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CEACAM-1 promotes myocardial injury following coxsackievirus infection by regulating the coxsackievirus-adenovirus receptor

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Abstract

Objective: To determine the effects and mechanism of carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM-1, CC1)-mediated regulation of the Coxsackie and Adenovirus Receptor (CAR) after Coxsackievirus B3 (CVB3) infection.

Methods: A mouse CC1 overexpression recombinant virus was constructed, followed by insertion of a pLVX-CEACAM 1-zsgreen-puro (rLV-CEACAM 1) plasmid into the recombinant retrovirus. Cardiac myocytes were assigned into different groups according to various treatments. The apoptosis rate and cell activity in each group were observed. Further, CAR expression and SYK, IL-1β, and p-SYK levels were measured.

Results: The recombinant retrovirus titer was measured as 1.5×10^8 TUs/ml. The apoptosis rate of cardiac myocytes in the CC1 overexpression plus CVB3 group was significantly elevated, and the relative expression of the *CAR* gene was the highest in the CC1 overexpression plus CVB3 group. TNF-α and IL-1β levels increased due to CC1 overexpression and further increased after CVB3 infection. CAR protein expression also changed along with the levels of CC1, SYK, and TNF-α after infection.

Conclusion: CC1 may promote CAR expression after CVB3 infection and regulate CAR protein expression by activating the CC1-SYK-TNF-α signaling axis during the infection process.

Abbreviations: BCA = bicinchoninic acid, CAR = Coxsackie and Adenovirus Receptor, CEACAM-1, CC1 = Carcinoembryonic antigen-related cell adhesion molecule 1, CVB3 = Coxsackievirus B3, DCM = Dilated cardiomyopathy, FBS = Fetal Bovine Serum, IL = Interleukin, LSD = least significant difference, PBS = Phosphate Buffered Saline, SYK = Spleen tyrosine kinase, TNF = Tumor Necrosis Factor.

Keywords: Carcinoembryonic antigen-related cell adhesion molecule 1, Coxsackie and Adenovirus Receptor, Coxsackievirus B3, Dilated Cardiomyopathy

1. Introduction

Dilated cardiomyopathy (DCM) is the predominant cause of heart failure in adults. One of the main contributors to the pathogenesis of DCM is Coxsackie and Adenovirus Receptor (CAR)-mediated Coxsackievirus B3 (CVB3) infection, which causes secondary myocardial injury.[1] Studies on numerous experimental mouse models of CVB3 infections have suggested the presence of 2 distinct stages in the course of myocarditis.[2] CVB3 replication can induce myocardial injury through apoptosis and necrosis, causing cardiomyopathy.[1,3] Complete inhibition of CAR can effectively reduce CVB3 infection and myocardial damage; however, this might have side effects whereby cardiac development and conduction may be affected.[4] Recently, research has gradually focused on the regulation of CAR, which is an ideal way to effectively control CVB3 infection and mitigate subsequent damage to the myocardium.

At present, research on CAR regulation mainly focuses on regulation of the variable conformation of its extracellular domain. The carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM-1, CC1) is deemed as an important regulator of virus-specific cellular functions in mice and humans, which may be involved in the process of CVB3 infection and regulation of CAR expression in cardiac tissues or cells.[5] Evidence has shown that the junctional adhesion molecule-like protein (JAML), which is expressed by polymorphonuclear neutrophils, regulates transmigration via binding interactions with epithelial CAR.[6] CC1 had similar variable conformations in the extracellular domain of CAR, and their interactions can affect the activity of related signaling molecules. Furthermore, CC1 can promote SYK dephosphorylation and inhibit TNF-α

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The authors declare no conflict of interest.

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secretion by neutrophils. Further, CAR expression could be down-regulated by stimulation of umbilical vein endothelial cells with inflammatory cytokines IL-1 and TNF-α.[7] Based on these findings, our study hypothesized that CC1 could regulate CAR expression by activating the CC1-SYK-TNF-α signaling axis.

2. Material and methods

2.1. Cell processing and grouping

The study was approved by Panyu Central Hospital Ethics Committee (2017–11). Specific-pathogen free (SPF) grade mice were sacrificed by cervical dislocation. Myocardial cells were cultivated and infected with the recombinant virus. The cells were assigned to the following groups, according to various treatments, after culture in a box with 5% CO_2 conditions for 48 hours: CC1 normal group, CC1 overexpression group, CC1 normal plus CVB3 group, and CC1 overexpression plus CVB3 group. The myocardial cells were infected with CVB3 after 2 hours of treatment with 1 μM BAY (SYK inhibitor), as necessary. Cells were also assigned to the CC1 normal plus SYK inhibitor group and CC1 overexpression plus SYK inhibitor group to explore the mechanism underlying CC1-mediated regulation of CVB3-induced myocardial injury. Additionally, the CC1 normal plus TNF-α receptor antagonist group was established to elucidate CVB3-mediated regulation of the TNF-α receptor signaling pathway.

2.2. Main experimental methods

cDNA was used as the template for PCR amplification of the target gene *CEACAM-1*, and the restriction sites of EcoRI and Not I were introduced at both ends of the primer. PCR reactions were performed according to the manufacturer's protocol. The DNA band of the target gene *CEACAM 1* (about 1377 bp) was recovered by 1% agarose gel electrophoresis. Subsequently, bands obtained after the EcoRI+Not I digestion were recovered after 3 hours of treatment with the pLVX-mCMV-ZsGreen-IRES-Puro Vector plasmid at 37°C in a water bath. The pLVX-mCMV-ZsGreen-IRES-Puro plasmid recycles the connection between the large segment and the CEACAM-1 segment. Single colonies were selected, inoculated in LB culture solution, and identified by enzyme cutting after plasmid extraction. The sequencing primer used was: CMV-F (5′-CGCAAATGGGCGGTAGGCGTG-3′). Following, transfection with the pLVX-CEACAM 1-ZsGreen-Puro (rLV-CEACAM 1) recombinant lentivirus plasmid was performed. After 48 hours of recombinant virus infection, the old culture medium was removed, and the cells were washed thrice with PBS. After the CVB3 virus solution was diluted appropriately, the cells were infected with the virus solution at a multiplicity of infection (MOI) of 10. After 1 hour of CVB3 infection, the cells were washed with PBS and cultured in fresh DMEM sugar culture medium containing 10% FBS at 37°C and 5% CO_2 in an incubator.

2.3. AnnexinV-PE/7-AAD staining and CCK8 assay

First, 50 μl of Binding Buffer and 7-ADD solution were added. After this reaction, 450 μl of PBS and 1 μl of Annexin V-PE were added to the cells. The PE excitation wavelength was Ex = 488 nm, and the emission wavelength was Em = 578 nm. For 7-AAD, the excitation and emission wavelengths were 546 nm and 647 nm, respectively. After 48 hours of CVB3 infection, 100 ml of culture medium containing 10% CCK8 solution were added, and the cells were cultured for 2 to 4 hours at 37°C and 5% CO_2. The OD450 was measured, and the apoptosis rate and cell proliferation activity were calculated.

2.4. ELISA

After preparing the mother liquor of the standard products, 100 μl standard samples were added. We washed each hole, and repeated the process twice for a total of 3 washes. Then we filled each hole with a wash buffer (200 μl) and removed the properties of the liquid after 2 minutes. After cleaning, we removed all residues by inhaling or decanting the wash buffer. We turned the board upside down and dried with a clean paper towel.100 μl each of HRP-anti-biotin protein, substrate, and termination solution were added. Subsequently, absorbance of the samples was measured at 450 nm. The curve was drawn after subtracting the value of blank holes from the standard and sample values. The data for TNF-α and IL-1β were analyzed using the ELISA Calc software.

2.5. Quantitative real-time PCR (qRT-PCR)

A solution containing 2 μg of RNA was added to each PCR tube. A quantitative PCR reaction was performed, and expression levels of *CAR* and *TNF-α* genes and relative loads of CVB3 were measured. Total RNA was extracted by adding TRIzol after CVB3 infection. After the RNA mass and concentration were determined, the cDNA was synthesized by reverse transcription reaction in a solution of 2 μg RNA. Using synthetic cDNA as a template, a quantitatve PCR was used to detect CAR and CVB3. The results were analyzed by $2^{-\Delta\Delta CT}$ method. The primers used to detect *CAR* genes were:CAR-F: 5'- GGAGTGTGTG-TAGCGTGTATTG-3', CAR-R: 5'- GCACAGCACCTGAAGA-GATTAG-3'; the primers used to detect CVB3 were: CVB3-F: 5'-CGGTACCTTTGTGCGCCTGT-3', CVB3-R: 5'- CAGGCCGC-CAACGCAGCC-3'; the primers of β-actin in the reference gene were: *β-actin-F*: 5'- GAGGTATCCTGACCCTGAAGTA-3', *β-actin-R*: 5'- CACACGCAGCTCATTGTAGA-3'.

2.6. Western blotting (WB)

A suitable amount of BCA liquid was prepared according to the sample quantity, and the protein concentration was calculated. After developing and fixing the protein bands, the optical density value of each target band was analyzed using the Image J software processing system. Expression of the CAR, SYK, and p-SYK proteins was measured. Interaction between CC1 and CAR was detected by protein immunoprecipitation.

2.7. Statistical analysis

All data are presented as the means ± SD. Differences between multiple groups were analyzed using one-way ANOVA, followed by the Fisher least significant difference (LSD) test. The significance level was set at $P < .05$.

3. Results

3.1. Recombinant virus packaging

The sequencing primer used was CMV-F (5′-CGCAAA TGGGCGGTAGGCGTG-3′). The CEACAM-1 overexpression

vector was constructed successfully. The recombinant virus titer measured at 1.5×10^8 TUs/ml (Fig. 1).

3.2. Effect of CEACAM-1 overexpression on CVB3-induced cardiac myocyte apoptosis

The CC1 overexpression plus CVB3 group showed the highest level of cardiac myocyte apoptosis, followed by the CC1 normal plus CVB3 and the CC1 overexpression groups. The CC1 normal group showed the lowest level of apoptosis. All differences were statistically significant ($P < .05$) (Fig. 2A, 2B, and Table 1).

3.3. Comparison of TNF-α and IL-1β levels by ELISA

TNF-α and IL-1β levels before infection were significantly higher in the CC1 overexpression group than in the CC1 normal group. Further, TNF-α and IL-1β levels were significantly higher in the 2 groups after CVB3 infection. The highest levels of TNF-α and IL-1β were seen in the CC1 overexpression group plus CVB3 group, and these differences between the 4 groups were statistically significant ($P < .05$) (Fig. 3A, 3B).

3.4. Comparison of CAR and TNF-α levels and quantification of CVB3 relative loads by qRT-PCR

The design and synthesis of primers are shown in Table 2. RNA amplification indicated that CAR mRNA expression in the CC1 overexpression group was significantly higher than that in the CC1 normal group. Further, CAR mRNA expression increased after infection with CVB3, and the relative load of CVB3 in the CC1 overexpression group was higher ($P < .05$) (Fig. 4A, 4B). CAR and TNF-α levels were the highest in the CC1 overexpression plus SYK inhibitor group and lowest in the CC1 normal group. All differences were statistically significant, indicating that CC1 could activate the CC1-SYK-TNF-α-CAR signaling axis ($P < .05$) (Table 3). Further, the CAR mRNA expression levels were higher in the SYK inhibitor and TNF-α receptor antagonist groups after CVB3 infection ($P < .05$) (Table 4).

3.5. Comparison of CAR, SYK, and p-SYK proteins by WB

Protein immunoprecipitation results demonstrated an interaction between CC1 and the CAR protein (Fig. 5A). CC1 overexpression increased expression of the CAR protein; however, expression of p-SYK and SYK proteins was decreased (Fig. 5B). After CVB3 infection, CAR protein expression changed according to differing levels of CC1, SYK, and TNF-α (Fig. 5C). These results revealed that CC1 regulated CAR expression by activating the CC1-SYK-TNF-α CAR pathway.

4. Discussion

Viral myocarditis (VMC) and DCM caused by CVB3 infection pose serious risks to human health.[8] Ongoing studies on the intervention of CVB3 infection to reduce myocardial cell damage have been reported.[9,10] CVB3 replication can induce myocardial injury through apoptosis and necrosis of cardiomyocytes. Proteins generated from translation of the viral genome can significantly affect the structure and functions of cellular proteins in various ways, including shutdown of host proteins, cleavage of transcription factors, etc.[1] Our experimental results showed that

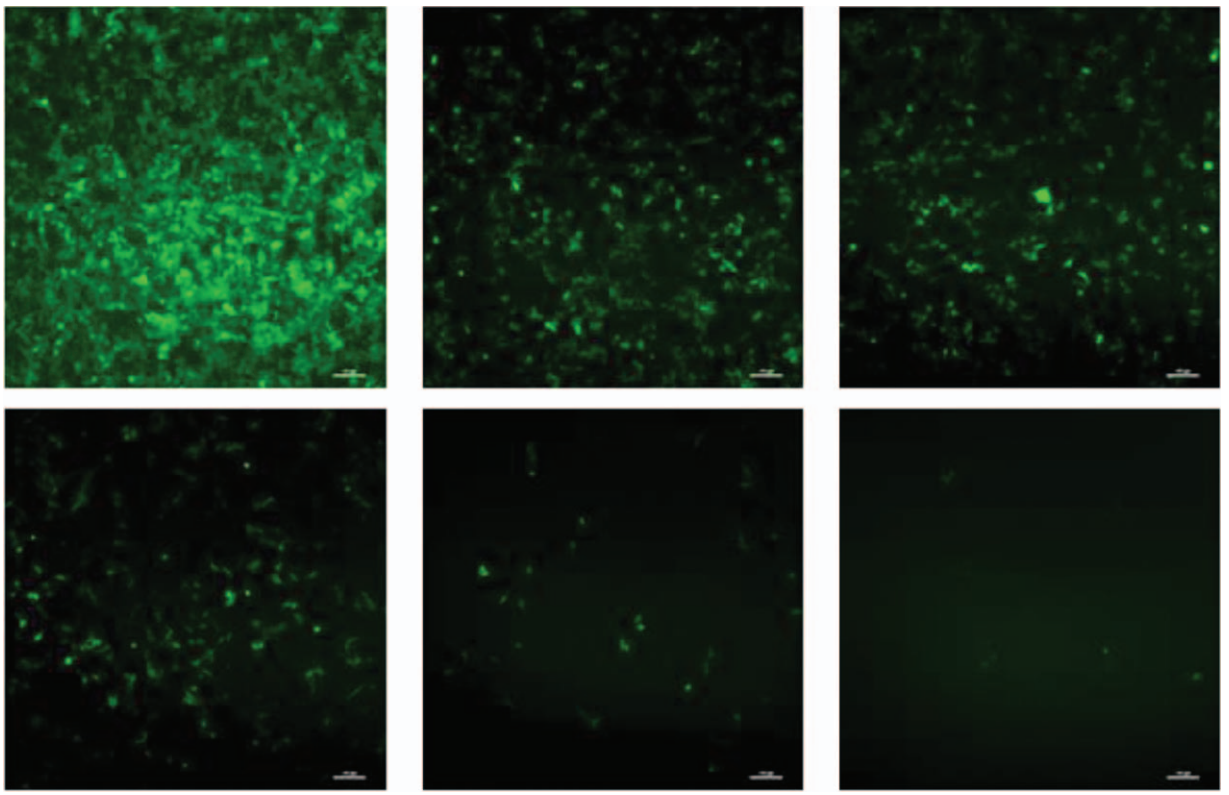

Figure 1. Determination of recombinant virus titer, The recombinant virus titer was found to be 1.5×10^8 TUs/ml.

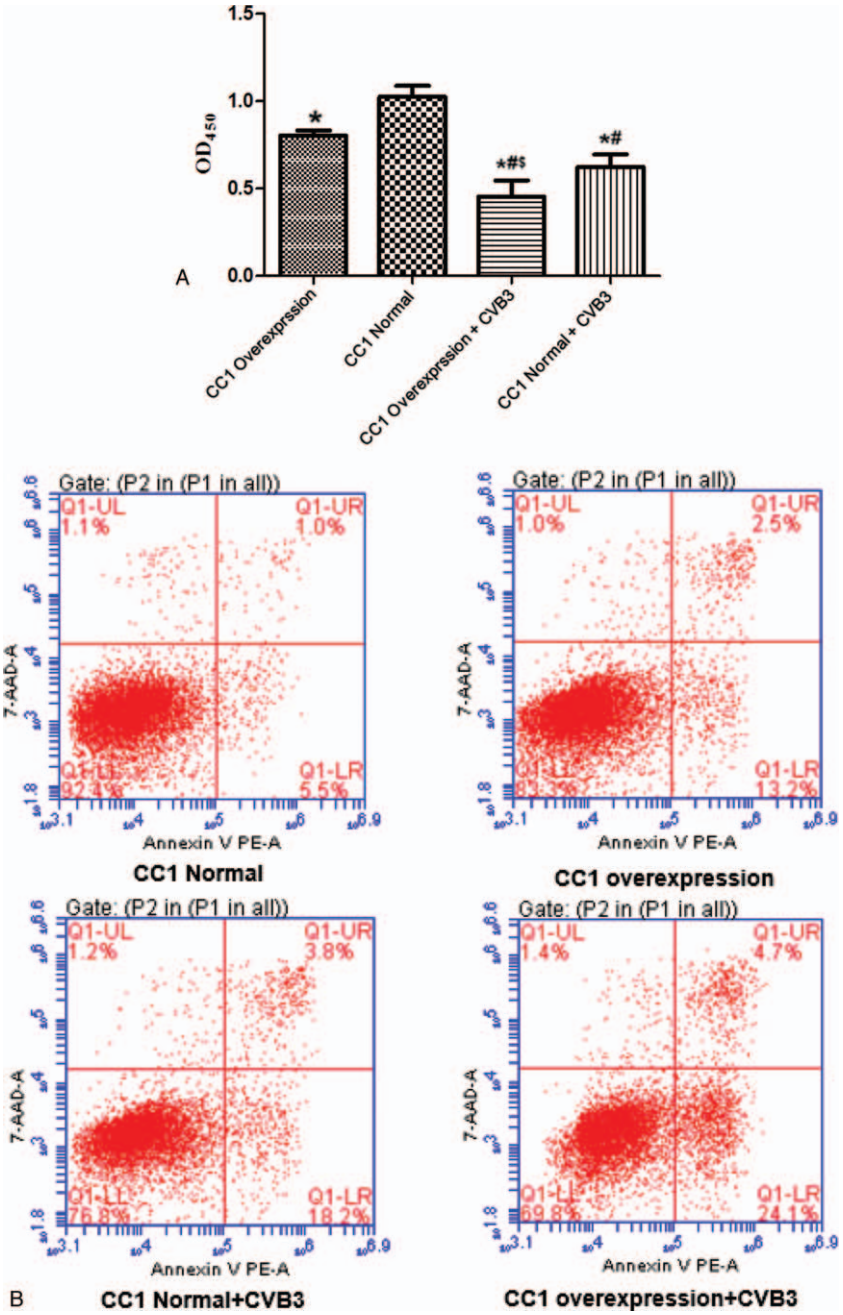

Figure 2. Effect of CEACAM-1 overexpression on CVB3-induced cardiac myocyte apoptosis. A. Cardiomyocyte proliferation measured by CCK8. B. Flow cytometry was applied to investigate the apoptosis rate after different kinds of treatment. n = 6. $^{*}P < .05$ vs CC1 normal group, $^{\#}P < .05$ vs CC1 overexpression group, $^{\$}P < .05$ vs CC1 normal +CVB3 group. $^{*}P = .038$; $^{\#}P = .026$; $^{\$}P = .031$.

Table 1

The OD450 of each group (Mean ± SD. n = 6).

A	B	C	D
0.804 ± 0.025	1.026 ± 0.057*	0.455 ± 0.084*	0.624 ± 0.066*

A = CC1 Overexpression group; B = CC1 Normal group; C = CC1 Overexpression+ CVB3 group; D = CC1 Normal + CVB3 group.
$P^{B} = .038$; $P^{C} = .026$; $P^{D} = .031$.
* $P < .05$ *vs* other groups.

the apoptosis rate of myocardial cells was significantly greater after CVB3 infection than that before, and the OD450 was decreased, suggesting that CVB3 induced secondary myocardial injury. Therefore, effective control of CVB3 infection remains the key to eliminate myocardial damage.

Since the mutability of CVB3 makes it difficult to control virus infection, the effect of viral infection can be limited by RNA interference.[11] Based on this method, regulating CAR may be an ideal way to interfere with its binding to viruses. Previous studies have shown that CC1 has variable conformation similar to the extracellular domain of CAR, and their interactions can affect the

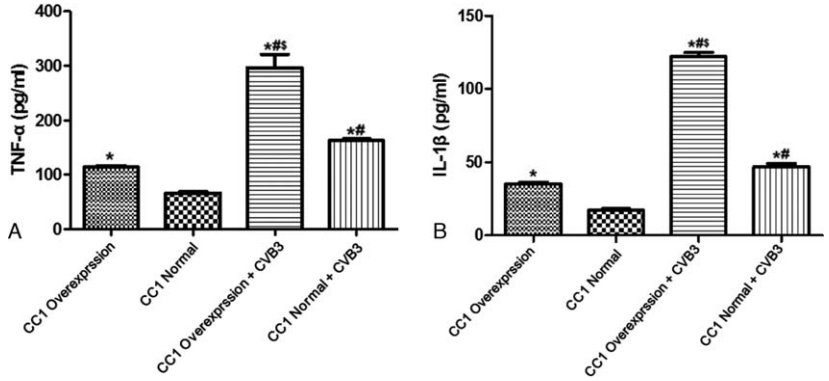

Figure 3. Comparison of TNF-α and IL-1β levels by ELISA. A. TNF-α level; B. IL-1β level. n = 6. *$P < .05$ vs CC1 normal group, #$P < .05$ vs CC1 overexpression group, $$P < .05$ vs CC1 normal+CVB3 group. TNF-α: *$P = .030$; #$P = .015$; $$P = .042$; IL-1β: *$P = .036$; #$P = .017$; $$P = .045$.

Table 2

Sequences of the primers.

Gene	ID		Sequences (5'-3')	Length (bp)
Beta-actin	NM_007393.5	Forward:	GAGGTATCCTGACCCTGAAGTA	104
		Reverse:	CACACGCAGCTCATTGTAGA	
CAR	NM_001025192.3	Forward:	GGAGTGTGTGTAGCGTGTATTG	130
		Reverse:	GCACAGCACCTGAAGAGATTAG	
CVB3	MG451802.1	Forward:	CGGTACCTTTGTGCGCCTGT	135
		Reverse:	CAGGCCGCCAACGCAGCC	
TNF-α	NM_013693.3	Forward:	CTGAGTTCTGCAAAGGGAGAG	107
		Reverse:	CCTCAGGGAAGAATCTGGAAAG	

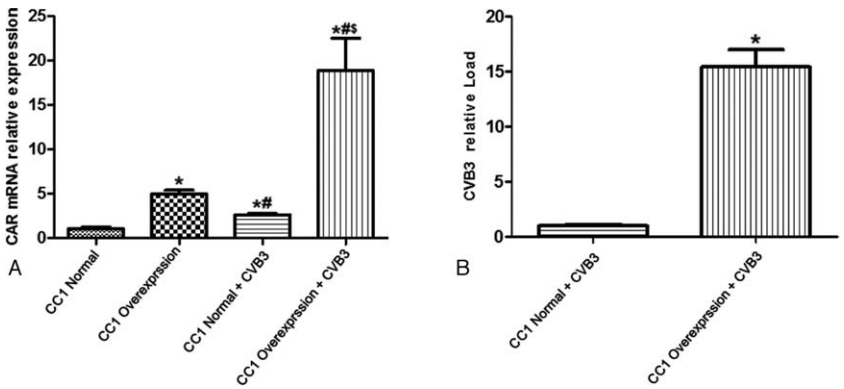

Figure 4. Comparison of the levels of CAR and the quantification of the relative loads of CVB3 by qRT-PCR. A. CAR mRNA relative expression in different groups; B. CVB3 relative load. n = 6. *$P < .05$ vs CC1 normal group, #$P < .05$ vs CC1 overexpression group, $$P < .05$ vs CC1 normal +CVB3 group. B. CVB3 relative load. *$P < .05$ vs CC1 normal +CVB3 group. CAR mRNA: *$P = .007$; #$P = .044$; $$P = .009$; CVB3 relative load: *$P = .008$.

Table 3

CAR and TNF-α expression measured by qPCR.

Gene	1	2	3	4
CAR	1.00 ± 0.12	5.46 ± 1.34*	2.33 ± 0.61*	7.03 ± 1.27*
TNF-α	1.01 ± 0.18	5.40 ± 1.49*	2.06 ± 0.37*	2.41 ± 0.78*

CAR: $P^2 = .016$; $P^3 = .033$; $P^4 = .036$.
TNF-α: $P^2 = .019$; $P^3 = .030$; $P^4 = .035$.
($2^{\wedge(-\triangle\triangle Ct)}$ Mean ± SD. n = 6).
* $P < 0.05$ νs other groups. 1 = CC1 Normal group; 2 = CC1 Overexpression group; 3 = CC1 Normal + SYK inhibitor group; 4 = CC1 Overexpression + SYK inhibitor group.

Table 4

CAR expression measured by qPCR after CVB3 infection.

Gene	a	b	c
CAR	1.02 ± 0.25	4.15 ± 0.36*	1.82 ± 0.46*

$P^b = .019$; $P^c = .034$.
($2^{\wedge(-\triangle\triangle Ct)}$ Mean ± SD. n = 6).
* $P < .05$ νs other groups. a = CC1 Normal group; b = CC1 Normal + SYK inhibitor group; c = CC1 Normal + TNF-α receptor antagonists group.

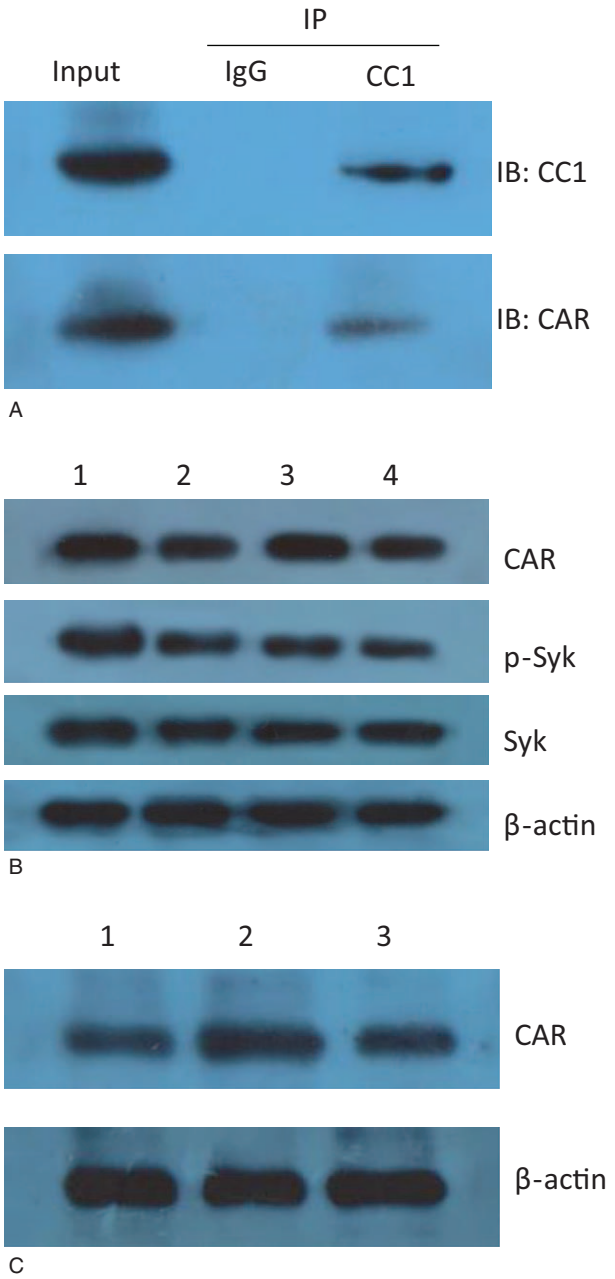

Figure 5. Comparison of the CAR, SYK, and p-SYK proteins by WB. A. Co-IP was used to detected the interaction between CC1 and CAR; B. Western blot was used to measure the level of CAR, p-SYK, and SYK. 1=CC1 normal group; 2=CC1 overexpression group; 3=CC1 normal+ inhibitor group; 4=CC1 overexpression+ inhibitor group; Western blot was used to measure the level of CAR in cardiomyocyte infected with CVB3. 1=CC1 normal group; 2=CC1 normal+ SYK inhibitor group; 3=CC1 normal+ TNF-α receptor antagonists group.

relevant signaling activity of CC1.[12] In this study, after adjusting CC1 expression in the CVB3 infection group, it was found that the apoptosis rate of CC1 overexpression was higher than that of myocardial cells with normal CC1 expression, while the apoptosis rate of myocardial cells increased due to CVB3 infection. Results of the CCK8 assay showed that the OD450 in the CC1 overexpression group was lower than that in the CC1 normal group, while that of the CC1 overexpression group was lowest among the 4 groups after CVB3 infection, suggesting that CC1 may participate in the process of CVB3 infection of cardiac cells.

In this study, CAR mRNA was detected by qPCR, and it was found that CAR mRNA levels in CC1-overexpressing cells were higher than cells with normal CC1 expression and uninfected cells after CVB3 infection. The relative expression of CVB3 in the CC1 overexpression group was more than the normal CC1 expression group. CAR protein expression as detected by WB was consistent with the CAR mRNA results. These results suggest that CC1 can directly affect CAR or indirectly regulate CAR expression by activating cell signaling pathways, promoting CVB3 infection of cardiac myocytes, and causing myocardial damage. Previous studies have indicated that CC1 in neutrophils can dephosphorylate SYK and inhibit TNF-α secretion.[13] CC1 can inhibit the inflammatory response of alveolar epithelial cells induced by *Neisseria gonorrhoeae*, inhibit activation of T lymphocytes and release of inflammatory factors, and become the intervention target of inflammatory bowel disease.[14] The level of inflammatory factors is closely related to CAR expression. Further, the expression of CAR can be down-regulated by stimulation of umbilical vein endothelial cells with cytokines IL-1 and TNF-α.[15] Similar results were found in our study. TNF-α and IL-1β levels were higher in the CC1 overexpression group before infection and significantly higher after CVB3 infection. Additionally, CAR protein expression varied along with changes in CC1, SYK, and TNF-α levels. Therefore, we concluded that CC1 may regulate CAR expression by activating the CC1-SYK-TNF-α signaling axis.

Limitation of our study is the sample size was relatively small compared to other studies. Despite this, our study supports CC1 might regulate CAR expression and activate the CC1-SYK-TNF-α signaling axis after CVB3 infection.

5. Conclusion

In this study, a CEACAM-1 overexpression vector was constructed, and the effects of CC1 overexpression post CVB3 infection were analyzed. The results suggested that CC1 may promote CAR expression in cardiomyocytes after CVB3 infection, and CAR may be a potential target for CC1 to regulate the CVB3-induced process of myocardial injury. CC1 might regulate CAR expression by activating the CC1-SYK-TNF-α signaling axis.

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Methodology: Mingcai Song, Yulan Zhang.
Project administration: Yulan Zhang.
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