Effect of interleukin-17A on anti-ANT antibodies as well as cytokines in viral myocarditis mice

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Introduction: To observe the effect of interleukin- 17 A (IL-17 A) on serum anti- mitochondrial endosomal ADP/ATP carrier autoantibody (anti-ANT antibody) levels and cytokines in viral myocarditis (VMC) mice.

Methods: Male wild-type (WT) and IL-17A knockout (IL-17A$^{-/-}$) BALB/c mice were intraperitoneally injected with coxsackievirus B3 (CVB3) to establish a VMC model (VMC-WT and VMC-IL-17A$^{-/-}$), while WT BALB/c mice were injected with PBS intraperitoneally to establish a normal control group (WT group). After 14 days, myocardial tissues were taken to calculate heart mass, and paraffin sections were prepared and stained with HE staining to observe the pathological changes and calculate the pathological score of myocardial tissues. Flow cytometry was used to detect the level of CD4^{+} T lymphocytes in peripheral blood, ELISA was used to determine the level of anti- ANT antibody, IL-17 and IL-23 in serum, and Western blotting was used to detect the expression level of IL-17 and IL-23 proteins in myocardial tissue.

Results: Viral myocarditis mice were successfully constructed. Mice in the WT group showed no abnormal activity; Mice in the VMC-WT group gradually showed behaviors such as huddling, shrugging, trembling, and poor response from the third day of injection; Mice in the VMC-IL-17A$^{-/-}$ group showed the above symptoms to a lesser extent. In the VMC-WT group, the HM and pathological score were 5.62 ± 0.27 g/kg and 3.12 ± 0.45 score. The HM and pathologic score in the VMC-IL-17A$^{-/-}$ group were lower than those in the VMC-WT group. The pathological examination of WT mice showed no inflammatory cell infiltration and patchy necrosis. The rats in VMC-WT group showed extensive inflammatory cell infiltration and large sheet necrosis of cardiomyocytes under microscope. Compared with the VMC-WT group, the VMC- IL-17 A$^{-/-}$ group showed focal myocardial necrosis and significantly reduced

inflammatory cell infiltration 14 days after CVB3 infection. At the same time, the percentage of $CD4^+$T was remarkedly lower in the VMC-WT mice compared with the WT mice. The percentage of $CD4^+$T was higher in the VMC-IL-$17A^{-/-}$ group (27.54 ± 3.62) than in the VMC-WT group (16.97 ± 2.18). In addition, anti-ANT antibody (1.48 ± 0.31 μg/L), IL-17 (33.47 ± 4.26 pg/mL) and IL-23 (32.42 ± 4.31 pg/mL) levels were significantly lower in serum of VMC-IL-$17A^{-/-}$ mice than in the VMC-WT mice. At the protein level, the expression of IL-17 and IL-23 showed consistent results.

Conclusion: Serum anti- ANT antibody, IL-17 and IL-23 levels were significantly elevated in VMC mice. IL-17A was involved in the production of anti-ANT antibody in VMC mice, and the knockdown of IL-17A gene could inhibit the development of myocardial inflammation, elevate the level of peripheral blood $CD4^+$ T lymphocytes, and ameliorate myocardial fibrosis in VMC mice.

Keywords: Interleukin-17 A; Viral myocarditis; Anti- ANT antibodies

INTRODUCTION

Viral myocarditis (VMC) is characterized by limited or diffuse inflammatory changes [1]. VMC is a nonspecific inflammatory lesion of the myocardium caused by viral infections, and more than 30 types of viruses are known to exist, with coxsackie virus B3 (CVB3) infection being the most common [2, 3]. CVB3 is a single-stranded positive-stranded small RNA virus, an enterovirus that infects humans via the digestive and respiratory tracts and is the main cause ofVMC in humans [4, 5]. CAR is a complex of CVB-specific receptors and decay-accelerating factors, and its expression is race- and tissue-specific, with high levels of expression in the human heart, pancreas, brain, and small intestine, where it is the main receptor for CVB infection of cardiomyocytes, and has an antigenic recognition and mediating role. The susceptibility to VMC is determined by the level of CAR expression and its distribution. Animal studies have shown that CAR expression is highest in embryonic and neonatal mice and decreases with age. This may be the main reason for the high susceptibility of neonates and children to VMC.DAF is a complement regulatory protein that promotes the binding of CVB to the CAR-DAF receptor complex, increasing the rate of CVB infection, and is thought not to be able to cause infection alone. However, it was found that CVB3 infects human intestinal epithelial cells via DAF rather than CAR, resulting in attenuation of anti-DAF antibody and CVB3 infection of CD4+ T cells. Myocardial fibrosis caused by CVB3 infection is one of the most important causes of cardiac insufficiency due to the lack of contractile function of the fibrous tissue, which changes the structure of the ventricle and affects the diastolic and contractile functions of the heart. In addition, the infectious virus that invades the organism can cause spasm or obstruction of microvessels in the heart through direct damage to vascular endothelial cells, leading

to myocardial ischemic injury and necrosis. Subsequent abnormal repair of myocardial tissue also further exacerbates pathological myocardial fibrosis. Although viral invasion of the myocardium directly destroys cardiomyocytes and causes cardiac tissue damage, a reactive immune response thereafter further leads to cardiomyocyte degeneration and necrosis and tissue damage. Autoimmune responses can be triggered by viral infection, which induces myocytes to express new antigens, as well as by the massive release of self-antigens from injured myocytes. Abnormal repair ofmyocardial tissue after immune injury can also aggravate myocardial fibrosis in VMC, leading to myocardial hypertrophy and cardiac dilatation, and ultimately causing cardiac insufficiency.

In addition to causing sudden death, some patients with VMC may develop chronic myocarditis and dilated cardiomyopathy. The mechanisms of myocardial injury in the pathogenesis of VMC are related to direct viral injury, myocardial injury by inflammatory factors, and immune-mediated myocardial injury caused by viral infections, including autoimmune responses [6]. During the acute phase of VMC, the virus directly invades the myocardium through the bloodstream, replicates and synthesizes viral proteins in large quantities in cardiomyocytes, leading to swelling, degeneration, and necrosis of cardiomyocytes, and then triggers an autoimmune response, which is an important reason for the prolonged and recurrent course of VMC [7, 8]. The clinical manifestations of VMC vary in severity, and the prognosis depends on the virulence ofthe infecting virus, the body's response status, the location and extent of the lesions, and the timeliness of interventions. VMC is most commonly seen in young adults under the age of 40 years, accounting for 75%-80% of the cases, and most of them are in the age group of 20-30 years [9]. VMC has a high mortality rate, which can reach 25% in children and 75% in infants [10]. The majority of adults with VMC recover with appropriate treatment. A small percentage ofpatients die in the acute phase of the disease due to acute heart failure, malignant arrhythmias, and cardiogenic shock. In a small number of patients, the disease continues to progress and gradually transforms into chronic viral myocarditis. The final stage is dilated cardiomyopathy, which is associated with severe arrhythmias, cardiac failure, and cardiogenic shock, resulting in a poor prognosis and a high mortality rate. According to the data, the incidence rate of VMC is 0.12%~12.00% in the world, and the incidence rate in China is slightly higher, which is 5%~15%, and the trend is increasing year by year. At present, clinical treatment is mainly symptomatic, and there is no effective drug. Clinical treatment for VMC still lacks specificity, so early intervention is crucial. Interleukin 17A (IL-17A) is an important inflammatory transmitter secreted by helper T cell 17 (Th17). Recently, IL-17A has been found to play a key role in a variety of viral infectious diseases. More and more studies have concluded that IL-17 can promote inflammation by stimulating T cells and other immune cells to produce various inflammatory cytokines, chemo activators, and cell adhesion molecules [11]. Liu indicated that IL-17A is involved in the pathogenesis of respiratory syncytial virus pneumonia, and the administration of IL-17A-neutralizing antibodies to RSV-infected mice resulted in a significant reduction in inflammatory response and a significant

decrease in viral load [12]. In a study of the relationship between IL-17A and influenza virus, IL-17A knockout mice were found to have higher viral loads and reduced morbidity and mortality [13]. Previous studies have indicated that IL-17A is involved in the progression of VMC in mice, and that the application of IL-17A antibody reduces CVB3 virus replication. However, the mechanism involved has not been fully confirmed. In this study, we used IL-17Aknockout mice (IL-17A$^{-/-}$) to block the action of IL-17A at the genetic level, and investigated the effect of IL-17A on CVB3 virus resurgence in VMC mice and its possible mechanisms by establishing a VMC model.

MATERIALS AND METHODS

1.1 Materials

Six-week-old SPF-grade male IL-17A$^{-/-}$ BALB/c mice and male wild-type (WT) BALB/c mice were purchased. All mice were housed in the animal house of the Experimental Center of the Second Hospital of Hebei Medical University. The CVB3 strain was provided by the Department of Microbiology, Hebei Medical University. ELISA kits were purchased from Suzhou Calvin Bio Co.

1.2 VMC model and grouping

Based on previous literature, male BALB/c mice were divided into VMC-WT group (10 mice), VMC-IL-17A$^{-/-}$ group (10 mice) and WT group (10 mice). Mice in VMC-WT group and VMC-IL-17A$^{-/-}$ group were injected with CVB3 virus solution 0.1-1.0 mg/kg with10^{-5} TCID50 into the abdomen. After 14 d, each group of mice was killed and the spleen and heart were aseptically removed, and the heart mass (HM)was recorded (HM= Heart weight/Body weight).

1.3 Histopathologic examination of myocardium

The myocardial tissues of each group of mice were stained with HE after 14 d. The myocardial tissues were examined under the microscope (×400). 5 fields ofview were randomly selected, and the ratio of the area of inflammatory cell infiltration in each field of view to the total area under the microscope was calculated. The statistical pathology score was 0 for no myocardial damage, 1 for lesion (<25%), 2 for lesion (25%-50%), 3 for lesion (50%-75%), and 4 for lesion (>75%).

1.4 Measurement of anti-ANT antibodies and cytokines

$CD4^+$ T-lymphocytes in peripheral blood were measured by flow cytometry. The levels of anti-ANT antibody and IL-17 and IL-23 in the serum of each group of mice were measured by ELISA according to the instructions of the kit, and the expression levels of IL-17 and IL-23 proteins in myocardial tissues were detected by Western blot.

1.5 Statistical methods

The obtained data were imported into SPSS27.0 software for analysis. Measurement

data were expressed as (x- ± s), and the independent sample t-test was used for the comparison between multiple groups, and the paired sample t-test was used for the comparison between two groups. Statistical significance was taken as $P < 0.05$.

RESULTS

1.6 Heart mass and myocardial histopathology score

Mice in the WT group showed no abnormal activity; Mice in the VMC-WT group gradually showed behaviors such as huddling, shrugging, trembling, and poor response from the third day of injection; Mice in the VMC-IL-17A$^{-/-}$ group showed the above symptoms to a lesser extent. The HM in the WT group was 4.42 ± 0.3 g/kg, and no myocardial damage was observed. In the VMC-WT group, the HM and pathological score were 5.62 ± 0.27 g/kg and 3.12 ± 0.45 score. The HM and pathologic score in the VMC-IL-17A$^{-/-}$ group were lower than those in the VMC-WT group (Table 1, $P < 0.05$).

Table 1 Results of HM and histopathology score

Group	HM (g/kg)	Histopathology score
VMC-IL-17A$^{-/-}$ (n=10)	4.66 ± 0.25**	1.24 ± 0.23**
VMC-WT (n=10)	5.62 ± 0.27$^{\#\#}$	3.12 ± 0.45$^{\#\#}$
WT (n=10)	4.42 ± 0.3	0.00 ± 0.00

1.7 Histopathologic manifestations of myocardial

In the WT group, there was no obvious enlargement of the myocardium and no inflammatory cell infiltration or sheet necrosis on pathological examination, while in the VMC-WT group, extensive inflammatory cell infiltration and large sheet necrosis of cardiomyocytes were observed under microscope. Focal necrosis of the myocardium was seen microscopically in the VMC-IL-17A$^{-/-}$ group 14 d after CVB3 infection, and the infiltrating inflammatory cells were remarkedly reduced compared with those in the VMC-WT group (Figure 1).

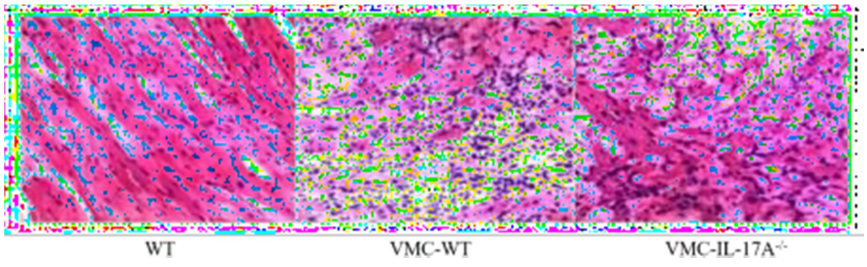

Figure 1 Histopathologic examination of myocardium (HE×400)

1.8 $CD4^+$ T-lymphocyte levels

The percentage of peripheral blood CD4+T in the WT group was 39.73 ± 4.92%. The CD4+T ratio was obviously lower in the VMC-WT group compared with the WT group. The CD4+T ratio was higher in the VMC-IL-17A-/- group than in the VMC-WT group (Table 2, Figure 2).

Table 2 Levels of $CD4^+T$

Group	$CD4^+T$ (%)
VMC-IL-17A$^{-/-}$ (n=10)	27.54 ± 3.62**
VMC-WT (n=10)	16.97 ± 2.18$^{\#\#}$
WT (n=10)	39.73 ± 4.92

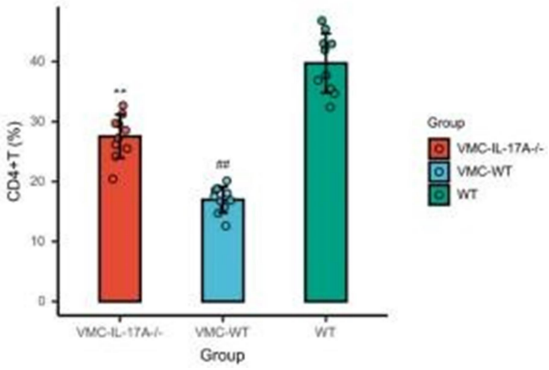

Figure 2 Levels of peripheral blood $CD4^+T$

1.9 Levels of Anti-ANT antibodies, IL-17 and IL-23 in serum

Serum anti-ANT antibody (3.46 ±1.13 μg/L), IL-17 (67.26 ± 8.35 pg/mL) and IL-23 (57.48 ± 6.31 pg/mL) levels were significantly increased in the VMC-WT group compared with the WT group (1.51 ± 0.48 μg/L; 29.52 ± 4.72 pg/mL; 21.37 ± 3.41 pg/mL). Anti-ANT antibody (1.48 ± 0.31 μg/L), IL-17 (33.47 ± 4.26 pg/mL) and IL-23 (32.42 ± 4.31 pg/mL) levels were obviously lower in the VMC-IL-17A$^{-/-}$ group than in the VMC-WT group (Table 3).

Table 3 Levels of Anti-ANT antibodies, IL-17 and IL-23 in serum

Group	Anti-ANT (μg/L)	IL-17 (pg/mL)	IL-23 (pg/mL)
VMC-IL-17A$^{-/-}$ (n=10)	1.48 ±0.31**	33.47 ± 4.26**	32.42 ± 4.31**
VMC-WT (n=10)	3.46 ±1.13$^{\#\#}$	67.26 ± 8.35$^{\#\#}$	57.48 ± 6.31$^{\#\#}$
WT (n=10)	1.51 ± 0.48	29.52 ± 4.72	21.37 ± 3.41

1.10 Expression levels of IL-17 and IL-23 proteins in myocardial tissue

At the protein level, the expression of IL-17 and IL-23 showed consistent results. The expression of IL-17 and IL-23 in myocardial tissues was obviously lower in the VMC-IL-17A$^{-/-}$ group than in the VMC-WT group.

Table 4 Expression levels of IL-17 and IL-23 in myocardial tissue

Group	IL-17	IL-23
VMC-IL-17A$^{-/-}$ (n=10)	0.35 ± 0.05**	0.4 ± 0.14**
VMC-WT (n=10)	0.56 ± 0.07$^{\#\#}$	0.64 ± 0.11$^{\#\#}$
WT (n=10)	0.21 ± 0.06	0.23 ± 0.04

DISCUSSION

Viral myocarditis is a clinical disease in which myocardial inflammation is caused mainly by CVB3 infection of cardiac tissue [14]. Some patients may develop severe arrhythmias, heart failure, and death from cardiogenic shock. In a small number of patients, the disease may develop into a prolonged or chronic viral myocarditis and eventually into dilated cardiomyopathy [15]. In addition, viral myocarditis is one of the main factors of sudden death in young adults. Viral myocarditis is a complex inflammatory response in which multiple cytokines are involved [16]. In addition to the classical Th1/Th2 cells that mediate the immune damage caused by viral myocarditis, the helper T lymphocyte subset 17 (Th17) cells also play an important role in viral myocarditis, mainly by regulating the levels of various inflammation-related factors, which leads to the destruction of tissues and the infiltration of inflammatory cells to promote the inflammatory response of tissues [17, 18]. Th17 cells can secrete IL-17, which is the main component of inflammation. Th17 cells can secrete various cytokines (such as IL-17 and IL-23), and IL-23 can promote Th17 activation and secretion of IL-17. In this study, we used IL-17A knockout mice to establish a VMC mouse model and

observed the effect of IL-17A on the level of serum anti-ANT antibody in VMC mice. The results suggested that IL-17A was involved in the pathogenesis of VMC mice by promoting the secretion of anti-ANT antibody.

The study concluded that the pathogenesis of VMC in mice can be divided into three stages [19]. In the first stage, the acute viral resuscitation phase, the virus causes direct damage to myocardial cells; in the second stage, the subacute immune response phase, which lasts from a few days to about 2 weeks after infection, the cellular and humoral immune responses are activated, the level of inflammation in the myocardial tissues reaches a peak, and the immune-inflammatory cascade mediates the pathological damage to myocardial tissues. Thereafter, the recovery phase is entered if there is no persistent viral infection. If the virus persists, the third stage, chronic cardiomyopathy, occurs, and the persistent immune-inflammatory response accelerates the process of cardiac remodeling, leading to DMC, which has a poor prognosis. Our results found that VMC-IL-17A-/- mice infected with CVB3 virus for 14 days showed focal myocardial necrosis and significantly reduced inflammatory cell infiltration compared with the VMC-WT group.

The proportion of various types of T lymphocytes in the subpopulation of T lymphocytes can reflect the immune function of the body, and the increase of $CD4^+$ T lymphocytes represents the enhancement of the immune function. The CD4+ T cells can be differentiated into subpopulations of cells such as Thl, Th2, Th17, regulatory T cells (Treg), etc., which participate in the inflammatory response through the secretion of cytokines, such as TNF-alpha, IL-6, IL-17, and so on. These cell subpopulations participate in the inflammatory response by secreting cytokines such as TNF-α, IL-6, and IL-17. Our results showed an obvious increase in the proportion of CD4+ T in IL-$17A^{-/-}$ VMC mice compared with VMC mice. ANT proteins are embedded in the inner mitochondrial membrane of cardiomyocytes ADP/ATP channel proteins, which play an important role in maintaining the homeostasis of energy metabolism in cardiomyocytes [20, 21]. ANT is organ-specific and exists specifically in the mitochondria of metabolically active cells such as heart, kidney, and liver. Its function is to transfer ATP synthesized in the mitochondria to the cytoplasm for cellular use, and at the same time transfer ADP generated after ATP metabolism to the mitochondria as raw material to synthesize ATP again by the mitochondria. therefore, ANT plays a particularly critical role in maintaining the cellular energy cycle and cellular function. In CVB3-induced VMC mice, because of the similar antigenic determinants between ANT and CVB3 viral proteins, the immune system produces CVB3 protein antibodies and at the same time cross-reacts with homologous epitopes on ANT to produce anti-ANT antibodies[22]. Anti-ANT antibody not only disrupts the balance of energy metabolism in cardiomyocytes, but also binds to calcium channel proteins on the cardiomyocyte membrane, causing intracellular calcium overload, which together lead to chronic injury of cardiomyocytes [23]. The role of anti-ANT antibody in myocardial injury is not clear. It is currently believed that mitochondrial dysfunction and cardiomyocyte calcium overload are important pathogenic mechanisms. Anti-ANT antibody directly

binds to the mitochondrial ANT of cardiomyocytes, forming antigen-antibody complex precipitation, which causes ANT to lose its normal activity, and the mitochondria cannot transport ATP to the cytoplasm, leading to the dysregulation of cellular energy metabolism. In this study, anti-ANT antibody can be an important indicator of the activity and severity of VMC. Our results showed that anti-ANT antibody levels were reduced in IL-17A$^{-/-}$VMC mice compared with wild-type VMC mice, suggesting that IL-17A is associated with the production of anti-ANT antibodies.

IL-23, a pro-inflammatory cytokine discovered in 2000, belongs to the IL-12 superfamily and is a heterodimeric molecule composed of P19 and P40 subunits bound by covalent disulfide bonds [24]. IL-23 and IL-12 both contain P40 subunits, but P19 is unique to IL-23 and is inactive when present alone.IL-23 is mainly expressed in dendritic cells and macrophages. Although IL-23 has various biological roles, its most important function is to promote the secretion of IL-17 by Th17 cells [25]. IL-17, produced mainly by Th17 cells, is a homodimer of 155 amino acids with a relative molecular mass of 3,000 and a signaling polypeptide with 19-23 amino acid residues at theN-terminal end, whose gene is localized on chromosome 2q31 [26]. In recent years, the gene is located on chromosome 2q31, and the gene is found on chromosome 2q31. Studies have shown that the IL-23/IL-17 inflammatory axis has a powerful pro-inflammatory effect, recruiting inflammatory cells such as neutrophils to the site of inflammation and stimulating the production of various pro-inflammatory transmitters by T-cells, fibroblasts, macrophages and epithelial cells, such as IL-1, IL-6, IL-8, tumor necrosis factor (TNF)-α, nitric oxide synthase 2, monocyte chemotactic protein-1, growth regulator alpha, metalloproteinases and chemoactivators, which further amplify the inflammatory response [27-29]. In addition, IL-17 can synergize with various inflammatory factors to enhance its inflammatory effects, such as promoting granulocyte-macrophage colony-stimulating factor secretion after interaction with TNF-α. Numerous studies have confirmed that cytotoxic T-lymphocyte-mediated cytotoxicity and inflammatory cytokines secreted by Th cells are important mechanisms leading to myocardial immune injury in VMC. We found that IL-17 and IL-23 expression was significantly elevated in serum and myocardial tissues of VMC-WT mice 14 d after virus inoculation. IL-17 and IL-23 expression was significantly lower in serum and myocardial tissue of IL-17A$^{-/-}$ mice compared with VMC-WT mice.

Conclusion

IL-17A knockout inhibited the generation of myocardial inflammation in VMC mice. HM and pathological scores were reduced and pathological injury was attenuated in IL-17A knockout mice. In addition, IL-17A$^{-/-}$ mice had increased levels ofperipheral blood CD4^{+} T lymphocytes, decreased serum levels of anti-ANT antibodies, and suppressed expression of IL-17 and IL-23 in serum and myocardial tissue.

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Data Availability

The experimental data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declared that they have no conflicts of interest regarding this work.

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