\( \gamma^d^+ \) T Cells Regulate Major Histocompatibility Complex Class II (IA and IE)-Dependent Susceptibility to Coxsackievirus B3-Induced Autoimmune Myocarditis

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Coxsackievirus B3 (CVB3) infection induces myocardial inflammation and myocyte necrosis in some, but not all, strains of mice. C57BL/6 mice, which inherently lack major histocompatibility complex (MHC) class II IE antigen, develop minimal cardiac lesions despite high levels of virus in the heart. The present experiments evaluate the relative roles of class II IA and IE expression on myocarditis susceptibility in four transgenic C57BL/6 mouse strains differing in MHC class II antigen expression. Animals lacking MHC class II IE antigen (C57BL/6 [IA+ IE-] and AB [IA- IE-]) developed minimal cardiac lesions subsequent to infection despite high concentrations of virus in the heart. In contrast, strains expressing IE (AB+ Eo [IA- IE+] and Bl.Tg.Eo [IA+ IE+]) had substantial cardiac injury. Myocarditis susceptibility correlated to a Th1 (gamma interferon-positive) cell response in the spleen, while disease resistance correlated to a preferential Th2 (interleukin-4-positive) phenotype. V\( \gamma^d^\) analysis indicates that distinct subpopulations of \( \gamma^d^+ \) T cells are activated after CVB3 infection of C57BL/6 and Bl.Tg.Eo mice. Depletion of \( \gamma^d^+ \) T cells abrogated myocarditis susceptibility in IE+ animals and resulted in a Th1→Th2 phenotype shift. These studies indicate that the MHC class II antigen haplotype controls myocarditis susceptibility, that this control is most likely mediated through the type of \( \gamma^d^+ \) T cells activated during CVB3 infection, and finally that different subpopulations of \( \gamma^d^+ \) T cells may either promote or inhibit Th1 cell responses.

Myocarditis is characterized as inflammation of the myocardium associated with microbial infections (7, 22, 31). Enteroviruses, including group B coxsackieviruses, are frequently implicated in this disease, yet only a small proportion of enterovirus-infected individuals contract clinical myocarditis. Various factors, including viral tropism, type and severity of cardiac infection (persistent versus nonpersistent), and characteristics of the host response to the virus, contribute to pathogenicity. The final outcome of the disease results from interactions between the virus, the infected cell, and the immune response. Studies using a murine model of coxsackievirus B3 (CVB3) myocarditis show that tissue injury depends predominantly on T-lymphocyte responses (11, 32). T-cell-deficient mice develop minimal cardiac damage even though virus continues to replicate in the heart. Furthermore, the type of T-cell response is crucial to pathogenicity. Mice can respond to infection with either Th1 or Th2 cell profiles (25). During Leishmania major infections, certain mouse strains dominant Th1 cells responses which are characterized by the production of interleukin-2 (IL-2), gamma interferon (IFN-\( \gamma \)), and tumor necrosis factor alpha; this pattern is associated with delayed hypersensitivity reactions (1, 12). Other strains preferentially develop Th2 cell responses, which are characterized by production of IL-4, IL-5, and IL-10, a pattern characteristic of T-cell-dependent humoral immunity and eosinophil-mediated inflammation. In cutaneous leishmaniasis, Th1 cell responses confer disease resistance whereas Th2 cell responses result in death. In experimental myocarditis, the opposite pattern holds true, with Th1 cells promoting susceptibility and Th2 cells conferring resistance. T cells expressing the \( \gamma^d^+ \) T-cell receptor (TcR) determine Th1 responsiveness possibly by selectively killing Th2 cells (12). The present study provides new evidence that \( \gamma^d^+ \) T-cell control of myocarditis susceptibility involves major histocompatibility complex (MHC) class II antigens.

MHC molecules are responsible for graft rejection, limit T-cell recognition and effector function, and serve as signaling receptors capable of triggering cell death (5, 21, 28). MHC class II alleles act as major genetic elements in susceptibility to a variety of autoimmune diseases (20). MHC class I molecules are expressed on all nucleated cells, whereas MHC class II molecules are constitutively expressed on dendritic cells, B cells, and monocytes, but expression can be induced on mesenchymal and epithelial cells under conditions of inflammation. The genetic background of the host, especially the allele of MHC class II (IA or IE in the mouse; HLA-DR, -DP, or -DQ in humans), affects the cytokine bias (immune deviation) of the T-cell response to antigenic stimulus in vivo, as demonstrated in several mouse systems of parasitic and autoimmune disease (1, 1a, 7a, 33). How class II molecules influence immune deviation is poorly understood. Epitope presentation by IA and IE antigens differ, and IE-restricted epitopes may be more apt to stimulate Th2 differentiation (33). Alternatively, IE expression could alter \( \gamma^d^+ \) T-cell repertoire through clonal selection in the thymus. Activating \( \gamma^d^+ \) T cells in myocarditis-susceptible mouse strains favors Th1 cell differentiation (12). In contrast, myocarditis resistance in other
mouse strains may result from activating different γδ T cells in these animals. In this report, we show that the γδ T-cell repertoires in the hearts and spleens of CVB3-infected C57BL/6 (IA^+ IE^-) and transgenic C57BL/6 mice expressing IE (Bl.Tg.Eα) differ substantially, that γδ T cells directly or indirectly determine myocarditis susceptibility to CVB3 infection, and that disease susceptibility depends upon the dominant CD4^+ Th phenotype in the animals.

MATERIALS AND METHODS

Mice. Genetically modified C57BL/6 mice (males, 5 to 7 weeks of age) were bred and housed at the University of Vermont Animal Care facility. MHC class II transgenic mice have been well characterized as described elsewhere (3, 4, 15, 16, 29). Briefly, C57BL/6 mice inherently lack MHC class II IE because of a naturally occurring defect in the Eα gene making these animals class II^+ IA^- MHC class II knockout (AB^o) mice were made by mutating the IA^o loci by homologous recombination, thus disrupting the gene. Clones of the disrupted IA^o gene were injected into C57BL/6 blastocysts, and chimeric males were bred to C57BL/6 females. Progeny were backcrossed to C57BL/6 mice (3, 4). Animals expressing class II IE (IA^+ IE^- or IA^- IE^+) were made by injecting a cloned Eα gene from A/J mice into male pronuclei of F2 hybrids from C57BL/6 × SJL animals. After the first generation of backcrossing transgenic Eα mice to C57BL/6 (IA^- IE^-) mice, the animals were bred with AB^o mice to make the IA^- IE^+ strain (15). Thus, all animals used in these studies are congenic to the C57BL/6 parental strain for variations in MHC class II.

Infection. Animals were infected by intraperitoneal (i.p.) injection in 0.5 ml of phosphate-buffered saline (PBS) containing 5 × 10^6 PFU CVB3 (H3 variant) derived from COxs transfected with the infectious CDNA of this virus (13).

Organ viral titer. Hearts were homogenized in 0.9 ml of RPMI 1640 containing penicillin (100 U/ml), streptomycin (100 μg/ml), and 5% fetal bovine serum (FBS). Cellular debris was removed by centrifugation at 1,045 × g for 10 min. The supernatant was titrated by the plaque-forming assay on HeLa cell monolayers as described previously (11).

Histology. Hearts were removed, fixed in 10% buffered formalin, paraffin embedded, sectioned, and stained with hematoxylin and eosin. Stained sections were used for image analysis in transmitted light mode with an Olympus BX50 compound light microscope (×4 objective lens; numerical aperture, 0.13). True color digital images (640 by 480 pixels) were captured with a Sony DXC-960MD/L LLP video camera connected via an RS170 cable to a video frame grabber on a Sun SPARCstation 5. Image processing and analysis were accomplished with IMIX software (Princeton Gamma Tech, Inc., Princeton, N.J.). Final percent cardiac injury was calculated by dividing the area of injury by the total area of the heart.

Preparation of lymphocytes. Mice were euthanized by injecting sodium pentobarbital (120 mg/kg of body weight in PBS) i.p. The spleens were removed, disrupted to produce single-cell suspensions, and washed in RPMI 1640 medium containing 5% FBS and antibiotics. After removal of tissue debris by sedimentation, the cells were centrifuged at 225 × g for 10 min at 5°C. The cell pellet was treated with RBC (erythrocyte) lysis solution (Sigma), washed with medium, resuspended in medium, and counted by trypan blue exclusion. For preparation of lymphoid cells infiltrating the heart, hearts were removed, minced finely with scissors, and digested sequentially three times with 1 ml of 0.4% collagenase. Cells in the supernatant were washed twice and centrifuged on Histopaque (Sigma). Lymphoid cells at the interface were retrieved, washed, and counted by trypan blue exclusion.

Antibodies. Isotype control and antigen-specific antibodies were obtained from Pharmingen (San Diego, Calif.) unless otherwise stated. These included fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-conjugated rat immunoglobulin G1 (IgG1) (clone R3-34); PE-anti-rat mouse IFN-γ (clone XMG 1.2); PE-anti-rat mouse IL-4 (clone BVD4-1D11); FITC-anti-rat mouse CD4 (clone GK 1.5); Red613-anti-mouse CD8 (clone 53-6.7; Gibco BRL); PE-mouse anti-IA^- (clone AF6-120.1); PE-mouse anti-IE^- (clone 14-4-5S); FITC-hamster IgG; and FITC-hamster anti-γδ TCR (clone GL3) antibodies. Additional FITC-conjugated antibodies to Vα1 (clone 21.1); Vγ1 (clone UCD3, V4; clone GL2), and V63 (clone 17C) were obtained from Rebeca O’Brien. Ascites antibody to γδ TCR was made by injecting approximately 10^8 GL3-3A hybridoma cells (clone originally obtained from Ralph Budd, Department of Medicine, University of Vermont) i.p. into BALB/c mice given 0.5 ml of 0.2% (2,6,10,14-tetramethylpentadecane) to 10 to 20 days earlier and 550 R on the day of inoculation. Immunoglobulin was purified by 50% ammonium sulfate precipitation and Sepharose S-200 chromatography. Protein determination was done by spectrophotometry at 280 nm.

Cell surface marker staining. Lymphocytes (10^7) were washed in PBS containing 1% bovine serum albumin (BSA) and 0.1% sodium azide (PBS-BSA) and resuspended in 0.1 ml PBS-BSA containing a 1:100 dilution of fluorochrome-labeled antibodies with 100 ng/ml of 0.1% BSA (PharMingen). After incubation for 30 min on ice, the cells were washed twice in PBS-BSA and fixed in 2% formaldehyde for flow analysis.

Intracellular cytokine staining. A modification of the method of Picker et al. (24) was used to evaluate intracellular cytokines in splenocytes. Briefly, 10^6 splenocytes were cultured in medium containing brefeldin A (10 μg/ml), phorbol myristate acetate (50 ng/ml), and ionomycin (500 ng/ml) (Sigma Chemical Co., St. Louis, Mo.) for 4 h at 37°C in 5% CO2. The cells were subsequently resuspended in medium containing rat polyclonal IgG (50 μg/ml; Zymed, San Francisco, Calif.) and brefeldin A, incubated for 10 min at 5°C, washed, and resuspended in medium containing Fc-Block (PharMingen) and either fluorochrome-labeled surface marker antibodies or appropriate immunoglobulin isotype controls. After incubation on ice for 30 min, the cells were washed in PBS-BSA/brefeldin A and fixed for 10 min in 2% paraformaldehyde. The cells were then washed once in PBS-BSA, incubated for 10 min in PBS-BSA containing 0.5% saponin, and stained for intracellular cytokines, using either PE-anti-IFN-γ or PE-anti-IL-4. Isotype control for intracellular staining was PE- rat IgG. All staining was performed in buffer containing Fc-Block and polyclonal rat IgG (50 μg/ml) to block nonspecific antibody binding. The cell membranes were permeabilized in saponin permeabilization solution. After incubation for 30 min, the cells were washed twice in PBS-BSA-saponin and once in saponin-free PBS to block membrane and then resuspended in PBS-oxide containing 1% paraformaldehyde.

Flow cytometry. We used a Coulter Epics Elite instrument with a single excitation wavelength (488 nm) and band filters for FITC (525 nm), and PE (565 nm). Each sample population was acquired for 10,000 events. Data acquisition was performed using the BD FACScanto software and analyzed using the BD CellQuest program. Data were displayed as contour plots of fluorescence intensity versus cell scatter (forward scatter) and complexity (side scatter) and gated on a population of interest; data for 10,000 cells were evaluated. Criteria for positive staining were established based on the intensity of the isotype controls. The results were expressed as the percentage of cells within the size/complexity gate that stained positively for each marker, or as the percentage of positive cells with gating on a second marker, after subtraction of the percent positive cells in the isotype control. The specificity of intracellular cytokine staining was demonstrated by negative controls or by decreased staining when brefeldin A was omitted. The saponin permeabilization step was omitted, or the cells were stained with unlabelled antibody before staining with fluorochrome-linked antibody (data not shown).

Each study was repeated at least two times, and the data from a representative experiment are presented.

Statistics. Statistical evaluation was performed by the Wilcoxon ranked score method.

RESULTS

Mortality and myocarditis in transgenic mice. Transgenic mice differing in class II MHC antigen expression were infected i.p. with CVB3. Surviving mice were euthanized 7 days later for evaluation of myocarditis. Figure 1 shows representative histological sections of infected mice. Table 1 summarizes the characteristics of the splenocyte populations in the various CVB3-infected transgenic mouse groups. As expected, no IE^- splenocytes were observed in AB^o mice; however, approximately 5% of these lymphoid cells expressed IA^o. This low-level class II antigen expression most likely explains the presence of small numbers of CD4^+ T cells in the spleen (1.5 × 10^6 cells, or approximately 14% of the number of CD4^- T cells in parental C57BL/6 mice). Total numbers of splenocytes in AB^o mice were only slightly lower...
FIG. 1. Myocarditis in MHC class II antigen transgenic mice. Male AB\(^{a}\) (IA\(^{-}\) IE\(^{-}\)) (A), C57BL/6 (IA\(^{+}\) IE\(^{-}\)) (B), BLT\(_{a}\) E\(_{a}\) (IA\(^{+}\) IE\(^{+}\)) (C), and AB\(^{a}\) E\(_{a}\) (IA\(^{-}\) IE\(^{+}\)) (D) mice were infected i.p. with \(10^4\) PFU of CVB3 and killed 7 days later. Heart sections were stained with hematoxylin and eosin. Magnification, ×80.
than in parental animals, due to increased proportions of other cell types such as CD8+ T cells in these animals (data not shown). IE+ strains (Bl.Tg.Ea and AB*Ea) generally had substantially fewer splenocytes than IE- strains (C57BL/6 and AB*). Interestingly, depletion of γδ+ T cells in Bl.Tg.Ea mice restored splenocyte numbers, implying that these effectors directly or indirectly might regulate lymphocyte numbers in peripheral lymphoid organs. Numbers of γδ+ T cells were unexpectedly lower in AB* (IA-IE-) mice than in other strains but tended to be higher in Bl.Tg.Ea mice. The reason for these differences is not known.

When Bl.Tg.Ea (IA-IE-) mice were treated with 200 μg of monoclonal antibody to anti-γδ TCR antibody, cardiac injury was substantially reduced, demonstrating the importance of these cell populations in viral pathogenesis (Table 1). Efficacy of cell depletion was determined to be greater than 90% in these cell populations in viral pathogenesis (Table 1). Efficacy was substantially reduced, demonstrating the importance of TcR antibody, cardiac injury (data not shown).

### Correlation of myocarditis susceptibility with preferential CD4+ Th1 cell responses

Earlier studies correlated myocarditis susceptibility in BALB/c mice infected with CVB3 with a preferential Th1 CD4+ cell phenotype (12). To evaluate the cytokine responses in C57BL/6 transgenic mice, splenocytes were isolated 7 days after infection and stained for CD4 and for intracellular IL-4 and IFN-γ (Table 4; Fig. 2). C57BL/6 mice had few IFN-γ-producing cells but more IL-4-producing cells than did IE-positive (Bl.Tg.Ea) animals. Depleting γδ+ T cells from Bl.Tg.Ea animals increased numbers of IL-4-producing cells, while numbers of IFN-γ-producing cells were only slightly decreased. Interestingly, most cytokine-producing cells in the spleen are CD4+. Evaluation of various additional lymphoid subsets indicates that approximately two-thirds of non-CD4 cytokine-positive cells are NK1.1+ and one-third are γδ+. Few CD8+ cytokine-producing cells were observed (data not shown). Thus, myocarditis susceptibility in MHC class II transgenic mice correlated with decreased Th2-like or increased Th1-like cell responses.

### DISCUSSION

This report demonstrates three points. First, MHC class II IE expression promotes myocarditis susceptibility in C57BL/6 mice. Second, myocarditis correlates with induction of a Th1

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**TABLE 1. Effect of depleting γδ+ T cells on CVB3-induced myocarditis a**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Antibody treatment</th>
<th>Cumulative mortality (day 7)</th>
<th>Mean virus titer (log 10 PFU) ± SEM</th>
<th>% Myocardium inflamed (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6 (IA+ IE-)</td>
<td>None</td>
<td>0</td>
<td>5.1 ± 0.7</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Anti-γδ TcR</td>
<td>0</td>
<td>5.5 ± 0.9</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>AB* (IA-IE-)</td>
<td>None</td>
<td>0</td>
<td>6.5 ± 1.4</td>
<td>0 ± 0</td>
</tr>
<tr>
<td></td>
<td>Anti-γδ TcR</td>
<td>0</td>
<td>7.1 ± 0.8</td>
<td>1.3 ± 0.8</td>
</tr>
<tr>
<td>AB*Ea (IA-IE-)</td>
<td>None</td>
<td>100</td>
<td>6.2 ± 0.9</td>
<td>5.1 ± 2.0b</td>
</tr>
<tr>
<td></td>
<td>Anti-γδ TcR</td>
<td>25</td>
<td>6.5 ± 0.7</td>
<td>1.8 ± 1.1c</td>
</tr>
<tr>
<td>BL.Tg.Ea (IA-IE-)</td>
<td>None</td>
<td>50</td>
<td>4.3 ± 0.5</td>
<td>8.3 ± 1.6b</td>
</tr>
<tr>
<td></td>
<td>Anti-γδ TcR</td>
<td>0</td>
<td>5.3 ± 0.4</td>
<td>1.7 ± 0.5c</td>
</tr>
</tbody>
</table>

a Male mice, 4 to 5 weeks of age, were injected i.p. with 100 μg of GL3-3A (anti-γδ) monoclonal antibody in 0.5 ml of PBS or isotype hamster IgG on days −1 and -2 relative to virus infection. Animals received 5 × 10^6 PFU of CVB3 on day 0, and surviving animals were euthanized on day 7. Hearts were removed from animals dying between days 5 and 7 for analysis. Hearts were divided, and the apex was formalin fixed, sectioned, and evaluated by image analysis for percentage of myocardium affected. The remaining tissue was titered by plaque-forming assay for virus. Groups consisted of four mice each.

b Significantly different from C57BL/6 at P ≤ 0.05.

c Significantly different from non-antibody-treated mice at P ≤ 0.05 by Wilcoxon ranked score.

d Anti-γδ treatment significantly reduced total γδ+ T cells in spleens of BL.Tg.Ea mice compared to isotype antibody-treated control animals of the same strain (P ≤ 0.05).

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**TABLE 2. Characterization of splenocyte populations in transgenic mice a**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Total splenocytes (10^6)</th>
<th>CD4+</th>
<th>γδ+</th>
<th>IA+</th>
<th>IE+</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SEM (n = 3–6 mice/group)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6</td>
<td>98.3 ± 5.5</td>
<td>10.88 ± 2.7 (11b)</td>
<td>4.0 ± 2.5 (4)</td>
<td>53.8 ± 10.4 (55)</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>AB*</td>
<td>77.6 ± 16.2</td>
<td>1.5 ± 0.48 (2)</td>
<td>1.7 ± 0.8 (2)</td>
<td>5.5 ± 1.2 (7)</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>AB*Ea</td>
<td>48.1 ± 9.2c</td>
<td>6.0 ± 0.70 (12)</td>
<td>2.6 ± 1.3 (5)</td>
<td>4.3 ± 1.2 (9)</td>
<td>23.6 ± 3.9 (49)</td>
</tr>
<tr>
<td>BL.Tg.Ea</td>
<td>Untreated</td>
<td>59.9 ± 18.4</td>
<td>8.2 ± 2.1 (14)</td>
<td>8.8 ± 3.9 (15)</td>
<td>43.2 ± 8.6 (72)</td>
</tr>
<tr>
<td></td>
<td>Treated with anti-γδ TcR</td>
<td>71.1 ± 10.3</td>
<td>10.6 ± 1.1 (15)</td>
<td>0.5 ± 0.1c (0.1)</td>
<td>ND</td>
</tr>
</tbody>
</table>

a Spleen cells from individual animals infected 7 days earlier with 5 × 10^6 PFU of CVB3 were treated with RBC lysing solution (Sigma) and counted by trypan blue exclusion, and aliquots of the cells were stained for the cell surface markers as described in Materials and Methods. ND, not determined.

b Percentage of total splenocytes positive for the marker.

c Total cells per spleen differ from C57BL/6 group at P ≤ 0.05 by Wilcoxon ranked score.

d Anti-γδ treatment significantly reduced total γδ+ T cells in spleens of BL.Tg.Ea mice compared to isotype antibody-treated control animals of the same strain (P ≤ 0.05).
cytokine phenotype whereas resistance correlates to a Th2 cell phenotype in these animals. Third, both myocarditis and susceptibility apparently depend on γδ+ T cells which may use MHC class II antigens to affect Th subset differentiation.

MHC class II molecules affect disease susceptibility through several distinct mechanisms. The best-known mechanism is through antigenic epitope selection and presentation to T lymphocytes, resulting in biasing of the T-cell repertoire. Thus, individuals of a particular MHC haplotype are more likely to develop pathogenic autoimmune responses due to the ability of their MHC molecules to bind specific self peptides. In this case, myocardial injury ought to be mediated by IE-restricted T cells, while mouse strains lacking the relevant IE molecules are incapable of generating pathogenic T-cell responses. However, while IE C57BL/6 mice are clearly resistant to CVB3-induced myocarditis, Henke et al. (8) demonstrated that CVB3 infection of C57BL/6 CD4 knockout mice resulted in severe myocarditis mediated by pathogenic CD8+ T-cell responses. This observation indicates that resistance in C57BL/6 mice results from preferential activation of immunoregulatory CD4+ T-cell responses which suppress pathogenic immunity. In this study we demonstrate that CVB3 infection of C57BL/6 mice stimulates primarily Th2-like (IL-4+) cell responses. Since CD4+ Th2 cells will suppress delayed hypersensitivity reactions, responses considered to be important in causing myocardial injury during myocarditis, this most likely explains the “immunosuppressive” CD4+ T cell described in the earlier publication. CVB3-infected Bl.Tg.Eα mice generate a predominant Th1-like response, but eliminating γδ+ T cells in these animals shifts the cytokine response to a Th2 cell phenotype and correlates to acquired resistance of these animals to myocarditis. Thus, the role of MHC class II IE in CVB3-induced myocarditis seems most likely to be in promoting immune deviation toward a Th1 phenotype rather than selecting specific IE-restricted CD4+ T-cell clones.

Studies using different antigenic stimuli variously report that γδ+ T cells promote immune deviation to either the Th1 or Th2 phenotype (2, 6, 9, 18, 34). We believe that the difference in γδ+ T-cell effect in C57BL/6 and Bl.Tg.Eα mice primarily reflects variations in γδ+ T-cell subtypes dominating in these two strains. Unlike T cells expressing the γδ TcR, γδ+ T cells usually react to antigen directly without requiring antigen processing. Frequently, γδ+ T-cell recognition either is MHC antigen independent or occurs in the absence of peptides bound to MHC molecules (27, 30). However, MHC class II IE molecules may determine the γδ+ T-cell repertoire in vivo presumably by affecting thymic differentiation or selection (14, 26). Thus, the subtypes of γδ+ T cells may differ between IE+ and IE− strains of mice. Should different subtypes of γδ+ T cells vary in the ability to influence Th cell differentiation, then the difference in effects of γδ+ T-cell depletion in C57BL/6 and Bl.Tg.Eα mice would reflect the γδ+ T-cell repertoire in these two strains. T cells expressing Vγ1 dominate in C57BL/6 mice. In contrast, cells expressing Vγ4 are more prevalent in Bl.Tg.Eα than parental mice. Differences in Vγ1+ Vγ4+ cell populations were especially evident in the heart as a proportion of the total γδ+ T-cell population. In absolute cell numbers, all γδ+ T-cell subpopulations were increased in hearts of IE+ mice because of the greater cardiac inflammation in this strain.

One potential problem with the γδ+ T-cell depletion studies is that the cells were depleted with monoclonal anti-γδ TcR. Animals required a total of 200 μg of the monoclonal antibody for effective γδ+ T-cell elimination over the 9 days of the experiment. While this treatment resulted in over 90% depletion of γδ+ T cells in the spleen at the end of the experiment, the numbers of γδ+ T cells in transgenic mice are significantly different from C57BL/6 at P < 0.05 by Wilcoxon ranked score.

### Table 3. Vγ/Vδ usage in transgenic mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of mice/group</th>
<th>Tissue</th>
<th>Total lymphoid cells (10⁶)</th>
<th>Total γδ+ (GL3-3A+) cells (10⁶)</th>
<th>Vγ1+</th>
<th>Vγ4+</th>
<th>Vδ4+</th>
<th>Vδ6.3+</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>3</td>
<td>Heart</td>
<td>8.7 ± 0.04</td>
<td>2.8 ± 0.9 (32)</td>
<td>1.6 ± 0.03 (18)</td>
<td>0.2 ± 0.1 (2)</td>
<td>0.5 ± 0.3 (6)</td>
<td>0.6 ± 0.2 (7)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Spleen</td>
<td>715.9 ± 63.5</td>
<td>45.2 ± 21.0 (6)</td>
<td>9.4 ± 3.5 (1)</td>
<td>2.1 ± 0.09 (0.3)</td>
<td>4.0 ± 1.6 (0.1)</td>
<td>4.8 ± 2.0 (0.1)</td>
</tr>
<tr>
<td>Bl.Tg.Eα</td>
<td>4</td>
<td>Heart</td>
<td>47.4 ± 1.8</td>
<td>18.2 ± 3.9 (39)</td>
<td>2.3 ± 0.4 (5)</td>
<td>5.2 ± 1.8 (11)</td>
<td>3.3 ± 0.8 (7)</td>
<td>1.4 ± 0.05 (3)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Spleen</td>
<td>405.06 ± 23.5</td>
<td>61.5 ± 16.6 (15)</td>
<td>4.7 ± 2.0 (1)</td>
<td>10.3 ± 2.7 (3)</td>
<td>3.2 ± 1.6 (1)</td>
<td>5.7 ± 2.3 (1)</td>
</tr>
</tbody>
</table>

*Lympoid cells were isolated from spleens and hearts of individual mice 7 days after infection with CVB3 and depleted of RBC as indicated in Materials and Methods. Aliquots of cells were stained for a total γδ+ cells or Vγ/Vδ subpopulations.

### Table 4. Th1/Th2 phenotype of CD4+ T cells in transgenic mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of mice/group</th>
<th>Total splenocytes (10⁶)</th>
<th>CD4+</th>
<th>IFN-γ+</th>
<th>IL-4+</th>
<th>CD4+ IFN-γ+</th>
<th>CD4+ IL-4+</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>4</td>
<td>86.5 ± 13.7</td>
<td>11.9 ± 2.5</td>
<td>0.7 ± 0.4 (0.39)</td>
<td>1.8 ± 0.4</td>
<td>0.1 ± 0.1 (0.13)</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>Bl.Tg.Eα</td>
<td>4</td>
<td>50.1 ± 12.4</td>
<td>6.0 ± 1.3</td>
<td>2.3 ± 0.9 (3.29)</td>
<td>0.7 ± 0.3</td>
<td>0.5 ± 0.2 (2.5)</td>
<td>0.2 ± 0.1v</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>68.1 ± 10.7</td>
<td>8.9 ± 2.2</td>
<td>1.6 ± 0.4 (0.84)</td>
<td>1.9 ± 0.5</td>
<td>0.3 ± 0.2 (0.60)</td>
<td>0.5 ± 0.2</td>
</tr>
</tbody>
</table>

*Bl.Tg.Eα mice were treated with a total of 200 μg of anti-γδ TcR antibody or hamster IgG as an isotype control. Spleen cells from individual mice obtained 7 days after infection with CVB3 were stained for CD4 and cytokines as described in Materials and Methods.

* Ratio of IFN-γ+ cells to IL-4+ cells.

* Number of positive cells significantly different from C57BL/6 at P < 0.05 by Wilcoxon ranked score.
it is probable that initial antibody-T cell interactions could have activated the \( \gamma^d \) T-cell population prior to its depletion. Thus, whether the apparent protection observed in Bl.Tg.Ea mice reflects the elimination or the initial activation of this cell population by the antibody is problematic. Because of this problem, animals were treated with the monoclonal antibody for several days prior to infection. This should allow elimination of the \( \gamma^d \) T cells and any transient effects caused by the antibody treatment before virus stimulation.

A separate question is how \( \gamma^d \) T cells modulate Th cell responses. Most studies suggest that these effectors release cytokines favoring one or the other type of Th cell response (6). Our studies indicate \( \gamma^d \) T-cell regulation in CVB3-induced myocarditis may depend on direct interactions between these effectors and the CD4\(^+\) T-cell population (10). Although many investigators have not demonstrated MHC class II antigen expression on activated CD4\(^+\) T cells, studies by Osborne and Rudikoff (23) show the presence of IA on this population. Our own experience indicates that MHC class II antigen expression on activated CD4\(^+\) T cells is restricted to IE molecules, while IA is often not induced. Thus, individuals staining only for IA could miss MHC class II upregulation in CD4\(^+\) T cells. Since a population of \( \gamma^d \) T cells are known to recognize IE (14, 26), \( \gamma^d \) T cells might influence modulation of immune deviation through the direct interaction of \( \gamma^d \) T cells and activated CD4\(^+\) T cells, using IE expressed by the latter cells.

While there clearly is a role for IE molecules in myocarditis, IA molecules must also have some impact. Both Bl.Tg.Ea (IA\(^+\) IE\(^+\)) and AB\(^o\)Ea (IA\(^-\) IE\(^+\)) mice develop significant cardiac lesions, but the lesions differ in nature. In the presence of IA, a highly cellular inflammatory lesion is observed, but in the absence of IA, the lesions are extremely necrotic yet have few infiltrating lymphoid cells. Furthermore, animal mortality is accelerated in IA\(^-\) IE\(^+\) mice. Thus, IA-dependent responses modulate the character of the myocardial disease, although these molecules appear inherently less important in conferring overall disease susceptibility. One interesting note is that AB\(^o\) mice, which should lack MHC class II molecules and be CD4\(^+\) T-cell deficient, are not myocarditis susceptible even though previous studies demonstrated that CD4 knockout mice developed myocarditis (8). One would think that MHC class II knockout and CD4 knockout mice should behave similarly. However, the AB\(^o\) strain appears slightly leaky for MHC class II antigen expression, and significant numbers of CD4\(^+\) T cells remain in the spleen. Because C57BL/6 mice have a natural defect in the E\(^a\) gene, and the AB\(^o\) strain was produced by disruption of the A\(^b\) gene, chimeric molecules are possible between the A\(^a\) and E\(^b\) chains (19). These chimeric molecules could allow some CD4\(^+\) T-cell selection, which might make the MHC class II knockout mice functionally different from CD4 knockout animals.

In conclusion, these studies are important because they demonstrate the complexity of genetic control of viral myocarditis. Clinically, HLA-DR4/1 and histidine at position 36 of the HLA-DQ \( \beta1 \) gene have been associated with increased susceptibility to myocarditis (17). Such associations can be con-
troversial, however, and may not be found in all studies. One factor which could complicate MHC associations is that specific MHC haplotypes could contribute to either susceptibility or resistance in distinct ways. Thus, while some MHC haplotypes may promote myocarditis through presentation of heart-specific antigens and stimulation of pathogenic autoimmune responses, other MHC haplotypes may modulate susceptibility through effects on other types of cells, such as the γδ T cell, or on immune deviation.

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