Original article

Antiviral and myocyte protective effects of IL-28A in coxsackievirus B3-induced myocarditis

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ABSTRACT

Objective: This study aimed to investigate whether interleukin-28A (IL-28A) plays a role in murine myocarditis induced by coxsackievirus B3 (CVB3), and to explore its possible mechanism involved.

Methods: Male BALB/c mice both infected and not infected by CVB3 were randomly divided into four groups: 1) untreated; 2) treated with different doses of IL-28A for 4 days, and then sacrificed on day 7 post-infection. The heart samples were collected for histopathologic examination. Cardiac viral load was determined by a plaque assay. Additionally, immuneobioanalysis, TUNEL assay, and immunohistochemistry were performed to examine the expression of signal transducer, activator of transcription 1 and 2 (STAT1 and STAT2), and STAT1 and STAT2 induced apoptosis and the expression of Bcl-2, BAX and Caspase-3.

Results: Compared to uninfected mice, the CVB3 infected mice exhibited higher mortality rate (p < 0.001), apparent inflammation and myocardial lesion (p < 0.01), and higher cardiac viral load (p < 0.01). After CVB3 infection, IL-28A treated mice presented no death (p < 0.001), reduced inflammation and myocardial lesion (p < 0.01), and lower viral load (p < 0.01) compared to untreated mice. Besides, treatment with IL-28A markedly increased the expressions of STAT1 and STAT2, and inhibited CVB3-induced apoptosis in myocardial cells with increased ratio of Bcl-2/BAX.

Conclusion: The antiviral and myocyte protective effects of IL-28A in CVB3-induced myocarditis are regulated by STAT1 and STAT2.

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Introduction

Myocarditis is a cardiac disease associated with inflammation and injury of the myocardium. Several types of viruses have been implicated in the development of human myocarditis. However, coxsackievirus B3 (CVB3) is considered the most common pathogen. CVB3-induced myocarditis is frequently complicated by cardiomyopathy, left ventricular dysfunction, and chronic heart failure. The murine model of CVB3-induced...
myocarditis shares many features with human disease. Despite decades of extensive efforts, the pathogenesis of CVB3-induced myocarditis is still not well defined. Although CVB3 causes cellular dysfunction either through induced cell apoptosis, shut down of cell RNA and protein synthesis or viral protease cleavage of contractile proteins,2 but the strong host Th1 immune responses may be more responsible for the course of myocyte damage, verified by the improvement of heart injury and function by immune modulating and inhibiting agents.8,9 Clinical studies have also found cytokines such as IL-1β, IL-6 and TNF-α that were involved in the course of cardiac dysfunction.11 Besides, the poor prognosis for more than half of patients presenting with clinical symptoms, since currently no effective therapy is available for this disease,12 much work on the development of vaccines and therapeutic agents against CVB3 has been done. 

Human IL-28A, IL-28B, and IL-29, also known as interferon (IFN)-λ2, λ3 and λ1, respectively, belong to the type III IFNs.14 Like other typical IFNs, these cytokines have been also demonstrated to play a key role in the negative regulation of cytopathic viruses such as encephalomyocarditis virus (EMCV),15 vesicular stomatitis virus (VSV),16 cytomegalovirus (CMV),16 influenza A virus (IAV),17 respiratory syncytial virus (RSV), hepatitis B virus (HBV),18 hepatitis C virus (HCV),18 and herpes simplex virus-2 (HSV-2).19 A growing number of previous reports have suggested that type III IFNs exert antiviral activity by the induced upregulation of IFN effector proteins such as STAT proteins.14,20 However, a recent study has shown that certain types of viruses can develop mechanisms to inhibit the antiviral activity of type III IFNs.21 This study aimed to investigate whether IL-28A plays a role in murine myocarditis induced by CVB3, and to explore its possible mechanism involved by determining the expressions of STAT1 and STAT2, Bcl-2, BAX, and Caspase-3 in CVB3-infected heart tissues. It will provide a reference for the development of vaccines and therapeutic agents against CVB3 that is human.

Materials and methods

Mice, CVB3 infection and IL-28A treatment

The research protocol for this study was approved by the ethical committee of Wuhan University School of Medicine. Animal handling was carried out according to the Wuhan Directives for Animal Research. A total of 240 healthy male BALB/c mice (4-week old), weighing 10–12 g, were purchased from the Animal Center of Hubei Province (Wuhan, Hubei, China) and had free access to food and water during the experiments. Among them, 160 randomly selected mice were inoculated intraperitoneally with CVB3 (Nancy strain) at a dose of 10⁵ plaque-forming units (pfu) in a volume of 100 µL using phosphate buffered saline (PBS) as a vehicle, and divided evenly into 4 groups (n = 40), including infected but untreated group, low-dose, mild-dose and high-dose IL-28A treated groups. Starting 1 h after infection, these CVB3 inoculated mice received intraperitoneal injection with PBS, 10, 20, or 40 µg/kg of recombinant murine IL-28A (Peprotech, Rocky Hill, NJ), respectively, followed by daily subcutaneous injection for 4 days. The 80 uninfected mice, divided evenly into two groups that were either intraperitoneally inoculated with PBS (n = 40, control group) or 40 µg/kg IL recombinant murine IL-28A (n = 40, IL-28A group). Ten mice from each group were euthanized on days 4 and 7 post-infection, respectively. The hearts were exposed, perfused with 5 mL PBS through the left ventricle, and then isolated. Approximately 10 mg of tissues from cardiac apex were excised and weighed for plaque assay, whereas the remainder of the heart was fixed in 10% neutral formalin for histopathologic examination as described below. The remaining 20 mice of each group were monitored for health status and survival analysis.

Histopathology

The hearts were fixed in 10% neutral formalin for 10 min, embedded in paraffin, and then cut into 4 µm-thick sections (about four sections per heart). Then the sections were stained with hematoxylin and eosin (H&E) and used to assess inflammatory infiltration and myocardial lesion or necrosis. The extent of inflammation and generalized or focal cardiac fiber necrosis were blindly scored by a pathologist based on the following scale: 0, absent; 0.5, rare; 1, 1–10% of myocardial area affected; 2, 25–50% area affected; 3, 50–75% area affected; 4, >75% area affected. The two scores reflecting the extent of inflammation and myocardial lesion were then averaged to yield an integrated pathology (IP) score as an indicator of overall injury.22

Viral plaque assays

The viral plaque assay was performed as previously described to determine cardiac viral load.22 One piece of heart apex (10 mg) was removed aseptically, weighed, and stored at −80 °C. Then the frozen heart tissue was homogenized in 200 µL PBS and centrifuged at 12,000 rpm for 5 min. Viral titer, expressed as pfu/mL, was determined from the supernatant of the heart homogenate by a plaque assay on HeLa cell monolayers according to previous study.23

Western blot analysis

Western blot analysis was performed as described in the literature to evaluate the expressions of STAT1 and STAT2.24 The ventricles of mice were homogenized in a lysis buffer consisting of 10 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM sodium-orthovanadate, 20 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 0.2% Nonidet P-40, 5 µg/mL leupeptin, and protease inhibitor cocktail (Sigma, St. Louis, MO). The homogenates were centrifuged at 12,000 rpm for 10 min at 4 °C, and supernatants were collected to determine the protein concentrations using the DC protein assay (Bio-Rad, Hercules, CA). The equal amounts of protein (40 µg) from each sample were separated by sodium dodecyl–poly acrylamide gel electrophoresis (SDS-PAGE) using 4–12% NuPAGE Novex precast Bis-Tris gradient gels (Invitrogen, Carlsbad, CA) and transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA). The membranes were blocked in 5% BSA for 40 min at room temperature and then incubated individually at 4 °C overnight with primary antibodies, including rabbit polyclonal antibodies against STAT1 (1:1000) and actin (1:2000), and mouse monoclonal antibodies against
STAT2 (1:200) (Cell Signaling Technology, Inc., Danvers, MA). After three washings with TBST, membranes were incubated at room temperature for 40 min with the secondary antibodies including horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:10,000) and goat anti-mouse IgG (1:5000) (Santa Cruz Biotechnology, Inc., Dallas, TX). The signals from the bound antibodies were detected using the SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce, Rockford, IL).

**Immunohistochemistry**

Immunohistochemistry was performed to assess the expressions of Bcl-2, BAX and Caspase-3 according to the instructions of the manufacturer. Briefly, paraffin-embedded myocardial sections were deparaffinized in xylene and rehydrated in a series of graded concentrations of ethanol. Protein antigenicity was enhanced by boiling the sections in a citrate buffer with microwave for 15 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in PBS for 20 min at 37 °C. Then the sections were washed with distilled water, and incubated with the primary antibodies against BAX (1:100), Bcl-2 (1:100), and caspase-3 (1:100) (Santa Cruz Biotechnology, Inc., Dallas, TX) at 4°C overnight, followed by staining with a streptavidin–biotin–peroxidase kit (Beijing Zhongshan Biotechnology Co., Ltd, Beijing, China). The immunoreactions were then visualized with a 3,3-diaminobenzidine solution (Beijing Zhongshan Biotechnology Co., Ltd, Beijing, China), counterstained with hematoxylin, and mounted. For each control tissue samples were visualized under a light microscope (Eclipse E800, Nikon, Japan) and five photomicrographs were taken from high power fields (400× magnifications) randomly. The average optical density (AOD) from the photomicrographs was then quantitated using the high resolution pathological image analysis system (HPIAS-1000; Qinhong Corporation, Wuhan, China) to measure protein expression levels of Bcl-2, BAX and Caspase-3. The ratio of BAX to Bcl-2 was determined individually from each photograph accordingly.

**TUNEL assay**

TUNEL (terminal deoxynucleotidyl transferase-mediated 20-deoxyuridine 5-triphosphate–biotin nick end labeling) staining on paraffin-embedded sections was used to evaluate the effect of IL-28A on CVB3-induced apoptosis according to the instructions provided by the manufacturer. Briefly, 4 μm-thick sections were dewaxed, rehydrated, and incubated with terminal deoxynucleotidyl transferase enzyme in a humectous chamber at 37 °C for 1 h. The reaction was detected by incubation with antidigoxigenin-peroxidase for 30 min in a humidified chamber at room temperature and visualized in a buffer containing diaminobenzidine. The specimens were counterstained by immersion in hematoxylin. The stained tissue samples were observed using a fluorescence microscope (Olympus BX 40, Japan) and five photomicrographs were obtained from high power fields (400× magnifications). The cells with apparent nuclear labeling were considered TUNEL positive. The percentage of TUNEL-positive cells in the total cell population was then quantitated to assess the apoptosis index.

**Fig. 1 – IL-28A protected mice from CVB3-induced myocarditis for surviving. The survival rate was analyzed by the Kaplan–Meier estimate. The IL-28A treated mice had higher survival rates than the untreated infected group following CVB3 infection (p < 0.001).**

**Statistical analysis**

Data were presented as means ± SD. One-way analysis of variance (ANOVA) was used to determine differences between individual mice for IP scores and myocardial viral titers. The survival rate was analyzed by the Kaplan–Meier estimate. All statistical analyses were performed using SPSS 13.0 (SPSS Inc., Chicago, IL). Any p-values <0.05 were considered statistically significant.

**Results**

**IL-28A treatment protects mice from CVB3 infection-associated death**

Uninfected BALB/c mice appeared healthy whereas CVB3-inoculated mice displayed signs of serious illness, including ruffled and dirty fur, soft feces, lethargy, and huddling behavior. Twelve mice in the infected group died after infection, with six, five and one mice dying on days 6, 9 and 12 post-infection, respectively. However, treatment with IL-28A improved mice’ general appearance, and completely prevented CVB3 infection-associated death up to 14 days post-infection, even with the lower dose of IL-28A (10 μg/kg). The Kaplan–Meier analysis clearly showed much higher survival rate in IL-28A treated mice than untreated mice (infected group) following CVB3 infection (Fig. 1) (p < 0.001).

**IL-28A treatment inhibits CVB3 infection-induced inflammation and myocardial lesion**

Histological examination of the H&E-stained slides showed evidence of lymphocyte infiltration and myocardial necrosis on day 7 after CVB3 infection (Fig. 2A) compared to controls.
IL-28A decreases the IP score of CVB3-infected mice. (A) Infected and CVB3-infected mice were treated with or without 40 μg/kg IL-28A for 4 days. Heart tissue was removed on day 7 post-infection and subjected to H&E staining (200× magnification) to examine myocardial inflammation and injury. The arrow indicated a site of lymphocyte infiltration in the heart tissue following CVB3 infection. (B) Infected BALB/c mice were treated with or without 40 μg/kg IL-28A. CVB3-infected mice were exposed to different doses of IL-28A for 4 days. Heart tissue was then removed on day 7 post-infection and subjected to H&E staining to quantitate IP scores. Treatment with IL-28A significantly reduced the IP scores in the hearts of IL-28A treated mice compared with infected mice (**p < 0.01).

However, the severity of myocardial inflammation was significantly reduced in IL-28A treated group (40 μg/kg). As shown in Fig. 2B, CVB3 infection increased the IP score to 2.81 ± 0.22, while in uninfected control and IL-28A groups the basal score was 0, with inflammation or generalized myocardial fiber necrosis undetected (N.D.). Despite no effect on uninfected mice, IL-28A treatment reduced the IP score of CVB3-infected mice in a dose-dependent manner (p < 0.01). A dose of 40 μg/kg almost completely eliminated cardiac inflammatory infiltration and protected cardiac myocytes from damage, with the average IP score on day 7 post-infection being reduced to 0.4 ± 0.1 (p < 0.01) compared with the infected group. Treatment with a low-dose IL-28A (10 μg/kg) also statistically reduced the IP score compared with the untreated infected group, but at a level inferior to that with 20 μg/kg IL-28A.

IL-28A reduces cardiac viral load

Inoculation of CVB3 in BALB/c mice resulted in considerably high levels of cardiac viral load (6432 ± 291 pfu/mg) detected by the plaque assay on day 4 post-infection (Fig. 3A), which then spontaneously declined to 550 ± 50 pfu/mg heart tissue on post-infective day 7 (Fig. 3B). As expected, treatment with IL-28A dose-dependently reduced cardiac viral load four days after CVB3 infections (p < 0.01), even at the low dose (10 μg/kg). On post-infective day 7, there was a further reduction in cardiac viral load in mice treated with IL-28A compared with that of untreated infected group (p < 0.01, Fig. 3B).

IL-28A upregulates the expression of STAT1 and STAT2

Treatment with 40 μg/kg IL-28A dramatically increased the protein levels of STAT1 and STAT2 in the heart tissues of mice 4 days after CVB3 infection (Fig. 4). No significant difference was observed in the levels of STAT1 and STAT2 between untreated infected and control groups.

IL-28A inhibits CVB3-induced apoptosis of the myocardial cells in mice

Following CVB3 infection, apoptosis was triggered with a significant increase in apoptosis index in the infected mice (0.7%) compared to control group (0.1%) (p < 0.01, Fig. 5). However, a significant decrease in apoptotic index was observed in IL-28A
Fig. 3 – IL-28A decreased viral load of heart tissue in CVB3-infected mice. Uninfected and CVB3-infected mice were treated with or without IL-28A for 4 days. Heart tissue of infected mice were removed on day 4 (A) and day 5 (B) post-infection and subjected to plaque assays. Treatment with IL-28A significantly reduced myocardial viral load compared with infected mice ("p < 0.01").

(0.39%) treated mice compared to the untreated infected group (p < 0.01, Fig. 5), suggesting that treatment with IL-28A could inhibit CVB3-induced apoptosis in myocardial cells.

As shown in Fig. 4, infection with the CVB3 substantially decreased the expression levels of Bcl-2 and upregulated the expressions of BAX and Caspase-3 compared to controls, while IL-28A treatment had a reverse effect on the expression levels of these apoptosis-related proteins, with increased expression levels of Bcl-2 and decreased expression of BAX and Caspase-3. The quantitative data further showed that CVB3 infection induced a 2.5-fold down-regulation of Bcl-2 expression, 5-fold upregulation of BAX expression, as well as a 10-fold decrease in the ratio of Bcl-2 to BAX compared with uninfected control mice (Fig. 6B and C). Treatment with IL-28A upregulated the expression of Bcl-2 by 5-fold, down-regulated the expression of BAX by 2.5-fold, and consequently increased the ratio of Bcl-2 to BAX by 10-fold compared to the untreated group following CVB3 infection (Fig. 6B and C).

Discussion

It was demonstrated that CVB3 infection in humans frequently progresses to cardiomyopathy, left ventricular dysfunction, and subsequent chronic heart failure. Despite the growing knowledge about viral myocarditis, it remained challenging to manage patients with viral myocarditis due to lack of effective antiviral therapeutic regimens. In this study, the results showed that IL-28A protected mice from CVB3-induced death, inhibited viral replication in heart tissues following CVB3 infection, and reduced the induced inflammation and myocardial lesion.

From the results, no inflammation or generalized myocardial fiber necrosis was detected (N.D.) in uninfected control and IL-28A groups (40 μg/kg IL), suggesting that this cytokine does not cause apparent side effects in mice. A relatively low dose (10 μg/kg) of IL-28A was sufficient to reduce CVB3-induced myocarditis in mice as evidenced by significantly decreasing IP scores and cardiac viral load, let alone treatment with 40 μg/kg IL-28A, which almost completely inhibited cardiac inflammatory infiltration and protected myocytes from injury with IP score decreased to 0.4 ± 0.1. It seemed that IL-28A was a highly effective and tolerable antiviral agent against CVB3-induced myocarditis, although further clinical trials will be conducted to ensure its effectiveness and safety in human patients.

Consistent with previous reports, this study also demonstrated that viral replication was a dominant pathological process in the early stages of CVB3 infection, as evidenced by a very high viral load with relatively low levels of inflammatory infiltration and tissue injury in the heart on day 4. This was followed by a spontaneous reduction of viral load but a further development of inflammation and injury in cardiac tissue on day 7, which would deteriorate general health, as evidenced by the presence of death on day 6 to 12. The spontaneous decay of viral load might be attributed to an activation
of the innate cellular defense mechanisms including the IFN system. As type III IFNs, including IL-28A, IL-28B and IL-29 are frequently upregulated in response to certain types of virus infections and exhibit antiviral activity in vitro. This study first documented the efficacy of IL-28A in inhibiting CVB3 replication in heart tissues of infected mice. Unlike type I IFNs using IFNAR (composed of subunits IFNAR1 and IFNAR2) as a receptor, type III IFNs signal through a heterodimeric receptor composed of IL-28A (IL-28R1 or CRF2-12) specific to IFN-λ1 and IL-10R (IL-10R2 or CRF2-4) shared with all other IL-10-related cytokines. However, upon binding their specific extracellular receptors, type I and type III IFNs trigger the same Jak/STAT signal transduction pathway, leading to the upregulation of many genes, called IFN-stimulated genes (ISGs).5,13

Our findings demonstrated that IL-28A markedly increased the expression of STAT1 and STAT2, indicating that these two proteins were key antiviral regulators in CVB3-infected heart tissues of mice. It was well documented that activation of STAT1 and STAT2 is essential for antiviral effect of IFNs. Following virus nucleic acids recognized by transmembrane toll-like receptors (TLRs), cytoplasmic DNA sensors and RNA helicases, kinases were activated. These kinases would promote the activation of transcription factors and their subsequent translocation to the nucleus where they stimulated IFN gene transcription. After binding to the cognate receptors, type I and type III IFNs induced the same Jak/STAT pathway to activate STAT1 and STAT2 transcription factors. Then phosphorylated forms of STAT1 and STAT2 were further associated with the DNA-binding protein IFN regulatory factor 9 (IRF-9) to form an IFN-stimulated gene factor 3 (ISGF3) complex, which was also a crucial mediator in the type I interferon receptor (IFNAR) signaling pathway. Then ISGF3 was translocated to the nucleus, bound to IFN-stimulated response elements (ISREs) within the promoters of interferon-stimulated genes (ISGs) and activated the transcription of ISGs. Although the complete signaling cascade initiated by the binding of IFN to its receptor had not yet been fully elucidated, current evidence indicates that the signaling pathways activated by IFN-α/β and IFN-λ may be similar or overlap. Thus, the findings about the expressions of STAT1 and STAT2 expression induced by IL-28A provided a reasonable mechanism responsible for IL-28A-mediated antiviral effect.

As apoptosis was implicated in virus-induced myocardial injury, the effect of IL-28A on CVB3-induced apoptosis was also determined. The results showed that treatment with IL-28A could inhibit CVB3-induced apoptosis in myocardial cells as evidenced by a significant decrease in apoptotic index in IL-28A (0.39%) treated mice compared to the untreated infected group (p<0.01). This could be attributed to the regulating
Fig. 6 – Immunohistochemistry staining of Bcl-2, BAX and caspase-3. IL-28A enhanced the expression of Bcl-2 ($p < 0.01$) and decreased the expression of BAX ($p < 0.01$) and caspase-3 ($p < 0.01$) in heart tissue. Uninfected and CVB3-infected mice treated with or without IL-28A (40 μg/kg) for 4 days, then heart tissue of mice were removed on day 4 post-infection and subjected to immunohistochemistry staining. The immunostained tissue samples were visualized and photographed under a light microscope (A). A total of five photomicrographs were taken from five high power fields (400× magnification) randomly. AOD from the photomicrographs was then quantitated to measure the protein expression levels of Bcl-2 and BAX (B) as described in the Section of Materials and Methods. The ratio of BAX to Bcl-2 was determined individually from each photograph (C).
effect of IL-28A on the expression of caspase-3, BAX and Bcl-2, which were key molecules regulating apoptosis.\textsuperscript{38-40} The Bcl-2 could inhibit apoptosis by binding to BAX while BAX promoted apoptotic cell death by binding to itself.\textsuperscript{41} Specifically, in cells with BAX overexpression, BAX/BAX homodimers predominated, and turning the cells susceptible to induced apoptosis. Conversely, in cells with Bcl-2 overexpression, BAX/Bcl-2 heterodimers predominated and the cells became resistant to apoptosis.\textsuperscript{41} Since BAX, a proapoptotic protein, antagonized the function of Bcl-2, it had been generally considered that the Bcl-2/BAX ratio could determine the susceptibility of a cell to apoptotic stimulus.\textsuperscript{42,43} Besides, caspase-3, a member of the cysteine-aspartic acid protease (caspase) family, acted as the executant of cellular apoptosis.\textsuperscript{44,45} According to the above reports, the result also showed that IL-28A reduced the expressions of BAX and caspase-3 but upregulated Bcl-2 expression, thereby resulting in an increase in the Bcl-2/BAX ratio and subsequent inhibition of CVB3-induced myocardial cell apoptosis in mice. Interestingly, previous studies demonstrated that the activation of STAT1 not only served to increase the expression of Bcl-2\textsuperscript{46} but also negatively regulated BAX expression.\textsuperscript{47} Therefore, it seemed that, in addition to its antiviral effect as discussed above, STAT1 activated by IL-28A might bind to the promoters of Bcl-2 or BAX, contributing to positive and negative regulation of Bcl-2 and BAX expression, respectively. Hence, IL-28A might increase the ratio of Bcl-2/BAX and consequently inhibit CVB3 infection-induced apoptosis of the myocardium in mice by the above mechanism.

In conclusion, this study demonstrated that IL-28A played an important role in the host innate defense against CVB3 infection and could inhibit CVB3-induced myocarditis in mice. The study also suggested that STAT1 and STAT2 are the key antiviral regulators in heart tissues of CVB3-infected mice, with the former IL-28A could increase the ratio of Bcl-2/BAX and consequently inhibit CVB3 infection-induced apoptosis of the myocardium. Thus it can be concluded that IL-28A had a great potential to become a therapeutic intervention for CVB3 infection in humans; however, the precise mechanisms by which IL-28A exerts the antiviral defense still need to be explored in the future.

**Conflicts of Interest**

The authors declare no conflicts of interest.

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