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IFN-γ-Producing $\gamma\delta$ T Cells Help Control Murine West Nile Virus Infection

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West Nile (WN) virus causes fatal meningoencephalitis in laboratory mice, thereby partially mimicking human disease. Using this model, we have demonstrated that mice deficient in $\gamma\delta$ T cells are more susceptible to WN virus infection. TCR$\delta^{-/-}$ mice have elevated viral loads and greater dissemination of the pathogen to the CNS. In wild-type mice, $\gamma\delta$ T cells expanded significantly during WN virus infection, produced IFN-γ in ex vivo assays, and enhanced perforin expression by splenic T cells. Adoptive transfer of $\gamma\delta$ T cells to TCR$\delta^{-/-}$ mice reduced the susceptibility of these mice to WN virus, and this effect was primarily due to IFN-γ-producing $\gamma\delta$ T cells. These data demonstrate a distinct role for $\gamma\delta$ T cells in the control of and prevention of mortality from murine WN virus infection. ***The Journal of Immunology*****, 2003, 171: 2524–2431.**

West Nile (WN)[3] virus is a mosquito-borne, single-stranded RNA flavivirus that emerged in the New York City metropolitan area in 1999 and has since spread throughout much of the United States and into other parts of North America (1–5). The clinical manifestations of human infection can range from asymptomatic seroconversion to fatal meningoencephalitis (4, 5). Seroprevalence in the geographic area of the 1999 New York City outbreak was estimated at 2.6% with ~30% of the seropositive individuals reporting a febrile illness (6). During the same outbreak, the case fatality rate among identified hospitalized patients was 12% with a bias toward mortality in the elderly (7). In the global experience of outbreaks in Romania, Israel, and the United States, hospitalized case fatality rates range from 4 to 14% and death most commonly occurs in the elderly and immunocompromised (8–10). Currently, there are no controlled clinical trials of antiviral medications and treatment is confined to supportive measures (10).

The viral pathogenesis and relevant components of the immune response to WN virus are incompletely understood but are central to the development of an effective treatment or vaccine. The murine model of WN virus infection partially mimics human disease and has been used to address these questions (11–14). Most inbred mice challenged with strains of WN virus, including both lineage I and II viral isolates, are susceptible to infection (15, 16). Following i.p. inoculation, the virus is initially found in the blood and lymphoid tissues and subsequently in the kidneys, heart, and CNS (17). Once the virus has breached the blood-brain barrier, the mice develop encephalitis and die shortly thereafter (12, 14, 17). Using this model system, several important components of the immune response have been delineated.

Passive transfer of antiserum specific to the WN virus envelope (E) protein confers protection to WN virus challenge in vivo (12, 18). Protection is enhanced with immunization with the recombinant WN virus E protein or DNA vaccination with constructs encoding the E and membrane (M) protein genes (12, 18). In addition, transfer of infected serum-protected B cell and Ab-deficient mice against WN virus induced mortality (19). Thus, while humoral immunity is sufficient to provide some level of protection against infection, the advantage of active vs passive immunization suggests a role for cell-mediated responses. In addition, flaviviruses can activate cytotoxic T lymphocytes and induce Ag-specific Th1 responses (20, 21), both of which may be important in viral defenses (22–24). Cell transfers have also been shown to confer protection against lethal flaviviral encephalitis (20). Collectively, these studies indicate that both cellular and humoral components of the immune system are engaged in a physiologic response to the virus and have protective capacity.

It has also been suggested that a robust early response to flaviviral infection is essential to protect against encephalitis, potentially allowing clearance before symptoms and severe pathology develop (25). This theory is supported by the fact that resistance mapping studies of the flavivirus susceptibility locus *Flv* and the closely associated WN virus-specific locus *Wnv* demonstrated a correlation between survival advantage with restriction of viral replication in the CNS (25, 26). In Ag-experienced animals, circulating Abs could provide a means of immediate protection by neutralizing the virus. We hypothesized that in the naive host, other mechanisms of early immune protection would be engaged in the response, in particular $\gamma\delta$ T cells.

$\gamma\delta$ T cells are a subset of T cells that comprise a minority of CD3$^+$ cells in the lymphoid tissue but are well represented in the peripheral blood and are abundant at epithelial and mucosal sites, including the gut and lung (27). Like $\alpha\beta$ T cells, they bear a TCR and can be polarized to produce Th1- or Th2-type cytokines (28–30), but in contrast they are more limited in TCR diversity (27), bear certain innate-like receptors such as NKG2D (31), and have more rapid kinetics of cell proliferation and effector function

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[3] Abbreviations used in this paper: WN, West Nile; Q-PCR, quantitative PCR.

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(L. Liu, personal communication). In addition, they can rapidly produce cytokines in response to microbial Ags (32) and have unique features including a lack of MHC restriction and the capacity to react with Ags without the requirement for undergoing conventional Ag processing, which together suggest a role in early pathogen control (33–35). Increased numbers of γδ T cells in the peripheral blood and/or localization to sites of infection have been documented in several human viral infections including HIV (36, 37), EBV (38, 39), and CMV (40). In murine models, γδ T cells have a protective role against HSV type 1, (41, 42), vaccinia virus (43), and influenza virus infection (44, 45).

Based on the importance of γδ T cells in other antiviral immune responses, their capacity to have early effector function and the potential importance of early control in preventing mortality in flavivirus infection, we investigated the role of γδ T cells in the murine immune response to WN virus challenge.

Materials and Methods

Mice

Female C57BL/6 (B6), TCR$\delta^{-/-}$, TCR$\beta^{-/-}$, IFN-$\gamma^{-/-}$, and TCR$\beta^{-/-}$IFN-$\gamma^{-/-}$ mice were bred under specific pathogen-free conditions. All the gene-deficient mice were bred on the B6 background and had been fully backcrossed. Experiments were performed with 6- to10-wk-old animals. Groups were age matched for each infection and were housed under identical conditions.

Virus

WN virus isolate 2741 was initially cultivated by Dr. J. Anderson at the Connecticut Agricultural Experiment Station (1). Mice were inoculated i.p. with 10^2 or 10^3 PFU of WN virus in 100 μl of PBS with 5% gelatin. A total of 10^2 PFU corresponds to the LD_{50} and 10^3 PFU represents the LD_{100} for this viral culture. Mice were observed for up to 16 days after in vivo challenge and the animals were checked twice daily for morbidity, including lethargy, anorexia, and difficulty in walking, and for mortality.

Adoptive transfer

Splenocytes from TCR$\beta^{-/-}$ or TCR$\beta^{-/-}$IFN-$\gamma^{-/-}$ mice were isolated and RBC were lysed. After washing, the cells were resuspended in PBS and transferred i.v. at 20×10^6 cells/mouse to TCR$\delta^{-/-}$ mice. TCR$\delta^{-/-}$ mice administered PBS were used as controls. Twenty-four hours posttransfer, mice were challenged with WN virus for the survival experiments and the reconstituted phenotype was delineated in parallel groups of animals.

Flow cytometry

Freshly isolated splenocytes and cells from the peritoneal cavity were used for staining with Abs specific for TCRαβ, TCRγδ, and CD3 (BD PharMingen, San Diego, CA). After staining, cells were fixed in PBS with 2% paraformaldehyde and examined using a FACSCalibur flow cytometer (BD Biosciences, San Diego, CA). Dead cells were excluded on the basis of forward and side light scatter. Data were analyzed using CellQuest or FlowJo software.

Intracellular IFN-γ and perforin staining

To measure cytokine production, splenocytes from WN virus-infected mice were isolated and cultured under one of two conditions. Half of the samples were incubated at 3×10^6 cells/tube at room temperature with no exogenous stimulation for 4 h and Golgi-plug was added for the final 2 h (BD PharMingen). These conditions were used as they have been described previously as optimal for spontaneous accumulation of cytokines after removal from the host (43). The remaining samples were stimulated at 3×10^6 cells/tube with 50 ng/ml PMA (Sigma-Aldrich, St. Louis, MO) and 500 ng/ml ionomycin (Sigma-Aldrich) for 4 h at 37°C and Golgi-plug (BD PharMingen) was added during the final 2 h (43). The cells were then harvested, stained with cell surface markers (CD3 (clone 145-2C11), TCRαβ (clone H57-597), or TCRγδ (clone GL3)) and fixed in 2% paraformaldehyde. The cells were then permeabilized with 0.5% saponin before adding PE-conjugated anti-IFN-γ mAb (BD PharMingen) or control PE-conjugated rat IgG1. To examine perforin production, permeabilized cells were stained with a rat anti-mouse perforin Ab (Kamaya, Seattle, WA) at 4°C for 15 min. The cells were then washed and incubated again with FITC-labeled goat anti-rat IgG (Sigma-Aldrich). Purified rat IgG2a (BD PharMingen) was used as an isotype control. Cells were examined using a FACSCalibur flow cytometer as described above.

Quantitative PCR (Q-PCR)

At days 2 and 6 of WN virus infection, RNA was extracted from the blood, spleen, and brain tissue from control and experimental mice using RNeasy extraction (Qiagen, Valencia, CA). The extracted RNA was eluted in a total volume of 60 μl of RNase-free water. Two hundred fifty nanograms of each extracted RNA sample was used to synthesize cDNA using the ProSTAR First-strand RT-PCR kit (Stratagene, Cedar Creek, TX). Forty nanograms of cDNA was then used for real-time PCR. The sequences of the primer-probe sets for WN virus were described earlier (46). The probe contained a 5′ reporter, FAM, and a 3′ quencher, TAMRA (Applied Biosystems, Foster City, CA). The reaction mixture contained a total volume of 50 μl including each primer pair at a concentration of 1 μM and a probe at a concentration of 0.2 μM. The assay was performed on an iCycler (Bio-Rad, Hercules, CA). The thermal cycling consisted of 95°C for 3.5 min and 48 cycles of 95°C for 30 s and 60°C for 1 min. To prepare the DNA standard for real-time PCR, the 1.5-kb E region was cloned into pBADTOPO as described earlier (12). To normalize the samples, the same amount of cDNA was used in the *β-actin* Q-PCR. The quantity of each sample was determined using the standard curve of each Q-PCR. The ratio of the amount of amplified *WNV-E* DNA compared with the amount of *β-actin* DNA represented the relative infection level of each sample.

Cytokine PCR

cDNA was prepared as described above. The PCR mixture contained 5 μl of 10× PCR buffer with $MgCl_2$, 1 μl of 10 mM dNTP, 4 μl of 20 μM primers, 0.5 μl of *Taq* polymerase (5 U/μl), and 2 μl of cDNA. The primers for *IFN-γ* were: forward primer, 5′-TGCATCTTGGCTTTG CAGCTCTTCCTCATGGC-3′; reverse primer, 5′-TGGACCTGTGGGT TGTTGACCTCAAACTTGGC-3′. *β-actin* PCR was used to normalize the cDNA. *β-actin* primers amplify a region of 300 bp with forward primer, 5′-AGCGGGAAATCGTGCGTG-3′; reverse primer, 5′-CAGGGTAC ATGGTGGTGCC-3′.

Histologic examination of tissues

Mice were euthanized, their chests were opened, and 30 ml of PBS was injected directly into their left ventricle. Four percent paraformaldehyde was then injected to perfuse and fix the bodies, and the brains were removed and placed in 4% paraformaldehyde. Subsequently, specimens were processed and histologic slides were prepared for staining with H&E.

Statistical analysis

Values of p were calculated with a nonpaired Student's t test. Survival curve comparisons were performed using Prism software (GraphPad Software, San Diego, CA) statistical analysis which uses the log rank test (equivalent to the Mantel-Haenszel test).

Results

TCR$\delta^{-/-}$ and TCR$\beta^{-/-}$ mice are more susceptible to WN virus infection than wild-type mice

To assess the role of γδ T cells in the host immune response to WN virus, wild-type B6, TCR$\delta^{-/-}$, and TCR$\beta^{-/-}$ mice were challenged with different doses of WN virus and examined daily for morbidity and mortality. When administered a LD_{100} of WN virus, TCR$\delta^{-/-}$ mice died rapidly within 6.5 ± 0.5 days, whereas the TCR$\beta^{-/-}$ and wild-type animals died at intervals of 9.5 ± 2.5 days and 9.0 ± 3.0 days, respectively (Fig. 1*a*). Differences between the survival rate of the T cell-deficient and control animals became more dramatic with a dose approximating the LD_{50} (Fig. 1*b*). Only 75% of wild-type controls survived whereas all of the TCR$\delta^{-/-}$ mice and 90% of the TCR$\beta^{-/-}$, mice died within 2 wk ($p < 0.01$, $n = 8$ and $p < 0.05$, $n = 8$, respectively, compared with wild-type controls). These data suggest that both αβ and γδ T cells are critical for host survival following WN virus infection and that their effects are not redundant.

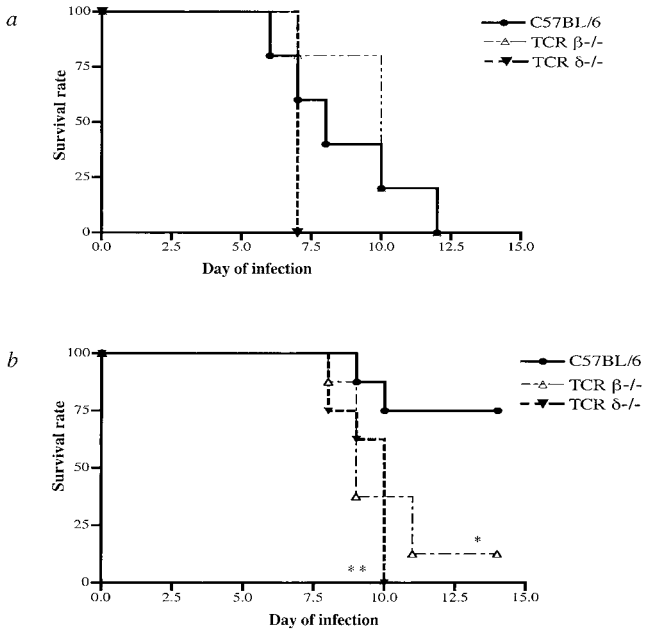

FIGURE 1. WN virus infection in TCR$\delta^{-/-}$ and TCR$\beta^{-/-}$ mice. Control and experimental mice were infected i.p. with a LD_{100} (*a*) or LD_{50} (*b*) of WN virus and monitored twice daily for mortality. Data shown are representative of three similar experiments. *, A value of $p < 0.05$ compared with the wild-type group and **, a $p < 0.01$ compared with the wild-type group.

TCRδ $^{-/-}$ mice have increased viral loads in the blood and lymphoid organs and more severe encephalitis than wild-type mice

To further study viral pathogenesis, wild-type, TCR$\beta^{-/-}$, and TCR$\delta^{-/-}$ mice were infected with WN virus (LD_{50}) and cDNA from different tissues was used to determine viral load by Q-PCR. Animals were viremic 2–3 days following i.p. infection and died within 7–10 days; therefore, we chose day 2 as the early stage of infection and day 6 as the later stage of infection. At day 2, TCR$\delta^{-/-}$ mice had an increased blood viral load and a higher viral burden in the spleen and brain compared with the wild-type and TCR$\beta^{-/-}$ mice (Fig. 2*a*). This difference persisted at day 6 in the blood and spleen (Fig. 2*b*) and in the brain compared with wild-type mice (Fig. 2*c*). Compared with the wild-type controls, TCR$\beta^{-/-}$mice also have enhanced levels of infection at both time points (except at day 6 in brain, Fig. 2*c*). Tissues were also examined histologically to further characterize the course of infection. At day 2, brain sections from wild-type mice did not have an inflammatory infiltrate (Fig. 3*a*). In TCR$\beta^{-/-}$ mice, rare mononuclear inflammatory cells were focally noted in the leptomeninges (Fig. 3*b*), while in TCR$\delta^{-/-}$ mice the lateral wall of the ventricles was infiltrated by mononuclear inflammatory cells in the subependymal area near the basal ganglia (Fig. 3*c*). At day 6, the histologic difference among the strains became even more apparent. TCR$\beta^{-/-}$ animals had a very mild mononuclear infiltrate in the leptomeninges whereas a moderate mononuclear inflammatory infiltration was noted in the leptomeninges of the TCR$\delta^{-/-}$ mice (Fig. 3, *e* and *f*). Inflammatory infiltrates were not evident in wild-type mice at this time point (Fig. 3*d*).

αβ and γδ T cells expand during WN virus infection

To examine T cell responses, we next assessed the magnitude and kinetics of αβ and γδ T cell expansion in B6 mice during WN virus infection. Splenocytes and peritoneal cavity cells were isolated and examined for the percentage and numbers of selected populations. Samples were assessed before infection (control) and at early (day 2) and later (day 6) intervals postinfection. Among splenocytes, the total numbers of both αβ and γδ T cells increased during infection (Fig. 4, *c* and *d*, respectively). γδ T cells expanded from $1.03 \pm 0.28 \times 10^6$ cells/spleen to $2.29 \pm 0.18 \times 10^6$ cells/spleen at day 2 and $1.95 \pm 0.19 \times 10^6$ cells/spleen at day 6 ($p < 0.05$). By contrast, αβ T cells expanded less dramatically from $24.2 \pm 0.6 \times 10^6$ cells/spleen to $31.1 \pm 2.8 \times 10^6$ cells/spleen at day 2 ($p < 0.05$) and $26.8 \pm 4.0 \times 10^6$ cells/spleen at day 6 (change not statistically significant). In addition, the percentage of γδ T cells in spleens increased from 4.2 ± 1.0 at baseline to 6.7 ± 0.5 at day 2 to 6.9 ± 0.7 at day 6 following infection ($p < 0.05$), while the percentage of αβ T cells did not significantly change (95.8 ± 3 at baseline to 94.0 ± 7 and 93.0 ± 13, $p > 0.05$) (Fig. 4, *a* and *b*). Similarly, in the peritoneal cavity (Fig. 4, *e* and *f*), the percentage of the αβ T cells did not change significantly (83 ± 4 at baseline to 78.2 ± 4 at day 2 and to 76.5 ± 4.5 at day 6, $p >$

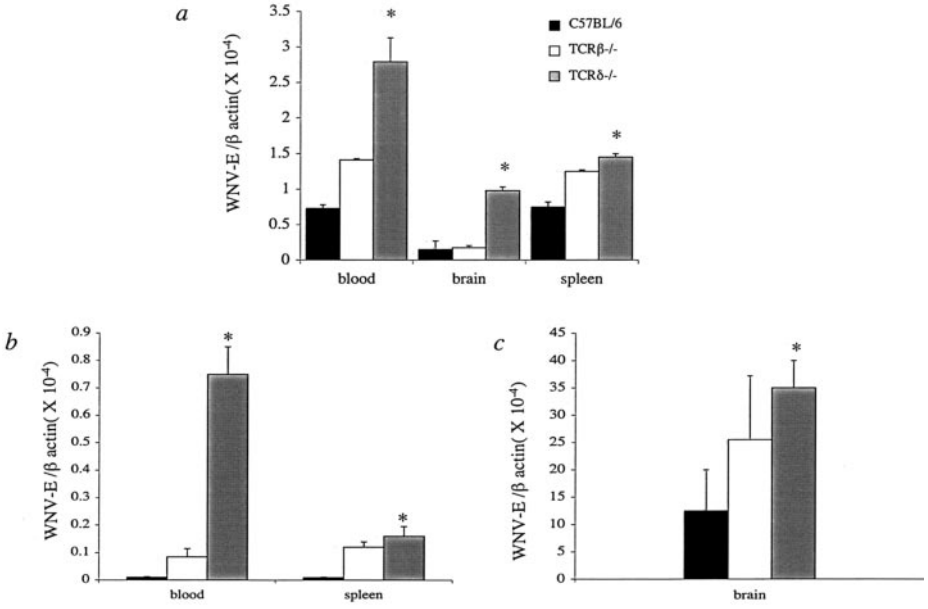

FIGURE 2. Viral load in TCR$\delta^{-/-}$ and TCR$\beta^{-/-}$mice. Viral loads were measured in the blood, spleens, and brains of mice infected with WN virus for 2 days (*a*) and 6 days (*b* and *c*). At each interval, two mice per group were analyzed. Equal volumes of cDNA were used for both *β-actin* and *WNV-E* Q-PCR as described in *Materials and Methods*. The *y*-axis depicts the ratio of the amplified *WNV-E* cDNA to *β-actin* cDNA of each sample, unit ($\times 10^{-4}$). Data shown are representative of three similar experiments. *, A value of $p < 0.05$ compared with the wild-type group.

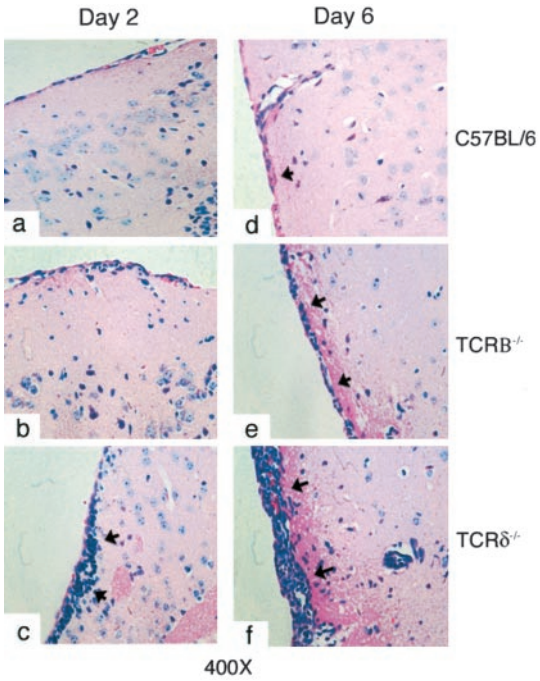

FIGURE 3. Histologic examination of WN virus-infected TCR$\delta^{-/-}$ and TCR$\beta^{-/-}$mice. Histolopathologic changes in the brains of mice infected with WN virus at 2 and 6 days after WN virus infection. *a–c*, Brain sections from wild-type, TCR$\beta^{-/-}$, and TCR$\delta^{-/-}$mice, respectively, at day 2 of infection; *d–f*, brain sections from wild-type mice, TCR$\beta^{-/-}$, and TCR$\delta^{-/-}$ mice at day 6 of infection. Two mice were examined from each group at both time points and representative sections are shown. Arrows point to the sites where inflammation can be found. Magnification, ×400.

0.05) while the percentage of γδ T cells markedly increased from 15.3 ± 0.7 at day 0 to 21.8 ± 1.9 at day 2 and 23.5 ± 2.3 at day 6 postinfection ($p < 0.05$) (see Fig. 4). Expansion patterns of the two subsets of T cells in the blood paralleled that seen in the spleen (data not shown). Taken together, these data indicate that although both αβ and γδ T cells increase in numbers in the spleen following WN virus infection, γδ T cell expansion is more dramatic and only these T cells have a percentage increase in the spleen and peritoneal cavity.

γδ T cells provide partial protection by producing IFN-γ

IFN-γ is known to be a major product of γδ T cells (30) and has multiple antiviral effects. Therefore, we examined the functional capacity of γδ T cells in response to WN virus infection by first specifically quantifying their capability to produce IFN-γ. Initially, IFN-γ-deficient (IFN-$\gamma^{-/-}$) mice were shown to be more susceptible to WN viral infection than control animals: a viral dose approximating the LD_{50} killed only 30% of controls within 2 wk, while 90% of the IFN-$\gamma^{-/-}$ group died of infection ($p < 0.01$) (Fig. 5*a*). Furthermore, TCR$\beta^{-/-}$IFN-$\gamma^{-/-}$ mice had an increased level of infection compared with TCR$\beta^{-/-}$ mice at both day 2 (Fig. 5*bi*) and day 6 of infection (Fig. 5*b*, *ii* and *iii*), as demonstrated by the viral load in all tissues examined (blood, spleen, and brain). This latter experiment suggested that IFN-γ produced by γδ T cells plays an important role in the control of WN virus.

We next determined whether splenic γδ T cells produced IFN-γ during WN virus infection using an ex vivo intracellular cytokine staining assay (Fig. 6, *a–c*). At day 2 postinfection in B6 animals, 15.7% of splenic γδ T cells produced IFN-γ compared with 4.9% of γδ cells from uninfected controls. By contrast, only 3.1% of the αβ T cells produced this cytokine compared with 0.8% in uninfected mice. In TCR$\beta^{-/-}$ mice, 12.4% of the γδ T cells produced IFN-γ vs 0.9% in the uninfected group. By contrast, only 0.8% of the αβ T cells in TCR$\delta^{-/-}$ mice produced IFN-γ at this time, comparable to levels produced by uninfected mice. Later in the course of infection (day 6), the γδ T cell response decreased, with 3.8% of cells from B6 animals producing IFN-γ compared with 0.7% of αβ T cells. At this later time point, γδ T cells from the TCR $\beta^{-/-}$ still produced significant amounts of IFN-γ (7.1%) and

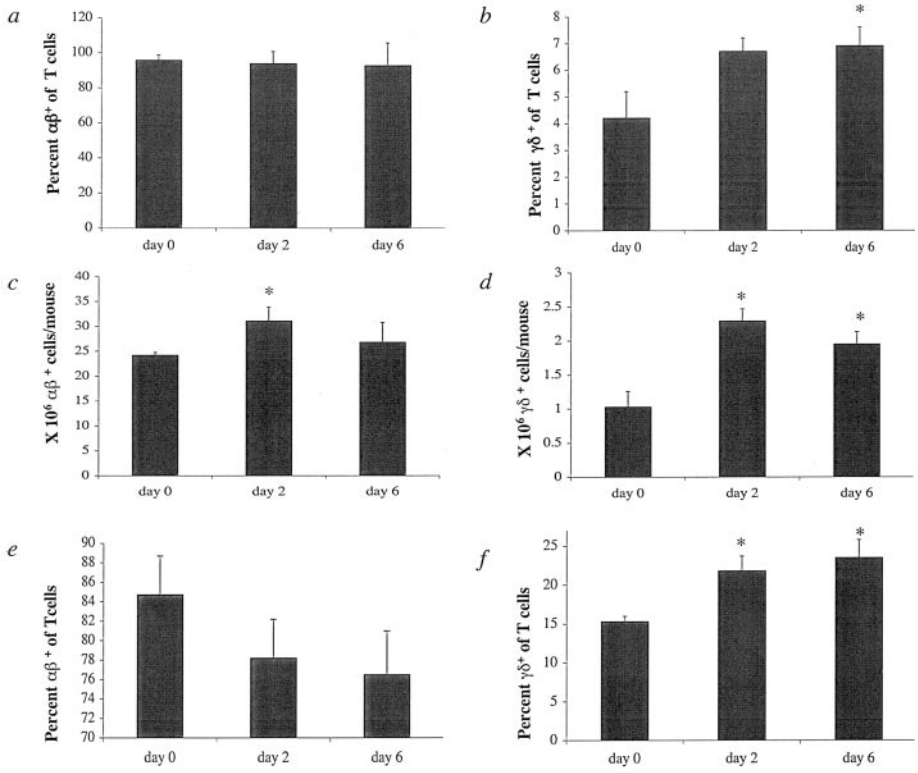

FIGURE 4. γδ T cells respond to WN virus infection. *a* and *b*, The percentage of αβ and γδ T cells in splenic T cells. *c* and *d*, Expansion of αβ and γδ T absolute cell numbers in the spleen (unit: $\times 10^6$ cells/mouse). *e* and *f*, The percentage of αβ and γδ T cells in peritoneal cavity. At each time point, two mice per group were used. Data shown are representative of three similar experiments. ∗, A value of $p < 0.05$ compared with the wild-type group.

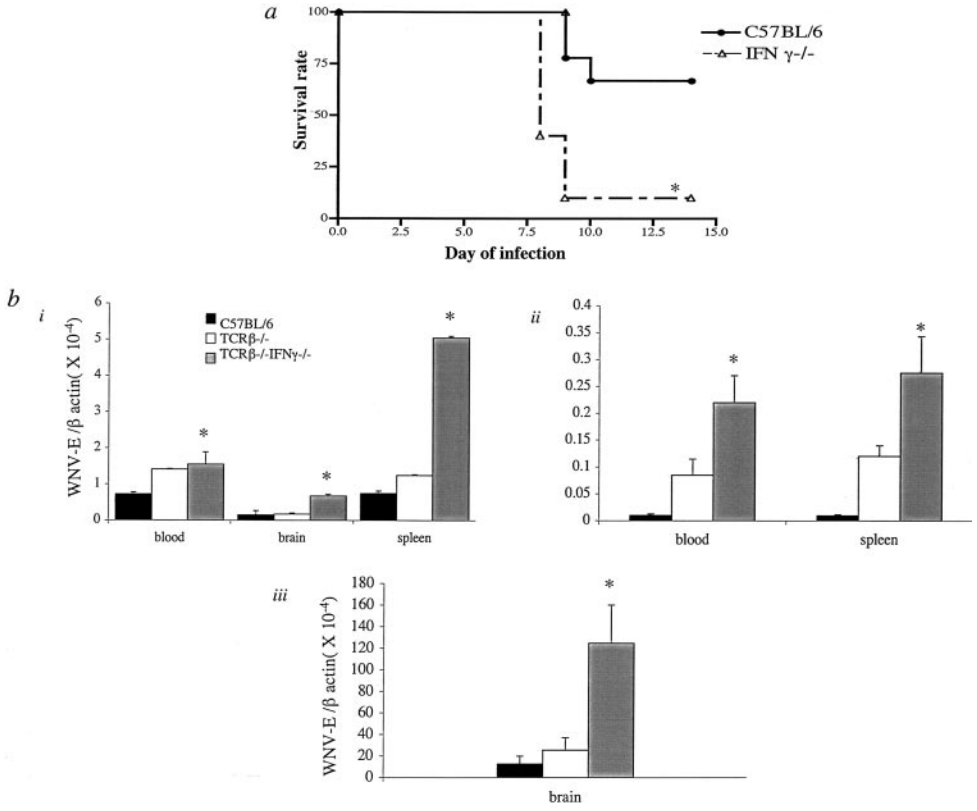

FIGURE 5. IFN-γ is an important factor for host resistance to WN virus infection. *a*, Control and IFN-$\gamma^{-/-}$ mice were infected i.p. with a LD_{50} of WN virus and survival was monitored. *b*, Viral load in the blood, spleens, and brains of mice infected with WN virus for 2 (*i*) and 6 days (*ii*, spleen and blood; *iii*, brain), unit: $\times 10^{-4}$) measured in control, TCR$\beta^{-/-}$ mice, and TCR$\beta^{-/-}$IFN-$\gamma^{-/-}$mice. ∗, A value of $p < 0.05$ compared with the wild-type group.

in TCR $\delta^{-/-}$ animals, 6.7% of $\alpha\beta$ T cells produced the cytokine. Together, these data suggested that γδ T cells are a major source of IFN-γ early during WN virus infection. In addition, the data indicate that the presence of γδ T cells promotes $\alpha\beta$ T cell production of IFN-γ as evidenced by the difference between $\alpha\beta$ T cell function between B6 control and TCR$\delta^{-/-}$ animals.

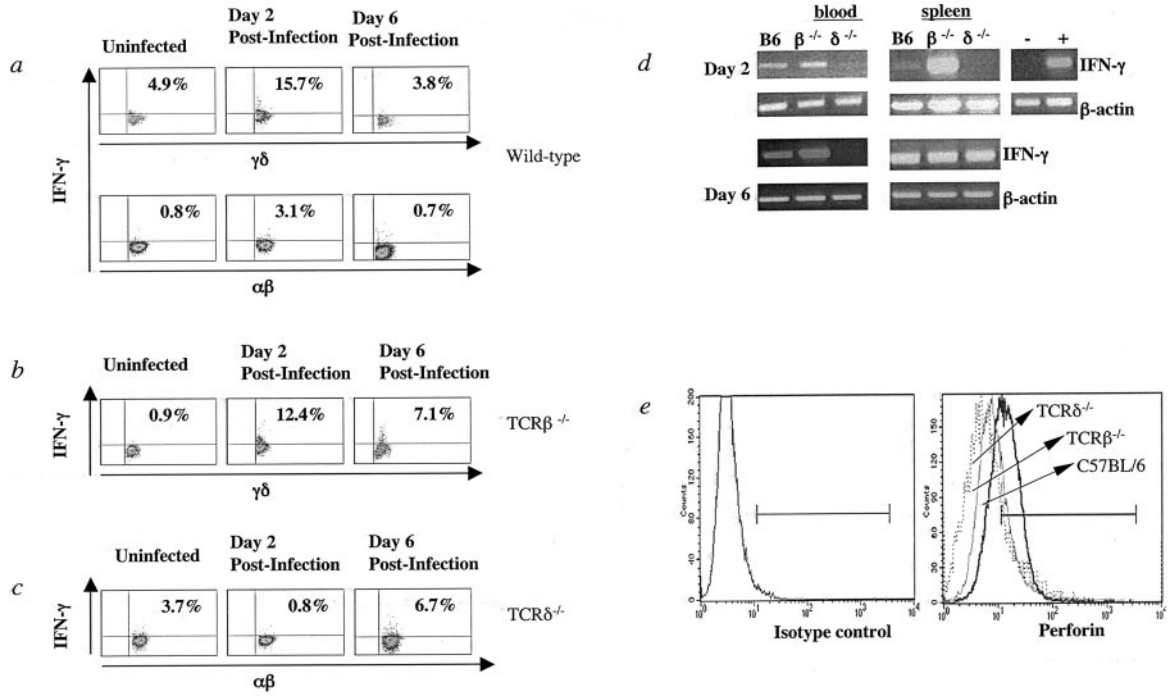

FIGURE 6. γδ T cells produce IFN-γ and promote perforin expression. Splenocytes from WN virus-infected mice were cultured ex vivo without exogenous stimulation as described in *Materials and Methods* and stained for IFN-γ, CD3, and TCR$\alpha\beta$ or TCRγδ. Cells were gated on $\alpha\beta^+$ or $\gamma\delta^+$ populations for analysis of IFN-γ production. The percentage of IFN-γ^+TCR$\alpha\beta^+$ or IFN-γ^+TCR$\gamma\delta^+$ is shown. *a*, Wild-type mice; *b*, TCR$\beta^{-/-}$ mice; and *c*, TCR$\delta^{-/-}$mice. *d*, IFN-γ and β-actin mRNA expression in blood and spleen from different strains of mice as detected by PCR. *e*, Perforin expression of T cells from different strains of mice at day 6 after WN virus infection. $CD3^+$ cells were gated for analysis of perforin expression. Histograms of perforin expression of the gated $CD3^+$ cells are shown. Data shown are representative of three similar experiments.

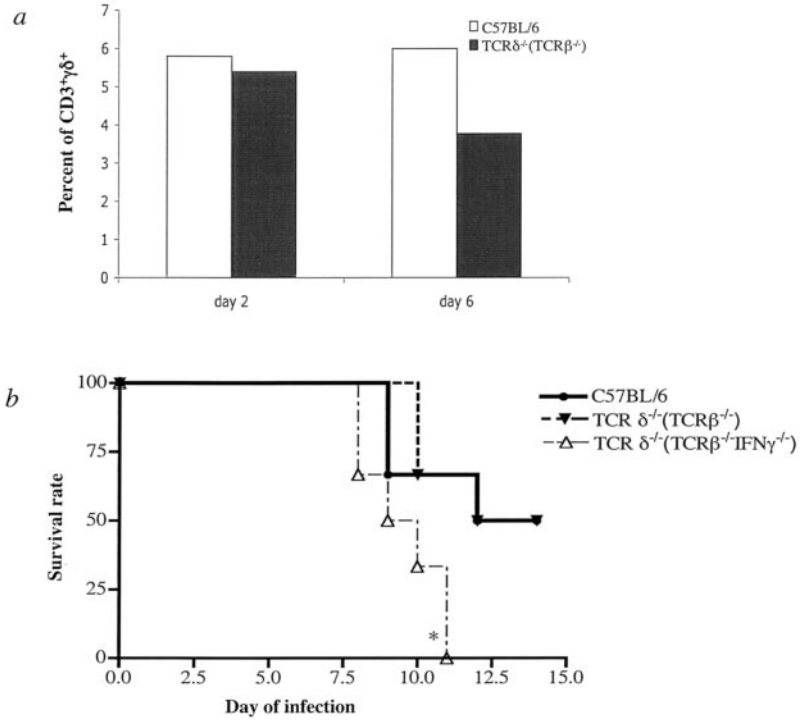

FIGURE 7. Adoptive transfer of splenocytes from TCR$\beta^{-/-}$ mice to TCR$\delta^{-/-}$mice. *a*, Following adoptive transfer of splenocytes from TCR$\beta^{-/-}$ mice to TCR$\delta^{-/-}$ mice, B6 mice, and TCR$\delta^{-/-}$mice with or without reconstitution were infected with a LD_{50} of WN virus. Splenocytes were harvested for phenotyping at days 2 and 6 after infection. *b*, B6 and TCR$\delta^{-/-}$ mice transferred with splenocytes from TCR$\beta^{-/-}$mice or TCR$\beta^{-/-}$IFN-$\gamma^{-/-}$ mice were infected with a LD_{50} of WN virus and survival was monitored for 2 wk. Data shown are representative of four similar experiments. *, A value of $p < 0.05$ compared with the wild-type group.

PCR was also used to assess IFN-γ production in murine tissues (Fig. 6*d*). At day 2, TCR$\delta^{-/-}$ mice produced less IFN-γ mRNA in the blood and spleen compared with wild-type and TCR$\beta^{-/-}$ mice. At day 6, total splenic IFN-γ mRNA in TCR$\delta^{-/-}$ mice increased to a level similar to that of the other two strains; however, in the blood there was still less IFN-γ mRNA. Other cells including NK cells may contribute to early IFN-γ production in the blood and spleen; however, these cell types are intact in the TCR$\delta^{-/-}$ mice. The fact that the IFN-γ PCR results are consistent with results from intracellular cytokine staining suggests that $\gamma\delta$ T cells are the major source of IFN-γ in early host response to WN virus infection.

$\gamma\delta$ T cells modulate cytotoxicity

$\gamma\delta$ T cells in IFN-$\gamma^{-/-}$ mice also expanded following WN virus infection (data not shown), suggesting that $\gamma\delta$ T cells might be involved in other mechanisms of viral protection. We investigated whether the increased susceptibility of TCR$\delta^{-/-}$ mice to WN virus was associated with impaired cytolytic activity of T cells. Here, we focused our studies on perforin, a cytolytic protein known to be expressed by $\gamma\delta$ T cells (27). Perforin is an intracellular protein found in NK and CTL cells and its expression reflects the CTL activity of these cells (47). To examine perforin expression, splenocytes were isolated from mice after infection and used for intracellular staining. T cells were then gated for analysis of perforin expression. Perforin expression of T cells in TCR$\delta^{-/-}$ mice was significantly down-regulated compared with wild-type and TCR$\beta^{-/-}$ mice at day 6 after infection (Fig. 6*e*), suggesting that $\gamma\delta$ T cells either directly produce perforin or, alternatively, are important for its expression by $\alpha\beta$ T cells.

Transfer of $\gamma\delta$ T cells to TCR$\delta^{-/-}$ mice affords partial protection against infection

To determine whether transfer of $\gamma\delta$ T cells could alter the course of infection in TCR $\delta^{-/-}$ animals, splenocytes from TCR$\beta^{-/-}$ mice were adoptively transferred to TCR$\delta^{-/-}$ mice 24 h before challenge with WN virus. To test the importance of $\gamma\delta$ T cell-produced IFN-γ, splenocytes from TCR$\beta^{-/-}$IFN-$\gamma^{-/-}$ mice were also transferred. The reconstituted mice are fully intact in cell composition, with the only defect in the IFN-γ-producing capacity of $\gamma\delta$ T cells. Initially, we examined whether $\gamma\delta$ T cells could be successfully transferred to and persist within TCR$\delta^{-/-}$ mice during infection. The transfer efficiency was assessed 2 and 6 days after challenge with a LD_{50} of WN virus (Fig. 7*a*). At day 2 postinfection, TCR$\delta^{-/-}$ mice administered cells from TCR$\beta^{-/-}$ mice had 5.4% $CD3^+\gamma\delta^+$ cells compared with 5.8% $CD3^+\gamma\delta^+$ cells in control wild-type mice and undetectable levels of $\gamma\delta^+$ cells in untransferred TCR$\delta^{-/-}$ animals. At day 6 postinfection, mice reconstituted with TCR$\beta^{-/-}$ splenocytes had 3.77% $CD3^+\gamma\delta^+$ cells whereas wild-type mice had 6% $CD3^+\gamma\delta^+$ cells.

In a parallel group of infected animals, mortality was recorded for 2 wk after viral challenge of these transferred mice. With a WN virus dose approximating the LD_{50}, ~60% of wild-type mice survived at 2 wk of infection, whereas 40% of the TCR$\delta^{-/-}$ mice that received TCR$\beta^{-/-}$ splenocytes survived ($p > 0.05$, compared with the wild-type group). By contrast, mice that received TCR$\beta^{-/-}$ IFN-$\gamma^{-/-}$ splenocytes all died after infection ($p < 0.05$, compared with the wild-type group, Fig. 7*b*). Collectively, the data from the transfer experiments suggest that $\gamma\delta$ T cells have a unique role in protection against WN virus and that this effect is mediated in part by IFN-γ production by $\gamma\delta$ T cells.

Discussion

We provide herein the first evidence for a unique role for $\gamma\delta$ T cells in the protective response against WN virus infection. Mice deficient in $\gamma\delta$ T cells had an increased mortality after WN virus infection that correlated with an elevated viral load and more severe encephalitis compared with control animals. We demonstrated that early following infection $\gamma\delta$ T cells increased in number and percentage, produced IFN-γ in ex vivo assays, and affected the level of perforin expression in splenocytes. Finally, we demonstrated that reconstituting TCR $\delta^{-/-}$ animals by transfers with

γδ T cells partially restored their resistance and that such protection was mediated at least in part by an IFN-γ-dependent mechanism. We acknowledge that NK cells may play a role in IFN-γ production, but given that we are comparing TCR $\delta^{-/-}$ and wild-type mice, effects seen in our experiments are from γδ T cells. Taken together, these data demonstrate that γδ T cells respond early in the course of infection with expansion and effector function and that this response limits viral titers and penetration to the CNS, thereby increasing survival rates.

IFN-γ is a major cytokine produced by γδ T cells in several viral infection systems (33, 43, 48–50) and has multiple mechanisms of viral control, including cell recruitment and activation, polarization of T cell responses, up-regulation of Ag processing and presentation, and direct antiviral action (reviewed in Ref. 51). IFN-γ has been implicated in control of some viral CNS infections (52–54), although it is not always essential (55). In a model of another flaviviral encephalitis, IFN-γ deficiency was associated with impaired recruitment of inflammatory cells to the CNS and with increased viral titers, although no survival difference was noted (56). In the present study, we have shown: 1) IFN-γ-deficient animals are more susceptible to infection, 2) γδ T cells produce IFN-γ early following infection, and 3) this is one of their essential effector functions as cells from TCR$\beta^{-/-}$IFN-$\gamma^{-/-}$ mice were unable to transfer protection. In the transfer experiment, the two groups of mice were identical in cell composition except that γδ T cells were unable to produce IFN-γ in TCR$\beta^{-/-}$IFN-$\gamma^{-/-}$ mice. The survival difference seen directly demonstrated that IFN-γ production by γδ T cells is important in protection.

Cytolytic function is another important mechanism of viral control that is primarily associated with $CD8^{+}\alpha\beta$ T cells, but has also been attributed to γδ T cells (42, 43, 57). We demonstrated that γδ T cell deficiency reduced levels of intracellular perforin in total splenocytes at day 6 postinfection, implying that they either directly or indirectly enhance cytolytic activity. In previous work, cytolytic activity against WN virus-infected target cells was detected at day 4, peaked on day 5, and declined rapidly after day 7 (23). Thus, while the initial differences between TCR $\delta^{-/-}$ and control animals were in cytokine production, the later differences in perforin expression correspond with the peak of this response, suggesting that the γδ T cell effect may have different functional significance at different points in the course of infection.

Our findings are consistent with and extend results from several other viral model systems in which γδ T cells have been assigned protective roles (41–45). Notably, in the case of WN virus the effect of γδ T cells is nonredundant and essential for survival even in the presence of αβ T cells. In addition, our results suggest both a direct mechanism of viral control through IFN-γ production and an indirect effect of γδ T cells to enhance αβ T cell function through modulation of cytokine production or perforin expression. The requirement for γδ T cell function not merely for viral control but for survival may reflect the particular importance of early responses in WN virus infection. γδ T cells may be uniquely capable of such early protective responses given their rapid and robust production of type I cytokines following pathogen challenge (30, 32). Immediate control may prevent CNS infection and the pursuant encephalitis, a factor that may be recapitulated in human disease. Although the pathogenesis of WN virus-induced encephalitis is still unclear, the enhanced viral load, higher level of brain inflammation, and increased mortality in TCR$\delta^{-/-}$ mice suggest that γδ T cells help the host to clear WN virus and delay the virus from crossing the blood-brain barrier and inducing encephalitis.

Although these findings provide novel insight into the function of γδ T cells, they also may provide important information on the pathogenesis of human WN virus infection. The relevance of these findings for understanding human disease is partially based on pathologic similarities in flavivirus-infected brains across vertebrate species (58) and further on the similarities of immune system function that have long supported murine immunologic studies. The importance of these findings lies in the implications for WN virus treatment and vaccine development. The target population of such a vaccine would be primarily the elderly, already thought to be somewhat immunocompromised, and any effective vaccine would have to elicit a robust response. The data presented in this report suggest that an early response involving γδ T cells is essential to preventing mortality. Experiments to determine whether this early response has an impact on memory development are ongoing. In addition, activation of γδ T cells by nonspecific immunomodulators offers a possible mechanism of boosting the immune response to acute infection, as demonstrated in other viral models (44).

This model system and the evidence that an early response by γδ T cells is critical to survival should help to further delineate the immunopathogenesis of WN virus infection and identify important factors in protection and prevention of mortality.

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