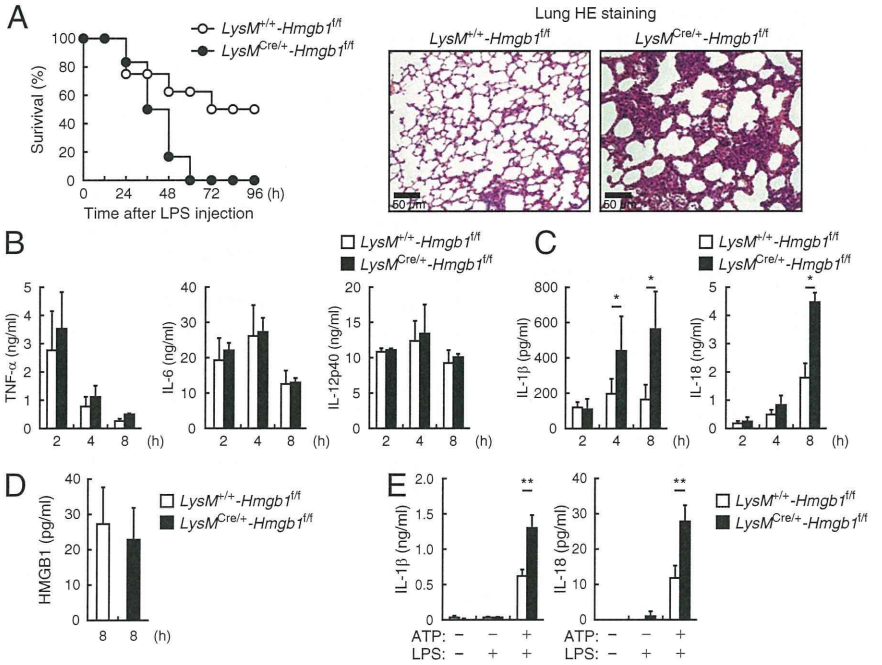

Fig. 2. Role of HMGB1 in myeloid cells in LPS-induced shock. (*A*) *LysM*$^{+/+}$-*Hmgb1*$^{f/f}$ ($n = 8$) or *LysM*$^{Cre/+}$-*Hmgb1*$^{f/f}$ ($n = 6$) mice were intraperitoneally injected with LPS (17.5 mg/kg). Mice survival was monitored (*Left*). Histological analysis of lungs from *LysM*$^{+/+}$-*Hmgb1*$^{f/f}$ or *LysM*$^{Cre/+}$-*Hmgb1*$^{f/f}$ mice injected with LPS, assessed by microscopy of sections stained with hematoxylin and eosin (*Right*). Original magnification, 40×. (Scale bars, 50 μm.) (*B–D*) Cytokine production in sera from *LysM*$^{+/+}$-*Hmgb1*$^{f/f}$ or *LysM*$^{Cre/+}$-*Hmgb1*$^{f/f}$ mice injected with LPS as in *A*. TNF-α, IL-6, and IL-12p40 (*B*) and IL-1β and IL-18 (*C*) protein levels were measured by ELISA. (*D*) HMGB1 levels in plasma 8 h after LPS injection were also measured. Data are shown as means ± SD; $*P < 0.05$. (*E*) Peritoneal macrophages from *LysM*$^{+/+}$-*Hmgb1*$^{f/f}$ or *LysM*$^{Cre/+}$-*Hmgb1*$^{f/f}$ mice were preincubated with ultrapure LPS (50 ng/mL) for 4 h followed by ATP stimulation (750 μM) for 1 h. IL-1β (*Left*) and IL-18 (*Right*) protein levels were measured by ELISA. We also observed that *Il1b* and *Il18* mRNAs were normally induced by LPS stimulation in peritoneal macrophages from *LysM*$^{Cre/+}$-*Hmgb1*$^{f/f}$ mice. Data are shown as means ± SD of triplicate determinants. $**P < 0.01$.

production, a hallmark of inflammasome activation. As shown in Fig. 2*E*, a marked increase of both IL-1β and IL-18 was observed in the *LysM*$^{Cre/+}$-*Hmgb1*$^{f/f}$ macrophages. In view of a previous report showing that caspase-1–deficient mice are highly resistant to LPS-induced endotoxemia (28), our observation indicates that intracellular HMGB1 in macrophages functions as a negative regulator of the inflammasome activation pathway, which may account for our observed LPS sensitivity in the *LysM*$^{Cre/+}$-*Hmgb1*$^{f/f}$ mice.

Critical Contribution of HMGB1 at the Interface Between Autophagy and Cell Death. It has been shown that the inflammasome pathway is regulated by autophagy which, as described above, has multiple immunological functions and influences immune responses to infection (13, 18, 19, 21–23). Because LPS can activate autophagy through the TLR4/Toll/IL-1 receptor domain–containing adapter-inducing interferon-β (TRIF)/p38 MAPK signaling pathway and autophagic proteins regulate caspase-1–mediated innate immune responses (29–31), we wished to examine whether enhanced inflammasome activation in the absence of HMGB1 (Fig. 2*E*) is a consequence of an unregulated autophagic process. We examined the status of microtubule-associated protein light chain 3 (LC3), a well-known marker of autophagosomes (32). As shown in Fig. 3*A*, LPS stimulation of *LysM*$^{Cre/+}$-*Hmgb1*$^{f/f}$ macrophages resulted in a significant decrease in the formation of two cleaved forms of LC3, LC3-I and LC3-II, which typically represent the induction of autophagosomes (32, 33), compared with *LysM*$^{+/+}$-*Hmgb1*$^{f/f}$ macrophages. These data indicate that HMGB1 is required for the autophagic process.

We also examined the in vivo regulation of macrophage fate following LPS administration in these mice. As shown in Fig. 3*B*, we observed a marked decrease of macrophage populations in *LysM*$^{Cre/+}$-*Hmgb1*$^{f/f}$ mice compared with *LysM*$^{+/+}$-*Hmgb1*$^{f/f}$ mice. Because suppression of autophagy results in cell death, this observation is consistent with the above in vitro results. Although TLR4 stimulation alone cannot induce cell death, it can if the pan-caspase inhibitor z-VAD-fmk is present (34, 35). Under this in vitro experimental setting, a marked enhancement of macrophage cell death was observed in the absence of HMGB1 (Fig. 3*C*). Thus, these observations in toto indicate that HMGB1 critically regulates autophagy and cell death in macrophages following TLR4 activation.

Because autophagy functions in a wide variety of cell types, we asked whether this observation can also be made in dendritic cells or hepatocytes, by respectively expressing the *cre* recombinase gene under the promoter of CD11c or albumin (*Cd11c*$^{Cre/+}$-*Hmgb1*$^{f/f}$ or *Alb*$^{Cre/+}$-*Hmgb1*$^{f/f}$ mice). In both cases, we observed a phenotype similar to that seen in *LysM*$^{Cre/+}$-*Hmgb1*$^{f/f}$ mice, namely, an elevation of serum IL-1β and IL-18 upon LPS stimulation, but no change in serum TNF-α, IL-6, or IL-12p40 levels (Fig. S4 *A* and *B*). Thus, HMGB1-mediated autophagy is likely to be a cell-type–independent mechanism.

Requirement of Macrophage HMGB1 in Antibacterial Responses. Because autophagy also participates in the host's response against infection by various pathogens, particularly the clearance of intracellular bacteria, we next examined *LysM*$^{Cre/+}$-*Hmgb1*$^{f/f}$ mice in response to a pathogenic bacterium *Listeria monocytogenes*. As shown in Fig. 4*A*, these mice showed a marked vulnerability to the bacterial infection compared with *LysM*$^{+/+}$-*Hmgb1*$^{f/f}$ mice. Expectedly, there was a marked elevation of bacterial burden seen in the spleen and liver of *LysM*$^{Cre/+}$-*Hmgb1*$^{f/f}$ mice (Fig. 4*B*), although mRNA levels of inflammatory cytokines remained essentially unchanged (Fig. S5).

Interestingly, the generation of LC3-II was significantly suppressed in peritoneal macrophages of infected *LysM*$^{Cre/+}$-*Hmgb1*$^{f/f}$ mice compared with those from *LysM*$^{+/+}$-*Hmgb1*$^{f/f}$ mice, indicating

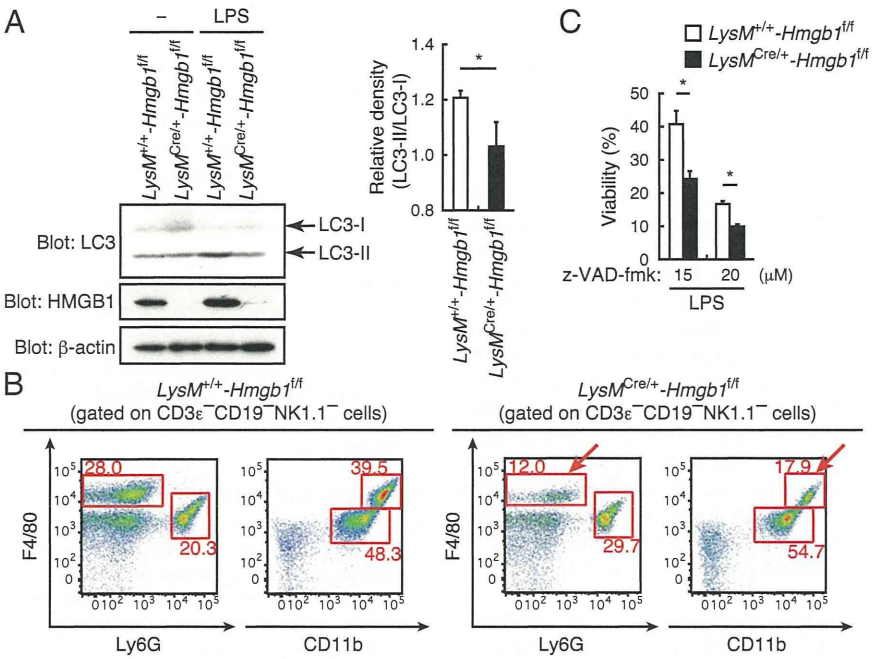

Fig. 3. Role of HMGB1 in myeloid cells to autophagy and cell death. (*A*) Bone marrow macrophages were stimulated with LPS (1 μg/mL) for 1 h. Whole cell lysates were prepared and subjected to immunoblot analysis. LC3-I/II, HMGB1, and β-actin were detected (*Left*). LC3-II/LC3-I relative band density ($n = 3$) is also shown (*Right*). $*P < 0.05$. (*B*) Single cell suspensions were prepared from the peritoneal cavity of *LysM*$^{+/+}$-*Hmgb1*$^{f/f}$ or *LysM*$^{Cre/+}$-*Hmgb1*$^{f/f}$ mice 12 h after i.p. LPS injection (17.5 mg/kg) and stained with the indicated combination of the following fluorochrome-conjugated antibodies: anti-F4/80 PerCP-Cy5, anti-CD11b APC, anti-Ly6G PE-Cy7, anti-CD19 PB, anti-NK1.1 PB, and anti-CD3ε PB antibodies. CD3ε$^-$CD19$^-$NK1.1$^-$ cells were gated and shown. The numbers represent the percentage of cells contained in each region. Arrows represent macrophage population. (*C*) Bone marrow macrophages were stimulated with LPS (500 ng/mL) for 24 h with z-VAD-fmk (15 or 20 μM), and cell viability was measured by the crystal violet staining method. $*P < 0.05$.

a critical role for HMGB1 in bacteria-induced autophagy in these cells (Fig. 4*C*). We also observed that upon bacterial infection of macrophages in vitro, there was a noticeable accumulation of HMGB1 in the cytosol, which also occurs upon LPS stimulation (36, 37) (Fig. 4*D*). Of note, cytosolic HMGB1 merged with bacterial DNA, suggesting an association of this protein with

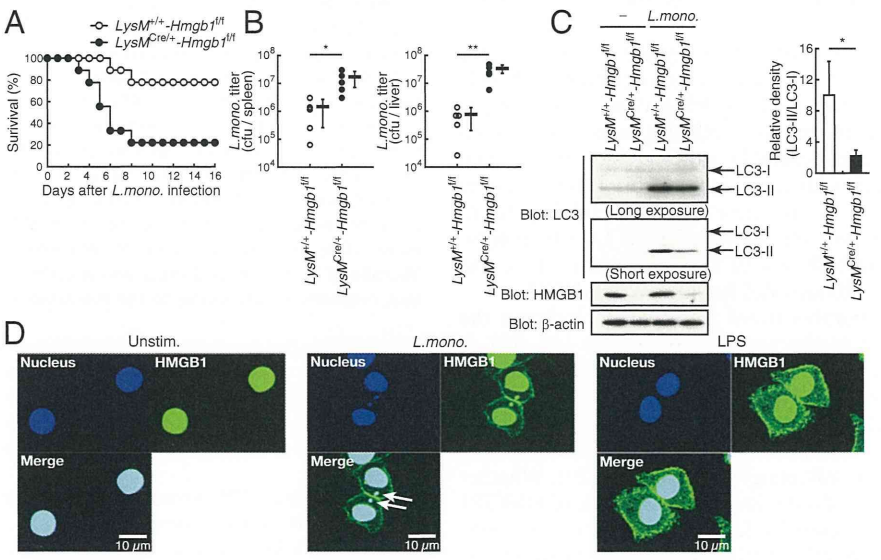

Fig. 4. *Hmgb1*-deficient mice in myeloid cells are vulnerable to *L. monocytogenes* infection. (*A*) *LysM*$^{+/+}$-*Hmgb1*$^{f/f}$ ($n = 9$) and *LysM*$^{Cre/+}$-*Hmgb1*$^{f/f}$ ($n = 9$) mice were infected with *L. monocytogenes* (5×10^5 cfu) intraperitoneally. Mice survival was monitored every 24 h. (*B*) *L. monocytogenes* titers in the spleen (*Left*) or liver (*Right*) from *LysM*$^{+/+}$-*Hmgb1*$^{f/f}$ ($n = 5$) and *LysM*$^{Cre/+}$-*Hmgb1*$^{f/f}$ ($n = 5$) mice infected for 3 d as in *A* were measured. Each symbol represents an individual mouse; long horizontal lines indicate the mean. $**P < 0.01$ and $*P < 0.05$. (*C*) Peritoneal macrophages were infected with *L. monocytogenes* [multiplicity of infection (MOI) of 5] for 1 h. Whole cell lysates were prepared and subjected to immunoblot analysis. LC3-I/II, HMGB1, and β-actin were detected (*Left*). LC3-II/LC3-I relative band density ($n = 3$) is also shown (*Right*). $*P < 0.05$. (*D*) Translocation of HMGB1 from nucleus to cytosol upon *L. monocytogenes* infection and LPS stimulation. RAW264.7 cells were untreated (*Left*), infected with *L. monocytogenes* (MOI of 1; *Center*), or stimulated with LPS (1 μg/mL; *Right*) for 1 h. Cells were then fixed and stained with anti-HMGB1 antibody and DAPI. Confocal images of HMGB1 (green) and nuclei (blue) are shown. (Scale bars, 10 μm.) Arrows show colocalization of HMGB1 and *L. monocytogenes*.

autophagosomes for autophagy-mediated bacterial eradication (Fig. 4*D*). Collectively, these results support the notion that HMGB1, released from the nucleus to the cytosol upon infection, participates in bacteria-induced autophagy, given that the absence of this protein severely affects bacterial clearance.

Discussion

In this study, we generated conditional knockout mice of HMGB1 to gain insight into its biological roles, specifically as it relates to immune responses, of this protein in vivo. These conditional knockout mice permitted the ablation of HMGB1 in a tissue- and cell-type–specific manner. Before our study, HMGB1 was identified as a multifunctional mediator of immune and inflammatory responses by virtue of its role as danger-associated molecular patterns (DAMPs) or alarmins upon its release from the cell, which occurs typically by cell death or a still poorly characterized mechanism (1–5). Intracellular HMGB1 is also an immune regulator, particularly in the cytoplasm where it functions as sensor and/or chaperone for immunogenic nucleic acids and mediates autophagy (13, 15, 17, 20). This study illustrates the in vivo validation of HMGB1 in myeloid cells, showing its protective role in the cytoplasm against LPS-induced endotoxemia and infection by *L. monocytogenes*.

Interestingly, previous studies have shown that HMGB1 functions as a crucial instigator of LPS-induced endotoxemia (1, 2, 11). Our present study by no means excludes this pathological aspect of HMGB1. However, we demonstrate that cells of myeloid lineage are not the main source of proinflammatory HMGB1, as serum HMGB1 levels, induced by LPS, were only marginally affected in $LysM^{Cre/+}$-$Hmgb1^{f/f}$ mice (Fig. 2*D*). Thus, it will be of great interest to clarify which cells are responsible for the proinflammatory HMGB1 as well as how it instigates inflammatory responses. In a similar context, the inflammatory function of hepatocyte-derived HMGB1 has been reported in ischemia–reperfusion injury. We therefore examined $Alb^{Cre/+}$-$Hmgb1^{f/f}$ mice but basically did not observe any overt differences compared with HMGB1-intact mice, suggesting that the hepatocyte-derived HMGB1 plays little, if any, role in this inflammatory pathogenesis (Fig. S6). Thus, the role and source of HMGB1 in this ischemia–reperfusion injury model also needs further clarification.

The antimicrobial functions of autophagy are known to serve as a series of barriers against invading microorganisms, wherein the antimicrobial function of LAP, which involves the engagement of the autophagic machinery while the bacterium is confined in the nascent and presumably intact phagosome, is well characterized. Our results indicate a critical role of HMGB1 in this process as revealed by an increased vulnerability to *L. monocytogenes* infection and the suppression of LC3-II generation in macrophages, the hallmark of LAP (Fig. 4*C*). Thus, this study reveals a unique, antibacterial function of HMGB1.

Our study also corroborates other reports that indicate the involvement of HMGB1 in the autophagic process (13, 20). It has been reported that stimuli that enhance reactive oxygen species promote cytosolic translocation of HMGB1 from the nucleus where it directly interacts with the autophagy-related (ATG) protein Beclin-1, a Bcl-2–homology (BH)-3 domain-only protein that is essential for autophagy induction (13, 20). Whether and/or how the suppression of autophagy in the absence of HMGB1 upon LPS stimulation or bacterial infection, revealed in this study, integrates to Beclin-1 and/or other molecules involved in autophagy remains to be determined further.

In summary, this study offers in vivo evidence for the protective aspect of cytosolic HMGB1 against endotoxemia and bacterial infection. The availability of the mice for conditional ablation of HMGB1 will provide new avenues of research for the investigation of this protein, which apparently shuttles from nucleus to cytoplasm to the extracellular environment. In particular, in view of the fact that the main source of serum HMGB1 upon LPS stimulation is not cells of myeloid cell lineage, further investigation by generation of additional Cre-loxP mutant mice will be an aide in the examination of the function of extracellular HMGB1 during endotoxemia.

It has been conventional wisdom that effector molecules are newly synthesized and released during immune or inflammatory responses typically starting at the level of transcriptional activation of the corresponding genes (23, 38, 39). The present findings together with previous reports may point to the existence of an additional mechanism, that is, the nucleus, wherein HMGB1 is abundantly accumulated without cellular stimulation, functions as "arsenal" for this and other effector molecules to be released into the cytosol and extracellular milieu so as to ensure prompt and effective cellular responses. Indeed, the newly generated $Hmgb1^{f/f}$ mice will allow further investigation to fully identify HMGB1's multiple functions during homeostasis and pathogenesis.

Materials and Methods

Mice. C57BL/6 mice were purchased from CLEA Japan, Inc. *LysM-Cre* knockin mice, *Alb-Cre* transgenic mice, CAG-*Cre* transgenic mice, and *Cd11c-Cre* transgenic mice were obtained from The Jackson Laboratory. All animal care and experiments conformed to the guidelines for animal experiments of the University of Tokyo, and were approved by the animal research committee of the University of Tokyo.

Construction of the Targeting Vector. A targeting vector was constructed to delete exons 2–4, which includes the translation initiation site of the *Hmgb1* gene. *Hmgb1* genomic DNA was amplified by PCR by using specific primers as follows: sense, 5′-actagtgcttgtctgtttcacagttttcgttac-3′ and antisense, 5′-gtcgacagttatcaagtataatcccctaacactgg-3′. The fragment was inserted between two loxP sites of the vector pKSTKNEOloxP, which has herpes simplex virus thymidine kinase and loxP-flanked pGK-neo. Then the 10.7-kb genomic fragment 5′ upstream of exon 2–4 and 3′ downstream 5-kb genomic fragment was inserted.

Reagents and Cells. LPS O55:B4 was purchased from Sigma. Ultrapure LPS and ATP were purchased from InvivoGen. z-VAD-fmk was purchased from Sigma. The mouse macrophage cell line RAW264.7 cells were cultured and maintained as described (13, 15, 17, 20). For preparing peritoneal macrophages, mice were injected with 2 mL of 4% (wt/vol) thioglycollate solution intraperitoneally. The peritoneal cavity was washed by PBS, containing 2% (vol/vol) FCS and 1 mM EDTA to collect peritoneal exudate cells 4 d after the injection, and cells were plated on Petri dishes for 2 h in RPMI, supplemented with 10% (vol/vol) FCS. After incubation, cells were recollected and seeded for each assay. Bone marrow cells were extracted from each mouse as described previously (17, 40), then seeded in 15-cm cell culture dishes and cultured in RPMI supplemented with 10% (vol/vol) FCS and M-CSF (100 ng/mL; Peprotech) for 6 d. M-CSF cultured bone-marrow–derived macrophages (BMMs) were replated for each assay. $CD3\varepsilon^+$ T cells and $CD19^+$ B cells were prepared by using a CD3ε MicroBead kit (Miltenyi Biotec) and a CD19 MicroBead kit (Miltenyi Biotec), respectively, according to the manufacturer's protocol.

LPS-Induced Lethal Shock. Mice were injected intraperitoneally with LPS (17.5 mg/kg weight) and monitored every 12 h. Whole blood samples were taken sequentially from tails, and sera were prepared by centrifugation for 5 min at 4 °C at 8,000 × *g* after incubation at room temperature for 30 min. For plasma sample preparation, blood samples were treated with 5 mM EDTA.

Statistical Analysis. Differences between control and experimental groups were evaluated with a Student *t* test.

Additional information can be found in *SI Materials and Methods*.

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