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Slam Haplotype 2 Promotes NKT But Suppresses Vγ4$^+$ T-Cell Activation in Coxsackievirus B3 Infection Leading to Increased Liver Damage But Reduced Myocarditis

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There are two major haplotypes of signal lymphocytic activation molecule (*Slam*) in inbred mouse strains, with the *Slam* haplotype 1 expressed in C57Bl/6 mice and the *Slam* haplotype 2 expressed in most other commonly used inbred strains, including 129 mice. Because signaling through Slam family receptors can affect innate immunity [natural killer T cell (NKT) and γ-δ T-cell receptor], and innate immunity can determine susceptibility to coxsackievirus B3 (CVB3) infection, the present study evaluated the response of C57Bl/6 and congenic B6.129c1 mice (expressing the 129-derived *Slam* locus) to CVB3. CVB3-infected C57Bl/6 male mice developed increased myocarditis but reduced hepatic injury compared with infected B6.129c1 mice. C57Bl/6 mice also had increased γδ$^+$ and CD8$^+$interferon-γ$^+$ cells but decreased numbers of NKT (T-cell receptor β chain + mCD1d tetramer$^+$) and CD4$^+$FoxP3$^+$ cells compared with B6.129c1 mice. C57Bl/6 mice were infected with CVB3 and treated with either α-galactosylceramide, an NKT cell-specific ligand, or vehicle (dimethyl sulfoxide/PBS). Mice treated with α-galactosylceramide showed significantly reduced myocarditis. Liver injuries, as determined by alanine aminotransferase levels in plasma, were increased significantly, confirming that NKT cells are protective for myocarditis but pathogenic in the liver. *(Am J Pathol 2013, 182: 401—409; http://dx.doi.org/10.1016/j.ajpath.2012.10.019)*

Myocarditis is an inflammation of the cardiac muscle that follows microbial infections.[1] Among viruses, enteroviruses including coxsackie B viruses are common etiologic agents.[2,3] Although infectious agents act as a trigger for myocarditis, there is considerable debate as to the actual mechanism(s) of myocardial injury. Viruses directly cause cellular dysfunction either through induced cell death, shut down of cell RNA and protein synthesis, or viral protease cleavage of contractile proteins.[4,5] In addition, cytokines such as IL-1β, IL-6, and tumor necrosis factor α, which are elicited from resident cells in the heart subsequent to infection, can suppress contractility, leading to cardiac dysfunction.[6] Finally, host immune responses to infection may kill myocytes, leading to cardiac stress. Host response can be directed specifically toward virally infected cardiocytes or infection can trigger autoimmunity to cardiac antigens (autoimmunity), which destroys both infected and uninfected myocytes.[7]

Host innate immune responses occur rapidly, subsequent to viral infections, and usually have broad specificity, unlike the classic adaptive immune response, which requires a week or more for development of a measurable response in the naive individual but is highly specific to the inducing pathogen. The innate immune response both helps to control microbe load before generation of the adaptive immune response and has a major impact on the phenotype and intensity of the adaptive response. Two types of T cells representing innate immunity are natural killer T cells (NKT) and T cells expressing the γ-δ T-cell receptor (γδ$^+$). A study by Wu et al[8] showed that *in vivo* administration of α-galactosylceramide, a ligand that specifically activates NKT cells, protects mice from coxsackievirus B3 (CVB3)-induced myocarditis. Prior studies have shown that signaling through Slam family receptors has a major impact on NKT cell development,[9—11] and that different *Slam* haplotypes can have distinct effects on NKT cell response and function.[9,12] There are two major *Slam* haplotypes, *Slam* haplotype 1 and *Slam* haplotype 2, that distinguish

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commonly used inbred mouse strains.[13,14] *Slam* haplotype 1 is present in C57Bl/6, and *Slam* haplotype 2 is present in most other commonly used mouse strains including 129S1/SvImJ and BALB/c mice. The congenic B6.129c1 mouse expresses the genetic region of chromosome 1 containing the 129-derived *Slam* haplotype 2 locus on the C57Bl/6 background and was used previously to show *Slam* haplotype control of liver NKT cell numbers and NKT cell cytokine production.[12] In addition, *Slam* haplotypes previously were shown to regulate macrophage tumor necrosis factor production in response to lipopolysaccharide.[12] Although less well studied, Slam family–receptor signaling also has been shown to affect $\gamma\delta^+$ T-cell development. Studies using human peripheral blood mononuclear cells stimulated *in vitro* with antibody to CD3 and either IL-2, anti-CD150 (SLAM), or IL-15 showed that all three stimulation protocols resulted in $\gamma\delta^+$ T-cell survival. However, co-culture with anti-CD3 and anti-CD150 resulted in selective proliferation of $CD8^+CD56^+\gamma\delta^+$ T cells expressing the Vδ1 chain, and cells co-cultured with anti-CD3 and IL-15 resulted in preferential generation of $CD8^-CD56^-\gamma\delta^+$ cells expressing the Vδ2 chain.[15] Therefore, SLAM signaling can impact the generation of a subpopulation of the total $\gamma\delta^+$ cell population in humans. Prior studies from the Huber laboratory have shown that a subpopulation of $\gamma\delta^+$ cells is crucial to myocarditis susceptibility subsequent to CVB3 infection[16] and that the relevant $\gamma\delta^+$ cell expresses both CD8 and the Vγ4 chain.[16,17] This raised the question of whether *Slam* haplotypes modulated selected $\gamma\delta^+$ cell subsets in the mouse, as it does in humans, and whether the *Slam* haplotype specifically could affect activation of the $CD8^+V\gamma4^+$ T cell, which is known to be pathogenic in CVB3-induced myocarditis.

CVB3 infection of mice results in multiple organ infection, including pancreas, liver, and heart with accompanying tissue injury in all tissues. There are well-established differences in disease susceptibility between BALB/c and C57Bl/6 mice, strains expressing the two distinct *Slam* haplotypes. C57Bl/6 mice are highly susceptible to type 2 autoimmune hepatitis and develop extensive hepatic inflammation, whereas BALB/c mice are resistant to this disease and show no inflammation.[18] In contrast, BALB/c mice are more susceptible to myocarditis[19–22] compared with the more resistant C57Bl/6 strain. However, there are many genetic differences between BALB/c and C57Bl/6 mice, which may influence disease development or development and activation of specific innate effectors such as NKT and γδ T cells. The goal of the current study was to determine whether *Slam* haplotype affected NKT and $V\gamma4^+$ T-cell responses subsequent to CVB3 infection using C57Bl/6 congenic mice in which the *Slam* locus alone differed between the mouse strains, and whether haplotype-dependent NKT/$V\gamma4^+$ cell response had a distinct effect in different organs infected with the virus in the absence of the many other genetic differences between BALB/c and C57Bl/6 mice.

Materials and Methods

Mice

Male C57Bl/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). B6.129c1 congenic mice have been described previously,[13] and a breeding colony of these mice was maintained at the University of Vermont. All mice were 5 to 7 weeks of age when infected. All of the studies were reviewed and approved by the University of Vermont Institutional Animal Care and Use Committee.

Virus

The H3 variant of CVB3 was made from an infectious cDNA clone as described previously.[23]

Infection of Mice

Mice were injected i.p. with 10^2 plaque-forming units of virus in 0.5 mL PBS. Animals were sacrificed when moribund or 7 days after infection.

Virus Titers

Hearts and livers were removed aseptically from the animals, weighed, and homogenized in RPMI 1640 medium containing 5% fetal bovine serum, L-glutamine, streptomycin, and penicillin. Cellular debris was removed by centrifugation at $300 \times g$ for 10 minutes. Supernatants were diluted serially using 10-fold dilutions and titered on Hela cell monolayers by the plaque-forming assay.[24]

Histology

Tissue was fixed in 10% buffered formalin for 48 hours, paraffin-embedded, sectioned, and stained by H&E. The percentage of the myocardium inflamed was determined on at least two heart sections per animal using a Nikon Eclipse 50i microscope and a grid overlay. The number of grid squares containing inflammation was divided by the total grid squares of cardiac tissue (×100) to determine the percentage. Analysis was performed blindly on coded slides. Evaluation of hepatic inflammation and necrosis was too diffuse to measure the percentage of liver. Therefore, liver was scored blindly on two sections per animal by using coded slides and a scoring system of 0 to 4, where 0 was no obvious inflammation or necrosis compared with liver from uninfected animals, and 4 represents widespread and diffuse lymphocytic infiltration and necrosis.

Isolation of Lymphocytes

Spleens were removed and pressed through fine mesh screens. Lymphoid cells were isolated by centrifugation of cell suspensions on Histopaque (Sigma-Aldrich, St. Louis, MO).

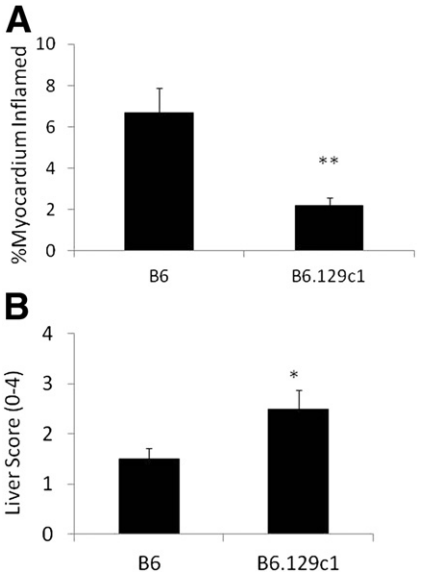

Figure 1 *Slam* locus polymorphisms control susceptibility to cardiac and liver damage after CVB3 infection. C57Bl/6 (B6) and B6.129c1 male mice were infected with CVB3 and sacrificed 7 days later. **A:** Hearts were evaluated by image analysis for the percentage of myocardium inflamed. **B:** Livers were scored using a scale of 0 to 4 for inflammation and hepatocyte necrosis. Results represent the mean ± SEM of 14 to 17 mice per strain. B6 data differ from B6.129c1 data at $*P < 0.05$ and $**P < 0.01$, respectively.

Flow Cytometry and Intracellular Cytokine Staining

Details for intracellular cytokine staining have been published previously.[25] Spleen cells (10^5) were cultured for 4 hours in RPMI 1640 medium containing 10% fetal bovine serum, antibiotics, 10 μg/mL Brefeldin A (Sigma), 50 ng/mL phorbol myristate acetate (Sigma), and 500 ng/mL ionomycin (Sigma). The cells were washed in PBS−1% bovine serum albumin (BSA; Sigma) containing Brefeldin A, incubated on ice for 30 minutes in PBS−BSA−Brefeldin A containing a 1:100 dilution of Fc Block (BD Biosciences, San Jose CA), Alexa647−anti-CD8a, and peridinin-chlorophyll proteins-Cy5.5 (PerCP-Cy5.5) anti-CD4 (clone GK1.5), or PerCP-Cy5.5 and Alexa647 rat IgG2b (clone A95-1, isotype controls). The cells were washed once with PBS−BSA−Brefeldin A, fixed in 2% paraformaldehyde for 10 minutes, and then resuspended in PBS−BSA containing 0.5% saponin, Fc Block, and 1:100 dilutions of phycoerythrin (PE) anti−interferon-γ (IFN-γ) (clone XMG1.2) or PE-rat IgG1 (clone R3-34). All antibodies were from BD Biosciences.

T regulatory cells were identified as $CD4^+FoxP3^+$ cell populations. Cells labeled with anti-CD4 in PBS−1% BSA containing Fc Block were washed, fixed, and permeabilized with PBS−BSA−saponin, and then incubated with anti-FoxP3 (PE−anti-FoxP3; clone FJK-16s; eBiosciences, San Diego, CA) and Fc Block for 2 hours at 4°C. The cells were washed once in PBS−BSA−saponin and once in PBS−BSA and then resuspended in 2% paraformaldehyde.

Evaluation of NKT cells was performed by labeling spleen cells with 1:100 dilutions of Fc Block, fluorescein

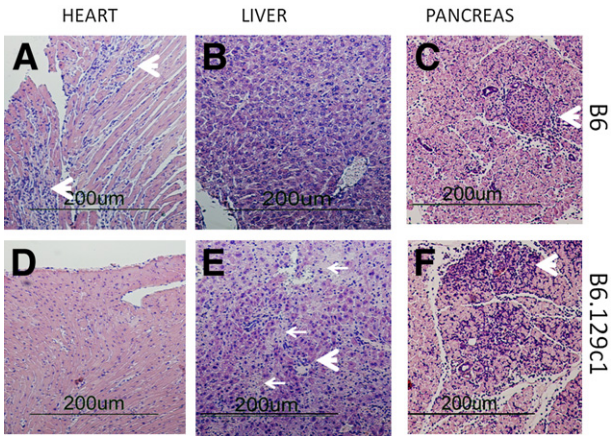

Figure 2 Representative histology of heart (**A** and **D**), liver (**B** and **E**), and pancreas (**C** and **F**) of CVB3-infected B6 (**A**, **B**, and **C**) and B6.129c1 (**D**, **E**, and **F**) mice, 7 days after infection. Tissue was formalin-fixed, paraffin-embedded, sectioned, and stained with H&E. **Thick arrows** indicate inflammation in the various organs. **Thin arrows** indicate hepatic necrosis.

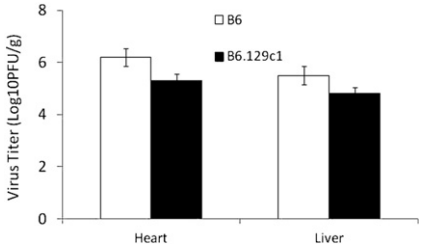

Figure 3 Cardiac and liver virus titers from mice in Figure 1. Hearts and livers were weighed, homogenized, and titered by the plaque-forming assay. Results represent the mean ± SEM. Plaque-forming units per gram of tissue from CVB3-infected B6 and B6.129c1 mice for which pathology was reported in Figure 1. No significant differences in virus titers were observed between the two mouse strains.

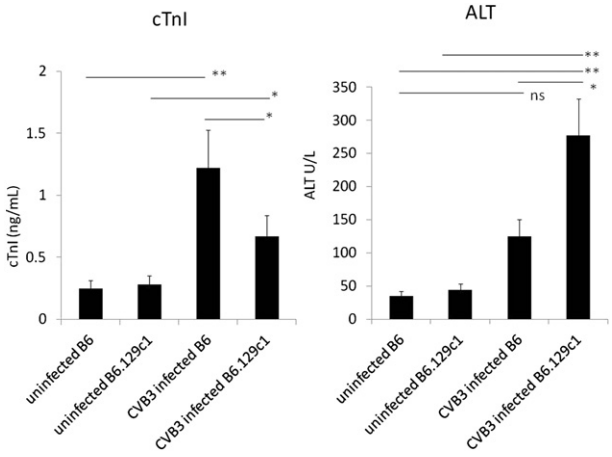

Figure 4 Plasma was obtained by cardiac puncture into EDTA-treated tubes and plasma was obtained by centrifugation. Aliquots of the plasma were evaluated using commercial ELISA kits for mouse ALT and mouse cTnI according to the manufacturer's directions. Results are the mean ± SEM of 5 to 10 mice per group. Significant differences were noted between indicated groups at $*P < 0.05$ and $** P < 0.01$, respectively.

isothiocyanate anti-CD4 (clone GK1.5), and PE-mCD1d/PBS57, which was supplied by the NIH Tetramer Core Facility at Emory University. $V\gamma4^+$ cells were evaluated by labeling spleen cells with 1:100 dilutions of Fc Block PE—anti-γδ T-cell receptor (clone GL3), fluorescein isothiocyanate—anti-Vγ4 (clone UC3-10A6), and PerCP—Cy5.5—anti-CD69. After incubation at 4°C for 30 minutes, cells were washed with PBS—BSA and resuspended in 2% paraformaldehyde.

Cells were analyzed using a BD LSR II flow cytometer (BD Biosciences) with a single excitation wavelength (488 nm) and band filters for PerCP-Cy5.5 (695/40 nm) and PE (575 nm). The excitation wavelength for Alexa 647 is 643 nm with a band filter of 660/20 nm. The cell population was classified for cell size (forward scatter) and complexity (side scatter). At least 10,000 cells were evaluated. Positive staining was determined relative to isotype controls. To determine the number of individual cell populations, the total number of viable cells was determined by trypan blue exclusion. After flow cytometry, the percentage of a subpopulation staining with a specific antibody was multiplied by the total number of cells.

Measurement of Alanine Aminotransferase

Mouse alanine aminotransferase (ALT) was determined in plasma using a commercial enzyme-linked immunosorbent assay (ELISA) kit for mouse ALT (MyBiosource, LLD, San Diego, CA) according to the manufacturer's directions. Kit sensitivity was between 1 and 250 U/L. Plasma was used at both undiluted and 1:2 dilutions in PBS to maintain values within the range of the kit. Sample values were read at an OD of 450 nm using an ELx 808 Ultra Microplate Reader (Bio-Tek, Inc, Winooski, VT) and concentrations were calculated against a standard curve using standards supplied in the kit.

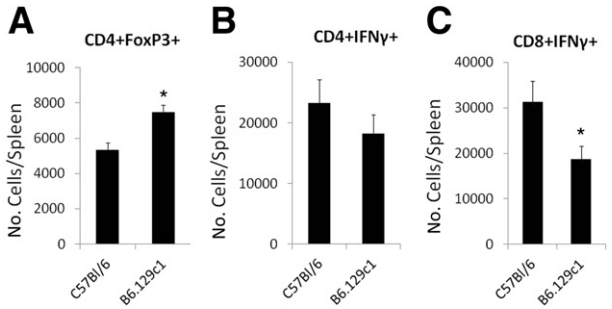

Figure 5 Number of T-regulatory ($CD4^+FoxP3^+$), CD4+IFN-γ^+, and $CD8^+$IFN-γ^+ cells in the spleens of B6 and B6.129c1 mice 7 days after CVB3 infection. For intracellular cytokine analysis, spleen cells were cultured for 4 hours in medium containing phorbol myristate acetate, ionomycin, and Brefeldin A, washed, and then labeled with antibodies to CD4 or CD8. The cells were fixed, permeabilized, and labeled with antibody to IFN-γ. For T-regulatory cell analysis, spleen cells were labeled with antibody to CD4, washed, fixed, permeabilized, and labeled with antibody to FoxP3. To determine the number of cells per spleen, the percentage of cells labeling positively for the indicated markers ($CD4^+FoxP3^+$; $CD4^+$IFN-γ^+ or $CD8^+$IFN-γ^+) was multiplied by the number of spleen cells. Results represent the mean ± SEM of 14 to 17 mice per strain. Data for B6.129c1 mice were either significantly ($*P < 0.05$) or not significantly different from C57Bl/6 animals.

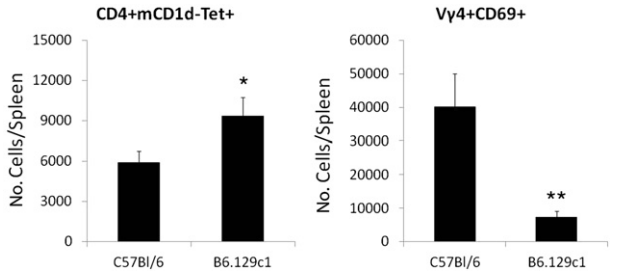

Figure 6 Evaluation of the number of NKT cells and activated $V\gamma4^+$ cells in the spleen. NKT cells were identified by labeling spleen lymphocytes with fluorescein isothiocyanate anti—T-cell receptor β and PE-mCD1d tetramer. Activated $V\gamma4^+$ cells were determined by labeling spleen cells with PE—anti-γδ TCR, FITC—anti-Vγ4, and PerCP—Cy5.5—anti-CD69, gating on the γδ TCR^+ cells, and determining the $V\gamma4^+$ $CD69^+$ population. The percentage of positive spleen cells for the indicated cellular antigens was multiplied by the number of spleen cells isolated per individual spleen to obtain the number of cells per spleen. Results are mean ± SEM of five to eight mice per group. $*P < 0.05$, $**P < 0.01$.

Measurement of Mouse Troponin I

Mouse cardiac troponin I (cTnI) was measured in plasma using a commercial kit from Life Diagnostics (West Chester, PA) according to the manufacturer's directions. The sensitivity of the kit was between 0.078 and 5 ng/mL. Values were read at 450 nm and determined against a standard curve using standards supplied in the kit.

Treatment with α-GalCer

α-Galactosylceramide (α-GalCer) was purchased from Avanti (Alabaster, AL), dissolved in dimethyl sulfoxide (Sigma) at a concentration of 1 mg/mL, and then diluted to a final concentration of 4 μg/mL in PBS. Mice were injected i.p. with 0.5 mL of the α-GalCer solution or equivalent dimethyl sulfoxide/PBS vehicle control on day 3 after infection.

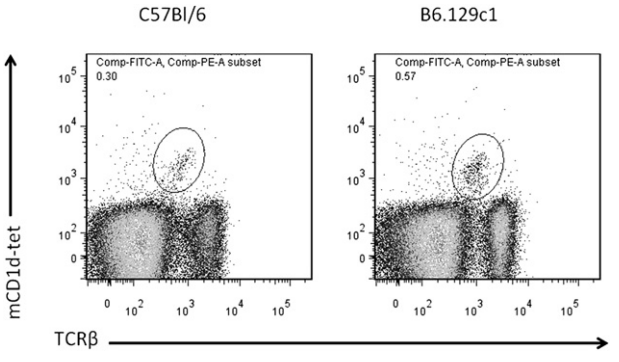

Figure 7 Histogram of NKT cells labeled with antibody to T-cell receptor (TCR)β and mCD1d tetramer. FITC, fluorescein isothiocyanate.

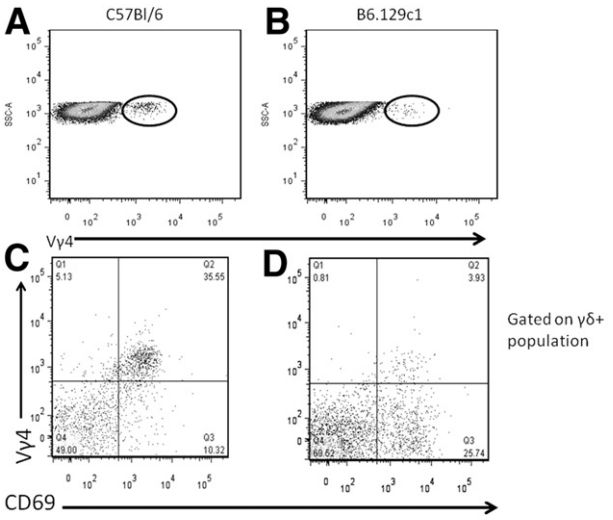

Figure 8 Histogram of $V\gamma4^+$ cells. **A**: Spleen cells were evaluated by side scatter and anti-Vγ4 antibody. **Circled** cells are $V\gamma4^+$. **B**: Spleen cells were gated on the T-cell receptor $\gamma\delta^+$ population and then evaluated for cells labeling positive for Vγ4 and CD69.

Statistics

Differences between groups were determined by the Wilcoxon ranked score or χ^2 analysis.

Results

Slam Haplotype Determines CVB3-Induced Pathology: Inverse Relationship between Myocarditis and Hepatitis

C57Bl/6 and B6.129c1 congenic mice were infected with CVB3 and surviving mice were sacrificed 7 days later. There was no significant difference in animal survival to day 7 after infection ($P > 0.05$ by χ^2 analysis) between B6 (17 surviving of 33 total animals infected; 51.5%) and B6.129c1 (14 surviving of 44 total animals infected; 31.8%) mice. Hearts and liver were evaluated histologically (Figures 1 and 2) and for virus titers (Figure 3). Myocardial inflammation was reduced significantly in B6.129c1 mice compared with B6 animals subsequent to infection (Figures 1A and 2D). In contrast liver inflammation (Figure 2E) and hepatocyte necrosis (Figure 2E) were increased in the B6.129c1 congenic animals compared with B6 animals. Figure 1B shows the summary of hepatic histology for all animals. Although there was a slight decrease in virus titers in both livers and hearts of the congenic mice compared with B6 animals, the difference was not statistically significant (Figure 3). Representative histology is shown for hearts (Figure 2, A and D), livers (Figure 2, B and E), and pancreas (Figure 2, C and F) of the two strains of mice. Acinar cells in the pancreas of both B6 and B6.129c1 mice were degranulated equally, but there was increased mononuclear cell infiltration in the pancreas

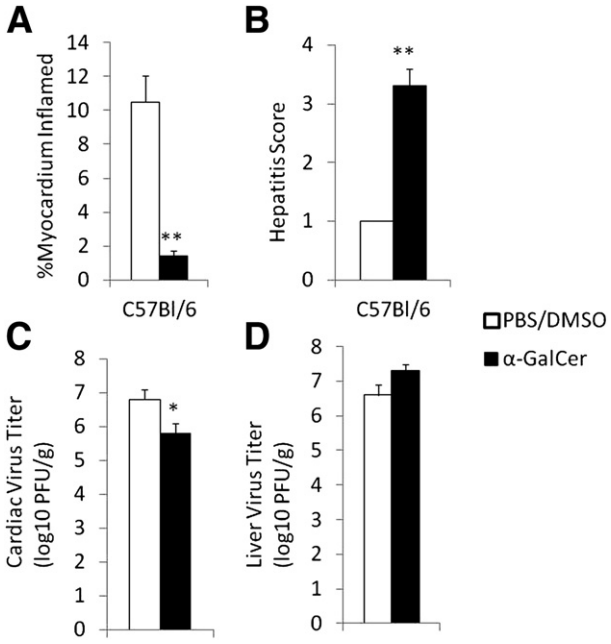

Figure 9 Treatment of CVB3-infected C57Bl/6 mice with either PBS/dimethyl sulfoxide (DMSO) (vehicle) or α-GalCer in PBS/DMSO. Mice were infected with CVB3 and then injected i.p. with either vehicle alone or 2 μg α-GalCer on day three after infection. Mice were sacrificed 7 days after infection and hearts (**A**) and livers (**B**) were evaluated for inflammation and necrosis using H&E—stained slides of the tissue. Hearts (**C**) and livers (**D**) also were homogenized and the supernatants were titered for virus by the plaque-forming assay. Results represent the mean ± SEM of 5 to 10 mice per group. Data for the α-GalCer—treated group were different from vehicle control at $*P < 0.05$ and $**P < 0.01$, respectively.

of the B6.129c1 mice (Figure 2F). Neither uninfected B6 nor B6.129c1 mice showed any pathology of liver, heart, or pancreas (data not shown). Injury to heart and liver was confirmed by measuring troponin I and ALT levels in plasma (Figure 4). ALT values were increased significantly in CVB3 wild-type and CVB3-infected B6.129c1 mice compared with uninfected control mice, but the levels in the infected B6.129c1 animals was more than twice that in infected B6 mice, confirming the increased liver injury in the B6.129c1 mice. In contrast, cTnI levels, although increased in both infected B6 and B6.129c1 animals, was significantly greater in B6 than in B6.129c1-infected animals.

Difference in Adaptive Immune Response Depending on *Slam* Haplotype

Because the *Slam* haplotype had a significant effect on the pathology associated with CVB3 infection, the subsequent question was whether activation of $CD4^+$ or $CD8^+$ T cells was different in the wild-type and congenic mouse strains. Spleen cells were isolated 7 days after infection. To evaluate T-regulatory cell response, cells were labeled with antibody to CD4, then intracellularly with antibody to FoxP3 (Figure 5A). There was a significant increase in the total

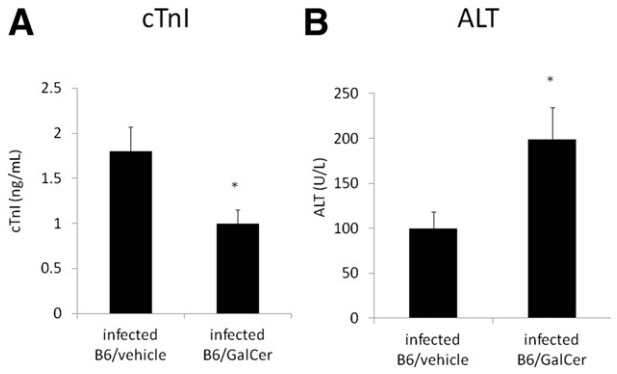

Figure 10 Plasma cTnI (**A**) and ALT (**B**) concentrations from mice described in Figure 9. Plasma from individual mice infected with CVB3 and treated 3 days after infection with either vehicle or α-GalCer was evaluated for mouse ALT and cTnI using commercial ELISA kits. Results represent the mean ± SEM of 5 to 10 mice per group. α-GalCer—treated mice were significantly different from vehicle controls at $*P < 0.05$.

number of T-regulatory cells in spleens of CVB3-infected B6.129c1 mice compared with infected B6 mice (40% increase). This change was specific to CVB3 infection because there was no difference observed in the total numbers of $CD4^+FoxP3^+$ cells in uninfected mice (data not shown). Spleen cells also were activated with phorbol myristate acetate and ionomycin in the presence of Brefeldin A to allow evaluation of cytokine-positive T cells. The cells were labeled with antibodies to CD4 and CD8, and then intracellularly labeled with antibody to IFN-γ (Figure 5, B and C). There was a slight, but not significant, reduction in $CD4^+IFN\text{-}\gamma^+$ cells but a significant reduction in $CD8^+IFN\text{-}\gamma^+$ cells in CVB3-infected B6.129c1 compared with B6 mice.

Differences in Innate Effector Response with *Slam* Haplotype

Prior studies have shown that although the total numbers of NKT cells were equivalent in thymus and spleen between B6 and B6.129c1 congenic mice, the B6.129c1 animals had significantly reduced numbers of NKT cells in the liver.[12] In addition, B6.129c1 mice produced significantly less serum cytokine in response to an *in vivo* lipopolysaccharide challenge compared with B6 animals.[12] As shown earlier, the cytokine response of B6.129c1 mice to CVB3 challenge also was reduced, whereas the T-regulatory cell response was increased. To investigate the effect of *Slam* haplotype on NKT and $\gamma\delta^+$ T-cell responses subsequent to CVB3 infection, spleen cells from infected B6 and B6.129c1 mice were labeled with antibodies to CD3, T-cell receptor δ, and Vγ4, or with antibody to T-cell receptor β and the mCD1d tetramer (Figure 6). These data showed that the total number of splenic NKT cells (T-cell receptor β^+CD1d tetramer$^+$) were increased significantly in B6.129c1 congenic mice compared with B6 animals, whereas the opposite result was observed with $V\gamma4^+$ T cells (greater numbers in spleens of B6 than B6.129c1 mice). Representative flow diagrams for NKT cells are shown in Figure 7, and for $V\gamma4^+$ cells are shown in Figure 8. In Figure 8, A and B, the percentage of $V\gamma4^+$ cells in the total lymphocyte population is shown for C57Bl/6 and B6.129c1 mice. In Figure 8, C and D, flow analysis was first gated on the total $\gamma\delta^+$ cell population, then the $V\gamma4^+$ and $CD69^+$ populations were analyzed for C57Bl/6 and B6.129c1 mice. CVB3 infection of B6 mice resulted in approximately a threefold increase in $V\gamma4^+$ cells as a percentage of total lymphocytes, but a nearly 10-fold increase as a percentage of total $\gamma\delta^+$ cells compared with B6.129c1 mice. No differences were noted in the percentage of $V\gamma4^+$ cells in uninfected B6 and B6.129c1 mice, indicating that the alteration in this subpopulation was a consequence of *Slam* haplotype and infection.

Treatment of Mice with α-GalCer Modulates Both Myocarditis and Hepatitis

The earlier-described data provide circumstantial evidence that NKT cells promote hepatic pathology in CVB3 infection while potentially suppressing myocarditis. To confirm this observation, C57Bl/6 mice were infected with CVB3 and injected i.p. with either 2 μg α-GalCer in dimethyl sulfoxide/PBS or with vehicle (dimethyl sulfoxide/PBS) alone (Figure 9). Myocarditis was reduced significantly in C57Bl/6 mice given α-GalCer compared with vehicle control, although hepatitis was increased significantly. Cardiac virus titers decreased modestly, but significantly, with α-GalCer treatment (Figure 9C). There was no significant change in virus titers in the liver (Figure 9D). Plasma levels of ALT and troponin I are shown in Figure 10. Treating infected B6 mice with α-GalCer resulted in a twofold increase in ALT plasma levels compared with infected mice treated with vehicle, as expected. In contrast, α-GalCer—treated mice showed a significant decrease in cTnI concentrations, correlating with the decrease in myocardial inflammation.

Discussion

This study showed that *Slam* haplotypes control both NKT and Vγ4 T-cell responses to CVB3 infection, leading to differences in severity of cardiac and hepatic injury from infection. There appears to be a limited effect of *Slam* haplotype on virus-induced acinar cell degranulation that was equivalent in infected C57Bl/6 and B6.129c1 mice. However, there was increased inflammatory cell infiltration of the pancreas in infected B6.129c1 mice. Pancreatitis is dependent on both T-cell—dependent immunity and virus-induced injury in coxsackievirus-infected mice, but the acute acinar degranulation may reflect viral injury alone whereas progression to chronic pancreatitis depends on proinflammatory host responses.[26–28] Because surviving

mice were sacrificed 7 days after infection, it was not possible to determine whether the increased lymphocytic infiltrate in B6.129c1 mice resulted in chronic pancreatitis; the acinar damage in B6 mice represents the acute form only. The histologic evidence for myocarditis and hepatitis was confirmed using assays evaluating c-troponin I and ALT levels in plasma from infected mice. As shown previously,[16] the increased number of $V\gamma4^+$ cells in infected C57Bl/6 mice correlates to myocarditis severity. Congenic B6.129c1 mice with the *Slam* haplotype 2 locus on the C57Bl/6 background showed significantly fewer $V\gamma4^+$ cells and myocardial inflammation, but had greater numbers of NKT cells and enhanced hepatic injury, as noted both by histologic evaluation of the liver and plasma ALT levels. These results strongly indicate that although the *Slam* locus regulates both NKT and $V\gamma4^+$ cell responses subsequent to CVB3 infection, it regulates these two innate effectors in a fundamentally different manner. Studies using peripheral blood mononuclear cells from healthy normal volunteers indicated that *in vitro* stimulation with anti-CD3 and anti-CD150 (SLAM) was effective in promoting the response of a subpopulation of $\gamma\delta^+$ T cells expressing CD8 and CD56, a cell surface marker found on natural killer cells.[15] Because the relevant $V\gamma4^+$ cell in CVB3-induced myocarditis has been shown to express CD8,[17] it seemed reasonable that Slam family signaling might prove important in activation of this subpopulation, as it was found to be in human cells *in vitro*.

The observation that decreased numbers of NKT cells correlated to poorer development of myocarditis is consistent with the report of Wu et al,[8] which showed that treating CVB3-infected mice with α-GalCer protected them from myocarditis and also reduced cardiac virus titers. Similar results were obtained in an experimental autoimmune myocarditis model in which *in vivo* administration of α-GalCer resulted in reduced cardiac inflammation accompanied by increases in T-regulatory cells.[29] The protective effects of NKT cells in autoimmune and CVB3-induced myocarditis is in contrast to Lyme carditis, in which infiltration of the myocardium with NKT cells is associated with local production of IFN-γ and inflammation.[30] No published reports address the presence or potential role of NKT cells, or of Slam family molecules, in clinical myocarditis.

A growing body of literature supports a role for NKT cells in hepatitis. It is now well accepted that concanavalin-A–mediated hepatitis, a classic mouse model of immune-mediated liver injury, is an NKT cell–dependent phenomenon.[31–33] In addition, NKT cells have been implicated in primary biliary cirrhosis in humans and in mouse models,[34–36] and in mouse models of nonalcoholic steatohepatitis.[37–40] A prominent role for NKT cells in liver pathology perhaps is not unexpected, given that NKT cells can comprise up to 50% of all liver αβ T cells. In this regard, it is interesting to note that liver NKT cell numbers are particularly variable among different inbred strains of mice.[41] Additional studies will be needed to determine whether liver NKT cells are involved directly in the hepatitis observed in B6.129c1 mice after CVB3 infection.

In the current study, both cardiac and liver virus titers were reduced per gram of tissue with α-GalCer, but the trend did not reach statistical significance. This suggests that the protection may not be mediated through direct NKT control of virus load but may result from other mechanisms. T-regulatory cells ($CD4^+FoxP3^+$) are increased in myocarditis-resistant mice whether the resistance results from infection with a non-myocarditic CVB3 variant or infection of female mice in which estrogen is protective.[42–46] Furthermore, although $V\gamma4^+$ cells selectively kill T-regulatory cells in CVB3-infected mice, leading to an increased proinflammatory response,[47,48] several studies have reported that NKT cells can promote T-regulatory cell responses.[49–52] Although not investigated, the protection in α-GalCer–treated CVB3-infected mice[8] would be consistent with increases in T-regulatory cell response, as well as with reduction in cardiac virus titers.

B6.129c1 congenic mice possess a region of chromosome 1 encompassing the 129-derived *Slam* haplotype 2 locus, as well as a number of other immunologically relevant 129-derived genes.[12,13] Given the previous reports linking Slam family receptors to innate immune function[53] and to NKT and $\gamma\delta^+$ T-cell development and function,[10,11,15] we speculate that Slam family receptors represent likely candidate genes to explain the phenotypes reported here. Although the mechanisms through which the congenic interval regulates NKT and $\gamma\delta^+$ T-cell numbers after CVB3 infection remains unclear, we note that previous reports suggest that, together with the signaling adapter protein Slam-associated protein, Slam receptors can regulate T-cell activation and proliferation[54,55] and that Slamf6 in particular has been shown to regulate restimulation-induced apoptosis in human T cells.[56] In addition, it previously was shown that expression of certain Slam family receptors on NKT cells differs significantly between B6 and B6.129c1 mice,[12] and that Slamf6 (Ly108) appears to regulate liver NKT cell number.[11,57] Given the previous work showing the roles of $\gamma\delta^+$ and NKT cells in the development of myocarditis,[9–11,15] it is possible that genetic differences in *Slam* family loci could regulate pathogenesis through the regulation of $\gamma\delta^+$ and NKT cell expansion and/or contraction after CVB3 infection.

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