Coxsackievirus B3 Induces T Regulatory Cells, Which Inhibit Cardiomyopathy in Tumor Necrosis Factor-α Transgenic Mice

Sally A. Huber, Arthur M. Feldman, Danielle Sartini

Abstract—Innate immunity promotes both the generation of autoimmunity and immunoregulation of adaptive immunity. Transgenic mice expressing the tumor necrosis factor-α (TNFα) gene under the cardiac myosin promoter (TNF1.6 mice) develop dilated cardiomyopathy. Transgenic mice show extensive cardiac inflammation, suggesting that immunopathogenic mechanisms may promote cardiomyopathy. Two coxsackievirus B3 (CVB3) variants infect and replicate in the heart. H3 variant is highly myocarditic, but H310A1 variant activates CD4+ T regulatory cells, which protect against viral myocarditis. T-cell depletion of TNF1.6 mice using monoclonal anti-CD3 or anti-CD4 antibody significantly reduced heart size and plasma troponin I concentrations compared with control TNF1.6 mice. Cardiomyopathy in TNF1.6 mice correlates to a CD4+Th1 response and autoimmune IgG2a antibodies. TNF1.6 mice infected with H310A1 virus reduced heart size and cardiac inflammation corresponding to the activation of CD4+CD25+Fox3+ (T regulatory cells). Immunosuppression is dependent on IL-10 but not TGFβ. Adoptive transfer of the CD4+CD25+ cells from H310A1-infected mice into uninfected TNF1.6 recipients abrogated cardiomyopathy. Exogenous administration of recombinant TNFα to H310A1-infected mice for 4 days abrogated immunosuppression. Cardiac enlargement in TNF1.6 mice is partly attributable to T-cell activation and humoral autoimmunity caused by cytokine expression. T regulatory cells induced by H310A1 virus abrogate autoimmunity caused by TNFα overexpression. H3 virus infection induces high levels of systemic TNFα, whereas H310A1 virus does not. The low TNFα response during H310A1 infections is likely responsible for the T regulatory cell response in these animals. (Circ Res. 2006;99:0-0.)

Key Words: myocarditis ■ inflammation ■ infection

Coxsackievirus B3 (CVB3) induces myocarditis and dilated cardiomyopathy.1-2 Cardiac injury results from both direct viral injury and host responses to infection.1 Cytokines, including tumor necrosis factor-α (TNFα), interleukin (IL)-2, IL-1β, and IL-6, cause cardiac dysfunction.3 Increased plasma concentrations of TNFα, a proinflammatory cytokine induced by infection and ischemic injury, are found in patients with congestive heart failure and dilated cardiomyopathy.4-6 Lymphoid cells, especially macrophage,7,8 and cardiac myocytes7 produce TNFα. TNFα decreases myocardial contractile efficiency, reduces ejection fraction, and induces biventricular dilation.5 TNFα causes acute disruption of calcium transients by affecting L-type channel–induced calcium influx through increases in sphingosine.5 Sustained contractile dysfunction results from TNFα induced desensitization of myofilaments to intracellular calcium through upregulation of nitric oxide.5 Other effects of TNFα on cardiac function include death of myocytes, disruption of excitation/contraction coupling, and induction of reactive oxygen species.5 As might be expected, a transgenic mouse containing the TNFα gene under the cardiac myosin promoter develops cardiomyopathy.9 The cardiomyopathy results from TNFα because anti-TNFα antibody treatment partially reverses cardiac failure.10 Nonetheless, the transgenic mice also have extensive cardiac inflammation,11 and this raises the question of whether immunopathogenic mechanisms also precipitate cardiomyopathy.

H3 and H310A1 CVB3 differ by a single nonconserved amino acid in the VP2 capsid protein.12 H3 causes severe myocarditis and induces a strong TNFα cytokine response. H310A1 is nonmyocarditic and fails to elicit TNFα. H310A1 preferentially induces a CD4+ T regulatory cell population that is not induced by H3.13 The CD4+ T regulatory cell effectively prevents H3-induced myocarditis when adoptively transferred before H3 virus infection. This communication demonstrates that the T regulatory cell is highly effective in abrogating dilated cardiomyopathy in TNFα transgenic mice (TNF1.6),10,11 showing that virus-induced T regulatory cells suppress already established heart disease as well as preventing the initiation of myocarditis.
Materials and Methods

Mice
Male TNF1.6 transgenic mice were used at 5 to 6 weeks of age. The TNF1.6 mice have cardiac-specific TNFα overexpression and are on the FVB/J background strain. FVB/J mice were purchased from The Jackson Laboratory (Bar Harbor, Me). The investigation conforms to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). The animal facility at the University of Vermont is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and the experiments were approved by the University Internal Animal Care and Use Committee.

Virus and Virus Titrations
The H3 and H310A1 variants of CVB3 were used. Adult mice were infected by IP injection with 10^5 plaque-forming units (pfu) of virus in PBS and killed 7 days later. Hearts were perfused with PBS, weighed, and then homogenized in 0.9 mL of RPMI medium 1640. Debris was removed by centrifugation at 300 g for 10 minutes, and the homogenate supernatant was titered using the plaque forming assay.

Debris was removed by centrifugation at 300 g in PBS and killed 7 days later. Hearts were perfused with PBS, stained with hematoxylin/eosin, and evaluated for myocyte apoptosis.

TNFα and Antibody Injections
Mice were injected IP with 0.5 mL if PBS containing either 300 ng of recombinant mouse TNFα (Pharmingen) or 100 μg of monoclonal anti-CD3 (clone 17A2; Pharmingen), anti-CD4 (clone GK1.5; American Type Culture Collection), or anti-CD8 (clone 2.43; American Type Culture Collection). Some H310A1-infected mice were injected IV through the tail vein with 0.25 mg of monoclonal anti–interferon-γ (clone JES5–2A5; Pharmingen) or anti-TGF-β (clone 1A12; R&D Systems) on days 2 and 5 after infection.

Histology
Hearts were fixed in 10% buffered formalin, paraffin embedded, sectioned, stained with hematoxylin/eosin, and evaluated for mycarditis using a 0 to 4 scale, where 0 represents no inflammation; 1 represents 1 to 10 inflammatory loci/section; 2 represents 11 to 20 inflammatory loci/section; 3 represents 21 to 40 inflammatory loci/section; and 4 represents >40 inflammatory loci/section as published previously.

Flow Cytometry
Inflammatory cells in the heart were isolated by digestion finely minced hearts with 0.4% collagenase II (Sigma Chemical Co, St Louis MO) and 0.25% pancreatic (Sigma). Spleens were pressed through fine mesh screens. The cellular debris was allowed to settle and the supernatant was centrifuged on Histopaque (Sigma). Blood was collected by intracardiac puncture in EDTA and centrifuged on Histopaque. The intracellular cytokine staining protocol has been published. Cells (10^6) were cultured for 4 hours with 10 μg/mL brefeldin A (BFA) (Sigma), 50 ng/mL phorbol myristate acetate (PMA) (Sigma), and 500 ng/mL ionomycin (Sigma); washed; incubated with a 1:100 dilution of Fc Block, APC-Cy7 rat-anti-mouse CD4 (clone GK1.5), and fluorescein isothiocyanate (FITC)-hamster anti-Vγ4 (clone UC3), APC-Cy7 rat IgG1 (clone R3-34), and FITC-hamster IgG (clone G253-2536). The cells were washed, fixed in 2% paraformaldehyde; resuspended in PBS-BSA containing 0.5% saponin, Fc Block, and 1:100 dilutions of phycoerythrin (PE)-rat IgG1 (clone R3–34), PE anti-FoxP3, or PE-rat IgG1 (clone R3–34); washed; and resuspended in 2% paraformaldehyde for flow cytometry. Additional cells (10^6 cells/well) were washed once with PBS containing 1% BSA and labeled with 1:100 dilutions of PE–anti-IFNγ (clone XMG 1.2), PE–anti-IFNα/β (clone H9253), or PE–anti-FoxP3, or PE-rat IgG1 (clone R3–34); washed; and resuspended in PBS containing 1% BSA and labeled with 1:100 dilutions of APC-Cy7 anti-CD11b (clone M1/70), PE anti-CD45R/B220 (clone RA3–6B2), and FITC-anti-CD3 (clone 17A2), FITC-anti-CD4 (clone GK1.5), and APC-Cy7 anti-CD25 (clone PC61). All reagents were purchased from Pharmingen/eBioscience.

Statistical Analysis
Data were analyzed for skewness and kurtosis using the SPPS for Windows program (Version 11.0, 2001; SPSS Inc, Chicago, Ill). Statistical analysis was done by Wilcoxon ranked score because variance was not normally distributed in many groups. Data represent 1 of at least 2 experiments.

Results
Heart-Specific Antibody and CD4+Th1 Cells in TNF1.6 Mice
Hearts, plasma, and spleen of uninfected TNF1.6 transgenic mice were assayed for TNFα (Figure 1A). TNFα expression in uninfected TNF1.6 transgenic mice is restricted to the heart (P<0.001 compared with hearts of FVB mice). Next, TNF1.6 mice were infected or killed 7 days earlier with 10^5 pfu H3 or H310A1 virus (Figure 1B). TNFα concentrations in plasma from H3-infected mice were significantly increased compared with uninfected or H310A1-infected animals. TNF1.6 mice develop dilated cardiomyopathy accompanied by the interaction of TNFα and other proinflammatory cytokines with CD4+Th1 cells.
with extensive myocardial inflammation.\textsuperscript{9} To determine whether inflammation contributes to cardiomyopathy, TNF1.6 mice were treated with 100 μg of monoclonal anti-CD4 or anti-CD8 antibodies twice 3 days apart and then killed 6 days after the last antibody treatment (Figure 1C to 1E). Heart weight was significantly reduced in only mice treated with anti-CD4 antibody. Analysis of CD4\textsuperscript{+}IFN\textgamma\textsuperscript{+} (Th1) and CD4\textsuperscript{+}IL-4\textsuperscript{+} (Th2) cells showed that Th1 cells predominate. Because previous studies showed IgG deposits in the myocardium of TNF1.6 mice,\textsuperscript{11} plasma was evaluated for cardiac myocyte-reactive antibody and showed high titers of IgG2a anti-myocyte antibodies but little IgG1 antibody.

**Figure 1.** A, Heart, spleen, and plasma were isolated from uninfected FVB and TNF1.6 mice. Heart and spleen were homogenized in 1 mL of medium. Homogenate supernatant and plasma were assayed for TNFα by ELISA using the Endogen Inc kit according to the directions of the manufacturer. Results are mean±SEM of 6 or more mice per group. B, Plasma from uninfected TNF1.6 mice and mice infected 7 days earlier with 10\textsuperscript{5} pfu of either H3 or H310A1 virus was evaluated for TNFα. Each dot represents an individual animal. In A, *significantly different from FVB at \( P<0.01 \) (A). In B, *indicated groups are significantly different at \( P<0.05 \). C, TNF1.6 mice were injected IP with 100 μg of rat IgG (isotype), monoclonal anti-CD4, or anti-CD8 antibody twice and killed 6 days after the last injection. Hearts were perfused and weighed. D, Peripheral blood mononuclear cells (PBMCs) were cultured with PMA, ionomycin, and BFA for 4 hours, and the cells were stained with antibodies to CD4 and intracellularly with antibodies to IFNγ and IL-4. E, Plasma was titered for antibody to cardiac myocytes as described in Materials and Methods. #Results are significantly different from isotype control at \( P<0.05 \). Groups consisted of 3 mice.
determined by heart weight and troponin I levels, whereas anti-TGFβ was minimally effective. These results demonstrate that IL-10 is the major immunoregulatory factor during H310A1 infection.

Next, inflammatory cells were isolated from the heart. Total numbers of recovered cells/heart (n=6/group) are given in Figure 3A. Inflammatory cells in uninfected TNF1.6 animals consisted mostly of CD11b+, CD3+, CD4+, and B220+ (B lymphocyte) cells (Figure 3B). H3 infection greatly increased numbers of all of these cells. H310A1 infection decreased CD11b+, CD3+, and CD4+ populations over uninfected mice but increased in CD4+CD25+ cells. All CD4+CD25+ cells in the heart are also FoxP3+. As expected, anti-CD3 treatment substantially reduced T cells in the heart, although numbers of CD11b+ cells were equivalent to uninfected TNF1.6 mice.

Peripheral blood mononuclear cells were isolated from H310A1-infected mice and infected mice treated with anti–IL-10 or anti-TGFβ (Figure 4). All CD4+CD25+ cells were FoxP3+ in H310A1-infected mice. Anti–IL-10 treatment significantly reduced the number of Treg cells, whereas anti-TGFβ treatment had no significant effect.

To confirm immunoregulation, CD4+CD25+ and CD4+CD25− cell populations were isolated from the spleens of uninfected, H3-infected, and H310A1-infected TNF1.6 mice and adoptively transferred into uninfected TNF1.6

Figure 2. A, Hearts were obtained from 6-week-old male uninfected FVB mice and TNF1.6 mice, which were either uninfected or infected for 7 days with 10^5 pfu H3 or H310A1 virus, were weighed in milligrams. Uninfected TNF1.6 mice were treated on 2 consecutive days with 100 μg of monoclonal anti-CD3 and killed 7 days after the last antibody treatment. H310A1-infected mice were treated on days 2 and 5 relative to infection with 0.25 mg of anti-IL-10 or anti-TGFβ. B, Animals were weighed in grams. C, Plasma murine troponin I concentrations in TNF1.6 mice. D, Splenocytes from TNF1.6 mice were stimulated with PMA and ionomycin for 4 hours in vitro, and the supernatants of the cultures were evaluated by ELISA for IL-10 and TGFβ. E, Photographs of hearts from representative mice. F, Histology of hearts from representative mice. Results represent mean±SEM of 6 mice per group. *Significantly different from uninfected FVB at P<0.05. #Mice are significantly different from uninfected and H3-infected TNF1.6 mice at P<0.05.
recipients (Table 1). The transferred cells were negative for virus as shown by titrating homogenized cells on Hela cell monolayers in the plaque forming assay. Figure 5 shows representative purity of the transferred cell populations. Recipient TNF1.6 mice were killed 7 days after cell transfer. Animals were evaluated for heart and body weight, plasma troponin I levels, and CD4+CD25+ cells in the spleen. CD4+CD25+ cells from H310A1-infected mice inhibited cardiomyopathy (reduced heart weight; decreased plasma troponin I concentrations) in recipient mice. Neither CD4+CD25+ cells from uninfected nor H3-infected donors were inhibitory. Giving CD4+CD25+ cells from H310A1-infected mice also increased splenic CD4+CD25+ cells in recipients. Plasma troponin I levels were increased in recipients given CD4+CD25+ cells from H310A1-infected donors, suggesting that a cardiopathic T cell is activated during H310A1 infection but inhibited by the CD4+CD25+ cells in the same animal.

**Discussion**

The immune system is divided into innate and adaptive immunity, in which the former involves rapid and broad
reactivity to a wide range of infectious agents, whereas the latter is highly specific to each inducing microbe. The innate system help control infections until the more effective and specific adaptive immune response develops and clears the microbes. However, the initial innate immune system also has a profound effect on the strength and type of adaptive immune response.19,20 Transgenic expression of TNFα, a proinflammatory cytokine usually induced by infection, initiates the development of cardiomyopathy.9 Depletion of CD4+ T cells in TNF1.6 mice is partially protective. The presence of IgG2a autoimmune anti-myocyte antibodies, substantial IgG deposits in the myocardium, and widespread myocardial inflammation in TNF1.6 mice11 is consistent with a role for humoral autoimmunity in cardiomyopathy. The ability of T regulatory cells to suppress existing dilated cardiomyopathy in the TNFα transgenic mice is also consistent with immunopathogenicity. Adoptive transfer of H310A1 immune CD4+CD25+ cells substantially reduces cardiomyopathy in TNF1.6 recipients, whereas the same populations from uninfected or H3 virus–infected mice have no inhibitory effects.

TNFα is a potent proinflammatory cytokine known to promote induction or progression of autoimmunity.21 The high levels of TNFα likely affect dendritic cells in the heart, resulting in self antigen processing and migration of the maturing dendritic cells to peripheral lymphoid tissues for autostimulation. High peripheral TNFα levels are clearly not necessary for autoimmunity induction because TNF1.6 mice have minimal plasma levels of this cytokine despite extremely high levels in the myocardium. The most surprising observation is that systemic TNFα is effective in abrogating T regulatory cell activity. T regulatory cells are important modulators of the adaptive immune response.22 There are three known types of T regulatory cells. T regulatory 1 (Tr1) cells produce high levels of IL-10 and IL-5 but either no or only moderate amounts of TGFβ.23 Tr1 cells express both CCR5 and T1-ST2. Because Th1 cells preferentially express CCR5 (CD195) over T1/ST2, whereas Th2 cells preferentially express T1-ST2 over CCR5, the expression of both markers on Th1 cells implies that these cells represent a distinct subset of CD4+ T cells,24,25 IL-10 is required for Tr1 induction and neutralization of IL-10 abrogates Tr1-mediated suppression.25 Other regulatory populations include Th3 cells, which express high levels of TGFβ,24 and natural T regulatory cells, which require direct cell/cell contact rather than cytokine secretion for suppression.26 The T regulatory cell induced during H310A1 infection probably is a Tr1 cell because IL-10 suppresses cardiomyopathy and Tr1 cells usually inhibit Th1 cell responses,24,27 the dominant CD4+ cells in TNF1.6 mice. IL-10 produced by dendritic cells

![Figure 5](image_url)

**Figure 5.** Representative original unsorted CD4+ (A) and sorted CD4+CD25+ (B) and CD4+CD25− (C) cell populations isolated from donor mice for adoptive transfer into uninfected TNF1.6 recipients. Top number is percentage of CD4+CD25+, and bottom number is percentage of CD4+CD25−.
promote the differentiation of Tr1 cells, possibly by upregulating costimulatory factors CD40, CD80, and/or CD86. Although TNFα can promote the induction of IL-10–producing Tr1 cells, in the CVB3 model, TNFα is highly effective in abrogating immunosuppression because exogenous administration of this cytokine substantially enhanced dilated cardiomyopathy and reduced CD4+CD25+FoxP3+ cells.

The reason(s) why TNFα promotes Tr1 cells in another system but abrogates Tr1 cells in H310A1 infection is probably complex. Although most studies find Tr1 cells suppress Th1 cells, some reports indicate they suppress Th2 responses. This means either that different Tr1 cell types exist or that Tr1 cell function varies depending on environmental and immunological conditions. Tr1 cells have been induced in response to both noninfectious (ovalbumin, nickel, or allograft immunity), and infectious (helminth, bacterial, and viral) stimuli. Both CD4+Th1 and Tr1 cells may respond to the same antigenic epitopes, indicating that antigen specificity is not unique for Tr1 cells but the conditions (high IL-10 prevalent during activation) cause Tr1 rather than Th1 responses. Coxsackievirus infection has many direct effects on cells through activation of nuclear factor κB, p38, extracellular signal-regulated kinase, and c-Jun N-terminal kinase signal pathways. TNFα might suppress Tr1 cell activation in CVB3 infections through effects on virus signaling combined with the effects of TNFα itself. Toll-like receptors (TLRs) activated during infections can modulate T regulatory responses. TLR4 activated by Bordetella pertussis initiates IL-10–dependent Tr1 responses, and CVB3 activates TLR4 and may induce Tr1 cells through this mechanism.

### Sources of Funding

The work was supported by NIH grants HL58583 and PO1 AI45666.

### Disclosures

None.

### References


### TABLE 2. Exogenous TNFα Treatment of H310A1-Infected Mice Abrogates Protection

<table>
<thead>
<tr>
<th>Virus</th>
<th>TNFα (300 ng)</th>
<th>Body Weight (mg)</th>
<th>Heart Weight (mg)</th>
<th>Heart’ Body</th>
<th>Cardiac Virus Titre (dil:lug)</th>
<th>CDA'+FoxP3+ (%)</th>
<th>CDA'+FoxP3+ (%)</th>
<th>CDA-CD25* (%)</th>
<th>CDA-CD25* (%)</th>
<th>IFNγ 4+ (%)</th>
<th>Which Am % IFNγ 4+ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>23664±3305</td>
<td>215±27</td>
<td>0.0087</td>
<td>0.2</td>
<td>1.9±0.9*</td>
<td>0.5±0.7*</td>
<td>0.1±0.1</td>
<td>0.3±0.1</td>
<td>16.7</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>300</td>
<td>22870±890</td>
<td>215±14</td>
<td>0.0094</td>
<td>0.2</td>
<td>2.9±0.5*</td>
<td>0.4±0.2*</td>
<td>0.1±0.1</td>
<td>0.4±0.1</td>
<td>25.0</td>
<td></td>
</tr>
<tr>
<td>10⁶ PFU H310A1</td>
<td>0</td>
<td>25030±1080</td>
<td>157±22</td>
<td>0.0063*</td>
<td>4.56±0.9</td>
<td>0.9±0.4*</td>
<td>2.7±0.5*</td>
<td>0.9±0.2*</td>
<td>0.4±0.2*</td>
<td>16.0</td>
<td></td>
</tr>
<tr>
<td>10⁶ PFU H3</td>
<td>0</td>
<td>20378±1219h</td>
<td>209±24</td>
<td>0.1010*</td>
<td>5.06±0.3</td>
<td>3.7±1.2*</td>
<td>0.8±0.4*</td>
<td>0.5±0.3</td>
<td>1.3±0.2*</td>
<td>52.0</td>
<td></td>
</tr>
<tr>
<td>0.9</td>
<td>0.9</td>
<td>19483±2032</td>
<td>234±31</td>
<td>0.0120</td>
<td>5.3±1.7</td>
<td>8.0±3.3</td>
<td>1.0±0.2</td>
<td>0.4±0.1</td>
<td>1.0±0.2</td>
<td>37.0</td>
<td></td>
</tr>
</tbody>
</table>


Coxsackievirus B3 Induces T Regulatory Cells, Which Inhibit Cardiomyopathy in Tumor Necrosis Factor-α Transgenic Mice
Sally A. Huber, Arthur M. Feldman and Danielle Sartini

Circ Res. published online October 12, 2006;

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/early/2006/10/12/01.RES.0000249405.13536.49.citation

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/