Deficiency of the novel high mobility group protein HMGXB4 protects against systemic inflammation-induced endotoxemia in mice

Xiangqin He[a,b,c,1], Kunzhe Dong[c,1], Jian Shen[c,d], Guoqing Hu[c], Jinhua Liu[c,e], Xiuhua Kang[c,e], Liang Wang[f], Reem T. Atawia[c], Islam Osman[c], Robert W. Caldwell[c], Meixiang Xiang[d], Wei Zhang[e], Zeqi Zheng[f], Liwu Li[g], David J. R. Fulton[c,h], Keyu Deng[a,b], Hongbo Xin[a,b,2], and Jiliang Zhou[c,2]

[a]The National Engineering Research Center for Bioengineering Drugs and Technologies, The Institute of Translational Medicine, Nanchang University, 330031 Nanchang, Jiangxi, China; [b]School of Life Sciences, Nanchang University, 330031 Nanchang, Jiangxi, China; [c]Department of Pharmacology and Toxicology, Medical College of Georgia, Augusta University, Augusta, GA 30912; [d]Department of Cardiology, The Second Affiliated Hospital, Zhejiang University School of Medicine, 310009 Hangzhou, China; [e]Department of Respiratory Medicine, The First Affiliated Hospital of Nanchang University, 330006 Nanchang, Jiangxi, China; [f]Department of Cardiology, The First Affiliated Hospital of Nanchang University, 330006 Nanchang, Jiangxi, China; [g]Department of Biological Sciences, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061-0910; and [h]Vascular Biology Center, Medical College of Georgia, Augusta University, Augusta, GA 30912

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Sepsis is a major cause of mortality in intensive care units, which results from a severely dysregulated inflammatory response that ultimately leads to organ failure. While antibiotics can help in the early stages, effective strategies to curtail inflammation remain limited. The high mobility group (HMG) proteins are chromosomal proteins with important roles in regulating gene transcription. While HMGB1 has been shown to play a role in sepsis, the role of other family members including HMGXB4 remains unknown. We found that expression of HMGXB4 is strongly induced in response to lipopolysaccharide (LPS)-elicited inflammation in murine peritoneal macrophages. Genetic deletion of *Hmgxb4* protected against LPS-induced lung injury and lethality and cecal ligation and puncture (CLP)-induced lethality in mice, and attenuated LPS-induced proinflammatory gene expression in cultured macrophages. By integrating genome-wide transcriptome profiling and a publicly available ChIP-seq dataset, we identified HMGXB4 as a transcriptional activator that regulates the expression of the proinflammatory gene, *Nos2* (inducible nitric oxide synthase 2) by binding to its promoter region, leading to NOS2 induction and excessive NO production and tissue damage. Similar to *Hmgxb4* ablation in mice, administration of a pharmacological inhibitor of NOS2 robustly decreased LPS-induced pulmonary vascular permeability and lethality in mice. Additionally, we identified the cell adhesion molecule, ICAM1, as a target of HMGXB4 in endothelial cells that facilitates inflammation by promoting monocyte attachment. In summary, our study reveals a critical role of HMGXB4 in exacerbating endotoxemia via transcriptional induction of *Nos2* and *Icam1* gene expression and thus targeting HMGXB4 may be an effective therapeutic strategy for the treatment of sepsis.

Sepsis is a life-threatening disorder triggered by an aberrant systemic response to bacterial infection. In sepsis, inflammatory and immune responses to invading pathogens are dysregulated, excessive, and fail to return to homeostasis, thus leading to a complex clinical syndrome which is characterized by tissue damage and ultimately organ failure (1). Despite decades of research, sepsis remains the most common cause of death in the intensive care units and a tremendous public health burden with an estimated 1.7 million cases and 270,000 deaths annually in the United States (2, 3). Currently, the therapeutic approach to sepsis is limited to antibiotics and supportive care, and there are no specific therapies targeting immune dysfunction. Therefore, there is a pressing need to improve our understanding of mechanisms involved in the pathogenesis of sepsis and to develop new therapeutic strategies.

Most cases of sepsis are triggered by infection with gram-negative bacteria, which release an endotoxin called lipopolysaccharide (LPS), a normal constituent of the bacterial wall of *Escherichia coli* (4). LPS induces a systemic inflammatory response via the pattern recognition receptor CD14 and Toll-like receptor (TLR4) present on the surface of macrophages (4, 5). This leads to activation of the nuclear factor-κB (NF-κB) transcription factor, which in turn, induces expression of proinflammatory cytokines such as TNF (tumor necrosis factor), IL1B (interleukin 1 beta), IL6 (interleukin 6), IFNG (interferon gamma), and NOS2 (inducible nitric oxide synthase 2) (6). Overproduction of these proinflammatory cytokines and high levels of nitric oxide (NO) generated by NOS2 are cytotoxic and lead to organ failure and mortality (7). The central role of cytokines and NO in this process suggest that manipulation of the production of cytokines or NO could be a potential therapeutic strategy for improving the outcome of sepsis. However, attempts to target cytokines in

Significance

Sepsis is a life-threatening disorder triggered by systemic inflammation. Currently, there are no effective therapies targeting immune dysfunction in sepsis. The high mobility group (HMG) proteins are chromosomal proteins with important roles in regulating gene transcription. We found that the HMGXB4 protein is strongly induced by lipopolysaccharide. Knockout of *Hmgxb4* protected against endotoxemia-induced lethality in mice. Mechanistically, HMGXB4 promoted expression of the proinflammatory genes, *Nos2 and Icam1*. Increased NOS2 expression promotes excessive NO that causes tissue damage, and increased ICAM1 encourages monocyte attachment to endothelial cells. Our study reveals a critical role of HMGXB4 in endotoxemia via excessive NO production and increased monocyte adhesion which advances the concept that strategies targeting HMGXB4 may be effective for the treatment of sepsis.

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[1]X.H. and K. Dong contributed equally to this work.

[2]To whom correspondence may be addressed. Email: xinhb@ncu.edu.cn or jizhou@augusta.edu.

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clinical trials have proven unsuccessful and led to considerable frustration (8), leaving only NO as a potential therapeutic target for sepsis treatment (9). In addition to binding to CD14 and TLR4 cell surface receptors, LPS can translocate to cytoplasm of cells where it directly binds to the intracellular receptor, caspase-11 protease in mice (and its human homologs caspase-4 and caspase-5), leading to pyroptosis, a highly inflammatory form of programmed cell death (10, 11). Consistently, deletion of caspase-11 protects against endotoxin-induced lethality in a murine endotoxemia model (12). Therefore, inhibiting the expression or activation of caspase-11/4/5 may be a potential strategy for the management of sepsis.

High mobility group box (HMGB) proteins are a diverse family of nonhistone chromosomal proteins and are composed of four members, including HMGB1, 2, 3, and 4 in mammals (13). HMGB1, 2, and 3 are evolutionarily conserved between species and contain two DNA-binding HMG domains and an acidic tail (14). HMGB4 is a less conserved mammal-specific protein that contains two HMG domains but lacks the acidic tail (15). HMGB proteins mainly function as architectural factors in the nucleus by binding to DNA in a DNA structure-dependent but nucleotide sequence-independent manner, to produce changes in DNA structure, such as bending and twisting (16). The function of HMGB1, 2, and 3 has been well studied using genetic mouse models and mice lacking these genes exhibit defects in glucose metabolism (17), spermatogenesis (18), and production of erythrocytes (19), respectively. Both HMGB1 and HMGB2 have also been shown to be major mediators of inflammation (20–23).

HMGXB4 (also known as HMG2L1) is a HMGB protein which contains a single HMG box domain. HMGXB4 was originally described in *Xenopus* where it was shown to negatively regulate the Wnt/β-catenin signaling pathway (24). Zhou et al. cloned the mammalian ortholog, HMGXB4, and demonstrate that HMGXB4 plays a critical role in modulating the phenotypic flexibility of vascular smooth muscle cells (25). Based on our previous findings of a close relationship between smooth muscle phenotypic switching and inflammation, we hypothesized that HMGXB4 may also have broader roles in inflammatory processes. Here we found that HMGXB4 plays an essential role in the inflammatory response in macrophages. We observed that HMGXB4 expression is induced in activated murine peritoneal macrophages upon treatment with TLR agonists and especially in LPS-elicited inflammation. We further demonstrated that *Hmgxb4* deficiency dramatically attenuates LPS- or cecal ligation and puncture (CLP)-induced endotoxemia and subsequent mortality by reducing tissue damage mediated by NOS2-dependent NO production and endothelial dysfunction. Our study uncovers a proinflammatory role of HMGXB4 in orchestrating systemic inflammatory response in vivo and suggests that HMGXB4 could be a potential therapeutic target for the treatment of sepsis.

Results

HMGXB4 Is Induced by LPS-Induced Inflammation. As macrophages play critical roles throughout all phases of the inflammatory response induced by pathogen-associated molecular patterns (PAMPs), we first examined the expression of HMGXB4 in murine peritoneal macrophages treated with an array of TLR agonists. We observed that HMGXB4 was significantly induced by all of the TLR agonists (Fig. 1 *A* and *B*), but especially by LPS-induced stimulation of TLR4. As LPS is responsible for triggering endotoxemia caused by infection with gram-negative bacteria, we further assessed HMGXB4 induction in murine peritoneal macrophages treated with LPS or vehicle control. We found that HMGXB4 was significantly induced by LPS treatment at both mRNA and protein levels in a time- (Fig. 1 *C–E*) and dose-dependent manner (Fig. 1 *F–H*), in parallel with the expression of well-known inflammatory markers NOS2 and PTGS2 (also known as COX-2). In contrast to the proinflammatory M1 phenotype induced by LPS, macrophages stimulated by IL4 switch to an M2 phenotype to resolve inflammation by secreting antiinflammatory cytokines (26). In contrast to LPS, IL4 treatment of murine macrophages had negligible effects on HMGXB4 expression at both mRNA and protein levels, whereas, IL4-induced expression of CHIL3 (Chitinase-3-like protein 3, also known as Ym1) and STAT6 activation confirmed the effectiveness of M2 pathway signaling (*SI Appendix*, Fig. S1). These data suggest that HMGXB4 is selectively induced in proinflammatory macrophages.

A previous study showed the HMG family protein HMGB1 can translocate from the nucleus to the cytoplasm and then be secreted into the serum as an inflammatory mediator (27). To test whether HMGXB4 can be similarly secreted, we examined the subcellular localization of HMGXB4 in peritoneal macrophages with or without LPS stimulation. Immunofluorescence (IF) results showed that HMGXB4 expression was exclusively restricted to the nucleus in resting macrophages and remained in the nucleus following LPS treatment (Fig. 1*I*). Western blot analysis of cytoplasmic and nuclear fractions of macrophages confirmed the exclusive nuclear localization of HMGXB4 regardless of LPS treatment and further demonstrated that LPS promotes HMGXB4 expression specifically in the nucleus (Fig. 1*J*). To examine whether HMGXB4 can be released from the nucleus into the supernatant during pyroptosis, we induced pyroptosis using the established approach of priming murine peritoneal macrophages with LPS followed by ATP (adenosine triphosphate) treatment. However, HMGXB4 was undetectable in the supernatant following induction of pyroptosis (Fig. 1*K*). Taken together, these observations suggest that HMGXB4 may exert its function via a novel nuclear regulatory mechanism.

***Hmgxb4* Deficiency Ameliorates LPS-Induced Mortality and Tissue Injury.** We next developed a *Hmgxb4* knockout (KO) mouse model to investigate the function of HMGXB4 in vivo. We first generated a targeted *Hmgxb4* gene trap allele mouse line with a cassette containing En2 splicing acceptor (SA), lacZ, and promoter-driven neomycin-resistant gene (neo) inserted between exons 2 and 3 in the *Hmgxb4* gene locus (herein referred to as *Hmgxb4* neo mice) (*SI Appendix*, Fig. S2 *A* and *B*). This insertion disrupts endogenous *Hmgxb4* expression up to 95% in macrophages, brain, and heart tissue obtained from adult $Hmgxb4^{neo/neo}$ mice (*SI Appendix*, Fig. S2 *C* and *D*). All $Hmgxb4^{neo/neo}$ animals were viable, fertile, and developed with normal body weight without any gross physical or behavioral abnormalities (*SI Appendix*, Fig. S2*E*). To obtain complete *Hmgxb4*-null mice, *Hmgxb4* neo mice were crossed with flippase recombinase-expressing mice to remove the En2/SA/LacZ/neo cassette. The resultant "conditional" (flox) mice were subsequently crossed with ubiquitous cytomegalovirus (CMV)-Cre mice to obtain global *Hmgxb4* heterozygous mice (*SI Appendix*, Fig. S2*F*). Finally, the heterozygous mice were intercrossed to generate mice with global deletion of *Hmgxb4* (*SI Appendix*, Fig. S2 *G* and *H*). qRT-PCR and Western blot analysis demonstrated that HMGXB4 expression was completely ablated in macrophages and tissues such as brain and heart harvested from KO mice (*SI Appendix*, Fig. S2 *I* and *J*). Similar to the *Hmgxb4* gene trap mice, both male and female *Hmgxb4*-null mice appear to be viable and fertile with no obvious difference in body weight compared to wild-type (WT) and *Hmgxb4* heterozygous mice (*SI Appendix*, Fig. S2*K*). Taken together, the generation of a *Hmgxb4*-null mouse line provides a valuable genetic tool to explore the functional role of HMGXB4 in vivo.

In WT and *Hmgxb4* KO mice (*SI Appendix*, Fig. S2), we first examined the effects of *Hmgxb4* deletion on the response to septic shock. We created two mouse models of endotoxemia by intraperitoneal injection of a lethal dose of LPS (30 mg/kg body weight) or CLP, which closely resemble the progression and characteristics of human sepsis. Age- and gender-matched WT mice served as controls for *Hmgxb4* KO mice. We found that the survival time of *Hmgxb4*-deficient mice was significantly

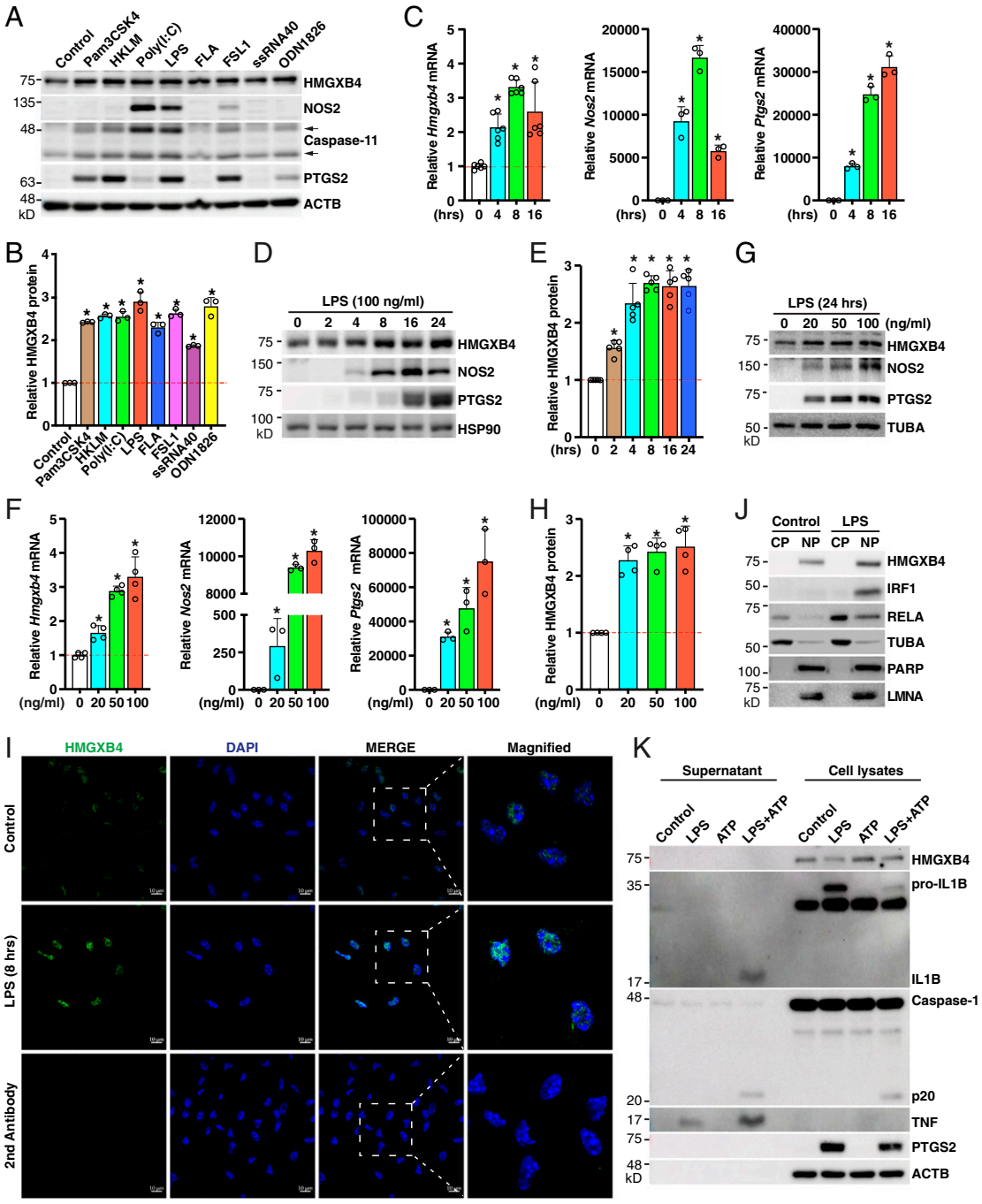

Fig. 1. HMGXB4 is induced by TLR agonist-elicited inflammation in murine peritoneal macrophages. (*A*) Cultured murine peritoneal macrophages were treated with TLR agonists (TLR1/2 agonist Pam3CSK4, TLR2 agonist HKLM, TLR3 agonist Poly(I:C), TLR4 agonist LPS, TLR5 agonist FLA, TLR6/2 agonist FSL1, TLR7 agonist ssRNA40, and TLR9 agonist ODN1826) for 16 h and cells were harvested for Western blot analysis. (*B*) Densitometric analysis of HMGXB4 protein levels shown in *A* normalized to the loading control ACTB and fold change relative to the signal of control (set to 1). $n = 3$; $^*P < 0.05$. (*C–E*) Cultured murine peritoneal macrophages were treated with LPS (100 ng/mL) for the indicated time and cells were harvested for (*C*) qRT-PCR or for (*D*) Western blot analysis. The relative mRNA levels were quantified and presented relative to time 0-h treatment (*C*, set to 1). $n = 3$–6; $^*P < 0.05$. (*E*) Densitometric analysis of HMGXB4 protein levels shown in *D* normalized to the loading control HSP90 and relative to signals at 0 h (set to 1). $n = 5$; $^*P < 0.05$. (*F* and *G*) Cultured murine peritoneal macrophages were treated with LPS at the doses indicated for 24 h and cells were harvested for (*F*) qRT-PCR or for (*G*) Western blot. The relative mRNA levels were quantified and presented relative to control without LPS treatment (*F*, set to 1). $n = 3$–4; $^*P < 0.05$. (*H*) Densitometric analysis of HMGXB4 protein levels in *G* normalized to the loading control TUBA and relative to the signal of the control group at 0 ng/mL (set to 1). $n = 4$; $^*P < 0.05$. (*I*) Cultured peritoneal macrophages in the absence (*Top*) or presence of LPS treatment (*Middle*) (100 ng/mL, 8 h) were analyzed by immunofluorescence staining for HMGXB4 (green). Nuclei were counterstained with DAPI (blue). Cells treated with LPS but only stained with second antibody served as negative control for staining (*Bottom*). The boxed areas are magnified to the *Right*. (*J*) Similar to *I*, except that cells were harvested for isolation of cytoplasmic and nuclear protein fractions and analyzed by Western blotting. IRF1 and RELA served as positive controls in response to LPS treatment. TUBA was used as cytoplasmic protein (CP) and PARP and LMNA were used as nuclear protein (NP) loading controls, respectively. HMGXB4 is constitutively expressed in macrophage nuclei which is unaffected by LPS treatment. (*K*) Cultured murine peritoneal macrophages were primed with or without LPS (0.2 μg/mL) for 3 h followed by treatment with or without ATP (5 mM) for 30 min. Supernatant and cells were subsequently harvested for Western blot analysis.

prolonged compared to that of WT mice in both endotoxemia mouse models (Fig. 2 *A* and *B*), suggesting a protective role of *Hmgxb4* deficiency against endotoxemia. We next challenged *Hmgxb4*-deficient mice and WT mice with a lower dose of LPS (20 mg/kg body weight) or PBS (phosphate-buffered saline) for 6 h to assess the effect of *Hmgxb4* deficiency on acute lung injury. We observed that although there was no significant difference in the serum levels of a set of proinflammatory cytokines, including IL6, TNF, and CCL2 between the *Hmgxb4* KO and WT mice (*SI Appendix*, Fig. S3*A*), the LPS-induced lung injury was significantly ameliorated in *Hmgxb4* KO mice compared to WT mice, as indicated by reduced alveolar congestion and infiltration of inflammatory cells (Fig. 2 *C* and *D*). The protective role of *Hmgxb4* deficiency on acute tissue injury induced by LPS was also observed in the kidney (*SI Appendix*, Fig. S3 *B* and *C*). Further examination of lung tissues revealed increased cell apoptosis (Fig. 2 *E* and *F*) and macrophage infiltration (Fig. 2 *G* and *H*) following LPS administration which were significantly attenuated in *Hmgxb4* KO mice as compared to WT mice. To further examine the effect of *Hmgxb4* deficiency on pulmonary permeability and disruption of the pulmonary vascular barrier, we injected Evans blue dye (EBD, 30 mg/kg body weight) in both *Hmgxb4* KO mice and WT mice with or without LPS treatment (10 mg/kg body weight) and then measured the extravasation of albumin-bound EBD in the lung. We found LPS-induced pulmonary vascular hyperpermeability is significantly reduced in *Hmgxb4* KO mice compared to WT mice (Fig. 2 *I* and *J*). Collectively, these observations suggest that *Hmgxb4* deficiency protects against septic shock-induced lethality and tissue injury in mice.

HMGXB4 Modulates LPS-Induced Gene Expression. To understand the molecular basis of the protective effects of *Hmgxb4* deficiency, we performed RNA-seq analysis on peritoneal macrophages isolated from *Hmgxb4* KO and WT mice with and without LPS stimulation. *Hmgxb4* deficiency caused a mild effect on gene expression in macrophages at baseline, as indicated by the identification of only 42 significantly up-regulated and 88 significantly down-regulated genes as compared to WT macrophages (fold change [FC] > 1.5 and false discovery rate [FDR] < 0.05) (*SI Appendix*, Fig. S4*A* and Dataset S1). These significantly changed genes were involved in a wide spectrum of functional categories, including inflammation-related pathways such as TNF and NF-κB signaling pathways that were suppressed by *Hmgxb4* deletion (*SI Appendix*, Fig. S4*B*). In response to LPS treatment, a total of 1,630 up- (defined as LPS-induced genes) and 1,549 down-regulated genes (defined as LPS-suppressed genes) were identified in WT macrophages (Fig. 3 *A*, *Left*), which included a list of previously defined LPS-response genes (annotated in the Gene Ontology term of "response to lipopolysaccharide," GO:0032496) such as *Il6* and *Nos2* (*SI Appendix*, Fig. S4*C*), confirming the reliability of our RNA-seq analysis. In *Hmgxb4*-deficient macrophages, substantially fewer up- (n = 1,345) and down-regulated genes (n = 1,201) were identified in response to LPS stimulation with the same criteria (FC >1.5 and FDR <0.05) (Fig. 3 *A*, *Right* and Dataset S1). Among these genes, the induction of 434 genes and the suppression of 556 genes in response to LPS treatment in WT macrophages were no longer significant in *Hmgxb4* KO macrophages (Fig. 3*B*). The majority of LPS-induced (n = 1,196) or suppressed (n = 993) genes identified in WT macrophages were still significantly up- or down-regulated in LPS-treated *Hmgxb4* KO macrophages (Fig. 3*B*). Their relative changes in expression were, however, significantly attenuated in *Hmgxb4*-null macrophages (Fig. 3*C*). Taken together, these results indicate that *Hmgxb4* deficiency affects both the breadth and degree of expression of genes regulated by LPS.

We next sought to determine which genes among the LPS-induced or -suppressed genes were regulated by HMGXB4. Differential analysis revealed 227 significantly up-regulated and 695 significantly down-regulated genes in LPS-treated *Hmgxb4*-deficient macrophages as compared to WT macrophages (*SI Appendix*, Fig. S4*D*), suggesting that expression of these genes in LPS-activated macrophages is dependent on HMGXB4. Further comparison with the LPS-responsive genes (Fig. 3*A*) revealed that the expression of 469 LPS-induced and 107 LPS-suppressed genes was significantly lowered and elevated, respectively, in *Hmgxb4*-deficient macrophages as compared to WT macrophages. These 469 and 107 genes were thus defined as *Hmgxb4*-dependent LPS-induced and LPS-suppressed genes, respectively (Fig. 3*D*). Pathway analysis showed that all of the top 10 significantly enriched pathways for the 1,630 LPS-induced genes in WT macrophages were associated with inflammation (e.g., TNF signaling pathway and Toll-like receptor signaling pathway; Fig. 3*E*). Interestingly, all of these pathways were affected by *Hmgxb4* expression, as evidenced by the significant enrichment of the 469 *Hmgxb4*-dependent LPS-induced genes (Fig. 3*E*). These data suggest that the major inflammatory pathways activated by LPS stimulation in macrophages are dependent on *Hmgxb4*.

To determine which genes were directly targeted by HMGXB4, we analyzed and annotated the HMGXB4 chromatin immunoprecipitation sequencing (ChIP-seq) peaks identified in human HepG2 cells obtained from the ENCODE database (https://www.encodeproject.org). This analysis revealed 20,911 HMGXB4 binding peaks in 9,620 genes (Dataset S2). Among these HMGXB4 targeted genes, 269 and 59 of HMGXB4-dependent LPS-induced and LPS-suppressed genes, respectively, had HMGXB4 binding peaks within their promoter regions (7 kb surrounding the transcription start site) (Fig. 3*F* and Dataset S2). Among these HMGXB4-dependent LPS-regulated genes, we focused on those highly expressed upon LPS stimulation (RPKM [reads per kilobase million] >100 in LPS-treated WT macrophages) (Fig. 3*G*). Of these genes, *Nos2* exhibited very high expression following LPS stimulation in WT macrophages (average RPKM = 139.4) and the transcriptional activation by LPS treatment was markedly inhibited by *Hmgxb4* deficiency (FC = 1.84, LPS-treated *Hmgxb4*-deficent vs. WT macrophages). The *Nos2* gene encodes for NOS2 protein, which constitutively generates high levels of NO. NO subsequently can react with superoxide anion to produce the potent oxidant, peroxynitrite ($ONOO^-$), which is a critical mediator of inflammation and tissue damage (28). We next tested whether *Nos2* accounts for the effect of *Hmgxb4* on LPS-induced gene expression. We examined the common genes shared by *Nos2*- and *Hmgxb4*-dependent LPS-induced genes by reanalyzing a publicly available RNA-array dataset from lung tissues that were harvested from WT and *Nos2* KO mice 7 h after stimulation with LPS (GSE130936) (29), which is comparable to the 6 h of challenge time of LPS in this study. This de novo analysis revealed that this dataset included 335 out of the 436 *Hmgxb4*-dependent LPS-induced genes and interestingly, the overall FC of these genes in comparison to LPS-treated *Nos2* KO versus WT mice was significantly lower than that of randomly selected genes (Fig. 3*H*). This bioinformatic analysis suggests that *Hmgxb4* and *Nos2* share a substantial portion of responding genes in response to LPS stimulation. Together with previous studies showing that inhibition of NOS2 or *Nos2* KO alleviates lung inflammation and ameliorates sepsis-induced pulmonary permeability and mortality in mice (30, 31), phenotypes similar to that observed in *Hmgxb4*-deficient mice in this study, we suggest that HMGXB4 likely drives inflammation, at least in part, by directly regulating expression of the *Nos2* gene.

HMGXB4 Mediates LPS-Induced NOS2 Expression and NO Production In Vitro. To validate the RNA-seq data, we selected *Nos2* together with other genes including *Tnf*, *Ccl2*, and *Il6*, whose expression were significantly induced by LPS but not affected by *Hmgxb4* deficiency. qRT-PCR and Western blot analysis confirmed that expression of *Nos2* was dramatically induced following

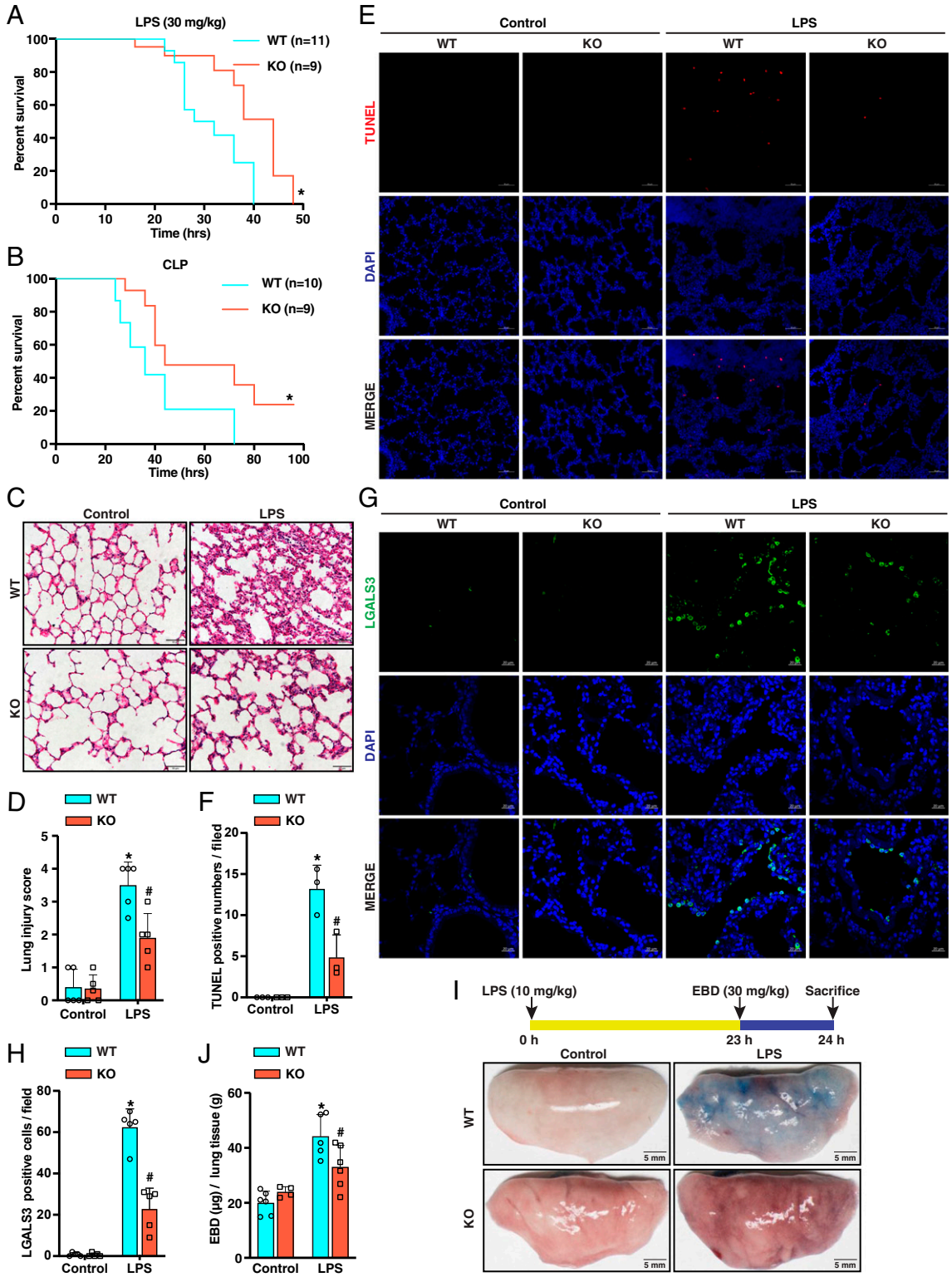

Fig. 2. Genetic deletion of *Hmgxb4* attenuates inflammatory responses. (*A*) Survival curves of WT and *Hmgxb4* KO mice following a lethal dose of LPS (30 mg/kg) or (*B*) subjected to CLP. $^{*}P < 0.05$. Log-rank (Mantel–Cox) test. (*C*) WT and KO mice were injected intraperitoneally with PBS (control) or LPS (20 mg/kg) for 6 h, and then lung tissues were harvested and sectioned for hematoxylin and eosin (H&E) staining. (*D*) Quantification of histopathological lung injury scores. $n = 5$; $^{*}P < 0.05$ vs. WT control; $^{\#}P < 0.05$ vs. WT LPS. (*E*) Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining (red) was performed using sections of lung tissues harvested from WT or KO mice treated with or without LPS. Nuclei were counterstained with DAPI (blue). (*F*) Quantification of the TUNEL-positive cells as shown in *E*. $n = 3$ mice each genotype; $^{*}P < 0.05$ vs. PBS-treated WT control mice; $^{\#}P < 0.05$ vs. LPS-treated WT mice. (*G*) Lung tissue sections were stained with macrophage marker LGALS3 antibody (green) and nuclei were counterstained with DAPI (blue). (*H*) Quantification analysis of the LGALS3-positive cell numbers as shown in *G*. $n = 5$; $^{*}P < 0.05$ vs. PBS-treated WT control mice; $^{\#}P < 0.05$ vs. LPS-treated WT mice. (*I*) WT and KO mice were intraperitoneally injected with PBS or LPS (10 mg/kg) for 23 h and then injected with EBD for an additional 1 h before mice were killed for harvesting lung tissues. Representative lungs with EBD staining (blue) indicate pulmonary vascular permeability. (*J*) Quantitative measurement of EBD-labeled albumin extravasation from lung tissues as shown in *I*. $n = 4$–6; $^{*}P < 0.05$ vs. PBS-treated WT control mice; $^{\#}P < 0.05$ vs. LPS-treated WT mice.

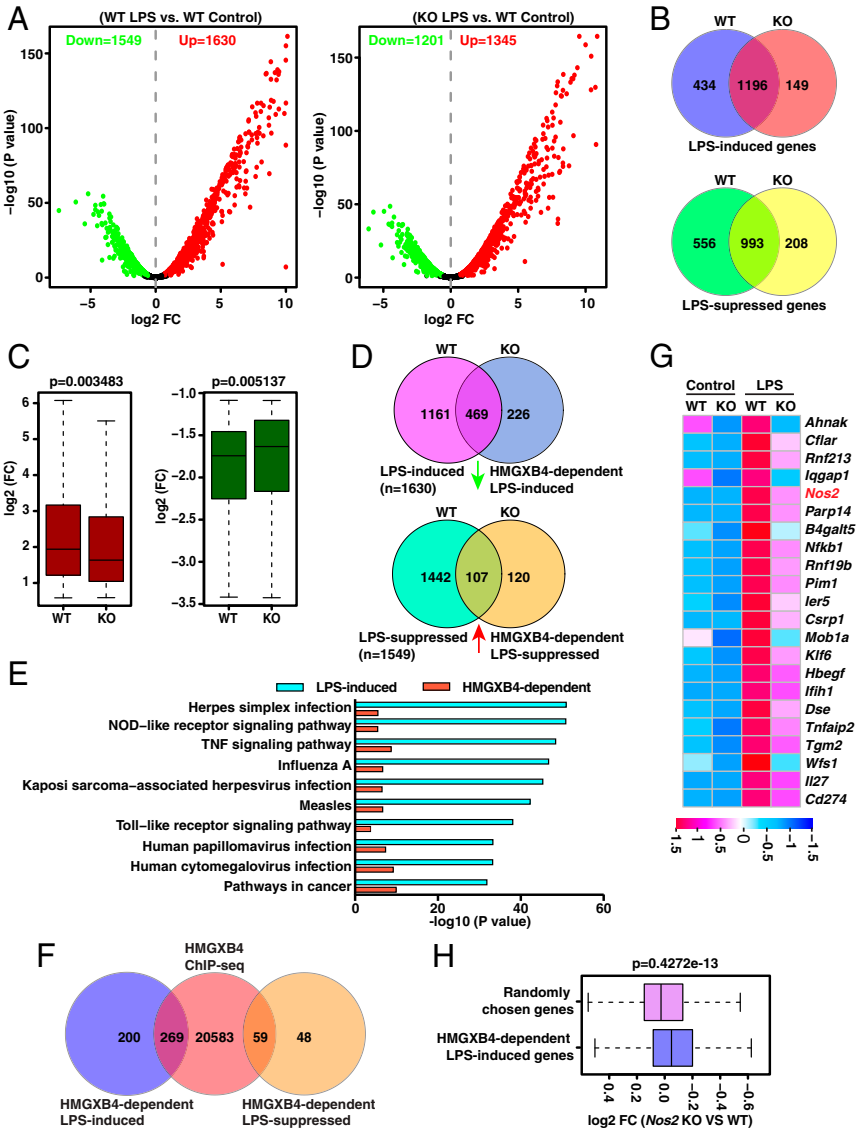

Fig. 3. HMGXB4 mediates LPS-induced gene expression in macrophages. (*A*) Volcano plots depicting the 1,630 LPS-induced and 1,549 LPS-suppressed genes in WT macrophages (*Left*) and 1,345 LPS-induced and 1,201 LPS-suppressed genes in *Hmgxb4* KO macrophages (*Right*). (*B*) Venn diagrams showing the overlap of LPS-induced (*Upper*) or suppressed genes (*Bottom*) between WT and *Hmgxb4* KO macrophages. (*C*) Box diagrams showing the fold change of the common LPS-induced (*Left*) or -suppressed (*Right*) genes in response to LPS treatment in WT vs. *Hmgxb4* KO macrophages. Student's *t* test. (*D*) Venn diagrams showing the identification of 469 and 107 HMGXB4-dependent LPS-induced (*Upper*) and LPS-suppressed genes (*Bottom*) by comparing LPS-responding genes with differentially expressed genes identified between LPS-treated *Hmgxb4*-deficient, and WT macrophages. (*E*) Pathway analysis showing that the 469 HMGXB4-dependent LPS-induced genes were also significantly enriched in the top 10 most significantly enriched pathways of the 1,630 LPS-induced genes. (*F*) Venn diagram showing 269 and 59 HMGXB4-dependent LPS-induced and LPS-suppressed genes containing HMGXB4 binding peaks within promoter region, respectively. The ChIP-seq data were generated in human HepG2 cells and obtained from the ENCODE database (ENCFF429KHK). (*G*) Heatmap showing the expression of the top 22 HMGXB4-dependent, LPS-induced genes with a threshold of RPKM >100 following LPS treatment in WT macrophage and containing HMGXB4 binding peaks within the promoter region. (*H*) RNA-array data (Gene Expression Omnibus [GEO] database: GSE130936) generated in lungs that were harvested from *Nos2* KO and WT mice subjected to LPS treatment for 7 h were de novo analyzed with the National Center for Biotechnology Information integrated GEO2R tool. Box diagram showing the FC of the 335 HMGXB4-dependent LPS-induced genes vs. randomly chosen genes between *Nos2* KO and WT mice. Mann–Whitney *U* test, $P = 0.427e\text{-}13$.

LPS treatment and the degree of induction was significantly reduced in macrophages lacking *Hmgxb4* (Fig. 4 *A*–*C*). Consistent with this, macrophages harvested from $Hmgxb4^{neo/neo}$ mice, in which *Hmgxb4* was ∼95% ablated, also showed similar inhibition of LPS-induced *Nos2* expression as compared to WT macrophages (*SI Appendix*, Fig. S5*A*). In contrast, the expression of *Tnf*, *Ccl2*, and *Il6* were not different between WT and *Hmgxb4*-null macrophages after LPS treatment, suggesting a specific effect of *Hmgxb4* deficiency on *Nos2* expression (*SI Appendix*, Fig. S5 *B*–*D*).

LPS-stimulated macrophages induce NOS2 expression which leads to the production of cytotoxic levels of NO (6). Excessive NO production during sepsis has a prominent role in undesirable vasodilatation, vascular hyperpermeability, and vascular leakage (32, 33). We next examined the effect of *Hmgxb4* on NO production in macrophages. We found that LPS-induced NOS2 expression and NO production in the macrophage culture medium in a time- and dose-dependent manner while *Hmgxb4* deficiency significantly blunted these effects (Fig. 4 *D*–*I*). Since LPS treatment can induce HMGXB4 expression in macrophages, we next sought to test whether overexpression of HMGXB4 can promote LPS-mediated NOS2 expression and NO production. We found that overexpression of HMGXB4 by adenoviral transduction in

peritoneal macrophages amplified LPS-induced NOS2 expression and NO production (Fig. 4 *J–L*). Together, these results demonstrate that HMGXB4 is required for efficient up-regulation of NOS2 expression and NO production in response to LPS in macrophage cells.

TLR4 is a cell surface receptor that mediates LPS-induced endotoxemia. In contrast, procaspase-11 is an intracellular LPS sensor that has also been shown to mediate endotoxemia by initiating pyroptosis independent of TLR4 signaling (34, 35). Capase-11 is also induced by double-stranded RNA, which can be mimicked by Poly(I:C). To test whether caspase-11 expression is HMGXB4 dependent, we treated WT or *Hmgxb4*-null peritoneal macrophages with or without the TLR3 agonist Poly(I:C) or the TLR4 agonist LPS. Data from Western blot assays revealed that both TLR3 and TLR4 agonists can significantly induce the expression of caspase-11 and HMGXB4 in a time-dependent manner (*SI Appendix*, Fig. S6 *A* and *B*). However, we found that *Hmgxb4* deficiency markedly reduces the increase in NOS2 expression while having no effect on the ability of TLR3/4 to induce caspase-11 expression, suggesting a specific effect of HMGXB4 in regulating *Nos2* gene expression (*SI Appendix*, Fig. S6 *A* and *B*).

HMGXB4 Transactivates the *Nos2* Gene. We next sought to determine the molecular mechanism by which HMGXB4 regulates *Nos2* gene expression. Although thus far there is no evidence that HMGXB4 acts as a transcription factor by directly binding to a specific DNA element, our de novo analysis of HMGXB4 ChIP-seq in HepG2 cells revealed a binding peak located in the ∼4.8-kb region upstream of the transcription start site of the human *NOS2* gene (Fig. 5 *A*, *Top*). Interestingly, this binding peak resides within an enhancer region as identified by two epigenetic transcriptional activation marks, H3K4me1 and H3K27ac (Fig. 5 *A*, *Bottom*), suggesting that this HMGXB4 binding region may be responsible for transcriptional regulation of the *NOS2* gene. To test this hypothesis, we amplified the ∼400-bp sequence surrounding the HMGXB4 binding site in the human and mouse *NOS2* genes (*SI Appendix*, Fig. S7 *A* and *B*) and cloned them into pGL2-promoter/luciferase vectors (Fig. 5*B*). Dual luciferase reporter assays revealed that overexpression of HMGXB4 significantly activated both the human and mouse *Nos2* enhancers (Fig. 5*C*), suggesting that the binding of HMGXB4 to the *Nos2* gene enhancer as revealed by ChIP-seq in human cells is both functional and conserved between human and mouse. By comparing the sequence of enhancer regions between human and mouse, we identified a conserved 84-bp sequence. To determine the importance of this conserved DNA fragment in mediating HMGXB4 transactivation of the *Nos2* gene, we generated a truncated luciferase reporter of human or mouse *Nos2* gene, harboring deletion of this conserved sequence (Fig. 5*D* and *SI Appendix*, Fig. S7 *A* and *B*). Data from luciferase assays demonstrated that truncation of this critical region almost completely abolished HMGXB4-mediated transactivation of both the human and mouse *Nos2* reporter genes (Fig. 5 *E* and *F*), suggesting that this conserved DNA region is required for *Nos2* activation. Taken together, these results advance the paradigm that HMGXB4 transactivates the *Nos2* gene via physically binding to a conserved region located in the *Nos2* gene enhancer and recruiting additional factors to drive *Nos2* gene transcription.

HMGXB4-Mediated Expression of NOS2 Is Critical for LPS-Induced Tissue Injury In Vivo. To extend the in vitro findings that HMGXB4 mediates NOS2 expression to in vivo settings of septic shock, we challenged both *Hmgxb4* KO and WT mice with a high dose of LPS (20 mg/kg body weight) for 6 h. Consistent with the results observed in macrophages, qRT-PCR and Western blot analysis revealed that HMGXB4 and NOS2 expression in lung tissues are induced by LPS treatment while NOS2 induction was significantly attenuated upon loss of *Hmgxb4*. Furthermore, consistent with previous finding that NOS3 (endothelial NOS or eNOS) is protective for LPS-mediated septic shock (36), *Hmgxb4* deficiency alleviates LPS-induced attenuation of both total and p-NOS3 expression (Fig. 6 *A–C*). The protective effects of *Hmgxb4* deficiency on LPS-induced lung damage was further supported by the inhibition of expression of inflammatory molecules, ICAM1 and VCAM1, in *Hmgxb4* KO mice as compared to WT mice (Fig. 6 *A–C*). Similar findings were also observed in liver tissues as revealed by qRT-PCR and Western blot analysis that showed that HMGXB4 was induced by LPS treatment while NOS2, ICAM1, and VCAM1 induction was significantly attenuated upon loss of *Hmgxb4* (Fig. 6 *D–F*).

By in vitro experiments we demonstrated that *Hmgxb4* deficiency decreased NO production by inhibiting NOS2 expression (Fig. 4 *D–I*). Given the fact that NO is responsible for tissue damage in sepsis (28, 37), we hypothesized that the *Hmgxb4* deficiency-mediated reduction of NO level was the most likely protective factor. To test this hypothesis, we treated mice with the pharmacological NOS2 inhibitor, 1400W, to inhibit NO production in vivo. We found that 1400W treatment significantly protects against LPS-induced lung injury in WT mice, but this protective effect was not seen in *Hmgxb4* KO mice after LPS treatment (Fig. 6 *G* and *H*). Consistently, compared to the WT LPS group, administration of 1400W in both the WT and *Hmgxb4* KO groups after LPS treatment prolonged the survival time. However, the survival time between the 1400W-treated WT LPS group and the *Hmgxb4* KO LPS group was not significantly different, suggesting that HMGXB4-mediated *Nos2* induction confers the susceptibility to endotoxemia-induced lethality in WT mice (Fig. 6*I*). Taken together, these data suggest that HMGXB4-mediated *Nos2* expression and NO production is critical for LPS-induced organ injury and lethality in mice.

HMGXB4 Is Critical for *NOS2* and *ICAM1* Expression in Both Human Pulmonary Epithelial and Endothelial Cells. We next sought to explore the translational relevance of this mechanism for sepsis management in humans by characterizing HMGXB4-mediated inflammatory responses in human cells. Previous studies have shown that isolated human macrophages and endothelial cells fail to express *NOS2* mRNA due to the epigenetic silencing around the human *NOS2* gene transcription start site (38, 39). However, in human lung epithelial cell line A549, the *NOS2* gene can be maximally induced by a mixture of the cytokines TNF, IL1B, and INFG (40, 41). To examine whether HMGXB4 regulates *NOS2* expression in human lung epithelial cells, A549 cells were transfected with control or *HMGXB4* silencing RNA duplexes followed by treatment with the TNF, IL1B, and INFG cytokine mixture (CM). qRT-PCR and Western blot analysis revealed that, in control cells, expression of *HMGXB4* and *NOS2*, together with the adhesive molecule ICAM1 are dramatically induced upon CM treatment. In contrast, silencing of *HMGXB4* significantly attenuated the expression of these genes at both the mRNA and protein levels (Fig. 7 *A–C*). ICAM1 plays a key role in mediating leukocyte–endothelial cell interactions and contributes to vascular permeability and septic shock (42). Since we found that induction of ICAM1 expression is dependent on HMGXB4 in human cells (Fig. 7 *A–C*), we hypothesized that the *ICAM1* gene may also be a direct transcriptional target of HMGXB4. Reanalysis of the HMGXB4 ChIP-seq dataset in human HepG2 cells indeed revealed two HMGXB4 binding peaks in the promoter region of *ICAM1* gene (Fig. 7*D*). To further examine whether ICAM1 is regulated by HMGXB4 in human endothelial cells, human pulmonary arterial endothelial cells (HPAECs) were transfected with control or *HMGXB4* silencing RNA duplexes followed by treatment with TNF. Consistent with the results in human epithelial cells, HMGXB4 knockdown significantly reduced TNF-induced ICAM1 expression (Fig. 7*E*). Moreover, reduced expression of ICAM1 in endothelial cells deficient in HMGXB4 significantly attenuated monocyte adhesion (Fig. 7 *F* and *G*). Together, these results suggest that HMGXB4 regulates the expression of both NOS2 and ICAM1 in multiple

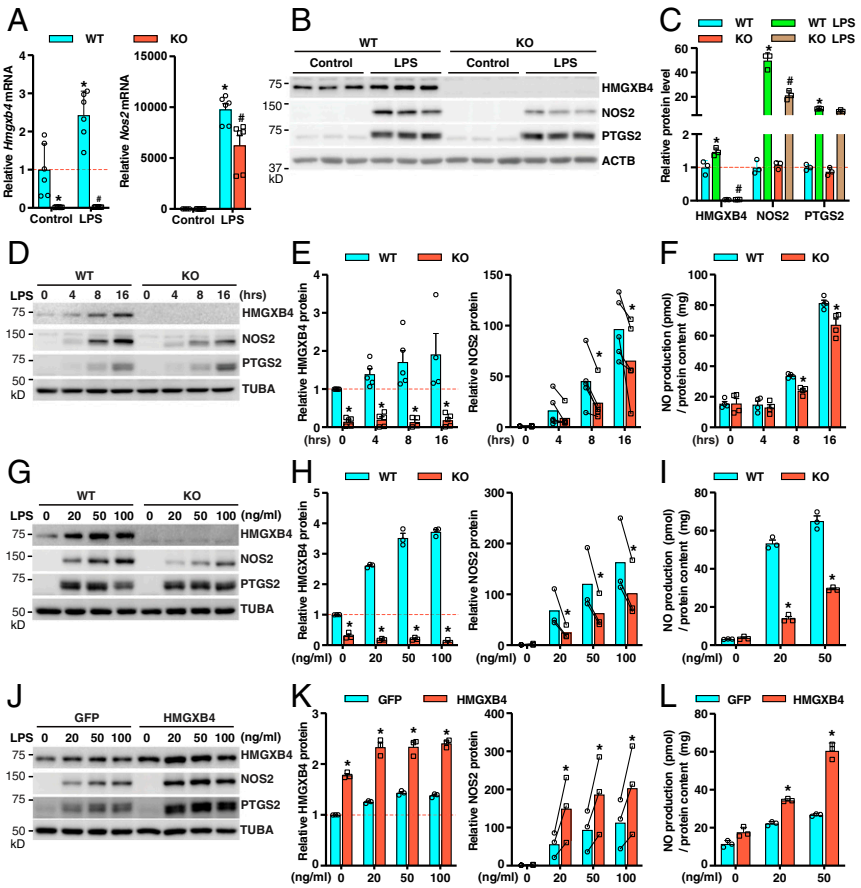

Fig. 4. Deletion of *Hmgxb4* attenuates LPS-induced *Nos2* expression and NO production in murine peritoneal macrophages. Cultured murine peritoneal macrophages harvested from WT and *Hmgxb4* KO mice were treated with LPS (100 ng/mL) for 6 h and cells were harvested for (*A*) qRT-PCR or for (*B*) Western blot analysis. The relative mRNA levels were quantified and presented relative to WT control (*A*, set to 1). $n = 6$; $^*P < 0.05$ vs. WT control; $^{\#}P < 0.05$ vs. WT LPS. (*C*) Densitometric analysis of relative protein levels as shown in *B*. $n = 3$; $^*P < 0.05$ vs. WT control; $^{\#}P < 0.05$ vs. WT LPS. (*D*) Cultured peritoneal macrophages harvested from WT and *Hmgxb4* KO mice were treated with LPS (100 ng/mL) for the time points as indicated and total proteins were extracted for Western blot analysis. (*E*) Densitometric analysis of relative HMGXB4 and NOS2 protein expression as shown in *D*. $n = 5$; $^*P < 0.05$ vs. WT. (*F*) Cell culture supernatant was collected from the cultured peritoneal macrophages after LPS treatment as shown in *D* to measure NO production. NO production was normalized to the protein content. $n = 4$; $^*P <$ 0.05 vs. WT. (*G*) Cultured peritoneal macrophages harvested from WT and *Hmgxb4* KO mice were treated with LPS at the doses indicated for 16 h and total proteins were extracted for Western blot analysis. (*H*) Densitometric analysis of relative HMGXB4 and NOS2 protein levels as shown in *G*. $n = 3$; $^*P < 0.05$ vs. WT. (*I*) Cell culture supernatant was collected from the cultured peritoneal macrophages as shown in *G* to measure NO production. Relative NO production is shown by normalization to the protein content. $n = 3$; $^*P < 0.05$ vs. WT. (*J*) Cultured peritoneal macrophages harvested from WT mice were transduced with GFP or HMGXB4 adenovirus for 48 h and then treated with LPS with the indicated doses for 16 h. Total protein was subsequently extracted for Western blot analysis. (*K*) Densitometric analysis of relative HMGXB4 and NOS2 protein levels as shown in *J*. $n = 3$; $^*P < 0.05$ vs. GFP. (*L*) Cell culture supernatant was collected from cultured peritoneal macrophages as shown in *J* to measure NO production. NO production was normalized to protein content. $n = 3$; $^*P < 0.05$ vs. GFP.

cell types, in both mouse and human, as part of the inflammatory response triggered by LPS or cytokines.

Discussion

In this study, we provide evidence that the HMG family protein HMGXB4 plays an important proinflammatory role in the context of systemic inflammation. We demonstrate that HMGXB4 expression is augmented by LPS or inflammatory cytokine-induced inflammation in macrophages, lung epithelial cells, and endothelial cells. The induction of HMGXB4 expression, which is likely through the NF-κB signaling pathway, in turn, transcriptionally activates *Nos2* gene expression, resulting in expression of constitutively active NOS2 and high levels of NO production. Meanwhile, HMGXB4 also promotes expression of the inflammatory adhesion molecule *ICAM1* in endothelial cells, leading to enhanced monocyte adhesion. The HMGXB4-dependent excessive production of NO and monocyte adhesion likely are responsible for vascular hyperpermeability, tissue damage, and organ failure in response to septic shock. Conversely, deletion of the *Hmgxb4* gene protects against LPS-induced systemic inflammation and tissue damage by attenuating the expression of ICAM1 and NOS2 and NO production, thereby ameliorating systemic inflammation-induced endotoxemia (Fig. 7*H*).

The HMG family of proteins has been implicated in various physiological and pathological pathways (19, 21, 43, 44), including inflammation (45). HMGXB4 is a member of the HMG family that we previously identified and cloned (25). Given the critical role of HMGB1 and 2 in inflammatory responses and our previous findings that HMGXB4 was induced in response to vascular injury, a process also associated with inflammation (25), we examined the possible role of HMGXB4 in regulating the inflammatory response in vivo. Interestingly, we found that HMGXB4 is induced in murine peritoneal macrophages activated by different TLR agonists, and especially by the major driver of septic shock, LPS (Fig. 1 *A*–*H*). Previous studies have reported that HMGB1 is released from various immune cells, including macrophages, monocytes, natural killer cells, dendritic cells, endothelial cells, and platelets (46–49), and is also passively released by necrotic or

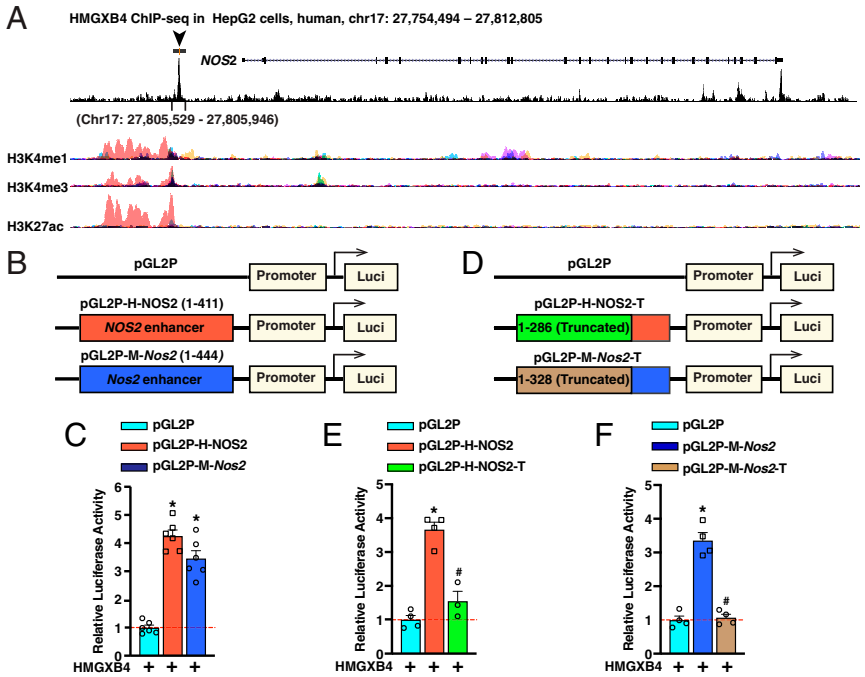

Fig. 5. HMGXB4 binds to and transactivates the *Nos2* gene. (*A*) Genomic tracks displaying the ChIP-seq binding peak of HMGXB4 and active transcription marks, H3K4me1, H3K4me3, and H3K27ac in the human *NOS2* gene locus in HepG2 cells. An arrowhead points to the HMGXB4 binding peak in the *NOS2* gene promoter and this binding region (numbered under the binding peak) was cloned for promoter activity analysis. (*B*) Schematic diagram shows the luciferase reporters that harbor HMGXB4 binding regions in human (H) and mouse (M) *Nos2* gene promoter as described in *A*. The empty pGL2P luciferase reporter served as a negative control. (*C*) pGL2P luciferase reporters carrying human or mouse *Nos2* gene promoter sequence was cotransfected into 10T1/2 cells with expression plasmid *Hmgxb4* and dual luciferase reporter assays were performed. Luciferase activity of the control (pGL2P) reporter cotransfected with *Hmgxb4* plasmid was set to 1. $n = 6$; $^*P < 0.05$. (*D*) Schematic diagram shows the generation of truncated (T) human and mouse *Nos2* promoter luciferase reporters, in which the putative HMGXB4 binding elements were deleted. (*E*) Human or (*F*) mouse truncated (T) *Nos2* gene luciferase reporters were cotransfected with HMGXB4 expression plasmid into 10T1/2 cells and dual luciferase reporter assays were performed. Luciferase activity of the control (pGL2P) reporter cotransfected with *Hmgxb4* plasmid was set to 1. $n = 3$–4; $^*P < 0.05$.

damaged cells (50–53). Furthermore, the proinflammatory property of HMGB1 depends on the translocation of HMGB1 from the nucleus to the extracellular milieu (54). Unlike HMGB1, we found that HMGXB4 is exclusively localized in the nucleus regardless of LPS stimulation or pyroptosis (Fig. 1 *J* and *K*), indicating a unique mechanism distinguishing HMGXB4-mediated proinflammatory effects.

Multiorgan dysfunction or failure, including acute lung injury, is a hallmark of sepsis. Administration of agents that specifically inhibit HMGB1 activity, such as antibodies and other pharmacological agents, has been shown to interrupt the progression of lung tissue injury and suppresses inflammatory responses (46, 55–58). Similarly, here we found that genetic deletion of *Hmgxb4* in mice significantly reduced LPS-induced mortality and tissue injury, as evidenced by increased survival time (Fig. 2 *A* and *B*), attenuated lung and kidney injury (Fig. 2 *C*–*H* and *SI Appendix*, Fig. S3 *B* and *C*), and pulmonary vascular permeability (Fig. 2 *I* and *J*) in *Hmgxb4*-deficient mice. Consistent with these observations, RNA-seq analysis showed that *Hmgxb4* depletion has a profound effect on LPS-activated gene programs in macrophages (Fig. 3 *A*–*C*). In addition to identifying the *Nos2* gene as a direct target of HMGXB4 in macrophages and lung epithelial cells, we also found that *Icam1* is also targeted by HMGXB4 and expression of ICAM1 promotes monocyte adhesion to endothelial cells. Thus, specific targets of HMGXB4 collectively confer a proinflammatory function in different cells in response to diverse inflammatory stimuli.

Tissue injury in sepsis is primarily caused by a cascade of autoamplifying cytokines and NO production. Multiple cytokines have been reported to be elevated in sepsis, including TNF (59, 60), CCL2 (61), and IL6 (62–64). However, all of the clinical trials targeting cytokine production have so far failed to produce beneficial results (7). Interestingly, we found that although *Hmgxb4* deficiency reduces mortality and protects against tissue damage caused by LPS-induced endotoxemia, the expression levels of these inflammatory cytokines were not different between WT and *Hmgxb4* KO mice (*SI Appendix*, Figs. S3*A* and S5 *B*–*D*). In contrast, we found that *Hmgxb4* specifically affects a subset of downstream inflammatory genes such as *Nos2*. Furthermore, we found that NOS2-mediated NO production is a key mechanism underlying HMGXB4-mediated inflammatory responses induced by LPS.

NO is produced by NOS and is a diffusible free radical gas with a wide range of chemical reactivities and bioactivities (65). There are three types of NOSs, including NOS1 (neuronal NOS or nNOS), NOS2 (inducible NOS or iNOS), and NOS3 (endothelial NOS or eNOS). In contrast to NOS1 and NOS3 which are constitutively expressed and dynamically regulated, NOS2 is only expressed on demand in activated cells and especially in proinflammatory macrophages.

In contrast to murine cells, methylation-induced silencing of the NOS2 gene in human monocytes and endothelial cells limited us from assessing a role for HMGXB4 in regulating NOS2 expression in these cells (38). However, we were able to confirm that HMGXB4 regulates NOS2 expression in human lung epithelial cells (Fig. 7 *A*–*C*) in a manner similar to that of rodent cells. NOS2 is the major source of excess NO observed in the serum of sepsis patients (66–68). In fact, in agreement with a previous report (36), we found that lung tissues have reduced NOS3 (eNOS) expression after septic shock (Fig. 6 *B* and *C*). These data support the concept that NO production in lung tissues during septic shock is mainly produced by NOS2. It remains controversial whether NO plays a beneficial or harmful role in inflammation (66, 67, 69). However, excessive production

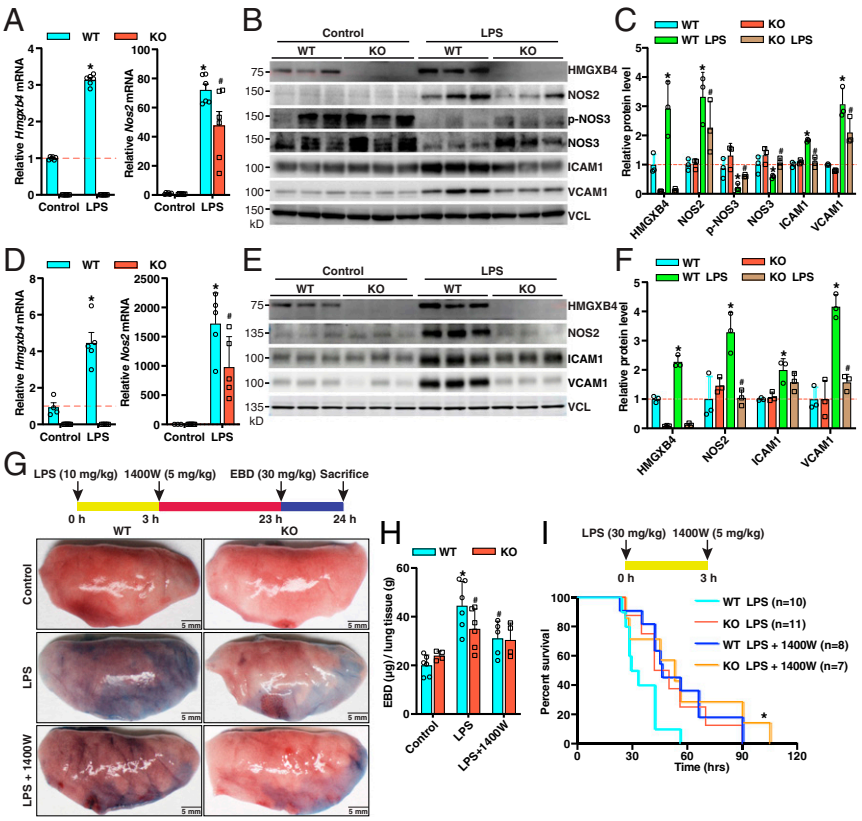

Fig. 6. HMGXB4 mediates LPS-induced NOS2 expression and tissue injury in vivo. WT and *Hmgxb4* KO mice were treated with LPS (20 mg/kg) for 6 h. Subsequently lung (*A*–*C*) and liver tissues (*D*–*F*) were harvested for (*A* and *D*) qRT-PCR or for (*B* and *E*) Western blot analysis. The relative mRNA levels were presented relative to WT without LPS treatment (*A* and *D*, set to 1). $n = 5$–6; $^{*}P < 0.05$ vs. WT control; $^{\#}P < 0.05$ vs. WT LPS. (*C* and *F*) Densitometric analysis of relative protein levels as shown in *B* and *E*, respectively. $n = 3$; $^{*}P < 0.05$ vs. WT control; $^{\#}P < 0.05$ vs. WT LPS. (*G*) WT or *Hmgxb4* KO mice were challenged with PBS (control) or LPS (10 mg/kg) for 3 h and then intraperitoneally injected with the NOS2 inhibitor 1400W (5 mg/kg) for additional 20 h. One hour prior to killing, mice were injected with EBD and lung tissues were then harvested to assess pulmonary vascular permeability. Representative images of lung tissues are shown. (*H*) Quantitative analysis of EBD-labeled albumin extravasation from the lungs of PBS-, LPS-, or 1400W-treated WT or *Hmgxb4* KO mice. $n = 4$–6; $^{*}P < 0.05$ vs. WT control; $^{\#}P < 0.05$ vs. WT LPS. (*I*) Survival curves of WT or *Hmgxb4* KO mice administered a lethal dose of LPS (30 mg/kg) for 3 h followed by treatment with or without the NOS2 inhibitor, 1400W (5 mg/kg). $n = 7$–11; $^{*}P < 0.05$, all other groups compared with WT treated with LPS group (cyan line); Log-rank (Mantel–Cox) test.

of NO can have deleterious consequences on the inflammatory process, causing cell death and tissue injury by reacting with superoxide anion to produce peroxynitrite ($ONOO^{-}$) (28, 37). Therefore, limiting NOS2-dependent NO production to an optimal range may be an attractive approach for treating sepsis. Along these lines, Baron et al. showed that a partial reduction of NOS2 expression during endotoxemia, rather than completely eliminating its expression, can improve the outcome of sepsis (70). Interestingly, our unbiased RNA-seq analysis clearly reveals that the *Nos2* gene, the major source of NO in sepsis, was attenuated in *Hmgxb4*-deficient macrophages after LPS treatment (Fig. 3*G*), suggesting that HMGXB4 is a critical regulator of *Nos2* gene expression and NOS2-derived NO production. Several additional lines of evidence further support a close association between *Hmgxb4*, *Nos2*, and inflammation. These include that 1) *Hmgxb4* and *Nos2* share a substantial portion of downstream target genes in response to LPS treatment (Fig. 3*H*); 2) inflammation-induced *Nos2* expression is dependent on HMGXB4 (Figs. 4 and 7); 3) LPS-induced NO production in macrophages is dependent on HMGXB4 (Fig. 4 *F*, *I*, and *L*); and 4) inhibition of NOS2 using a selective pharmacological inhibitor dramatically attenuates LPS-induced pulmonary vascular permeability (Fig. 6 *G* and *H*), which resembles the protective effect observed in *Nos2*$^{-/-}$ (30) and *Hmgxb4*-deficient mice. Together, these results collectively suggest that manipulation of the HMGXB4-NOS2-NO axis may be a promising therapeutic approach for treatment of sepsis.

De novo analysis of the publicly available HMGXB4 ChIP-seq datasets revealed widespread binding of HMGXB4 across the human genome (Fig. 3*F*). On closer inspection we identified a HMGXB4 binding peak within an enhancer region of *NOS2* (Fig. 5*A*) and we further demonstrated that this binding region is functional in mediating HMGXB4-induced *Nos2* expression in both mouse and human (Figs. 5 *B*–*F* and 7 *A*–*C*). Since HMGXB4 is not known to contain a canonical DNA binding motif, it is unclear at this time whether HMGXB4 acts as a transcription factor by directly binding to this region or whether it acts as a transcription cofactor through the binding of other transcription factors. In cells, we found that deletion or overexpression of *Hmgxb4* only affected LPS-mediated *Nos2* expression but not its baseline expression (Fig. 4 *B* and *J*), suggesting other factors could be involved. Furthermore, in luciferase reporter assays *Hmgxb4* was able to stimulate *Nos2* enhancer activity when adjacent to a minimal promoter. It is well known that LPS stimulates transcription of the *Nos2* gene mainly through activation of TLR4/NF-κB pathway via RELA (P65) which binds to the κB binding motif located within the *Nos2* proximal promoter (71–73). Since the major function of HMG proteins is to induce conformational changes in DNA (16) and our data suggest that HMGXB4 transactivates a distal region in the *Nos2* gene promoter (Fig. 5), we speculate that HMGXB4 may act as a DNA binding protein which facilitates the interaction of the remote region with the proximal promoter of the *Nos2* gene. Alternatively, HMGXB4 may bind to

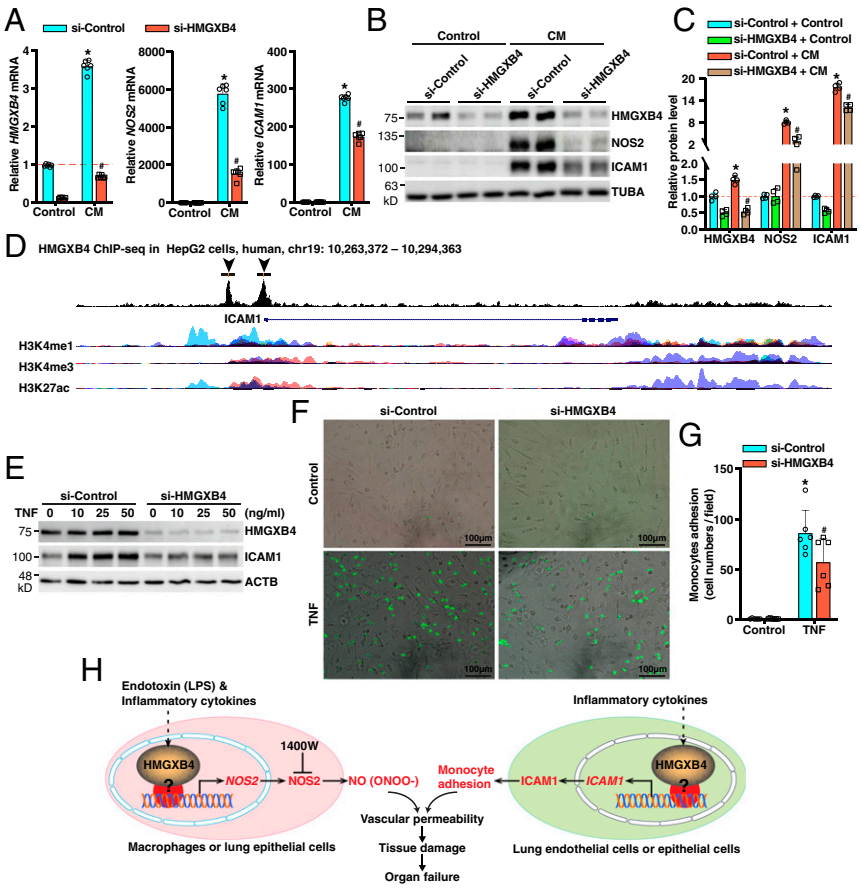

Fig. 7. HMGXB4 regulates inflammatory responses in human cells. (*A*) Following transfection with control or HMGXB4 silencing RNA duplexes for 48 h, human lung epithelial cells (A549 cells) were treated with or without a cytokine mixture (CM, 10 ng/mL TNF, 10 ng/mL IL1B, and 100 ng/mL INFG) for 6 h and were then harvested for qRT-PCR to examine *HMGXB4*, *NOX2*, and *ICAM1* mRNA expression. The relative mRNA levels are presented relative to silencing control without CM treatment (set to 1, red dashed line). $n = 6$; $^{*}P < 0.05$ vs. si-control + control; $^{\#}P < 0.05$ vs. si-control + CM. (*B*) Similar to *A* except A549 cells were treated with or without CM for 16 h; cells were harvested for Western blot analysis. (*C*) Densitometric analysis of relative protein levels as shown in *B*. $n = 4$; $^{*}P < 0.05$ vs. si-control + control; $^{\#}P < 0.05$ vs. si-control + CM. (*D*) Genomic tracks displaying ChIP-seq binding peaks of HMGXB4 and active transcription marks, H3K4me1, H3K4me3, H3K27ac in the *ICAM1* gene locus in human HepG2 cells. Arrows point to the binding peaks of HMGXB4 in human *ICAM1* gene promoter. (*E*) Human pulmonary artery endothelial cells (HPAECs) were transfected with control or HMGXB4 silencing RNA duplexes for 48 h and then were treated with TNF at the doses indicated for 24 h. Cells were then harvested for Western bot analysis. (*F* and *G*) Representative images and quantification of cellular adhesion of THP-1 monocytes (green) to activated HPAECs transfected with control or HMGXB4 silencing RNA duplexes, followed by treatment with 10 ng/mL TNF for 6 h. $n = 6$; $^{*}P < 0.05$ vs. si-control + control; $^{\#}P < 0.05$ vs. si-control + TNF. (*H*) Schematic diagram outlining the key findings of this study. We found that HMGXB4 expression is induced by LPS or inflammatory cytokines. Subsequently HMGXB4 transactivates the target genes *NOS2* or *ICAM1* via direct or indirect binding (as shown by a question mark). The overexpression of *NOS2* and *ICAM1*, in turn, leads to excessive NO production and monocyte adhesion, which increases vascular permeability, causing tissue damage and eventually fatal organ failure. Conversely, deletion of the *Hmgxb4* gene protects against LPS or cytokine-induced systemic inflammation, endothelial system dysfunction, and tissue damage by attenuating the induction of *Nos2* and *Icam1* gene expression.

RELA to synergistically activate the *Nos2* gene upon LPS treatment. These proposed working models, however, need to be confirmed by future studies.

In summary, our study provides evidence that HMGXB4 plays an important proinflammatory role in endotoxin-induced systemic inflammation, through transcriptional activation of the *Nos2* and *Icam1* genes. Our results suggest that inhibition of HMGXB4 expression or its ability to bind enhancer regions of inflammatory genes could serve as a therapeutic approach for management of sepsis.

Materials and Methods

A targeted *Hmgxb4* allele mouse line harboring a gene-trap knockout, lacZ-tagged insertion with conditional potential was generated by Cyagen. A conditional (flox, F) allele was subsequently achieved by crossing the gene-trapped *Hmgxb4* allele female mice with male flippase recombinase expressing mice (The Jackson Laboratory, stock no. 009086) to remove the En2 SA/LacZ/neo cassette flanked by two FRT (flippase recognition target) sites. To generate the global *Hmgxb4* KO mice, female mice harboring the conditional allele, $Hmgxb4^{F/F}$, were bred with male mice ubiquitously expressing Cre (CMV-Cre, The Jackson Laboratory, stock no. 006054) under the transcriptional control of a ubiquitous human CMV minimal promoter to delete loxP-flanked exon (E) 3 to 5 of the *Hmgxb4* gene in all cell types/tissues. The resultant heterozygous mice were subsequently intercrossed to generate mice with global deletion of *Hmgxb4*. All mice used in this study were maintained on a C57BL/6J background. Both male and female mice were used in this study. The use of experimental animals has been approved by the Institutional Animal Care and Use Committee and Biosafety Committee at Augusta University in accordance with NIH guidelines. More detailed methods and materials are included in *SI Appendix*.

Data Availability. RNA-seq data have been deposited in Sequence Read Archive at the National Center for Biotechnology Information (PRJNA565447).

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