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Icariin ameliorates viral myocarditis by inhibiting TLR4-mediated ferroptosis

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ABSTRACT

Objective: To explore the mechanism by which icariin alleviates viral myocarditis.

Methods: CVB3-induced cardiomyocytes were used as an *in vitro* model of viral myocarditis to assess the effects of icariin treatment on cell viability, inflammation, and apoptosis. Moreover, the effects of icariin on ferroptosis and TLR4 signaling were assessed. After AC16 cells were transfected with TLR4 overexpression plasmids, the role of TLR4 in mediating the regulatory effect of icariin in viral myocarditis was investigated.

Results: Icariin significantly elevated cell viability and reduced inflammatory factors TNF-α, IL-1β, IL-6, and IL-18. Flow cytometry revealed that icariin decreased apoptosis rate, and the protein expression of Bax and cleaved caspase 3 and 9 in CVB3-induced cardiomyocytes. Additionally, it suppressed ferroptosis including lipid peroxidation and ferrous ion, as well as the TLR4 signaling. However, TLR4 overexpression abrogated the modulatory effects of icariin.

Conclusions: Icariin mitigates CVB3-induced myocardial injury by inhibiting TLR4-mediated ferroptosis. Further animal study is needed to verify its efficacy.

KEYWORDS: Viral myocarditis; CVB3; TLR4; Ferroptosis; Icariin

1. Introduction

Viral myocarditis is a form of infectious cardiomyopathy characterized by cardiomyocyte degeneration, necrosis, and interstitial inflammation, resulting from virus infection[1]. Localized or diffuse acute or chronic inflammatory lesions in the myocardium lead to varying degrees of cardiac dysfunction, from nonspecific symptoms such as shortness of breath to more severe symptoms resembling acute coronary syndrome[2]. In severe cases, it can progress to dilated cardiomyopathy or even sudden cardiac death[3]. Notably, the incidence of viral myocarditis has been on the rise among children and adolescents in recent years[4]. Due to its asymptomatic early stage and the virus's ability to replicate rapidly

Significance

The incidence of viral myocarditis has been on the rise in children and adolescents, making it a common cause of sudden death. Our team's previous pathological studies have indicated that icariin can reduce myocardial damage and inflammatory infiltration in rats with viral myocarditis. This study shows that icariin reduces CVB3-induced myocardial injury by inhibiting TLR4-mediated ferroptosis, suggesting its potential in alleviating viral myocarditis.

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within myocardial cells during vigorous exercise or insufficient rest, it has emerged as a common cause of sudden death among young individuals[5]. The clinical presentation of viral myocarditis varies greatly, even if the myocardium is damaged, and the lack of specific clinical manifestations makes timely clinical or virological diagnosis challenging, resulting in an underestimation of its incidence. To date, the effectiveness of targeting viral infections in treating acute viral myocarditis has not been established in randomized clinical trials[6].

Previous pathological studies conducted by our team have shown that icariin (ICA) can reduce myocardial damage and inflammatory infiltration in rats with viral myocarditis[7]. Icariin is the primary active ingredient in *Epimedium* (*Herba epimedii*) and is one of the most frequently used prescriptions of traditional Chinese medicine for the treatment of osteoporosis[8]. Icariin is a flavonoid compound and its structural similarity to estradiol allows it to compete with estradiol for binding to estrogen receptors, exerting estrogen-like biological effects[9]. Coxsackie virus is a major contributor to viral myocarditis and tends to be more prevalent and severe in males. Studies using myocarditis mouse models infected with coxsackie virus B3 (CVB3) have demonstrated that estrogen receptor α promotes type I interferon (IFN), activates natural killer cells[10] and plays a cardioprotective role. Additionally, icariin has been found to have various pharmacological effects, including increasing cardiovascular and cerebrovascular blood flow, enhancing hematopoietic and immune functions, and exerting anti-aging effects[11,12]. Based on the finding from Super-PRED website[13], Toll-like receptor 4 (TLR4) is a potential target of icariin and TLR4 and inflammasome are key signaling pathways that exacerbate inflammation during myocarditis[14].

Therefore, we hypothesized that modulating the TLR4 signaling pathway is one of the pivotal mechanisms through which icariin alleviates viral myocarditis. In this study, CVB3-induced cardiomyocytes were used as an *in vitro* model to address this hypothesis. In addition, given that CVB3 is known to induce lipid peroxide accumulation and iron overload[15], this study also explored the effect of icariin on cardiomyocyte ferroptosis.

2. Materials and methods

2.1. Cell culture

Human cardiomyocytes AC16 (Millipore) were cultured in DMEM/F-12 supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin solution in a 37℃ and 5% CO_2 atmosphere. AC16 cells were stimulated with CVB3 (100 $TCID_{50}$, Center for Type Culture Collection, Wuhan, China) to mimic viral myocarditis *in vitro*. Icariin (Yuanye, Shanghai, China) at concentrations of 5, 10, and 20 μM was applied to treat AC16 cells for 24 h[16].

2.2. Cell transfection

AC16 cells were transfected with TLR4 overexpression plasmids (Ablife, Wuhan, China). AC16 cells were seeded in a six-well plate the day before transfection. After culturing overnight, plasmids were mixed with the transfection reagent (Thermo Fisher, Shanghai, China) and the mixture was added to the wells. Cells were harvested to assess transfection efficiency after 48 h of transfection.

2.3. CCK8

Cells were treated with icariin or CVB3 for 24 h. CCK8 reagent (Meilunbio, Shanghai, China) was added to the wells and they were incubated for another 2 h. The absorbance in each well was recorded. Cell viability was calculated based on the standard curve.

2.4. Lactate dehydrogenase (LDH) content

The cell supernatant was removed, and the diluted LDH release reagent provided with the kit (Beyotime, Shanghai, China) was added. One hour later, the cell culture plates were then centrifuged at 400 ×*g* for 5min. The supernatant of each well was taken and the absorbance was measured at 490 nm.

2.5. ELISA assay

The levels of inflammation factors TNF-α, IL-1β, IL-6, and IL-18 were assessed using ELISA kits (Beyotime, Shanghai, China). Cell supernatant was collected by centrifugation at 300 ×*g* for 5 min. The supernatant was transferred to the wells and the absorbance was recorded at 450 nm. The levels were calculated based on the standard curve.

2.6. Western blotting analysis

Proteins obtained from cell lysates were quantified and then separated based on molecular weight. Then the proteins were transferred to PVDF membranes (Bio-Rad, Tokyo, Japan), followed by blocking with 5% skimmed milk. The membranes were incubated with specific primary antibodies overnight at 4℃. HRP-conjugated secondary antibody was applied to a couple primary antibodies for 1.5 h at room temperature. Information on the antibodies used is included in Supplementary Table 1. Blots were visualized using an ECL reagent (Hanbio Biotechnology, Shanghai, China) and semi-quantified using ImageJ software (version 1.8.0).

2.7. Flow cytometry

AC16 cells were suspended in 1× binding buffer, incubated with

Annexin V-FITC (Vazyme, Nanjing, China) in the dark for 10 min, and then propidium iodide (Vazyme, Nanjing, China) in 1× binding buffer was added. Cell apoptosis was analyzed using flow cytometry (BD Biosciences) and FlowJo software (version 10.6.2).

2.8. Lipid peroxidation

C11 BODIPY probe (FushenBio, Shanghai, China) was used to indicate lipid peroxidation. Briefly, cells were incubated with C11 BODIPY for 30 min. Following washing twice with phosphate buffered saline, cells were observed under a fluorescence microscope (Nikon, Tokyo, Japan) at 200× magnification.

2.9. Ferrous iron concent

The ferrous iron content in the cells was determined using iron colorimetric assay kits (Elabscience, Wuhan, China) according to the manufacturer's instructions.

2.10. RT-qPCR analysis

Total RNA from cells was precipitated using isopropanol and gathered. Reverse transcription and the qPCR reaction were carried on with a cDNA Synthesis Kit (Bio-Rad, Tokyo, Japan) and the SYBR Green RT-qPCR kit (Takara, Kusatsu, Japan). The amplification program was as follows: 95 ℃ for 30 s; 40 cycles of 95 ℃ for 5 s and 60 ℃ for 31 s. Relative mRNA levels were measured using the ΔΔCt method after normalization to *β-actin*. Primer sequences are included in Supplementary Table 2.

2.11. Molecular docking

The structure of icariin was hydrogenated and converted into a mol2 format file. The structure of TLR4 (PDB: 4G8A) was downloaded from the RCSB PDB website. The water molecules in the protein file were deleted and the structure was optimized. The molecular docking was operated in AutoDock software (version 4.2.6, Scripps).

2.12. Statistical analysis

All data are presented as mean ± SD, and comparisons were analyzed by one-way ANOVA and Tukey's test in the SPSS 26 software. $P<0.05$ is considered statistically significant.

3. Results

3.1. Effects of icariin treatment on cell viability, inflammation, and apoptosis

CCK8 assays were used to assess the effect of icariin on the viability of AC16 cells in the presence and absence of CVB3. Icariin at concentrations of 5, 10, and 20 μM did not affect AC16 cell viability (Figure 1A). After stimulation with CVB3, cell viability was markedly decreased ($P<0.001$). However, icariin effectively mitigated CVB3-induced declined cell viability ($P<0.01$) (Figure 1B). Moreover, LDH content was significantly increased in CVB3-treated cells ($P<0.001$), which was significantly reduced by all concentrations of icariin ($P<0.01$) (Figure 1C). ELISA results showed that CVB promoted AC16 cells to secrete inflammatory factors TNF-α, IL-1β, IL-6, and IL-18. Icariin significantly reduced the levels of these inflammatory cytokines in a concentration-dependent manner (Figure 2A). Western blotting analysis also demonstrated that icariin lowered CVB3-induced protein levels of Cox2 and iNOS ($P<0.05$), further highlighting icariin's anti-inflammatory properties (Figure 2B). Flow cytometry analysis reflected that cell apoptosis was significantly increased in the CVB-induced group ($P<0.001$), which was effectively reduced by icariin treatment ($P<0.05$) (Figure 2C). This result was corroborated

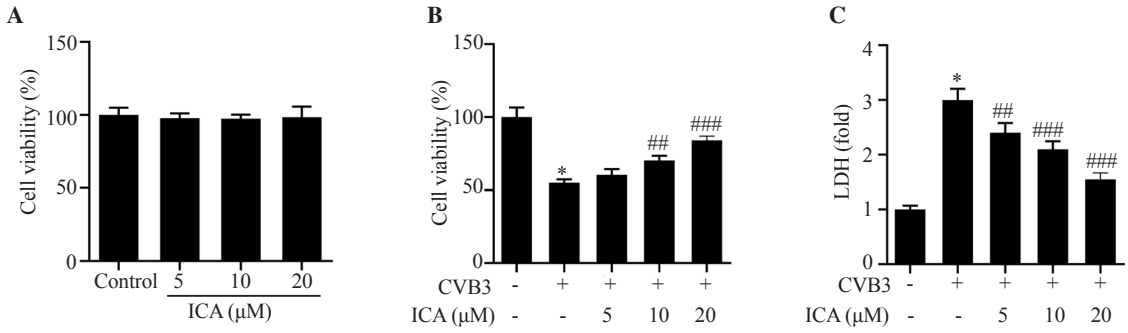

Figure 1. (A) Effect of icariin (ICA) treatment on cell viability. (B-C) Effect of ICA on CVB3-stimulated AC16 cell viability and LDH content. $^{*}P<0.001$ *vs*. the control; $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ *vs*. the CVB3 group. LDH: lactate dehydrogenase; CVB3: coxsackie virus B3.

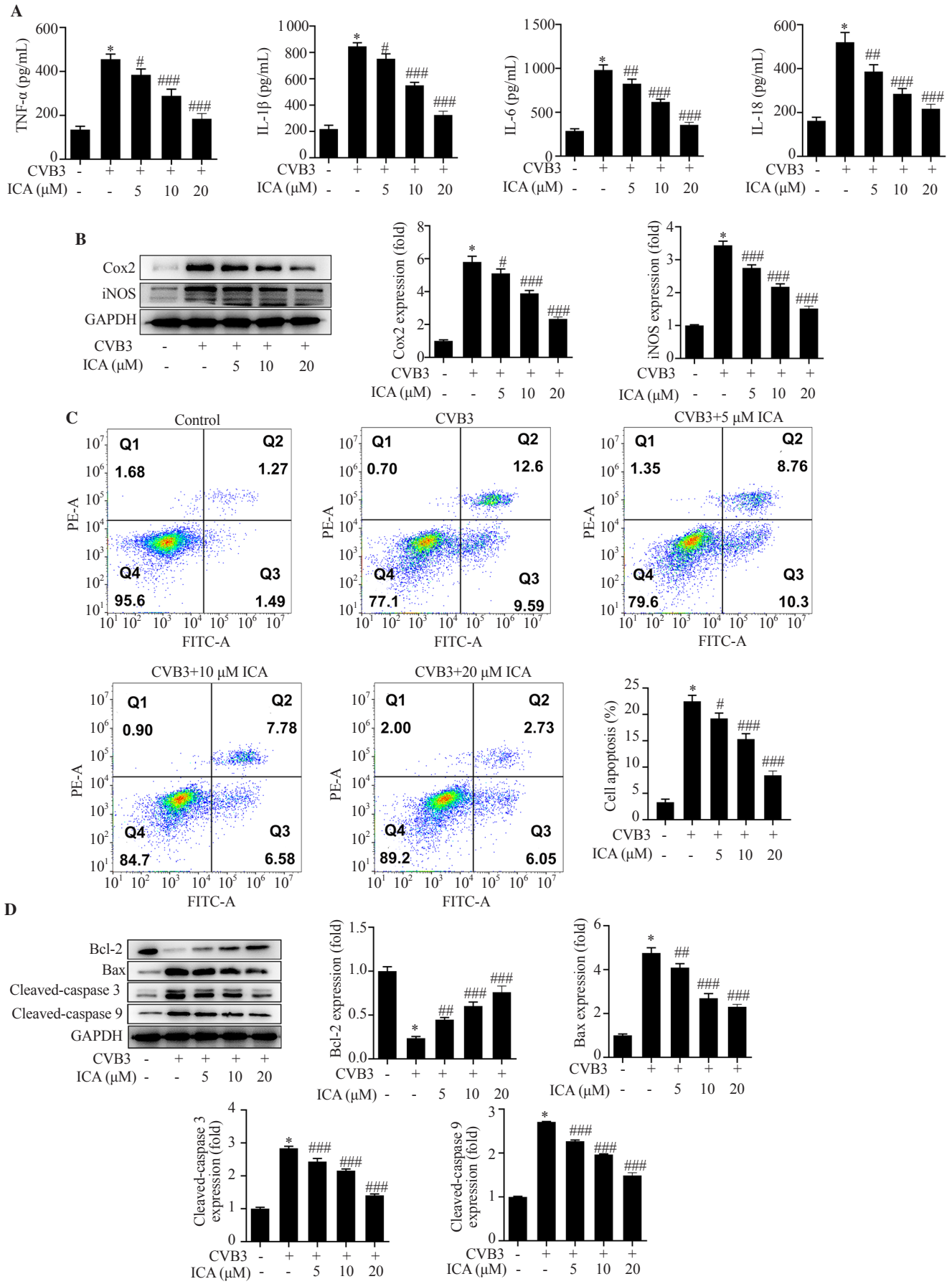

Figure 2. Effects of ICA treatment on the inflammation and apoptosis. (A) Effect of ICA and CVB3 on the secretion of inflammatory factors measured by ELISA kits. (B) Levels of inflammation-related proteins determined by Western blotting. (C) Cell apoptosis determined by flow cytometric analysis. (D) Levels of apoptosis-related proteins determined using Western blotting. $^{*}P<0.001$ *vs*. the control; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ *vs*. the CVB3 group.

by Western blotting, which revealed the decreased levels of pro-apoptotic proteins Bax, cleaved-caspase 3, and cleaved-caspase 9 and an increase in the anti-apoptotic protein Bcl2 following icariin treatment (Figure 2D).

3.2. Effects of icariin treatment on ferroptosis and TLR4 signaling

BODIPY 581/591 indicated the level of intracellular lipid

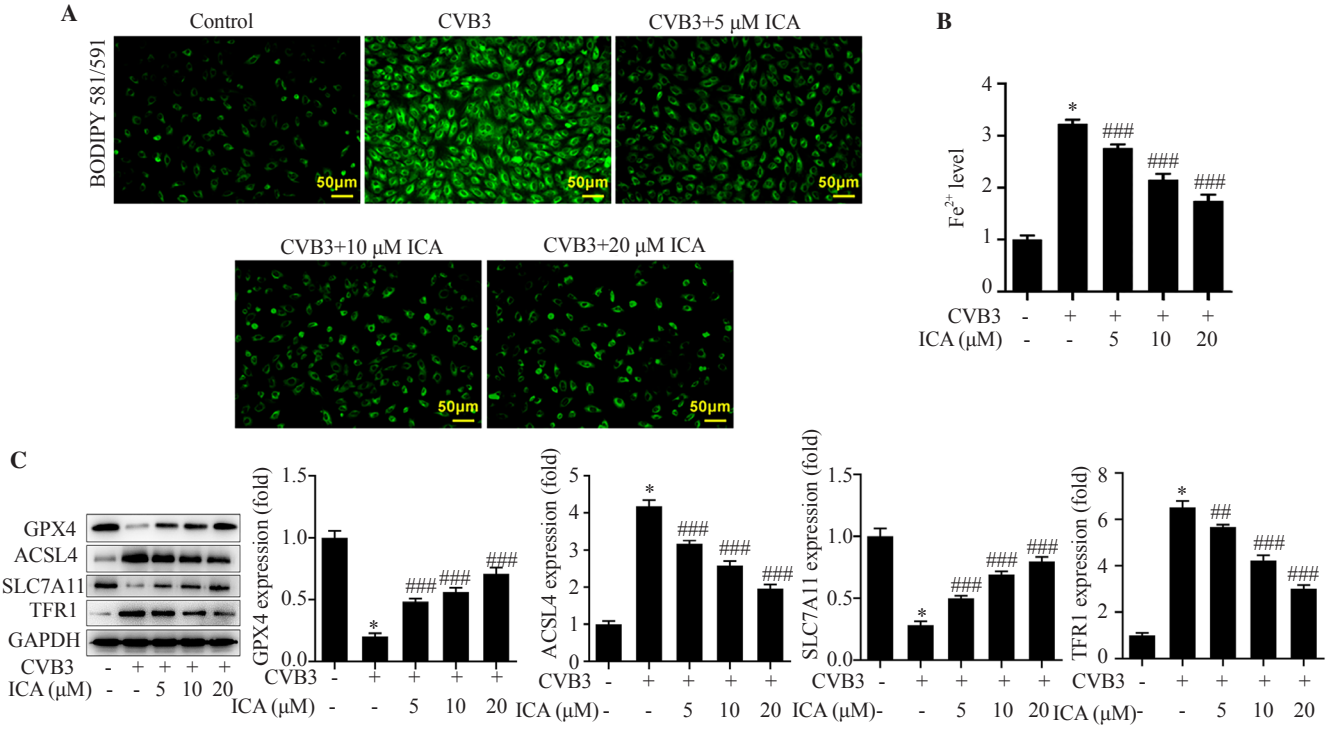

Figure 3. Effect of ICA treatment on ferroptosis. (A) Level of intracellular lipid peroxidation. (B) Effect of ICA on the intracellular iron level in CVB3-induced cells. (C) Levels of ferroptosis-related proteins determined using Western blotting. $^{*}P<0.001$ *vs*. the control; $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ *vs*. the CVB3 group.

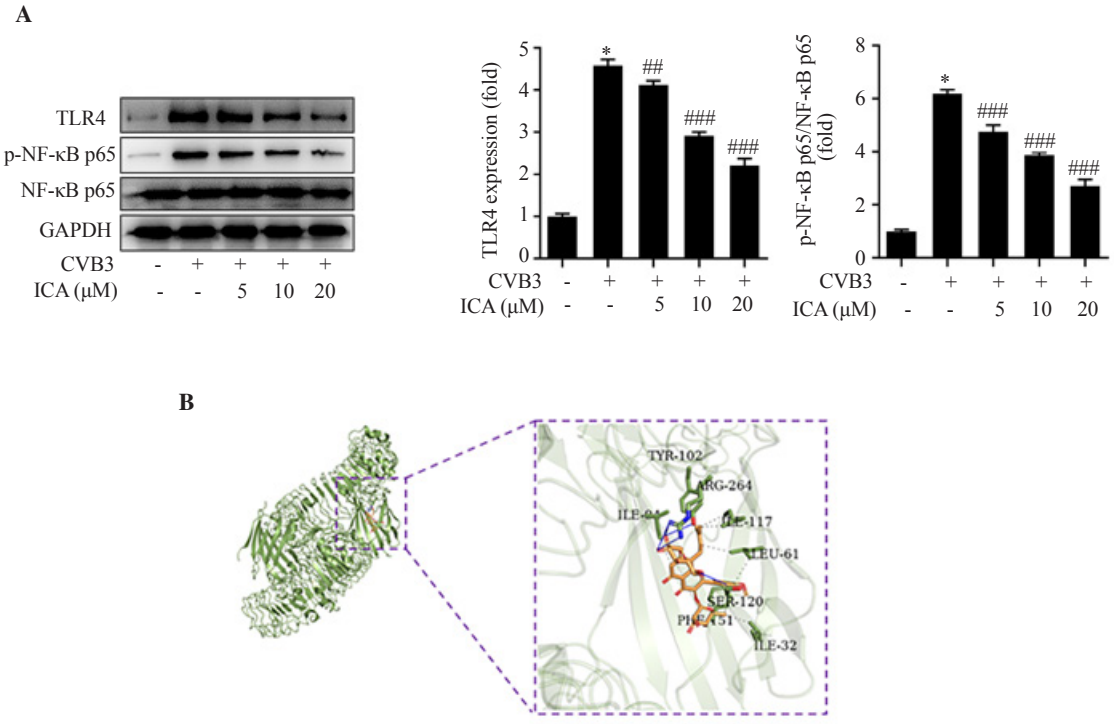

Figure 4. Effect of ICA treatment on the TLR4 signaling. (A) Effect of ICA on CVB3-induced TLR4 and p-NF-κB p65 levels determined using Western blotting. (B) Molecular docking of ICA as a ligand to TLR4 receptor. The left picture is an overall view, and the right picture is a partially enlarged view. The yellow skeleton is ICA, the green is the amino acids in the TLR4 protein, and the blue solid line represents the hydrogen bond. $^{*}P<0.001$ *vs*. the control; $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ *vs*. the CVB3 group.

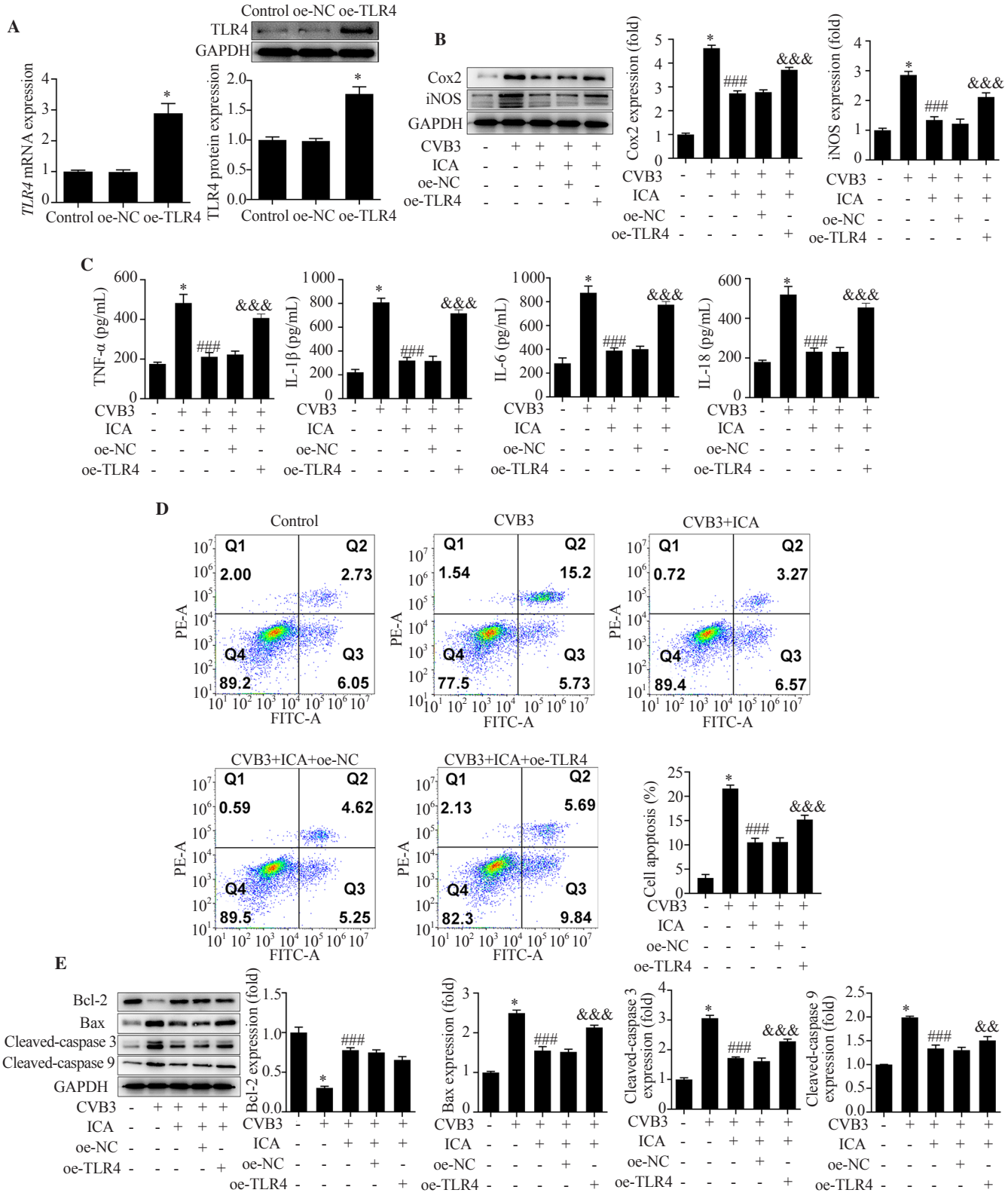

Figure 5. Effect of ICA on CVB3-induced inflammation and apoptosis *via* regulating TLR4. (A) Intracellular expression of TLR4 determined by RT-qPCR and Western blotting. (B) Effect of TLR4 overexpression on the levels of inflammation-related proteins evaluated using Western blotting. (C) Effect of TLR4 overexpression on the secretion of inflammatory factors evaluated using ELISA kits. (D) Cell apoptosis determined by flow cytometric analysis. (E) Effect of TLR4 overexpression on the levels of apoptosis-related proteins evaluated using Western blotting. $^{*}P<0.001$ *vs*. the control; $^{\#\#\#}P<0.001$ *vs*. the CVB3 group; $^{\&\&}P<0.01$, $^{\&\&\&}P<0.001$ *vs*. the CVB3 + ICA + oe-NC group.

peroxidation. CVB3 induced intracellular lipid accumulation, while icariin significantly reduced lipid peroxidation (Figure 3A). CVB3 also induced intracellular iron overload, which was concentration-dependently decreased by icariin (P<0.001) (Figure 3B). Western blotting was employed to detect intracellular ferroptosis-related proteins. CVB3 significantly decreased the protein levels of GPX4 and SLC7A11 in AC16 cells and increased the protein levels of ACSL4 and TFR1. Icariin reversed the effect of CVB3 on the levels of these proteins (Figure 3C). In addition, CVB3 promoted an increase in TLR4 protein levels and the phosphorylation of NF-κB p65 (P<0.001), while icariin exhibited an inhibitory effect on TLR4 and p-NF-κB p65 in a concentration-dependent manner (P<0.01) (Figure 4A). Molecular docking results displayed that the oxygen atoms in icariin formed hydrogen bonds with the hydrogen atoms in Tyr, Arg, and Ser amino acids, indicating that icariin could bind to TLR4 based on the three-dimensional structure (Figure 4B).

3.3. TLR4 overexpression reverses icariin-induced alleviative effects

To further investigate the role of TLR4, the intracellular expression of TLR4 was enhanced by transfection (Figure 5A). The inflammatory response of TLR4-overexpressing cells after CVB3 or icariin treatment was evaluated. Compared to the oe-NC group, TLR4 overexpression promoted the secretion of inflammatory cytokines (Figure 5C) and an increase in inflammation-related protein levels (Figure 5B). Flow cytometry (Figure 5D) and Western blotting results (Figure 5E) indicated that TLR4 overexpression promoted apoptosis and reversed the anti-apoptotic effects of icariin. Furthermore, it led to increased intracellular lipid peroxidation (Figure 6A) and iron overload (Figure 6B), accompanied by an increase in the pro-ferroptosis proteins ACSL4 and TFR1, and a decrease in the superoxide-scavenging proteins GPX4 and SLC7A11 (Figure 6C).

4. Discussion

CVB belongs to the enterovirus genus and the picornaviridae family. CVB3 infection is a significant cause of sudden cardiac death[17], dilated heart disease, and heart transplantation, with a detection rate of about 50%. CVB3 binds to the coxsackie-

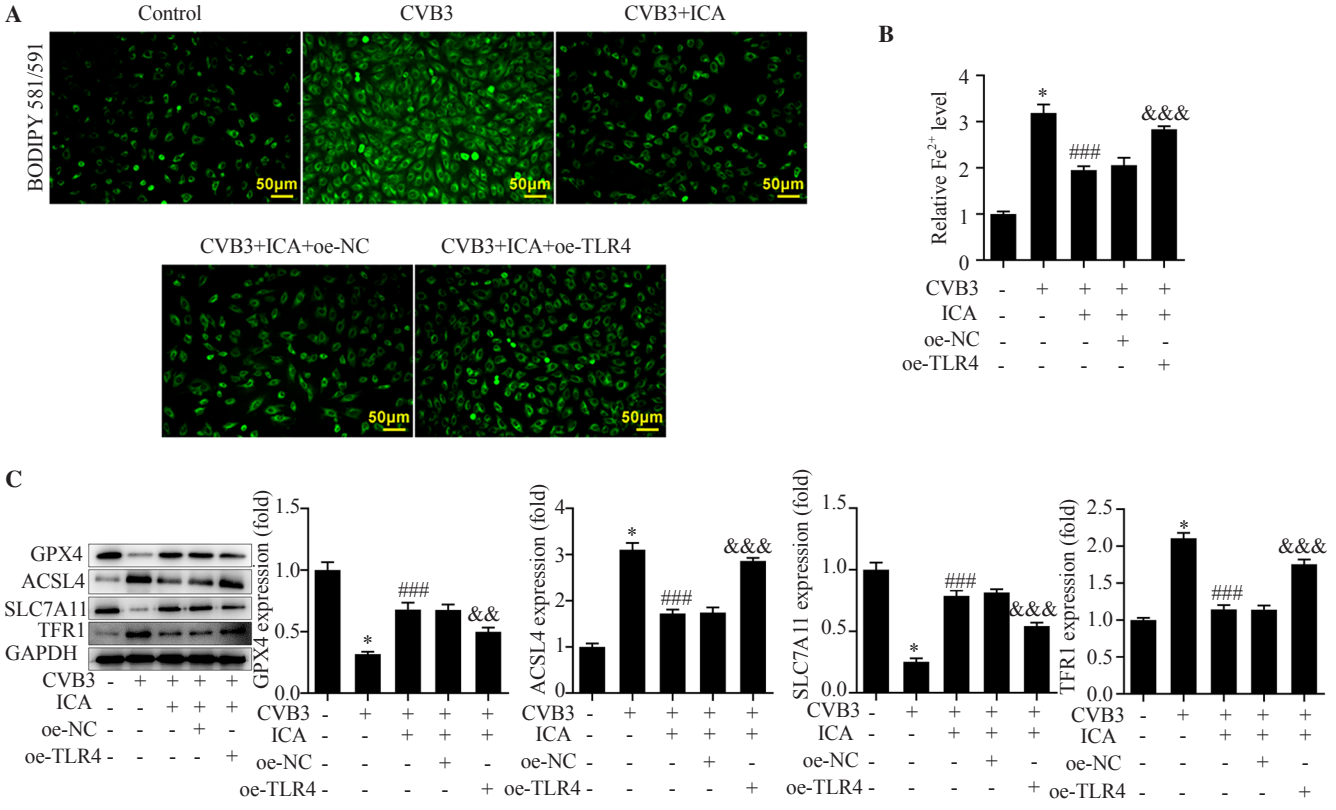

Figure 6. Effect of ICA on CVB3-induced ferroptosis *via* regulating TLR4. (A) Level of intracellular lipid peroxidation after TLR4 overexpression. (B) Effect of TLR4 overexpression on the intracellular iron level determined using an iron colorimetric assay kit. (C) Effect of TLR4 overexpression on the levels of ferroptosis-related proteins determined using Western blotting. $^{*}P$<0.001 *vs*. the control; $^{\#\#\#}P$<0.001 *vs*. the CVB3 group; $^{\&\&}P$<0.01, $^{\&\&\&}P$<0.001 *vs*. the CVB3 + ICA + oe-NC group.

adenoviral receptor on the surface of susceptible cells such as cardiomyocytes[18] and then enters the intercellular tight junction. Alterations in the membrane structure occur upon receptor-mediated release of viral RNA into the cytoplasm, and then RNA takes over the gene expression machanism in the host cell for replication[19]. IFN has a certain therapeutic effect on inflammatory cardiomyopathy and viral myocarditis caused by enteroviruses or adenoviruses. Trial participants with enterovirus-positive myocarditis showed viral clearance after IFNβ treatment[20]. Antiviral drugs like pocapavir and pleconaril, along with immunoglobulin injections, have proven effective in neonates with enteroviral myocarditis[21,22]. Novel technologies and emerging therapies are expected to address current limitations soon.

Increasing evidence suggests that the inflammatory response caused by the body's innate immunity and adaptive immune response triggered by viral infection play a pivotal role in aggravating myocardial inflammatory injuries[23]. The irreversible myocardial damage caused by the release of abnormal cytokines during the innate immune response may be a critical factor. Following infection, innate immune cells as well as cardiomyocytes are activated through recognition by pattern recognition receptors, including TLR4[24]. Activated innate immune cells and cardiomyocytes release cytokines, and chemokines, leading to further activation and recruitment of innate immune cells to the heart. While innate immune response activation in the heart can be beneficial due to its antiviral effects, excessive or persistent activation can lead to chronic inflammatory processes, triggering myocardial destruction and remodeling, ultimately leading to cardiac dysfunction[25]. In this study, icariin was found to inhibit TLR4 and reduce the levels of various inflammatory cytokines. This suggests that icariin can block key pro-inflammatory pathways in cardiomyocytes. Additionally, icariin can effectively reduce CVB3-induced iron-dependent peroxidation, jointly promoting cell protection. The involvement of ferroptosis in the pathology of viral myocarditis has been verified in humans. For example, ferroptosis markers were detected in myocardial tissue from cardiogenic shock triggered by COVID-19[26]. Ferroptosis inhibitors have been found to reduce the viral load of human enterovirus and coronavirus[27]. Nevertheless, this study is still in its initial stage, and animal experiments are required to verify the mechanism.

Taken together, this study demonstrates that icariin reduces CVB3-induced inflammation and ferroptosis in AC16 cardiomyocytes by inhibiting TLR4. These findings suggest the potential of icariin in alleviating viral myocarditis.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Data availability statement

The data supporting the findings of this study are available from the corresponding authors upon request.

Authors' contributions

WL and YL contributed to conceptualization, investigation, visualization, and draft. JHD, PL, YH, WXL, and CLH contributed to investigation and analysis. All authors approved the final version.

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