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Chronic cold stress-induced myocardial injury: effects on oxidative stress, inflammation and pyroptosis

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ABSTRACT

Background: Hypothermia is a crucial environmental factor that elevates the risk of cardiovascular disease, but the underlying effect is unclear.
Objectives: This study examined the role of cold stress (CS) in cardiac injury and its underlying mechanisms.
Methods: In this study, a chronic CS-induced myocardial injury model was used; mice were subjected to chronic CS (4°C) for three hours per day for three weeks.
Results: CS could result in myocardial injury by inducing the levels of heat shock proteins 70 (HSP70), enhancing the generation of creatine phosphokinase-isoenzyme (CKMB) and malondialdehyde (MDA), increasing the contents of tumor necrosis factor-α (TNF-α), high mobility group box 1 (HMGB1) interleukin1b (IL-1β), IL-18, IL-6, and triggering the depletion of superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH). Multiple signaling pathways were activated by cold exposure, including pyroptosis-associated NOD-like receptor 3 (NLRP3)-regulated caspase-1-dependent/Gasdermin D (GSDMD), inflammation-related toll-like receptor 4 (TLR4)/myeloid differentiation factor 88 (MyD88)-mediated nuclear factor kappa B (NF-κB), and mitogen-activated protein kinase (MAPK), as well as oxidative stress-involved thioredoxin-1/thioredoxin-interacting protein (Txnip) signaling pathways, which play a pivotal role in myocardial injury resulting from hypothermia.
Conclusions: These findings provide new insights into the increased risk of cardiovascular disease at extremely low temperatures.

Keywords: Hypothermia; myocardial injury; pyroptosis; inflammation; Signal Transduction

INTRODUCTION

The environmental temperature is an essential factor that contributes to high levels of death and disease that have raised public health concerns [1]. Cold temperatures are largely responsible for the current burden of total mortality in many areas [2]. As a common stressor in livestock, cold stress (CS) hampers animal husbandry in cold climates, which affects the neuroendocrine, reproductive, and cardiovascular systems in animals [3]. Cold-induced

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Conflict of Interest
The authors declare no conflicts of interest.

Data Availability Statement
The original data analyzed during the current study are available from the corresponding author upon reasonable request.

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cardiovascular risks, such as blood stickiness, arrhythmia, and blood pressure, are common in the general population [4,5]. It is unclear whether cardiovascular disease morbidity and mortality are associated with extreme cold weather [6]. Accordingly, it is crucial to investigate the effects of CS on cardiac injury.

Increasing evidence suggests that chronic cold exposure can promote autophagy, inflammatory responses, and oxidative stress in different organs, including the heart [7-9]. However, few studies have examined CS and myocardial pyroptosis. Pyroptosis is a sort of regulated necrotic cell death characterized by the formation of pores in the cell membrane leading to cell enlargement, cell lysis, and the release of interleukin (IL)-1β and IL-18 [10]. Pyroptosis occurs through two typical pathways: the caspase-1-dependent canonical pathway, and the caspase-4/5/11-dependent noncanonical pathway [11,12]. Both caspase-1/11 (or caspase-4/5) are processed by protein hydrolysis Gasdermin D (GSDMD), which produces an N-terminal fragment (GSDMD-N) that opens a pore in the plasma membrane, resulting in serious inflammation reaction and pyroptosis [13]. Accumulating evidence shows that the occurrence of pyroptosis is nearly associated with the activation of the nucleotide-binding oligomerization domain (NOD)-like receptors protein 3 (NLRP3) [14]. The NLRP3 inflammasome recruits and activates caspase-1 either directly or through the adaptor protein ASC (apoptosis-associated speckle protein), which is then stimulated by pro-IL-1β/18 and cleaved by Gasdermin D (GSDMD), leading to severe inflammatory responses and pyroptosis [15].

More importantly, NLRP3 inflammasome activation is involved in multiple signaling pathways, including toll-like receptor 4 (TLR4)/nuclear factor-κB (NF-κB) and thioredoxin-1/thioredoxin-interacting protein (Txnip) [16,17]. Thioredoxin-1 (Trx-1), an essential anti-inflammatory and antioxidant protein, has been reported to bind to Txnip under basal conditions, while the Trx-1/Txnip complex dissociates under stress conditions, and Txnip binds NLRP3 to activate NLRP3 inflammasome [18,19]. On the other hand, TLR4 is one of the important pattern recognition receptors (PRRs) in the TLR family, which play a crucial role in various inflammatory diseases [20]. TLR4 can stimulate the NLRP3 inflammasome by recruiting myeloid differentiation factor 88 (MyD88) adaptors to activate NF-κB [20,21]. Previous research has shown that chronic and acute CS could induce the liver and hippocampus in mice via the activation of TLR4/MyD88/NF-κB and NLRP3 inflammasome signaling pathway [22,23]. Nevertheless, it is unclear whether chronic CS causes myocardial damage by altering pyroptosis in the NLRP3-related pathway. The current study examined the following: (i) pyroptosis and inflammation under prolonged CS in mouse cardiac tissues and (ii) the effects of pyroptosis and the inflammation-related NLRP3 inflammasome, Trx-1/Txnip, and TLR4/MyD88 signaling pathway.

MATERIALS AND METHODS

Reagents

The primary antibodies of HSP70, HSP90, TLR4, MyD88, NLRP3, ASC, caspase-1, caspase-11, IL-18, IL-1β, HMGB1, COX-2, iNOS, and caspase-3 were supplied by ABclona Technology (China). Moreover, p-JNK, p-ERK, p-p38, p-IκBα/IκBα, and p-NF-κB (p65) were purchased from Cell Signaling (USA); GSDMD, Bax Trx-1, α-Tubulin, and Txnip were afforded by Abcam (USA). Additionally, CK-MB, CAT, GSH, MDA, and SOD test kits were supplied by Nanjing Jiancheng Biotech. Co., Ltd. (China). Unless specified otherwise, all other reagents were obtained from Sigma–Aldrich (USA).

Animals

Male C57BL/6 mice, six weeks old, 18–22 g, were supplied by Liaoning Changsheng Technology Industrial Co., Ltd. (Certificate SCXK2010-0001; China). All mice were maintained for one week under controlled environmental conditions (temperature 24°C ± 2°C, humidity 40%, light/dark cycle 12 h). All studies were performed in accordance with the International Guiding Principles for Biomedical Research Involving Animals, which has been agreed with the Institutional Animal Care and Use Committee of Heilongjiang Bayi Agricultural University (JXY2022037).

Experimental protocol

To establish chronic CS-induced myocardial damage, the mice were divided randomly into two groups (n = 5 mice per group), including the room temperature (RT, 24°C ± 2°C) group and the chronic CS group (4°C). Briefly, the mice in the CS group were placed in a climatic chamber at 4°C for three hours per day, and transferred back to RT; the chronic CS process lasted for three weeks. After a final cold stimulation, the mice were euthanized, and the heart tissue samples and serum were collected for biochemical index determination, hematoxylin and eosin (H & E) staining, quantitative real time polymerase chain reaction (RT-qPCR), and western blot analysis.

Measurement of myocardial function index and oxidative indicators

The C57BL/6 mice were kept at RT or CS (4°C) for three hours per day for three weeks. The heart tissues and blood from the mice were harvested to determine the different biochemical analyses. The levels of creatine kinase MB isoenzyme (CK-MB), MDA, CAT, SOD, and GSH in serum were analyzed using the appropriate assay kit according to the manufacturer′s instructions (Jiancheng Bioengineering Institute, China).

H & E staining

To evaluate the cardiac function, fresh cardiac tissues were fixed with 4% paraformaldehyde, dehydrated with a graded series of ethanol, embedded in paraffin wax, and cut into 5-μm-thick sections. These sections were stained with H & E to observe the pathological changes in the heart by optical microscopy (OM, Nikon, Japan).

Staining of Masson′s trichrome

Staining was performed using the Masson's Trichrome Stain (Masson) kit (Sigma-Aldrich) according to the manufacturer's instructions. The images were captured by OM at 200× magnification. Two individuals blinded to the experimental design analyzed the images using Image-Pro Plus software (version 6.0, Media Cybernetics, USA) for semi-quantitative analysis. Each section was selected randomly from five different areas for analysis, and the average value was taken as the final value.

RNA extraction and qRT-PCR analysis

The total RNA was extracted from the macrophage lysates and mouse heart tissues using Trizol reagent (Invitrogen, USA) according to the manufacturer′s instructions. The isolated RNA was reverse transcribed to cDNA using PrimeScript™ RT reagent Kit with a dDNA Eraser (TaKaRa). The cDNA was subjected to qRT-PCR using SYBR Green Supermix (Bio-Rad, USA). The primers were synthesized by Shanghai Shenggong and are listed in **Table 1**. β-actin was used to normalize mRNA expression. The fold change in the relative mRNA levels was calculated using the $2^{-\Delta\Delta CT}$ method. The PCR reactions were carried out in an SYBR green working solution and measured quantitatively using the Applied Biosystems 7300 qRT-PCR

Table 1. Primers used for quantitative real time polymerase chain reaction

Genes	Sequences
TNF-α	Forward primer: 5′-GCAACTGCTGCACGAAATC-3′
	Reverse primer: 5′-CTGCTTGTCCTCTGCCCAC-3′
HMGB1	Forward primer: 5′-GTTCTGAGTACCGCCCCAAA-3′
	Reverse primer: 5′-TAGGGCTGCTTGTCATCTGC-3′
IL-6	Forward primer: 5′-ACATCGACCCGTCCACAGTAT-3′
	Reverse primer: 5′-CTTGGGACTGATGCTGGTGACAAC-3′
β-actin	Forward primer: 5′-CTACCTCATGAAGATCCTGACC-3′
	Reverse primer: 5′-CACAGCTTCTCTTTGATGTCAC-3′

system and software (Applied Biosystems, USA). The following thermal cycler parameters were used: 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 60°C for 30 s.

Western blot analysis

Equal amounts of protein (20 μg) were separated on 10% SDS-polyacrylamide gel and transferred electrophoretically to a polyvinylidene fluoride membrane. The membranes were immersed in 5% skim milk powder for 1 h and incubated overnight at 4 °C with multiple specific primary antibodies, including HSP70, HSP90, TLR4, MyD88, NLRP3, ASC, caspase-1, caspase-11, IL-18, IL-1*β*, HMGB1, COX-2, iNOS, HMGB1, IL-6, TNF-α, p-JNK, p-ERK, p-p38, p-IκBα/IκBα, Bax, Txnip, NLRP3, caspase-1, caspase-11, GSDMD, ASC, IL-1*β*, IL-18, caspase-3, and α-Tubulin. Subsequently, the membrane was washed three times with PBST and incubated for 1 h with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5,000). Finally, the membranes were again washed and visualized by enhanced chemiluminescence (ECL, Bio-Rad) Western blotting detection system. The band intensities were quantified using Image J gel analysis software. All experiments were carried out in triplicate.

Statistical analysis

All data above were calculated as the means ± SEM and analyzed using SPSS19.0 (IBM, USA) software. Comparisons between each group were conducted using one-way analysis of the variance (ANOVA), whereas the least significant difference was used for multiple comparisons analysis. The value of $p < 0.05$ or < 0.01 were considered significant.

RESULTS

Chronic CS-induced myocardial damage in C57BL/6 mice

The pathological features of heart tissues caused by cold stimulation were analyzed by HE and Masson staining to determine if chronic CS could induce myocardial damage in mice. The CS group manifested severe myocardial architecture disruption, hemorrhage, and inflammatory cell infiltration compared to the RT group (**Fig. 1A-B**). Moreover, compared to the RT group, a significant increase in CK-MB was examined in the chronic CS-exposed group (**Fig. 1C**). The stress-related protein expression of HSP90 and HSP70 was detected by western blot. Chronic CS could enhance the expression of HSP70 significantly but had little effect on HSP90 protein expression compared to the RT group (**Fig. 1D-E**). In addition, western blot analysis indicated that chronic cold exposure could effectively promote the expression of cleaved-caspase-3 and Bax apoptosis-related protein in the heart tissues (**Fig. 1F-G**). These findings showed that chronic cold exposure resulted in myocardial injury.

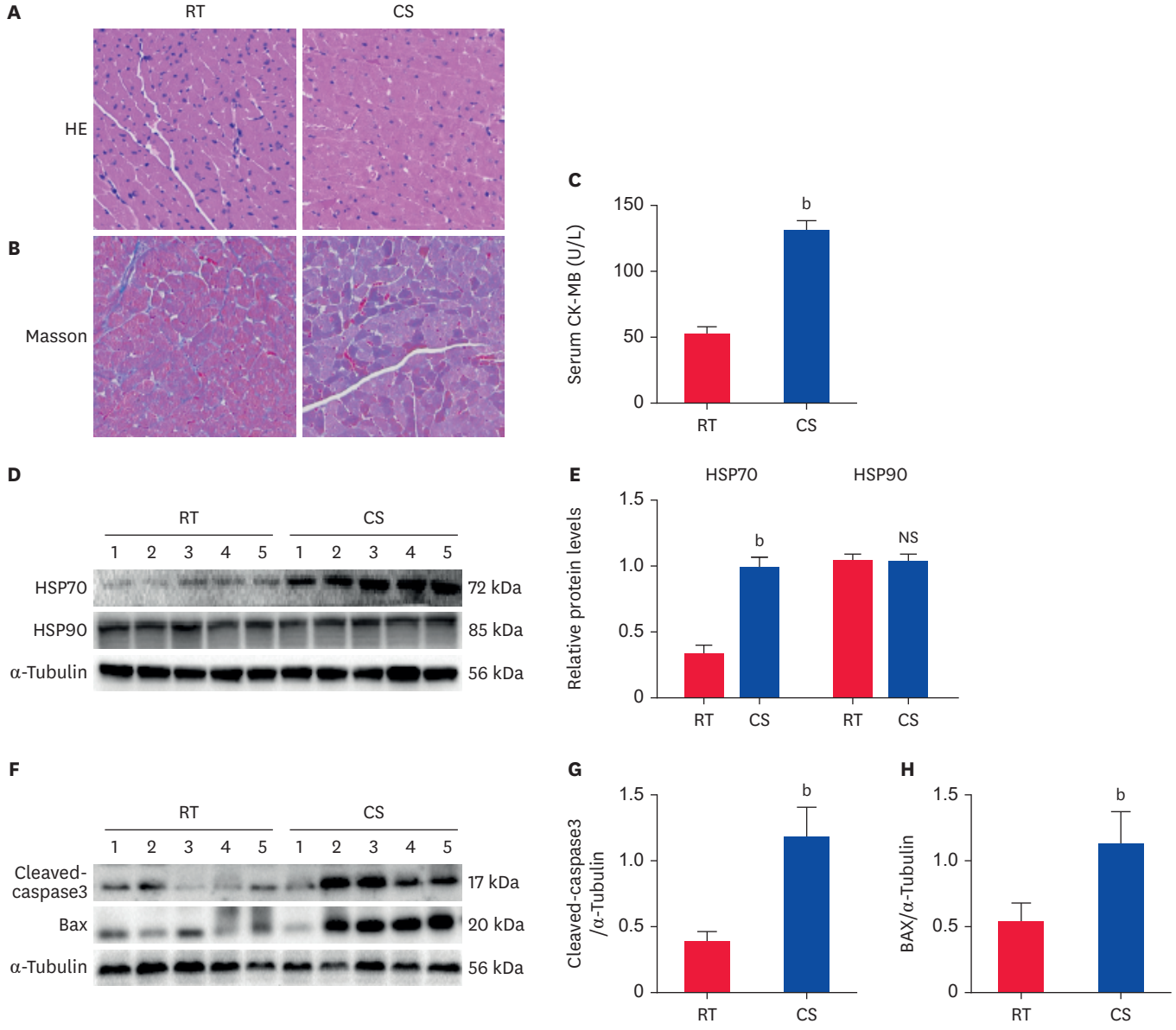

Fig. 1. Chronic cold stress-induced myocardial damage in C57BL/6 mice. C57BL/6 mice were kept at RT or CS (4°C) for three weeks, three hours per day. (A) Representative histological sections of the heart were stained with hematoxylin and eosin (magnification×400). (B) Representative histological sections of the heart were stained with Masson-stain (magnification×400). (C) Myocardial injury was detected through the serum levels of CK-MB and lactate dehydrogenase using the enzyme-linked immunosorbent assay method. (D and F) Effects of low temperature on the level of stress-related HSP70 and HSP90 proteins, and apoptosis-related cleaved-caspase3 and BAX protein. (E and G) The relative protein expression was quantified by densitometric analysis. Similar results were obtained from three independent experiments. All data are presented as the means ± SEM (n = 5/group).
RT, room temperature; CS, cold stress; CK-MB, creatine kinase isoenzyme MB.
[a]$p < 0.05$ and [b]$p < 0.01$ vs. CS group; NS, not significant.

Chronic cold exposure triggered oxidative stress in the heart tissues of mice

Given that an oxidative insult is considered a vital factor of tissue damage in CS-induced mice, this study examined whether chronic cold exposure could induce oxidative myocardial damage. As shown in **Fig. 2**, chronic cold exposure increased the excessive accumulation of MDA and the depletion of GSH and SOD, suggesting severe oxidative stress in the heart tissues of mice caused by CS.

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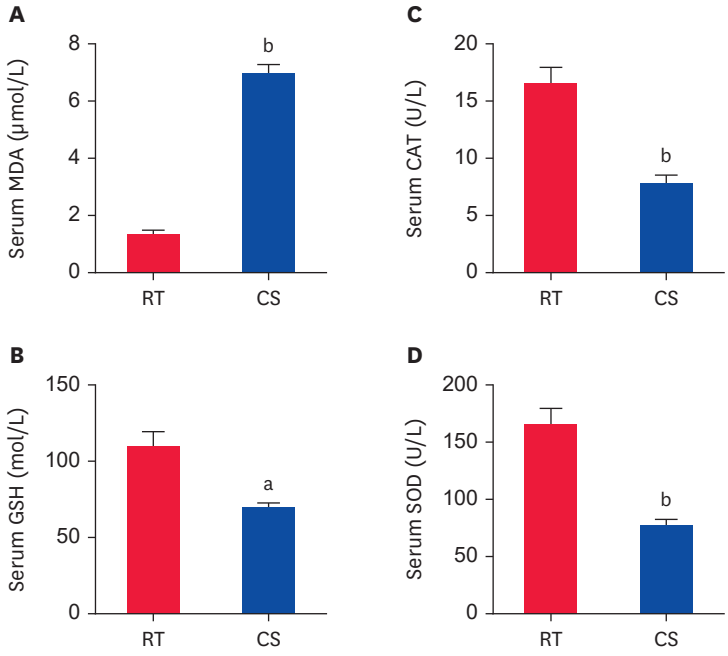

Fig. 2. Effect of chronic cold exposure on levels of oxidative markers in mice. The C57BL/6 mice were kept at RT or CS (4°C) for three weeks, three hours per day. (A-D) Heart tissues of mice were gathered to measure the MDA, CAT, SOD, and GSH levels. Similar results were obtained from three independent experiments. All data are presented as the means ± SEM (n = 5/group).
MDA, malondialdehyde; RT, room temperature; CS, cold stress; CAT, catalase; GSH, glutathione; SOD, superoxide dismutase.
[a]$p < 0.05$ and [b]$p < 0.01$ vs. CS group.

Chronic CS led to the release of inflammatory mediators in mice

Inflammatory mediators are major contributors to various heart diseases. Therefore, the expression of iNOS, COX-2, TNF-α, IL-6, and HMGB1 was assessed after the three-week chronic cold exposure period. CS led to the tremendous expression of HMGB1, IL-6, and TNF-α protein, whereas the iNOS and COX-2 levels were similar to those in the RT groups (**Fig. 3A-F**). Simultaneously, the RT-qPCR results showed that CS dramatically induced the levels of HMGB1, IL-6, and TNF-α mRNA expression in myocardial tissue (**Fig. 3G-I**), indicating that chronic cold exposure promoted the cardiac inflammatory response.

Chronic CS-induced NLRP3-caspase1-GSDMD pyroptosis signaling pathway in mice

Inflammation is closely associated with pyroptosis. Therefore, this study examined whether CS could excite the pyroptosis-related signaling pathway using western blot. As shown in **Fig. 4**, chronic cold exposure induced the increased expression of NLRP3, ASC, cleaved-caspase1, GSDMD-N, mature-IL-1β, and mature-IL-18 protein, while cleaved-caspase11 protein expression was moderate, suggesting that CS activates the GSDMD-mediated pyroptosis signaling pathway and is regulated by NLRP3 in a caspase-1-dependent, non-caspase-1-dependent manner.

Chronic CS activated NF-κB and MAPK signaling pathways in mice

The underlying mechanism of how chronic cold exposure promoted the cardiac inflammatory response was assessed. The classical inflammation-related NF-κB and MAPK signaling pathways were detected by western blot. Compared to the RT group, CS

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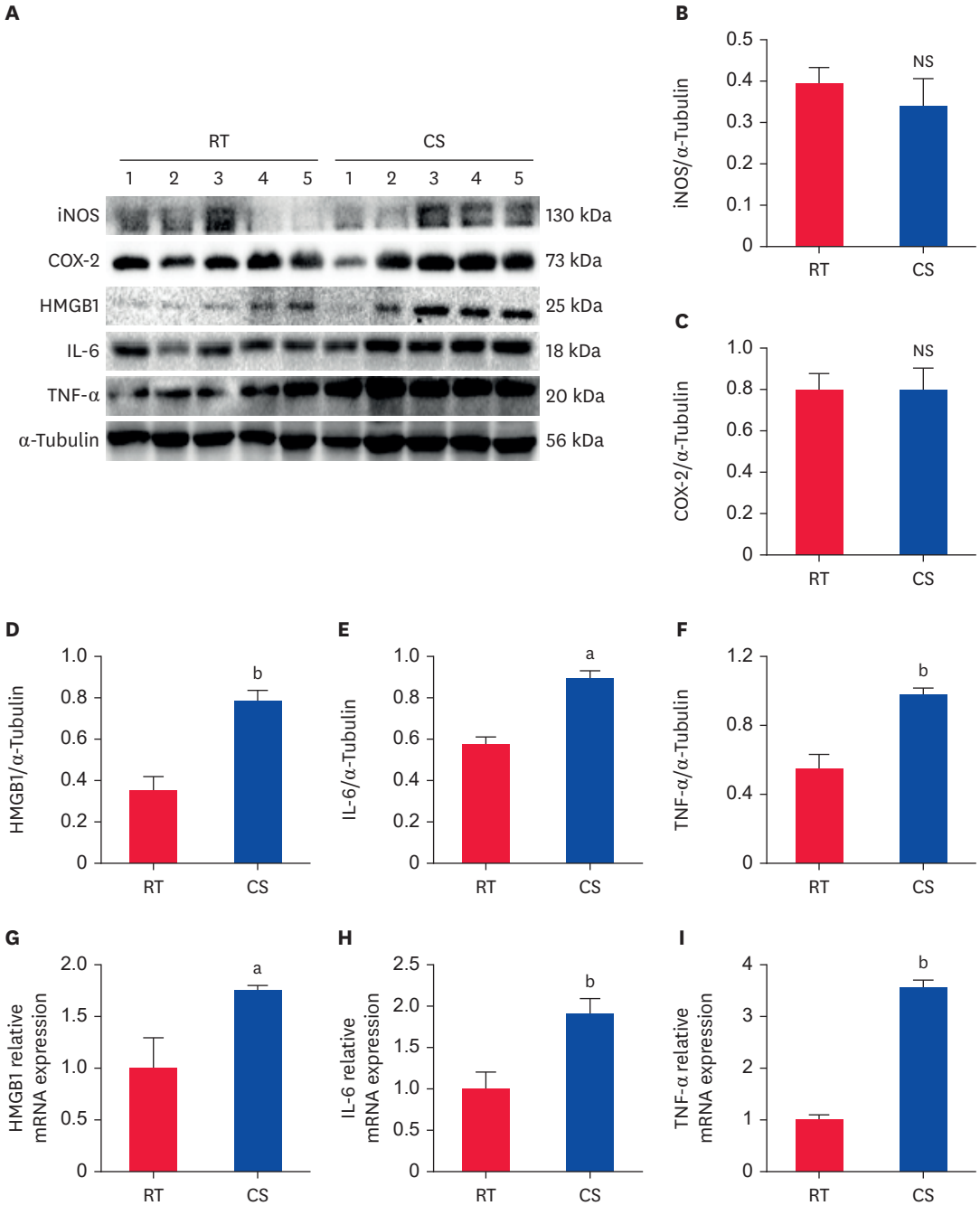

Fig. 3. Effect of chronic cold exposure on the release of inflammatory mediators in mice. C57BL/6 mice were kept at RT or CS (4°C) for three weeks, three hours per day. The heart tissues of the mice were gathered and analyzed by western blot and RT-qPCR. (A) Effects of cold exposure on the protein levels of iNOS, COX-2, HMGB1, IL-6, and TNF-α in the heart tissues of mice. (B-F) Quantification of relative protein expression was performed by densitometric analysis. α-Tubulin was used as an internal control. (G-I) The mRNA levels of TNF-α, IL-6, and HMGB1 were detected by RT-qPCR. Similar results were obtained from three independent experiments. All data are presented as the means ± SEM (n = 5/group).
RT, room temperature; CS, cold stress; IL, interleukin; TNF-α, tumor necrosis factor-α; CAT, catalase; RT-qPCR, quantitative real time polymerase chain reaction.
[a]$p < 0.05$ and [b]$p < 0.01$ vs. CS group; NS, not significant.

increased the phosphorylation of NF-κB (P65), JNK, ERK, and p38 MAPK and elevated the phosphorylation and degradation of IκBα, suggesting that CS could effectively activate NF-κB and MAPK signaling pathway (**Fig. 5**).

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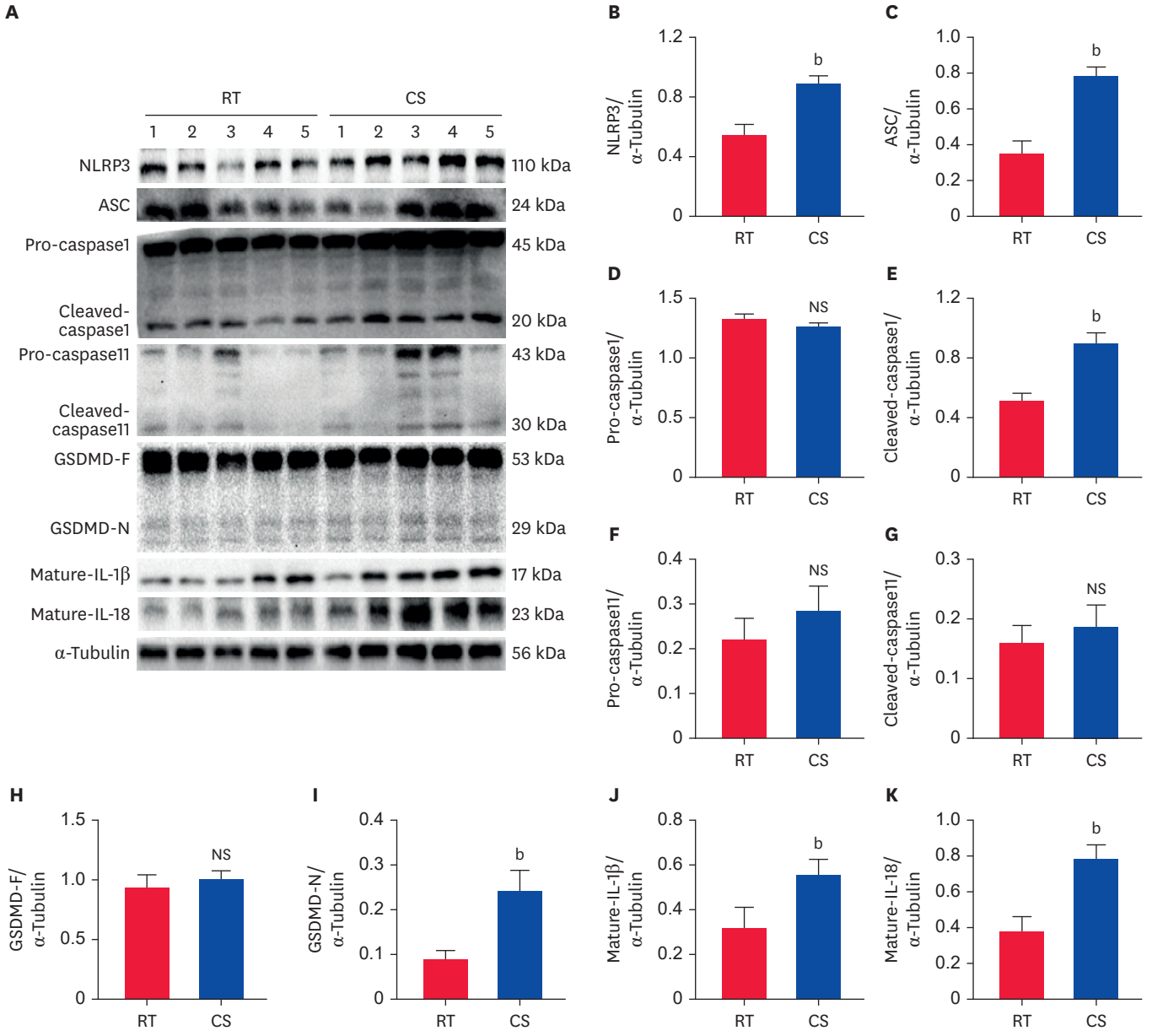

Fig. 4. Effect of chronic cold exposure on NLRP3-caspase1/11-GSDMD pyroptosis signaling pathway in mice. C57BL/6 mice were kept at RT or CS (4°C) for three weeks, three hours per day. The heart tissues of the mice were collected and analyzed by western blot. (A) Effects of cold exposure on the protein levels of NLRP3, ASC, caspase1, caspase11, GSDMD, IL-1β, and IL-18 in heart issues of mice. (B-K) Quantification of relative protein expression was performed by densitometric analysis. α-Tubulin was used as an internal control. Similar results were obtained from three independent experiments. All data are presented as the means ± SEM (n = 5/group).
RT, room temperature; CS, cold stress; NLRP3, NOD-like receptor 3; IL, interleukin.
[a]$p < 0.05$ and [b]$p < 0.01$ vs. CS group; NS, not significant.

Chronic CS enhanced TLR4 and MyD88 protein levels in mice

Several studies showed that TLR4/MyD88, a critical inflammation regulator, is upstream of the NF-κB and MAPK signaling pathway. Thus, TLR4 and MyD88 protein expression under cold stimulation was examined using western blot. As shown in (**Fig. 6**), chronic cold exposure could result in the TLR4 and MyD88 protein expression in contrast to the RT group. These results suggest that the CS-induced cardiac inflammatory reaction may partially be due to the regulation of the TLR4/MyD88-NF-κB and -MAPK signaling pathways.

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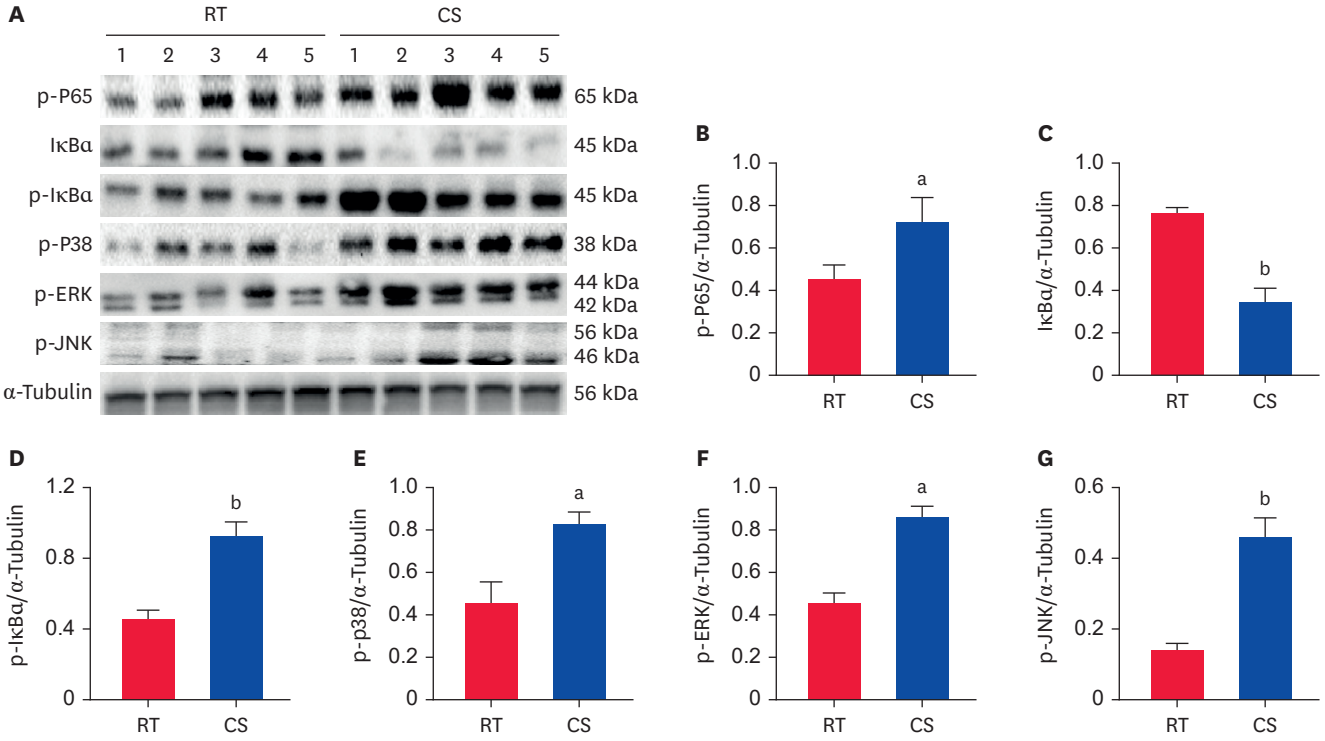

Fig. 5. Effect of chronic cold exposure on nuclear factor-κB and MAPK signaling pathway in mice. C57BL/6 mice were kept at RT or CS (4°C) for three weeks, three hours per day. The heart tissues of mice were gathered and analyzed by western blot. (A) Effects of cold exposure on the protein levels of p-P65, IκBα, p-IκBα, p-JNK, p-ERK, and p-p38 MAPK in heart issues of mice. (B-G) Quantification of the relative protein expression was performed by densitometric analysis. α-Tubulin was used as an internal control. Similar results were obtained from three independent experiments. All data are presented as the means ± SEM (n = 5/group).
RT, room temperature; CS, cold stress; MAPK, mitogen-activated protein kinase.
[a]$p < 0.05$ and [b]$p < 0.01$ vs. CS group.

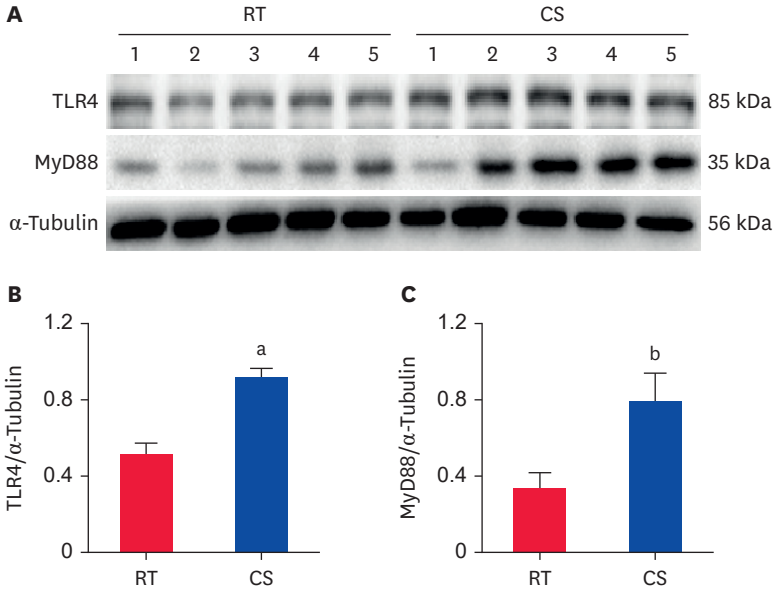

Fig. 6. Effect of chronic cold exposure on TLR4 and MyD88 protein levels in mice. C57BL/6 mice were kept at RT or CS (4°C) for three weeks, three hours per day. The heart tissues of mice were collected and analyzed by western blot. (A) Effects of cold exposure on the protein levels of TLR4 and MyD88 in the heart issues of the mice. (B-C) Quantification of relative protein expression was performed by densitometric analysis. α-Tubulin was used as an internal control. Similar results were obtained from three independent experiments. All data are presented as the means ± SEM (n = 5/group).
RT, room temperature; CS, cold stress; TLR4, toll-like receptor 4.
[a]$p < 0.05$ and [b]$p < 0.01$ vs. CS group.

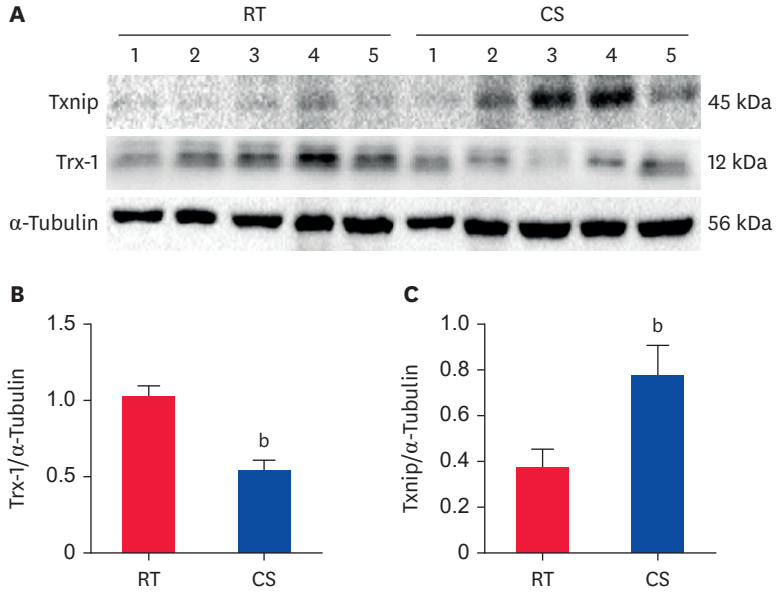

Fig. 7. Effect of chronic cold exposure on the Trx-1 and Txnip protein levels in mice. C57BL/6 mice were kept at RT or CS (4°C) for three weeks, three hours per day. The heart tissues of the mice were gathered and analyzed by western blot. (A) Effects of cold exposure on the protein levels of Trx-1 and Txnip in the heart issues of the mice. (B-C) Quantification of relative protein expression was performed by densitometric analysis. α-Tubulin was used as an internal control. Similar results were obtained from three independent experiments. All data are presented as the means ± SEM (n = 5/group).
RT, room temperature; CS, cold stress.
[a]$p < 0.05$ and [b]$p < 0.01$ vs. CS group.

Chronic cold exposure caused a decrease in Trx-1 and an increase in the Txnip protein levels in mice

The potential mechanisms of inflammasome NLRP3 activation caused by chronic cold exposure were examined further. The Trx-1/Txnip signal plays an essential role in modulating inflammasome NLRP3. Therefore, the levels of Trx-1 and Txnip protein expression were detected by western blot. Expression of the Txnip protein was elevated by cold stimulation compared to the RT group (**Fig. 7**), but the expression of the Trx-1 protein was decreased in heart tissues.

DISCUSSION

Increasing evidence has shown that various cardiovascular diseases are strongly associated with extremely low temperatures [24,25]. Pyroptosis and inflammatory responses play vital roles in many heart disorders, particularly myocardial ischemia/reperfusion (I/R) injury [26], but a few studies examined the underlying mechanisms of CS and cardiac injury. Accordingly, this study examined the level of pyroptosis and inflammatory responses in mouse myocardial tissue under chronic CS conditions. The results showed that chronic cold exposure could induce a pyroptosis and inflammation reaction in the heart tissue of mice by regulating the NLRP3 inflammasome, Trx-1/Txnip, and TLR4/MyD88 signaling pathway.

The cardiac structure and level of myocardial enzymes in the serum are considered important indicators of cardiac injury [27]. Several studies reported that CS could cause cardiac remodeling and cardiac dysfunction [28]. In the present study, chronic CS also caused myocardial injury with an elevation of the creatine kinase isoenzyme-MB (CK-MB) levels, which is consistent with previous reports. Moreover, heat shock proteins (HSP) are key stress

proteins, including HSP70 and HSP90, which play an essential role as molecular chaperones in stress protection and have important regulatory roles in inflammation and oxidative stress [29,30]. The increase in HSP70 protein expression, but not HSP90, was induced by chronic cold stimulation. Moreover, cold exposure could increase the expression of apoptosis-related proteins, which are closely associated with myocardial functional impairment [31]. Previous studies showed that cold exposure provoked hippocampal neuroinflammation and hepatic oxidative stress by promoting the generation of inflammatory factors, decreasing the content of the antioxidant enzyme, and increasing the content of the oxidation product [22,32]. Western blot and qRT-PCR analysis showed that chronic hypothermia exposure resulted in the obvious expression of HMGB1, IL-6, and TNF-α protein and mRNA. In addition, biochemical indicators analysis indicated that the antioxidant enzymes CAT, SOD, and GSH were efficiently depleted, while the lipid oxidation product MDA increased under chronic cold exposure conditions. These investigations suggested that chronic CS could lead to cardiac injury by promoting cell apoptosis, inflammation, and oxidative stress.

Pyroptosis is a newly discovered pro-inflammatory type of programmed cell death (PCD) resulting from gasdermin D (GSDMD)-mediated membrane pore formation, cell swelling, and rapid lysis, which leads to the massive secretion of inflammatory cytokines, such as IL-1β and IL-18 [33,34]. Pyroptosis is strongly involved in cardiovascular disease (CVD), including atherosclerosis, myocardial ischemia/reperfusion injury (MI/RI), and myocardial infarction [35]. Although cold exposure may result in cardiac injury [32], it is unclear if it is associated with cellular pyroptosis. The present findings showed that the release of mature-IL-1β and IL-18 and the formation of N-terminal cleavage product (GSDMD-N) were induced by CS. The effects of cold exposure on caspase-1 and caspase-11 activation were examined further because GSDMD, a key pyroptosis executor, is a common substrate for caspase-1 and caspase-11 in mice [36]. Caspase-1 was activated, while caspase-11 was not efficiently changed. Moreover, new evidence indicated that NLRP3 inflammasome could first recognize various stimuli, and then activate pro-caspase-1 cleavage and an adaptor protein, like apoptosis-associated speck-like protein containing a CARD (ASC) recruitment to assemble inflammasomes, leading to inflammation and pyroptosis [37,38]. Indeed, NLRP3 was significantly activated in chronic CS-induced pyroptosis death and inflammation. Overall, chronic CS can induce pyroptosis mainly via the NLRP3-regulated caspase-1-dependent canonical pathway.

NLRP3 activation is closely related to multiple signaling pathways, including Trx-1/Txnip and TLR4-mediated NF-κB pathway [16,21]. More importantly, abundant research has shown that the TLR4 and Txnip pathways play a critical role in various myocardial injuries [26,39]. Inhibition of the TLR4 pathway reduces myocardial inflammation and improves the cardiac function [26]. In the present study, western blot analysis showed that chronic cold exposure activated the NF-κB and MAPK signaling pathways by promoting the TLR4/MyD88 signal. Moreover, Txinp was reported to be an endogenous inhibitor of the Trx-1 pathway, which is involved in some pathological consequences of myocardial ischemia /reperfusion injury [40]. The present results showed that chronic cold exposure could decrease Trx-1 protein expression and increase Txnip protein expression. These findings indicated that the chronic CS-induced NLRP3/caspase1/GSDMD signal might be associated with the Trx-1/Txnip and TLR4/MyD88 pathways.

In summary, as shown in **Fig. 8**, chronic CS triggers cell apoptosis, oxidative stress, inflammation, and pyroptosis, which leads to cardiac injury, with possible mechanisms

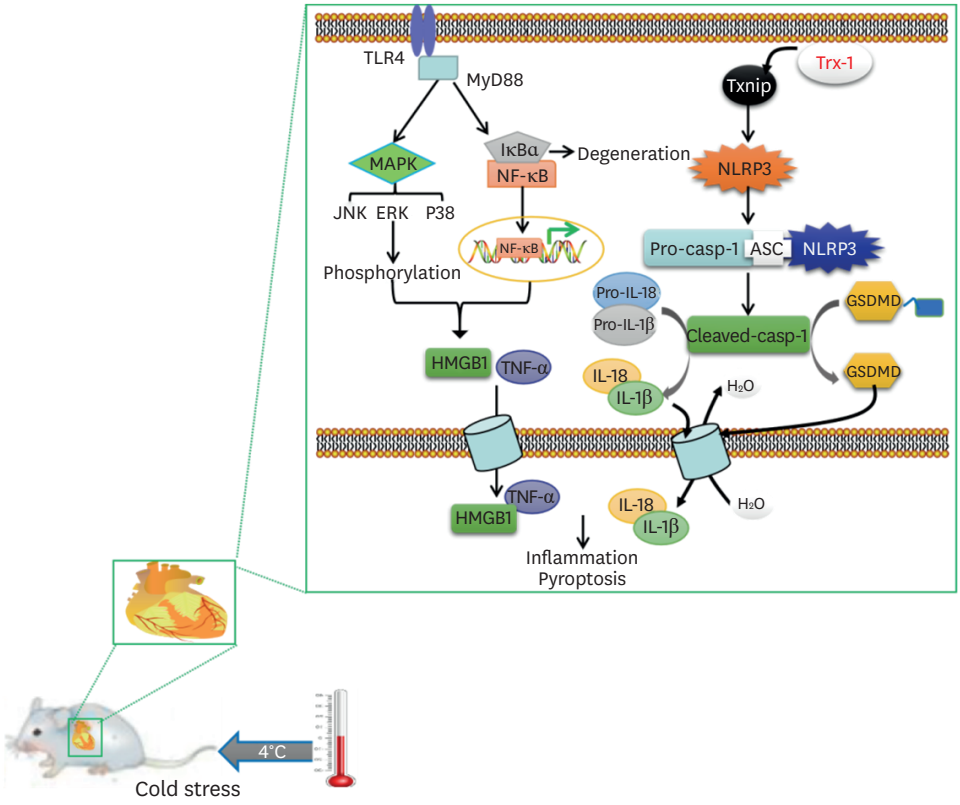

Fig. 8. Schematic diagram summarizing chronic cold stress-induced oxidative stress, inflammation, and pyroptosis through the regulation of TLR4/MyD88, Trx-1/Txnip, and NLRP3/caspase1/GSDMD signaling pathways.
TLR4, toll-like receptor 4; MAPK, mitogen-activated protein kinase; NLRP3, NOD-like receptor 3; IL, interleukin; TNF-α, tumor necrosis factor-α.

related to the NLRP3-mediated caspase-1-dependent canonical pathway, Trx-1/Txnip, and TLR4/MyD88 pathway. These investigations provide insights into the increased risk of cardiovascular disease with cold exposure.

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