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γδ T lymphocytes kill T regulatory cells through CD1d

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Summary

Coxsackievirus B3 (CVB3) induces myocarditis, an inflammation of the myocardium, in C57Bl/6 male mice but not in mice lacking γδ+ T cells [γδ knockout (γδKO)]. Suppression of myocarditis in γδKO mice corresponds to an increase in $CD4^+$ $CD25^+$ $FoxP3^+$ T regulatory cells. A subpopulation of the T regulatory cells in infected γδKO mice expressed high levels of CD1d, a non-classical major histocompatibility complex class 1-like molecule. Adoptive transfer of $CD1d^+$ and $CD1d^-$ $CD4^+$ $CD25^+$ cells into infected C57Bl/6 recipients showed that the $CD1d^+$ subpopulation is substantially more suppressive than the $CD1d^-$ subpopulation. T cells expressing the γδ T-cell receptor comprised approximately 30–50% of the infiltrating lymphoid cells in the hearts of myocarditic C57Bl/6 mice and approximately half of the γδ+ cells expressed the Vγ4 T-cell receptor. The Vγ4+ cells lysed T regulatory cells from γδKO mice but not from wild-type (C57Bl/6) animals. Lysis was inhibited by antibody to CD1d and zVAD-fmk, a pan-caspase inhibitor. The Vγ4− γδ+ cells were not lytic to T regulatory cells and did not promote myocarditis. These results demonstrate that Vγ4+ cells selectively abrogate T regulatory cells through recognition of CD1d expressed on the regulatory cells and caspase-dependent apoptosis.

Keywords: infection; inflammation; innate immunity; myocarditis

Introduction

Coxsackievirus B3 (CVB3) is a picornavirus that has been implicated as a significant aetiological agent in myocarditis and dilated cardiomyopathy.[1,2] Virus infection causes cardiac injury through multiple mechanisms, including direct cardiocyte death through virus replication,[3] induction of cytokines that suppress cardiac function,[4] virus protease cleavage of contractile proteins,[5] and induction of virus-specific and autoimmune responses resulting in cardiocyte death.[6,7] Previous studies from this laboratory have shown that, in our mouse model of CVB3-induced myocarditis, autoimmune $CD8^+$ T cells, which are specific to cardiac antigens, are the primary effectors causing myocarditis.[7–9] The major question is how CVB3 infection induces activation of these autoimmune $CD8^+$ T cells. Evidence now indicates that the initial interaction between the virus and the virus receptor, decay-accelerating factor (CD55), causes intracellular calcium flux and nuclear factor of activated T cells (NFAT) activation leading to up-regulation of CD1d.[10]

CD1d is one isoform of a family of CD1 molecules. These are major histocompatibility complex (MHC) class 1-like molecules that are most closely associated with innate immunity.[11] There are currently five known CD1 molecules, CD1a, CD1b, CD1c, CD1d and CD1e, but only CD1d is expressed in mice.[12] Invariant natural killer T (iNKT) cells are the innate effector most closely associated with CD1d restriction.[13–15] iNKT cells have a constant Vα14-Jα18 T-cell receptor combined with one of several different Vβ chains.[16,17] These effectors are potent producers of cytokines, including interferon (IFN)-γ and interleukin (IL)-4,[18,19] activate classical natural killer (NK) cells,[20] and can promote antimicrobial or antitumour immunity.[21–23] iNKT cells also regulate autoimmunity as these cells promote systemic tolerance[24] and promote $CD4^+$ $CD25^+$ T regulatory cell potency and proliferation.[25] Despite the close association between iNKT cells and CD1, these are not the only innate effectors that are CD1 restricted. T cells expressing the γδ T-cell receptor (γδ+ T cells) also recognize CD1 molecules.[26–28] Previously, we have shown that $CD4^+$ $CD25^+$ T regulatory cells can suppress CVB3-induced myocarditis,[29] and *in vivo* depletion of γδ+ T cells results in increased numbers of T regulatory cells in infected mice.[30] These results indicate that CD1d-restricted γδ+ T cells may balance the

tolerogenic aspects of CD1d-restriced iNKT cells and promote both autoimmunity and inflammation through their ability to modulate the T regulatory cell population. In this communication, results demonstrate that γδ+ T cells directly kill $CD4^+$ $CD25^+$ T regulatory cells through CD1d expressed on a subpopulation of the regulatory cell population. Furthermore, the $CD1d^+$ T regulatory cells are shown to be more suppressive on a per cell basis than the $CD1d^-$ T regulatory cells.

Materials and methods

Mice

Male C57Bl/6 and B6·129P2-*Tcrd*^{tm1Mom}/J [γδ knockout (γδKO)] mice were purchased from Jackson Laboratories (Bar Harbor, ME). All mice were 5–7 weeks of age when infected. All of the experiments have been 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.[31]

Infection of mice

Mice were injected intraperitoneally (i.p.) with 10^2 plaque-forming units (PFU) of virus in 0·5 ml of phosphate-buffered saline (PBS). Animals were killed when mortibund or 7 days after infection.

Organ virus titres

Hearts were aseptically removed from the animals, weighed, and homogenized in RPMI-1640 medium containing 5% fetal bovine serum (FBS), L-glutamine, streptomycin and penicillin. Cellular debris was removed by centrifugation at 300 ***g*** for 10 min. Supernatants were diluted serially using 10-fold dilutions and titred on HeLa cell monolayers using the plaque-forming assay.[32]

Histology

Tissue was fixed in 10% buffered formalin for 48 hr, paraffin embedded, sectioned and stained with haematoxylin and eosin. Image analysis of cardiac inflammation was performed as described previously.[31]

Isolation of lymphocytes

Spleens were removed and pressed through fine-mesh screens. Inflammatory cells in the heart were isolated by perfusing individual hearts with PBS, mincing finely, and digesting the hearts with 0·4% collagenase II (Sigma Chemical Co, St Louis, MO) and 0·25% pancreatin (Sigma). Lymphoid cells were isolated by centrifugation of cell suspensions on Histopaque (Sigma). Purified Vγ4+ T cells were obtained by sterile sorting. Lymphoid cells from the heart were labelled with phycoerythrin (PE)-anti-δ T-cell receptor antibody (clone GL3) and fluorescein isothiocyanate (FITC)-anti-Vγ4 antibody (clone UC3-10A6) and then sorted using a BD FACS Aria (BD Biosciences, San Jose, CA) at the Flow Cytometry Facility at the University of Vermont.

Flow cytometry and intracellular cytokine staining

Details of the intracellular cytokine staining have been published previously.[33] Spleen cells (10^5) were cultured for 4 hr in RPMI-1640 medium containing 10% fetal bovine serum, antibiotics, 10 μg/ml of brefeldin A (BFA; Sigma), 50 ng/ml phorbol myristate acetate (PMA; Sigma), and 500 ng/ml ionomycin (Sigma). The cells were washed in PBS-1% bovine serum albumin (BSA; Sigma) containing BFA, and incubated on ice for 30 min in PBS-BSA-BFA containing a 1 : 100 dilution of Fc Block, and peridinin chlorophyll protein (PerCP)-Cy5.5 anti-CD4 (clone GK1.5) or PerCP-Cy5.5 rat immunoglobulin G2b (IgG2b) (clone A95-1). The cells were washed once with PBS-BSA-BFA, fixed in 2% paraformaldehyde for 10 min, and then resuspended in PBS-BSA containing 0·5% saponin, Fc Block and 1 : 100 dilutions of PE-anti-IFN-γ (clone-XMG1.2) or PE-rat IgG1 (clone R3-34) and incubated for 30 min on ice. All antibodies were from BD Biosciences/Pharmingen (Franklin Lakes, NJ). FoxP3 labelling was performed using the eBioscience kit from BD Biosciences (Franklin Lakes, NJ) according to the manufacturer's directions. Cells were labelled with Alexa647 anti-CD4, PerCP-Cy5.5 anti-CD25 (clone PC61) and FITC-anti-CD1d (clone 1B1) in PBS-1%BSA containing Fc Block, washed, fixed and permeabilized, and then incubated with PE-anti-FoxP3 and Fc Block overnight at 4°. The cells were washed once in PBS-BSA-saponin and once in PBS-BSA, and then resuspended in 2% paraformaldehyde. Cells were analysed using a BD Biosciences LSR II flow cytometer with a single excitation wavelength (488 nm) and band filters for PerCP-Cy5.5 (695/40 nm), FITC (525 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.

Adoptive transfer of $CD4^+$ $CD25^+$ cells

Purified $CD4^+$ $CD25^+$ cells were isolated from spleens of mice infected 7 days earlier using the Dynabeads FlowComp Mouse $CD4^+$ $CD25^+$ Treg kit (Invitrogen, Carlsbad,

CA) according to the manufacturer's directions. The cells were washed and labelled with FITC-anti-CD1d antibody and then sorted into $CD4^+$ $CD25^+$ $CD1d^+$ and $CD4^+$ $CD25^+$ $CD1d^-$ populations. Cell concentrations indicated in the text were injected intravenously (i.v.) in 0·2 ml of PBS into the tail veins of recipients 1 day after infection with virus. To confirm that virus was not transferred with the cells, 10^6 $CD4^+$ $CD25^+$ cells from infected mice were homogenized and titred using the plaque-forming assay on HeLa cells and showed no virus.

Cytotoxicity assay

Purified $CD4^+$ $CD25^+$ cells were isolated as described above and incubated with 100 μCi ^{51}Cr ($Na^{51}CrSO_4$; New England Nuclear, Wellesley, MA) in 1 ml of RPMI-1640–5% FBS for 90 min at 37°. The cells were washed and added (0·01 ml; 5×10^5 cells) to the wells of 96-well round-bottom tissue culture plates (Fischer Scientific, Pittsburg, PA). Vγ4+ cells were isolated by sterile sorting from hearts of infected C57Bl/6 mice as described above and co-cultured (0·1 ml; 5×10^4 cells) with the $CD4^+$ $CD25^+$ cells. Some cultures contained 50 μM zVAD-fmk (Promega Inc, Madison, WI) and others contained 1 μg/ml anti-CD1d (clone 1B1; BD Biosciences). Cultures were run in quadruplicate. After 12 hr of incubation at 37°, 100 μl of supernatant was removed and ^{51}Cr release [counts per minute (c.p.m.)] determined using a gamma counter. Medium control cultures were ^{51}Cr-labelled $CD4^+$ $CD25^+$ cells cultured without Vγ4+ effector cells. Maximum ^{51}Cr release was determined by diluting the content of select wells 1 : 1 with HCl (3·0 N) and evaluating radioactivity in the supernatant. The per cent specific lysis was calculated as follows:

$$[(\text{experimental c.p.m.} - \text{spontaneous release c.p.m.})/(\text{maximum release c.p.m.} - \text{spontaneous release c.p.m.})] \times 100.$$

Statistics

Data were analysed for skewness and kurtosis using the SPSS for Windows program (version 11.0; SPSS, Inc., Chicago, IL), which showed that variance was not normally distributed for several groups. Statistical analysis was performed using the non-parametric Mann–Whitney test in SPSS for Windows. The threshold for significance was 0·05 or better.

Results

Mice lacking $\gamma\delta$+ T cells are resistant to CVB3-induced myocarditis

C57Bl/6 and $\gamma\delta$KO mice were either infected with 10^2 PFU CVB3 or uninfected and evaluated for myocarditis and cardiac virus titre 7 days later (Figs 1 and 2). As expected, no inflammation or virus was observed in the hearts of uninfected mice. Infected C57Bl/6 animals developed significant cardiac inflammation (Fig. 2, arrows) and had high virus titres in the heart. In contrast, infection of $\gamma\delta$KO animals resulted in significantly reduced myocarditis but higher cardiac virus titres than in infected C57Bl/6 animals. Next, spleen lymphocytes were evaluated by flow cytometry for T helper type 1 (Th1) ($CD4^+$ IFN-γ^+) and T regulatory ($CD4^+$ $FoxP3^+$) cells. Infected C57Bl/6 mice had significantly greater numbers of Th1 cells than uninfected C57Bl/6, uninfected $\gamma\delta$KO or infected $\gamma\delta$KO mice (all $P < 0{\cdot}01$). Numbers of $CD4^+$ $FoxP3^+$ cells were significantly higher in infected $\gamma\delta$KO mice than in uninfected C57Bl/6, $\gamma\delta$KO or infected C57Bl/6 mice (all $P < 0{\cdot}05$) (Fig. 3). No differences were observed in numbers of $CD4^+$ $FoxP3^+$ cells between uninfected C57Bl/6 and $\gamma\delta$KO animals. These results indicate that the presence of $\gamma\delta$+ T cells suppresses the T regulatory cell response.

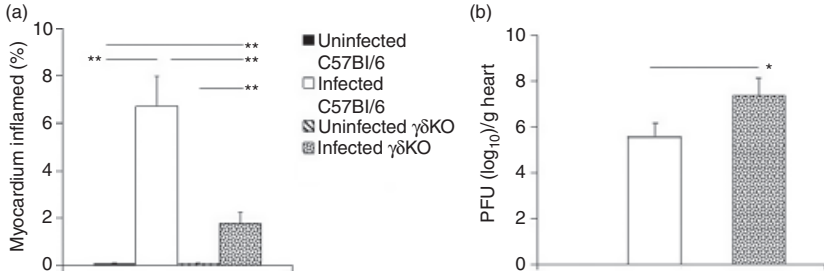

Figure 1. C57Bl/6 mice lacking $\gamma\delta$+ T cells are resistant to coxsackievirus B3 (CVB3)-induced myocarditis. C57Bl/6 and $\gamma\delta$ knockout ($\gamma\delta$KO) male mice were either infected intraperitoneally (i.p.) with 10^2 plaque-forming units (PFU) of CVB3 or uninfected, and then killed 7 days later for evaluation of (a) the percentage of myocardium inflamed, using image analysis, and (b) cardiac virus titres, using the plaque-forming assay. Results are given as mean ± standard error of the mean (SEM) for four to six mice per group. * and **, significantly different at $P < 0.05$ and $P < 0.01$, respectively.

T regulatory cells from γδKO mice express increased CD1d

Next, the $CD4^+$ $FoxP3^+$ subset was evaluated for expression of CD1d (Fig. 4). Although few in number, the $CD4^+$ $FoxP3^+$ cells derived from uninfected C57Bl/6 mice expressed no detectable CD1d ($0{\cdot}3 \pm 0{\cdot}05 \times 10^6$ $CD4^+$ $FoxP3^+$ cells/spleen). Infection of C57Bl/6 mice induced low CD1d expression on a small number of cells ($0{\cdot}84 \pm 0{\cdot}15 \times 10^6$ $CD4^+$ $FoxP3^+$ cells/spleen; $0{\cdot}07 \pm 0{\cdot}01 \times 10^6$ $CD4^+$ $FoxP3^+$ $CD1d^+$ cells/spleen). Infection of γδKO mice resulted in a substantially greater number of $CD1d^+$ T regulatory cells ($2{\cdot}1 \pm 0{\cdot}25 \times 10^6$ $CD4^+$ $FoxP3^+$ cells/spleen; $1{\cdot}85 \pm 0{\cdot}46 \times 10^6$ $CD4^+$ $FoxP3^+$ $CD1d^+$ cells/spleen). As the T regulatory cells from infected γδKO mice show increased CD1d expression, one question was whether the γδ+ cells activated in infected C57Bl/6 mice eliminate the $CD1d^+$ population. In the absence of γδ+ cells, the $CD1d^+$ population might then survive. To address this question, $CD4^+$ $CD25^+$ cells were isolated from spleens of infected C57Bl/6 and γδKO mice, labelled with ^{51}Cr and co-cultured (5×10^5) with 5×10^4 purified Vγ4+ T cells derived from the hearts of infected C57Bl/6 donor mice. Figure 5 demonstrates the purity of the Vγ4+ cell population. Figure 6a shows that nearly all $CD4^+$ $CD25^+$ cells were $FoxP3^+$ and Fig. 6b shows that Vγ4+ T cells were highly cytolytic to $CD4^+$ $CD25^+$ cells from the γδKO mice but showed little cytolytic activity to $CD4^+$ $CD25^+$ cells from infected C57Bl/6 donors. Killing was attributable to apoptosis as $CD4^+$ $CD25^+$ cells cultured with 50 μM zVAD-fmk and Vγ4+ cells were not lysed. Killing also depended on CD1d expression as incubation of $CD4^+$ $CD25^+$ cells with 10 μg/ml monoclonal anti-CD1d antibody suppressed cytolysis.

Increased suppressive activity of $CD1d^+$ T regulatory cells

The relative suppressive activity of the $CD1d^+$ and $CD1d^-$ T regulatory cells was determined by purifying the $CD4^+$ $CD25^+$ $CD1d^+$ and $CD4^+$ $CD25^+$ $CD1d^-$ cell populations from spleens of infected γδKO mice and adoptively transferring 0, 10^3 or 10^5 cells i.v. into C57Bl/6 mice on day +1 relative to infection (Fig. 7). Hearts were evaluated 7 days after infection for myocarditis. When 10^3 cells were transferred, the $CD4^+$ $CD25^+$ $CD1d^+$ cell population produced significantly more suppression while the $CD4^+$ $CD25^+$ $CD1d^-$ population was ineffective. When 10^5 cells were transferred, both $CD4^+$ $CD25^+$ $CD1d^+$ and $CD4^+$ $CD25^+$ $CD1d^-$ cells resulted in less myocarditis than C57Bl/6 mice not given T regulatory cells, although, again, the $CD1d^+$ T regulatory cells were more protective than the $CD1d^-$ cells ($P < 0{\cdot}05$).

Vγ4+ cells promote myocarditis susceptibility

The experiments detailed above indicate that Vγ4+ cells selectively kill $CD4^+$ $CD25^+$ T regulatory cells *in vitro*. To confirm that this population restores myocarditis susceptibility in γδKO mice, 10^5 purified Vγ4+ and Vγ4− cells

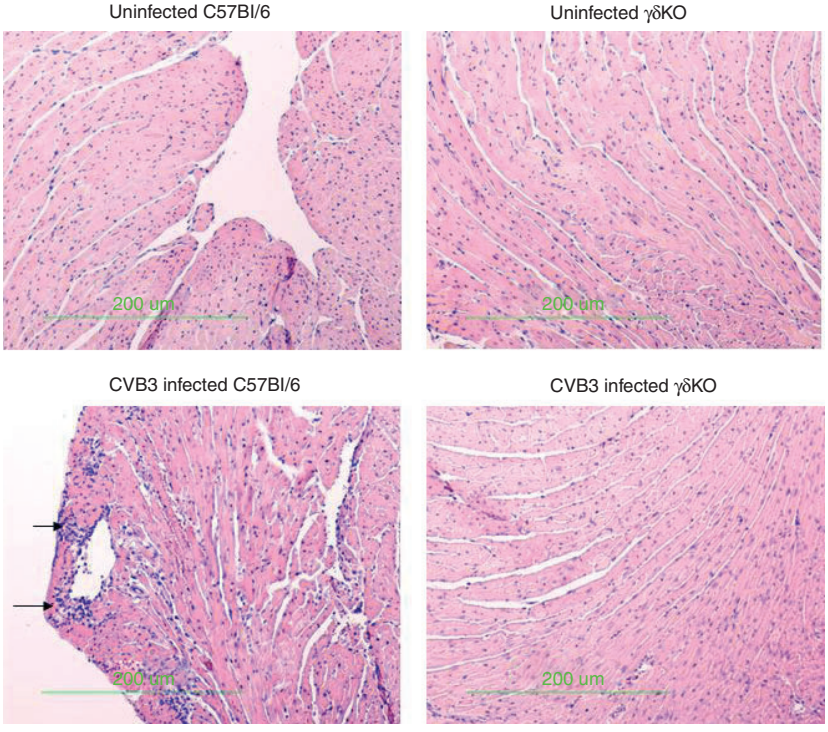

Figure 2. Representative histology of hearts described in Fig. 1. Hearts were fixed in formalin, sectioned and stained with haematoxylin and eosin. Inflammation is indicated by the arrows.

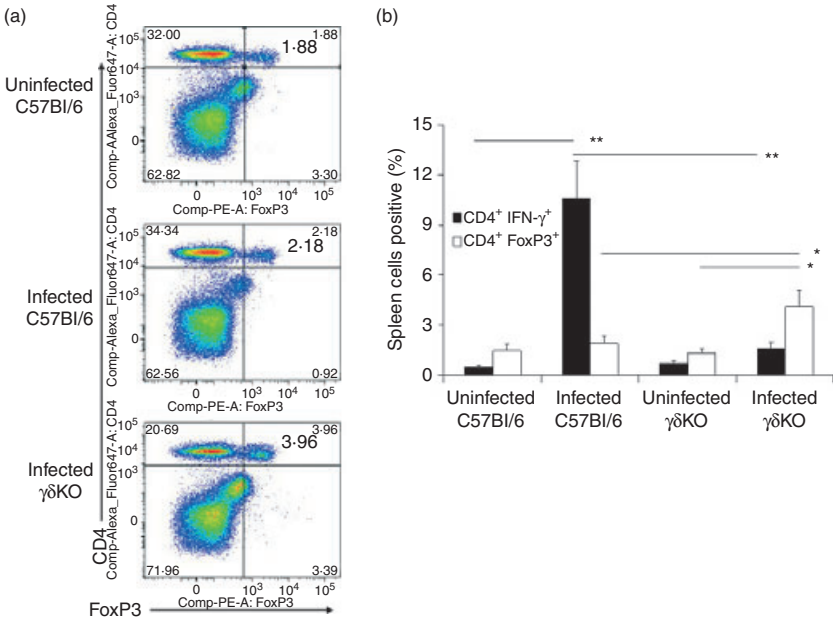

Figure 3. $CD4^+$ IFN-γ^+ and $CD4^+$ $FoxP3^+$ cells in uninfected and infected C57Bl/6 and $\gamma\delta$ knockout ($\gamma\delta$KO) mice. Spleen lymphocytes were isolated from individual mice described in Fig. 1. Cells (1×10^6) were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin in the presence of brefeldin A for 4 hr, labelled with antibody to CD4, fixed with paraformaldehyde, permeabilized and labelled with antibody to interferon (IFN)-γ. An additional 10^6 cells were labelled with antibody to CD4 and CD1d, fixed, permeabilized and labelled with antibody to FoxP3. (a) Representative flow diagrams of cells labelled with anti-CD4 and anti-FoxP3. (b) Mean per cent positive splenocytes ± standard error of the mean (SEM) for four to six mice per group. * and **, significantly different at $P < 0.05$ and $P < 0.01$, respectively comp, compensated value; PE, phycoerythrin.

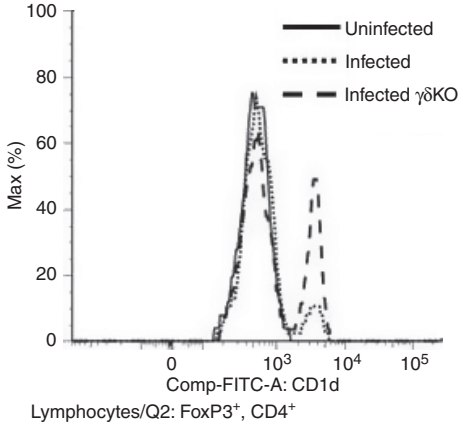

Figure 4. $CD4^+$ $FoxP3^+$ cells from infected $\gamma\delta$ knockout ($\gamma\delta$KO) mice show increased expression of CD1d. Gating on the $CD4^+$ $FoxP3^+$ cells (Lymphocytes/Q2, upper right quadrant of histograms in Fig. 3a), cells were evaluated for CD1d expression comp, compensated value; FITC, fluorescein isothiocyanate.

isolated from hearts of infected C57Bl/6 donor mice were injected i.v. into $\gamma\delta$KO mice infected with 10^2 PFU CVB3 on the same day as infection (Fig. 8). Hearts were evaluated 7 days after infection for myocarditis and spleens were evaluated for $CD4^+$ $CD25^+$ $FoxP3^+$ cells. Only the Vγ4+ cells promoted myocarditis in recipients and suppressed the T regulatory cell response. The Vγ4− population was ineffective in modulating myocarditis.

Discussion

Several publications have reported an inverse relationship between activated $\gamma\delta$+ T cells and tolerance or immunosuppression.[30,34–38] However, the mechanisms by which $\gamma\delta$+ T cells abrogate immunosuppression have not been fully elucidated. Several distinct mechanisms have been reported. Reports indicate that $\gamma\delta$+ T cells inhibit IL-10 expression by a population of $FoxP3^-$ $CD4^+$ T cells;[37] and that $\gamma\delta$+ T cells, which produce IL-10, are directly immu-

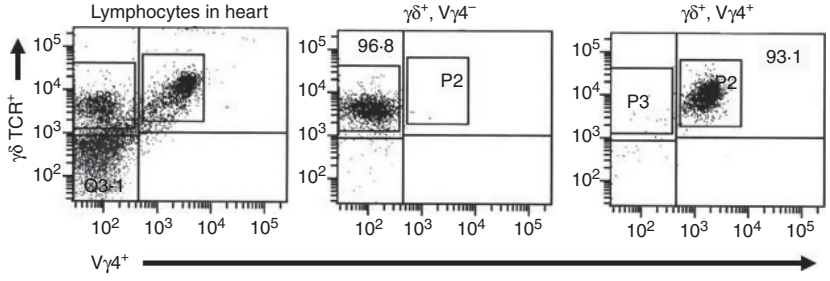

Figure 5. Isolation of purified Vγ4+ cells by sterile sorting. C57Bl/6 mice were infected with 10^2 plaque-forming units (PFU) of coxsackievirus B3 (CVB3) and killed 7 days later. Hearts were aseptically removed and infiltrating lymphoid cells were isolated as described in the Materials and methods section. The cells were labelled with antibody to the $\gamma\delta$ T-cell receptor (TCR) and Vγ4 and sorted into the $\gamma\delta$+ Vγ4− and $\gamma\delta$+ Vγ4+ cell populations. The purity of the isolated cells is indicated in the relevant quadrant.

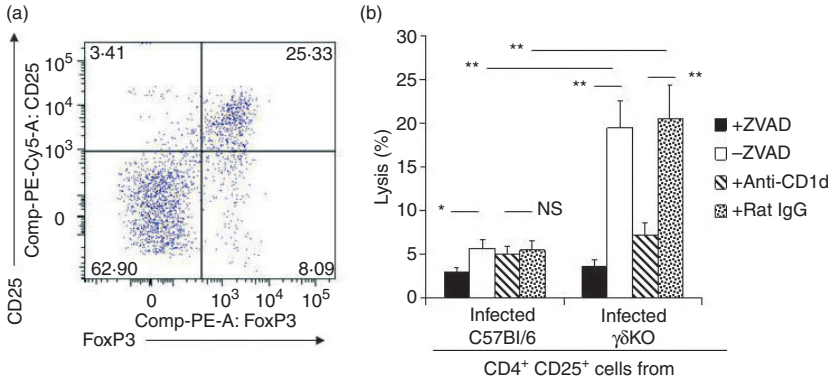

Figure 6. Vγ4+ cells lyse $CD4^+$ $CD25^+$ cells through caspase-dependent and CD1d-dependent mechanisms. (a) Spleen lymphocytes from an infected C57Bl/6 mouse 7 days after infection were labelled with antibody to CD4 and CD25, and then fixed, permeabilized and labelled with antibody to FoxP3. Gating on the $CD4^+$ cells by flow cytometry, expression of CD25 and FoxP3 was determined. (b) $CD4^+$ $CD25^+$ cells were isolated using negative selection as described in the Materials and methods section and resulted in > 94% pure $CD4^+$ $CD25^+$ cells. $CD4^+$ $CD25^+$ cells were labelled with ^{51}Cr and then 5×10^5 cells were co-cultured with 5×10^4 Vγ4+ cells for 12 hr in medium alone or medium containing 50 μM zVAD-fmk (ZVAD) or 1 μg/ml anti-CD1d. Controls were $CD4^+$ $CD25^+$ cells cultured in medium without Vγ4+ cells. Results represent the mean percentage lysis ± the standard error of the mean (SEM) of four replicate cultures per group. * and **, significantly different at $P < 0.05$ and $P < 0.01$, respectively comp, compensated value; IgG, immunoglobulin G; PE, phycoerythrin; NS, not significant.

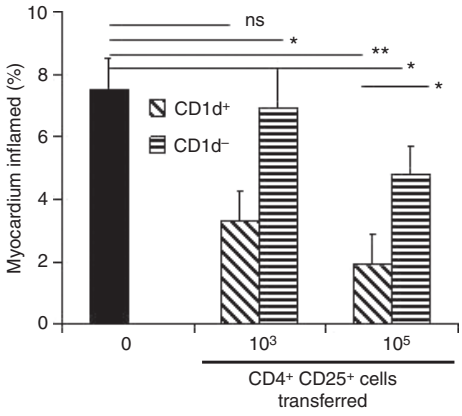

Figure 7. $CD1d^+$ T regulatory cells are more suppressive for myocarditis than $CD1d^-$ T regulatory cells. $CD4^+$ $CD25^+$ cells were isolated from spleens of γδ knockout (γδKO) mice 7 days after infection with 10^2 plaque-forming units (PFU) of coxsackievirus B3 (CVB3) using the Dynabeads FlowComp Mouse $CD4^+$ $CD25^+$ Treg kit, followed by labelling of the $CD4^+$ $CD25^+$ cells with anti-CD1d antibody. The cells were washed and sorted into $CD4^+$ $CD25^+$ $CD1d^+$ and $CD4^+$ $CD25^+$ $CD1d^-$ populations by sterile sorting. The purity of the $CD4^+$ $CD25^+$ cell population was 93.1%. C57Bl/5 mice were infected with 10^2 PFU of CVB3 and 1 day later were injected with 0.2 ml of phosphate-buffered saline (PBS) alone (0 $CD4^+$ $CD25^+$ cells) or 10^3 and 10^5 $CD4^+$ $CD25^+$ $CD1d^+$ or $CD4^+$ $CD25^+$ $CD1d^-$ cells intravenously (i.v.) through the tail vein. * and **, significantly different at $P < 0.05$ and $P < 0.01$, respectively.

nosuppressive.[39] Additionally, γδ+ T cells can interact with dendritic cells enhancing maturation and antigen presentation.[40] As immature dendritic cells may be important for $CD4^+$ $CD25^+$ $FoxP3^+$ T regulatory cell activation,[41] the ability of γδ+ T cells to induce dendritic cell maturation may indirectly lead to a reduced T regulatory cell response. This laboratory previously reported that depletion of γδ+ T cells in CVB3-infected mice results in increased $CD4^+$ $CD25^+$ $FoxP3^+$ cells *in vivo* and suppression of virus-induced myocarditis in mice.[30] We had previously shown that the specific γδ+ T-cell subpopulation that promotes CVB3-induced myocarditis, Vγ4+, recognizes CD1d,[26] a non-classical MHC class 1-like molecule associated with innate immunity.[15,21] In this communication, we have demonstrated not only that infection of γδKO mice resulted in an increased number of $CD4^+$ $CD25^+$ $FoxP3^+$ T regulatory cells, but that a subpopulation of the T regulatory cells showed increased CD1d expression. Few or no $CD1d^+$ $FoxP3^+$ cells were observed in infected wild-type (γδ+ cell-containing) or uninfected mice. Most importantly, activated Vγ4+ cells isolated from the hearts of infected wild-type mice were highly cytolytic to the T regulatory cells derived from infected γδKO donors and this cytotoxicity was inhibited both by anti-CD1d monoclonal antibody and zVAD-fmk, indicating that γδ+ cells kill T regulatory cells through recognition of CD1d and caspase-dependent mechanisms. The activated γδ+ cells showed minimal killing of T regulatory cells isolated from infected wild-type C57Bl/6 mice. This is an expected result as the $CD4^+$ $CD25^+$ cells from infected C57Bl/6 mice expressed minimal CD1d.

The next question was whether there was a significant functional difference between $CD1d^+$ and $CD1d^-$ T regulatory cells. When these two populations were isolated and transferred into infected C57Bl/6 mice (Fig. 7), the $CD1d^+$ T regulatory cells were consistently more suppressive than the $CD1d^-$ population whether 10^3 or 10^5 cells

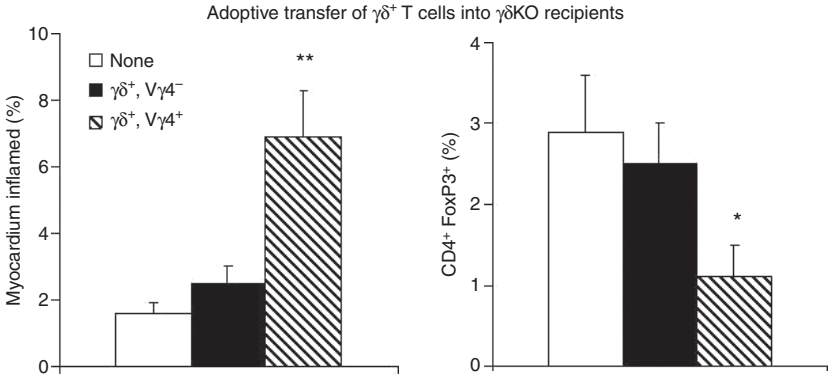

Figure 8. Vγ4+ cells promote myocarditis and suppress the T regulatory cell response. $\gamma\delta$+ Vγ4+ and $\gamma\delta$+ Vγ4− cells were isolated by sterile sorting (see Fig. 5) from hearts of C57Bl/6 mice infected with 10^2 plaque-forming units (PFU) of coxsackievirus B3 (CVB3) 7 days earlier. 10^5 of the purified cells were injected intravenously (i.v.) through the tail vein into $\gamma\delta$ knockout ($\gamma\delta$KO) recipient mice infected with 10^2 PFU CVB3 on the same day as the lymphocyte transfer. Recipients were killed 7 days after infection and evaluated for myocarditis (a). Spleen lymphocytes were isolated and evaluated for $CD4^+$ $CD25^+$ $FoxP3^+$ T regulatory cells (b). Results are mean ± the standard error of the mean (SEM) for five mice per group. * and **, significantly different at $P < 0.05$ and $P < 0.01$, respectively.

were transferred. Thus, the $CD1d^+$ T regulatory cell population is more effective in abrogating pro-inflammatory responses than the $CD1d^-$ population. One question is whether the expression of CD1d on the T regulatory cells is simply an indication of the activation state of the T regulatory cells or whether the $CD1d^+$ population represents a distinct suppressor cell subpopulation. Currently, this question cannot be conclusively answered. However, activated $FoxP3^+$ T regulatory cells express the early activation marker CD69,[42] and there was a significant difference in $CD69^+$ $CD4^+$ $CD25^+$ cells in the $CD1d^+$ population (63% of $CD1d^+$ $CD4^+$ $CD25^+$ cells) compared with the $CD1d^-$ population (18% of $CD1d^-$ $CD4^+$ $CD25^+$ cells). This strongly implies that a higher proportion of recently activated T regulatory cells are $CD1d^+$ while quiescent T regulatory cells are preferentially $CD1d^-$. This would also suggest that $\gamma\delta$+ cells may be more effective in eliminating recently activated T regulatory cells while leaving non-activated T regulatory cells alone.

Vγ4+ cells comprise a substantial proportion of the lymphoid cells infiltrating the heart during CVB3-induced myocarditis. In most infected mice developing myocarditis, approximately 30–50% of lymphoid cells were $\gamma\delta$+ and approximately 60–80% of the $\gamma\delta$+ cells were Vγ4+ cells (Fig. 5). Both Vγ4+ and Vγ4− cells were isolated from the heart (Fig. 5) and adoptively transferred into infected $\gamma\delta$KO recipients (Fig. 8). While the Vγ4+ $\gamma\delta$+ cells were highly effective in promoting myocarditis susceptibility and elimination of T regulatory cells, the Vγ4− $\gamma\delta$+ population was incapable of inhibiting the T regulatory cell response or restoring myocarditis susceptibility. The Vγ4+ and Vγ4− subpopulations in the heart expressed equivalent levels of CD69 (72% and 68%, respectively), indicating equivalent levels of activation. Thus, the ability of the Vγ4+ cells to selectively abrogate the T regulatory cell response is not a consequence of the activation state of these innate effectors but represents a distinct functional characteristic of the subpopulation. As previous studies have shown that only the Vγ4+ population recognizes CD1d,[43] the selective ability of the Vγ4+ cells to abrogate immunosuppression corresponds to the predominant role of $CD1d^+$ T regulatory cells in preventing myocarditis.

This is not the first report implying a role for CD1d in T regulatory cell responses. Previous studies have shown that T regulatory cells are not generated in $CD1d^{-/-}$ mice.[24] In these reports, the CD1d-restricted NKT cell promoted T regulatory cell generation through the production of high levels of transforming growth factor (TGF)-β and IL-10.[24,44,45] One question has always been why both iNKT and $\gamma\delta$+ cells recognize CD1d in innate immunity. While it is possible that iNKT and $\gamma\delta$+ cells have redundant functions, it would be more reasonable if each innate effector had a separate contribution. The data presented in this communication suggest that iNKT and $\gamma\delta$+ cells balance each other, with the former cell promoting and the latter abrogating immunoregulation. What is not clear is how the balance between iNKT and $\gamma\delta$+ cells is maintained; that is, how in certain circumstances T regulatory cells increase in number, presumably through a dominance of iNKT cells, while in other circumstances inflammation dominates, presumably through a dominance of $\gamma\delta$+ cells. One possibility is that these two effectors act at different levels of the T regulatory cell response. iNKT cells probably act via cytokine-mediated alterations in dendritic cells leading to enhanced T regulatory cell generation.[46] In contrast, $\gamma\delta$+ cells in the CVB3 model of myocarditis apparently have less effect on the generation of T regulatory cells than on the survival of T regulatory cells once generated.

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