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*J. Immunol.* 2010;185;4004-4010; originally published online Aug 27, 2010; doi:10.4049/jimmunol.1001718

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Th17 Cells Contribute to Viral Replication in Coxsackievirus B3-Induced Acute Viral Myocarditis

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Acute viral myocarditis (AVMC) is characterized by virus-triggered myocardial inflammation, and Coxsackievirus B3 (CVB3) is the primary pathogen. We previously proved that Th17 cells, besides having proinflammatory effects, were involved in AVMC by enhancing humoral response. However, the relationship between Th17 cells and CVB3 replication remains unknown. In this experiment, we infected BALB/c mice with CVB3 for establishing AVMC models and then found that, with the increase of viral replication, the expressions of splenic Th17 cells, serum IL-17, and cardiac IL-17 mRNA were elevated significantly, accompanied by the progressive cardiac injuries of AVMC. Furthermore, on day 5, the peak time for viral replication, correlation was positive between cardiac IL-17 mRNA and CVB3 RNA (correlation index = 0.835; p < 0.01). Although the expressions of Th1 and CD8 T cells, which could secrete the antiviral cytokine IFN-γ and damage the heart, were also elevated, along with Th17 cells, in AVMC, the neutralization of IL-17 further upregulated the percentages of splenic Th1 and CD8 T cells and the levels of cardiac IFN-γ mRNA. The cardiac pathological changes were obviously improved after neutralization, with reduced viral replication followed by decreases in the cardiac inflammatory cytokines IL-17, TNF-α, and IL-1β. These data suggest that Th17 cells contribute to CVB3 replication in AVMC, and that IL-17 might be an important target for regulating the balance of antiviral immunities. The Journal of Immunology, 2010, 185: 4004–4010.

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cute viral myocarditis (AVMC), a precursor of dilated cardiomyopathy leading to heart failure, is a triphasic disease involving an initial viral infection, followed by autoimmune response and finally remodeling of cardiac structure and function (1). Coxsackievirus B3 (CVB3), an enterovirus of the Picornaviridae family, is the primary pathogen of viral myocarditis (2). Although an excessively activated immune response triggered by the virus is the dominant cause of myocyte injury, the virus itself also plays an important role in the development of AVMC through direct attack on myocardial cells (3).

Recently, the unique CD4 Th cell subset called Th17 cells have been shown to be involved in several inflammatory diseases, including experimental autoimmune myocarditis, experimental autoimmune encephalomyelitis, and collagen-induced arthritis (4). Besides the proinflammatory effect of Th17 cells, our earlier research suggested that Th17 cells could facilitate the production of autoantibodies in the development of AVMC (5, 6). However, the relationship between Th17 cells and CVB3 replication remains unknown in AVMC.

Therefore, in the current study, we investigated the dynamic alterations of Th17 cells, viral replication, and related inflammatory factors in the course of AVMC. At the same time, the IL-17 mAb was given to AVMC mice to further explore whether and how Th17 cells are associated with viral replication in CVB3-induced AVMC.

Materials and Methods

Mice

Male BALB/c mice aged 4 wk were purchased from the experimental animal research center (Hubei province, China). All animals were kept in the pathogen-free mouse room in the experimental animal center (Tongji Medical College of Huazhong University of Science and Technology, Wuhan, China), and all experiments were carried out in accordance with guidelines for the Care and Use of Laboratory Animals (Science and Technology Department of Hubei Province, China, 2005).

Virus

The CVB3 (3m strain) was prepared by passage through Hela cells. The virus titer determined by PFU assay of HeLa cells was 1 × 107. BALB/c mice were infected by an i.p. injection of 0.2 ml RPMI 1640 (Life Technologies, Carlsbad, CA) containing ~105 PFUs of the virus for establishing AVMC models.

Interventions and groups

BALB/c mice were randomly divided into four groups: 1) in the AVMC group (n = 24), mice were treated with CVB3 and saline (100 μl per mouse) i.p. on day 0; 2) in the IL-17 mAb group (n = 24), mice were treated with CVB3 and anti-mouse IL-17 mAb (100 μg per mouse; eBioscience) i.p. on day 0; 3) in the isotype control group (n = 24), mice were treated with CVB3 and isotype control IgG1Ab (100 μg per mouse; eBioscience) i.p. on day 0; and 4) in the normal group (n = 24), BALB/c mice were not administered anything else. On days 3, 5, 7, and 10 of the experiment, six animals of each group were randomly assigned to each intervention.

Abbreviations used in this paper: AVMC, acute viral myocarditis; cTn, cardiac troponin I; CVB3, Coxsackievirus B3; HW/BW, heart weight to body weight; R, correlation index; RORγt, retinoic acid-related orphan receptor γt; T-bet, transcription factor T-box expressed in T cells.
chosen to be killed separately, and their hearts and spleens were removed aseptically as fresh specimens to be measured. Meanwhile, the ratio of heart weight to body weight (HW/BW) was also assessed. Before the mice were killed, their blood was collected via retro-orbital bleeding and the serum was then prepared for study.

**Histopathology**

The ventricular tissues of the heart were fixed in 10% phosphate-buffered formalin, then trimmed and embedded routinely in paraffin. Next, 5-μm sections were cut longitudinally and stained with H&E. For CVB3-induced myocarditis, the severity of impairment was assessed as the percentage of the heart section with inflammation compared with the overall size of the heart section, using a microscope eyepiece grid with magnification ×200 according to the following scoring system: grade 0, no involvement; grade 1, <25% of the heart section is involved; grade 2, 25–50%; grade 3, 50–75%; grade 4, >75% (7). The assessment was scored by two independent researchers separately in a blinded manner.

**ELISA**

The levels of serum IL-17 and IFN-γ in mice were determined by ELISA kits (Bender MedSystems, Vienna, Austria), according to the manufacturer’s instructions. The sensitivity of ELISA kits for IL-17 and IFN-γ was 1.6 pg/ml and 5.3 pg/ml, respectively, and no cross-reactivity was observed in detection. All samples were measured in triplicate.

The determination of serum cardiac troponin I (cTnI), the early sensitive indicator of myocardial injury, was also completed using a high-sensitivity mouse cardiac troponin-I ELISA kit (Life Diagnostics, West Chester, PA), according to the manufacturer’s instructions. The sensitivity of this ELISA kit was 0.156 ng/ml. All samples were measured in triplicate.

**Plaque-forming assay**

Part of the heart was weighed and homogenized in 2 ml PBS. After three freeze-thaw cycles and centrifuging at 2000 rpm for 10 min, the supernatant was absorbed and sequential 10-diluted in RPMI 1640 medium. The

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**FIGURE 1.** Evaluation of the severity of AVMC. A, The ratio of HW/BW in different groups. B, Representative of histopathologic images in heart tissue (H&E, original magnification ×200). C, The pathological scores in different groups. D, The levels of serum cTnI in different groups. Six mice per group were analyzed for each time point. *p < 0.01 versus normal group; **p < 0.05 versus normal group; †p < 0.01 versus AVMC group; ‡p < 0.05 versus AVMC group. Values are mean ± SEM.
HeLa cell monolayers were incubated with the supernatant for 1 h at 37˚C, 5% CO2, in six-well plates, washed in PBS, and covered with 2 ml 0.4% agar, RPMI 1640, and 10% FCS (Life Technologies). After 72 h of cultivation, the monolayers were fixed in 10% phosphate-buffered formalin and stained in crystal violet, and the numbers of plaques were counted. Viral titers were determined by standard plaque formation assay and expressed per organ weight (in grams).

Flow cytometry

Splenic mononuclear cell suspension was prepared from each mouse when it was killed. After three washings, the cells were collected and resuspended at a density of $1.5 \times 10^6$/ml. Then they were stimulated with PMA (20 ng/ml) and ionomycin (1 μg/ml) plus monensin (2 μmol/l) at 37˚C, 5% CO2 of a 24-well culture plate (Corning Costar, Corning, NY) in RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS. At 5 h later, the cells were harvested and stained with FITC-labeled anti-mouse CD4 or FITC-labeled anti-mouse CD8 Ab (eBioscience). After washing, fixing, and permeabilizing according to the manufacturer’s instructions (eBioscience), the cells were stained intracellularly with PE-labeled anti-mouse IFN-γ or allophycocyanin-labeled anti-mouse IL-17A Ab (eBioscience). Incubated at 4˚C for 30 min, the cells were finally washed and measured by FACScalibur flow cytometry (BD Biosciences). Meanwhile, isotype-matched Ab (eBioscience) controls were used to ensure the specificity of the staining. The data were analyzed with CellQuest software (BD Biosciences).

Real-time RT-PCR

The total RNA of the heart tissues was extracted with TRizol reagent (Invitrogen, Carlsbad, CA) and reverse transcribed into cDNA, according to the manufacturer’s protocol. Primers for CVB, IL-17, IFN-γ, IL-1β, TNF-α, and the housekeeping gene β-actin are shown in Table I. After an initial denaturation step at 94˚C for 3 min, a three-step cycle procedure was carried out (denaturation, 94˚C, 30 s; annealing, 58˚C, 30 s; and extension, 72˚C, 30 s) for 40 cycles. All reactions were performed in at least duplicate for each sample. The relative mRNA expressions were normalized to the level of β-actin transcripts. The analysis was conducted in an ABI 7700 Sequence Detection System (Applied Biosystems, Foster City, CA).

Western blot

The total proteins of the heart tissues were extracted with the Total Protein Extraction Kit (Pierce/Thermo Scientific, Rockford, IL). Protein concentration was determined by the BCA Protein Assay Kit (Pierce). Samples containing 50 μg proteins were separated on a 10% SDS-PAGE and electrotransferred onto nitrocellulose membranes. Membranes were sequentially blocked in TBST containing 5% skim milk and then incubated with primary Abs against transcription factor T-box expressed in T cells (T-bet) (1:500; eBioscience), retinoic acid-related orphan receptor γt (RORγt) (1:500; eBioscience), and β-actin (1:1000; Abcam, Cambridge, MA) at 4˚C.

Table I. Sequences of primers for real-time RT-PCR

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<thead>
<tr>
<th>Molecule</th>
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<tr>
<td>CVB sense</td>
<td>CGGTACCTTTTGTGCCGCTGT</td>
</tr>
<tr>
<td>CVB anti-sense</td>
<td>CAGGCCGCGCAACGCAGCC</td>
</tr>
<tr>
<td>IL-17 sense</td>
<td>CCTCAGAATCACTCTCAACCGCT</td>
</tr>
<tr>
<td>IL-17 anti-sense</td>
<td>TCCATGTTGCTGTTCCAGCTTT</td>
</tr>
<tr>
<td>IFN-γ anti-sense</td>
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</tr>
<tr>
<td>IFN-γ sense</td>
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<tr>
<td>TNF-α anti-sense</td>
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<td>IL-1β anti-sense</td>
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<tr>
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FIGURE 2. The levels of serum IL-17 and IFN-γ in AVMC mice, measured by ELISA. A, The levels of serum IL-17 in different groups. B, The levels of serum IFN-γ in different groups. Six mice per group were analyzed for each time point. *p < 0.01 versus normal group; **p < 0.05 versus normal group; *p < 0.01 versus AVMC group. Values are mean ± SEM.

FIGURE 3. The levels of viral replication. A, The levels of cardiac CVB3 RNA on days 3, 5, 7, and 10. B, The levels of cardiac CVB3 titers on day 5. Data represent mean values of CVB3 PFU per gram of heart. Six mice per group were analyzed for each time point. *p < 0.01 versus normal group; **p < 0.05 versus normal group; *p < 0.01 versus AVMC group. Values are mean ± SEM.
overnight. After washing, the membranes were then further incubated with HRP-conjugated secondary Ab (1:5000, 37°C, 2 h). The target bands were finally washed and developed with super ECL reagent (Thermo Scientific) and semiquantitatively analyzed using densitometric methods.

**Statistical analysis**

Data were shown as the mean ± SEM. Statistical analyses of the data were performed with one-way ANOVA, and the correlation between two variables was tested by bivariate correlation analysis using SPSS11.0; p < 0.05 was considered statistically significant.

**Results**

**Evaluations for the severity of AVMC**

On days 3, 5, 7, and 10, no changes were observed in normal controls. The ratio of HW/BW and the pathological scores of heart sections in the AVMC, IL-17 mAb, and isotype control groups were elevated significantly, compared with those in the normal group (all p < 0.05), accompanied by progressive cardiac inflammatory lesions. However, the ratio of HW/BW, the pathological scores of heart sections, and the cardiac injuries in the IL-17 mAb group were lower or less than those in AVMC and isotype control groups (all p < 0.05), and no significant difference was found between AVMC and isotype control groups (Fig. 1A–C).

On days 5 and 7, the levels of serum cTnI in AVMC, IL-17 mAb, and isotype control groups were all elevated, compared with those in the normal group (all p < 0.05). Nevertheless, the levels of serum cTnI in the IL-17 mAb group were lower than those in the AVMC and isotype control groups (all p < 0.01), and no obvious difference was found between the AVMC group and isotype control group (Fig. 1D).

**Measurements of the levels of serum IL-17 and IFN-γ**

No changes were seen in serum IL-17 and IFN-γ in the normal group on days 3, 5, 7, and 10. The levels of serum IL-17 in the AVMC, IL-17 mAb, and isotype control groups were increased dramatically, compared with those in the normal mice, especially on days 7 and 10 (all p < 0.05). However, the levels of serum IL-17 in the IL-17 mAb group were lower than those in the AVMC and isotype control groups (all p < 0.01), and no significant difference was seen between the AVMC group and isotype control group (Fig. 2A).

The levels of serum IFN-γ in the AVMC, IL-17 mAb, and isotype control groups were also obviously raised, compared with those in the normal group, on days 3, 5, 7, and 10, especially on day 7 (all p < 0.01). Moreover, the levels of serum IFN-γ in the IL-17 mAb group were even higher than those in the AVMC and isotype control groups on days 3 and 5 (all p < 0.01), and there was no significant difference between the AVMC and isotype control groups (Fig. 2B).

**Assays for viral replication**

On days 3, 5, 7, and 10, the levels of cardiac CVB3 RNA in the AVMC, IL-17 mAb, and isotype control groups were elevated significantly, compared with those in the normal group, especially for the alteration of CD4+ Th17 cells in different groups. Ad. The results of statistical analysis for the alteration of CD4+ Th1 cells in different groups. Ba. Representative pictures of flow cytometry from each treatment group for CD8+ T cells. Numbers in the right quadrants (upper plus lower) indicate the percent of CD8+ T cells. Bb. The results of statistical analysis for the alteration of CD8+ T cells. Six mice per group were analyzed for each time point. *p < 0.01 versus normal group; **p < 0.05 versus normal group; p < 0.01 versus AVMC group; *p < 0.05 versus AVMC group. Values are mean ± SEM.
on day 5 (all \( p < 0.01 \)). However, the levels of cardiac CVB3 RNA in the IL-17 mAb group were lower than those in the AVMC and isotype control groups on days 3 and 5 (all \( p < 0.05 \)), and no difference was noted between the AVMC group and isotype control group (Fig. 3A, Table I).

On day 5, the levels of cardiac CVB3 titers in the AVMC, IL-17 mAb, and isotype control groups were higher than those in the normal group (\( p < 0.01 \)), and that in the IL-17 mAb group was lower than those in the AVMC and isotype control groups (\( p < 0.05 \)). No difference was found between the AVMC and isotype control groups (Fig. 3B).

At the same time points, cardiac CVB3 was not detected in normal controls (Fig. 3A, 3B).

Investigations for percentages of CD4\(^+\) Th17, CD4\(^+\) Th1, and CD8\(^+\) T cells

On days 3, 5, 7, and 10, the percentages of CD4\(^+\) Th17 cells in the AVMC, IL-17 mAb, and isotype control groups were increased markedly, compared with those in the normal group, especially on days 7 and 10 (all \( p < 0.05 \)). However, the percentages of CD4\(^+\) Th17 cells in the IL-17 mAb group were lower than those in the AVMC and isotype control groups (all \( p < 0.05 \)), and there was no significant difference between the AVMC group and isotype control group (Fig. 4A).

On days 3, 5, and 7, the percentages of CD4\(^+\) Th1 cells in the AVMC, IL-17 mAb, and isotype control groups were also elevated, compared with those in the normal group, especially on day 7 (all \( p < 0.05 \)). Furthermore, on days 3 and 5, the percentages of CD4\(^+\) Th1 cells in the IL-17 mAb group were even higher than those in the AVMC and isotype control groups (all \( p < 0.05 \)), and no significant difference was noted between the AVMC group and isotype control group (Fig. 4A).

On days 3, 5, and 7, the percentages of CD8\(^+\) T cells in the AVMC, IL-17 mAb, and isotype control groups rose significantly, compared with those in the normal group (all \( p < 0.05 \)). Moreover, on day 3, the percentages of CD8\(^+\) T cells in the IL-17 mAb group were even higher than those in the AVMC and isotype control groups (\( p < 0.05 \)), and no difference was observed between the AVMC and isotype control groups (Fig. 4B).

Throughout the course of the experiment, there were no changes in the percentages of CD4\(^+\) Th17 cells, CD4\(^+\) Th1 cells, and CD8\(^+\) T cells in normal mice (Fig. 4A, 4B).

\[ \text{FIGURE 5.} \quad \text{The levels of cardiac IL-17, IFN-}\gamma, \text{TNF-}\alpha, \text{and IL-1}\beta \text{mRNA in AVMC mice detected by real-time PCR.} \]

A. Cardiac IL-17 mRNA on days 3, 5, 7, and 10. B. The correlation analysis of cardiac IL-17 mRNA and cardiac CVB3 RNA on day 5. Each point represents an individual mouse; \( R = 0.874; p < 0.01 \). C. Cardiac IFN-\gamma mRNA on days 3, 5, 7, and 10. D. Cardiac TNF-\alpha mRNA on days 3, 5, 7, and 10. E. Cardiac IL-1\beta mRNA on days 3, 5, 7, and 10. F. The correlation analysis of cardiac IFN-\gamma mRNA and cardiac CVB3 RNA on day 5. Each point represents an individual mouse; \( R = -0.827; p < 0.01 \). Six mice per group were analyzed for each time point. \(^* \text{all } p < 0.01 \text{ versus normal group; } ^{\#} \text{p } < 0.01 \text{ versus AVMC group; } ^{\#\#} \text{p } < 0.05 \text{ versus AVMC group. Values are mean } \pm \text{ SEM.} \]
Examinations for cardiac protein levels of IL-17, IFN-γ, RORγt, and IL-1β mRNA

On days 3, 5, 7, and 10, the levels of cardiac IL-17 mRNA in the AVMC, IL-17 mAb, and isotype control groups were elevated dramatically, compared with those in the normal group, especially on days 7 and 10 (all p < 0.01). However, the levels of cardiac IL-17 mRNA in the IL-17 mAb group were lower than those in the AVMC and isotype control groups (all p < 0.01), and no significant difference was observed between the AVMC group and isotype control group (Fig. 5A). In addition, on day 5, the levels of cardiac IL-17 mRNA were positively correlated with the levels of cardiac CVB3 RNA (correlation index [R] = 0.835; p < 0.01; Fig. 5B).

On days 3, 5, 7, and 10, the levels of cardiac IFN-γ, TNF-α, and IL-1β mRNA in the AVMC, IL-17 mAb, and isotype control groups were also apparently increased, compared with those in the normal group (all p < 0.05). The levels of cardiac IFN-γ mRNA in the IL-17 mAb group were higher than those in the AVMC and isotype control groups on days 3 and 5 (all p < 0.01), whereas the levels of cardiac TNF-α and IL-1β mRNA in the IL-17 mAb group were lower than those in the AVMC and isotype control groups on days 5, 7, and 10 (all p < 0.05), and no significant difference was noted between the AVMC group and isotype control groups on days 3 and 5 (all p > 0.05). However, the levels of cardiac IFN-γ mRNA were negatively correlated with the levels of cardiac CVB3 RNA (R = −0.827; p < 0.01; Fig. 5F).

In the course of the experiment, no changes in the levels of cardiac IL-17, IFN-γ, TNF-α, and IL-1β mRNA were found in the normal group (Fig. 5A–E).

Examinations for cardiac protein levels of IL-17, IFN-γ, RORγt, and T-bet

On day 5, the levels of cardiac IL-17, RORγt, IFN-γ, and T-bet in the AVMC, IL-17 mAb, and isotype control groups were higher than those in the normal group (all p < 0.05). The levels of cardiac IL-17 and RORγt in the IL-17 mAb group were lower than those in the AVMC and isotype control groups (p < 0.05), whereas the levels of cardiac IFN-γ and T-bet were higher in the IL-17 mAb group than in the AVMC and isotype control groups (p < 0.05), and there was no significant difference between the AVMC and isotype control groups (Fig. 6A, 6B).

Discussion

In this experiment, we proved that Th17 cells were enriched after CVB3 infection and that neutralization of IL-17 improved measures of the severity of disease, including the HW/BW, serum level of cTnI, and pathological scores of heart sections throughout the course of AVMC. Consistent with these improvements were decreased viral replication in mice with AVMC. Furthermore, on day 5, the levels of cardiac IL-17 mRNA were positively correlated with the levels of cardiac CVB3 RNA. These data suggested that Th17 cells might contribute to viral replication in CVB3-induced AVMC. At the same time, we demonstrated that neutralization of IL-17 promoted Th1 cell function and efficiently controlled virus replication, along with reduction of IL-17, and neutralization of IL-17 increased T-bet expression and decreased RORγt expression in cardiac tissues on day 5, which implied that Th17 cells might facilitate CVB3 replication by the inhibitory effect of IL-17 on Th1 cell differentiation.

The differentiation of each Th lineage is partly dependent on the suppressive effects on other lineages. IFN-γ and IL-12, the characteristic cytokines of Th1 cells, are confirmed to inhibit Th17 cell differentiation (13). Recently, a few studies in vitro have reported that IL-17 might inhibit IL-12–induced Th1 cell differentiation (14, 15), however, the mechanism of which is still not fully understood. To further clarify the Th1/Th17 change by neutralization of IL-17, we measured the key signal transduction proteins for Th1 (T-bet) and Th17 (RORγt) differentiation. We showed that neutralization of IL-17 increased T-bet expression and decreased RORγt expression in cardiac tissues on day 5, which implied that IL-17 might inhibit Th1 differentiation via T-bet pathways in vivo.

In the development of AVMC, the CD8+ T cell is another important immune cell acting against viral infection by secreting antiviral cytokines (such as IFN-γ and IL-12) or inducing cytolyis
of infected cells via Fas and perforin pathways (16, 17). Martin-Orozco et al. (18) considered that Th17 cells could amplify CD8+ T cell activation in lung melanoma. Conversely, other investigations showed that Th17 cells could downregulate CD8+ T cell function by repressing the production of IL-12 and IFN-γ (12, 19). In addition, IL-17 could increase the expression of antiapoptotic proteins of the Bcl-2 family though the NF-kB signaling pathway, which might impair the Fas pathway of CD8+ T cells via protecting the infected cells from apoptosis (12, 20). In this study, we observed that neutralization of IL-17 enhanced the production of CD8+ T cells and suppressed the levels of CVB3 replication on day 3, which provided evidence that Th17 cells might contribute to CVB3 replication by inhibiting CD8+ T cell activity.

Although CD4+ Th1 cells and CD8+ T cells perform a function in antiviral immunity, they are also believed to exacerbate myocarditis by autoimmune association with excessive release of proinflammatory cytokines, such as TNF-α and IL-1β. Thus, a balance between antiviral immunity and autoimmune might exist in AVMC (21, 22). To clarify whether the increased proportions of Th1 cells and CD8+ T cells would lead to aggressive myocarditis in this experiment, we detected the expression of TNF-α and IL-1β, which were the dominant inflammatory mediators of myocardial injury in the inflammatory phase of CVB3 infection in AVMC (23, 24).

Unlike the elevation in percentages of CD8+ T cells and CD4+ Th1 cells, the levels of cardiac TNF-α and IL-1β mRNA declined along with the reduced IL-17 and decreased CVB3 replication, which diminished the severity of AVMC. We then could conclude that IL-17 produced by Th17 cells might play a role in regulating the balance between antiviral immunity and autoimmune in CVB3-induced AVMC and that IL-17 would be a novel therapeutic target for AVMC.

Acknowledgments
We thank all the members of the experimental animal center of the Tongji Medical College (Huazhong University of Science and Technology) for excellent animal care.

Disclosures
The authors have no financial conflicts of interest.

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