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PCSK9 promotes the secretion of pro-inflammatory cytokines by macrophages to aggravate H/R-induced cardiomyocyte injury *via* activating NF-κB signalling

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Abstract. The upregulation of proprotein convertase subtilisin/kexin type 9 (PCSK9) was reported to be involved in regulating the levels of inflammatory markers and apoptosis in macrophages. This study aims to investigate the function and regulation of PCSK9 in myocardial ischaemia. The results of our study showed dramatically increased expression of PCSK9 induced by hypoxia/reoxygenation (H/R) stress rather than by apoptosis in primary murine cardiomyocytes and HL-1 cells. Moreover, PCSK9 promoted H/R-induced pro-inflammatory cytokine release from macrophages, while silencing of PCSK9 inhibited the expression of the pro-inflammatory cytokines TNF-α, IL-6 and IL-1β. Additionally, PCSK9 facilitated the release of pro-inflammatory cytokines from macrophages under H/R conditions, which decreased cardiomyocyte viability and promoted apoptosis of cardiomyocytes. For the underlying mechanisms, we identified PCSK9-induced NF-κB activation as being involved in the cardiomyocyte apoptosis, which was blocked by the NF-κB inhibitor BAY 11-7082. Collectively, this study provides new insights into the therapeutic possibility of regulating PCSK9 in cardiomyocytes for the treatment of ischaemic cardiomyopathy.

Key words: PCSK9 — Cardiomyocytes — Macrophages — Pro-inflammatory cytokines — NF-κB signalling

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FBS, foetal bovine serum; H/R, hypoxia/reoxygenation; I/R, ischaemia/reperfusion; IL-6, interleukin 6; LDLR, low-density lipoprotein receptor; LPS, lipopolysaccharide; MI, myocardial infarction; NF-κB, nuclear factor kappa B; PCSK9, proprotein convertase subtilisin/kexin type 9; TBS, tris-buffered saline; TLR4, toll like receptor 4; TNF-α, tumour necrosis factor alpha; TRAF, TNF-receptor associated factor; TRAIL, tumour necrosis factor-related apoptosis-induced ligand.

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Introduction

Myocardial ischaemia has become one of the most common causes of death and disability in modern society (Berliner et al. 2018). Clinical ischaemic heart disease is frequently accompanied by angina pectoris and myocardial infarction (Choi et al. 2019). A recent study demonstrated that myocardial ischaemia contributed to myocardial necrosis as well as cardiomyocyte apoptosis (Ran et al. 2015). Additionally, it is noteworthy that inflammation in the border zone is a prominent feature of myocardial ischaemia that reflects an early response to injury (Ding et al. 2018). Moreover, emerging evidence suggests that pro-inflammatory cytokines, such as tumour necrosis factor alpha (TNF-α), can result in additional injury to the cardiomyocytes (Nian et al. 2004; Chiong et al. 2011; Xie et al. 2014). Nonetheless, inhibition of inflammatory factors was indicated to diminish ischaemic injury in myocardial ischaemia (Kain et al. 2014). Inflammation has been revealed to be an important step during the process of tissue repair after ischaemic insult. Specifically, the recruitment and local expansion of myeloid cells (especially macrophages) is crucial for cardiac healing after myocardial ischaemia (Lorchner et al. 2015). Although the crosstalk between macrophages and cardiomyocytes has been previously studied in ischaemic heart disease (Frantz and Nahrendorf 2014), the underlying molecular mechanisms responsible for the secretion of pro-inflammatory cytokines by macrophages that facilitates ischaemia-induced cardiomyocyte injury have not been clearly investigated.

Pro-inflammatory cytokines secreted by macrophages can promote apoptosis of cardiomyocytes, which is an important cause of ischaemia-induced cardiomyocyte injury. Once ischaemia/reperfusion injury (I/R) occurs in cardiomyocytes, such as in the case of myocardial infarction (MI), neutrophils and monocytes are immediately recruited to the ischaemic tissue through chemokine/chemokine receptor interaction (Frantz and Nahrendorf 2014). Inflammatory macrophages are strongly associated with I/R-induced injury to cardiomyocytes. Activated macrophages can trigger myeloid differentiation factor 88-dependent or independent activation of E3 ubiquitin ligase (Kuang et al. 2018), which results in activation of nuclear factor kappa B (NF-κB)-mediated secretion of pro-inflammatory cytokines such as interleukin 6 (IL-6), IL-1β and TNF-α (Kuang et al. 2018). Evidence revealed that the elevated levels of pro-inflammatory cytokines in ischaemic cardiomyocytes resulted in acute myocardial dysfunction and, more importantly, apoptosis of myocardial cells (Sun et al. 2004). Therefore, the present study investigated the regulation of macrophage-secreted inflammatory cytokines as well as the underlying molecular pathways during the progression of myocardial ischaemia.

The proprotein convertase subtilisin/kexin type 9 (PCSK9) is encoded by the gene *PCSK9*, and it is synthesized primarily by the liver and undergoes autocatalytic cleavage in the endoplasmic reticulum (Benjannet et al. 2004). PCSK9 is identified as a regulator of cholesterol homeostasis, which plays a crucial role in the degradation of the low-density lipoprotein receptor (LDLR) (Tang et al. 2017). The function of PCSK9 in regulating pro-inflammation mediators was primarily suggested because of the finding that increased LDLR expression was involved in protection against severe sepsis (dos Santos and Marshall 2014). It was also found that circulating PCSK9 regulated the inflammatory responses within the atheroma *via* apoE-dependent and independent pathways (Overton et al. 2007). Notably, the overexpression of PCSK9 in macrophages was reported to be associated with decreased levels of anti-inflammatory markers such as IL-10 and Arg-1, while Ricci et al. (2018) have indicated that the suppression of PCSK9 induces a direct pro-inflammatory response on macrophages. Additionally, gain-of-function mutations in PCSK9 were suggested to be related to higher susceptibility to coronary heart disease, while the silencing of PCSK9 significantly inhibited THP-1-mediated macrophage apoptosis (Liu et al. 2009). Interestingly, a recent study reported that expression of PCSK9 was found to be strong in the myocardium, especially between the infarct area and normal area of the heart in MI (Ding et al. 2018). Given the above-mentioned roles of PCSK9 in other diseases and its impacts on the levels of inflammatory markers and apoptosis in macrophages, it is possible that PCSK9 regulates pro-inflammatory cytokine secretion from macrophages in inflammatory responses that occur during the progression of myocardial ischaemia. However, the effect of PCSK9 on cardiomyocytes as well as the underlying molecular pathway responsible for its regulatory function in myocardial ischaemia remains unclear.

Therefore, the aim of this study was to assess the regulatory role of PCSK9 in the secretion of pro-inflammatory cytokines by macrophages in a hypoxic/reoxygenation environment. Additionally, the underlying signalling pathway responsible for PCSK9-induced cardiomyocyte injury after hypoxia/reoxygenation (H/R) was investigated in the current study. Our results show that PCSK9 was upregulated in HL-1 under H/R conditions and that it subsequently promoted the secretion of pro-inflammatory cytokines by macrophages, which caused further cardiomyocyte injury. Moreover, PCSK9 stimulation after H/R activated NF-κB signalling promoted the secretion of pro-inflammatory cytokines by macrophages and eventually resulted in ischaemic injury of the cardiomyocytes. Taken together, these data suggest a potential therapeutic role for inhibiting the expression of PCSK9 in the treatment of myocardial ischaemia.

Materials and Methods

Ethics statement

All animal experiments were conducted in accordance with the guidelines of Hunan University of Chinese Medicine.

Primary cultures

Primary murine cardiomyocytes were isolated from neonatal C57BL/6J mice purchased from Shanghai Super-B&K Laboratory Animal Corp. Ltd., Shanghai. Mice were anesthetized with isoflurane (5%) in ventilating equipment. Heart tissues were then isolated after lateral thoracotomy. After washed with saline, heart tissues were dissociated with 0.05% trypsin (Gibco, MD, USA) plus 0.08% type II collagenase (Gibco, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, MD, USA) contained 10% foetal bovine serum (FBS, Invitrogen, USA) and 0.1% penicillin-streptomycin for 90 min, at 37°C in a 5% CO_2 incubator. Then, unadherent cells were plated onto 6-well plate at the concentration of 1×10^6 cells/well for the using in the subsequent experiments. For lipopolysaccharide (LPS) treatment, the isolated primary murine cardiomyocytes were stimulated with LPS (10 μg/ml) (Sigma-Aldrich Co, USA) in DMEM for the indicated period of time.

Cell culture and treatment

Mouse cardiomyocyte cell line HL-1 and murine macrophage cell line RAW 264.7 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured in DMEM (Gibco, MD, USA) containing 10% FBS (Invitrogen, USA). Cells were maintained in a humidified atmosphere of 5% CO_2 and 95% O_2 at 37°C. For exposure to H/R conditions, all cells were cultured until they reached 80% confluence and then were transferred to a humidified hypoxic chamber (Thermo, MA, USA) with 94% N_2, 5% CO_2 and 1% O_2 for 4 h. Reoxygenation was established by removing the cell cultures from the hypoxic chamber and returning them to the humidified normoxic atmosphere (95% O_2, 5% CO_2, 37°C) for 1 h. The RAW 264.7 cells were pretreated with anti-TNF-α-neutralizing antibody (1 μg/ml) for 1 h before the exposure to hypoxic conditions. Each independent experiment was carried out in triplicate.

Co-culture experiments

HL-1 cells and RAW 264.7 cells were incubated in a 12-well plate (5×10^5 cells/well). After exposure to normoxia (4 h) or H/R (3 h/1 h), the cell culture media was collected and analysed for cytokine analysis as described below. According to previous methods, HL-1 cells and RAW264.7 cells were mixed in 1:1 ratio (Cambier et al. 2017; Hitscherich et al. 2019). The co-cultured HL-1 cells and RAW 264.7 cells were exposed to normoxia for 4 h or exposed to H/R atmosphere for 3 h/1 h at a density of 2.5×10^5 cells (of each cell type) *per* well for 15 h; then, the cells were washed with PBS before exposure to normoxia (4 h) or H/R (3 h/1 h). The cell culture media was then collected for subsequent cytokine analysis.

Cell transfection

HL-1 cells were transfected with a PCSK9 small interfering RNA (si-PCSK9) or a negative control (si-NC) (GenePharma Co., Ltd, Shanghai, China) using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's protocol. The PCSK9 RNA sequence was found in GenBank (accession No. NM_174936). The si-PCSK9 sense strand was 5'-GGCAGAGACUGAUCCACUUdTdT-3', and the antisense strand was 3'-dTdTCCGUCU CUGACUAGGUGAA-5'; the si-NC was provided by the same company. The transfection efficiency was determined using qRT-PCR after 48 h of incubation.

RNA extraction and qRT-PCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, CA, USA) following the manufacturer's instructions. An ultraviolet spectrophotometer (Eppendorf, Hamburg, German) was used to determine the concentration of total RNA. Based on the gene sequence source in the GenBank database, primers were designed as follows: PCSK9 = 5'-TTGCAGCAGCTGGGAACTT-3'-forward, 5'-CCGACTGATGACCTCTGGA-3'-reverse; IL-6 = 5'-AGGATACCACTCCCAACAGACCT-3'-forward, 5'-CAAGTGCATCATCGTTGTTCATAC-3'-reverse; IL-1β = 5'-CAAATCTCGCAGCAGCACA-3'-forward, 5'-TCATGTCCTCATCCTGGAAGG-3'-reverse;TNF-α = 5'-CTTCTCCTTCCTGATCGTGG-3'-forward, 5'-GCTGGTTATCTCTCAGCTCCA-3'-reverse; and GAPDH = 5'-GGGTCTTTGCAGCGTATGG-3'-forward, 5'-ACCTCCTGTTTCTGGGGACT-3'-reverse.

cDNA was synthesized using two micrograms of RNA with MMLV Reverse Transcriptase Kit (Takara, China) according to the manufacturer's protocol. Quantitative real-time PCR was performed using SYBR Green PCR Master Mix (Toyobo, Japan) on a Mastercycler ep Realplex (Eppendorf, Germany). GAPDH was used as an internal reference to normalize the relative abundance of mRNA. Then, the relative expression levels were calculated using $2^{-\Delta\Delta CT}$ method. All experiments were performed in triplicate.

MTT assay

The viability of HL-1 cells was measured with the MTT assay (Beyotime, Shanghai, China). HL-1 cells transfected with si-PCSK9 or si-NC were co-cultured in a 96-well plate with RAW 264.7 macrophages. The supernatants were then collected as culture media for maintaining HL-1 cells. After incubation under normoxic and H/R conditions for the indicated times, MTT (5 mg/ml, Sigma) was added to each well. Cells were maintained for an extra 4 h before 100 μl dimethyl sulfoxide (Sigma, MO, USA) was added *per* well

of the plate. Absorbance was measured at a wavelength of 490 nm using a microplate reader (Bio-Tek, VA, USA). All experiments were performed in triplicate.

Flow cytometry analysis

Flow cytometry analysis of the apoptotic rate was measured in HL-1 cells after the following treatments: transfection with si-PCSK9, co-culture with RAW 246.7 macrophages, after treatment with hPCSK9 (BPS Biosciences, CA, USA) and/or BAY11-7082 (Beyotime, Shanghai, China) under normoxic and H/R conditions. Apoptosis was detected using an Annexin V FITC apoptosis kit (KeyGenBioTECH, KGA107). The cells were digested with 2.5% trypsin followed by centrifugation at 1000 r.p.m. for 5 min. After discarding the supernatant, a single cell suspension at a density of 1×10^6 cells/ml was prepared in a PBS solution containing calcium. A 100 μl cell suspension was added to the test tube supplemented with propidium iodide and RNase A (both at 10 mg/ml), followed by incubation at 4°C for 30 min. The cells were immediately analysed using flow cytometry (Becton, New Jersey, USA) after adding 400 μl staining buffer. Annexin V-positive cells were determined to be apoptotic cells.

ELISA analysis

The supernatants from cell cultures were collected following the establishment of the normoxia and H/R model. The supernatants were centrifuged before being analysed with commercially obtained ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The experiments were conducted and analysed in triplicate.

Western blot analysis

Total protein was prepared with Radio Immunoprecipitation Assay (RIPA, Beyotime, Shanghai, China), and the concentration was determined using a BCA protein assay kit (Thermo Scientific, MA, USA). Equivalent amounts of protein were separated on by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis before transferring them to a polyvinylidene fluoride membrane (Millipore, MA). Five percent non-fat milk in Tris-buffered saline (TBS) supplemented with 0.1% Tween-20 (TBST) was used to block the membrane at room temperature for 2 h, followed by incubation at 4°C overnight with the following primary antibodies: phospho-p65 (1:1000, 8242, CST), phospho-IκBα (1:1000, 2859, CST), and phospho-IKKα/β (1:1000, 2697, CST). β-actin (1:1000, ab49900, Abcam) was used as an internal reference. TBST was used to wash the membrane three times, and the membrane was incubated with a goat anti-rabbit IgG antibody (1:10000 dilution, #7054, Cell Signaling Technology, MA, USA) linked to horseradish peroxidase at room temperature for 2 h. The signal was detected using enhanced chemiluminescence reagent (ECL, CA, USA) and visualized using a Bio-Rad Gel Doc EZ imaging system (Gel Doc EZ Imager, CA, USA). ImageJ software was applied to analyse the signals for the immunoreactive bands.

Statistics

Statistical analysis was performed using SPSS 21.0 statistical software (SPSS, Illinois, USA). All data are represented as the mean ± standard deviation (SD). A single comparison between two independent groups was performed with an unpaired, two-tailed Student's *t*-test. Differences among multiple groups were analysed by Tukey post hoc analysis of variance (ANOVA) test. Statistical significance was determined when $p < 0.05$.

Results

H/R induced an increase in PCSK9 produced by cardiomyocytes

The mRNA and protein level of PCSK9 in primary murine cardiomyocytes were measured by qRT-PCR and ELISA. Primary murine cardiomyocytes were exposed to normoxia or H/R as illustrated in Fig. 1A–C, mRNA and protein level of PCSK9 significantly increased in H/R-exposed group compared with that in normoxia-exposed group. To confirm whether the elevated PCSK9 expression was caused by apoptosis in cardiomyocytes or not, primary murine cardiomyocytes were stimulated by LPS. As it was shown in Fig. 1D, apoptosis was induced by LPS stimulation compared with control. Fig. 1E–F demonstrated that no significant difference on PCSK9 expression was observed between the LPS stimulated group and control. The results suggested that H/R stimulated PCSK9 secretion in primary murine cardiomyocyte.

Cardiomyocytes enhanced the secretion of pro-inflammatory cytokines by macrophages in a co-culture system under H/R conditions

RAW 264.7 and HL-1 cells were exposed to H/R or normoxic conditions in separate media or in mixed co-culture. Subsequently, qRT-PCR and ELISA were performed to detect the mRNA and protein levels of pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β), respectively. As shown in Fig. 2A–F, in the HL-1 and RAW 264.7 co-culture system, the mRNA and protein levels of TNF-α, IL-6 and IL-1β were increased dramatically compared to those of single HL-1 or RAW

264.7 cultures. Whilst in RAW264.7 or HL-1 separate media, increase in both mRNA and protein levels of TNF-α, IL-6 and IL-1β can be observed, in H/R-exposed group compared with normoxia-exposed group, this increase in co-culture system was more significant. The results suggested that H/R stimulation, along with the co-culture of cardiomyocytes and macrophages, contributed to the elevated secretion of pro-inflammatory cytokines.

PCSK9 produced by cardiomyocytes enhanced pro-inflammatory cytokine secretion by H/R-induced macrophages

To determine the effects of PCSK9 on the secretion of pro-inflammatory cytokines in RAW 264.7 cells, non-transfected HL-1 cells, HL-1 cells transfected with a PCSK9 siRNA (si-PCSK9 HL-1) or siRNA negative control (si-NC HL-1) were co-cultured with RAW 264.7 cells under H/R conditions. Firstly, the efficiency of transfection was confirmed by qRT-PCR (Fig. 3A), the results of which indicated that PCSK9 was silenced by si-PCSK9. Further, the mRNA levels of pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) were measured by qRT-PCR. The results showed that the expression of pro-inflammatory cytokines was significantly decreased in RAW 264.7 cells co-cultured with si-PCSK9-transfected HL-1 cells compared to RAW 264.7 cells co-cultured with si-NC-transfected HL-1 cells under H/R conditions (Fig. 3B). In line with the mRNA expression trends, the protein levels of the above-mentioned cytokines were observed and these proteins were dramatically downregulated in the co-culture

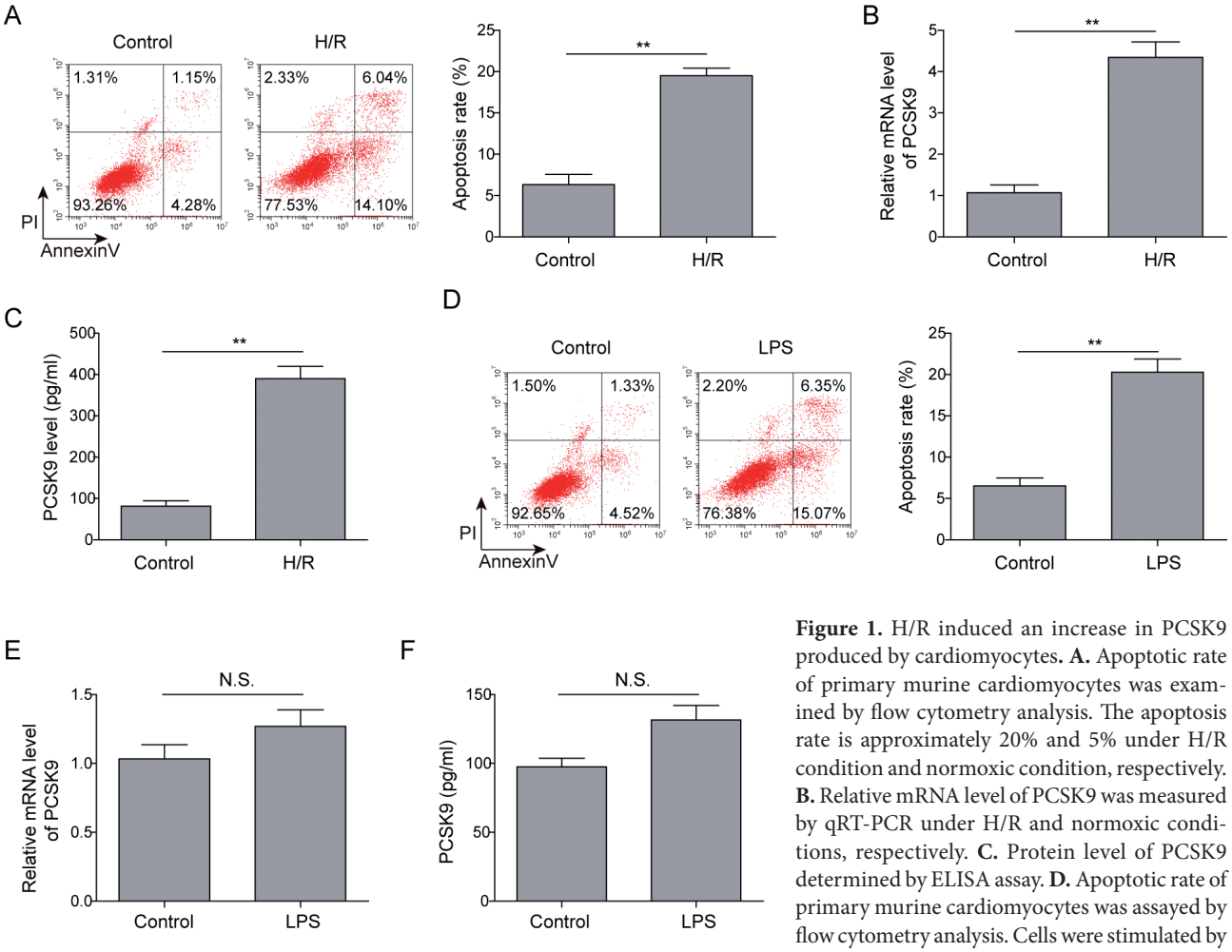

Figure 1. H/R induced an increase in PCSK9 produced by cardiomyocytes**. A.** Apoptotic rate of primary murine cardiomyocytes was examined by flow cytometry analysis. The apoptosis rate is approximately 20% and 5% under H/R condition and normoxic condition, respectively. **B.** Relative mRNA level of PCSK9 was measured by qRT-PCR under H/R and normoxic conditions, respectively. **C.** Protein level of PCSK9 determined by ELISA assay. **D.** Apoptotic rate of primary murine cardiomyocytes was assayed by flow cytometry analysis. Cells were stimulated by LPS (10 μg/ml) or cultured in regular media. **E.** Relative mRNA level of PCSK9 was measured by qRT-PCR under LPS stimulation or regular culture. **F.** Protein level of PCSK9 determined by ELISA assay. Each experiment was repeated three times. The results are presented as the mean ± SD. ** $p < 0.01$. Control, primary murine cardiomyocytes cultured in DMEM medium; H/R, hypoxia/reoxygenation-exposed primary murine cardiomyocytes; LPS, primary murine cardiomyocytes cultured in DMEM medium treatment with LPS (10 μg/ml). PI, propidium iodide; N.S., non-significant.

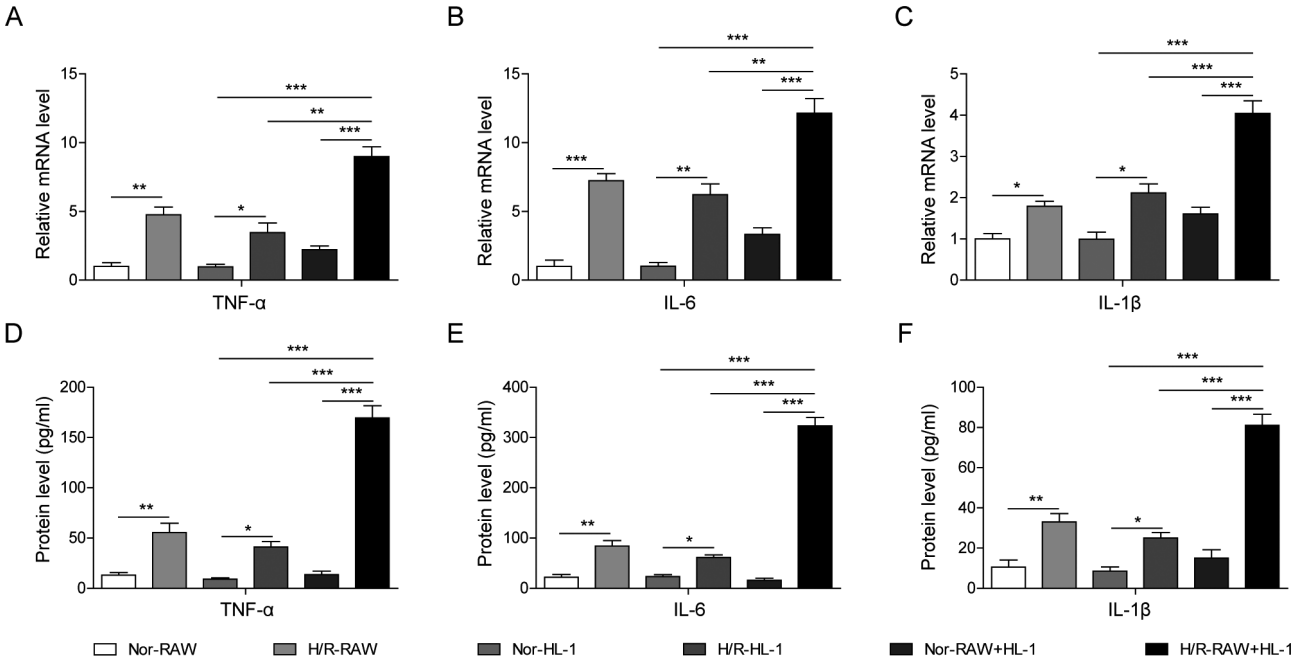

Figure 2. Cardiomyocytes promoted the secretion of pro-inflammatory cytokines from macrophages in the co-culture system under H/R conditions. Relative mRNA levels of TNF-α (**A**), IL-6 (**B**), and IL-1β (**C**) were measured by qRT-PCR. Protein levels of TNF-α (**D**), IL-6 (**E**), and IL-1β (**F**) were determined by ELISA. Each experiment was repeated three times. The results are presented as the mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Nor-RAW, normoxia-exposed RAW 264.7; H/R-RAW, H/R-exposed RAW 264.7; Nor-HL-1, normoxia-exposed HL-1 cells; H/R-HL-1, H/R-exposed HL-1 cells; Nor-RAW+HL-1, co-culture of normoxia-exposed RAW 264.7 and HL-1 cells; H/R-RAW+HL-1, co-culture of H/R-exposed RAW 264.7 and HL-1 cells; H/R, hypoxia/reoxygenation.

system where HL-1 was transfected with si-PCSK9 after H/R (Fig. 3C) compared with the values from the si-NC HL-1.

PCSK9 produced by cardiomyocytes promoted H/R-induced cardiomyocyte injury in co-culture system

To evaluate the viability and apoptosis of HL-1 cardiomyocytes, the supernatants from the previous co-culture experiments (non-transfected HL-1, si-NC HL-1, si-PCSK9 HL-1) were collected to culture HL-1 cardiomyocytes under normoxic or H/R conditions. The results of the MTT assay demonstrate that the viability of HL-1 cells was significantly attenuated when they were maintained in co-culture media from H/R groups (Fig. 4A). The viability of HL-1 cells was partially enhanced when cultured in media from RAW 264.7 cells co-culture with si-PCSK9 HL-1 cells with in comparison with media from the parental HL-1 and RAW 264.7 cell co-cultured (Fig. 4A). Additionally, apoptosis of HL-1 cardiomyocytes was detected by flow cytometry, as shown in Fig. 4B and the apoptotic rate was quantitatively expressed in Fig. 4C. To note, in the co-culture media, the silencing of PCSK9 in HL-1 was significantly associated with the lower apoptotic rate of HL-1 cells (H/R-HL-1+si-PCSK9 HL-1) (approximately 35%) compared to HL-1 transfected with si-NC (H/R-HL-1+si-NC HL-1) (approximately 48%) under H/R condition (Fig. 4C). These data suggested that PCSK9 knockdown in cardiomyocytes can decrease pro-inflammatory cytokines and alleviate cardiomyocyte injury in cardiomyocytes and macrophages co-culture system.

PCSK9 activated NF-κB signalling to promote the secretion of pro-inflammatory cytokines in macrophages

The RAW 264.7 macrophages that underwent exposure to H/R were treated with human recombinant PCSK9 (hPCSK9) and/or BAY 11-7082 (NF-κB inhibitor) to assess the impacts of PCSK9 on the NF-κB signalling pathway. According to the results of Western blot in Fig. 5A, the hPCSK9 treatment significantly upregulated NF-κB signalling-associated proteins (p-p65, p-IκBα, p-IKKα/β), but they were decreased following treatment with the BAY11-7082. Further, the mRNA levels of pro-inflammatory cytokines were measured by qRT-PCR, as shown in Fig. 5B. The mRNA expression levels of TNF-α, IL-6 and IL-1β were significantly increased in RAW 264.7 cells treated with hPCSK9 alone, and they were dramatically decreased in RAW 264.7 cells treated with hPCSK7 plus BAY11-7082. In addition, the protein levels of TNF-α, IL-6 and IL-1β were upregulated

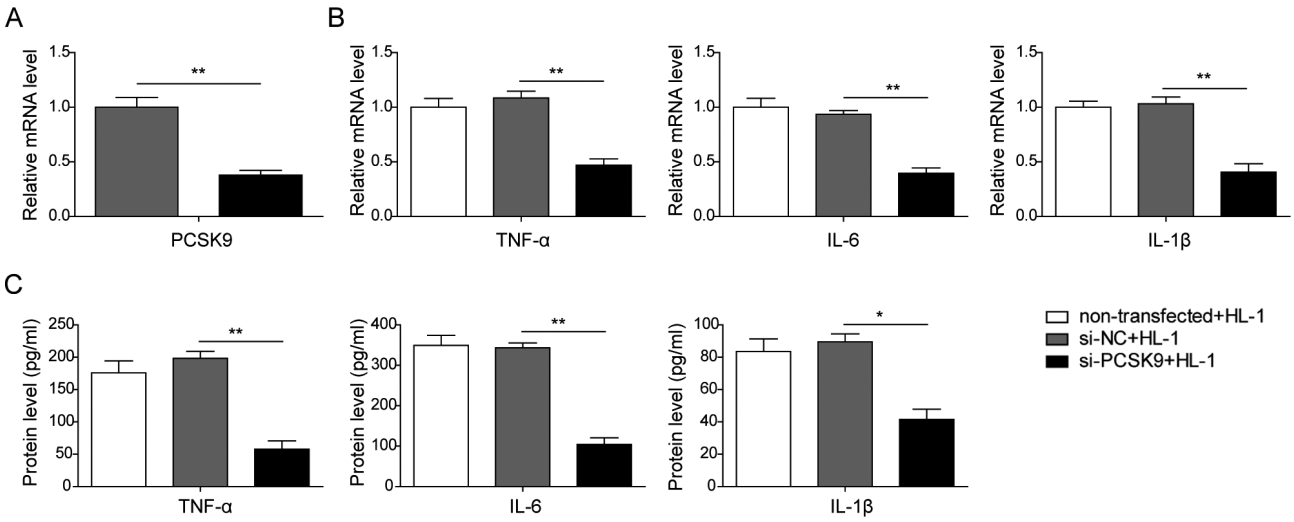

Figure 3. PCSK9 produced by cardiomyocytes enhanced pro-inflammatory cytokine secretion by H/R-induced macrophages. **A.** The efficiency of si-PCSK9 transfection was validated by measuring the relative mRNA level of PCSK9 in HL-1 cell lines by qRT-PCR. **B.** Relative mRNA expression levels of TNF-α, IL-6, and IL-1β in the HL-1 cells of the co-culture system were measured by qRT-PCR under H/R condition. **C.** Protein levels of TNF-α, IL-6, and IL-1β in the HL-1 cells of the co-culture system under H/R condition were determined by ELISA. Each experiment was repeated three times. The results are presented as the mean ± SD. * $p < 0.05$; ** $p < 0.01$. non-transfected HL-1, co-culture of H/R-exposed RAW 264.7 and non-transfected HL-1; si-NC HL-1, co-culture of H/R-exposed RAW 264.7 and HL-1 transfected with si-NC; si-PCSK9 HL-1, co-culture of H/R-exposed RAW 264.7 and HL-1 transfected with si-PCSK9.

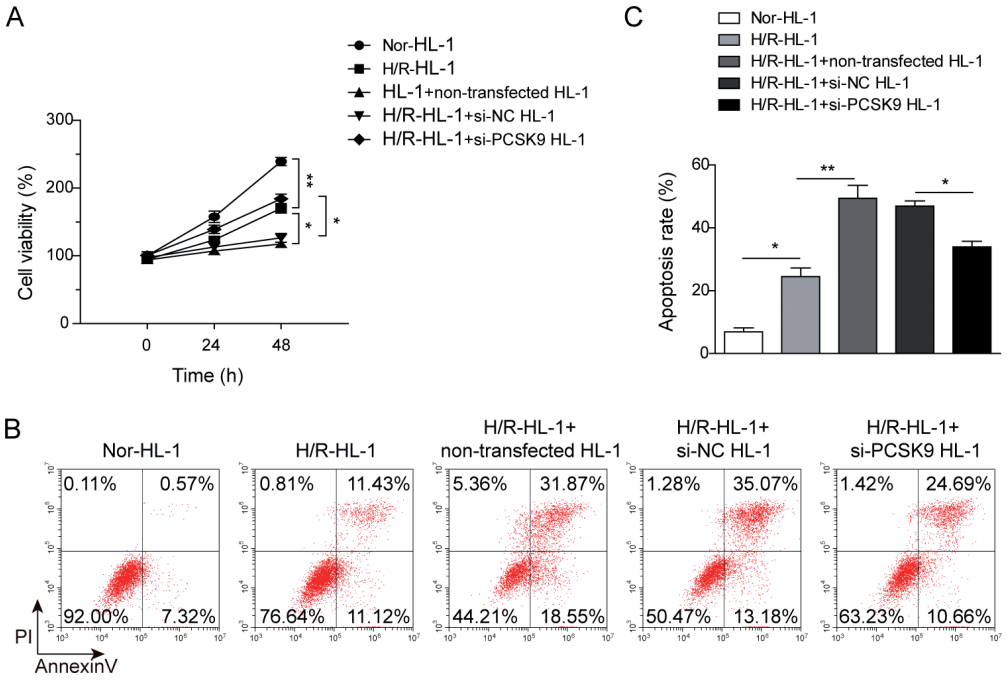

Figure 4. PCSK9 produced by cardiomyocytes promoted pro-inflammatory cytokine secretion of macrophages to make the H/R-induced cardiomyocyte injury worse. **A.** Viability of HL-1 cells was assessed after 0 h, 24 h and 48 h under normoxic or H/R conditions by MTT assay. **B.** The apoptotic rate of HL-1 cells was assayed by flow cytometry analysis. The apoptosis rates were approximately 8, 23, 50, 48 and 35%. **C.** Quantitative analysis of the apoptotic rate in HL-1 cells is presented in the histogram. Each experiment was repeated three times. The results are presented as the mean ± SD. * $p < 0.05$, ** $p < 0.01$. Nor-HL-1, normoxia-exposed HL-1 cells; H/R-HL-1, H/R-exposed HL-1 cells; H/R-HL-1+non-transfected HL-1, H/R-exposed HL-1 cells cultured in the supernatant collected from the co-culture of H/R-exposed non-transfected HL-1 and RAW264 cells; H/R-HL-1+si-NC HL-1, H/R-exposed HL-1 cells cultured in the supernatant collected from the co-culture of H/R-exposed HL-1-transfected with si-NC and RAW264 cells; H/R-HL-1+si-PCSK9 HL-1, H/R-exposed HL-1 cells cultured in the supernatant collected from the co-culture of H/R-exposed HL-1-transfected with si-PCSK9 and RAW264 cells.

following treatment of RAW 264.7 cells with hPCSK9 alone compared with H/R-RAW group, while the treatment of BAY 11-7082 reversed this effect (Fig. 5C). The results indicated that PCSK9 can promote secretion of pro-inflammatory cytokines by macrophages after H/R stimulation *via* activating NF-κB signalling.

PCSK9 activated NF-κB signalling to promote macrophage inflammatory cytokine secretion, which made the cardiomyocyte injury worse after H/R

HL-1 cells were maintained in the supernatants collected from the previous experiments (H/R-RAW, H/R-RAW+hPCSK9, H/R-RAW+hPCSK9+BAY) or were cultured alone under normoxic or H/R conditions to measure the impacts of PCSK9 on H/R-induced cardiomyocyte injury. According to the results of the MTT assay (Fig. 6A), the viability of HL-1 cells was significantly attenuated in the H/R-HL-1+H/R-RAW+hPCSK group compared with the H/R-HL-1+H/R-RAW group, while the administration of BAY 11-7082 reversed this inhibitory effect of hPCSK9. Furthermore, the apoptotic rate of HL-1 cells was determined based on flow cytometry analysis. The results shown in Fig. 6B–C demonstrate that there was a significant increase in apoptosis in the H/R-HL-1+H/R-RAW+hPCSK9 group (approximately 53.27%) compared with the H/R-HL-1+H/R-RAW group (28.31%). The administration of BAY 11-7082 significantly reversed the effect of hPCSK9 on apoptosis in HL-1 cells (approximately 35%). Collectively, it was indicated that PCSK9 can enhance the H/R-induced cardiomyocyte injury and this could be associated with the increased secretion of pro-inflammatory cytokines by macrophages *via* the activation of NF-κB signalling combining the previous findings from Figure 5.

Discussion

Cardiac cell death is associated with various cardiac diseases, such as MI, angina pectoris and I/R (Chiong et al. 2011). MI contributes to cardiomyocyte apoptosis, which is an important type of cell death. Hence, studying protective pathways against ischaemia-induced cell apoptosis and inflammation injury is crucial for the treatment of myocardial ischaemia. The current study demonstrated that PCSK9 was highly expressed in H/R-stimulated primary murine

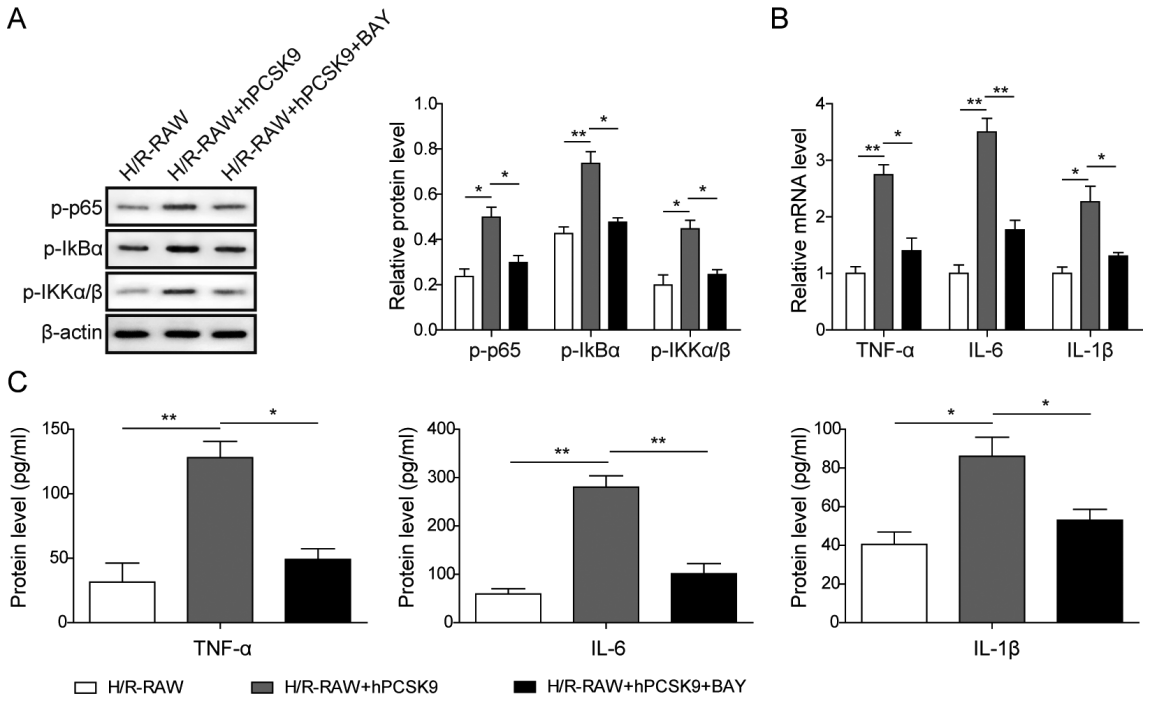

Figure 5. PCSK9 activated NF-κB signalling to promote the secretion of pro-inflammatory cytokines in macrophages. **A.** Protein levels of p-p65, p-IκBα, p-IKKα/β in RAW 264.7 cells under H/R conditions were measured by Western blot. **B.** Relative mRNA levels of TNF-α, IL-6, and IL-1β in RAW 264.7 cells were measured by qRT-PCR. **C.** Protein levels of TNF-α, IL-6, and IL-1β in RAW 264.7 were determined by ELISA. Each experiment was repeated three times. The results are presented as the mean ± SD. * $p < 0.05$, ** $p < 0.01$. H/R-RAW, H/R-exposed RAW 264.7; H/R-RAW+hPCSK9, H/R-exposed RAW 264.7 cells treated with hPCSK9; H/R-RAW+hPCSK9+BAY, H/R-exposed RAW 264.7 cells treated with hPCSK9 and BAY 11-7082.

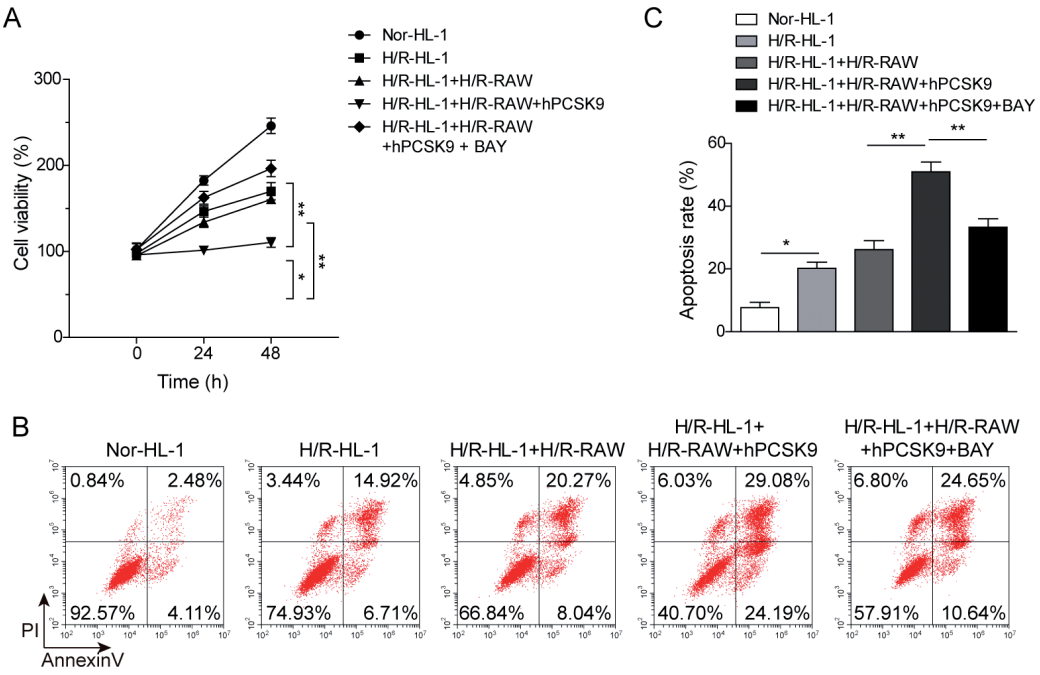

Figure 6. PCSK9 activated NF-κB signalling to promote macrophage inflammatory cytokine secretion and enhanced cardiomyocyte injury after H/R. **A.** Cell viability of HL-1 cells under normoxic or H/R conditions was measured at the time points of 0, 24 and 48 h. **B.** The apoptotic rate of HL-1 cells was assayed by flow cytometry analysis. The apoptosis rates were 6.59, 21.63, 28.31, 53.27 and 35.29%. **C.** Quantitative analysis of the apoptotic rate in HL-1 cells is presented in the histogram. Each experiment was repeated three times. The results are presented as the mean ± SD. * $p < 0.05$, ** $p < 0.01$. Nor-HL-1, normoxia-exposed HL-1 cells; H/R-HL-1, H/R-exposed HL-1 cells; H/R-HL-1+ H/R-RAW, H/R-exposed HL-1 cells cultured in the supernatant collected from H/R-exposed RAW 264.7 cells; H/R-HL-1+H/R-RAW+hPCSK9, H/R-exposed HL-1 cells cultured in the supernatant collected from H/R-exposed RAW 264.7 cells treated with hPCSK9; H/R-HL-1+H/R-RAW+hPCSK9+BAY, H/R-exposed HL-1 cells cultured in the supernatant collected from H/R-exposed RAW 264.7 cells treated with hPCSK9 and BAY 11-7082.

cardiomyocytes. The co-culture of cardiomyocytes and macrophages under H/R conditions promoted the secretion of pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β). We revealed that PCSK9 produced by cardiomyocytes enhanced the secretion of pro-inflammatory cytokines by H/R-stimulated macrophages, which made the cardiomyocyte injury worse. Moreover, analysis of the underlying molecular pathways indicated that PCSK9 activated NF-κB signalling to promote pro-inflammatory cytokine secretion by macrophages and thus made the H/R-induced injury in cardiomyocytes worse.

PCSK9 was previously demonstrated to be intensively expressed in the ischaemic region of the heart (Ding et al. 2018). In line with the findings of previous study, the current study found that PCSK9 was significantly upregulated in H/R-stimulated primary murine cardiomyocytes compared with cells grown in normoxic environments. Furthermore, our finding excluded the impact of apoptosis on inducing the upregulation of PCSK9 in primary cardiomyocytes as Figure 1 indicated. Macrophages have been previously reported to make pulmonary I/R injuries worse by secreting the pro-inflammatory cytokine TNF-α (Sharma et al. 2007). Similarly, we reported in this study that the secretion of pro-inflammatory cytokines (TNF-α, IL-6, IL-1β) by macrophages was a distinct result observed in cardiomyocytes after H/R stimulation, and this was increased further by overexpression of PCSK9 in cardiomyocytes. Our finding is also consistent with a previous study in which PCSK9 was suggested to promote a significant increase in the release of pro-inflammatory cytokines, including TNF-α, IL-6, IL-1β, C-X-C motif ligand 2 and Monocyte Chemoattractant Protein-1, from macrophages implicated in atherogenesis (Ricci et al. 2018). Further, the present study demonstrated that pro-inflammatory cytokines secreted by RAW 264.7 cells attenuated cell viability and promoted apoptosis in H/R-induced HL-1 cells. This result is also consistent with the previous report that inflammatory macrophages produce cytokines, such as TNF-α, that initiate the intrinsic cell death pathway in cardiomyocytes and thus induce cardiomyocyte apoptosis (Haudek et al. 2007; Frantz and Nahrendorf 2014). Evidence shows different subsets of macrophages in the myocardium may exert opposite functions. The resident macrophage is believed to play a critical role in tissue recovery by inducing cardiomyocyte proliferation and promoting angiogenesis in response to injury (Duffield et al. 2005; Strassheim et al. 2019); whilst $CCR2^{+}$ monocyte-derived cell is the predominant subset of macrophage that leads to early pro-inflammatory response in heart fol-

lowed by cardiac injury (Epelman et al. 2014). This study was mainly focused on exploring the effects of PCSK9 on monocyte-derived macrophages from the aspect of secreting pro-inflammatory cytokines after ischemic injury. The protective roles of macrophages in primary cardiomyocyte would be further explored in future study. Collectively, our findings suggested that PCSK9 produced by cardiomyocytes promoted pro-inflammatory cytokine released from cardiac macrophages to make H/R-induced cardiomyocyte injuries worse under H/R conditions.

The current experiments that analysed HL-1 cell viability and apoptosis revealed that enhanced cardiomyocyte injury was caused by pro-inflammatory cytokine release from macrophages through PCSK9-induced activation of NF-κB signalling. This finding supported previous research that reported that PCSK9 activated the Toll Like Receptor (TLR4)/NF-κB pathway in RAW 264.7 macrophages in a mouse atherosclerotic inflammation model (Tang et al. 2017). NF-κB refers to a family of five transcription factors: RelA (p65), RelB, c-Rel, NF-κB1 (p105/p50) and NF-κB2 (p100/p52) (Paul et al. 2018). In normal cells, NF-κB signalling has crucial roles in inflammation, immune system control and apoptosis. Importantly, NF-κB signalling is overexpressed at sites of inflammation in diverse human diseases and can play a central role in inducing the transcription of pro-inflammatory cytokines such as IL-1, IL-6, IL-8 and TNF-α. Usually, extracellular ligands such as the TNF-receptor associated factor (TRAF) family members, can initiate DNA damage responses that result in the dissociation of DNA-binding protein dimers from IκB molecules and initiate the activation of NF-κB signalling (Paul et al. 2018). Additionally, tumour necrosis factor-related apoptosis-induced ligand (TRAIL) expressed by immune cells can result in the activation of NF-κB through recruiting TRAF, receptor interacting protein, TNFR1-associated death domain protein and NF-κB essential modulator (Kimberley and Screaton 2004). It was shown that TRAIL-induced NF-κB signalling could mediate the secretion of macrophage cytokines (IL-1β, TNF-α) as well as chemotactic factors (C-C Motif Chemokine Ligand 2, Intercellular cell adhesion molecule-1, intercellular cell adhesion molecule) following H/R stimulation in rats, and this process was regulated by ER stress (Jiang et al. 2017). Further in human monocytes, it was also reported that the most common activated form of NF-κB, p65, can trigger activation of inflammatory gene (IL-1, TNF-α) expression (Tak and Firestein 2001). In line with the previous findings, the present study confirmed that the activation of the NF-κB pathway promoted the secretion of the pro-inflammatory cytokines TNF-α, IL-6 and IL-1β by macrophages under H/R stress. Moreover, our results demonstrated that PCSK9 can promote secretion of these pro-inflammatory cytokines by macrophages after H/R stimulation *via* activating NF-κB signalling. This finding was also supported by a previous study in which a novel function of PCSK9 is to promote inflammation in atherosclerosis through the TLR4/NF-κB pathway (Tang et al. 2017).

In summary, our present data suggested that PCSK9 was upregulated in cardiomyocytes under H/R stress. Furthermore, PCSK9 facilitated pro-inflammatory cytokine release from macrophages *via* activating NF-κB signalling, and it contributed to H/R-induced cardiomyocyte injury. Nevertheless, limitations exist in the current study. The study was performed in an in vitro environment instead of an *in vivo* model; hence, the complicated interplay between cardiomyocytes, macrophages and other inflammatory cells in a real clinical setting of myocardial ischaemia cannot be fully addressed here. In light of the previous studies that mainly focused on the secretion of pro-inflammatory cytokines by macrophages in cardiomyocytes after ischemic injury, this study thus did not examine the expression of anti-inflammatory cytokines such as IL-10 under H/R condition, which worth investigation in the future study (Tak and Firestein 2001; Sharma et al. 2007; Tang et al. 2017; Ricci et al. 2018). Additionally, even though human PCSK9 was used in the experiment to make it more relevant to human conditions, future studies should be performed in human cell lines. Generally, this study provides new insights into the role of PCSK9 inhibition in cardiomyocytes as a therapeutic target for the treatment of ischaemic cardiomyopathy.

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Authors contributions. CLY conceived and YDZ designed the experiments. ZXH guaranteed the integrity of the research and made clinical studies. HL defined intellectual content, YDZ performed literature research, CLY performed data analysis and HL contributed statistical analysis. CLY and YDZ were responsible for the editing of the manuscript. All authors read and approved the final manuscript. HL revised and validated the manuscript.

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