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Role of CD1d in Coxsackievirus B3-Induced Myocarditis

Sally Huber, Danielle Sartini,* and Mark Exley†

The myocarditic (H3) variant of Coxsackievirus B3 (CVB3) causes severe myocarditis in BALB/c mice and BALB/c mice lacking the invariant Jα281 gene, but minimal disease in BALB/c CD1d−/− animals. This indicates that CD1d expression is important in this disease but does not involve the invariant NKT cell often associated with CD1d-restricted immunity. The H3 variant of the virus increases CD1d expression in vitro on neonatal cardiac myocytes whereas a nonmyocarditic (H310A1) variant does not. Vγ4+ T cells show increased activation in both H3-infected BALB/c and Jα281−/− mice compared with CD1d−/− animals. The activated BALB/c Vγ4+ T cells from H3-infected mice kill H3-infected BALB/c myocytes and cytotoxicity is blocked with anti-CD1d but not with anti-MHC class I (Kd/Dd) or class II (IA/IE) mAbs. In contrast, H3 virus-infected CD1d−/− myocytes are not killed. These studies demonstrate that CD1d expression is essential for pathogenicity of CVB3-induced myocarditis, that CD1d expression is increased early after infection in vivo in CD1d+ mice infected with the myocarditic but not with the nonmyocarditic CVB3 variant, and that Vγ4+ T cells, which are known to promote myocarditis susceptibility, appear to recognize CD1d expressed by CVB3-infected myocytes. The Journal of Immunology, 2003, 170: 3147–3153.

Myocarditis is an inflammation of the myocardium, which often follows microbial infections (1). Two variants of Coxsackievirus B3 (CVB3) have been used to study myocarditis in BALB/c mice. One variant, designated H3, causes severe cardiac inflammation and high animal mortality (2). The second variant, designated H310A1, differs from the H3 virus by a single amino acid mutation in the VP2 capsid protein and induces minimal myocarditis and mortality (3, 4). Why this single amino acid difference between H3 and H310A1 viruses should have such a strong biological effect on disease susceptibility is not clearly understood, but may partially depend upon differences in activation of innate immune responses by proinflammatory cytokines and γδ T cells. H3 infection of cultured monocytes induces strong TNF-α secretion which is not observed with H310A1 (5). TNF-α is necessary for CVB3 pathogenicity (6–8). A second difference between H3 and H310A1 virus infections is that only the former virus activates Vγ4+ T cells, which promote myocarditis susceptibility (9). γδ T cells comprise up to 50% of the T cell infiltrate in the hearts of H3-infected animals (8, 10). As with αβ TCR+ cells, γδ T cells can be distinguished by their variable (V) gene usage. Vγ1+ T cells induce myocarditis resistance while Vγ4+ T cells induce susceptibility (9, 11). In the present studies, we show that H3 virus infection up-regulates expression of CD1d (a nonpolymorphic MHC I-like molecule often associated with innate immunity and control of infectious agents (12–18) both in vitro on infected myocytes and in vivo in the heart. Vγ4+ cells isolated from H3 virus-infected BALB/c mice kill H3 virus-infected myocytes by recognition of CD1d in vitro. Since the classical invariant NKT (Jα281+γδ) cells are not involved in CVB3-induced myocarditis and since Vγ4+ cells have already been shown to determine myocarditis susceptibility, we hypothesize that the requirement for CD1d in this disease is probably functioning at the level of the Vγ4+ cells.

Materials and Methods

Mice

Male BALB/cJ, BALB/c-CD1d KO (CD1d−/−), and BALB/c Jα281−/− mice, 6–8 wk of age, were used in these experiments. BALB/cJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Breeding pairs of BALB/c CD1d−/− mice were kindly supplied by Dr. M. Grusby (Harvard School of Public Health, Boston, MA); breeding pairs of BALB/c Jα281−/− mice were kindly supplied by Dr. M. Taniguchi (Chiba University, Chiba, Japan, and currently RIKEN, Japan).

Virus, virus infection, and virus titration

Animals were infected by i.p. injection of 0.2 ml of PBS containing 104 PFU of CVB3 H3 or H310A1 variants as described previously (9). Virus titers in tissues or in in vitro cell populations were determined by the plaque-forming assay as described earlier. Infectious center assay was used to determine the percentage of myocytes infected (19).

Antibodies

Ab class control (isotype control) and Ag-specific Abs were obtained from BD PharMingen (San Diego, CA). These included: PE-conjugated anti-CD3 (clone 17A2); purified rat anti-mouse CD16/CD32 (Fc Block; clone 2.4G2); CyChrome, FITC, and PE-rat IgG1 (clone R3-34); FITC- and PE-conjugated anti-mouse IFN-γ (clone XMG 1.2); PE-rat anti-mouse IL-4 (clone BVD4-1D11); PE-hamster anti-mouse CD69 (clone H1.2F3); CyChrome-rat anti-mouse CD4 (clone GK 1.5); purified, FITC, and PE-hamster IgG; purified and FITC anti-mouse CD1d (clone 1B1); purified anti-mouse H-2D4 (clone 34-2-12); purified anti-mouse H-2Kb (clone SF1-1.1); purified anti-mouse IA/IE (clone 2G9); and FITC-anti-δ TCR (clone GL3). FITC-conjugated mAbs to Vγ1 (clone 2.11) and Vγ4 (clone UC3) were prepared and tested in the laboratory of Dr. R. O’Brien (National Jewish Medical and Research Center, Denver CO). PE-streptavidin was purchased from BD PharMingen.

Isolation of splenic lymphocytes

Isolation of cell populations has been described previously (9). Briefly, spleens were pressed through fine mesh screens, centrifuged on Histopaque-1077 (Sigma-Aldrich, St. Louis, MO), to remove RBC, and incubated on nylon wool for 30 min at 37°C. The nonadherent cells were retrieved. To isolate enriched Vγ1+ or Vγ4+ cells, splenocytes after nylon wool incubation were depleted of CD4+ and anti-IA/IE Ab treatment of the cells followed by incubation with anti-rat IgG-conjugated magnetic particles. The remaining
cells were divided into three different tubes and incubated in 500 µL of PBS-1% BSA containing a 1/50 dilution of either FITC anti-Vy4 and PE anti-CD3 (tube 1) or anti-Vy1 and PE anti-CD3 (tube 2) for 20 min on ice, washed, and resuspended in RPMI 1640 containing 10% FBS (Life Technologies, Grand Island, NY) and sterile sorted.

Isolation of neonatal myocytes

Isolation of neonatal mouse myocytes has been described previously (20). Briefly, hearts are obtained from mice within 72 h of birth, minced finely, and subjected to sequential digestion with 0.25% pancreatin (Life Technologies) for 20 min at 37°C. The cells were washed once in PBS-BSA-saponin containing 1/100 dilutions of FITC anti-Vy1, Vy4, CD69, and either biotynlated anti-Vy3 (clone UC3) and CyChrome-streptavidin. Results represent mean ± SEM of three to five mice per group.

Confocal microscopy

BALB/c and CD1d−/− mice were uninfected or were infected i.p. with H3 or H310A1 virus and then killed 3 days later. Hearts were perfused with PBS, snap frozen in Tissue Freezing Medium (TBS; Triangle Biological) and sectioned, and stained with H&E. Stained sections were evaluated for myocarditis as described previously (9).

Histology

Hearts were removed, fixed in 10% buffered formalin, paraffin embedded, sectioned, and stained with H&E. Stained sections were evaluated for myocarditis as described previously (9).

Statistics

Statistical evaluation was performed using the Wilcoxon ranked score method. Each experiment was repeated at least two times.

Results

Myocarditis in BALB/c, BALB/c CD1d−/−, and BALB/cJ Jo281−/− mice

Male mice were infected with H3 virus and surviving animals were euthanized 7 days later. Cumulative mortality was 67% (10/15) for wild-type, 50% (5/10) for Jo281−/−, and 25% (2/8) for CD1d−/− mice. No significant difference was observed between the strains in cardiac virus titers (Table I); however, myocarditis was significantly reduced in CD1d−/− mice (0.5% of the myocardium inflamed compared with 9.3% for BALB/c mice). CD1d−/− mice also had decreased numbers of CD4+ IFN-γ+ cells, but greater numbers of CD4+ IL-4+ cells in the spleen (Table I). Total numbers of splenic CD4+ cells were not significantly different between wild-type (38.9%) and CD1d−/− (42.1%) mice, indicating that reduced CD4+ IFN-γ+ numbers do not reflect selective loss of CD4+ cells.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Myocarditis (% myocardium inflamed)</th>
<th>Cardiac Virus Titer (104)</th>
<th>% CD4+ Spleen Cells</th>
<th>% Spleen Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>9.3 ± 1.2</td>
<td>6.68 ± 2.32</td>
<td>14.5 ± 3.8</td>
<td>1.1 ± 0.7</td>
</tr>
<tr>
<td>BALB/c CD1d−/−</td>
<td>0.5 ± 0.2</td>
<td>7.05 ± 5.62</td>
<td>2.9 ± 2.2b</td>
<td>1.7 ± 0.4b</td>
</tr>
<tr>
<td>BALB/c Jo281−/−</td>
<td>8.6 ± 2.5</td>
<td>3.45 ± 2.04</td>
<td>11.0 ± 2.5</td>
<td>1.8 ± 1.3</td>
</tr>
</tbody>
</table>

* BALB/c and transgenic BALB/c male mice 6–8 weeks of age were infected with 106 PFU of H3 virus and euthanized 7 days later. Hearts were removed and evaluated for the percentage of myocardium inflamed and for virus titers. Spleens were removed, centrifuged (1000 × g for 5 min), and the lymphoid population was surface stained with CyChrome anti-mouse CD4 and intracellularly stained for IFN-γ and PE anti-IL-4. For evaluation of Vy1+ cell populations, spleen cells were stained with FITC-anti- Vy1 (clone 2.11) or Vy4 (clone UC3) and CyChrome-streptavidin. Results represent mean ± SEM of three to five mice per group.

* Significantly different from BALB/c at p ≤ 0.05.
in the CD1d\(^{-/-}\) mouse. We have previously shown that V\(\gamma\)4\(^+\) cells promote myocarditis susceptibility (9). Wild-type BALB/c and Jc281\(^{-/-}\) mice showed increased numbers of V\(\gamma\)4\(^+\) cells compared with infected CD1d\(^{-/-}\) mice, and most of the V\(\gamma\)4\(^+\) cells are positive for the early activation marker CD69. Uninfected BALB/c, Jc281\(^{-/-}\), and CD1d\(^{-/-}\) mice had <0.01% CD69\(^+\) cells, indicating that infection is necessary for activation marker induction. The data shown in Table I represents one of three experiments performed. Results obtained in replicate experiments was similar. These studies demonstrate that H3 virus fails to activate V\(\gamma\)4\(^+\) cells or induce myocarditis in mice lacking CD1d, whereas mice lacking the classical Jc281 NKT cell as well as wild-type mice are susceptible to myocarditis.

**CD1d expression in vivo and in vitro during H3 and H310A1 virus infections**

Since H3 only induces myocarditis in CD1d\(^+\) mice, the next studies investigated whether CD1d expression alters in vivo and in vitro during H3 and H310A1 infection. In initial studies, neonatal cardiac myocytes were isolated, infected with 100 PFU of virus/cell for 18 h, then labeled with PE-anti-\(\alpha\)-actin and FITC anti-CD1d. After gating on the \(\alpha\)-actin-positive population, the percentage of myocytes positive for CD1d was determined (Fig. 1). Approximately 37.2% of H3 virus-infected BALB/c myocytes expressed CD1d compared with <2% of either uninfected or H310A1 virus-infected cells. To confirm specificity of the CD1d labeling, myocytes were isolated from CD1d\(^{-/-}\) mice and infected with H3 virus. None of these myocytes were labeled with anti-CD1d Ab above the isotype control level.

To confirm similar virus infectivity as myocytes, replicate cultures were infected with virus for 18 h, the cells were retrieved by incubation in EDTA and manual scraping, counted, and frozen and thawed three times. The supernatant from four replicate wells for each group was titrated for virus. Titters (per 10⁴ myocytes) were 0 PFU for uninfected BALB/c, 2.4 ± 0.7 × 10⁵ PFU for H3-infected BALB/c, 3.1 ± 0.4 × 10⁵ PFU for H3-infected CD1d\(^{-/-}\), and 1.9 ± 0.6 × 10⁵ PFU for H310A1 virus-infected BALB/c myocytes. There was no significant difference in virus titers between any of the infected myocyte groups. Infectious center assays were performed to determine the percentage of infected myocytes. Results indicate that 48 ± 12% of BALB/c myocytes were infected with H3, 39 ± 8% BALB/c myocytes were infected with H310A1, and 55 ± 18% of CD1d\(^{-/-}\) myocytes were infected with H3 virus in four replicate wells for each group (no significant difference). These studies were repeated three times with similar results in each experiment.

Next, BALB/c and CD1d\(^{-/-}\) mice were infected with either H3 or H310A1 virus. On day 3 after infection, the hearts were perfused with PBS, snap frozen, and sectioned for confocal microscopy (Fig. 2). The myocardium was visualized using anti-\(\alpha\)-actin and Cy3-conjugated anti-mouse IgG Ab (red, stains myocardial cells). Virus-infected cells were identified using monoclonal anti-CVb3 Ab (mouse IgM) and Cy5-anti-mouse IgM (\(\mu\)-chain specific; blue), CD1d expression was determined using anti-CD1d (rat IgG) and Alexa 488 anti-rat IgG Ab (green). Uninfected BALB/c mice showed actin staining but neither virus nor CD1d. Both H3-infected CD1d\(^{-/-}\) and H310A1-infected BALB/c hearts showed infected myocytes (purple due to colocalization of blue and red), but no significant CD1d expression. In contrast, hearts from H3-infected BALB/c mice showed substantial numbers of CD1d\(^+\) cells. Although CD1d is associated with virus-positive cells, other CD1d\(^+\) cells are not myocytes and do not appear to be infected. These may represent CD1d inflammatory cells rapidly infiltrating the heart during H3 virus infection. Double-color analysis of CD1d (green) and virus (blue) provides better visualization of virus-positive and virus-negative CD1d\(^-\) cells without the complication of \(\alpha\)-actin staining (Fig. 3). Analysis of hearts at a later time (day 6 after infection) showed more widespread CD1d\(^+\) cells in H3-infected BALB/c mice but few virus-positive cells. Again, few CD1d\(^+\) cells were seen in H310A1-infected BALB/c hearts at this time (data not shown).

**Lysis of H3-infected myocytes by V\(\gamma\)4\(^+\) cells**

The following studies investigated whether V\(\gamma\)1\(^+\) or V\(\gamma\)4\(^+\) effector cell populations could directly kill infected myocyte targets in vivo. V\(\gamma\)1\(^+\) and V\(\gamma\)4\(^+\) effector cell populations were isolated from uninfected BALB/c mice or from BALB/c or CD1d\(^{-/-}\) mice infected 7 days earlier with either H3 or H310A1 virus. Fig. 4 shows representative purity of the sorted effector cell populations. Effectors were assayed on \(^{51}\)Cr-labeled H3 virus-infected myocyte targets. Fig. 5A shows that only V\(\gamma\)4\(^+\) cells from H3-infected BALB/c mice showed cytotoxicity to the infected targets. Neither V\(\gamma\)1\(^+\) cells isolated from H3-infected BALB/c mice nor V\(\gamma\)4\(^+\) cells from uninfected BALB/c mice were cytolytic. This indicates that not all \(\gamma\)6\(^+\) cell subpopulations from H3 virus-infected mice kill cardiac myocytes and that virus infection of V\(\gamma\)4\(^+\) cell donors is required to activate cytotoxic potential. In a second set of experiments, V\(\gamma\)4\(^+\) cells were isolated from H3-infected BALB/c

![FIGURE 1](http://www.jimmunol.org/Downloadedfrom/http://www.jimmunol.org)  
CD1d expression in neonatal myocytes. Myocytes were cultured from 3-day-old BALB/c (A–D) and CD1d\(^{-/-}\) (E) neonatal mice. Some cells were uninfected (B) while the remaining cells were infected with 100 multiplicity of infection of H3 virus (A, C, and E) or H310A1 virus (D) for 18 h. Cells were labeled with FITC-anti-CD1d, fixed with paraformaldehyde, and then resuspended in buffer with saponin and mouse anti-\(\alpha\)-actin and PE-anti-CD1d. Flow analysis was gated on the \(\alpha\)-actin-positive cell population which represented >80% of the total cells. Graphs indicate CD1d staining of these \(\alpha\)-actin-positive cells. The isotype control represents infected myocytes incubated with FITC-rat IgG and was set so that between 1 and 2% of cells fall in the positive range. Numbers in B–E indicate the percentage of CD1d\(^+\) cells above isotype control.
donors and assayed for cytotoxicity against BALB/c (uninfected, H3, or H310A1 infected) and CD1d<sup>−/−</sup> (H3 infected) myocyte targets (Fig. 5B). The Vγ4<sup>+</sup> effectors were cytolytic to the H3-infected BALB/c (CD1d<sup>+</sup>) but not to CD1d<sup>−/−</sup> myocytes. Also, no cytotoxicity was observed to either uninfected or H310A1 virus-infected BALB/c myocytes. One possibility is that Vγ4<sup>+</sup> cells from H3-infected mice do not kill H310A1 virus-infected targets due to viral specificity of the Vγ4<sup>+</sup> cell populations. In Fig. 5C, Vγ4<sup>+</sup> cells were isolated from H310A1-infected BALB/c mice and assayed for cytotoxicity to both H3 and H310A1 virus-infected myocytes. The Vγ4<sup>+</sup> cells from H310A1 virus-infected donors were not significantly lytic to either infected target. Thus, not only do the effectors need to come from H3-infected donors, but these cells can distinguish between uninfected and H3-infected targets.

Since H3 infection increases CD1d expression on myocytes, Vγ4<sup>+</sup> cells may need this molecule for myocyte killing. To investigate this possibility, Vγ4<sup>+</sup> cells from H3 virus-infected BALB/c donors were assayed for cytotoxicity on H3 virus-infected myocytes in the presence or absence of mAbs (1 μg/ml) to CD1d, MHC class I K<sup>d</sup> or D<sup>d</sup> Ags, or MHC class II IA/IE Ag to the cultures (Fig. 6). Only the anti-CD1d blocked cytotoxicity.

Finally, studies were done to determine whether Vγ4<sup>+</sup> cells kill myocyte targets through Fas-dependent or perforin-dependent mechanisms (Fig. 7). Vγ4<sup>+</sup> cells were isolated from H3-infected BALB/c donors and assayed on H3 virus-infected BALB/c myocyte targets in medium containing either CMA to block perforin-dependent killing or Fas-Fc to block Fas-dependent killing. Only the Fas-Fc inhibited Vγ4<sup>+</sup> cell-mediated cytotoxicity.

**Discussion**

These studies show the following major points: 1) CD1d is important in myocarditis susceptibility during H3 virus infection; 2)
CD1d expression is increased in H3-infected neonatal myocytes in vitro and in the myocardium in vivo; 3) H310A1, differing from the H3 variant by a single amino acid mutation in the VP2 capsid protein (4), does not increase CD1d expression on neonatal myocytes in vitro or in the heart in vivo; and 4) Vγ4+ cells kill CD1d+ but not CD1d−/−infected myocytes in vitro.

MHC class I-like CD1 molecules are involved in innate immunity to many infectious agents (12–15, 22–25). Most often, CD1d-restricted cells belong to the invariant NKT subpopulation (12–15) or Th2 (IL-5, IL-4) restricted immune responses in the mouse (29, 30). CD1d-restricted murine CTL belonging to the γδ T cell population extends current knowledge regarding these diverse types of CD1d-restricted immune responses in the mouse (29, 30). CD1d-restricted T cells can make either Th1 (IFN-γ) or Th2 (IL-5, IL-4) cytokines, depending upon the model system used (14, 26, 30, 31). Although cytokine bias of the CD1d-restricted effector might reflect an inherent characteristic of the T cell, CD1d is expressed on dendritic and other APCs, and the APC may be the determining factor in T cell cytokine bias (23, 32, 33). Our studies in CVB3-induced myocarditis resemble those in hepatitis C virus-infected humans (30), in which CD1d-restricted intrahepatic lymphocytes were increased in patients with chronic hepatitis C infections, but cells expressing the invariant TCR were rare. The CD1d-restricted intrahepatic lymphocytes were strongly Th1 biased and cytolytic. Despite TCR utilization by NKT cells for CD1d recognition, it is not certain whether CD1d recognition by Vγ4+ T cells is through the TCR. This point will need further investigation.

Generally, CD1 molecules bind hydrophobic lipids or peptides (17). In bacterial infections, the lipid Ags can derive from the bacterial membrane. This cannot be true of CVB3 because these are nonenveloped viruses. However, CVB3-infected cells might release glycolipid Ags, which could be exogenously taken up by target CD1d+ cells, or glycolipids endogenous to the CD1d+ cell might provide the relevant Ag. A hydrophobic virus peptide is also a possible candidate for the CD1d-restricted Ag. A large number of CD1d+ cells rapidly appear in the hearts of H3-infected BALB/c mice. The nature of these cells is not known, but presumably represent various lymphoid cells infiltrating the heart and may even be the γδ T cells themselves (16, 34).

Vγ4+ effectors could directly induce cardiac injury since these cells are cytolytic to infected myocytes in vitro. It is not clear how
much of the total myocyte destruction results from the Vp4+ effector. Certainly, γδ+ cells comprise a large proportion of the inflammatory cells in myocarditis (8). We have shown that up to 50% of the lymphoid cells recoverable at the time of peak myocarditis are γδ+ TCR+ and that these effectors are strongly positive for Fas ligand expression. However, maximum Coxsackievirus replication is observed between 3 and 4 days after infection and the infectious virus is rapidly cleared by day 10 (35). Since Vp4+ cells kill infected but not uninfected myocytes in vitro, presumably damage mediated by this effector should be limited to periods of virus infection. If persistent virus infections are established, as has been reported by some investigators in CVB3-induced heart disease (36), the amount of cytotoxicity may be significant. Vp4+ cells kill CD1d+ H3-infected neonatal myocytes in vitro. It is not known whether they can also kill adult myocytes. Unfortunately, isolation of viable adult myocytes is extremely difficult, if not impossible. Although we infer that Vp4+ cells also kill adult cells of the same type, this may not be true. An alternative hypothesis is that Vp4+ cells affect myocarditis susceptibility in more than one way. As reported earlier and as shown in Table I, myocarditis susceptibility correlates to preferential activation of CD4+ Th1 (IFNγ+) cell responses (9). Both NKT and γδ+ cells are known to produce pro- and anti-inflammatory cytokines as part of the innate immune response and influence developing adaptive immunity (14, 30, 37, 38). The Vp4+ cells in H3 virus-infected BALB/c mice are strongly IFNγ- and production of this cytokine is important for their ability to bias CD4+ T cell responses toward a Th1 phenotype (9). It is presently not known whether Vp4+ cells induced in CD1d+ mice and apparently recognizing CD1d Ag are primarily pathogenic through their direct ability to kill infected cells or through their ability to modulate adaptive immunity through production of IFN-γ early in the infection.

The cytolytic Vp4+ cell population is primarily (>80%) CD4−CD8+. The Vp1+ cell population is primarily CD4+CD8− (95%). However, in both populations, a minority of other phenotype cells are present (~20% CD4+CD8− Vp4+ cells and ~5% CD4−CD8−Vp1+ cells). No CD4 expression was noted in either sorted cell population. It is not known what proportion of the total Vp4+ cells isolated from H3-infected BALB/c mice actually modulate myocarditis. This is likely to be a heterogeneous population with regard to Vδ gene usage and only a specific Vp4+ Vδ population might prove pathogenic. Positive selection of NKT cells during ontogeny involves CD1d (39, 40), like conventional T cell MHC restriction (41, 42). Although Vp4+ cells are found in CD1−/− mice, it is possible that the specific CD1d-reactive Vp4+ subpopulation may be absent rather than not activated.

A remaining question is why the single amino acid mutation between the H3 and H310A1 viruses had such a dramatic effect on infectivity of cardiocytes. It is not known whether these factors could increase CD1d expression.

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