Regulatory T Cells Protect Mice Against Coxackievirus-Induced Myocarditis Through the Transforming Growth Factor β–Coxsackie-Adenovirus Receptor Pathway

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Background—Coxsackievirus B3 infection is an excellent model of human myocarditis and dilated cardiomyopathy. Cardiac injury is caused either by a direct cytopathic effect of the virus or through immune-mediated mechanisms. Regulatory T cells (Tregs) play an important role in the negative modulation of host immune responses and set the threshold of autoimmune activation. This study was designed to test the protective effects of Tregs and to determine the underlying mechanisms.

Methods and Results—Coxsackievirus B3 immediately after the last cell transfer. Transfer of Tregs showed higher survival rates than transfer of CD4+ T cells (P=0.0136) but not compared with the PBS injection group (P=0.0589). Interestingly, Tregs also significantly decreased virus titers and inflammatory scores in the heart. Transforming growth factor-β and phosphorylated AKT were upregulated in Tregs-transferred mice and coxsackie-adenovirus receptor expression was decreased in the heart compared with control groups. Transforming growth factor-β decreased coxsackie-adenovirus receptor expression and inhibited coxsackievirus B3 infection in HL-1 cells and neonatal cardiac myocytes. Splenocytes collected from Treg-, CD4+ T-cell–, and PBS-treated mice proliferated equally when stimulated with heat-inactivated virus, whereas in the Treg group, the proliferation rate was reduced significantly when stimulated with noninfected heart tissue homogenate.

Conclusions—Adoptive transfer of Tregs protected mice from coxsackievirus B3–induced myocarditis through the transforming growth factor β–coxsackie-adenovirus receptor pathway and thus suppresses the immune response to cardiac tissue, maintaining the antiviral immune response. (Circulation. 2010;121:2624-2634.)

Key Words: coxsackieviruses B • cardiomyopathy • heart failure • inflammation • myocarditis • viruses

Coxsackievirus B3 (CVB3) infections have been shown to cause acute or chronic myocarditis and dilated cardiomyopathy.1 Cardiac injury is caused by a direct cytopathic effect of the virus, an immune response to viral infection, or autoimmune triggerd by viral infection.2,3 The disease consists of 3 distinct clinical processes: a viral phase characterized by viral entry and proliferation, an immune response involving both innate and adaptive immunity, and a chronic phase of repetitive repair and remodeling that leads to heart failure. The ultimate immune response involved in the disease is T cell–mediated cytotoxicity, which is central for disease progression against not only the virus but also self-antigen triggering autoimmunity.4 A modulation strategy that processes the antiviral immunity without excessive autoimmune tissue injury would be beneficial in treating CVB3-associated autoimmune myocarditis.

Editorial see p 2609
Clinical Perspective on p 2634

Suppressor T cells, now renamed regulatory T cells (Tregs), are important negative immune modulators and constitute 5% to 10% of peripheral CD4+ T cells in naïve mice and humans. They express CD25 (interleukin [IL]-2 receptor α chain) and play an important role in the negative regulation of immune responses.5 Tregs can suppress the activation, proliferation, differentiation, and functions of many cell types, including CD4 and CD8 T cells, B cells, natural killer cells, natural killer T cells, and dendritic cells, thus controlling immune responses to self-antigens and to pathogens, alloantigens, and tumors.6

Tregs are crucial for preventing autoimmune diseases and maintaining self-tolerance, and autoimmune lesions could

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occur when the so-called natural Tregs were absent. Studies have shown that natural Tregs also influence immunity to infectious agents, including viruses. In mice infected with herpes simplex virus, for example, Tregs help limit herpes simplex virus–induced immunopathological lesions in the eye. In HIV, Tregs limit the chronic immune activation that may precede collapse of the immune system. However, when infections lead directly to tissue damage, excessive Treg cell activity may become a double-edged sword, preventing damage to self on the one hand while contributing in some instances to viral persistence and chronic disease (eg, chronic viral hepatitis B and C) on the other. In this study, we tested the protective effect of Tregs in a myocarditis model by adoptively transferring naturally occurring Tregs to mice before infection with CVB3.

Methods

Animals
Specific pathogen-free C57Bl/6 mice (Jackson Laboratory, Bar Harbor, Me) were housed under standard conditions at the Animal Facility of the University Health Network and were treated in accordance to our institutional animal care and ethics regulations. During the experiment, the mice were fed a normal chow diet and tap water ad libitum.

Cell Separation and Flow Cytometry
Splenocytes were isolated from uninfected mice, and non-CD4+ T cells were depleted with the Dynal Mouse CD4 Negative Isolation Kit (Invitrogen, Carlsbad, Calif). Then the CD4+ cells were costained with PE-CD4 (BD PharMingen, Franklin Lakes, NJ), APC-CD25 (BD PharMingen), FITC-FoxP3 (intracellular staining; Thermo Scientific, Chicago, Ill), and PE-Cy7-CD127 (eBioscience, San Diego, Calif). Singly positive CD4+ (naïve CD4+ T cells) and CD4-CD25+ T cells (Tregs) were sorted, and the purity of the cells was examined with an Aria-RITT BRV cell sorter (Becton Dickinson, San Jose, Calif).

In Vivo Adoptive Transfer
Sorted T cells were labeled with 2 μmol/L carboxyfluorescein diacetate, succinimidyl ester (CFSE; Vybrant CFDA SE Cell Trace Kit, Invitrogen) at 37°C for 30 minutes, and ~3×10⁵ cells were transferred via retroorbital injection. The labeled cells or PBS was infused for 3 times at 2-week interval. Immediately after the third infusion, the mice were challenged with CVB3.

Virus
A cardiovirulent strain of CVB3-CG (a generous gift from Dr Charles Gauntt) was propagated in HeLa cell cultures, titered by standard plaque formation assays, and stored at ~80°C. Aliquots from the same stock were used for all animals. After aseptic removal, hearts were stored individually at ~80°C. Viral titers in the hearts were determined by standard viral plaque assay as described previously.

Viral Challenge
Six- to 8-week-old male mice were injected intraperitoneally with 10⁴ plaque-forming units of CVB3. Mice were observed everyday for the development of clinical morbidty and mortality until day 14. To determine pathological and molecular end points, additional animals were randomly assigned to be killed on postinfection days 7, 10, and 14 and their tissue harvested.

Histopathology
Transverse midsections of hearts were fixed in zinc fixative, embedded in paraffin, sectioned at 5 mm, and processed for hematoxylin and eosin or immunohistochemical staining. Pathological grading of inflammation of the myocardium was evaluated by a previously established method with slight modifications (0=no inflammatory infiltrates; 1=small foci of inflammatory cells between myocytes or <5% of cross section involved [mild]; 2=5% to 10% of cross section involved [moderate]; 3=10% to 25% of cross section involved [moderate]; and 4=>25% of cross section involved [severe]). Pancreatitis was evaluated according to previously described criteria. The antibodies for immunohistochemical staining were anti-CD4 and anti-CD8 (ABD Serotec, Oxford, UK), CD11c (BD PharMingen), and CD94 (Lifespan Biosciences, Seattle, Wash). The primary antibody binding was probed with a goat anti–mouse IgG conjugated with horseradish peroxidase (VECTASTAIN ABC Kit, Vector Laboratories, Burlingame, Calif). For immunofluorescent staining, the antibodies were mouse anti-fluorescein (Roche, Mannheim, Germany), CVB3 (Light Diagnostics. Millipore Corp. Billerica, Mass), CD45, PE-FoxP3, and APC-CD25 (BD PharMingen). Fluorescent images were acquired with the FV1000 confocal microscope (Olympus IX81).

Preparation of Neonatal Rat Cardiac Myocytes
Ventricular myocytes were isolated from 2-day-old neonatal Sprague-Dawley rats (Charles River Canada Inc, Quebec, Quebec, Canada) as previously described. The cardiomyocytes were cultured at 37°C in 5% carbon dioxide and 95% air.

Cell Culture of HL-1 Cardiomyocytes
HL-1 mouse atrial tumor–derived cardiomyocytes were originally obtained from Dr W. Claycomb (Louisiana State University Medical Center, New Orleans, La) and cultured as described earlier.

Coxsackie-Adenovirus Receptor Expression and CVB3 Infection in Cell Culture
Cardiomyocytes were serum starved for 8 hours before being treated with or without recombinant tumor necrosis factor-α (TNF-α; Chemicon International, Temecula, Calif) and purified human transforming growth factor-β (TGF-β; R&D Systems, Minneapolis, Minn) with a final concentration of 20 μg/mL and 10 μg/mL, respectively. The treated cells were harvested at 48 hours for immunostaining or protein extraction. For CVB3 infection, the cells were incubated with the virus at 100 multiplicity of infection for 1 hour; then the inoculum was replaced with fresh medium with or without TNF-α (20 μg/mL) and/or TGF-β (10 μg/mL) and incubated for 48 hours.

Statistical Analysis
Survival was analyzed by the Kaplan-Meier method, and differences between groups were tested by the log-rank test. Data from different treatment groups at different time points were subjected to 2-way ANOVA analysis and Tukey-Kramer multiple subgroup comparisons. The data that were not normally distributed (eg, virus titer, cell count, and plasma cytokines) were logistically transformed to remove variance heterogeneity before statistical testing. Comparison of the ratio of heart weight to body weight was analyzed with nonparametric Kruskal-Wallis test. The data-analyzing software was JMP 8.0 (SAS Institute, Inc, Cary, NC). Values are expressed as mean±SEM, with values of P<0.05 considered significant.

Results

Adaptively Transferred Cells Were Present in the Spleen and Heart
In preparation for adoptive transfer, T cells from spleen were isolated and stained with CD4 and CD25 antibodies. Through fluorescence-activated cell sorting, ~8.7% of the CD4+ T cells were identified as CD4+CD25+, and the purity of the Tregs was recorded at 96.1%, of which 83% were also positive for FoxP3 and >96% were negative for CD127 (Figure 1A). CD127 has been shown to be a negative marker for Tregs. Before retroorbital injection of the isolated cell
Figure 1. Isolation, CFSE label, and transfer of CD4\(^+\) and Tregs. A, Spleen cells from uninfected mice were CD4 positively selected, stained with anti–CD4-PE and CD25-APC, and sorted to CD4\(^+\)CD25\(^-\) (naïve CD4\(^+\) T cells) and CD4\(^+\)CD25\(^+\) cell (Treg) populations. The purity of the Tregs and staining for Foxp3 and CD127 were then checked again after sorting. B, The sorted naïve CD4\(^+\) T cells or Tregs were stained with CFSE and confirmed by flow cytometry. C, Using anti-CFSE antibody, we visualized the transferred cells in the spleen with brown (original magnification ×200). Transferred Tregs were observed under confocal microscope in spleen (D) and heart (E) at postinfection (pi) day 7 (green for CFSE, blue for DAPI staining nuclei, red for FoxP3, APC-CD25 was acquired at 633 nm with the pseudocolor of magenta). Positive staining of transferred Tregs therefore is white (arrows). Insets are low-magnification images. Bars=17 μm.
population into the recipients, they were stained with CFSE (Figure 1B). The transfer was deemed successful because cells labeled with CFSE were detected by anti-CFSE antibody (Figure 1C). The phenotype of Tregs was maintained after transfer because the CFSE-positive cells were also positive for CD25 and FoxP3 in both spleen (Figure 1D) and heart (Figure 1E) at postinfection day 7.

Treg Transfer Protected Mice From CVB3 Myocarditis

After virus inoculation, Kaplan-Meier analyses showed that the Treg-treated group had a significantly higher 14-day survival rate with 76% of mice surviving compared with the naïve CD4+ T cell–treated animals (CD4 group) with only 40% surviving ($P=0.0136$), but this was not statistically different compared with the PBS-treated group with 48% surviving ($P=0.0589$; Figure 2A). At postinfection day 7, when myocardial necrosis is usually apparent on pathology, the body weight of all infected mice was found to be lower than at day 0 by 16% to 30%. The ratio of heart weight to body weight was greatest in mice with the transfer of CD4+ T lymphocytes compared with the PBS and Treg groups, whereas the Treg transfer group showed a significantly lower ratio of heart weight to body weight compared with the CD4 group ($P<0.05$; Figure 2B) at postinfection day 7.

Pathological analysis showed evidence of myocardial damage consisting of myocytolysis (necrosis) and inflammatory cell infiltration (Figure 3) in accordance with the Dallas criteria.16 There were no histological abnormalities in the hearts of either the control mice or the cell-transfered mice on day 4 (Figure 3A and 3C), although CVB3 was already present in the hearts (Figure 3B). At postinfection days 7, 10, and 14, Treg transfer significantly reduced inflammatory score compared with the CD4 and PBS groups (Figure 3A). The cardiac viral titer peaked at day 7 when inflammation became apparent. A significant reduction in cardiac viral load was also found in the Treg transfer group at postinfection days 7 and 10 compared with the CD4 and PBS groups (Figure 3B).

To investigate further the nature of the inflammatory cell infiltration, the mononuclear cells were stained for subtype of immune cells. The results showed that macrophages were the dominant cell type at postinfection day 7. However, there was a significant reduction in the macrophage population in the Treg group compared with the PBS and CD4 groups (Figure 4). On the other hand, CD4+ T lymphocytes were also observed in the lesion, whereas CD8+ T cells were absent. Naturally killer cells and dendritic cells were not found in the lesion at postinfection days 7, 10, and 14 (data not shown). In the Treg group, from days 7 through 14, staining for both the virus and inflammatory cells was greatly reduced compared with the PBS and CD4+ groups ($P<0.05$; Figure 5). These findings, along with the better survival rate, decreased inflammatory scores, and virus titers, indicate that adoptively transferred Tregs protect mice from CVB3 infection by suppressing inflammation in the heart and by decreasing the cardiac viral load.

Treg Transfer Promoted Expression of Prosurvival Gene Phosphorylated AKT and Antiinflammatory Cytokine TGF-β and Decreased Coxsockie-Adenovirus Receptor Level in the Host Heart

It is known that Tregs can suppress the immune response through both direct cell contact and cytokine production.5,17 Therefore, we measured plasma cytokine levels from infected mice throughout the experiment. As shown in Figure 6, the levels of IL-10, interferon-γ, and TNF-α at postinfection day 4 were significantly higher in the CD4 group than in the PBS and Treg groups. Treg transfer did not significantly reduce cytokine levels compared with the PBS group except for IL-10 at days 4, 7, and 10 ($P<0.05$; Figure 6A). This suggested that Treg transfer does have very specific cytokine suppressive effect rather than broad cytokine suppression.

To further investigate the protective mechanisms of Tregs in the heart, we extracted protein from heart tissue at postinfection day 7 to assess cell survival signaling, cytokine and inflammation pathways, and coxsackie-adenovirus receptor (CAR) expression (Figure 6B). We first examined the expression of prosurvival gene phosphorylated AKT and found that the Treg group showed a significantly increased ratio of phosphorylated AKT to AKT ($P<0.05$). Activator of nuclear factor-κB ligand is also known to be important for osteoclast and endothelial cell survival by promoting upregulation of PI3-kinase and the phosphorylated AKT pathway.18 In this study, however, neither activator of nuclear factor-κB ligand nor nuclear factor-κB showed a significant difference in expression between the experimental groups. Although naturally occurring Tregs have been demonstrated to be able...
to produce both TGF-β and IL-10. We found that only TGF-β was significantly increased in the hearts of mice in the Treg transfer group (*P* < 0.05).

CAR is the primary receptor for CVB3 infection. Expression of CAR protein was significantly decreased in the Treg-treated group (*P* < 0.05; Figure 6B). Immunostaining of heart sections also demonstrated CAR presence from postinfection day 7 to 14 in the PBS and CD4 groups, whereas the Treg group showed significantly less staining (Figure I in the online-only Data Supplement). Expression of protein kinases associated with virus replication such as p56LCK and ERK1/2 was not affected (Figure 6B).

**TGF-β Negated CAR Expression Induced by TNF-α and Inhibited CVB3 Infection in Cardiac Myocytes**

To test whether the protective effect of Treg transfer is through increased TGF-β production, an in vitro study was performed with HL-1 cells and rat neonatal cardiac myocytes. HL-1 cells are the only cardiomyocyte cell line that have been used to study normal cardiomyocyte function with regard to signaling, electric, metabolic, and transcriptional regulation.19 TNF-α caused increased expression of CAR in both HL-1 cells and rat neonatal myocytes, whereas TGF-β had no effect on CAR expression but reduced the CAR levels induced by TNF-α (Figure 7A and 7B). Because cardiac myocytes can produce TNF-α after various stresses, including hypoxia and infection,20–22 increased CAR expression was expected in response to CVB3 infection. We next investigated the susceptibility of these cells to CVB3 infection with or without TGF-β treatment. Both immunostaining for CVB3 and virus titer analysis (Figure 7C and 7D and Figure II in the online-only Data Supplement) confirmed that TGF-β treatment significantly inhibited virus replication in the cardiac myocytes (*P* < 0.05). CAR has been reported to mediate
connexin 45 expression in the murine heart\textsuperscript{23,24}; however, the connexin 45 levels in rat neonatal myocytes were not changed by the treatment (Figure III in the online-only Data Supplement).

**Tregs Inhibited Immune Responses Against Autoantigen of the Heart Tissue**

To analyze the effects of Tregs on the host immune response, spleen cells were isolated, cultured, stimulated with either inactivated CVB3 or noninfected heart tissue homogenate (from the same mouse genotype), and quantified by splenocyte proliferation (Figure 8). The CD4\textsuperscript{+} and Treg transfer groups showed moderately higher response against inactivated CVB3 compared with the PBS group. Interestingly, both the PBS and CD4\textsuperscript{+} groups showed a strong response to heart tissue, whereas splenocytes from Treg-transferred mice showed a significantly weaker response ($P<0.05$). This suggested that Tregs had a broad suppressive effect against activation to self-antigen.

**Tregs Attenuate Inflammation and Viral Presence in the Pancreas and Spleen**

Histological analysis was also performed to examine the dissemination of the virus and inflammation in other organs. The pancreas showed extensive lymphocytic infiltration in the exocrine portion at postinfection day 7 (Figure IV in the online-only Data Supplement), whereas the islets were spared. Adoptive transfer of Tregs also significantly diminished the severity of the pancreatic disease at day 14 (Figure IV in the online-only Data Supplement) in contrast to the CD4\textsuperscript{+} and PBS groups. Immunostaining detected virus throughout postinfection day 14 in the pancreas and spleen (Figure V in the online-only Data Supplement). The Treg-treated group again showed significantly decreased CVB3 antibody–positive staining in pancreas (versus the PBS and CD4 groups at day 10 and versus the CD4 group at day 14, $P<0.05$) and spleen (versus CD4 group at day 14, $P<0.05$). There was no significant difference in virus positivity in the spleen between the PBS and Treg groups throughout the 14-day experiment. Tregs may not have an effect on CVB3 infection of immune cells because of undetectable expression of CAR in these cells.\textsuperscript{22} These data are consistent with those seen in the heart with broad effect of Tregs in suppressing both inflammation and viral proliferation.

**Discussion**

Viral myocarditis involves the processes of viral proliferation in host tissue balanced by the host immune response in eliminating the virus while minimizing damage to the host tissue. The host response is complicated by the immune activator through autoimmunity related to either molecular mimicry between epitopes shared by viral and host antigens or exposure to host cardiac antigens.\textsuperscript{2} Excessive autoimmune activation can lead to persistent severe host myocardial destruction despite a very significant reduction in viral titers. The ability to control the viral proliferation while minimizing host autoimmunity is the key to a favorable outcome.

Previous studies suggested that Treg cells can protect the host from autoimmune disease and exaggerated cellular immune responses. A recent study by Huber et al\textsuperscript{25} demonstrated that Tregs can inhibit cardiomyopathy caused by
TNF-α overexpression. The question that remains is whether Tregs can suppress host immune response. If so, is it at the expense of more severe viral proliferation?

In this study, we decided to compare the transfer of CD4<sup>+</sup>CD25<sup>+</sup> Tregs and naïve CD4<sup>+</sup> T cells in a CVB3-induced myocarditis model. CD4<sup>+</sup><sup>+</sup> rather than CD8<sup>+</sup> T cells<sup>28</sup> have been shown to be the key cell population mediating broad myocardial injury in murine models of myocarditis.

We have shown that the adoptive transfer of CD4<sup>+</sup>CD25<sup>+</sup> Tregs protected mice from early mortality after CVB3 infection through a reduction in cardiac viral load, inflammatory response, and autoimmunity against self-antigen. The transfer of naïve CD4<sup>+</sup> T cells alone exacerbated myocarditis with greater inflammatory scores, higher virus titers, and increased plasma levels of proinflammatory cytokines than in the PBS and Treg groups. Although transfer of naïve CD4 T cells showed accentuated inflammation in the heart histologically, the effect on survival was not significant compared with PBS-treated mice. Naïve CD4<sup>+</sup> T cells can differentiate into diverse effector and regulatory subsets to establish peripheral tolerance. Besides Th1 and Th2 effector subsets, which produce interferon-γ and IL-4, respectively, naïve T cells can differentiate into proinflammatory Th17 cells<sup>29</sup>. Even though naïve T cells are not antigen specific, they were more potent than memory CD4 cells in causing graft-versus-host dis-
ease. Although the mechanisms are unclear, further study suggests that naïve CD4 T cells constitutively express CD40L and augment autoreactive B-cell survival, and exposure to activating stimuli can further induce CD4 T-cell CD40L messenger RNA and protein expression by at least 10-fold, therefore amplifying autoimmune responses. The CD40L-CD40 pathway has frequently been implicated in the development of autoimmunity, including lupus and other autoantibody-mediated diseases, and the inhibition of CD40L was found to have therapeutic benefits in mouse disease models and in human lupus patients. This finding may partially explain why transfer of naïve CD4 T cells tended to increase response to heart homogenate and enhanced inflammation in the heart in our study.

Figure 6. Plasma cytokine levels and protein expression in heart tissue of CVB3-infected mice. A, Plasma IL-10, interferon-γ (IFN-γ), and TNF-α concentrations (log transformed) in PBS, CD4
t cells, and Treg mice (n=3 for each time points of nonrepeated samples) were measured by ELISA, and data were analyzed by 2-factor ANOVA. B, Protein was extracted from heart homogenates at postinfection day 7 and probed with antibodies as indicated after Western blotting (n=3). Data are expressed as mean±SEM. *P<0.05 vs CD4 (naïve CD4 T cells) groups; †P<0.05 vs PBS group.
In this study, TGF-β was highly expressed in the heart after Treg transfer in response to CVB3 infection. IL-10, which is produced by another subtype of inducible Tregs, was not significantly increased in the heart of infected mice after Treg transfer. After infection, in the Treg group, there was also significantly increased expression of phosphorylated AKT, which is critical for cell survival. However, activator of nuclear factor-κB ligand and nuclear factor-κB, which are upstream of the activation of AKT, showed no difference in expression between groups. It is likely that TGF-β was directly responsible for AKT activation in this model in that it plays a role in epithelial cell survival via AKT-dependent regulation of FKHRL1.

Although CVB3 can exploit leukocyte-specific protein tyrosine kinase and ERK-1/2 to promote its replication in T cells and myocytes as reported by our group earlier, we did not find a significant change in ERK-1/2 phosphorylation between the 3 groups. However, the reduction in viral titers is likely related to the significantly decreased expression of the viral receptor CAR in the hearts of the Treg-treated group. This is potentially attributable to the suppressed inflammation exerted by Tregs and high TGF-β production. A previous study has found that Tregs inhibit cardiomyopathy by suppressing TNF-α production. Our present study using both HL-1 cells and rat neonatal cardiac myocytes showed that TGF-β reduced CAR levels induced by TNF-α and inhibited CVB3 infection in these cells. TGF-β has also been shown to reduce CAR levels in cancer cells. CAR expression is positively related to the extent of inflammation in the cardiac myosin–induced myocarditis model. In our previous study, knockout of MyD88, an adaptor involved in toll-like receptor signaling, causes reduced cardiac expression of CAR and proinflammatory cytokines.

Spleen cells from the Treg group tolerated the heart tissue antigen efficiently, whereas spleen cells from the CD4 or PBS groups did not (Figure 8). This is in the context of Tregs having a strong response to CVB3, suggesting that transfer of Tregs promotes an antiviral immune response while sparing native host tissue from autoimmune-activated inflammatory damage. Antibodies against myosin heavy chain, actin, and tropomyosin have been found in CVB3-infected mice; cardiac myosin has been demonstrated to be an autoimmune antigen that can induce myocarditis and plays a pathogenic role in postinfectious myocarditis. In contrast to immunosuppressive therapy, which causes nonspecific inhibition to a broad repertoire of immune responses, Tregs have a large
repertoire of self-specific T-cell receptors and suppress via contact-dependent or high TGF-β/IL-10 production. Tregs likely increase the threshold of the immune response and can attenuate local inflammation by providing a mechanism for tissue-specific regulation of the balance between immune activation and suppression.40

Conclusions
The adoptive transfer of Tregs adjusts the appropriate immune response to CVB3 in heart tissue, thus confirming the regulatory role of Tregs in CVB3-induced myocarditis. In the future, manipulating Tregs through small molecules or biological agents may provide a novel therapeutic strategy for viral myocarditis and other similar viral infection to the host.

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Disclosures
None.

References


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SUPPLEMENTAL MATERIAL
Methods

Cell Separation and Flow Cytometry

Four uninfected C57Bl/6 mice (8 weeks) were euthanized with isoflurane for splenocyte isolation. Non-CD4+ T cells were depleted using Dynal Mouse CD4 Negative Isolation Kit (Invitrogen, CA, USA). Then the CD4+ cells were costained with PE-CD4 (BD PharMingen, NJ, USA), and APC-CD25 (BD PharMingen). For fluorescence-activated cell sorting (FACS), 2 x 10^7 to 3 x 10^7 cells/ml in full RPMI-1640 media (Invitrogen) with no phenol red were analyzed on a Aria-RITT BRV cell sorter (Becton Dickinson, CA, USA). CD4+CD25+ T cells and CD4+ cells were sorted and the purity of the cells was examined. Small portion of the sorted cells were further stained with PE-Cy7-CD127 (eBioscience, CA, USA), then fixed and permeabilized with solution containing 1xHBSS, 10 mM HEPES, 4% paraformaldehyde, and 0.05% saponin before incubating with FITC-FoxP3 (Thermo Scientific. IL. USA).

In Vivo Adoptive Transfer

The sorted cells were pooled before transfer to recipient mice. For labeling, the sorted T cells were incubated with 2 µM Carboxyfluorescein diacetate, succinimidyl ester (CFSE) (Vybrant CFDA SE cell Tracer Kit, Invitrogen) at 37°C for 30 minutes and washed with PBS. Recipient mice were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg). About 3 x 10^5 cells in 100 µl PBS were transferred to recipient mouse via retro-orbital injection. The labeled cells or PBS were infused for 3 times at 2 weeks interval. Immediately after the 3rd infusion, the mice were challenged with CVB3 under anesthesia.
**Immunofluorescent staining**

Frozen sections were used to stain transferred cells with fluorescence-conjugated antibodies. The frozen sections were air-dried for 20 minutes then fixed in cold acetone for 10 minutes before application of APC-CD25 and PE-FoxP3 antibodies (BD Pharmingen). The sections were mounted with DAPI antifade (Sigma). Fluorescent images were acquired using the confocal microscope FV1000 (Olympus IX81) with lasers of UV (405 nm), Argon (448 nm, 488 nm, and 515 nm), HeNe (543 nm, 633 nm). The filters used for these experiments: (1) CFSE filter (green pseudocolor) set: excitation 488 nm, emission 514 nm; (2) PE filter (red pseudocolor) set: excitation 488 nm, emission 575 nm; (3) APC filter (magenta pseudocolor) set: excitation 633 nm, emission 660 nm; (4) DAPI filter (blue pseudocolor) set: UV laser, emission 455 nm.

**ELISA Measurement of Plasma Cytokines**

Blood was collected at days 4, 7, 10 and 14 post infection and plasma cytokine levels were measured with ELISA kits. IL-10, TNF-α, IFN-γ were purchased from eBioscience, MCP-1 and GM-CSF were from R&D Systems (Minneapolis, MN, USA).

**Cell Proliferation Assay**

Splenocytes were prepared and co-cultured with either heart tissue homogenates (29.3 µg/ml) or heat-inactivated CVB3 at multiplicity of infection (MOI) 4:1. The concentration of the heart tissue homogenates and virus were optimized after several preliminary experiments. To prepare the heat-inactivated CVB3, the virus was heated at 90°C for 30 minutes. The cell proliferation assay was carried out using a
Vybrant CFDA SE cell Tracer Kit (Invitrogen). According to the manufacture’s instruction, CFSE labeling is distributed equally between the daughter cells, which are therefore half as fluorescent as the parents. As a result, halving of cellular fluorescence intensity marks each successive generation in a population of proliferating cells, and this was readily followed by flow cytometry, Coulter Cytomics FC500 with CXP software (Becton Dickinson).

**Western Blot Analysis**

Protein was extracted by homogenization from the heart tissue and detected with primary antibodies as follows: AKT and phospho-AKT (Cell Signaling, Danvers, MA), TGF-β (MBL, MA. USA), RANKL (IMGENEX, CA, USA), Rabbit anti-LCK polyclonal antibody (abcam, Cambridge, MA), NF-κB (Sanata Cruz, CA, USA), IL-10 (ABD Serotic), Rabbit anti-CAR polyclonal and goat anti-Cx45 (Santa Cruz), Lck (Abcam, Cambridge, MA), ERK1/2 and Phospho-ERK1/2 (Cell Signaling), GAPDH (Santa Cruz).

**Supplemental Figure 1.** Coxsackievirus adenovirus receptor (CAR) expression in heart tissue of CVB3-infected mice at days 4, 7, 10, and 14 postinfection. A. Positive staining cardiomyocytes are apparent as a brown color in the cytoplasm. Original magnification, ×200. B, Quantitative analysis of intensity of CAR expression in heart sections. * indicates $P<0.05$ vs CD4 groups (naïve CD4$^+$ T cells); † indicates $P<0.05$ vs PBS group. Data are expressed as mean ± SEM.

**Supplemental Figure 2.** CVB3 infection in HL-1 cells. A, Immunostaining of CVB3 in HL-1 cells. The cells were incubated with 100 MOI of CVB3 for 1 hour, then the inocula was replaced with fresh medium with treatment of TNF-α or TGF- β for 48.
Cells then were fixed and stained with anti-CAR antibody (red color). The nuclei were counterstained with DAPI (blue color). B, Infected cell homogenates were applied to plaque assay for virus titers. Original magnification of photographs is 200x.* indicates \( P<0.05 \) vs TNF-\( \alpha \) treatment. Data are expressed as mean ± SEM.

**Supplemental Figure 3.** Western blotting and quantitative analysis for connexin 45 (Cx45) expression in rat neonatal cardiac myocytes. Data are expressed as mean ± SEM.

**Supplemental Figure 4.** Treg transfer also attenuated inflammation in the pancreas. Representative photographs of H&E sections (original magnification, \( \times200 \)). Long arrows show necrotic lesions with mononuclear cell infiltration. Short arrows show spared islets of Langerhans. Inflammatory scores were determined in H&E stained sections and shown in the graph. Quantitative data are expressed as mean ± SEM. * indicates \( P<0.05 \) vs PBS and CD4 groups (naïve CD4\(^+\) T cells). * indicates \( P<0.05 \) vs CD4 groups; † indicates \( P<0.05 \) vs PBS group.

**Supplemental Figure 5.** Detection of CVB3 in pancreas and spleen at day 7, 10, and 14 post-infection. A, Virus in the cytoplasm stained brown. Pancreas sections include islets of Langerhans and acini. Area around periarterial lymphatic sheath are shown in the spleen sections (original magnification, \( \times200 \)). B, Quantitative analysis of intensity of CVB3 positive cells. * indicates \( P<0.05 \) vs CD4 groups (naïve CD4\(^+\) T cells); † indicates \( P<0.05 \) vs PBS group. Data are expressed as mean ± SEM.
Supplemental Figure 1
A  

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B  

**Plaque assay of HL-1 cells**

![Graph](image13.png)

Supplemental figure 2
Supplemental Figure 3

Neonatal myocytes

![Image of a graph showing Cx45 and GAPDH expression levels with PBS, TNF-α, and TGF-β treatments]
Supplemental Figure 4
Supplemental Figure 5