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Innate Immunity to Viruses: Control of Vaccinia Virus Infection by γδ T Cells

Liisa K. Selin, Paul A. Santolucito, Amelia K. Pinto, Eva Szomolanyi-Tsuda, and Raymond M. Welsh

The existence of γδ T cells has been known for over 15 years, but their significance in innate immunity to virus infections has not been determined. We show here that γδ T cells are well suited to provide a rapid response to virus infection and demonstrate their role in innate resistance to vaccinia virus (VV) infection in both normal C57BL/6 and β TCR knockout (KO) mice. VV-infected mice deficient in γδ T cells had significantly higher VV titers early postinfection (PI) and increased mortality when compared with control mice. There was a rapid and profound VV-induced increase in IFN-γ-producing γδ T cells in the peritoneal cavity and spleen of VV-infected mice beginning as early as day 2 PI. This rapid response occurred in the absence of priming, as there was constitutively a significant frequency of VV-specific γδ T cells in the spleen in uninfected β TCR KO mice, as demonstrated by limiting dilution assay. Also, like NK cells, another mediator of innate immunity to viruses, γδ T cells in uninfected β TCR KO mice expressed constitutive cytolytic activity. This cytotoxicity was enhanced and included a broader range of targets after VV infection. VV-infected β TCR KO mice cleared most of the virus by day 8 PI, the peak of the γδ T cell response, but thereafter the γδ T cell number declined and the virus recrudesced. Thus, γδ T cells can be mediators of innate immunity to viruses, having a significant impact on virus replication early in infection in the presence or absence of the adaptive immune response. The Journal of Immunology, 2001, 166: 6784–6794.

The host response to viral and bacterial infections is biphasic, with innate effectors such as IFN, NK cells, and macrophages (1) being critical in the early phase, and with slower-developing adaptive Ag-specific αβ T and B cell responses often being essential for clearance of the pathogen and establishment of immunity. Although the existence of γδ T cells has been known for over 15 years, their significance in this paradigm of protective immunity is still being determined. Some work has suggested that γδ T cells may play a role in the control of parasitic infections such as malaria and Eimeria veriformis and bacterial infections such as Listeria monocytogenes and Klebsiella pneumonia (2–11), but information about these cells in viral infections remains rudimentary (12–19). Increases in γδ T cell number appear in the peripheral blood of individuals infected with EBV and HIV infections (13, 14), and some human γδ T cell clones preferentially lyse targets infected with vaccinia virus (VV) (15) or HSV (16). In localized ocular HSV-1 infection of mice, the absence of γδ T cells have been correlated with enhanced neurological disease late in infection; however, the role for γδ T cells in controlling HSV titers is hidden, as the antiviral function of γδ T cells can be substituted for and masked by αβ T cells (10, 16, 17); both αβ TCR knockout (KO) and γδ T CR KO mice clear HSV, but this infection is fatal in β + δ TCR KO mice. These observations are in contrast with many other viruses such as VV, lymphocytic choriomeningitis virus (LCMV), and influenza, which the host is unable to clear in the absence of αβ T cells. Infections with the parasite E. veriformis also demonstrate a γδ T cell effector function that is masked by αβ T cells, as β + δ TCR KO mice are more susceptible to primary infection with this pathogen than β TCR KO mice, but there is no difference in clearance of this parasite in γδ-deficient mice when compared with normal controls (7, 10).

γδ T cells accumulate in the lung late after influenza infection of mice, but their function in murine influenza is unknown. It has been suggested that they may target heat-shock protein-displaying macrophages and play a housekeeping role during the resolution phase of the inflammatory response (18, 20, 21). γδ T cells may also have a role in the pathogenesis of various autoimmune diseases and diseases of unknown etiology, as elevated levels of γδ T cells have been reported in rheumatoid arthritis (22), multiple sclerosis (23, 24), pulmonary sarcoidosis (25), inflammatory bowel disease (26), and polymyositis (27). Some of these diseases might be precipitated or exacerbated by viral infections, and recent evidence has indicated that γδ T cells may contribute to myocarditis in mice infected with encephalomyocarditis virus (19).

Although we previously have predicted, based on a few preliminary observations, that γδ T cells may participate in protection against VV infection (28), in no viral infection to date has a clear role for γδ T cells in innate immunity to virus infection been definitively documented. Here we report that γδ T cells not only were constitutively cytolytic, but VV-specific γδ T cells were present at significant levels in the unprimed host, rapidly expanding in number, secreting IFN-γ, and having enhanced cytotoxic activity on VV infection. All of these features make them well fitted for providing early resistance to VV infection.

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3 Abbreviations used in this paper: VV, vaccinia virus; KO, knockout; LCMV, lymphocytic choriomeningitis virus; PEC, peritoneal exudate cell; LDA, limiting dilution assay; pCTL, precursor CTL; PI, postinfection.

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Materials and Methods

**Mice**

Male C57BL/6 (H-2b), δ TCR KO, β TCR KO, β + δ TCR KO, and SCID mice all on the C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME) and used at 2–26 mo of age. The 129 × C57BL/6 δ TCR KO and +/- controls (6) were donated to us by P. Mombaerts and S. Tonegawa (Whitehead Institute, Boston, MA).

**Viruses**

The WR strain of VV was propagated in vero cells obtained from the American Type Culture Collection (ATCC, Manassas, VA; Ref. 29). LCMV, cells was propagated in BHK21 baby hamster kidney cells (29). These viruses were purified over sucrose gradients and diluted in PBS. Purified viruses were used to prevent activation of γδ T cells by tissue culture contaminants. Control uninfected mice were either left uninjected or injected with mock-infected culture supernatant sedimented on tissue culture contaminants. Control uninfected mice were either left uninfected or injected with mock-infected culture supernatant sedimented on sucrose gradient. The control mice were always age-matched to the experimental group to prevent under the same pathogen-free conditions as the experimental group for the identical time period. All mice used were healthy, with no evidence of any underlying disease. For acute virus infections, mice were inoculated i.p. with 4 × 10^3 PFU of VV or 1–2 × 10^4 PFU of VV.

**Cell lines**

KO (H-2b), a SV40-transformed kidney cell line derived from a C57BL/6 mouse (30) and provided to us by Sativar Tevethia (Pennsylvania State Medical Center, Hershey, PA), was propagated in DMEM (Life Technologies). NCTC929 cells (ATCC) are a variant of L cells derived from CHH mouse liver (H-2b). YAC-1 cells are a Moloney virus-transformed T lymphocyte line from strain A mice, classically used as an NK-sensitive target in cytotoxicity assays. KO and MC57G cells were infected with LCMV at a multiplicity of infection of 0.01–0.02 PFU/cell and incubated for 2 days at 37°C. KO, MC57G and NCTC929 cells were infected with VV at a MOI of 4 for 4–5 h at 37°C. KO cells were infected with HSV at a multiplicity of infection of 4 and incubated overnight at 37°C. All cell lines were cultured in medium supplemented with 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, 2 mM l-glutamine, 10 mM HEPES, and 10% heat-inactivated (56°C, 30 min) FBS (Sigma, St. Louis, MO).

**FACS staining**

Freshly isolated splenocytes, peritoneal exudate cells (PEC), and T cells derived from limiting dilution assays (LDA) were stained for fluorescence, as described previously (29). The following Abs obtained from BD Pharmingen (San Diego, CA) were used: anti-γδ TCR-TDI clone (biotinylated) (clone GL3), anti-β TCR bioin (clone H57-597), anti-Vγ2 TDI clone (clone UC3-104A6), anti-Vδ4 bioin (clone IT10) FITC-conjugated (clone GL2), anti-CD4 FITC-conjugated (clone H129.19), anti-CD8a FITC-conjugated (clone 53-6.7), and anti-γδ TDI FITC-conjugated (clone 53-2.1). Secondary stains used included PE- and FITC-conjugated streptavidin (Becton Dickinson, San Jose, CA) and tri-color-conjugated streptavidin (Calbio, Burlingame, CA).

**Depletion of NK or γδ T cells in vivo**

A carefully titrated dose of antiserum to asialo Gmp (5 μl; Wako Chemical, Dallas, TX) that depleted NK cells but not γδ T cells was injected i.p. with 50 ng/ml PMA (Sigma) and 500 ng/ml ionomycin (Sigma) for 4 h at 37°C or left untreated as described previously (37). The unstimulated cells were kept at room temperature during processing, as this was found to help retain spontaneous IFN-γ production after removal from the host. The cells were stained with anti-NK1.1 biotin and tricolor-conjugated streptavidin and FITC-conjugated anti-CD3 and fixed with 2% paraformaldehyde. They were permeabilized with 0.5% saponin before adding PE-conjugated rat anti-mouse IFN-γ mAb (BD Pharmingen, San Diego, CA) or control PE-conjugated rat IgG1 isotype. They were analyzed on either a FACS 440 (Becton Dickinson) or FACSStarTM. The purity of these populations was >90%. These enriched γδ T cell populations then were used in cytotoxicity assays. The particular anti-CD3e (clone 145-2C11) used for sorting did not inhibit or enhance lysis of targets when directly placed in cytotoxicity assays with unsorted PEC lymphocyte populations from day 8 VV-infected mice or TCR KO mice. Similar experiments with anti-γδ TCR mAb resulted in enhancement of lysis of certain targets, including VV-infected MC57G, NCTC929, and VV-infected NCTC929. Therefore, anti-γδ TCR mAb could not be used for sorting experiments.

**Intracellular IFN-γ staining**

Splenocytes or PECs at 2 × 10^6 per tube were stimulated with 50 ng/ml PMA (Sigma) and 500 ng/ml ionomycin (Sigma) for 4 h at 37°C or left untreated as described previously (37). The unstimulated cells were kept at room temperature during processing, as this was found to help retain spontaneous IFN-γ production after removal from the host. The cells were stained with anti-NK1.1 biotin and tricolor-conjugated streptavidin and FITC-conjugated anti-CD3 and fixed with 2% paraformaldehyde. They then were permeabilized with 0.5% saponin before adding PE-conjugated rat anti-mouse IFN-γ mAb (BD Pharmingen, San Diego, CA) or control PE-conjugated rat IgG1 isotype. They were analyzed on either a FACS 440 (Becton Dickinson) or FACSStarTM (Becton Dickinson).

**Virus titration**

The virus load in organs was determined by plaque assays on vero cells with a 10% tissue homogenate taken from individual mice. Results were expressed as the geometric mean titers, i.e., the arithmetic averages of the log_{10} titers for four or five animals, plus or minus the SEM. Titers reported are log_{10} PFU per whole spleen, liver, or both abdominal fat pads.

**Results**

Mice lacking γδ T cells are more susceptible to VV-induced mortality

The importance of γδ T cells in protection against VV infection was examined in γδ T cell-deficient mice. At a high dose of VV (5 × 10^5 PFU), the δ TCR KO mice began to die much more rapidly (day 3) than normal C57BL/6 mice (day 7; Fig. 1A) and were all dead by day 8. In contrast, 43% of the normal C57BL/6 mice survived the VV infection. With the usual sublethal dose of
Increased VV-induced mortality in mice lacking γδ T cells. A. C57BL/6 and δ TCR KO mice were infected with a high dose of VV (5 × 10⁶ PFU), and their time of death after VV infection was recorded. The difference in mean time to death between the two groups is significant (Student’s t test, p < 0.05; n = 7). B, β + δ TCR KO and β TCR KO mice were infected with a sublethal dose of VV in normal mice (1 × 10⁶ PFU), and their time of death after VV infection was recorded. The difference in mean time to death between the three groups when compared with each other is significant (Student’s t test, p < 0.05; n = 5). These results are representative of two similar experiments.

VV (1 × 10⁶ PFU), both normal C57BL/6 and δ TCR KO mice survived. Studies comparing β TCR KO to β + δ TCR KO mice also suggested that γδ T cells played a role in decreasing mortality after VV infection (Fig. 1B). At the sublethal dose of VV in normal C57BL/6 mice (1 × 10⁶ PFU), only 20% of the β + δ TCR KO mice were still alive by 12 days after VV infection as compared with 80% of the β TCR KO mice. However, without the presence of αβ T cells, these mice all eventually died by 17 days after VV infection. SCID mice, which lack both γδ and αβ T cells as well as B cells, died more rapidly than the β + δ TCR KO mice after VV infection, suggesting that T cell-independent Abs produced by B cells may also play a significant role in mice that lack T cells, as has been shown previously with polyoma virus infections (38).

Enhanced replication of VV early after infection in the absence of γδ T cells

Table I shows that at day 3 PI there was a significant 2.5- to 3-fold higher VV titer in three organs (spleen, fat pads, and liver) of the γδ TCR KO mice in comparison to normal mice. This increased to a 10- to 79-fold difference at day 4 and a 5- to 20-fold difference at day 5. There was a 10- to 63-fold increase in VV titers 3 days PI in another γδ T cell-deficient strain of mouse, 129 x C57BL/6, when compared with a +/− (129 x C57BL/6) control. VV titers were elevated when γδ T cells were depleted by using anti-γδ TCR mAb in vivo in C57BL/6 mice. There was a 25-, 10-, and 6-fold higher VV titer in the spleen, fat pad, and liver, respectively, of the γδ T cell-depleted mice 4 days after VV infection. In contrast, it should be noted that the titers of LCMV in δ TCR KO mice on day 4 after LCMV infection were the same as in control C57BL/6 mice, suggesting that γδ T cells do not play an overt role in protection against LCMV infection.

Similar experiments demonstrated that on day 4 after VV infection the β + δ TCR KO mice had 25- and 8-fold higher VV titers and on day 8, 25- and 158-fold higher VV titers in the spleen and liver, respectively than the β TCR KO mice (Table I). The fat pads were not titrated at day 8, as the fat pads in the β + δ TCR KO mice had virtually disappeared, a phenomenon that we have observed previously with very high dose VV infections in normal mice.

Recruitment and activation of γδ T cells by VV infection in C57BL/6 mice

There was a moderate but significant increase in γδ T cells in the spleen of normal C57BL/6 mice starting 2 days after VV infection, with 80% of the γδ T cells having disappeared by day 4. In contrast, γδ T cells were still detectable in the spleen of TCR KO mice at day 4, but had virtually disappeared by day 8, a phenomenon that we have observed previously with very high dose VV infections in normal mice.

Table I. Enhanced vaccinia virus replication in the absence of γδ T cells

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Strain*</th>
<th>Days PI</th>
<th>Virus</th>
<th>Spleen (mean log₁₀ PFU/organ ± SEM)*</th>
<th>Fat pads</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C57BL/6</td>
<td>Day 3</td>
<td>VV</td>
<td>4.7 ± 0.2*</td>
<td>4.8 ± 0.2</td>
<td>4.6 ± 0.1†</td>
</tr>
<tr>
<td>2</td>
<td>δ TCR KO</td>
<td>Day 4</td>
<td>VV</td>
<td>4.9 ± 0.3†</td>
<td>3.9 ± 0.3†</td>
<td>3.3 ± 0.6†</td>
</tr>
<tr>
<td>3</td>
<td>C57BL/6</td>
<td>Day 5</td>
<td>VV</td>
<td>&lt;2.0 ± 0.0†</td>
<td>2.9 ± 0.4†</td>
<td>&lt;2.0 ± 0.0†</td>
</tr>
<tr>
<td>4</td>
<td>δ TCR +/− (129 × B6)</td>
<td>Day 3</td>
<td>VV</td>
<td>4.9 ± 0.2†</td>
<td>4.4 ± 0.15†</td>
<td>ND†</td>
</tr>
<tr>
<td>5</td>
<td>C57BL/6 + control IgG</td>
<td>Day 4</td>
<td>VV</td>
<td>4.9 ± 0.2†</td>
<td>3.5 ± 0.6†</td>
<td>&lt;2.3 ± 0.2†</td>
</tr>
<tr>
<td>6</td>
<td>β TCR KO</td>
<td>Day 4</td>
<td>VV</td>
<td>3.4 ± 0.4</td>
<td>4.5 ± 0.4</td>
<td>3.1 ± 0.3</td>
</tr>
<tr>
<td>7</td>
<td>β + δ TCR KO</td>
<td>Day 8</td>
<td>VV</td>
<td>3.1 ± 0.3</td>
<td>5.0 ± 0.1</td>
<td>3.1 ± 0.3</td>
</tr>
<tr>
<td>8</td>
<td>C57BL/6</td>
<td>Day 4</td>
<td>LCMV</td>
<td>5.6 ± 0.1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>9</td>
<td>δ TCR KO</td>
<td>Day 4</td>
<td>LCMV</td>
<td>5.4 ± 0.3</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Four to six mice used per group. δ TCR KO mice are on a C57BL/6 background except in Expt. 4, where they are on a 129 × C57BL/6 background. The +/− (129 × C57BL/6) mice are heterozygous for the δ TCR KO.

† VV and LCMV were titrated on vero cell monolayers using a 3-day and 5-day plaque assay, respectively.

§ Statistically significant difference between C57BL/6 and δ TCR KO mice (Student’s t test). *, p = 0.09; †, p < 0.05; ‡, p = 0.055; §, p = 0.07. Each experiment is representative of two or three similar experiments.

ND, Not done.
increasing from 2.1% before infection to 5.0% by 6 days PI (Fig. 2A, i). This represents a gradual increase in total γδ T cell number of 4-, 6.2-, and 9.5-fold at days 2, 4, and 6 PI, respectively (Fig. 2C, ii). In normal C57BL/6 mice, the percentage of γδ T cells in the peritoneal cavity, the initial site of virus replication, increased from 1.2% to 9.1% at 2 days PI, and remained still elevated at

**FIGURE 2.** A, Increased percentage of (i) γδ T cells and (ii) IFN-γ-secreting γδ T cells in the spleen of VV-infected C57BL/6 mice. Splenocytes from C57BL/6 mice that were infected with VV were harvested at various times after infection and directly stained with anti-γδ TCR mAb and anti-CD3 mAb. They also were assessed by gating on the γδ TCR+CD3+ cells after intracellular IFN-γ staining following PMA and ionomycin stimulation for IFN-γ production as described in Materials and Methods. B, Increased percentage of IFN-γ-secreting γδ T cells in the peritoneal cavity of VV-infected C57BL/6 mice. PECs from C57BL/6 mice infected with VV were harvested at various times after infection and directly stained with anti-γδ TCR mAb and anti-CD3 mAb. They also were assessed by gating on the γδ TCR+CD3+ cells after intracellular IFN-γ staining following (i) PMA and ionomycin stimulation or (ii) no stimulation, for IFN-γ production as described in Materials and Methods. C, Increase in the total number of γδ T cells and IFN-γ-secreting γδ T cells in the (i) peritoneal cavity and (ii) spleen of C57BL/6 mice after VV infection. C57BL/6 mice were infected with VV, and at various time points after infection, the total number of γδ T cells and PMA-induced IFN-γ-secreting γδ T cells was determined by FACS staining, as described in Materials and Methods. All the data in this figure are representative of three similar experiments where cells from three mice were pooled for each group.
7.2% and 4.9% at 4 and 6 days, respectively. Because the total number of leukocytes increased, this represents a dramatic 31-, 81-, and 63-fold increase in total γδ T cell number at days 2, 4, and 6 PI, respectively, compared with uninfected mice (Fig. 2Ci).

VV is known to be very sensitive to IFN-γ (39). Therefore, we examined whether γδ T cells were activated by VV infection to produce IFN-γ. In C57BL/6 mice, by day 2 of VV infection, 12% of the γδ T cells in the peritoneal cavity produced IFN-γ on PMA and ionomycin stimulation as compared with 1.0% in uninfected mice (Fig. 2Bi). This increased level of γδ T cells capable of producing IFN-γ persisted even at day 6 of infection. In fact, by day 2, there had been a 300-fold increase in the total number of γδ T cells producing IFN-γ after stimulation, and they continued to increase at day 4 and day 6 of VV infection (Fig. 2Ci). This treatment with PMA and ionomycin measures the potential of cells to make IFN-γ, but to determine whether there were signals in their environment to elicit IFN-γ, we tested cells in the absence of PMA and ionomycin and showed at 2 days PI that 5.6% of the peritoneal γδ T cells produced IFN-γ spontaneously (Fig. 2Bii). Comparable results were seen in the spleen after PMA and ionomycin stimulation, but with a more gradual increase in γδ T cells capable of producing IFN-γ (to 5% by day 6 (0.5% in uninfected mice) PI (Fig. 2Ai, ii and C, ii).

Recruitment and activation of γδ T cells by VV infection in β TCR KO mice

In the above experiments shown with C57BL/6 mice, we could not rule out that some IFN-γ-producing αβ T cells may have contaminated the γδ T cell population, especially at day 6, and confound our results. Because of this, and because β TCR KO mice demonstrated an ability to control VV better than β + δ TCR KO mice (Table I), we also examined expansion and activation of γδ T cells in β TCR KO mice. Fig. 3A, i demonstrates a representative example of the gradual percentage increase in γδ T cells in the peritoneal cavity at consecutive time points after VV-infection in β TCR KO mice.

It should be noted that in the absence of αβ T cells the kinetics of the γδ T cell response were altered. β TCR KO mice had at least 13- and 6-fold more γδ T cells present in the uninfected peritoneal cavity (1.0 × 10^5 vs 0.08 × 10^5) and spleen (3.8 × 10^6 vs 0.6 × 10^6), respectively, than normal C57BL/6 mice. They also had 2-fold higher numbers of γδ T cells in the peritoneal cavity and spleen at the peak of their response (Figs. 2C and 3B). In β TCR KO mice there also was a more gradual increase in γδ T cells in the peritoneal cavity after VV infection, with a 6-fold increase by day 5 PI and peaking at day 8 PI, with a 14-fold increase in γδ T cell number (Fig. 3B). This slight delay in γδ T cell increase as
compared with normal C57BL/6 mice may relate to the already high numbers of γδ T cells in the peritoneal cavity of uninfected mice, and the continued increase most likely relates to the greater difficulty these mice have in completely clearing the virus. There was a more moderate 2.5-fold increase in the total number of γδ T cells in the spleen, peaking at day 5 of infection.

In comparison to the C57BL/6 mice, a much greater percentage (50–60%) of γδ T cells recruited to the peritoneal cavity were capable of producing IFN-γ in response to PMA and ionomycin, even in the uninfected host (Fig. 3A, ii). This suggests that γδ T cells may be at a higher state of activation in the absence of αβ T cells. There was a 7-fold increase in IFN-γ-producing γδ T cells in the peritoneal cavity at day 5 PI, ultimately peaking with a 20-fold increase at day 8 PI (Fig. 3B, i). Day 5 of VV infection was the peak of the γδ T cell response in the spleen, with ~50% of the γδ T cells producing IFN-γ, representing a 4-fold increase in total number of IFN-γ-secreting γδ T cells when compared with uninfected mice (Fig. 3B, ii). Peritoneal γδ T cells after VV infection also spontaneously produced IFN-γ without in vitro stimulation with PMA and ionomycin (Fig. 3B, iii). This spontaneous production also appeared to peak slightly earlier, at day 5 of VV infection, rather than at day 8, which was seen with PMA and ionomycin stimulation. Consistent with this increased ability of γδ T cells in β TCR KO mice to produce IFN after VV infection was the observation that serum of the β TCR KO mice was shown by ELISA to have 7-fold higher IFN-γ levels than β+δ TCR KO mice at 8 days after VV infection (2470 ± 931 vs 350 ± 50; n = 4; p < 0.05, Student’s t test). It should also be noted that there was very little recruitment of γδ T cells into the peritoneal cavity of β TCR KO mice infected with LCMV (data not shown), a virus whose replication in vivo was not influenced by the presence or absence of γδ T cells (Table I).

Available variable chain Abs were used to examine the dynamics of VV-induced γδ T cell subpopulations in the β TCR KO mice. In one representative experiment at day 8 after VV infection, 5.8% of the splenocytes were γδ T cells, and of those, 28% (of the CD3+γδ population) stained with Vy2 and 22% stained with Vy4. Only a very small subpopulation (0.4%) of the CD3+, γδ+ cells expressed both Vy2 and Vy4. The spleen showed comparable increases in both subsets on VV infection with 2-fold (day 0, 1.5 × 10^6 vs day 5, 2.6 × 10^6) and 3-fold (day 0, 0.7 × 10^6 vs day 5, 2.0 × 10^6) increases in Vy2 and Vy4, respectively, at day 5, the peak of the splenic γδ T cell response. There was a more dramatic increase in both subsets in the peritoneal cavity by day 8 of VV infection with a 4-fold increase in Vy2 (day 0, 0.6 × 10^5 vs day 8, 2.4 × 10^5) and a 9-fold increase in the Vy4 subpopulations (day 0, 0.2 × 10^5 vs day 8, 1.8 × 10^5). These results suggest that there was an expansion of both of these two common peripheral subsets of γδ T cell populations on VV infection.

**Cytotoxic activity of γδ T cells**

**Potential cytotoxic lymphocyte populations in VV-infected β TCR KO mouse.** To examine whether VV infection could also activate γδ T cells into cytotoxic effectors, we used β TCR KO mice, which had high γδ T cell yields, and with which we would not have to distinguish γδ T cell from αβ T cell cytotoxic activity. To design these experiments we had to carefully consider the types of potentially cytotoxic effector cells in the β TCR KO mouse spleen. Fig. 4A demonstrates a representative splenic lymphocyte population at day 8 of VV infection in β TCR KO mice. It consisted of 35% B cells (CD19+), 21% NK cells (NK1.1+CD3−) and 16.7% γδ T cells (TCRγδ+CD3+). Some of these γδ T cells coexpressed molecules that are classically associated with NK and αβ T cells. Approximately 23% of the γδ T cells expressed NK1.1, and 18% expressed CD8α. All γδ T cells coexpressed Thy1.2, which at day 8 of VV infection separated into a dim and a bright population of Thy1.2 expression. The peritoneal cavity at day 8 of VV infection showed very similar results (data not shown). The γδ T cell coexpression of NK1.1 and CD8 was the same in the spleen and peritoneal cavity, whether or not the mouse was infected. Thus, the presence of NK cells and γδ T cells that coexpressed NK1.1 had to be taken into consideration in designing our experiments to examine γδ T cell cytotoxic function in vivo.

γδ T cells from β TCR KO mice constitutively lyse sensitive targets. Although γδ T cells have often been shown to display cytotoxic activity after several days of in vitro stimulation with various Ags, it has been difficult to show that freshly isolated γδ T cells are cytotoxic (2). By using naive resting C57BL/6 (H2b) β
TCR KO mice, we showed that an allogeneic target, NCTC929 (H2k), was very sensitive to lysis, and its sensitivity was further augmented after infection with VV. Fig. 4B, i shows that splenocytes from naïve β TCR KO mice lysed targets in the hierarchy of NCTC929 + VV > YAC-1 > NCTC929. Because NK cells in uninfected mice are constitutively cytotoxic and capable of lysing YAC-1 cells (40), we needed to determine whether NK cells in β TCR KO mice contributed to the constitutive lysis of these three targets. Because a significant portion of γδ T cells was shown to express NK1.1 (Fig. 4A) we were not able to use anti-NK1.1, which is commonly used to deplete NK cells in C57BL/6 mice. Instead, we treated mice with a dose of anti-asialo GMI, that we have routinely used to selectively deplete NK cells but not T cells in C57BL/6 mice and in mouse strains that do not express the NK1.1 molecule (31, 32). Treatment of uninfected β TCR KO mice with anti-asialo GMI resulted in the selective loss of YAC-1 killing and in the retention of killing of VV-infected and uninfected NCTC929 cell targets (Fig. 4B, ii). There was no lysis of the syngeneic target MC57G (H2b) or VV-infected MC57G by lymphocytes from these uninfected mice (Fig. 4B). These results suggested that γδ T cells in β TCR KO C57BL/6 mice (H2b) constitutively lysed NCTC929 cells (H2k) and that VV infection of these cells further enhanced their lysis. It should be noted that it is possible that γδ T cells may not be in this activated state in the presence of αβ T cells in the normal host.

Splenic cytotoxic activity against all tested targets was greatly elevated by day 8 after VV infection (data not shown), but when VV-infected β TCR KO mice were treated with anti-asialo GMI there was a significant decrease in the cytotoxic activity against all of the targets (data not shown). Residual activity resembled that of an uninfected mouse treated with anti-asialo GMI (Fig. 4B). FACS staining demonstrated that activated γδ T cells increased their expression of asialo GMI (data not shown), a phenomenon that previously has been described for whole spleen cell populations after viral infections (31). Thus, in VV-infected mice, depletions with this Ab could not be used to distinguish the effector populations. Therefore, we used FACS staining to analyze the γδ T cells and then tested the cytotoxic activity of the remaining cells.

Significant frequency of VV-specific γδ pCTL in unprimed β TCR KO mice as quantitated by LDA

To examine the presence of VV-specific γδ CTL clones in β TCR KO mice we stimulated splenocytes from uninfected and VV-infected mice in vitro in LDAs. Consistent with the cytotoxicity data, we were able to detect a significant frequency of VV-specific γδ pCTL in the unprimed uninfected spleen, as 1/3781 splenocytes was able to lyse a VV-infected target (Fig. 6A, i). Because a naive spleen has ~4.0 ± .5% (n = 7) γδ T cells, this means 1/151 of the

FIGURE 5. A. Lysis of VV-infected and uninfected targets by peritoneal exudate cells from VV-infected (day 8) β TCR KO mice. Peritoneal lymphocytes were harvested and tested in cytotoxicity assays. B. Lysis of targets by sorted γδ T cells from the peritoneal cavity lymphocytes from VV-infected (day 8) β TCR KO mice. Peritoneal lymphocytes harvested and pooled from six to eight VV-infected mice were labeled with anti-CD3 and anti-NK1.1 and sorted for CD3+ NK1.1+ or CD3+ NK1.1+ populations and then tested in cytotoxicity assays against VV-infected allogeneic or syngeneic targets. These results are representative of three similar experiments.
γδ T cells was able to lyse a VV-infected target. Thus, unlike VV-specific αβ T cells, there is constitutively a high frequency VV-specific γδ T cells in the unexposed naive host, and it was further increased after VV infection (Fig. 6A, ii).

This LDA technique also was used to grow out and analyze these cells in vitro. We pooled the microwells after 5 days of growth and stained the cells. Fig. 6B shows that these pooled cells were virtually all CD3+, Thy1.2+, γδ TCR-expressing cells, with levels of coexpression of NK1.1 and CD8 similar to that of the in vivo splenic and peritoneal γδ T cells. We were unable to detect the expression of β TCR, or the B cell marker CD19 on these cells, nor were there significant numbers of NK1.1+ cells not expressing CD3 or γδ TCR. These cells also were stained with Abs for the γδ TCR variable chains to examine subpopulations of VV-induced γδ T cells (Fig. 6C). There were similar proportions of the two common peripheral γδ T cell subsets, Vγ2 (30%) and Vδ4 (17.4%). There also was a small fraction (5%) of γδ T cells that express both Vγ2 and Vδ4.

Kinetics of VV clearance in β TCR KO mice
Young (4 mo) and older (8 mo) β TCR KO mice were examined for their abilities to control VV infection. Both groups of mice were able to bring the virus transiently under control by day 8 of VV infection. The clearance of VV from the spleen, liver, and fat pads varied somewhat, dependent on the age of the mice (Fig. 7).

Younger mice (4 mo) had virus titers that were high at day 4, lower at day 6, and, except in the fat pad, very low at day 8, at the peak of the γδ T cell response. This indicates that a host innate response mechanism almost completely cleared the virus in the absence of a normal αβ T cell response. However, Fig. 7 shows that there was a recrudescence in viral titers at day 12, in parallel with the decline in the γδ T cell number, as presented in Fig. 1. In these younger mice we were unable to titrate the virus in the fat pads at day 12, as the fat pads had virtually completely disappeared. The older mice (8 mo) initially had much lower virus titers than the younger mice, had almost completely cleared virus by day 6, and had undetectable virus in all organs at day 8; however, viral titers started
to recrudesce by day 12. Young β TCR KO mice died at about day 14 of VV infection, whereas older mice survived for 3–6 mo before dying. This indicates that VV was transiently brought under control without the participation of αβ T cells, but when the γδ T cells declined in number, the virus recrudesced. These results clearly indicate that these mechanisms of innate immunity can have a significant impact on virus load in the absence of the adaptive T cell immune response and may in part be age dependent.

Discussion

The innate components of the host’s immune system provide pre-existing and/or rapidly inducible barriers to an invading pathogen. Innate immunity plays an important role in pathogenesis by retarding the proliferation of the pathogen, thereby providing time for the expansion and differentiation of αβ T cell and Ab-producing B cell clones, which together usually clear the infection and provide lasting immunity. Cytotoxic IFN-γ-secreting lymphocytes are uniquely suited for the control of virus infections, and innate immunity against some viruses, such as murine CMV, has been shown by us to be effectively mediated by NK cells activated by type I IFNs early in infection (1). Recently, we also have reported that memory αβ T cells specific to previously encountered pathogens can infiltrate virus-infected tissue early in infection and play protective and/or immunopathological roles in antiviral immunity in response to heterologous viruses (42). Here we provide evidence that a third class of cytotoxic, IFN-γ-producing lymphocytes, γδ T cells, can in immunologically naïve mice rapidly respond to a viral infection and curtail titers by 3 to 5 days PI. Hence, γδ T cells can be effectors of innate immunity to viruses.

Our results suggest that the γδ T cells control VV infection early but transiently, and that αβ T cells have the capacity to permanently clear the virus. δ TCR KO mice had much higher VV titers than normal control mice early in infection, but they did ultimately clear the virus. VV infection was gradually brought under control in mice lacking αβ T cells by day 8 of infection, but it then recrudesced, leading to death of the hosts. In contrast, in other reports of γδ T cells altering the course of bacterial, parasitic, or viral infections, the protective role of γδ T cells could be substituted for and masked by αβ T cells. For instance, mice deficient in αβ T cells clear ocular HSV-1, L. monocytogenes, K. pneumonia, and E. veriformis as efficiently as normal mice (6, 7, 11, 16). Only in the absence of αβ T cells could a protective role for γδ T cells be identified in ocular HSV-1 (16) and E. veriformis infection (7); mice deficient in both αβ and γδ T cells were more susceptible to either infection than were αβ T cell-deficient mice. Interestingly, in K. pneumonia infection, both γδ T cell- or αβ + γδ T cell-deficient mice were more susceptible than normal intact or αβ T cell-deficient mice (11). Our results with VV infection were clearly different, as αβ T cells could not substitute for γδ T cells, and γδ T cells could not substitute for αβ T cells.

γδ T cells appear to have features that make them well suited for mediating innate immune responses to VV. For instance, γδ T cells were rapidly expanded and recruited to the site of virus replication and activated to produce IFN-γ, to which VV is extremely sensitive (39). The activation of IFN-γ-secreting γδ T cells has been observed in a number of parasite and bacterial models such as with Plasmodium chabaudi or Salmonella choleraesuis (3, 4) but IFN-γ production by γδ T cells had not been observed previously in viral infections. The finding that γδ T cell expansion peaks early during VV infection and then decreases sharply is a feature common to γδ T cell behavior in other antigenic systems, such as infection with L. monocytogenes or E. veriformis (10). The rapid increase of γδ T cells in the peritoneal cavity on VV infection, where there is such a low frequency of γδ T cells, may relate to both recruitment and expansion. γδ T cells, much like NK cells and memory CD8 T cells appear to be constitutively in a partially activated state and therefore are easily stimulated (28). It is possible that most γδ T cells exist de facto as circulating memory cells after activation by foreign- or self-ligands early in the host’s development, and hence are able to respond rapidly. However, there are no clear high-affinity, pathogen-specific γδ T cells with recall ability defined (10). Because their specificity is broad, it also would be important that they be under stringent immunological control and be rapidly down-regulated when they have accomplished their function.

We also observed a second characteristic of γδ T cells that would be important in mediators of innate immunity. γδ T cells from naïve β TCR KO mice were constitutively cytolytic against a sensitive allogeneic target, and this target’s sensitivity to γδ T cells increased on VV infection. This type of cytolyis by cells from a resting naïve mouse is analogous to NK cell lysis of YAC-1 cells and suggests that the cytolytic machinery is partially turned on constitutively and is further enhanced with virus challenge. Studies in human infections with viruses such as EBV and HIV have focused predominantly on demonstrating that the primary function of γδ T cells after a period of in vitro stimulation is to kill and eliminate virus-infected targets (12, 43–45). However, freshly isolated γδ T cells are usually reported to be devoid of cytotoxic activity (2, 44, 46), with the exception of one report of freshly
isolated γδ T cells from healthy uninfected donors never previously infected with tuberculosis being able to lyse monocytes infected with *Mycobacterium tuberculosis* (47). Experiments assessing fresh ex vivo cytotoxic activity are difficult studies to do, as γδ T cells are low in number and appear to lose their cytolytic activity very quickly after harvest.

The presence of constitutively active cytotoxic γδ T cells capable of lysing VV-infected targets suggests that VV-specific CTL are present at a high frequency without any priming with VV, and this would be another important feature for mediating innate immunity. In fact, we were able to directly quantify by LDA that there was a high frequency (∼1/150) of γδ T cells in the unprimed uninfected host capable of lysing VV-infected targets. The further activation and expansion of these cells by VV infection would make them even more useful in the control of VV. This observation is compatible with the concept that γδ T cells have been reported to recognize very ubiquitous Ags shared by different pathogens or host proteins induced by infection or cell damage. Some of the Ags so far identified include allo-Ags (48–50), heat-shock proteins (51, 52), and microbial Ags such as phosphoantigens (2, 10, 53–55). γδ TCR recognition is reported to be more Ig-like than αβ TCR-like, suggesting that γδ T cells can respond to Ags that would not be recognized by αβ T cells (2, 10, 56). γδ T cells can also mediate rapid cellular immune functions, as they don’t appear to require Ag processing and presentation (2, 10, 56). Although at this time it is unclear what specific Ags are involved in the activation and cytotoxicity of γδ T cells during VV infection, the rapidity and broad specificity of the response are consistent with the above predicted γδ T cell Ag recognition paradigm and implicate γδ T cells as potentially important effectors of innate immunity.

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