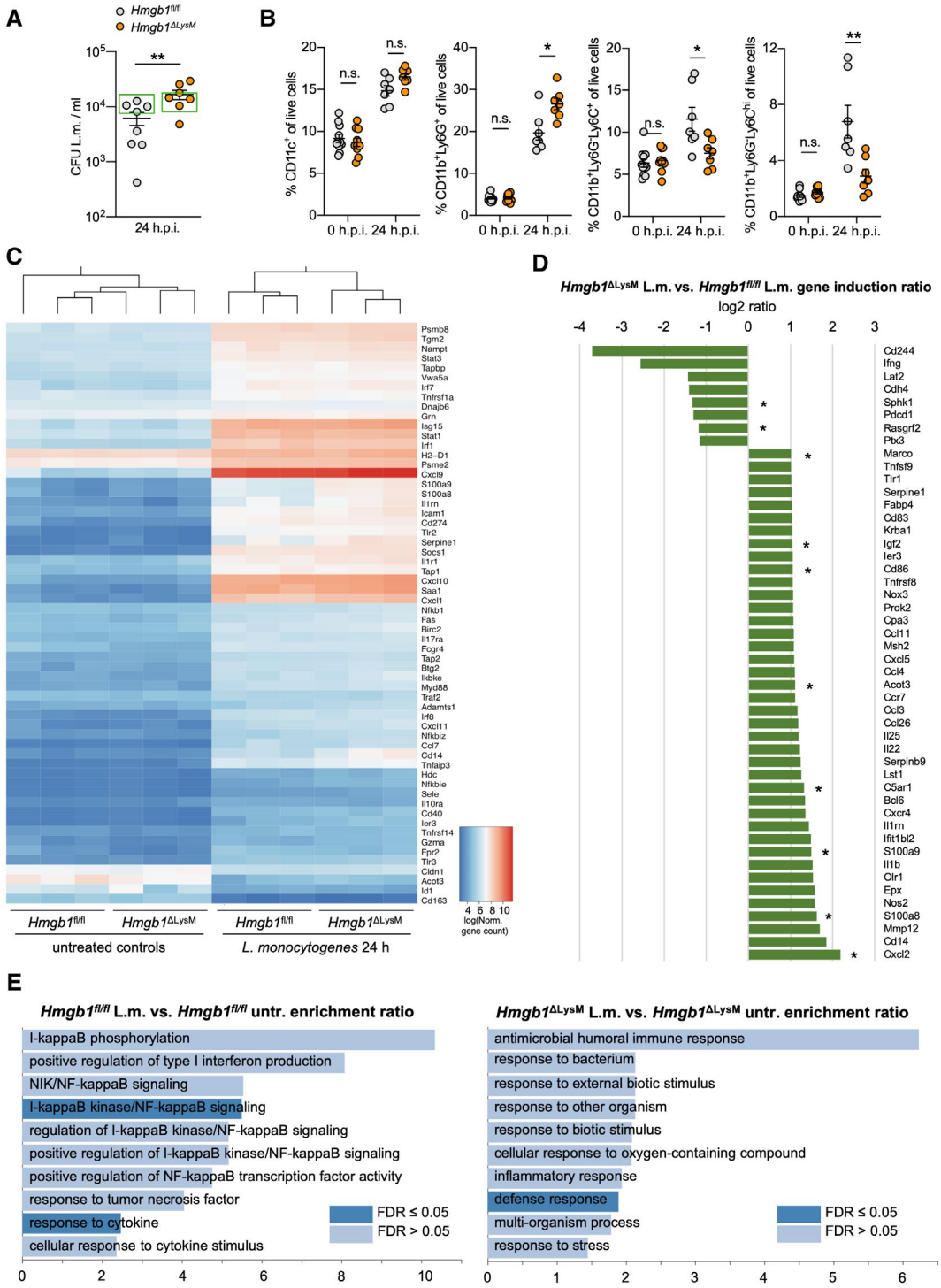

FIG. 5. Impaired monocyte recruitment and differential immune pathway activation in *Hmgb1*$^{\Delta LysM}$ mice early after infection with *L. monocytogenes*. (A) Hepatic bacterial burden in *Hmgb1*$^{fl/fl}$ and *Hmgb1*$^{\Delta LysM}$ mice 24 hours after infection (n = 7-8 animals per group) with 2×10^4 CFU *L. monocytogenes*. (B) FACS analysis of hepatic CD11c$^+$ dendritic cells, CD11b$^+$Ly6G$^+$ neutrophils, and CD11b$^+$Ly6C$^+$ and CD11b$^+$Ly6C^{high} monocytes 24 hours after infection. (C) Heat map analysis of hepatic gene expression of *Hmgb1*$^{fl/fl}$ and *Hmgb1*$^{\Delta LysM}$ 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 *Hmgb1*$^{fl/fl}$ and *Hmgb1*$^{\Delta LysM}$ mice. (E) Overrepresentation enrichment analysis of gene induction in *Hmgb1*$^{fl/fl}$ and *Hmgb1*$^{\Delta LysM}$ 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-κB)-related pathways was highly enriched in *Hmgb1*$^{fl/fl}$ livers but largely absent in the livers of *Hmgb1*$^{\Delta LysM}$ animals (Fig. 5E). In contrast, qPCR analysis from severely infected livers revealed timely induction of key proinflammatory genes *Tnfα*, *Nos2*, and *Cxcl2*, 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.[26] To further elucidate the relative contributions of HMGB1 from liver-resident phagocytes and bone marrow-derived immune cells, we generated *Hmgb1*$^{\Delta LysM}$ bone marrow chimeric mice. Both WT mice replenished with *Hmgb1*$^{\Delta LysM}$ bone marrow and *Hmgb1*$^{\Delta LysM}$ mice with WT bone marrow exhibited impaired clearance of *Listeria*, with an attenuated disease phenotype compared to *Hmgb1*$^{\Delta LysM}$>*Hmgb1*$^{\Delta LysM}$ 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*$^{\Delta LysM}$ and *Hmgb1*$^{\Delta LysM}$>*Hmgb1*$^{fl/fl}$ after 3 days (Fig. 6C). Defects in bacterial clearance were marginally more pronounced in WT mice reconstituted with *Hmgb1*$^{\Delta LysM}$ 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.[2] Intuitively and demonstrably, elevated circulating DAMP levels can also be detected during infections with protozoans,[33] fungi,[34] viruses,[35] and bacteria.[6] The extent of evolutionary conservation and remarkably high expression levels of the prototypical DAMP candidate HMGB1 in virtually all mammalian tissues[3] 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[4,11] and promotes tissue injury inferred by gram-positive infections.[6,7] 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.[36] In part due to the early postnatal lethality of global HMGB1 knockout animals,[37] 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.[10]

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),

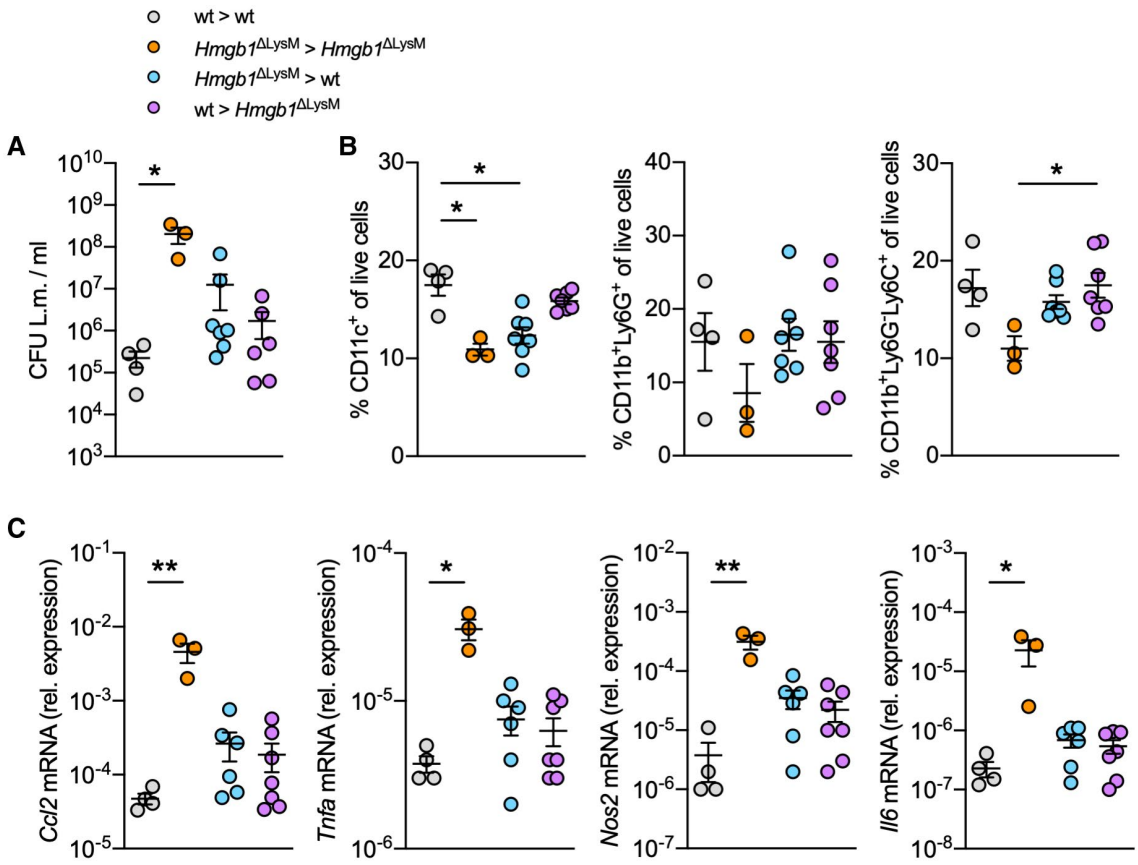

FIG. 6. Bone marrow chimera reveal contributions of HMGB1 from bone marrow and liver-resident immune cells to successful bacterial clearance. (A) Hepatic bacterial titers of the indicated animals 72 hours after injection of 2×10^4 CFU *L. monocytogenes* (n = 3-7 animals per group). (B) FACS analysis of hepatic $CD11c^+$ dendritic cells, $CD11b^+Ly6G^+$ neutrophils, and $CD11b^+Ly6C^+$ monocytes 72 hours after infection. (C) qPCR of hepatic gene expression of proinflammatory *Ccl2*, *Tnfα*, *Nos2*, and *Il6*. Single dots indicate individual animals, and vertical bars represent mean ± SEM. (A-C) Kruskal-Wallis test with Dunn's post hoc test; $^*P < 0.05$, $^{**}P < 0.01$. Abbreviation: rel., relative.

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.[38] 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[13] and LPS-induced shock.[11] 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 *Hmgb1*$^{\Delta hep}$ 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[10] in which the increased sensitivity

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.[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.[12] Similarly, in the present study, we did not observe autophagy defects in BMDMs or livers of *Hmgb1*$^{\Delta LysM}$ mice as a potential mechanism of impaired pathogen clearance following intravenous infection. While we did observe hepatic accumulation of p62 in *Hmgb1*$^{\Delta LysM}$ 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[42,43] and may reflect a defense mechanism by directing bacteria toward degradation in hepatocytes.[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.[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*$^{\Delta LysM}$ 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*$^{\Delta LysM}$ 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,[45] and subsequently enters the liver and spleen to mediate immune responses.[29,30,46] Consequently, profiling of hepatic transcriptional responses to infection in *Hmgb1*$^{fl/fl}$ and *Hmgb1*$^{\Delta LysM}$ 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*$^{\Delta LysM}$ livers despite identical pathogen burden.

These findings likely reflect differential leukocyte recruitment to the liver early during infection (as coevidenced 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*$^{\Delta LysM}$ 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*$^{fl/fl}$>*Hmgb1*$^{\Delta LysM}$ 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*$^{\Delta LysM}$ 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[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*-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.

REFERENCES

1) Medzhitov R. Origin and physiological roles of inflammation. Nature 2008;454:428-435.
2) Rock KL, Latz E, Ontiveros F, Kono H. The sterile inflammatory response. Annu Rev Immunol 2010;28:321-342.
3) Sims GP, Rowe DC, Rietdijk ST, Herbst R, Coyle AJ. HMGB1 and RAGE in inflammation and cancer. Annu Rev Immunol 2010;28:367-388.
4) Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, et al. HMG-1 as a late mediator of endotoxin lethality in mice. Science 1999;285:248-251.
5) Yang H, Ochani M, Li J, Qiang X, Tanovic M, Harris HE, et al. Reversing established sepsis with antagonists of endogenous high-mobility group box 1. Proc Natl Acad Sci U S A 2004;101:296-301.
6) Achouiti A, van der Meer A, Florquin S, Yang H, Tracey KJ, van 't Veer C, et al. High-mobility group box 1 and the receptor for advanced glycation end products contribute to lung injury during Staphylococcus aureus pneumonia. Crit Care 2013;17:R296.
7) Höhne C, Wenzel M, Angele B, Hammerschmidt S, Häcker H, Klein M, et al. High mobility group box 1 prolongs inflammation and worsens disease in pneumococcal meningitis. Brain 2013;136(Pt. 6):1746-1759.
8) Stevens NE, Chapman MJ, Fraser CK, Kuchel TR, Hayball JD, Diener KR. Therapeutic targeting of HMGB1 during experimental sepsis modulates the inflammatory cytokine profile to one associated with improved clinical outcomes. Sci Rep 2017;7:5850.
9) Andersson U, Yang H, Harris H. Extracellular HMGB1 as a therapeutic target in inflammatory diseases. Expert Opin Ther Targets 2018;22:263-277.
10) **Yanai H**, **Matsuda A**, An J, Koshiba R, Nishio J, Negishi H, et al. Conditional ablation of HMGB1 in mice reveals its protective function against endotoxemia and bacterial infection. Proc Natl Acad Sci U S A 2013;110:20699-20704.
11) **Deng M**, **Tang Y**, Li W, Wang X, Zhang R, Zhang X, et al. The endotoxin delivery protein HMGB1 mediates caspase-11-dependent lethality in sepsis. Immunity 2018;49:740-753.e7.
12) **Huebener P**, **Gwak G-Y**, Pradere J-P, Quinzii CM, Friedman R, Lin C-S, et al. High-mobility group box 1 is dispensable for autophagy, mitochondrial quality control, and organ function in vivo. Cell Metab 2014;19:539-547.
13) **Huebener P**, **Pradere J-P**, **Hernandez C**, Gwak G-Y, Caviglia JM, Mu X, et al. The HMGB1/RAGE axis triggers neutrophil-mediated injury amplification following necrosis. J Clin Invest 2015;125:539-550. Erratum in: J Clin Invest 2019;130:1802.
14) **Hernandez C**, **Huebener P**, Pradere J-P, Antoine DJ, Friedman RA, Schwabe RF. HMGB1 links chronic liver injury to progenitor responses and hepatocarcinogenesis. J Clin Invest 2018;128:2436-2451. Erratum in: J Clin Invest 2019;129:1803.
15) Constien R, Forde A, Liliensiek B, Gröne H-J, Nawroth P, Hämmerling G, et al. Characterization of a novel EGFP reporter mouse to monitor Cre recombination as demonstrated by a Tie2 Cre mouse line. Genesis 2001;30:36-44.
16) Postic C, Shiota M, Niswender KD, Jetton TL, Chen Y, Moates JM, et al. Dual roles for glucokinase in glucose homeostasis as determined by liver and pancreatic beta cell-specific gene knock-outs using Cre recombinase. J Biol Chem 1999;274:305-315.
17) Clausen BE, Burkhardt C, Reith W, Renkawitz R, Förster I. Conditional gene targeting in macrophages and granulocytes using LysMcre mice. Transgenic Res 1999;8:265-277.
18) Lecuit M. Listeria monocytogenes, a model in infection biology. Cell Microbiol 2020;22:e13186.
19) **Liu K**, **Mori S**, Takahashi HK, Tomono Y, Wake H, Kanke T, et al. Anti-high mobility group box 1 monoclonal antibody ameliorates brain infarction induced by transient ischemia in rats. FASEB J 2007;21:3904-3916.
20) Wang D, Liu K, Wake H, Teshigawara K, Mori S, Nishibori M. Anti-high mobility group box-1 (HMGB1) antibody inhibits hemorrhage-induced brain injury and improved neurological deficits in rats. Sci Rep 2017;7:46243.
21) Pamer EG. Immune responses to Listeria monocytogenes. Nat Rev Immunol 2004;4:812-823.
22) Tsung A, Sahai R, Tanaka H, Nakao A, Fink MP, Lotze MT, et al. The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia-reperfusion. J Exp Med 2005;201:1135-1143.
23) Khambu B, Huda N, Chen X, Antoine DJ, Li Y, Dai G, et al. HMGB1 promotes ductular reaction and tumorigenesis in autophagy-deficient livers. J Clin Invest 2018;128:2419-2435. Erratum in: J Clin Invest 2019;129:2163.
24) Blériot C, Dupuis T, Jouvion G, Eberl G, Disson O, Lecuit M. Liver-resident macrophage necroptosis orchestrates type 1 microbicidal inflammation and type-2-mediated tissue repair during bacterial infection. Immunity 2015;42:145-158.
25) Kobayashi SD, Braughton KR, Whitney AR, Voyich JM, Schwan TG, Musser JM, et al. Bacterial pathogens modulate an apoptosis differentiation program in human neutrophils. Proc Natl Acad Sci U S A 2003;100:10948-10953.
26) Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, Acevedo Arozena A, et al. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). Autophagy 2016;12:1-222. Erratum in: Autophagy 2016;12:443.

27) Chittaranjan S, Bortnik S, Gorski SM. Monitoring autophagic flux by using lysosomal inhibitors and western blotting of endogenous MAP1LC3B. Cold Spring Harb Protoc 2015;2015: 743-750.
28) Witter AR, Okunnu BM, Berg RE. The essential role of neutrophils during infection with the intracellular bacterial pathogen Listeria monocytogenes. J Immunol 2016;197:1557-1565.
29) Serbina NV, Salazar-Mather TP, Biron CA, Kuziel WA, Pamer EG. TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection. Immunity 2003;19:59-70.
30) Serbina NV, Kuziel W, Flavell R, Akira S, Rollins B, Pamer EG. Sequential MyD88-independent and -dependent activation of innate immune responses to intracellular bacterial infection. Immunity 2003;19:891-901.
31) Bianchi ME. HMGB1 loves company. J Leukoc Biol 2009;86:573-576.
32) Yoshida H, Lareau CA, Ramirez RN, Rose SA, Maier B, Wroblewska A, et al.; Immunological Genome Project. The cis-regulatory atlas of the mouse immune system. Cell 2019;176:897-912.e20.
33) **Higgins SJ**, **Xing K**, Kim H, Kain DC, Wang F, Dhabangi A, et al. Systemic release of high mobility group box 1 (HMGB1) protein is associated with severe and fatal Plasmodium falciparum malaria. Malar J 2013;12:105.
34) Cunha C, Carvalho A, Esposito A, Bistoni F, Romani L. DAMP signaling in fungal infections and diseases. Front Immunol 2012;3:286.
35) **Alleva LM**, **Budd AC**, Clark IA. Systemic release of high mobility group box 1 protein during severe murine influenza. J Immunol 2008;181:1454-1459.
36) Andersson U, Tracey KJ. HMGB1 is a therapeutic target for sterile inflammation and infection. Annu Rev Immunol 2011;29:139-162.
37) Calogero S, Grassi F, Aguzzi A, Voigtländer T, Ferrier P, Ferrari S, et al. The lack of chromosomal protein Hmg1 does not disrupt cell growth but causes lethal hypoglycaemia in newborn mice. Nat Genet 1999;22:276-280.
38) **Kader M**, **El Andaloussi A**, Vorhaour J, Tamama K, Nieto N, Scott MJ, et al. Interferon type i regulates inflammasome activation and high mobility group box 1 translocation in hepatocytes during Ehrlichia-induced acute liver injury. Hepatol Commun 2020;5:33-51.
39) **Huang H**, **Nace GW**, McDonald K-A, Tai S, Klune JR, Rosborough BR, et al. Hepatocyte-specific high-mobility group box 1 deletion worsens the injury in liver ischemia/reperfusion: a role for intracellular high-mobility group box 1 in cellular protection. Hepatology 2014;59:1984-1997.
40) Kang R, Tang D, Schapiro NE, Loux T, Livesey KM, Billiar TR, et al. The HMGB1/RAGE inflammatory pathway promotes pancreatic tumor growth by regulating mitochondrial bioenergetics. Oncogene 2014;33:567-577.
41) **Zhu X**, **Messer JS**, Wang Y, Lin F, Cham CM, Chang J, et al. Cytosolic HMGB1 controls the cellular autophagy/apoptosis checkpoint during inflammation. J Clin Invest 2015;125:1098-1110.
42) Homma T, Ishibashi D, Nakagaki T, Satoh K, Sano K, Atarashi R, et al. Increased expression of p62/SQSTM1 in prion diseases and its association with pathogenic prion protein. Sci Rep 2014;4:4504.
43) Lee H-M, Yuk J-M, Kim K-H, Jang J, Kang G, Park JB, et al. Mycobacterium abscessus activates the NLRP3 inflammasome via Dectin-1-Syk and p62/SQSTM1. Immunol Cell Biol 2012;90:601-610.
44) Zheng YT, Shahnazari S, Brech A, Lamark T, Johansen T, Brumell JH. The adaptor protein p62/SQSTM1 targets invading bacteria to the autophagy pathway. J Immunol 2009;183:5909-5916.
45) Kurihara T, Warr G, Loy J, Bravo R. Defects in macrophage recruitment and host defense in mice lacking the CCR2 chemokine receptor. J Exp Med 1997;186:1757-1762.
46) Shi C, Velázquez P, Hohl TM, Leiner I, Dustin ML, Pamer EG. Monocyte trafficking to hepatic sites of bacterial infection is chemokine independent and directed by focal intercellular adhesion molecule-1 expression. J Immunol 2010;184:6266-6274. Erratum in: J Immunol 2010;185:4508.
47) Peng H-H, Liu Y-J, Ojcius DM, Lee C-M, Chen R-H, Huang P-R, et al. Mineral particles stimulate innate immunity through neutrophil extracellular traps containing HMGB1. Sci Rep 2017;7:16628.

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