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Chloroquine Inhibits HMGB1 Inflammatory Signaling and Protects Mice from Lethal Sepsis

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Abstract

Sepsis is caused by an overwhelming immune response to bacterial infection. The discovery of high mobility group box 1 (HMGB1) as a late mediator of lethal sepsis has prompted investigation into the development of new therapeutics which specifically target this protein. Here, we show that chloroquine, an anti-malarial drug, prevents lethality in mice with established endotoxemia or sepsis. This effect is still observed even if administration of chloroquine is delayed. The protective effects of chloroquine were mediated through inhibition of HMGB1 release in macrophages, monocytes, and endothelial cells, thereby preventing its cytokine-like activities. As an inhibitor of autophagy, chloroquine specifically inhibited HMGB1-induced I κ -B degradation and NF- κ B activation. These findings define a novel mechanism for the anti-inflammatory effects of chloroquine and also suggest a new potential clinical use for this drug in the setting of sepsis.

Keywords

HMGB1; chloroquine; sepsis; autophagy; NF- κ B; Beclin 1

1. Introduction

Sepsis, also known as systemic inflammatory response syndrome, is a life threatening condition and the most common cause of death in intensive care units [1]. Bacterial infections are the most common cause of sepsis. Sepsis is pathologically mediated by the release of multiple proinflammatory cytokines such as tumor necrosis factor (TNF) and high mobility group box 1 (HMGB1) from immune cells. TNF is released early in the

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CONFLICT OF INTEREST

The authors declare no conflicts of interest or financial interests.

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development of sepsis [2], and anti-TNF- α neutralizing antibodies, although efficacious, appear to have a narrow therapeutic window in human patients [3]. HMGB1 functions as a late mediator of lethal endotoxemia and circulating HMGB1 is elevated in a delayed fashion in septic mice [4]. Once released, HMGB1 binds to cell-surface receptors (e.g. TLR4 and RAGE) and mediates various cellular responses, including activation of the NF- κ B signaling pathway and subsequent translation and release of additional proinflammatory cytokines such as TNF and IL-6 [5]. Mice treated with anti-HMGB1 antibodies or HMGB1 antagonists are protected against lethal endotoxemia and sepsis [4, 6–9]. In addition, a high concentration of HMGB1 in the plasma of patients with severe sepsis correlates with an increased likelihood of mortality [10]. Therefore, HMGB1 is a molecular target which provides an opportunity for clinical intervention in sepsis and perhaps other inflammatory diseases [11, 12].

Chloroquine is a 4-aminoquinoline drug long used in the treatment or prevention of malaria [13] and is also a widely used inhibitor of autophagy being evaluated for usefulness in cancer therapy regimens [14]. Autophagy is a process of “self-eating”, in which cytoplasmic material (e.g. organelles and proteins) is delivered to lysosomes for degradation [15, 16]. Recent developments reveal an important role for autophagy in immunity and inflammation [17, 18]. Chloroquine raises the lysosomal pH and inhibits lysosome-autophagosome fusion [19]. Chloroquine also has been used as a non-steroidal anti-inflammatory drug to treat rheumatoid arthritis, lupus erythematosus, and multiple sclerosis [20–22]. Moreover, one study showed that chloroquine protects from sepsis-induced acute kidney injury and pro-inflammatory cytokine TNF release by decreasing TLR9 protein level [23]. However, it was previously unknown if chloroquine could inhibit HMGB1 signaling in experimental sepsis. Here, we show that chloroquine prevents fatality in mice with established lethal sepsis and systemic inflammation. Inhibition of autophagy with chloroquine reduced lipopolysaccharide (LPS)-mediated release of HMGB1, as well as HMGB1-mediated pro-inflammatory signaling responses in immune cells by inhibition of NF- κ B activation.

2. Materials and methods

2.1 Reagents

The antibodies to LC3 (#NB100-2220), beclin 1 (#NB500-249), and ULK1 (#NB110-74844) were obtained from Novus (Woburn, MA, USA). The antibodies to I κ -B (#4814), p65 (#4764), tubulin (#2128), and actin (#3700) were obtained from Cell Signaling Technology (Danvers, MA, USA). The antibodies to HMGB1 (#ab18256) and fibrillarin (ab4566) came from Abcam (Cambridge, MA, USA). LPS (*Escherichia coli* LPS 0111:B4; #L4391), chloroquine (#C6628), and hydroxychloroquine (#H0915) were obtained from Sigma (St. Louis, MO, USA). Recombinant HMGB1 protein with endotoxin content 200 pg/mg was kindly provided by Dr. Kevin J. Tracey (The Feinstein Institute for Medical Research, Manhasset, NY 11030) [24]. NE-PER Nuclear and Cytoplasmic Extraction Kits (#78835) were obtained from Thermo Fisher Scientific (Rockford, IL, USA).

2.2 Cell culture

Murine macrophage-like RAW 264.7 cells and human umbilical vein endothelial cells (HUVECs) were obtained from American Type Culture and were cultured in RPMI medium 1640 or DMEM supplemented with 10% heat-inactivated fetal bovine serum and 2 mM glutamine. Human peripheral blood mononuclear cells (HUPBMCs) were isolated from the blood of healthy donors by density gradient centrifugation through Ficoll and cultured in RPMI 1640 supplemented with 10% heat-inactivated human serum and 2 mM glutamine, as previously described [25].

2.3 Animal model of endotoxemia and sepsis

Endotoxemia was induced in Balb/C mice (male, 7–8 weeks old, 20–25 g weight) by intraperitoneal (i.p.) injection of bacterial endotoxin (LPS, 5 mg/kg), as previously described [4, 8]. Sepsis was induced in male Balb/C mice (male, 7–8 week old, 20–25 g weight) by cecal ligation and puncture was performed as previously described [4, 8]. Chloroquine was administered i.p. in mice at indicated doses and time points. Blood was collected at indicated time points, allowed to clot for two hours at room temperature, and then centrifuged for 15 minutes at 1,500×g. Serum samples were stored at 20°C before analysis. Mortality was recorded for up to two to three weeks after injection to ensure that no additional late deaths occurred. All animal experiments strictly followed the guidelines of the Institutional Review Board.

2.4 Cytokine measurements

Commercially available enzyme linked immunosorbant assay (ELISA) kits were used to measure the concentrations of HMGB1 (Shino Test Corporation, Tokyo, Japan, #ST51011), TNF (R&D Systems, Minneapolis, MN, USA, #DRT100, #MRT20), and IL-6 (R&D Systems, #D6050, #M6000B) in serum or the culture medium according to the manufacturer's instructions.

2.5 RNAi

Transfection with beclin 1-shRNA (SHCLNG-NM_019584), ULK1- shRNA (SHCLNG-NM_009469), and control shRNA (SHC001) from Sigma, were performed using the FuGENE® HD Transfection Reagent (Roche Applied Science, 04709705001) according the manufacturer's instructions.

2.6 Western blot analysis

Proteins in cell lysates was first resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, then transferred to nitrocellulose membrane and subsequently incubated with the primary antibody, as previously described [26]. After incubation with peroxidase-conjugated secondary antibodies, the signals were visualized by enhanced chemiluminescence (Pierce, Rockford, IL, USA, # 32106) according to the manufacturer's instructions. Relative band intensity was quantified using the Gel-pro Analyzer® software (Media Cybernetics, Bethesda, MD, USA).

2.7 NF-κB activation assay

Cells were transiently transfected in a 12-well plate with an NF-κB luciferase reporter plasmid or control empty plasmid using the Lipofectamine 2000 reagent, as previously described [27]. After 24–48 hours, the cells were exposed to various agents. Luciferase activity was determined using the luciferase assay system with the reporter lysis buffer (#E2000) obtained from Promega (Madison, WI, USA). The results are expressed as relative NF-κB activity after normalizing to the control empty plasmid.

2.8 Quantitative real time polymerase chain reaction

cDNA from various cell samples were amplified by real-time quantitative polymerase chain reaction (PCR) with specific primers from SABiosciences (Frederick, MD, USA) as previously described [28]. The control group was set as 100%.

2.9 Statistical analysis

Data are expressed as means ± SEM of two or three independent experiments. Significance of differences between groups was determined by analysis of variance (ANOVA) least

significant difference (LSD) test. The Kaplan-Meier survival analysis with Logrank significance test was used to compare the differences in mortality rates between groups. A p -value < 0.05 was considered statistically significant.

3. Results

3.1 Pretreatment with chloroquine/hydroxychloroquine protects mice against lethal endotoxemia and inhibits release of TNF and HMGB1

We evaluated the protective effect of chloroquine in a standard murine model of endotoxemia. Administration of a single dose of the chloroquine (50 mg/kg, i.p., or 5 mg/kg, i.p.) was followed 30 minutes later by an injection of LPS (5 mg/kg LPS, i.p.). Pretreatment with chloroquine at 50 mg/kg conferred significant protection from lethal endotoxemia (survival in chloroquine-treated mice = 12/20; survival in saline-treated mice = 4/20; $P < 0.05$) (Fig. 1A). All of these mice were observed for at least two weeks, and no late deaths occurred, indicating that chloroquine does not merely delay the onset of LPS lethality, but provides long-lasting protection. To gain insight into the protective mechanism of chloroquine against lethal endotoxemia, we evaluated its effects on the systemic accumulation of TNF- α (peaking between one to two hours) and HMGB1 (peaking between 16–32 hours) [2, 4]. Pretreatment of endotoxemic mice with chloroquine (50 mg/kg, i.p.) significantly attenuated the serum levels of both TNF- α at one hour (Fig. 1B) and HMGB1 at 20 hours (Fig. 1C) after LPS treatment, indicating that it prevented endotoxemia by attenuating the release of early and late cytokine release. Since hydroxychloroquine is far less toxic to the retina in clinic, we examined the protective effects of hydroxychloroquine in lethal endotoxemia. Like chloroquine, its derivative, hydroxychloroquine, protects mice against lethal endotoxemia (Fig. 1D) and inhibits release of TNF- α (Fig. 1E) and HMGB1 (Fig. 1F).

3.2 Delayed administration of chloroquine protects mice against lethal endotoxemia and inhibits release of HMGB1

To investigate whether chloroquine treatment could be delayed until after administration of LPS and still maintain its protective effects, we injected chloroquine four hours after LPS (followed by additional doses at 12, 24, and 36 hours after LPS), a time at which the clinical manifestations of LPS-induced toxicity, including diarrhea, piloerection, and depressed spontaneous activity were evident. This delayed treatment conferred significant protection against lethal endotoxemia (Fig. 2A). All of these mice were observed for at least two weeks, and no late deaths occurred. Notably, treatment of endotoxemic mice with chloroquine beginning four hours after LPS injection significantly attenuated the systemic release of HMGB1 measured at 30 hours after the onset of endotoxemia (Fig. 2B). To demonstrate that the protective effect of chloroquine occurs via inhibition of HMGB1 release, we treated mice with HMGB1 protein after LPS insult and chloroquine treatment. The reduced lethality (Fig. 2A) and TNF- α production (Fig. 2C) in response to chloroquine is partially reversed. These findings indicate that delayed treatment with chloroquine confers significant protection partly by inhibition of HMGB1 release.

3.3 Delayed administration of chloroquine protects mice against lethal sepsis and inhibits release of HMGB1

Although endotoxemia provides a useful model for investigating complex cytokine cascades, more clinically relevant animal models are necessary to explore the therapeutic benefit of various agents in the treatment of human sepsis. One well-characterized, standardized animal model of sepsis is induced by surgical perforation of the cecum (CLP). After cecal ligation, the first dose of chloroquine was given 24 hours after the onset of sepsis and followed by additional doses at 48 and 72 hours post-sepsis. Compared to the saline-

treated group, chloroquine at 50 mg/kg significantly increased the animal survival rate (Fig. 3A). ELISA analysis of serum prepared from blood obtained 30 hours after cecal puncture revealed that chloroquine significantly reduced circulating HMGB1 levels (Fig. 3B). These findings indicate that chloroquine confers significant protection in sepsis by inhibition of HMGB1 release.

3.4 Chloroquine inhibits the cytokine activity and release of HMGB1

Recombinant HMGB1 has potent cytokine-like stimulating activity in macrophages, monocytes, and endothelial cells [5, 24, 29]. To explore whether chloroquine inhibits the cytokine activity of HMGB1, we treated the murine macrophage cell line RAW264.7 with different doses of chloroquine after rHMGB1 treatment. Indeed, HMGB1-induced TNF and IL-6 release was dose-dependently inhibited by chloroquine in these cells (Fig. 4A). We further confirmed its HMGB1-inhibiting activities using primary HUPBMCs and HUVECs. In HUPBMCs (Fig. 4B) and HUVECs (Fig. 4C), chloroquine abrogated HMGB1-induced TNF and IL-6 release. Furthermore, chloroquine inhibited HMGB1-induced TNF and IL-6 mRNA upregulation in RAW264.7 cells (Fig. 4D) and HUVECs (data not shown). To explore whether the inhibition of autophagy is required for HMGB1-induced TNF and IL-6 mRNA upregulation, we knocked down the essential autophagy gene beclin 1 (the mammalian homolog of yeast ATG6) [30] in RAW264.7 cells. Knockdown of beclin 1 inhibited HMGB1-induced TNF and IL-6 mRNA upregulation (Fig. 4E). We and others recently demonstrated that autophagy regulates starvation- and nigericin (inflammasome agonist)- induced HMGB1 release in fibroblasts and macrophages [31, 32]. Similarly, chloroquine or knockdown of beclin 1 inhibited LPS-induced HMGB1 release in RAW264.7 cells (Fig. 4F and 4G). Taken together, these data suggest that inhibition of autophagy by chloroquine or genetic approaches is capable of effectively inhibiting the cytokine activity and release of HMGB1 in immunity cells.

3.5 Chloroquine inhibits HMGB1-mediated NF- κ B activation

HMGB1-induced NF- κ B activation has been recognized as a key contributor to the proinflammatory response [11, 12, 33]. To study chloroquine's effects on this pathway, we next analyzed the influence of chloroquine on HMGB1-mediated NF- κ B activation. Chloroquine significantly inhibited HMGB1-induced NF- κ B activity as measured by luciferase reporter assay and NF- κ B p65 nuclear translocation by Western blot assay (Fig. 5A and B). In unstimulated cells, NF- κ B is sequestered in the cytoplasm by I κ B inhibitory proteins. NF- κ B-activating agents can induce I κ B degradation and release NF- κ B to enter the nucleus, where it regulates pro-inflammatory gene expression and cytokine release. Chloroquine inhibited HMGB1-induced I κ B degradation in RAW264.7 and HUVECs by Western blot analysis (Fig. 5C), suggesting that autophagy is involved in regulation of I κ B degradation. To explore whether the inhibition of autophagy is required for I κ B degradation, we knocked down the essential autophagy gene beclin 1 and ULK1 (the mammalian homolog of yeast ATG1) in RAW264.7 cells (Fig. 5D). Inhibition of these genes significantly blocked HMGB1-induced microtubule-associated protein 1 light chain 3 (LC3)-II expression (Fig. 5D), an indicator of autophagosome formation [34]. Notably, knockdown of beclin 1 and ULK1 decreased HMGB1-induced I κ B degradation (Fig. 5D), NF- κ B activity (Fig. 5E), and TNF release (Fig. 5F). Taken together, these data suggest that autophagy-mediated I κ B degradation is required for HMGB1-mediated NF- κ B activation.

4. Discussion

These studies indicate that the anti-malarial drug chloroquine significantly inhibits the systemic release of both early (TNF) and late (HMGB1) cytokines, which are known to mediate the lethality of sepsis and systemic inflammation in human patients. The molecular

mechanism of chloroquine's protective effects occurs in part by inhibition of HMGB1-induced I κ B degradation and NF- κ B activation.

HMGB1 functions as a DNA-binding protein and regulator of intracellular transcription within the nucleus as a universal cytosolic sensor of nucleic acids and as an extracellular cytokine/inflammatory mediator [12, 35, 36]. HMGB1 can be released into the extracellular milieu under two conditions: 1) HMGB1 is actively secreted from activated immune cells [4]; and 2) HMGB1 is passively released by dying or injured cells [37]. When released into the extracellular compartment, HMGB1 can cause pathological inflammatory responses, including acute lung injury and epithelial barrier dysfunction [29, 38, 39]. Compared to TNF, HMGB1 has a more delayed kinetic profile and provides a wider therapeutic window for clinical use. Therefore, agents capable of selectively attenuating systemic HMGB1 accumulation may prove beneficial in the treatment of lethal sepsis. In this study, we demonstrated that chloroquine is a potential inhibitor of HMGB1 release and activity. More importantly, delayed and repeated administration of chloroquine, beginning at 24 hours after onset of sepsis, rescued mice from lethal sepsis, supporting a therapeutic role for chloroquine in the clinical management of human sepsis.

Activation of the NF- κ B plays a central role in inflammation through its ability to induce production and release of multiple proinflammatory cytokines [40]. HMGB1 is an inducer of classical NF- κ B (p50/p65) pathway activation [11, 12, 33]. In the nucleus, the NF- κ B p65 subunit is a strong activator for a wide variety of genes. NF- κ B is kept in an inactive form by the inhibitory subunit I κ B in most cell types [41]. The ubiquitin-proteasome system (UPS) and autophagy are two functionally linked major degradation pathways. Previous studies have indicated that UPS is important for LPS-induced degradation of I κ B in inflammatory cells [42]. In this study, we demonstrated that autophagy is required for HMGB1-induced I κ B degradation. Inhibition of autophagy by chloroquine or likewise, knockdown of critical autophagy genes (e.g. beclin 1 and ULK1) reduced HMGB1-mediated I κ B degradation and resulted in the inhibition of NF- κ B-mediated cytokine (e.g. TNF and IL-6) release. A study recently demonstrated that autophagy is also required for TNF-induced I κ B degradation in the long term [43]. The precise means by which autophagy mediates I κ B degradation is an area of ongoing investigation.

In eukaryotic cells, autophagy is constitutively active at low levels, whereas significant upregulation occurs in response to a multitude of stresses [15]. The complex relationship between autophagy and inflammation is dependent on cell type and context [17, 18, 44, 45]. For example, genetic ablation of the autophagy regulator Atg16L1 enables LPS-dependent inflammasome activation [46]. In contrast, autophagy facilitates IFN- γ -induced Signal Transducers and Activators of Transcription (STAT)-1 activation and cellular inflammation [47]. In the tumor microenvironment, autophagy promotes the production of inflammatory mediators (e.g. IL-6, IL-8, and GM-CSF) [48]. In addition, the interplay between autophagy and NF- κ B signaling pathways is very complex [49–51]. Our findings indicate that HMGB1-mediated autophagy is required for cytokine release in inflammatory cells, partly via regulation of I κ B degradation. Our recent studies indicate that HMGB1 is a positive regulator of autophagy in fibroblasts and cancer cells by both transcription-dependent and independent pathways [31, 52–54], but it is unknown whether HMGB1-mediated autophagy was involved in the regulation of inflammation and immunity. In addition, one recent study indicates that autophagy regulates tanshinone IIA sodium sulfonate-mediated HMGB1 degradation in macrophages [55].

In summary, we demonstrate here that chloroquine, as well as its derivative hydroxychloroquine, may have therapeutic potential for lethal sepsis and systemic inflammation. The molecular target of chloroquine action is enigmatic, but it is an effective

inhibitor of HMGB1 release and pro-inflammatory function in macrophage/monocyte/endothelial cells *in vitro* and in animal models of lethal endotoxemia and sepsis *in vivo*. Moreover, autophagy-dependent I κ -B degradation pathways were reported. It will be of interest to assess the pharmacological activity of chloroquine as an inhibitor of HMGB1 signaling in other animal models of local and systemic inflammation. Given the inflammatory genomic response in mice is significantly different to that observed in humans [56], it is of great interest to explore whether chloroquine also inhibits HMGB1 inflammatory signaling and protects lethal sepsis in humans.

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Figure 1. Chloroquine/hydroxychloroquine pretreatment prevents endotoxin lethality by attenuating TNF and HMGB1 release *in vivo*

(A) Mice (n=20 per group) were injected with a single dose of chloroquine ("Chlo") as indicated, followed 30 minutes later by a lethal infusion of endotoxin (LPS, 5 mg/kg, i.p.). The Kaplan-Meier method was used to compare the differences in survival rates between groups. *, $P < 0.05$ versus saline group. (B, C) In parallel, serum levels of TNF at 1–4 hours (B) and HMGB1 at 20 hours (C) were measured by ELISA (n=4 animals per group, values are mean $\pm$ SEM, *, $P < 0.05$). (D) Mice (n=20 per group) were injected with hydroxychloroquine ("HChlo", 100 mg/kg), followed 30 minutes later by a lethal infusion of endotoxin (LPS, 5 mg/kg, i.p.). The Kaplan-Meier method was used to compare the differences in survival rates between groups. *, $P < 0.05$ versus saline group. (E, F) In parallel, serum levels of TNF- α at 1 hour (E) and HMGB1 at 20 hours (F) were measured by ELISA (n=4 animals per group, values are mean $\pm$ SEM, *, $P < 0.05$).

Figure 2. Delayed administration of chloroquine attenuates the lethality of endotoxemia and sepsis HMGB1

(A) Mice (n=20 per group) received a lethal infusion of endotoxin (LPS, 5 mg/kg, i.p.) and were treated with chloroquine ("Chlo", 50 mg/kg) 4, 12, 24, and 36 hours later with or without HMGB1 (100 μ g per mouse, i.p., followed at 48 hours after LPS). The Kaplan-Meier method was used to compare the differences in survival rates between groups. *, $P<0.05$ versus saline group; #, $P<0.05$ versus chloroquine group. (B, C) In parallel, serum levels of and HMGB1 at 30 hours and TNF- α at 50 hours was measured by ELISA (n=4 animals per group, values are mean $\pm$ SEM, *, $P<0.05$).

Figure 3. Chloroquine protects mice against lethal sepsis and inhibits HMGB1 release

(A) The cecal ligation and puncture technique was used to induce intraabdominal sepsis in mice ($n = 20$ per group). Repeated administration of indicated chloroquine at 24, 36, 48, 60, and 72 hours after cecal ligation and puncture significantly increased survival, as compared to the saline group (*, $P < 0.05$), as measured by the Kaplan-Meier test. (B) In parallel, serum levels and HMGB1 at 30 hours was measured by ELISA ($n = 5$ animals per group, values are mean $\pm$ SEM, *, $P < 0.05$).

Figure 4. Chloroquine effectively abrogated HMGB1-induced expression and release of cytokines in immunity cells
 (A–C) RAW 264.7 cells (A), primary human peripheral blood mononuclear cells (HUPBMCs) (B), and human umbilical vein endothelial cells (HUVECs) (C) were stimulated with HMGB1 (1 μ g/ml) in the absence or presence of chloroquine at indicated concentrations. At 16 hours after HMGB1 stimulation, levels of TNF and IL-6 in the culture medium were determined by ELISA. Values are mean $\pm$ SEM of three independent experiments. * $p < 0.05$ versus HMGB1 group. (D) In parallel, the mRNA expression level of TNF- α and IL-6 in RAW264.7 cells was detected by real time PCR analysis. Values are mean $\pm$ SEM of two independent experiments. * $p < 0.05$ versus HMGB1 group. (E) RAW 264.7 cells were transfected with beclin 1 shRNA and control shRNA for 48 hours, and then

treated with HMGB1 (1 μ g/ml) for 16 hours. The mRNA expression levels of beclin 1, TNF- α , and IL-6 in RAW264.7 cells were detected by real time PCR analysis. Values are mean $\pm$ SEM of two independent experiments. * $p < 0.05$. (F) RAW 264.7 cells were stimulated with LPS (200 ng/ml) in the absence or presence of chloroquine at 100 μ M. At 24 hours after LPS stimulation, levels of HMGB1 in the culture medium were determined by ELISA. Values are mean $\pm$ SEM of three independent experiments. * $p < 0.05$. (G) RAW 264.7 cells were transfected with beclin 1 shRNA and control shRNA for 48 hours and then treated with LPS (200 ng/ml) for 24 hours. Levels of HMGB1 in the culture medium were determined by ELISA. Values are mean $\pm$ SEM of three independent experiments. * $p < 0.05$.

Figure 5. Chloroquine inhibits HMGB1-mediated NF-κB activation

(A–B) Indicated cells were stimulated with HMGB1 (1 μg/ml, 16 hours) in the absence or presence of chloroquine (“Chlo”, 50 μM), and the activity of NF-κB was assayed by luciferase reporter analysis (A) and p65 level in cytoplasm (“Cyt”)/nucleus (“Nuc”) was assayed by Western blot (B). Values are mean ± SEM of three independent experiments in duplicate. * $p < 0.05$. Fibrillarin and tubulin were used as a loading control. AU=arbitrary unit. (C) In parallel experiments, the level of IκB in whole cell lysate was assayed by Western blot. Actin was used as a loading control. (D) RAW 264.7 cells were transfected with beclin 1 shRNA, ULK1 shRNA, and control shRNA for 48 hours, and then treated with HMGB1 (1 μg/ml) for 16 hours. The indicated proteins were assayed by Western blot. AU=arbitrary unit. (E–F) In parallel, the activity of NF-κB and TNF release were assayed by luciferase reporter analysis and ELISA, respectively. Values are mean ± SEM of two independent experiments. * $p < 0.05$.