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Intracellular Hmgb1 Inhibits Inflammatory Nucleosome Release and Limits Acute Pancreatitis in Mice

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Abstract

BACKGROUND & AIMS: High mobility group box 1 (HMGB1) is an abundant protein that regulates chromosome architecture and also functions as a damage-associated molecular pattern molecule. Little is known about its intracellular roles in response to tissue injury or during subsequent local and systemic inflammatory responses. We investigated the function of Hmgb1 in mice following induction of acute pancreatitis.

METHODS: We utilized a Cre/LoxP system to create mice with pancreas-specific disruption in *Hmgb1* (Pdx1-Cre; HMGB1^{fllox/fllox} mice). Acute pancreatitis was induced in these mice

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Full Methods and any associated references are available in the **SUPPLEMENTAL MATERIALS AND METHODS**.

(HMGB1^{flox/flox} mice served as controls) following injection of L-arginine or cerulein. Pancreatic tissues and acinar cells were collected and analyzed by histologic, immunoblot, and immunohistochemical analyses.

RESULTS: Following injection of L-arginine or cerulein, Pdx1-Cre; HMGB1^{flox/flox} mice developed acute pancreatitis more rapidly than controls, with increased mortality. Pancreatic tissues of these mice also had higher levels of serum amylase, acinar cell death, leukocyte infiltration, and interstitial edema than controls. Pancreatic tissues and acinar cells collected from the Pdx1-Cre; HMGB1^{flox/flox} mice following L-arginine- or cerulein injection demonstrated nuclear catastrophe with greater nucleosome release when compared with controls, along with increased phosphorylation/activation of RELA Nfkb, degradation of Ikb, and phosphorylation of Mapk. Inhibitors of reactive oxygen species (N-acetyl-L-cysteine) blocked L-arginine-induced DNA damage, necrosis, apoptosis, release of nucleosomes, and activation of Nfkb in pancreatic tissues and acinar cells from Pdx1-Cre; HMGB1^{flox/flox} and control mice. Exogenous genomic DNA and recombinant histone H3 proteins significantly induced release of HMGB1 from mouse macrophages; administration of antibodies against H3 to mice reduced serum levels of HMGB1 and increased survival following L-arginine injection.

CONCLUSIONS: In 2 mouse models of acute pancreatitis, intracellular HMGB1 appeared to prevent nuclear catastrophe and release of inflammatory nucleosomes to block inflammation. These findings indicate a role for the innate immune response in tissue damage.

Keywords

DNA damage; pancreatitis; oxidative stress; Nfkb

Introduction

Innate immune cells orchestrate both the physiologic and pathologic inflammatory immune response following engagement by pattern-recognition receptors including pathogen-associated molecular pattern (PAMPs) or damage-associated molecular pattern (DAMPs) molecules¹⁻³. Many DAMPs are derived from the nucleus, and are thus collectively termed nuclear DAMPs (nDAMPs). Examples of nDAMPs include high mobility group box 1 (HMGB1)^{4,5} and components of the nucleosome (e.g., DNA⁶ and histones⁷). Within the nucleus, HMGB1 maintains chromosomal structure and regulates DNA damage responses⁸. Under a variety of stressful situations, however, HMGB1 translocates to the cytosol, where it sustains autophagy, and then is released into the extracellular space. There, it coordinates inflammation, immunity, and other local cellular processes⁹. Pathologic nDAMP release is increasingly being recognized as an etiology for a variety of human inflammatory diseases and represents an emergent target for therapy^{10,11}. A better understanding of the intricate mechanisms underlying the release and response to nDAMPs will aid in this effort.

Acute pancreatitis (AP) is a poorly understood inflammatory disease, responsible for significant human morbidity and mortality each year worldwide¹². In patients and animals with AP, serum levels of HMGB1 are significantly increased and positively correlate with the severity of the disease¹³⁻¹⁵. Inhibiting the release or cytokine activity of HMGB1 confers protection against experimental AP¹⁶⁻¹⁸. The precise role of HMGB1 during acute pancreatitis-induced tissue injury and subsequent local and systemic inflammation is poorly understood. Here, we show, in contrast to neutralization of HMGB1 in the serum, that conditional knockout of HMGB1 in the pancreas rendered mice dramatically more susceptible to experimental AP. Deficiency of endogenous pancreatic HMGB1 resulted in accelerated tissue injury and lethality. This enhanced severity was associated with increased nuclear catastrophe and nucleosome (histone and DNA) release and increased recruitment and activation of inflammatory cells. Interestingly, the subsequent activation of innate

immune effectors was associated with increased HMGB1 release into the circulation. Neutralizing extracellular histone and/or HMGB1 conferred protection against AP in conditional pancreas-specific HMGB1 knockout mice. Thus, intracellular nuclear HMGB1 serves as a previously underappreciated negative regulator of inflammation-limiting nuclear catastrophe following injury with resultant release of other nDAMPs. This work improves our understanding of the complex role of HMGB1 in protecting against cellular injury and subsequent activation of innate immune responses following sterile tissue damage.

Materials and Methods

Reagents

The antibodies to p-RELA, RELA (also known as p65), p-I κ b, I κ b, IKK α , IKK β , IKK γ , p100, RELB, p-MAPK14, MAPK14 (also known as p38), p-MAPK8, MAPK8 (also known as JNK), p-MAPK1, MAPK1 (also known as ERK), H3, H4, γ -H2AX, C-Casp3, C-PARP, and actin were obtained from Cell Signaling Technology (Danvers, MA, USA). The antibody to HMGB1 came from Novus (Littleton, CO, USA). The antibody to 4-Hydroxy-2-nonenal came from Alpha Diagnostic (San Antonio, TX, USA). Mouse genomic DNA and DNase I came from New England Biolabs (Ipswich, MA, USA). Recombinant calf thymus histone H3 came from Roche (Madison, WI, USA). Recombinant HMGB1 proteins were generated as previously described¹⁹. Contaminating endotoxin was removed from protein by Triton X-114 extraction. Mouse HMGB1 neutralizing antibody (IgG2B) was generated as previously described²⁰. Rabbit H3 neutralizing antibody came from Abcam. Control mouse IgG2B and rabbit IgG came from R&D Systems (Minneapolis, MN, USA). Unless otherwise stated, all other reagents were purchased from Sigma (St. Louis, MO, USA).

Mouse strains

The protocol for animal use was reviewed and approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Pancreatic specific HMGB1 knockout mice were prepared and bred in our laboratory by crossing floxed HMGB1 (*HMGB1^{flox/flox}*) and *Pdx1-Cre* transgenic mice. In the floxed mice, exons 2 and 3 of HMGB1 gene were flanked by two lox/p sites that enable the recombination of the HMGB1 loci in the presence of cre recombinase (Fig.S1A). *Pdx1-Cre* transgenic mice on C57BL/6 background were obtained from the MMHCC/NCI Mouse Repository. As both mouse strains were on the B6 background, all progeny used in our study were generated with B6 mice with pure genetic backgrounds. Specifically, F1 offspring of an initial *Pdx1-Cre* \times *HMGB1^{flox/flox}* intercross mating were genotyped to confirm the presence of both the *Pdx1-Cre* and floxed HMGB1 alleles by standard polymerase chain reaction (PCR). To generate mice homozygous for the floxed HMGB1 allele, F1 generation mice were backcrossed with the *HMGB1^{flox/flox}* line (Fig.S1B). *Pdx1-Cre; HMGB1^{flox/flox}* (termed CH mice) offspring were identified by genotyping for the presence of both the *Pdx1-Cre* and floxed HMGB1 alleles and the absence of the wild-type HMGB1 allele. Recombination/deletion of the HMGB1 gene in pancreatic cells was confirmed by genotyping analysis (Fig.S1C). The presence of the *Pdx1-Cre* transgene was detected by PCR amplification with primers 5'-CTGGACTACATCTTGAGTTGC-3' and 5'-GGTGTACGGTCAGTAAATTTG-3'; the identification of floxed (700bp) and wild type (635bp) HMGB1 with 5'-TGATGCGAACACGGCGTGCTCTA' and 5'-GCACAAAGAATGCATATGAGGAC-3'. In parallel, HMGB1 level in pancreatic tissue or extracts from the pancreatic acinar cells of CH mice was assayed by immunofluorescent staining (Fig.S1D) and Western blot (Fig.S1E), respectively.

Experimental animal models of acute pancreatitis

For L-arginine-induced pancreatitis, a sterile solution of L-arginine hydrochloride (8%; Sigma) was prepared in normal saline and the pH was adjusted to 7.0. Mice received two hourly intraperitoneal (i.p.) injections of L-arginine (4 g/kg), while controls were administered saline i.p. as a control as described previously²¹. For cerulein-induced pancreatitis, mice received seven hourly i.p. injections of 50 µg/kg cerulein (Sigma) in sterile saline, while controls were given saline as described previously²². Animals were sacrificed at the indicated time by CO₂ asphyxia, and a blood sample and tissue were collected. Blood samples were collected in heparinized syringes and centrifuged at 10,000 g for 10 minutes at 4°C. Following centrifugation, the plasma was aspirated and used for measurement of amylase, lactate dehydrogenase (LDH), nucleosomes, HMGB1, and other cytokines by ELISA. Tissue samples were collected, snap frozen in liquid nitrogen, and stored at -80°C for analysis of myeloperoxidase (MPO) activity. Formalin-fixed pancreas samples were processed, and 5 µm thick paraffin sections were stained with hematoxylin and eosin (H&E) for histological analysis.

Results

Pancreas-Derived HMGB1 Protects Against Experimental Acute Pancreatitis

Because global HMGB1 knockout mice die shortly following birth²³, we generated transgenic mice with conditional knockout of HMGB1 within the pancreas (CH mice) (Fig. S1A-1C). The expression of HMGB1 is essentially missing from pancreatic tissue (Fig.S1D) or cultured pancreatic acinar cells from CH mice (Fig.S1E). CH mice demonstrated phenotypes similar to wild type and/or HMGB1^{flox/flox} control mice (F/F mice) in pancreas size (Fig.S1F), exocrine function (serum amylase level and pancreatic trypsin activity) (Fig.S1G-H), endocrine function (blood glucose level) (Fig.S1I), and pancreatic histology (Fig. S1J). These findings suggest that HMGB1 does not itself affect pancreatic development.

Severe AP was induced with i.p. injection of L-arginine or cerulein as previously described^{21, 22}. The CH mice were substantially more susceptible to AP with significantly higher mortality rates compared to F/F mice (Fig. 1A). Histological assessment of pancreatic damage revealed exaggerated acinar cell death, leukocyte infiltration, and interstitial edema in the CH mice compared to F/F mice (Fig. 1B). The level of serum amylase, the most commonly used biochemical marker of established AP, was also significantly higher in CH mice (Fig. 1C). Consistently, pancreatic neutrophil recruitment (as measured by the pancreatic MPO activity, Fig. 1D) and pancreatic necrosis (as reflected by the serum LDH activity, Fig. 1E) were significantly higher in the CH mice as well. Intrapancreatic conversion of trypsinogen to trypsin is an important step in the development of acute pancreatitis¹². The loss of HMGB1 within pancreatic tissue from CH mice accelerated L-arginine-induced pancreatic trypsin activity when compared with F/F mice (Fig. 1F). Severe AP is often associated with acute lung injury, a significant cause of morbidity and mortality in this disease. L-arginine-induced, AP-associated acute lung injury was worse in CH mice (Fig. S2). Similarly, cerulein-induced experimental AP was also associated with increased acinar cell injury (Fig. S3A), increased serum amylase (Fig. S3B), increased pancreatic MPO activity (Fig. S3C), and increased pancreatic trypsin activity (Fig. S3D) in CH mice when compared with F/F control mice. These results demonstrate that, in contrast to neutralization or prevention of HMGB1 release within the serum, loss of endogenous HMGB1 within the injured pancreatic acinar cell worsens the severity of experimental AP.

Pancreas-Derived HMGB1 Inhibits Inflammatory Response in Acute Pancreatitis

Excessive inflammation contributes to the pathogenesis of AP through activation of multiple signal transduction pathways involving nuclear factor κ B (Nf κ b) and mitogen-activated protein kinases (MAPKs)^{24, 25}. Loss of HMGB1 in the pancreas led to a significant enhancement of L-arginine-induced Nf κ b RELA phosphorylation/activation (Fig. 2A, 2B, 2C), I κ b degradation (Fig. 2B), and MAPK phosphorylation (Fig. 2B and Fig. S4) in the pancreas or pancreas extracts. In parallel, the serum levels of TNF- α and IL-6 were significantly increased at 48 and 72 hours in the CH mice when compared with F/F control mice following administration of L-arginine (Fig. 2D) or cerulein (Fig. S3E and 3F). The intra-pancreatic increase in cytokines and chemokines are early and much more pronounced than found in the blood. Loss of HMGB1 in the pancreas led to a significant enhancement of L-arginine-induced intra-pancreatic cytokine (e.g., IL-6) and chemokine (monocyte chemoattractant protein 1, MCP-1) expression at 24 hours (Fig. 2E). Thus, loss of HMGB1 in the acinar cell created an exaggerated local and systemic inflammatory response.

Intracellular HMGB1 Prevents DNA Damage, Cell Death, and nDAMP Release in Acute Pancreatitis

Nuclear HMGB1 is a high affinity DNA-binding and bending protein that directly interacts with the nucleosome to maintain chromosomal structure, function, and stability²⁶⁻³⁰. The extracellular release of HMGB1 and other nDAMPs (including individual nucleosomal components including DNA and histone proteins H3 and H4) triggers rigorous inflammatory responses^{6, 7, 31-34}. Following L-arginine stimulation, a large number of HMGB1-nucleosomal complexes were observed within the nuclei of pancreatic acinar cells from F/F control mice (Fig. S5), which may represent sites of DNA damage and repair. Notably, the release of nDAMPs, including the nucleosomal octamer histone proteins H3 and H4, was significantly higher in primary cultured CH pancreatic acinar cells following stimulation with L-arginine (Fig. 3A and 3B) or cerulein (Fig. S6). Consistent with this finding, exposure of CH mice to L-arginine produced significantly higher serum nucleosome levels (Fig. 3C). Since DNA damage promotes nDAMP release, we investigated whether the loss of HMGB1 would exaggerate DNA damage in AP. The loss of HMGB1 within pancreatic acinar cells accelerated L-arginine-induced phosphorylation of H2AX (namely γ -H2AX), a specific marker for DNA damage (Fig. 3B and 3D). Consistently, both necrosis (Fig. 1E and Fig. S7) and apoptosis (Fig. 3B, 3E and Fig. S7) were significantly increased in pancreatic tissue and/or acinar cells from CH mice in response to L-arginine. These findings suggest that intracellular HMGB1 prevents DNA damage, cell death, and nDAMP release in the setting of AP.

Oxidative Stress Mediates Experimental Acute Pancreatitis in HMGB1 Conditional Knockout Mice

The generation of reactive oxygen species (ROS) directly or indirectly provokes additional DNA damage³⁵, thereby resulting in the so-called mitotic catastrophe³⁶. In addition, ROS is an important mediator of Nf κ b activation during pancreatitis^{37, 38}. We have previously demonstrated that HMGB1 is essential for mitochondrial quality control, and loss of HMGB1 leads to excessive ROS production³⁹. Consistent with these previous observations, ROS inhibitors (e.g., N-acetyl-L-cysteine, NAC) effectively blocked L-arginine-induced DNA damage, necrosis, and apoptosis, as well as the release of nucleosomes and Nf κ b activation by pancreatic acinar cells derived from F/F and CH mice (Fig. 4A). NAC protected against L-arginine-induced AP in F/F and CH mice (Fig. 4B), as indicated by a decrease in oxidative injury (Fig. 4C) and serum amylase (Fig. 4D) or nucleosomes (Fig. 4E). Similarly, administration of NAC also decreased cerulein-induced oxidative injury (Fig. S8A), serum amylase (Fig. S8B), or nucleosome release (Fig. S8C) in both F/F and CH mice. Thus, oxidative stress is a critical component of experimental AP.

Neutralization of Extracellular Histone and HMGB1 Confers Protection against Acute Pancreatitis in HMGB1 Conditional Knockout Mice

We anticipated that CH mice would demonstrate lower serum HMGB1 levels following administration of L-arginine as a consequence of diminished release from injured pancreatic acinar cells deficient in the molecule (Fig. 3B). We observed, however, higher serum levels of HMGB1 accumulation in the CH mice when compared to controls (Fig. 5A). HMGB1 is released utilizing two predominant pathways: 1) passive release by dying or stressed cells^{5, 40}, or 2) active secretion by innate immune or parenchymal cells^{4, 41, 42}. To identify the source of circulating HMGB1 in CH mice, we evaluated the effect of pancreatic acinar cell-derived products on macrophage cell migration using a trans-well system (Fig. 5B). The supernatant of L-arginine-treated acinar cells from CH mice was significantly more potent in inducing macrophage migration (Fig. 5C) and HMGB1 release (Fig. 5D) when compared to F/F control acinar cells. Addition of a DNA inhibitor (Dnase I) or H3-neutralizing antibodies significantly impaired macrophage migration (Fig. 5C) and HMGB1 release (Fig. 5D), suggesting that the release of pancreatic nucleosomal DNA and histone may be important in triggering HMGB1 release by infiltrated leukocytes. As expected, exogenous genomic DNA and recombinant histone H3 proteins significantly induced HMGB1 release in mouse primary peritoneal macrophages (Fig. 5E) and the macrophage-like RAW 264.7 cell line (Fig. S9). However, exogenous genomic DNA and recombinant histone H3 proteins did not induce HMGB1 release in acinar cells from F/F mice (Fig. 5E), suggesting that activated immune cells, but not acinar cells, release HMGB1 into the extracellular space during nDAMP stimuli. Consistent with these *in vitro* observations (Fig. 5D), anti-H3 antibody significantly suppressed serum HMGB1 levels (Fig. 6A) and improved survival following L-arginine administration in F/F and CH mice (Fig. 6B). To determine whether serum HMGB1 is responsible for L-arginine-induced pancreatitis in CH mice, we injected anti-HMGB1 neutralizing antibody two hours after L-arginine (followed by additional doses at 12 and 24 hours after L-arginine administration). This delayed anti-HMGB1 treatment conferred significant and long-lasting protection against lethal pancreatitis (Fig. 6C). Consistently, serum levels of tissue enzymes (amylase, LDH, and MPO) and the pro-inflammatory cytokine TNF- α were significantly reduced by repetitive administration of anti-HMGB1 or anti-histone H3 antibodies (Fig. 6D).

Discussion

Our current knowledge of the development of pancreatitis can be described as progression from an initial injury of the exocrine pancreas which then progresses to local and systemic inflammatory responses⁴³. Our findings demonstrate that HMGB1 plays two major pathological roles in experimental AP: 1) Pancreatic-derived intracellular HMGB1 limits the severity of the disease by protecting cells from Nf κ b activation, DNA damage, cell death, and release of nucleosomes from injured acinar cells and 2) Extracellular HMGB1 released from innate immune cells exacerbates the disease by enhancing pathologic inflammatory responses (Fig. 7).

In experimental models of AP, acinar cells die as a consequence of both necrosis and apoptosis¹². Our findings demonstrate that loss of HMGB1 in pancreatic tissues increased cell death *in vivo* and *in vitro*. HMGB1 is the most abundant non-histone protein within the nucleus and is demonstrably a DNA damage sensor during stress⁸. Loss of HMGB1 in pancreatic acinar cells increases Nf κ b activation and DNA damage. This enhanced Nf κ b activation and DNA damage is mediated in part by ROS signaling, as the free radical scavenger NAC reverses the effects of HMGB1 loss on cellular injury *in vivo* and *in vitro*. Oxidative stress plays a pivotal role in the development of AP. The functional relationship between ROS and AP is admittedly complex. In many AP cases, ROS is responsible for cell

death. In some cases, ROS generation within acinar cells may be a protective response as NAC stimulates necrosis in models of AP⁴⁴. The key molecular events that eventually determine whether ROS is protective or destructive still remain largely unknown.

Nfkb is responsible for regulating the transcription of a wide variety of genes involved in inflammation and cell death in the development and progression of AP⁴⁵. Mechanisms mediating the upstream signal of Nfkb activation involve calcium, ROS, and MAPK activation. Loss of HMGB1 in the pancreatic acinar cells led to a significant enhancement of L-arginine-induced Nfkb phosphorylation/activation, suggesting that both inflammatory cell-dependent and -independent mechanisms may contribute to the effects of HMGB1. A number of studies have demonstrated that pharmacologic inhibition of Nfkb activation (e.g., Bay 11-7082 and NAC) diminish the inflammatory response and limit the severity of pancreatitis³⁸. Of note, Nfkb activation may function as a stress-induced self-defense mechanism to protect AP. Thus, blocking of Nfkb activation (e.g., pyrrolidine dithiocarbamate and NAC) increased cerulein-induced injury³⁷. Activation of the pancreas-specific Nfkb pathway in transgenic mice by overexpressing Nfkb RELA (termed LSL-RELA/Cre mice) or ablation of Ikb α (termed Ikb $\alpha^{\Delta\text{panc}}$ mice) had distinct phenotypes in AP. Cerulein- and L-arginine-induced AP is protected in Ikb $\alpha^{\Delta\text{panc}}$ mice⁴⁶, whereas it is aggravated in LSL-RELA/Cre mice⁴⁷. These studies suggest that the key components of the Nfkb pathway may have differing roles in the regulation of AP development. For this reason, further exploration of these pathways should be carried out.

Despite the loss of HMGB1 in pancreatic tissues, CH mice with experimentally induced AP demonstrate enhanced disease severity that is neutralized by anti-HMGB1 antibodies. In fact, serum levels of HMGB1 were higher in CH mice than in control mice. Indeed, tissue leukocyte infiltration and serum levels of pro-inflammatory cytokines (e.g., TNF- α and IL-6) were significantly higher in CH mice compared to their wild-type control littermates. Thus, in our model, extracellular HMGB1 acts as a pro-inflammatory cytokine and its release by innate immune effectors is enhanced in the setting of increased nDAMP release. A recent study showed that extracellular DAMP mediated inflammation in AP partly through TLR9 signaling⁴⁸. RAGE may also contribute to DAMP-TLR9 signaling, serving as a vehicle for internalization of DNA/protein complexes, which has been described in the setting of necrosis in plasmacytoid dendritic cells⁴⁹. Thus, the functional interplay between DAMP molecules and their individual receptors may provide clues to developing new therapeutic strategies for patients with AP.

While it has been well established that DAMPs contribute to cell death-induced inflammation¹⁻³, it has not been previously demonstrated that these same molecules can also serve as intracellular mediators of cellular stress. Many DAMPs are nuclear or cytosolic proteins. Our observation that loss of HMGB1 in pancreatic acinar cells enhanced cell death and promoted release of other nDAMP such as nucleosomes (DNA and histone) suggests a new paradigm whereby the same molecules that serve to protect cells against cellular stress also serve as extracellular signaling molecules when recognized by innate immune effectors. While there is not likely to be a physiologic setting where cells will have complete loss of HMGB1, we have previously shown that inflammation and stress induce HMGB1 translocation from the nucleus to the cytosol^{50, 51}. We hypothesize that relative loss of HMGB1 from the nucleus will render cells more sensitive to DNA damage and subsequent cell death. This observation may explain increased DNA damage in the setting of inflammation and cancer.

In summary, our observations describe for the first time the existence of an HMGB1-mediated nuclear regulatory mechanism for the control of nuclear homeostasis and nDAMP release that modulates the magnitude of the subsequent inflammatory response following

tissue injury. Significant loss of nuclear HMGB1 could result in a rapid escalation of local inflammation through destabilization of the nucleus and enable rapid DNA and histone release. These findings significantly add to our understanding of the complex regulatory mechanisms and functional consequences of the innate immune response to tissue damage mediated through nDAMPs. In addition, to our knowledge, our CH model is the first HMGB1 conditional knockout disease model, which will accelerate the study of HMGB1 function and provide direct evidence for its role *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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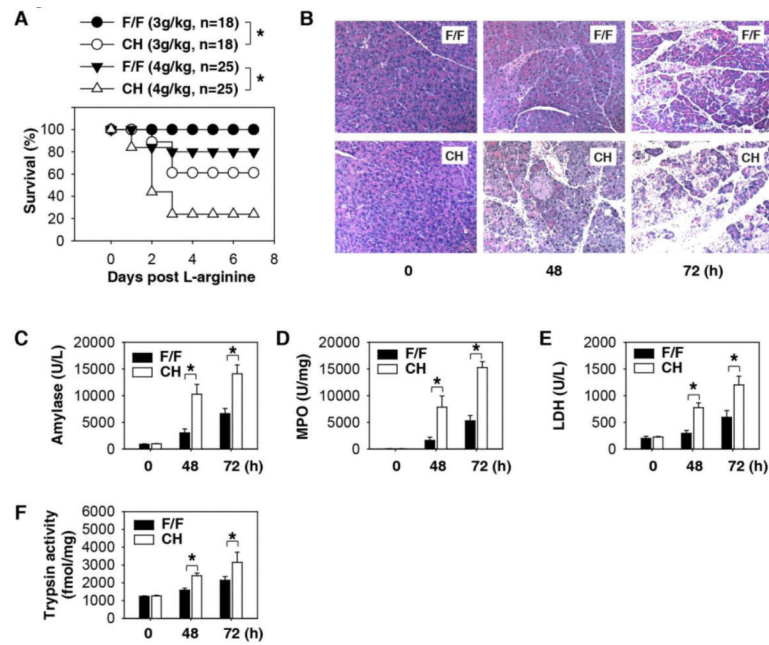


Fig.1. HMGB1 protects against L-arginine-induced acute severe pancreatitis
 (A) HMGB1^{fllox/fllox} (“F/F”) and pancreas-specific conditional HMGB1 knockout mice (“CH”) received a lethal L-arginine dose (3-4 g/kg × 2, i.p.). The Kaplan-Meier method was used to compare the differences in survival rates between groups. *, *P*<0.05. (B-D) Hematoxylin and eosin -stained pancreatic sections (B), plasma amylase activity (C), pancreatic MPO activity (D), plasma LDH activity (E), and pancreatic trypsin activity (F) at the indicated time points following administration of L-arginine (4 g/kg × 2, n=3-5 mice/group, *, *P*<0.05).

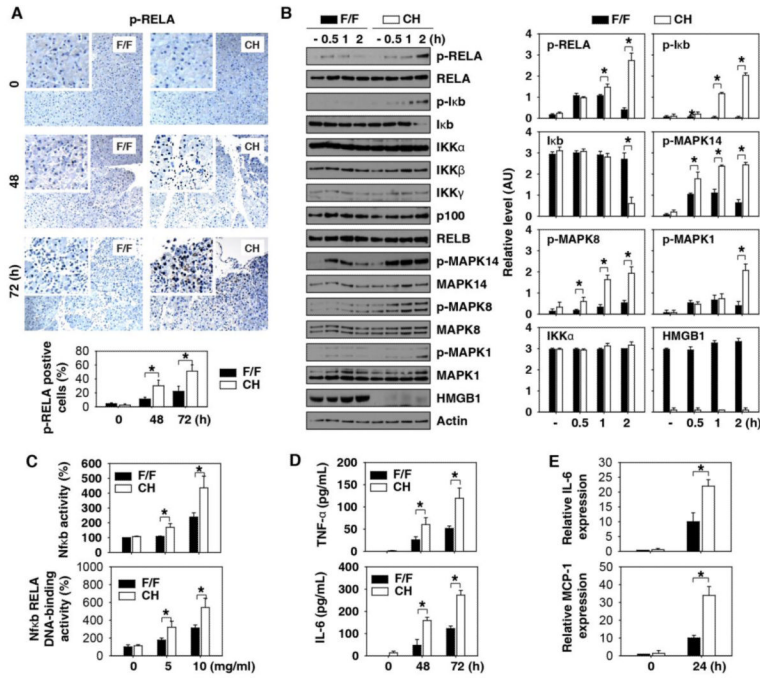


Fig.2. Loss of HMGB1 promotes Nfkb and MAPK inflammatory signaling pathways *in vitro* and *in vivo*

(A) Immunohistochemistry and quantitative analysis of phosphorylation of RELA in pancreatic tissue sections from F/F and CH mice at indicated times following administration of L-arginine (4 g/kg× 2). (B) Western blot analysis of the indicated proteins in L-arginine (10 mg/ml) treated pancreatic acinar cells from F/F and CH mice *in vitro*. (C) Nfkb transcriptional activity and Nfkb RELA DNA-binding activity analysis in L-arginine (10 mg/ml) treated pancreatic acinar cells from F/F and CH mice *in vitro* (n=3, *, P<0.05). (D) Serum TNF- α and IL-6 levels in F/F and CH mice at indicated time points following administration of L-arginine (4 g/kg× 2, n=3-5 mice/group, *, P<0.05). (E) Intra-pancreatic IL-6 and MCP-1 protein expression levels in F/F and CH mice at 24 h following administration of L-arginine (4 g/kg× 2, n=3-5 mice/group, *, P<0.05).

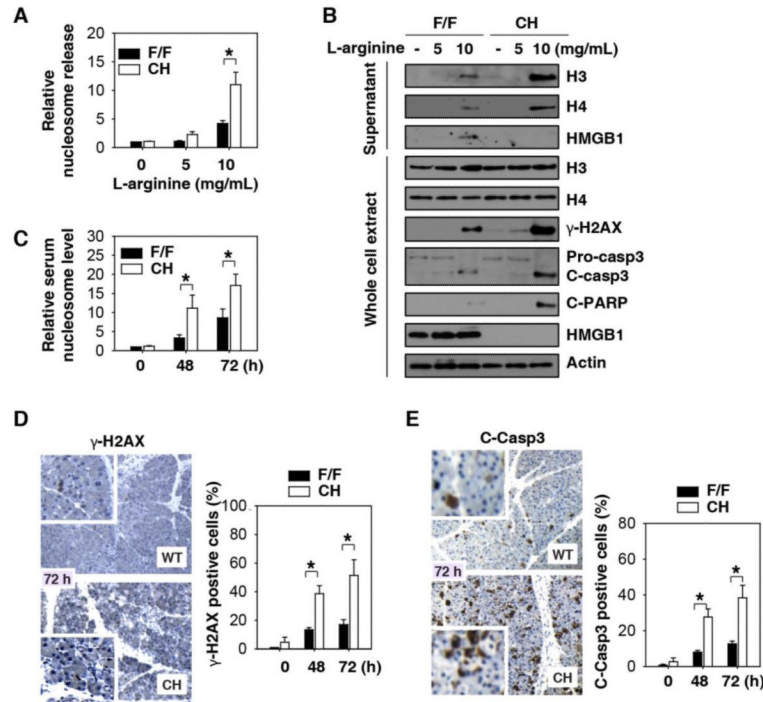


Fig.3. Loss of HMGB1 promotes DNA damage, cell death, and nDAMP release *in vitro* and *in vivo*

(A) Relative extracellular nucleosome levels in L-arginine (24 hours) treated pancreatic acinar cells from F/F and CH mice *in vitro* (n=3, *, $P < 0.05$). (B) Western blot analysis of whole- cell lysates and supernatants from L-arginine (24 hours) treated pancreatic acinar cells from F/F and CH mice. γ -H2AX is a marker of DNA damage. Cleaved-caspase 3 (“C-casp3”) and cleaved-PARP (“C-PARP”) are markers of apoptosis. (C-E) Relative serum nucleosome level (C), pancreatic γ -H2AX (D), and C-casp3(C) in F/F and CH mice following administration of L-arginine (4 g/kg \times 2, n=3-5 mice/group, *, $P < 0.05$).

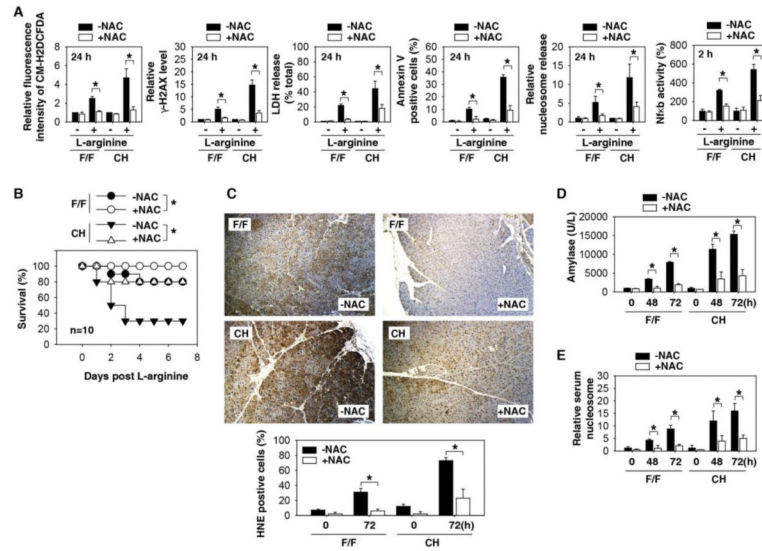


Fig.4. NAC protects against L-arginine-induced AP

(A) The fluorescence intensity of CM- H2DCFDA, γ -H2AX level, LDH release, percentage of annexin V-positive cells, nucleosome release, and Nf κ b activation in L-arginine (10mg/mL) treated cultured pancreatic acinar cells derived from F/F and CH mice with or without addition of 50 mM NAC ($n=3$, *, $P<0.05$). (B) F/F and CH mice received a lethal L-arginine dose (4 g/kg \times 2, i.p.) with or without NAC (300 mg/kg i.p.). The Kaplan-Meyer method was used to compare the differences in survival rates between groups ($n=10$ mice/group, *, $P<0.05$). (C-E) In parallel, oxidative injury by staining for HNE (the marker of oxidative damage) (C), plasma amylase activity (D), and relative level of serum nucleosome (E) at indicated time points were assayed (*, $P<0.05$).

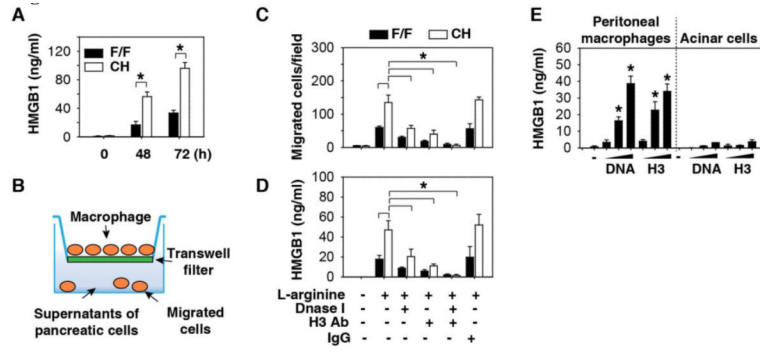


Fig.5. Extracellular histone and DNA induce HMGB1 release in macrophages
 (A) Serum HMGB1 levels in F/F and CH mice following administration of L-arginine (4 g/kg × 2, n=3-5 mice/group, *, $P < 0.05$). (B) Schematic diagram of the transwell assay. (C) Transwell assay shows the number of mouse RAW264.7 cells that migrated towards the supernatants of L-arginine treated pancreatic cells obtained from F/F and CH mice with or without treatment with Dnase I (10 U/ml), H3 antibody (10 μ g/ml), and control IgG (n=3, *, $P < 0.05$). (D) In parallel, HMGB1 release by RAW264.7 cells was analyzed by ELISA (n=3, *, $P < 0.05$). (E) Peritoneal macrophages or acinar cells from F/F mice were treated with genomic DNA and recombinant histone H3 proteins for 24 hours. HMGB1 release was analyzed by ELISA (n=3, *, $P < 0.05$).

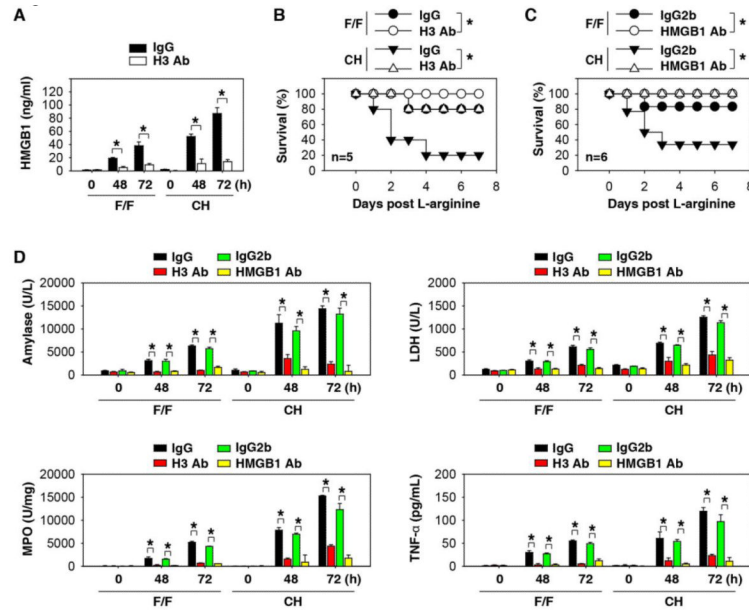


Fig.6. Neutralization of extracellular histone and HMGB1 confer protection against acute pancreatitis

(A, B) F/F and CH mice received anti-histone H3 (20 mg/kg) or rabbit isotype control IgG by i.p. injection two hours after completion of the L-arginine-induced pancreatitis protocol. Serum HMGB1 levels (A) and animal survival rates (B) were assayed (*, $P < 0.05$). (C) F/F and CH mice received anti-HMGB1 (20 mg/kg) or mouse isotype control IgG2B by i.p. injection two hours after the end of the L-arginine-induced pancreatitis procedure (followed by additional doses at 12 and 24 hours after L-arginine). The Kaplan-Meier method was used to compare the differences in survival rates between groups. *, $P < 0.05$. (D) In parallel, plasma amylase activity, plasma LDH activity, pancreatic MPO activity, and serum TNF- α levels at the indicated time points after administration of L-arginine with or without indicated antibody in F/F and CH mice were assayed ($n = 3-5$ mice/group, *, $P < 0.05$).

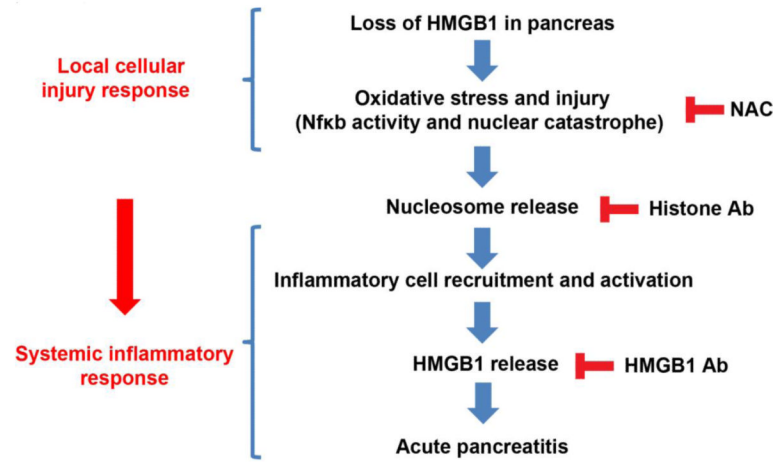


Fig.7. Endogenous HMGB1 protects against severe acute pancreatitis by preventing nuclear catastrophe and proinflammatory nucleosome release

Illustrated is our current model of the role of HMGB1 in limiting nucleosomal release and the subsequent inflammatory response.