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+FoxP3+ (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 autoimmune damage 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.1,3 Cytokines, including tumor necrosis factor-α (TNFα), interleukin (IL)-2, IL-1β, and IL-6, cause cardiac dysfunction.1,3 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.

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From the Department of Pathology (S.A.H., D.S.), University of Vermont, Colchester; and Department of Medicine (A.M.F.), Center for Translational Medicine, Thomas Jefferson University, Philadelphia, Pa.

Correspondence to Dr Sally Huber, Department of Pathology, University of Vermont, 208 South Park Dr, Ste 2, Colchester, VT 05446. E-mail Sally.Huber@uvm.edu.

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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, and the supernatant was rehomogenized in 0.9 mL of RPMI medium 1640. Hearts were fixed in 10% buffered formalin, paraffin embedded, sectioned, stained with hematoxylin/eosin, and evaluated for myocarditis.

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Flow Cytometry
Inflammatory cells in the heart were isolated by digesting 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 (Sigma). Histopaque. The intracellular cytokine staining protocol has been published previously.

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

Statistical Analysis
Data were analyzed for skewness and kurtosis using the SPPS for Windows program (Version 11.0. 2001; SPSS Inc. Chicago, Il). 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.
with extensive myocardial inflammation. 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+ IFNγ (Th1) and CD4+ IL-4 (Th2) cells showed that Th1 cells predominate. Because previous studies showed IgG deposits in the myocardium of TNF1.6 mice, plasma was evaluated for cardiac myocyte-reactive antibody and showed high titers of IgG2a anti-myocyte antibodies but little IgG1 antibody.

H310A1 Virus Infection Abrogates Cardiomyopathy

TNF1.6 transgenic mice were uninfected or infected for 7 days with 10⁵ pfu of either H3 or H310A1 virus (Figure 2). H310A1 virus does not induce myocarditis but infects and replicates in the myocardium to levels similar to those of the pathogenic H3 virus. Some uninfected TNF1.6 mice received 2 IP injections of 100 μg of monoclonal anti-CD3 antibody 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+ IFNγ (Th1) and CD4+ IL-4 (Th2) cells showed that Th1 cells predominate. Because previous studies showed IgG deposits in the myocardium of TNF1.6 mice, plasma was evaluated for cardiac myocyte-reactive antibody and showed high titers of IgG2a anti-myocyte antibodies but little IgG1 antibody.

Anti-CD3 antibody treatment also reduced heart weights. Troponin I concentrations were determined in plasma from individual mice (Figure 2C). Anti-CD3-treated uninfected TNF1.6 mice and H310A1-infected TNF1.6 mice had significantly less troponin I than uninfected TNF1.6 animals. No significant difference in troponin I levels occurred between uninfected and H3-infected TNF1.6 mice. Uninfected and H3-infected hearts had a globular morphology (Figure 2E) compared with normal morphology for H310A1 infected and anti-CD3–treated hearts. Uninfected TNF1.6 mice have a diffuse inflammatory infiltrate but minimal myocyte necrosis (Figure 2F). H3-infected TNF1.6 mice have substantial myocyte necrosis and increased inflammatory cell infiltrates. H310A1 infected and anti-CD3–treated hearts show reduced inflammation and no necrosis. Cardiac virus titers showed no significant difference between infected groups (5.6 ± 1.0 log10 pfu/heart for H3-infected animals and 4.9 ± 1.3 log 10 pfu/heart for H310A1-infected mice).

H310A1 infection actively protected TNF1.6 mice from cardiomyopathy, raising the question of immunoregulation. Two likely mediators of immunoregulation would be IL-10 or TGFβ. Splenocytes from uninfected, H3-infected, and H310A1-infected TNF1.6 mice were activated with PMA/ ionomycin. The supernatants from H310A1 immune cells had increased IL-10 and TGFβ concentrations (Figure 2D). H310A1-infected TNF1.6 mice were injected IV with either 250 μg of monoclonal anti–IL-10 or anti-TGFβ on days 2 and 5 days then killed 7 days after infection (Figure 2A through 2C). Anti–IL-10 treatment abrogated protection as

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 10⁵ 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 mice.
recipients (Table 1). The transferred cells were negative for virus as shown by titering 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.

**TNFα Inhibits T Regulatory Cell Response**

H3 virus induces TNFα expression and does not induce T regulatory cells, and exogenous TNFα treatment of CVB3 resistant mice restores myocarditis susceptibility. The following study evaluated whether TNFα modulates the T regulatory cell response during viral infection. Uninfected and H310A1-infected TNF1.6 mice were treated with 300 ng of recombinant TNFα daily from days 0 to 3 relative to infection. Additional mice were infected with H3 virus. All mice were killed 7 days after infection (Table 2). TNF1.6 mice infected with H310A1 virus showed reduced cardiomyopathy compared with uninfected and H3-infected mice, but TNFα treatment resulted in increased heart weight. Cytokine treatment of uninfected TNF1.6 mice had no effect on heart weight. Cardiac virus titers were not significantly different between any of the infected groups. However, H310A1-infected mice had fewer CD4+IFNγ and increased numbers of CD4+CD25+FoxP3+ cells compared with H3-infected or uninfected groups. These H310A1-infected mice also had fewer activated V4+IFNγ cells, a cell population known to be important in myocarditis induction.

**Discussion**

The immune system is divided into innate and adaptive immunity, in which the former involves rapid and broad
high levels of TNF
tent with immunopathogenicity. Adoptive transfer of
development of cardiomyopathy.9 Depletion of
proinflammatory cytokine usually induced by infection, ini-
tiates the development of cardiomyopathy.19,20 Transgenic expression of TNF
a profound effect on the strength and type of adaptive
system help control infections until the more effective and
microbes. However, the initial innate immune system also has
specific adaptive immune response develops and clears the
latter is highly specific to each inducing microbe. The innate
presence of IgG2a autoimmune anti-myocyte antibodies,
substantial IgG deposits in the myocardium, and widespread
myocardial inflammation in TNF1.6 mice11 is consistent with
the 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.23 The
high levels of TNFα likely affect dendritic cells in the heart,
resulting in self antigen processing and migration of the
maturinig 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 ex-
tremely 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 preferen-
tially express T1-ST2 over CCR5, the expression of both
markers on Tr1 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](http://circres.ahajournals.org/)

**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-.

**TABLE 1. Adoptive Transfer of CD4+CD25+ and CD4+CD25- Cell Populations Into TNF
1.6 Mice**

<table>
<thead>
<tr>
<th>Donor Cells</th>
<th>Body Weight (g)</th>
<th>Heart Weight (mg)</th>
<th>Troponin I (ng/mL plasma)</th>
<th>CD4+CD25+ (%), CD25+ (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected CD4+CD25+</td>
<td>28.3±0.5</td>
<td>265±12</td>
<td>1.28±0.32</td>
<td>0.5±0.3</td>
</tr>
<tr>
<td>Uninfected CD4+CD25-</td>
<td>25.8±0.5</td>
<td>244±15</td>
<td>1.35±0.26</td>
<td>0.3±0.1</td>
</tr>
<tr>
<td>H3 infected CD4+CD25+</td>
<td>26.4±0.5</td>
<td>274±19</td>
<td>1.23±0.13</td>
<td>0.6±0.2</td>
</tr>
<tr>
<td>H3 infected CD4+CD25-</td>
<td>24.3±0.5</td>
<td>280±0</td>
<td>1.28±0.19</td>
<td>0.5±0.1</td>
</tr>
<tr>
<td>H310A1 infected CD4+CD25+</td>
<td>27.3±0.8</td>
<td>185±8*</td>
<td>0.67±0.19*</td>
<td>1.4±0.5*</td>
</tr>
<tr>
<td>H310A1 infected CD4+CD25-</td>
<td>28.1±0.6</td>
<td>249±14</td>
<td>2.75±0.99*</td>
<td>0.5±0.3</td>
</tr>
</tbody>
</table>

Purified CD4+ populations were isolated from spleens of uninfected TNF1.6 and TNF1.6 males infected 7 days
earlier with 10^5 pfu of either H3 or H310A1 virus. Uninfected TNF 1.6 recipients received 3×10^6 CD4+CD25+ or
1×10^6 CD4+CD25- cells IV and were killed 7 days later. Results are mean±SEM of 3–11 mice per group.

*Significantly different at P<0.05 from uninfected donor cell recipients of same cell type (CD4+CD25+ or
CD4+CD25-).
promote the differentiation of Tr1 cells, possibly by upregulating costimulatory factors CD40, CD80, and/or CD86.28,29 Although TNFα can promote the induction of IL-10–producing Tr1 cells,30 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.24,27 This means either that different Tr1 cell types exist or that Tr1 cell function varies depending on environmental and viral conditions. Tr1 cells have been induced in response to both noninfectious (ovulamin, nickel, or allograft immunity),31–33 and infectious (helmenth, bacterial, and viral)25,34–35 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.35 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.36–38 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.39 CVB3 activates TLR440 and may induce Tr1 cells through this mechanism.

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Disclosures
None.

References
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