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The role of Th17 cells and regulatory T cells in Coxsackievirus B3-induced myocarditis

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ABSTRACT

IL-17-producing (Th17) and regulatory T (Treg) cells have been well established in the pathogenesis of many inflammatory diseases. To assess whether Th17 and Treg were altered in acute virus-induced myocarditis (AVMC) mice, we assessed Th17/Treg functions on different levels in AVMC. It was shown that the expression of splenic Th17 cells and Th17-related cytokines (IL-17A, IL-21) markedly increased. Interestingly, the expression of splenic Treg cells and Treg-related cytokines (TGF-β, IL-10) also significantly increased. Using neutralization of IL-17 in the AVMC, we found that Treg cells roughly decreased compared with isotype control mice. However, T cells and perforin dramatically increased, followed by a marked reduction in CVB3 replication. The results suggested that Th17 cells possibly contributed to viral replication through the action of Treg cells in mediating T cells and perforin response in AVMC.

Introduction

Viral myocarditis is a frequent cause of sudden death in young adults and can progress to chronic dilated cardiomyopathy, a major cause of heart failure associated with an autoimmune response (Feldman and McNamara, 2000). Coxsackievirus B3 (CVB3)-induced acute myocarditis occurs from day 7 to 14 post-infection in susceptible BALB/c mice (Fairweather et al., 2001). CVB3-induced myocarditis is related to a direct cytopathic effect of the virus or immune-mediated mechanisms. Meanwhile, the negative modulation of host immune responses and the autoimmune inflammatory activation is regulated by primary T lymphocyte cells (Afanasyeva et al., 2001). Strikingly, cytotoxic T cells are able to induce apoptosis of the virus-infected cells through perforin, which acts by punching holes in cells that have been invaded by viruses. The holes allow toxic enzymes entering into the cells, which in turn destroy the cells. Interleukin-17 (IL-17) and Th17 cells have been shown to play important roles in inflammation and immune response. Recently, a general consensus has developed that Th17 cells play an important role in autoimmune diseases, such as systemic lupus erythematosus (SLE) and experimental autoimmune myocarditis (EAM) (Doreau et al., 2009; Korn et al., 2009). Previous studies have observed that systematic depletion of IL-17 in MyHC-α/CFA immunized mice both ameliorated the recruitment of inflammatory cells and relieved disease severity by an anti-IL-17 antibody or by active vaccination, indicating a direct pathogenic role of IL-17 in EAM (Sonderegger et al., 2006). Moreover, regulatory T (Treg) cells are a thymus-derived distinct lineage of T cells that recognize and suppress the expansion and function of potential self-reactive T cell clones, thus maintaining peripheral self-tolerance (Vahlenkamp et al., 2005). It is well accepted that Treg cells play a pivotal role in maintaining immune homeostasis and preventing autoimmune disease. Th17 and Treg cells are subsets of the $CD4^+$ T cell compartment which are vital modulators of the innate and adaptive immune response in many immune diseases (Chauhan et al., 2009; Fantini et al., 2007; Ratajczak et al., 2010). Previous investigations have shown that the generation of induced Treg and Th17 cells is reciprocally regulated in autoimmune disease (Bettelli et al., 2006). TGF-β has the ability to induce both RORγt and Foxp3 expression in T cells in vitro, which depending on the concentration (Zhou et al., 2008). Moreover, accumulating evidence indicates that Th17 cells retain potential developmental plasticity (Zhou et al., 2009), especially, Ye et al. (2011) provided the first evidence that human Th17 cells can differentiate into Treg cells at the clonal level. However, the expression and function of Th17 and Treg cells in CVB3-infected myocarditis are still unclear. It is natural to investigate the relationship between the levels of CVB3 replication and Th17 and Treg cells. It is also important to evaluate the effects of neutralization of IL-17A on differentiate into Treg cells, which may be associated with Treg-mediated T cells and perforin response. To better understand the effect of neutralization of IL-17 on $Foxp3^+$ Treg cells during acute viral infections, we examined the effects of activation of immunosuppressive $Foxp3^+$ Treg cells. Finally, activation of these cells was capable of inhibiting anti-viral $CD4^+$, $CD8^+$ T cells and perforin immune responses on day 7 in acute CVB3-induced myocarditis. It was shown in the present study that viral load was possibly associated with the changes of function and expression of Treg cells in

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myocarditis. Therefore, Th17 and Treg cells presumably control over viral replication in CVB3-induced acute myocarditis.

Results

Proportion of Th17 and Treg cells in viral myocarditis

Compared with those in the control group, the percentages of $CD4^+$ Th17 cells in AVMC markedly increased on days 7, 10, and 14. Specifically, the percentages of $CD4^+$ $IL\text{-}17^+$ T cells on day 10 in myocarditis were remarkably higher than those on days 7 and 14 (all $p<0.01$) (Figs. 1A and C). The percentages of $CD4^+CD25^+$ Treg cells in the AVMC group were higher than those in the control group ($p<0.01$) (Figs. 1B and D), however, no obvious difference could be observed among days 7, 10 and 14 in the AVMC group.

Detection of cardiac IL-17A, TGF-β mRNA and CVB3 RNA on day 7 in viral myocarditis

On day 7, the mRNA expression of cardiac IL-17 in AVMC was markedly elevated compared to the normal control group ($p<0.01$, Fig. 2A). Moreover, the mRNA expression of cardiac IL-17 positively correlated with that of cardiac CVB3 (correlation index [R] = 0.897; $p<0.01$; Fig. 2C). Similarly, the levels of cardiac TGF-β in the AVMC apparently increased compared to the normal control group ($p<0.01$, Fig. 2B). The mRNA expression of cardiac TGF-β negatively correlated with the RNA expression of cardiac CVB3 ($R=-0.913$; $p<0.01$; Fig. 2D).

Relationships among pathological score, the proportion of $CD4^+$, $CD8^+$, $CD4^+/CD8^+$ T cells, $CD4^+$ $Perforin^+$ T cells, $CD8^+$ $Perforin^+$ T cells, $CD4^+CD25^+Foxp3^+$ cells and CVB3 copies in spleen upon neutralization of IL-17.

The percentage of $CD4^+$ $CD25^+Foxp3^+$ T cells increased greatly in the AVMC, isotype control and anti-IL-17 groups compared to the control group. Additionally, the percentages of $CD4^+$ $CD25^+Foxp3^+$ T cells were lower in the anti-IL-17 group than those in isotype control group (all $p<0.01$) (Fig. 3A). Accordingly, the percentages of $CD4^+$ $Perforin^+$ and $CD8^+$ $Perforin^+$ T cells increased greatly in the AVMC, isotype control and anti-IL-17 groups compared to the control group. However, these percentages were higher in the anti-IL-17 group than those in the isotype control group (all $p<0.01$) (Fig. 3B). A marked reduction was observed in the $CD4^+$ and $CD8^+$ T cell percentages and the ratios of $CD4^+/CD8^+$ T cells in the AVMC, isotype control and anti-IL-17 groups compared to the control group. However, the percentages of $CD4^+$ and $CD8^+$ T cells were higher in the anti-IL-17 group than those in the isotype control group ($p<0.05$), However, no difference was observed in terms of the ratios of $CD4^+/CD8^+$ T cells between the anti-IL-17 and isotype control groups (Figs. 3C and D). Interestingly, the pathological score significantly increased in the AVMC, isotype control and anti-IL-17 groups compared to the control group, whereas the pathological score decreased in the anti-IL-17 group than that in the isotype control group ($p<0.05$) (Fig. 3E). Moreover, no marked difference was detected in the proportion of $CD4^+$, $CD8^+$, $CD4^+/CD8^+$ T cells, $CD4^+Foxp3^+$, $CD4^+Perforin^+$ and $CD8^+$ $Perforin^+$ T cells between the AVMC and isotype groups (Fig. 3). In addition, the levels of cardiac CVB3 RNA were obviously higher in the AVMC, isotype control and anti-IL-17 groups than in the control group (all $p<0.01$). However, these levels were markedly lower in the anti-IL-17 group than those in the isotype control group ($p<0.01$), and no marked difference was observed between the AVMC and isotype control group (Fig. 3F).

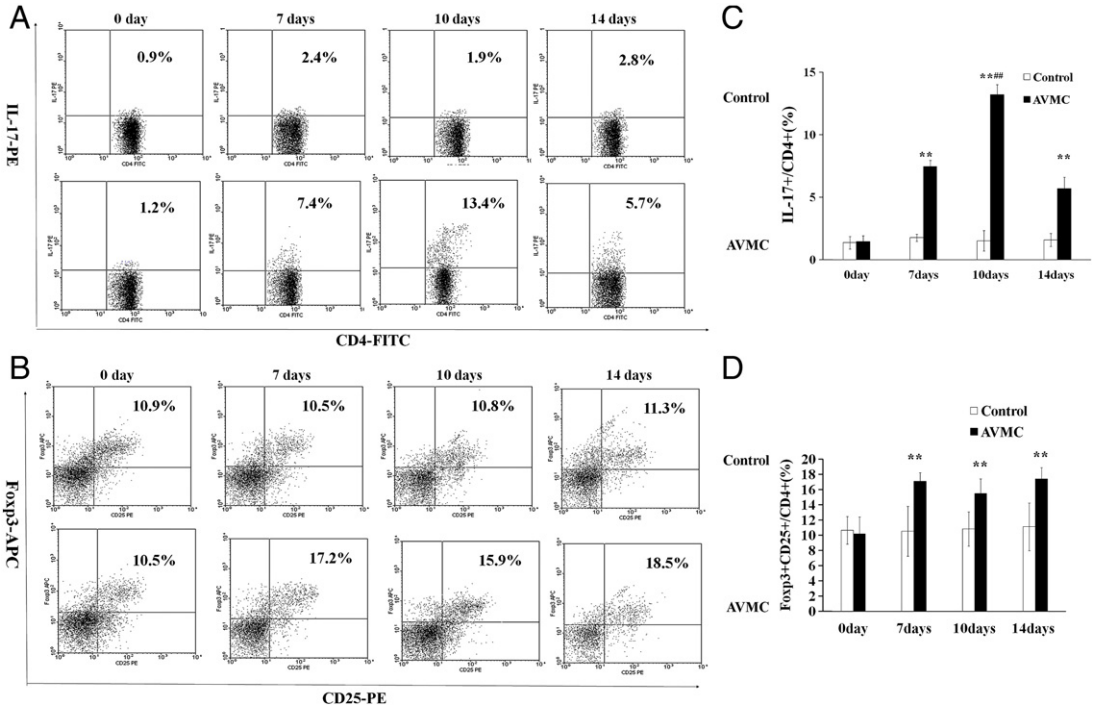

Fig. 1. The percentages of $CD4^+$ $IL\text{-}17^+$ T cells, $CD25^+$ $Foxp3^+$ T cells were investigated by flow cytometry in control and AVMC groups on days 0, 7, 10 and 14. A Representative pictures for Th17 ($CD4^+$ $IL\text{-}17^+$) cells gated on $CD4^+$ T cells by flow cytometry in each group. Numbers in upper right quadrants and lower right quadrants indicate the separate percentages of Th17 cells and $CD4^+$ T cells, respectively. B. Representative pictures for the analysis of $CD25^+$ $Foxp3^+$ Treg cells by flow cytometry. The percentage of $Foxp3^+$ $CD25^+$ cells was gated on $CD4^+$ T cells. Numbers in upper right quadrants and lower left quadrants indicate the separate percentages of $CD25^+$ $Foxp3^+$ Treg cells and $CD4^+$ T cells, respectively. C. Statistic analysis showed the variation of $CD4^+$ $IL\text{-}17^+$ T cells at all time points in each group (all $p<0.01$). D. Statistic analysis showed the variation of $CD4^+$ $CD25^+$ $Foxp3^+$ T cells at all time points in each group ($p<0.01$). The data from eight mice per group are showed as mean ± SEM, the difference between control and AVMC group at different time points was analyzed by two-way ANOVA, individually. ** $p<0.01$ vs control group, ## $p<0.01$ day 10 vs days 7 and 14 in the AVMC group (n = 6).

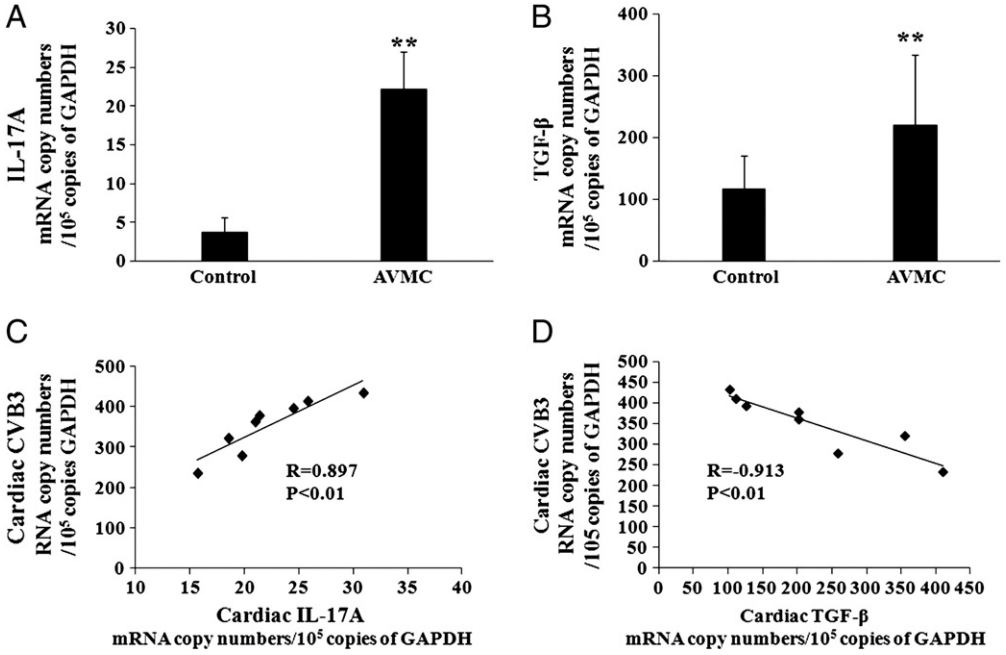

Fig. 2. The mRNA expression of related cytokines and CVB3 RNA in the myocardial tissues on day 7 after viral inoculation. The hearts tissues in the control and AVMC group mice (n = 8) were partly removed on days after CVB3 infection. A, The mRNA levels of IL-17A (A), TGF-β (B) in myocardial tissues were measured by real time PCR. C. The correlation analysis of cardiac IL-17 mRNA and cardiac CVB3 RNA on day 7 post-infection (R = 0.897, $p<0.01$). D. The correlation analysis of cardiac TGF-β and cardiac CVB3 RNA on day 7 (R = −0.913, $p<0.01$). Each point represents an individual mouse. GAPDH gene primer sets were used as an internal control. ** $p<0.01$ vs control group.

Measurements of cardiac protein levels of IL-17, TGF-β, IFN-γ and VP1 upon neutralization of IL-17

The protein levels of cardiac IL-17A, TGF-β and VP1 were higher in the AVMC, isotype control and anti-IL-17 groups than in the control group (all $p<0.01$). However, the levels of these proteins were markedly lower in the anti-IL-17 group compared to the isotype control group ($p<0.05$, $p<0.05$ and $p<0.01$, respectively) (Figs. 4A and B). In addition, the level of the IFN-γ protein was higher in the AVMC, isotype control and anti-IL-17 groups than in the control group ($p<0.05$, $p<0.05$ and

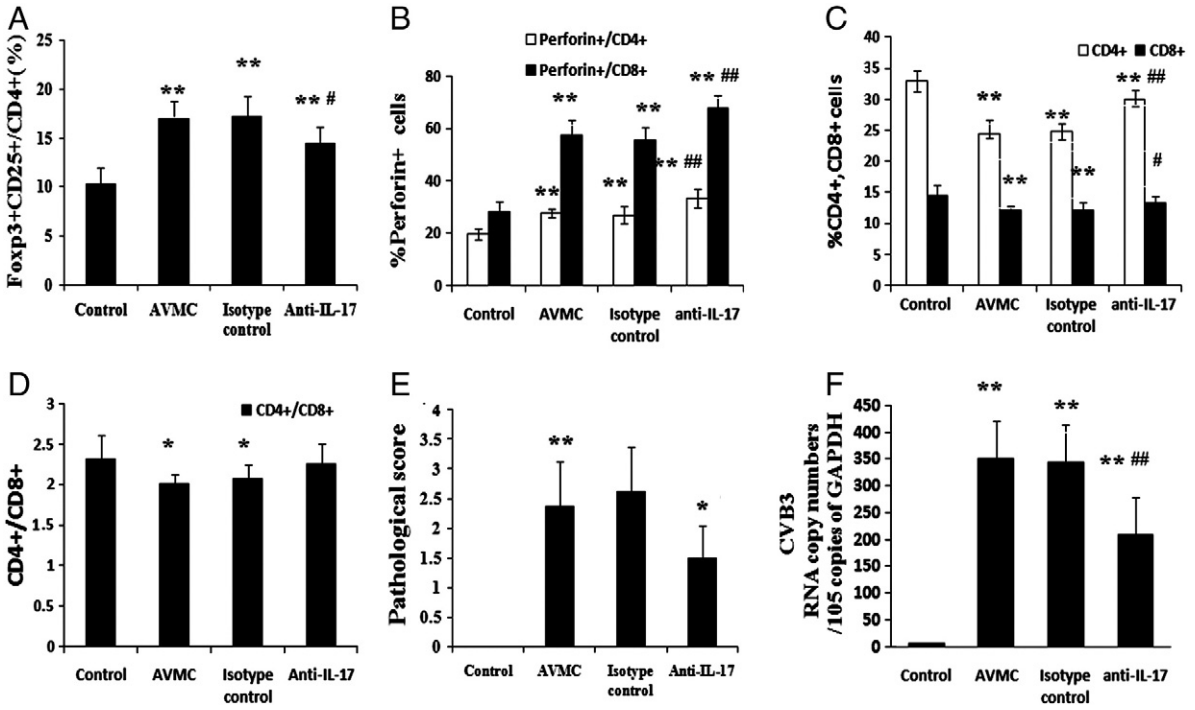

Fig. 3. Relationships among pathological score, CVB3 copies and the proportion of $CD4^+$, $CD8^+$, $CD4^+/CD8^+$ T cells, $CD4^+Perforin^+$ T cells, $CD8^+$ $Perforin^+$ T cells and $CD4^+CD25^+Foxp3^+$ cells in spleen upon neutralization of IL-17. A. Statistic analysis showed the percentage of $CD25^+Foxp3^+$ cells gated on $CD4^+$ T cells by flow cytometry in each group. B. Statistic analysis showed the percentages of $CD4^+$ $Perforin^+$ and $CD8^+$ $Perforin^+$ T cells in each group. C. The percentages of $CD4^+$ and $CD8^+$ T cells gated on $CD4^+$ and $CD8^+$ T cells were analyzed by flow cytometry in each group. D. Statistic analysis of the ratio of $CD4^+/CD8^+$ T cells in each group. The data from eight mice per group are showed as mean ± SEM and analyzed by one-way analysis of variance, individually. E. The pathological scores of virus myocarditis are shown for different groups by HE staining in the midventricular regions. The differences in the pathological scores were evaluated using the Mann–Whitney U-test. F. The mRNA levels of CVB3 were measured by real time PCR on day 7 after CVB3 infection. ** $p<0.01$ vs control group, * $p<0.05$ vs control group, ## $p<0.01$ or # $p<0.05$ vs isotype control group (n = 6).

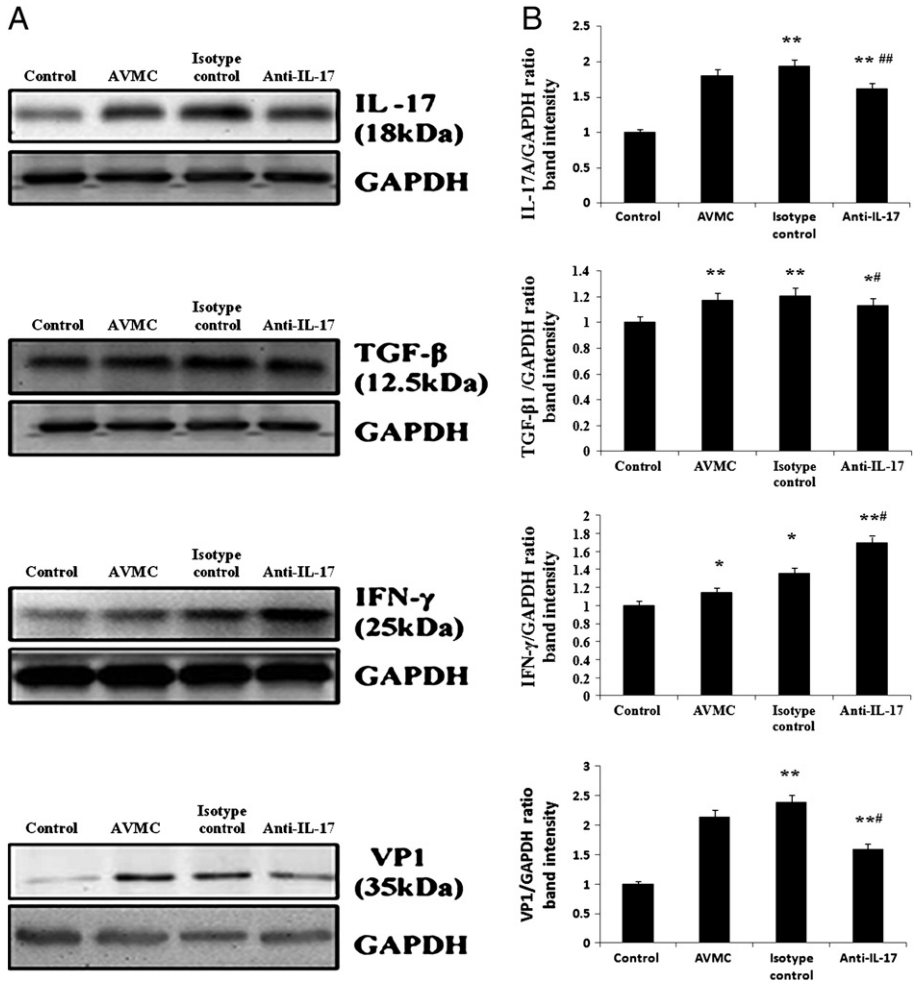

Fig. 4. The proteins expression of cardiac protein levels of IL-17A, TGF-β, IFN-γ and VP1 examined by Western blot on day 7 in myocarditis upon neutralization of IL-17. A Representative images for protein expression of cardiac IL-17A, TGF-β, IFN-γ and VP1 in different group. In the pictures of Western blot, the treatment group from left to right is control, AVMC, Isotype control and Anti-IL-17. B. Statistical analysis for the levels of cardiac IL-17A, TGF-β, IFN-γ and VP1 protein. Six mice per group were analyzed on days. ** $p<0.01$ vs control group, * $p<0.05$ vs control group, ## $p<0.01$ vs isotype control group, # $p<0.05$ vs isotype control group (n = 6).

$p<0.01$, respectively), while the IFN-γ protein level was markedly higher in the anti-IL-17 groups than in the isotype control group ($p<0.05$). No significant difference was detected between the AVMC and isotype control groups (Figs. 4A and B).

Expression of Th17 and Treg-related cytokines upon neutralization of IL-17A

The serum levels of Th17 and Treg-related cytokines, such as IFN-γ, IL-10, IL-2, IL-6, IL-21 and IL-17A were markedly increased in the AVMC and isotype control groups than in the control group, while they were fully decreased (except for IFN-γ) in the anti-IL-17 group compared to the isotype control group. Other cytokines could not be detected or there exists no remarkable differences between the isotype control and anti-IL-17 groups (Fig. 5).

Discussion

Th17 cells show pro-inflammatory functions and play a critical role in various autoimmune disorders in mice and humans. In contrast, Treg cells are important in the maintenance of immune homeostasis in many infectious diseases (Korn et al., 2009; Shi et al., 2010). Th17 and Treg cells control injury and inflammation in the EAM and may be important in virus-induced myocarditis. In this study, we mainly clarified whether the Th17/Treg functional imbalance exists during acute CVB3-induced myocarditis. The results showed that acute myocarditis exhibited an apparent increase in Th17 cells and Th17-related cytokines (IL-17A, IL-21). These results indicate that Th17 cells play a critical role in the formation and development of acute viral myocarditis. Moreover, marked increases were observed in Treg cells and Treg-related cytokines (TGF-β, IL-10 and IL-6) in AVMC mice. CVB3-infected myocarditis induced an early immunosuppressive response that correlated with a marked increase in the frequency of Treg cells and the related cytokines TGF-β and IL-10. It indicates that Treg cells may contribute to viral persistence by prematurely limiting the antiviral immune response. The maintenance of long-term Treg cell activation could contribute to the progressive loss of $CD4^+$ and $CD8^+$ T cell immune function (anergy) and promote the development of myocarditis. Intriguingly, our studies further showed that the replication of CVB3 was associated with the levels of cardiac IL-17 and TGF-β mRNA. Meanwhile, the results indicated that the Treg cells and related cytokine TGF-β somewhat decreased upon treatment with the anti-mouse IL-17Ab to block IL-17 on day 7 in AVMC mice. Conversely, $CD4^+$ $Perforin^+$ and $CD8^+$ $Perforin^+$ T cells were markedly elevated, followed by a marked reduction in CVB3 replication and a direct decrease in the cardiac inflammatory cytokines IL-17A, IL-10, IL-2 and IL-21. These data suggest that a Th17/Treg function exists and is intimately associated with viral replication in myocarditis. The results further suggest that Th17 cells would contribute to viral replication possibly by Treg cell-mediated T cell and perforin response in Coxsackievirus B3-induced acute myocarditis.

Many viruses, including Coxsackieviruses, have been implicated as main causes of myocarditis, which is defined as inflammation of the

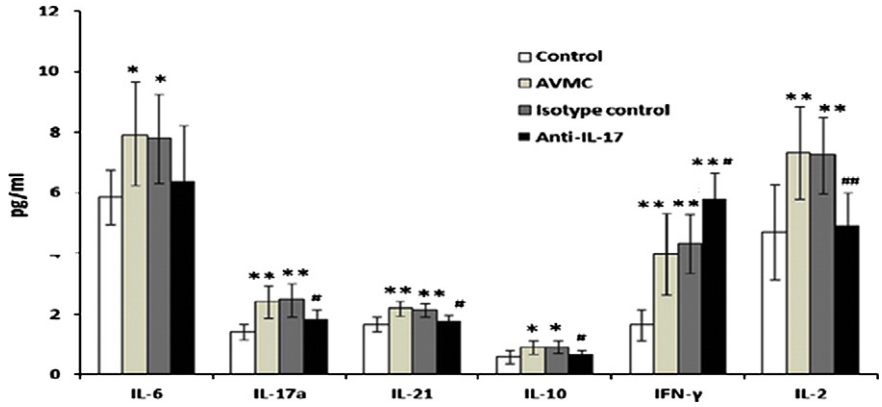

Fig.5. The production of related cytokines of IL-6, IL-17A, IL-21, IL-10, IFN-γ, IL-2 in the serum of different group mice by Flowcytomix bead assay. Results are mean values ± SD of six independent mice. One-way ANOVA was used to analyze the data. ** $p<0.01$ vs Control group, * $p<0.05$ vs control group, ## $p<0.01$ vs isotype control group, # $p<0.05$ vs isotype control group (n = 6).

heart. The viral genome has a vital role in the progression of the state by maintaining an immune response through both innate and adapted immunity. Moreover, early investigations have suggested that local heart tissue damage associated with viral infection was mediated by primary T cells (Afanasyeva et al., 2001). $CD4^+$ T cells play a critical role in the induction of autoimmune myocarditis (Blauwet and Cooper, 2010). Furthermore, deficiencies in $CD4^+$ T cells are associated with virus reactivation, generalized susceptibility to opportunistic infections and poor vaccine efficacy (Whitmire, 2011). The marked reduction of inflammation in infected mice after depletion of $CD4^+$ T cells highlights a potential contribution of $CD4^+$ T cells in the progression of myocarditis in CVB3-infected mice (Huber et al., 2002; Neu et al., 1993). In this study, we clarified that the percentage of $CD4^+$ T cells markedly decreased in AVMC mice. Previous studies have indicated that Th1 cells play a pivotal role in the adaptive immune response, mainly because these cells can secret cytokines and chemokines for the effective clearance of viral infections and can down-regulate the immune response in the progression of myocarditis. In addition, IFN-γ, the prototypic cytokine associated with Th1 immune responses, was formerly considered to be essential for the development of EAM (Fairweather et al., 2005). However, many experiences have shown that IFN-γ has a protective effect in the pathology of autoimmune disease, and this effect had been ascribed to the deregulation of IL-17, resulting from the regulation of the Th17-associated transcription factor RORγt (Ivanov et al., 2007). Our data showed that expression of splenic Th17 cells and Th17-related cytokines (IL-17A) increased in AVMC mice. Furthermore, cardiac IL-17A mRNA positively correlated with cardiac viral replication. These data suggested that Th17 cells might contribute to viral replication in CVB3-induced AVMC. Most importantly, the results demonstrated that the neutralization of IL-17 promoted higher production of IFN-γ, which promoted Th1 cell function and efficiently controlled virus load, along with the reduction of IL-17A and IL-21, indicating that Th17 cells might contribute to virus load by the inhibitory effect of IL-17 on Th1 cell differentiation. Estes et al. (2006) reported that simian immunodeficiency virus (SIV) infection of Macaques induced an early immunosuppressive response that correlated with a marked increase in frequency of $CD4^+CD25^+Foxp3^+$ Treg cells and TGF-β and IL-10 positive T cells, indicating that Treg cells and/or anti-inflammatory cytokines may possibly contribute to viral persistence by prematurely limiting the antiviral immune response. Also, a role for $CD4^+CD25^+Foxp3^+$ Treg cells on the $CD4^+$ and $CD8^+$ T cells anergy to HIV peptide stimulation in vitro (Aandahl et al., 2004; Eggena et al., 2005; Wherry et al., 2006). In short, HIV-specific T cells response during acute stage infection may involve epitope specific clonal anergy rather than clonal deletion, and this anergy may be mediated by active Treg cells. However, several chronic viral infections, including HCV, HIV, HTLV-1, FIV, Friend virus and the murine form of AIDS, have additionally been linked to unregulated Treg cell activation and suppressor function (Vahlenkamp et al., 2005). In the course of investigating the relationship between Th17 and Treg cells in the AVMC model, we found that the expression of splenic Treg cells and Treg-related cytokines (TGF-β, IL-6, IL-10) levels remarkably increased in acute virus myocarditis. In addition, $CD4^+$ T cells, $CD8^+$ T cells and the ratio of $CD4^+/CD8^+$ T cells were fully down-regulated in AVMC mice. From the studies described above, viral infections are clearly characterized by an early and sustained activation of immunosuppressive $Foxp3^+$ Treg cells that are capable of inhibiting anti-viral $CD4^+$ and $CD8^+$ T cell immune responses, which allows for the establishment of long-term infection. Furthermore, cardiac TGF-β mRNA was negatively correlated with cardiac viral replication. The present study showed that CVB3-infected myocarditis induced an early immunosuppressive response that correlated with the frequency and levels of Treg-related cytokines, and indicating that Treg cells may contribute to viral persistence by prematurely limiting the antiviral immune response. These data suggest that Treg cells, which attained a distinct immunosuppressive property in various aspects of immune response and maintained self-tolerance, might also contribute to viral replication in CVB3-induced acute myocarditis.

Th17 and Treg cells and the developmental programs of T cells are reciprocally interconnected. This discovery was initially based on the observation that upon TCR stimulation, a naïve T cell can be driven to express Foxp3 and become a Treg cell in the presence of TGF-β. However, in the presence of TGF-β plus IL-6 or IL-21, the Treg developmental pathway is abrogated, and instead of the T cells developing into Th17 cells (Veldhoen et al., 2006). Certainly, TGF-β is a regulatory cytokine with a pleiotropic function in T cell development, homeostasis and tolerance (Korn et al., 2009; Mesa et al., 2010). Moreover, TGF-β has certainly been recognized as a hub between Th17 and Treg cells. In addition, IL-2 is required for the generation and maintenance of Treg in many infectious diseases (Laurence et al., 2007; Papiernik et al., 1998). Increasing evidence suggests that Th17 cells and Treg cells have evolved greater developmental plasticity than Th1 and Th2 subsets (Lee et al., 2009). Previous studies provided that Th17 clones can differentiate into IFN-γ-producing and $Foxp3^+$ populations after multiple in vitro TCR stimulations and expansions, and that these expanded Th17 clones convert into Treg cells possessing potent suppressive activity (Ye et al., 2011). The functional roles, the regulatory mechanisms of Th17 cells, Treg cells and Th1 cells in vivo under different pathological conditions will be needed to further investigate. Certainly, we have observed that Th17 cells and Treg cells increased drastically and had played a vital role in the pathological condition of CVB3-induced myocarditis. Whether altering the Th17-cell-polarizing conditions upon neutralization of IL-17

significantly decreased differentiation of Treg cell and affect CVB3 replication remained a question to be addressed. In this study, an anti-IL-17 antibody was used to block IL-17 on day 7 in AVMC mouse model. We found that Treg cells possessing potent suppressive activity somewhat decreased upon neutralization of IL-17, but importantly, a direct decreased in the cardiac inflammatory cytokines which local environment of differentiation of Th17 and Treg such as TGF-β, IL-6, and IL-2. We speculated that neutralization of IL-17 could alter the pathological conditions of acute myocarditis and decrease the percentage of Th17-derived Treg cells. Thus, it could alleviate the immunosuppressive response of activated Treg, in turn leading to clonal expansion of cytotoxic T cells and the development of an effective immune response in the early acute stage of the CVB3-induced myocarditis, in which cytotoxic T cells are able to induce apoptosis of the virus-infected cells through a perforin-mediated apoptosis pathway. In this apoptotic mechanism, perforin polymerizes and forms a transmembrane pore that allows the delivery of granzymes into the cytosol, where they initiate various apoptotic death pathways. Unlike relatively redundant individual granzymes, functional perforin is absolutely essential for cytotoxic lymphocyte function and immune regulation in the host (Voskoboinik et al., 2010). In conclusion, these data showed that the Th17 and Treg cells are involved in CVB3-induced acute autoimmune myocarditis, and this study partially elucidated that anti-IL-17 antibody and/or Treg cells may be considered as a potential immune therapy in viral myocarditis. Clinical trials showed that immunosuppressive agents had no significant benefit for outcome of human myocarditis. New therapeutic strategies in viral myocarditis might be taken into account the interaction of Th17 and Treg cells. Taken together, pan-immune suppression, perhaps, is not the better treatment of acute CVB3-induced myocarditis. Consequently, more effective and targeted approaches to mediate the immune function of Th17 and Treg cells are needed in the course of viral myocarditis.

Materials and methods

Virus

The CVB3 (Nancy strain) was prepared by passage through HeLa cell cultures and then titered by a plaque assay and stored at −80 °C. The virus titer was determined by the median tissue culture infective dose (TCID50 = 10^7, Nancy strain) in 100 μl of Eagle's minimal essential medium (EMEM, GIBCO) (Chen et al., 2011).

Mice

BALB/c mice (4–6 weeks old) were purchased from the Joint Ventures Sipper BK Experimental Animal Company. All animal experiments were performed in accordance with the National Institute of Health guide for the Care and Use of Laboratory Animals, with the approval of the Animal Care Committee of the Zhongshan Hospital affiliated with Fudan University (Shanghai, China). BALB/c mice were randomly divided into four groups: (1) Normal control group ($n = 60$) mice were injected intraperitoneally (*i.p.*) with 100 μl of EMEM, (2) AVMC group ($n = 60$) mice were injected *i.p.* with 100 μl of CVB3 diluted in EMEM on day 0 to induce acute viral myocarditis after days 7, 10 and 14, (3) Isotype control group ($n = 25$) mice were treated with CVB3 *i.p.* on day 0 and isotype control IgG1 Ab (100 μg per mouse; eBioscience) *i.p.* on days 3 and 5, and (4) IL-17mAb group ($n = 25$) mice were treated with CVB3 *i.p.* on day 0 and anti-mouse IL-17 mAb (100 μg per mouse; R&D Systems, Inc) *i.p.* on days 3 and 5 (Baldeviano et al., 2010). The mice in isotype control group and anti-mouse IL-17mAb group were sacrificed on day 7 post-infection (Chen et al., 2009). All mice were kept in specific pathogen-free conditions.

Histopathology

Paraffin-embedded hearts were cut into 5 μm-thick tissue sections and stained with hematoxylin & eosin (H&E) and Masson trichrome staining to assess myocardial injury and inflammation. Pathological grading of inflammation of the myocardium was evaluated by giving the samples a myocarditis score from 0 to 4 according to a previous study (Valaperti et al., 2008) with slight modifications (score: 0, no inflammatory infiltrates; 1, small foci of inflammatory cells between myocytes; 2, 5% to 10% of a cross-section involved; 3, 10% to 25% of a cross-section involved; 4, >25% of a cross-section involved). The slides were graded independently in a masked fashion by two observers. For immunohistochemical analysis, paraffin-embedded sections were first deparaffinized and hydrated, then microwave antigen retrieval was performed, endogenous peroxidase activity was blocked by incubation of the slides in 0.3% H_2O_2, and non-specific binding sites were blocked with 4% BSA (Sigma).

Flow cytometric analysis

Splenocytes were isolated and suspended in RPMI 1640 containing 10% fetal bovine serum, and red blood cells were lysed by 3 min incubation in ACK lysis buffer (TIANGEN, China). Cells were collected and resuspended at a density of 1.0×10^6/ml. Then, the cells were stimulated for 4 h with 50 ng/ml phorbol 12-myristate 13 acetate (PMA) and 1 μg/ml ionomycin (all from Sigma-Aldrich, St. Louis, MO), and cytokine secretion was blocked using 10 μg/ml brefeldin A (Sigma-Aldrich) at 37 °C, 5% CO_2 in 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% FBS. Surface markers were stained with FITC-labeled anti-mouse CD4 or CD8 (eBiosciences, San Diego, CA), or PE-conjugated anti mouse CD25 (eBiosciences). After washing, fixing and permeabilizing according to the manufacturer's instructions (eBiosciences), the cells were stained intracellularly with APC-conjugated anti-mouse Foxp3 (eBiosciences), PE-labeled anti-mouse IL-17A (TC11-18H10, BD Biosciences, San Jose, CA) or anti-mouse perforin (eBiosciences). After incubation at 4 °C for 30 min, the samples were finally washed in staining buffer and measured by flow cytometry on a FACSCalibur machine. The data were analyzed with CellQuest software (BD Biosciences).

Real time PCR

Total RNA was isolated from each preparation using Trizol® reagent (Invitrogen, USA) according to the manufacturer's instructions and converted into cDNA using M-MuLV reverse transcriptase (Fermentas International Inc, Canada). cDNA was amplified with a SYBR green master mix (TaKaRa, China) using an ABI7500 thermocycler (Applied Biosystems). The $2^{-\Delta CT}$ method (Schmittgen and Livak, 2008) was used to normalize transcription to GAPDH mRNA and calculate the fold induction relative to control. The primer pairs that were used are shown in Table 1.

Western blot

The total proteins of the heart tissues were lysed in RIPA lysis buffer, which contained a protease and phosphatase inhibitor cocktail (AG Scientific). Protein concentration was quantified using a bicinchoninic (BCA) protein assay kit (Pierce). Samples containing 50 μg of proteins were separated on an 8% to 12% SDS-PAGE gel and transferred to polyvinylidene difluoride membranes. Membranes were blocked for 2 h at room temperature with 2–5% BSA, reacted with anti-IFN-γ antibody (1:500, Santa Cruz Biotechnology, CA USA), anti-IL-17A antibody (1:1000, Cell Signaling Technology), anti-TGF-β antibody (1:500, Santa Cruz Biotechnology), anti-VP1 antibody (1:300, Leica Microsystems) or anti-GAPDH antibody (1:1000, Kangchen Biotech, China) at

Table 1
Sequences of the primers for real time PCR*.

Genes	Sequences(5′-3′)
IL	CTGTGTCTCTGATGCTGTT
IL	TGGAACGGTTGAGGTAGT
TGF	GCAACAACGCCATCTATG
TGF	CAAGGTAACGCCAGGAAT
CVB3 sense	CGGTACCTTTGTGCGCCTGT
CVB3 anti-sense	CAGGCCGCCAACGCAGCC
GAPDH sense	AGTATGATGACATCAAGAAGG
GAPDH anti-sense	ATGGTATTCAAGAGAGTAGGG

* IL-17A = interleukin-17A; TGF-β = transforming growth factor-β; CVB3 = Coxsackieviruses B3.

4 °C overnight, and then incubated with a secondary HRP-conjugated antibody (1:2000, Kangchen Biotech) for 1 h at room temperature. After incubation with the secondary antibody, the blots were detected with ECL (Thermo Scientific). The bands obtained were analyzed using "Quantity One" software (Bio-Rad). Ratios were compared using the expression of the bands in a ratio with GAPDH expression for the same sample.

Multiple cytokine detection

Serum was collected after centrifugation and stored at −80 °C. A bead-based multiplexed assay (FlowCytomix mouse Th1/Th2/Th17/Th22 13plex Sample Kit; Bender MedSystems) that quantifies multiple cytokines was used for analysis of cytokine expression patterns (Figueiredo et al., 2009). Cytokine levels were measured according to the manufacturer's instructions. All samples were measured in triplicate.

Statistics

Comparisons of different treatment groups were performed by one-way ANOVA. Data from control and AVMC group at different time points were subjected to two-way ANOVA analysis. The differences in the pathological scores were evaluated using a Mann–Whitney U-test. The statistical package employed was SPSS 16.0 for Windows. Values are expressed as mean ± SEM, with values of $p<0.05$ considered significant.

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