Beclin1 controls caspase-4 inflammsome activation and pyroptosis in myocardial reperfusion-induced microvascular injury

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Research

Keywords: Ischemia/Reperfusion, Caspase-4 inflammasome, Pyroptosis, Beclin1

Posted Date: April 6th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-384461/v1

Abstract

Background Myocardial reperfusion injury is often accompanied by cell death and inflammatory reactions. Recently, pyroptosis is gradually recognized as pivotal role in cardiovascular disease. However, little is known about the regulatory role of beclin1 in the control of caspase-4 activation and pyroptosis. The present study confirmed whether beclin1 regulates caspase-4 mediated pyroptosis and thereby protects Cardiac microvascular endothelial cells (CMECs) against injury.

Methods TTC and Evan's blue dye, western blot, immunofluorescence and immunohistochemistry staining were performed in wild mice and transgenic mice with overexpression of beclin-1(BECN1-Tg). CMECs were transfected with a beclin1 lentivirus. The cell cytotoxicity was analyzed by LDH-Cytotoxicity Assay Kit. The protein levels of autophagy protein (Beclin1, P62 and LC3II/LC3I) and caspase-4/GSDMD pathway were determined by western blot. Autophagic vacuoles in cells were monitored with RFP-GFP-LC3 using fluorescence microscope.

Results I/R caused caspase-4 activity and gasdermin D expression increase in vivo and in vitro. Overexpression of beclin-1 in heart tissue and CMECs suppressed the caspase-4 activity and decreased the levels of gasdermin D; meanwhile beclin1 overexpression also reduced IL-1β levels, promoted autophagy (P62 expression was inhibited while LC3II expression was increased) in the heart and CMECs. Interestingly, beclin1 overexpression increased animal survival and attenuated myocardial infarct size post-myocardial ischemia reperfusion.

Conclusions Induction of beclin-1 signaling can be a potential therapeutic target in myocardial reperfusion-induced microvascular injury.

1 Background

Microvascular dysfunction is a critical mediator of myocardial ischemia reperfusion injury that may influence patient outcomes. Baars et al demonstrated that attenuating endothelial damage could improve long-term outcomes in patients and animals who suffer from myocardial infarction [1]. Moreover, studies also indicated that apoptosis of endothelial cells occurred earlier than that of cardiomyocytes during MI/R [2]. However, it remains unclear whether cardiac microvascular endothelium injury is associated with pyroptosis during MIR. Thus, exploring the molecular mechanisms of CMECs' damage/death is critical to the search of clinically therapeutic strategies to minimize myocardial ischemia reperfusion injury.

Caspase-4 is a cysteine protease that is identified to promote cell pyroptosis, an inflammatory programmed cell death mediated by gasdermin D (GSDMD) [3]. It is activated by a cleavage that is mediated by caspase-4/511, thereby releasing amino-terminal gasdermin-N and carboxy-terminal gasdermin-C domains [4]. N-terminal fragment of GSDMD translocates to the plasma membrane, where it binds with phospholipids to form pores in membranes, leading to lytic cell death [5, 6]. Continuous activation of inflammasomes leads to excessive maturation of GSDMD, membrane pore formation and,

ultimately, pyroptosis. However, whether caspase-4 contributes to cell pyroptosis through the cleavage of GSDMD in cardiovascular disease and promotes microvascular endothelium remains to be fully clarified.

Autophagy influences a diverse of cellular responses, such as cell fate and inflammation [7, 8]. Beclin1, an essential protein for autophagy, have been documented to act as a contact point between autophagy and cell death [9]. But the precise mechanisms of beclin1-dependment autophagy in regulating inflammation and cell death remain elusive. It has been indicated that promoting autophagy decreases the apoptosis rate and protects the heart from the MI/R injury [10]; Yu et al suggested that inhibition of autophagy could augment pyroptosis in doxorubicin-treated human melanoma cells [11]. Recently, it has been reported that caspase-1-medicated pyroptosis is linked to autophagy [12]. However, the underlying interaction between beclin1-dependmemt autophagy and pyroptosis in CMECs and whether autophagy can affect caspase-4-mediated pyroptosis has not been elucidated.

In this study, we report a pathway by which beclin1-driven autophagy regulates caspase-4 mediated pyroptosis and presents a new potential molecular biomarker and therapeutic target for the potential treatment of myocardial reperfusion-induced microvascular injury.

2 Materials And Methods

2.1 Ischemia-reperfusion model

Adult male Wild-type (WT) and BECN1-Tg C57BL/6J mice (18–22 g) used in the study were purchased from Cyagen Biosciences and housed under specific pathogen-free conditions. The mice were anesthetized with 1% Pentobarbital. After anesthesia, the hearts were then exposed between the fourth and fifth ribs and the left anterior descending (LAD) coronary artery was ligated 6 – 0 silk suture. It was released for 1 h, 6 h and 12 h after occlusion for 45 min. In sham-operated animals, a silk suture was passed under LAD without ligation.

2.2 Assessment of myocardial infarct size

The region of infarct size was measured by double staining with TTC and Evan's blue dye which proceeded as previously described [13]. The area at risk (AAR) portion of the LV was stained red and white, whereas the infarct size (IS) was stained white and normal myocardium stained dark blue. The heart slices were photographed digitally. After this procedure, the images were analyzed using Image J, the IS and AAR were calculated as a percentage of the LV.

2.3 Immunofluorescence and immunohistochemistry

Briefly, fresh heart, spleen and liver tissues were fixed in 4% paraformaldehyde. After this, tissue were dehydrated with a graded ethanol series and embedded in paraffin. The sections were subsequently incubated through 0.3% hydrogen peroxide in PBS to block endogenous peroxidase activity. They were then treated with 10 mM citrate buffer (pH 6.0) to retrieve the antigen, followed by rinsing in phosphate buffered saline (PBS). Thereafter, the sections were blocked with 5% goat serum in PBS for 2 h, and incubated overnight at 4°C with the following primary antibodies: rabbit polyclonal anti-GSDMD antibody

(1:100, Cell Signaling Biotechnology), anti-F4/80 (1:200, Cell Signaling Biotechnology). After three washes with PBS, tissues were incubated with secondary for an additional 2 h at room temperature. Captured images were further analyzed by software Image J (version 1.37; National Institutes of Health, Bethesda, MD, USA). For cytofluorescense staining and immunohistochemistry were proceeded as previously described [13].

2.4 Cells culture and hypoxia/reoxygenation

Human cardiac microvascular endothelial cells line (HCMECs) were purchased from the Bei Na Chuanglian Biotechnology (BNCC, Wuhan, China). The passage number we used for this study was the second or third. Cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM: Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (BI: Israel, Middle East) and 100 IU/ml penicilin and 100IU/ml streptomycin (Hyclone, Logan, UT, USA). The cells were cultured in a humidified environment composed of 95% air and 5% CO_2 at 37℃. hypoxia/reoxygenation establishment was performed as previously described [13].

2.5 Lentivirus transfection

CMECs were cultured in six-well plate, On the following day, the medium was replaced with new medium containing lentivirus carrying beclin1 gene (Len-Becn1) and lentiviral vector carrying green fluorescence (Len-GFP), which were, respectively, stably transfected into CMECs for 8 h; puromycin (Thermo Scientific, A1113802) was added for further selection. Two days after transfection, H/R model of CMECs was established as described above.

2.6 Autophagic flux measurement

Autophagic flux was monitored by transfection with RFP-GFP-LC3. CMECs were cultured in six-well plate for 24 h and subsequently transfected with RFP-GFP-LC3 lentivirus for 8–10 h. After culturing for 24–48 h, cells were fixed with 4% paraformaldehyde for 15 min. Finally, the cell nuclei were stained with DAPI. Fluorescence images were captured using a fluorescence microscope (Olympus, Tokyo, Japan) for detection of autophagosomes (yellow puncta in fusion images) and autolysosomes (red puncta in fusion images).

2.7 Western blot analysis

Total protein was extracted from CMECs and heart, spleen and liver tissues. Protein concentration was examined by the BSA protein assay (Thermo Fisher, USA). Cell and tissue lysates were separated by SDS–PAGE using a Tris–Glycin system, and the protein bands then transferred to a PVDF membrane. The membrane was blocked with 5% fat-free milk in Tris buffered saline (TBS) for 2 h at room temperature and subsequently incubated overnight at 4℃ with the following primary antibodies: caspase-4 (1:1000, Abclonal), GDMDM (1:1000, Cell Signaling Technology), IL-1β (1:1000, Santa Cruz biotechnology), beclin1 (1:2000, Abclonal), LC3 (1:2000, Cell Signaling Technology), P62 (1:10000, Abcam) and β-actin (1:2000, Santa Cruz biotechnology). After washing three times with TBST, membranes were incubated with horseradish peroxidase (HRP)-conjugated IgG (goat anti-rabbit secondary antibody and goat anti-mouse) secondary antibody for 2 h at room temperature. Proteins were

visualized by ECL procedure (Amersham Imager 600). The expression of target proteins was standardized by β-actin.

2.8 LDH and myocardial enzymatic assay

LDH activity in cell supernatants was evaluated using LDH-Cytotoxicity Assay Kit (Beyotime, C0017). Briefly, CMECs were plated in 96-well plates before exposure to H/R (2/2 h), H/R + 3-methyladenine (3-MA) or H/R + VX-765. LHD activity was measured at 490 nm by a microplate reader following the manufacturer's instructions. The serum levels of creatine kinase (CK), lactate dehydrogenase (LDH) were measured using automatic biochemical analyzer (cobas c 311, Mannheim, Germany).

2.9 Statistical analysis

Data are presented as mean ± SD and were analyzed using GraphPad Prism 6. Differences between groups were assessed by *student's t*-tests (one measured variable) or by a two-way ANOVA with Bonferroni *post hoc* testing. P 0.05 was considered statistically significant.

3 Results

3.1 Ethical approval

Animal experiments were reviewed and approved by the Animal Research Ethics Committee of the Kunming Medical University.

3.2 Beclin1 overexpression attenuates myocardial reperfusion injury in mice

Beclin1 overexpression increased animal survival and decreased the levels of serum LDH and CK (Fig. 1A, 1B, $P<0.05$) compared to WT-I/R group. Fig. 1C showed the effect of BECN1-Tg on the myocardial infarct size at 6 h after reperfusion. The ratio of myocardial infarct size to area at risk (AAR) in the I/R group was larger than that of in the Sham group (Fig. 1D, $P<0.05$). However, the ratio of infarct size to AAR in BECN1-Tg-I/R group was smaller than that of the WT-I/R group (Fig. 1D, P 0.05). The AAR as the percentage of the LV (AAR/LV) in the BECN1-Tg- I/R group was significantly decreased in comparison with WT-I/R group (Fig. 1D, $P<0.05$). Our data also revealed that BECN1-Tg attenuated $F4/80^+$ macrophages and $CD11b^+$ neutrophils infiltration in the heart, spleen and liver compared to that of the WT group after MI/R (Fig. 2A-F, $P<0.05$).

3.3 Beclin1 overexpression enhances autophagic flux in CMECs

The beclin1 content of CMECs transfected with Len-GFP and Len-Becn1 was evaluated by western blotting: The results showed beclin1 up-regulation in Len-Becn1 group (Fig. 3A, 3B, $P<0.05$). Following this, the effect of beclin1 overexpression on autophagy flux was determined. CMECs were cultured for 2 h in the absence or presence of Bafilomycin A1 (Baf). The results indicated that beclin1 overexpression turnover the process of Baf inhibiting autophagy flux (Fig 3A-D). Staining of endogenous LC3-positive vesicles in Baf-treated CMECs supports these data (Fig. 3E, 3F).

3.4 I/R induces caspase-4 inflammasome activation and pyroptosis *in vivo* and *in vitro*

Studies have reported the time-dependent activation of the NLRP3 inflammasome in the heart after MI/R [14, 15]. However, recent studies have indicated that caspase-4 is a specific molecular cell marker to trigger cell pyroptosis [16]. But the role of caspase-4-dependent pyroptosis in MI/R was unclear. Therefore, we examined the levels of caspase-4, IL-1β, GSDMD in the heart and CMECs at different reperfusion time points. The results showed that I/R induced caspase-4 activation, IL-1β secretion and resulted in higher levels of CMECs or heart pyroptosis, as determined by high expression of GSDMD in vivo and in vitro, especially at after 45min ischemia/6 h reperfusion in vivo and 2 h of hypoxia/2 h of reoxygenation in vitro significantly increased (Fig. 4A-G, P<0.05). Therefore, I/R (45min/6 h) or H/R□2/2 h□was used in subsequent experiments. The results indicate that caspase-4 -dependent pyroptosis may play an important role in the pathophysiology of MI/R injury.

3.5 Beclin1 overexpression suppresses caspase-4 inflammasome activation and pyroptosis by enhancing autophagic flux in mice

To examine whether up-regulation of beclin1 and autophagy levels influences caspase-4 activation mediated pyroptosis in mice. The levels of LC3, P62, caspase-4, IL-1β and GSDMD in WT and BECN1-Tg the heart were assessed by western blotting. Our data indicated that the basal levels of caspase-4 and IL-1β were significantly lower in BECN1-Tg group than WT group (Fig. 5A, 5B, P<0.05). Next, to further establish MI/R model and observe the changes of above proteins, the results showed that the levels of LC3-II/LC3□ were significantly increased (Fig. 5A, 5B, P<0.05); while P62, caspase-4, IL-1β and GSDMD expression were significantly decreased in BECN1-Tg-I/R group compared to WT-I/R group (Fig. 5A, 5B, P<0.05). We also investigated expression of GSDMD in the spleen and liver after MI/R. The results were consistent with the heart tissue (Fig. 5C, 5D, P<0.05). Immunofluorescence analysis also indicated the expression of GSDMD was attenuated in BECN1-Tg-I/R group not only in the heart but also in the spleen and liver (Fig. 5E-H, P<0.05). These observations suggest that beclin-1-dependent autophagy is a protective response in MI/R through suppressing capase-4-dependent pyroptosis.

3.6 Impaired autophagic flux promotes caspase-4 inflammasome activation in CMECs

To investigate the effect of autophagy on caspase-4 activation, CMECs were subjected to 2 h hypoxia followed 2 h re-oxygenation. In needed, H/R significantly increased caspase-4 activation as well as secretion of IL-1β (Fig. 6A, 6B, P<0.05); meanwhile the levels of beclin1, LC3-II/LC3□, P62 were also elevated (Fig. 6A, 6B, P<0.05). Furthermore, CMECs were stimulated with the autophagy inhibitor 3-MA for 2 h. The results indicated that following the treatment with 3-MA, the expression of beclin1, LC3-II/LC3□ and P62 was decreased; whereas caspase-4 and IL-1β expression were further increased in CMECs (Fig. 6A, 6B, P<0.05). On the other hand, to explore the impact of caspase-4 on autophagy activity, caspase-4 inhibitor VX-765 was treated with CMECs for 2 h. The results showed that VX-756 pre-treatment suppressed caspase-4 activation, IL-1β secretion as well as pyroptosis; whereas further increased the expression of beclin1, LC3-II/LC3□ and decreased P62 in CMECs (Fig. 6A, 6B, 6D, 6E, P<0.05). LDH release

assay indicated H/R-induced cell death. In this connection, LDH release in CMECs were blocked by VX-765 and activated by 3-MA (Fig. 6C, $P<0.05$).

Given our findings above, the effect of autophagic flux of regulating the caspase-4 activation was further confirmed. Autophagic flux comprises induction, maturation and degradation of autophagosome. To monitor autophagic flux, RFP-GFP-LC3 was transfected into CMECs using fluorescence microscope to detect the puncta. CMECs without H/R showed a basal level of autophagy as exhibited by a few autophagosomes and autolysosomes puncta. CMECs exposed to H/R or treatment Baf (inhibition lysosomal degradation) had massive autophagosomes and few autolysosomes puncta, suggesting autophagic fiux was impaired. 3-MA treatment resulted in fewer autophagosomes and autolysosomes puncta. However, VX-765 treatment resulted in more autolysosomes and fewer autophagosomes, indicating that VX-765 treatment enhanced autophagic flux (Fig. 6F, 6G, $P<0.05$). To confirm that caspase-4 activation regulated by autophagic flux during H/R, Baf was treated with CMECs. The result showed that Baf treatment significantly increased LC3II/LC3Ⅰ and P62 expression; while caspase-4 expression was further increased (Fig. 6H, 6I, $P<0.05$). These observations revealed that impaired autophagic flux may promote caspase-4 activation.

3.7 Beclin1 overexpression driven autophagic flux inhibits caspase-4 inflammasome activation and pyroptosis in CMECs

Next, to determine the effect of up-regulation of beclin1 and autophagy on caspase-4 medicated pyroptosis, Len-Becn1 was transferred into CMECs. The levels of LC3, P62, caspase-4, and GSDMD in CMECs were assessed by western blot. The results indicated that Len-Becn1 group showed increased levels of LC3-II/LC3Ⅰ compared to Len-GFP group (Fig. 7A, 7B, $P<0.05$), whereas P62 expression had no altered. However, LC3II/LC3I expression was up-regulated, while P62 expression was decreased in Len-Becn1 group during H/R (Fig. 7A, 7B, $P<0.05$). Furthermore, caspase-4 activation and GSDMD expression was lower in Len-Becn1 group during H/R (Fig. 7A, 7B, $P<0.05$). In addition, LDH Cytotoxicity Assay demonstrated that beclin1overexpression blocked LDH release in CMECs exposed to H/R (Fig. 7C, $P<0.05$). To further ensure beclin1 overexpression affects caspase-4-dependent pyroptosis during H/R. Cells was treated with Baf. The results showed accumulation of LC3 and P62 compared to in Len-GFP group (Fig. 7D, 7E, $P<0.05$). Nevertheless, transfection with Len-Becn1 in CMECs treated with Baf is able to restore autophagic flux compared to Len-GFP+Baf group (Fig. 7D, 7E, $P<0.05$). These results report that up-regulation beclin1 may inhibit caspase-4-dependment pyroptosis and ameliorate H/R induced CMECs injury by enhancing autophagic flux.

4 Discussion

This study indicates that beclin1 overexpression alleviates microvascular injury by promoting autophagic flux. Beclin1 overexpression enhances autophagic flux to inhibiting caspase-4 inflammasome activation to decrease cell pyroptosis and consequently increasing animal survival and attenuating myocardial infarct size and inflammatory cells infiltration post-MI/R.

Evidence from recent studies have indicated that pyroptosis contributes to the pathogenesis of cardiovascular diseases [17-19]. Recent publication suggested that pyroptosis mediated by NLRP3/caspase-1 pathway exacerbated cardiomyocytes injury during I/R [20]; A recent report from our laboratory demonstrated that NLRP3/caspase-1-dependment pyroptosis aggravated microvascular endothelium damage [13]. Surprisingly, caspase-4 mediated pyroptosis in CMECs has not been characterized in MI/R. In the present study, caspase-4 activation and pyroptosis in both the heart and CMECs during MI/R have been observed. Suggesting caspase-4 mediated pyroptosis indeed plays a pivotal role in the pathophysiology of MI/R.

Several studies have demonstrated that autophagy is a multifaceted modulator of cell death [8, 21]. But, at present, we do not fully understand how autophagy regulation of pyroptosis protects CMECs against MI/R injury. Beclin1 is a pivotal molecule in the autophagy pathway and is essential for autophagosome formation [9]. Here, we have found that up-regulation beclin1 in CMECs and mice result in enhancement autophagic flux. This is consistent with previous report demonstrating increase autophagy in cardiac from transgenic mice with cardiac-specific overexpression of beclin-1 [22]. Numerous reports have indicated that autophagy is involved in regulation inflammatory [23, 24]. Inflammatory response would cause heart injury by inducing cardiomyocytes/endothelial cells death [25-27]. Ghulam Ilyas et al reported that decreasing macrophage autophagy facilitated liver injury by promoting inflammasome activation [28]. In light of published data, we hypothesized that beclin-1 driven autophagy might inhibit pyroptosis by regulating caspse-4 inflammasome activation during MI/R. To support this hypothesis, we analyzed that whether up-regulation in beclin-1 could suppress caspase-4 activation and pyroptosis in MI/R model established by using BECN1-Tg mice. In addition, our observations in the spleen and liver were consistent with that in the heart showing decreased pyroptosis after MI/R. Taken together with our present results, it can be stated that beclin-1 overexpression suppressed caspase-4-dependment pyroptosis induced by MI/R through enhancing autophagy.

To gain a deeper understanding of the potential mechanism of autophagy that regulates caspase-4-dependent pyroptosis in vitro. Previous experimental studies have highlighted the cross-talk between autophagy and inflammasome activation, the cellular machinery is related to the elimination of cellular components and maintenance of intracellular homeostasis [29, 30]. Previous study manifested that H/R induced accumulation of autophagosomes, LC3-II, p62 and decreased autolysosomes [31], suggesting that H/R may impair autophagic flux in cells. Our data demonstrated that H/R lead to caspse-4 activation and pyroptosis may be related to impair autophagic flux in CMECs. In order to further determine the interaction between autophagic flux and caspse-4 activation on the molecular level. Addition of the autophagy blocker 3-MA or Baf resulted in more increased expression of caspase-4 and IL-1β after H/R. In addition, RFP-GFP-LC3 was transfected to monitor the autophagic flux, the results also indicated that autophagic flux was suppressed by 3-MA or Baf. These data illustrate that inhibition of autophagy further promotes caspse-4 activation and IL-1β production. A recent study shed light on inhibition of inflammasome activation which promotes autophagy [32]. Our data indicated that inhibition of caspase-4 increased CMECs autophagy after H/R. CMECs transfected with RFP-GFP-LC3 was elucidated that autophagic flux was increased by VX-765 pretreatment. Our observations suggest that enhancing

autophagic flux may contribute to protect CMECs against H/R injury mediated by caspase-4 inflammasome activation.

Our above data have suggested up-regulation beclin1 can alleviate caspase-4 activation, IL-1β release and pyroptosis in vivo. Judith Houtman et al showed that reduction in beclin1 resulted in enhanced NLRP3 activation and IL-1β release [33]. Sun et al revealed that beclin-1 protected the heart during sepsis [34]. Based on these data, we further explored whether increased beclin1 levels would have an effect on caspase-4-dependment pyroptosis in CMECs during H/R. Our data demonstrate that beclin1 overexpression inducing autophagy and enhancing (increased LC3II and decreased P62 expression) suppressed caspase-4-mediated pyroptosis which may protect CMECs against H/R injury.

In summary, our data have provided the molecular mechanism whereby beclin1-driven autophagy may regulate caspase-4-mediated pyroptosis. This would be helpful to determine the potential molecular biomarker and therapeutic targeting of autophagic process for amelioration myocardial reperfusion-induced microvascular injury.

Abbreviations

NLRP3: NOD-like receptor protein 3; Caspase-1: Cysteinyl aspartate-specific proteinase-1; IL-1β: Interleukin 1β; 3-MA: 3-Mthyladenine; CMECs: Cardiac microvascular endothelial cells; LC3: Microtubule-associated protein light chain 3; LDH: Lactate dehydrogenase; CK: Creatine Kinase; TTC: Tetracycline; WT: Wild type; Becn1-Tg: Transgenic mice with overexpression of Becn1

Declarations

Acknowledgements

The authors thank Prof. Ling Eng-Ang for their generous help in the spelling, semantic, syntactic and grammar revision.

Authors' contributions

DL and LS conceived and designed the study; WJS, HQL, SJD and NW performed the experiments; LMTW and YZ analyzed the data; DL, LS and WJS wrote the manuscript. All authors read and approved the final manuscript.

Finding

This work was supported in part by National Natural Science Foundation of China (grant No. 31760292; grant No. 81760087; grant No. 81560050; grant No. 81260061.), and Department of Science and Technology of Yunnan Province (grant No. 2019ZF011-2; grant No. 2017FA035; grant No. 2017FE467 (-008).

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article files as well as materials prepared are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The authors declare that they have no conflicts of interest to disclose. The study was approved by the Experimental Animal Center of Kunming medical university, Kunming, China. All procedures followed were in accordance with the ethical standards of the Ethics Committee of the Kunming medical university.

Consent for publication

All authors consent to publication.

Competing interests

The authors declare that they have no competing interests.

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Figures

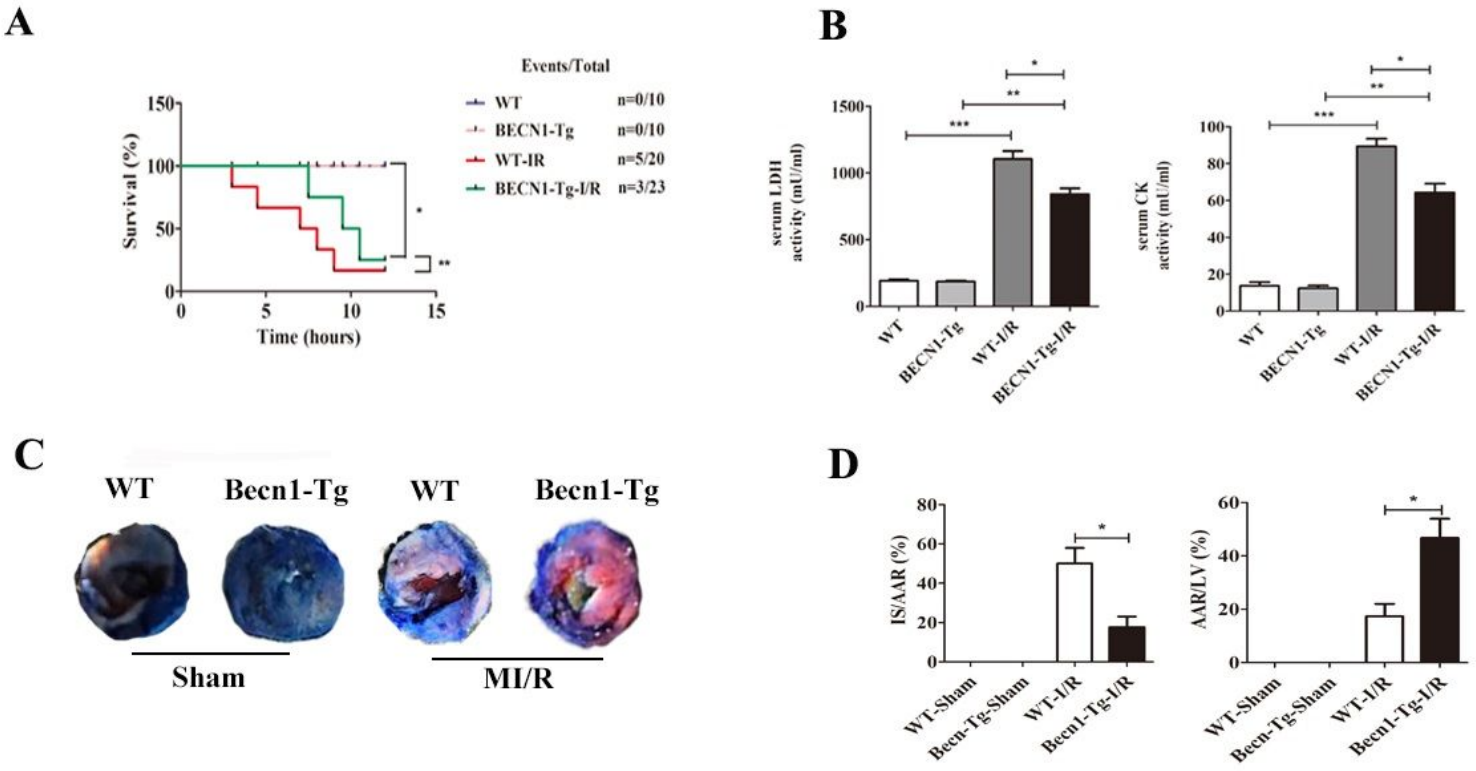

Figure 1

Beclin1 overexpression alleviates myocardial reperfusion injury in mice. (A) Survival analysis for mice subjected to 45 min ischemia and following 6 h perfusion (*P < 0.05 vs. Sham group, **P < 0.05 vs. WT-I/R group, Kaplan-Meier survival analysis). (B) Serum concentrations of LDH and CK in each group were measured using automatic biochemical analyzer. Data are expressed as mean ± SD (n=5 for each). *P < 0.05 vs. WT-I/R group, **P < 0.05 vs. BECN1-Tg group, ***P < 0.05 vs. WT group. (C) Representative TTC-Evan's Blue stained sections of heart from each group. Brick red-stained area represents the area at risk (AAR), whereas the white area indicates the infarcted size (IS). (D) Ratio of IS/LV, AAR/LV. Data are expressed as mean ± SD (n=5 for each). *P < 0.05 vs. WT group, **P < 0.05 vs. WT-I/R group.

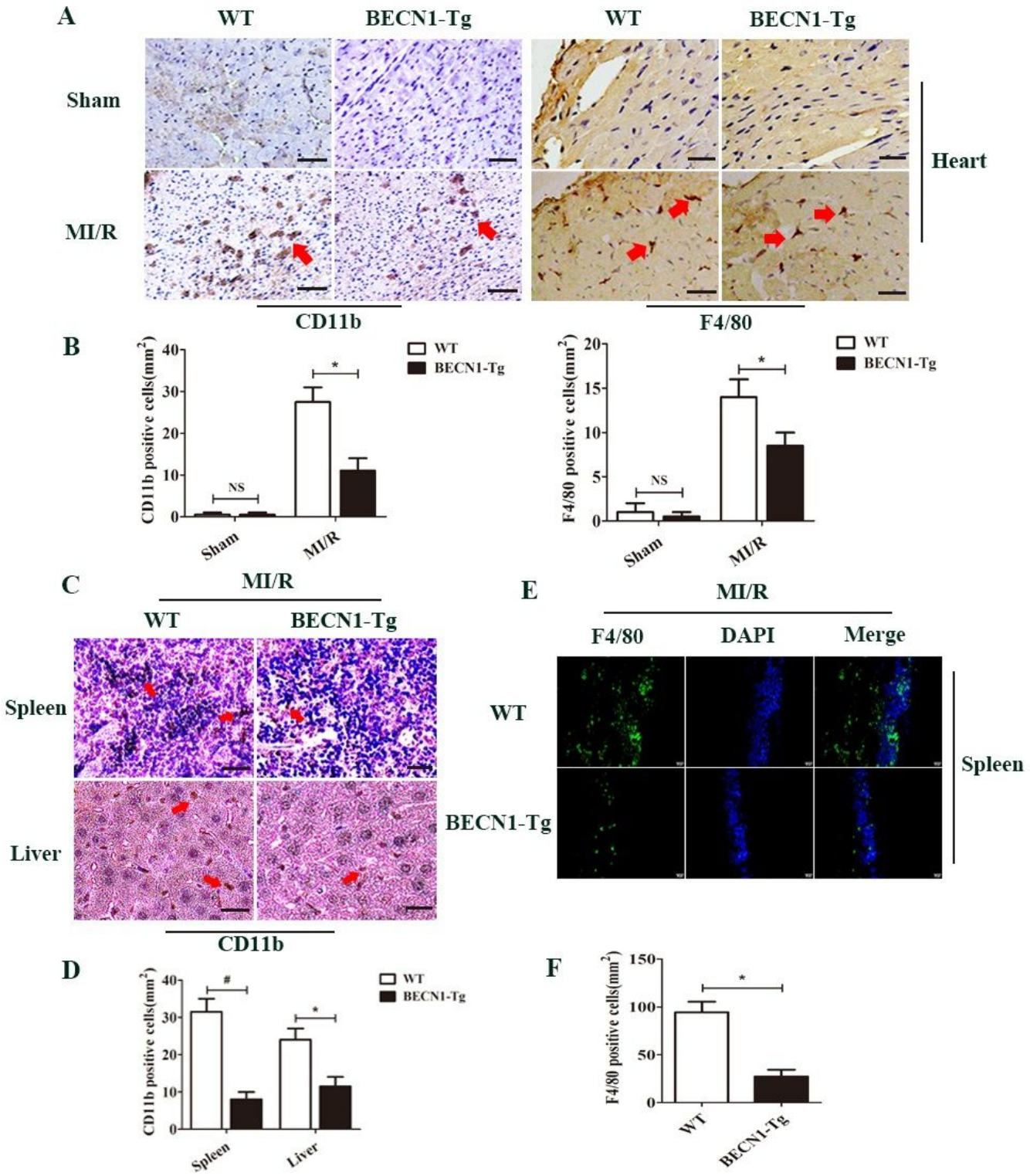

Figure 2

Beclin-1 overexpression regulates autophagic flux in CMECs. (A-D) Beclin-1 and LC3 expression in Len-GFP and Len-Becn1 CMECs treated with or without Bafilomycin A1 (Baf, 50 nM) was quantified by western blotting. Data are expressed as mean ± SD (n=3). *P < 0.05 vs. Len-GFP group. (E) Endogenous LC3 expression in Len-GFP and Len-Becn1 CMECs treated with or without Bafilomycin A1 (50 nM). (F)

Quantification of LC3-positive vesicles in CMECs. Data are expressed as mean ± SD (n=3). *P < 0.05 vs. Len-GFP group, **P < 0.05 vs. Len-GFP+Baf group.

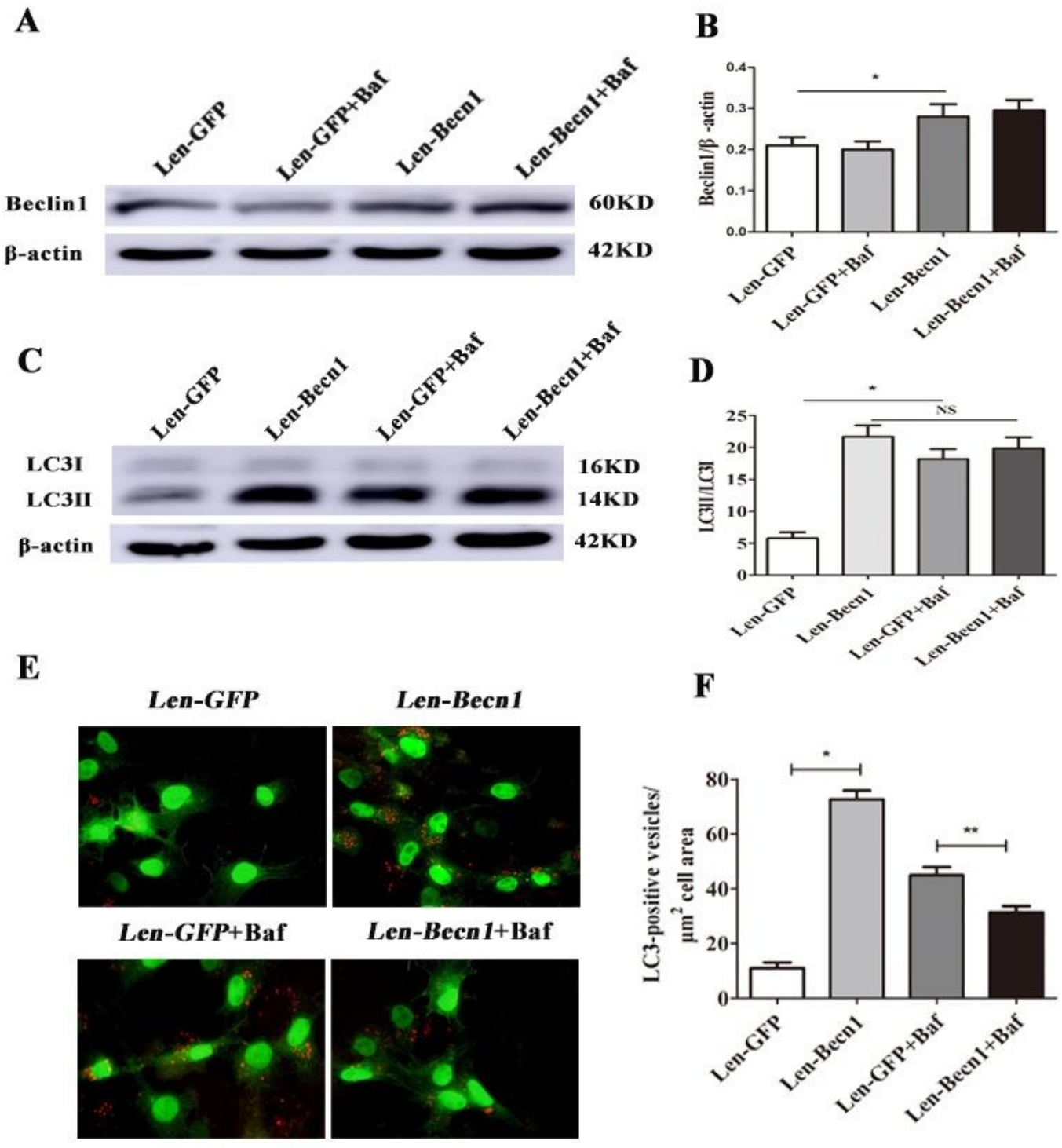

Figure 3

Caspase-4 inflammasome and pyroptosis-associated proteins are activated in heart and CMECs after MI/R or H/R. (A-D) Representative western blotting bands and densitometric quantification of GSDMD, caspase-4 and IL-1β in heart at different myocardial ischemia/reperfusion (MI/R) time points. Data are expressed as mean ± SD (n=3). *P < 0.05 vs. Sham group. (E-F) Representative western blotting bands

and densitometric quantification of caspase-4, IL-1β and GSDMD were assessed in CMECs at different hypoxia/reoxygenation (H/R) time points. Data are expressed as mean ± SD (n=3). #P < 0.05 vs. control group. (G) Immunofluorescence staining for GSDMD in CMECs at 2 h of hypoxia/2 h of re-oxygenation. Scar bar=20 μm.

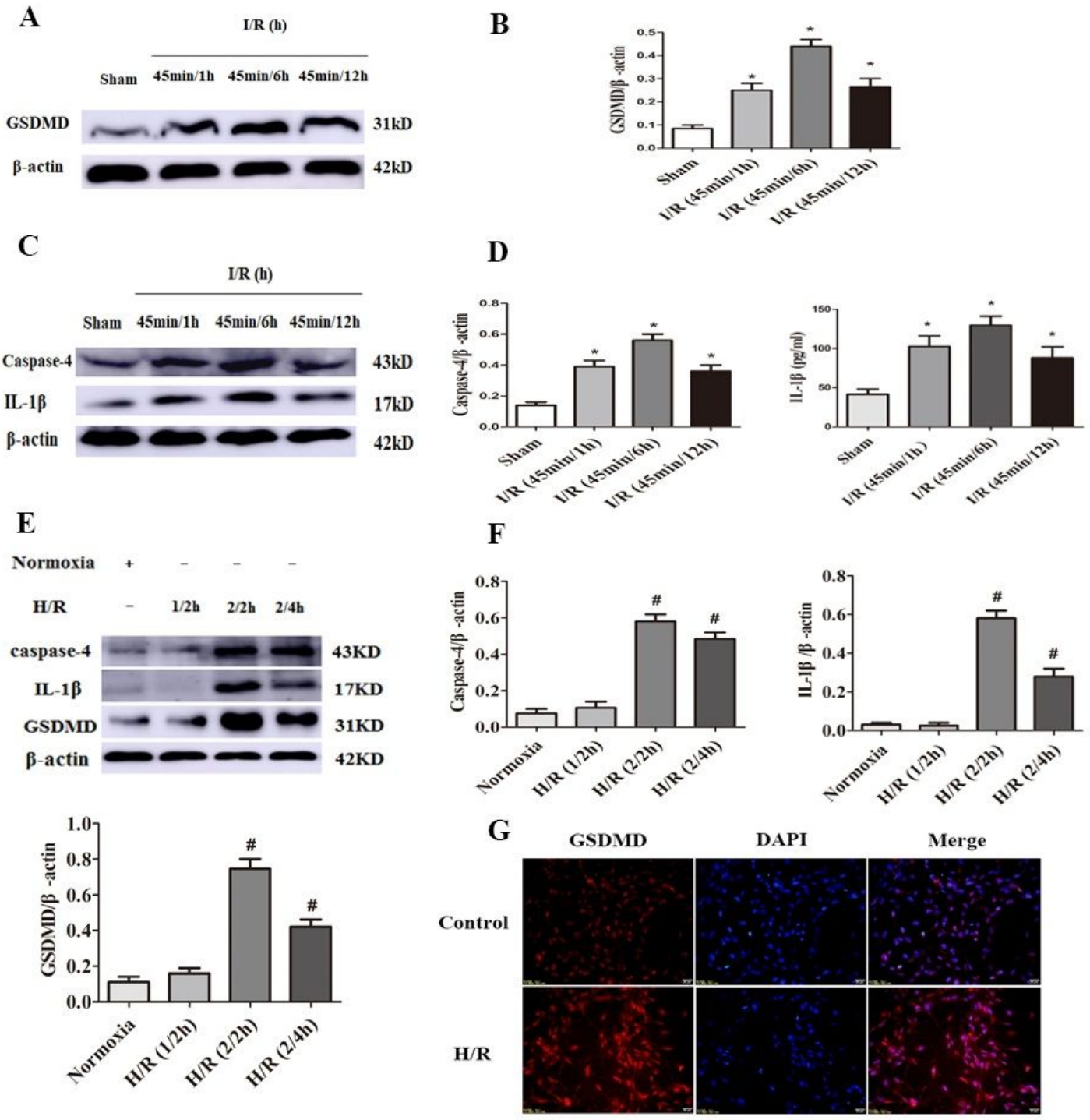

Figure 4

Enhanced beclin1 expression results in suppressed caspase-4 activation and pyroptosis in mice. (A) Beclin1, LC3, p62, caspase-4, IL-1β and GSDMD expression in WT and BECN1-Tg heart under Sham or 45min ischemia/6 h of reperfusion was assessed by western blot analysis. (B) Densitometric

quantification of Beclin1, LC3Ⅱ/LC3Ⅰ, p62, caspase-4, IL-1β and GSDMD in heart. Data are expressed as mean ± SD (n=3). *P < 0.05 vs. WT group, **P < 0.05 vs. WT-I/R group. (C) GSDMD expression in WT and BECN1-Tg spleen and liver after 45min myocardial ischemia/6 h of reperfusion as assessed by western blot analysis. (D) Densitometric quantification of GSDMD in spleen and liver. Data are expressed as mean ± SD (n=3). *P < 0.05 vs. WT group. (E-F) Immunofluorescence staining and quantification of GSDMD in WT and BECN1-Tg heart in Sham or 45min ischemia/6 h of reperfusion. Data are expressed as mean ± SD (n=3). Scar bar=20 μm. *P < 0.05 vs. WT-I/R group. (G) Expression of GSDMD in WT and BECN1-Tg spleen and liver after 45min myocardial ischemia/6 h of reperfusion as determined by immunofluorescence. (H) Quantification showed a remarkably decreased expression of GSDMD in BECN1-Tg group. Data are expressed as mean ± SD (n=3). Scar bar=20 μm. *P < 0.05 vs. WT group.

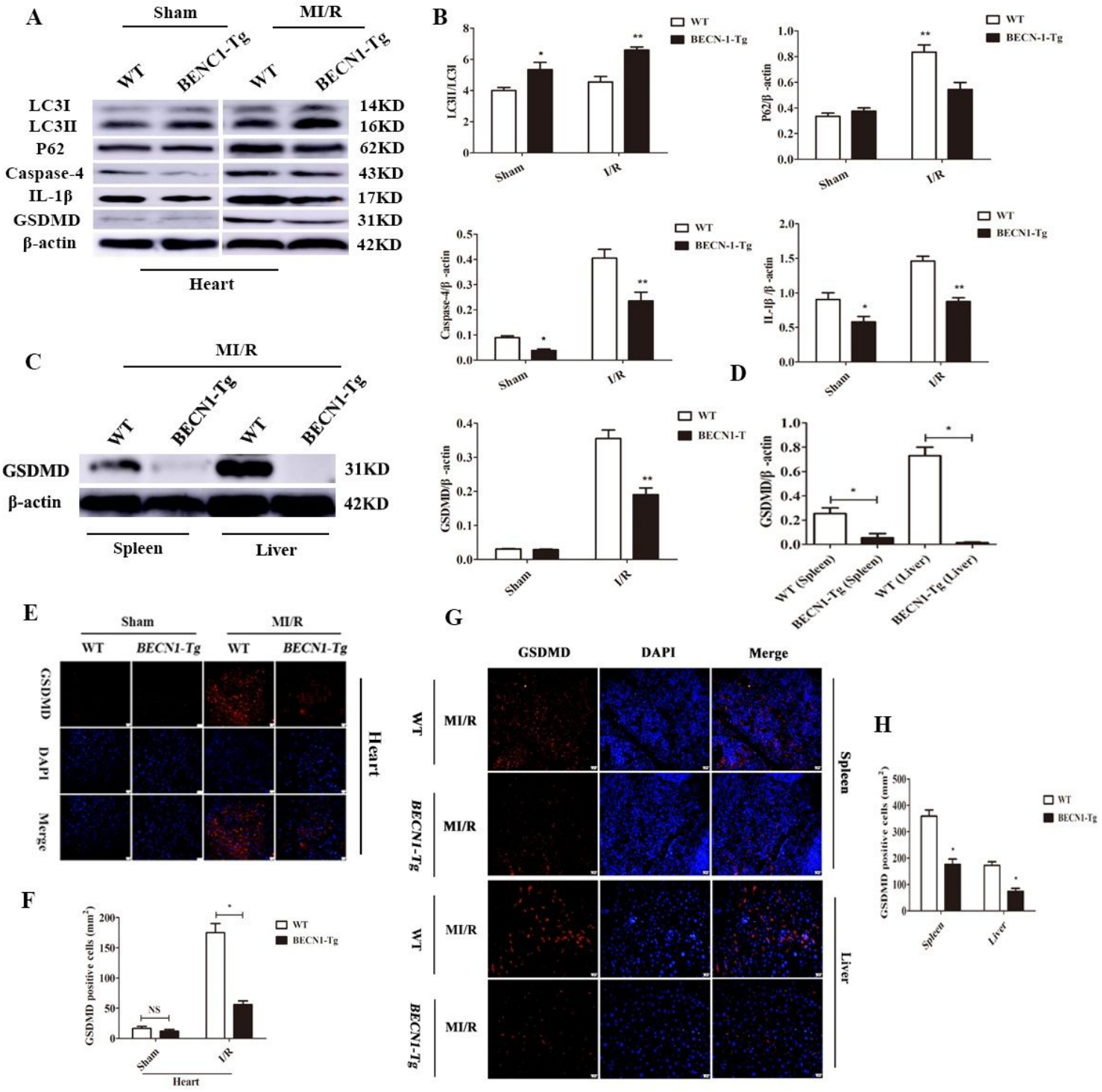

Figure 5

Beclin-1 regulates autophagy flux through inhibiting caspase-4-mediated pyroptosis in CMECs. (A) Expression of beclin1, LC3, p62, caspase-4, IL-1β in CMECs under normoxia or 2 h hypoxia/ 2 h of re-oxygenation ± 3-MA (5 mM) or VX-765 (10 μM) was assessed by western blot analysis. (B) Densitometric quantification of Beclin1, LC3Ⅱ/LC3Ⅰ, p62, caspase-4 and IL-1β in CMECs. Data are expressed as mean ± SD (n=3). *P < 0.05 vs. control group, **P < 0.05 vs. 3-MA+H/R group, ***P < 0.05 vs. VX-765+H/R group. (C) Analysis of LDH release in CMECs under normoxia or 2 h hypoxia/ 2 h of re-oxygenation ± 3-MA (5

mM) or VX-765 (10 μM). Data are expressed as mean ± SD (n=5). *P < 0.05 vs. control group, **P < 0.05 vs. 3-MA+H/R group, ***P < 0.05 vs. VX-765+H/R group. (D) CMECs were cultured for 24 h after transfection with lenovirus harbouring tandem fluorescent RFP-GFP-LC3. CMECs carring RFP-GFP-LC3 subjected to H/R were treated with or without 3-MA (5 mM) or VX-765 (10 μM). Representative immunofluorescence images of CMECs expressing RFP-GFP-LC3; cell nuclei were stained with DAPI (blue). Red punta represent autophagosomes; yellow punta in merged picture represent autolysosomes. (B) Semi-quantitative analysis of autophagosomes and autolysosomes in each group. (F) Expression of LC3, p62, caspase-4 and GSDMD in Len-GFP and Len-Becn1 CMECs under normoxia or 2 h hypoxia/ 2 h of re-oxygenation. (G) Densitometric quantification of LC3Ⅱ/LC3Ⅰ, p62, caspase-4 and GSDMD in CMECs. Data are expressed as mean ± SD (n=3). *P < 0.05 vs. control group, #P < 0.05 vs. Len-GFP group, ## P < 0.05 vs. Len-GFP+H/R group. (H) Analysis of LDH release in Len-GFP and Len-Becn1 CMECs under normoxia or 2 h hypoxia/ 2 h of re-oxygenation. Data are expressed as mean ± SD (n=5). ## P < 0.05 vs. Len-GFP+H/R group.

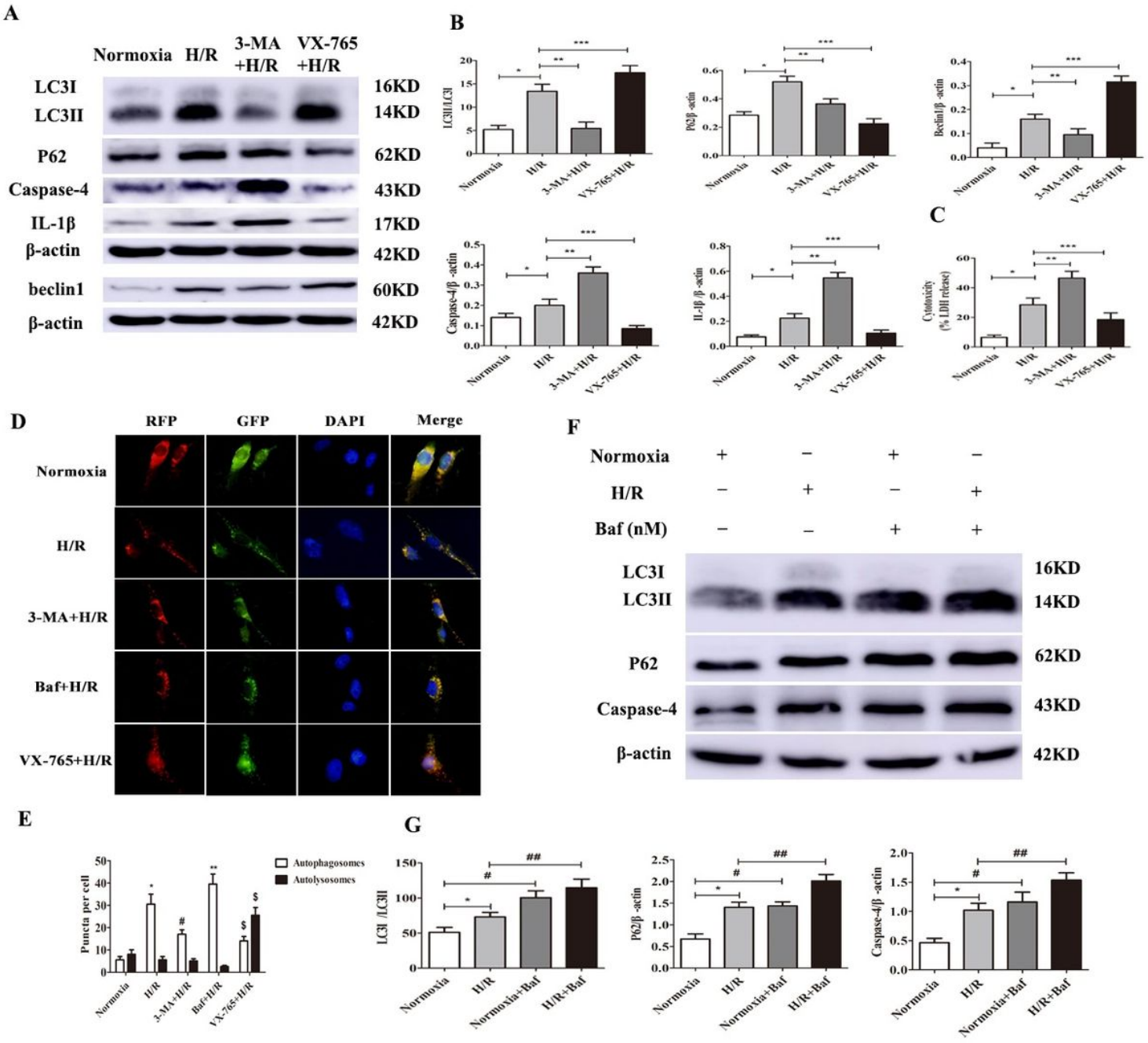

Figure 6

Autophagy regulates caspase-4 inflammasome and yroptosis as cardiac microvascular endothelial cells (CMECs) responses to I/R. (A) In resting CMECs, autophagy component beclin-1 is complexed with caspase-4, inhibiting pyroptosis. (B, C) In response to I/R, caspase-4 inflammasome activation in CMECs, when caspase-4 inflammasome activation is exceeded, an increase in expression of beclin-1promotes autophagy activity to orchestrate caspase-4 activation and pyroptosis, which protects cells from I/R injury.

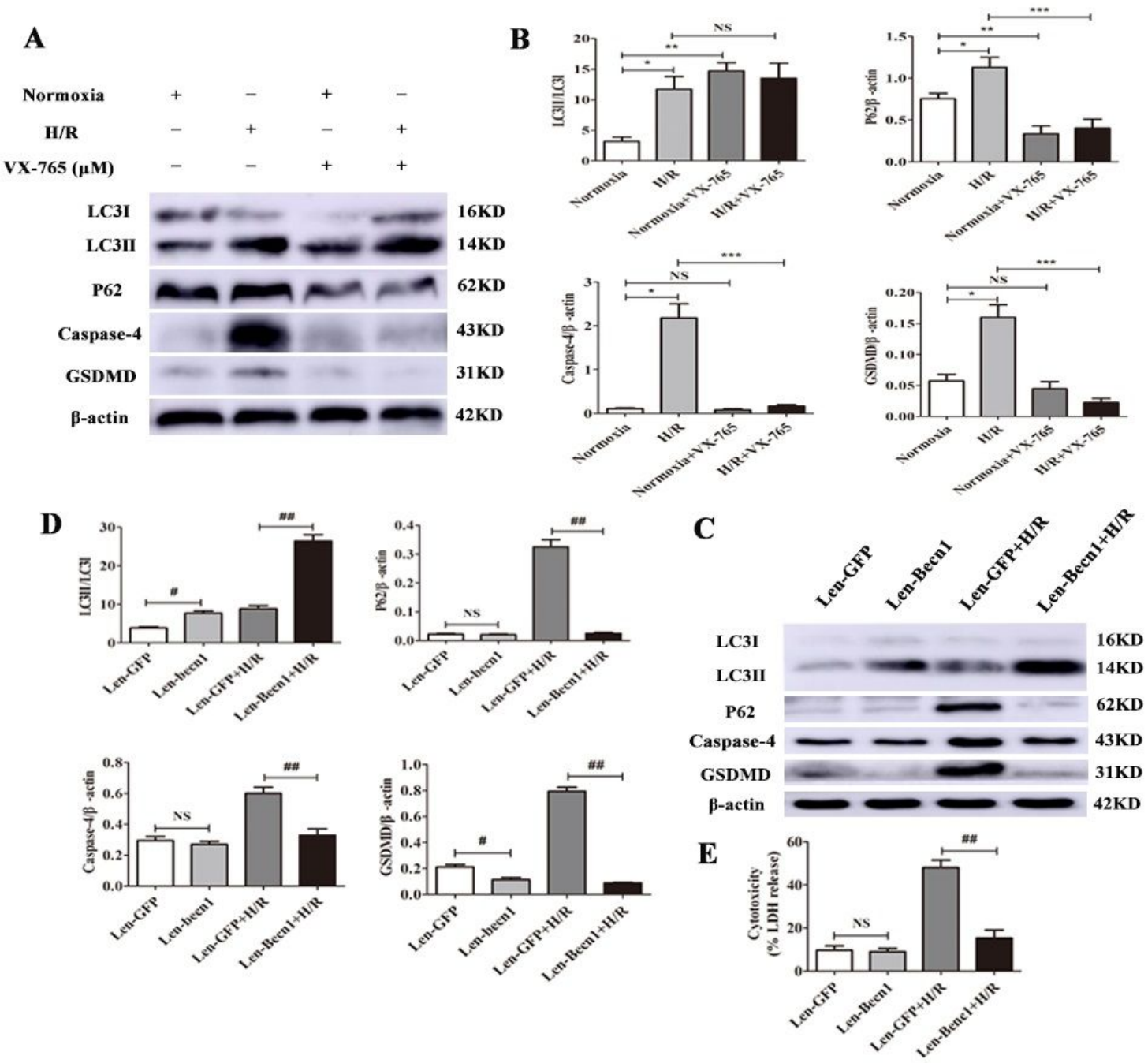

Figure 7

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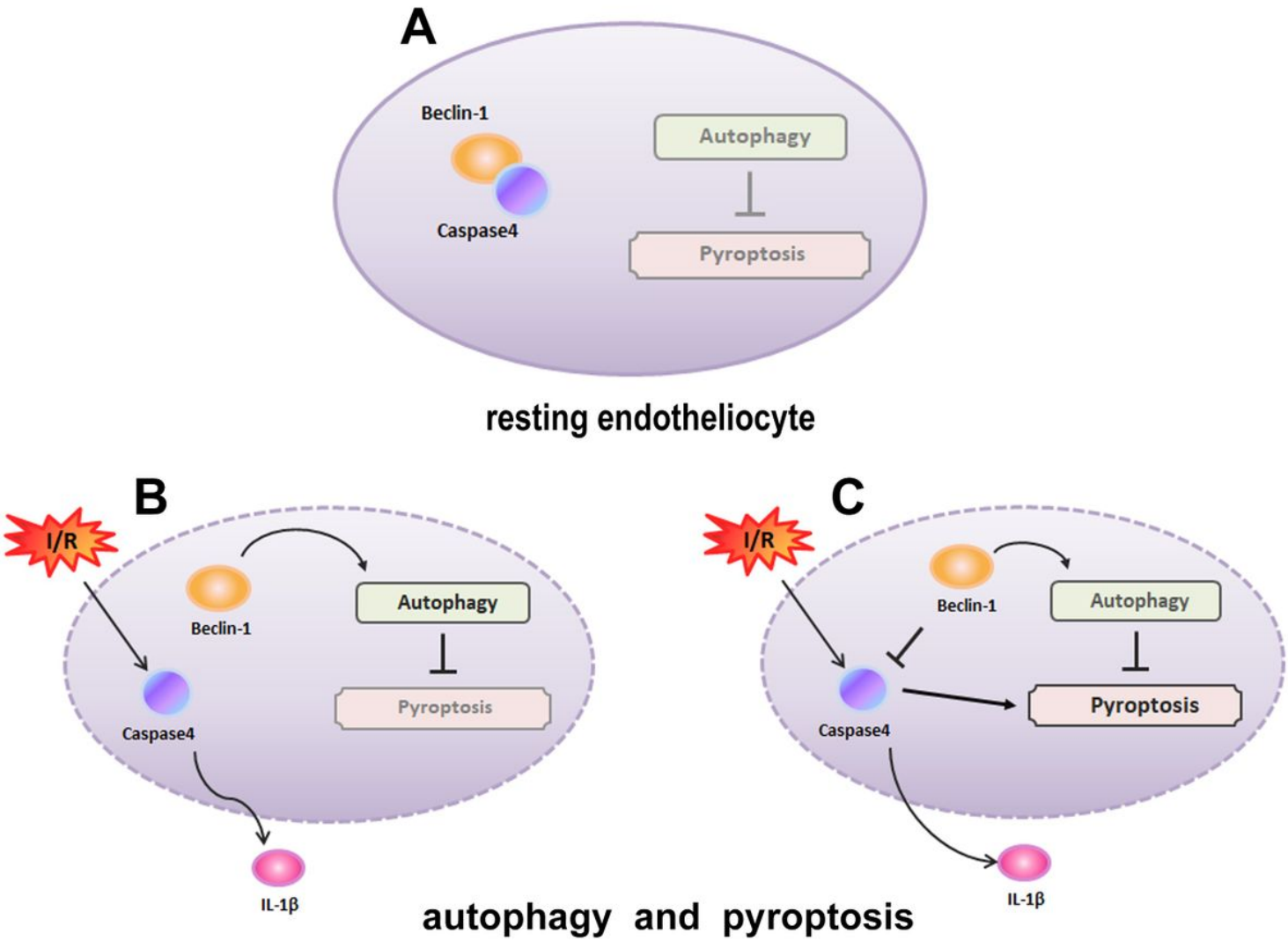

Figure 8

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