CD8\(^{+}\) T cell-mediated immune responses in West Nile virus (Sarafend strain) encephalitis are independent of gamma interferon

Yang Wang, Mario Lobigs, Eva Lee, Aulikki Koskinen and Arno Müllbacher

Division of Immunology and Genetics, The John Curtin School of Medical Research, The Australian National University (ANU), PO Box 334, Canberra, ACT 2601, Australia

The flavivirus West Nile virus (WNV) can cause fatal encephalitis in humans and mice. It has recently been demonstrated, in an experimental model using WNV strain Sarafend and C57BL/6 mice, that both virus- and immune-mediated pathology is involved in WNV encephalitis, with CD8\(^{+}\) T cells being the dominant subpopulation of lymphocyte infiltrates in the brain. Here, the role of activated WNV-immune CD8\(^{+}\) T cells in mouse WNV encephalitis was investigated further. Passive transfer of WNV-immune CD8\(^{+}\) T cells reduced mortality significantly and prolonged survival times of mice infected with WNV. Early infiltration of WNV-immune CD8\(^{+}\) T cells into infected brains is shown, suggesting a beneficial contribution of these lymphocytes to recovery from encephalitis. This antiviral function was not markedly mediated by gamma interferon (IFN-\(\gamma\)), as a deficiency in IFN-\(\gamma\) did not affect mortality to two strains of WNV (Sarafend and Kunjin) or brain virus titres significantly. The cytolytic potential, as well as precursor frequency, of WNV-immune CD8\(^{+}\) T cells were not altered by the absence of IFN-\(\gamma\). This was reflected in transfer experiments of WNV-immune CD8\(^{+}\) T cells from IFN-\(\gamma^{-/-}\) mice into WNV-infected wild-type mice, which showed that IFN-\(\gamma^{-/-}\)-deficient T cells were as effective as those from WNV-immune wild-type mice in ameliorating disease outcome. It is speculated here that one of the pleiotropic functions of IFN-\(\gamma\) is mimicked by WNV-Sarafend-mediated upregulation of cell-surface expression of major histocompatibility complex antigens, which may explain the lack of phenotype of IFN-\(\gamma^{-/-}\) mice in response to WNV.

INTRODUCTION

CD8\(^{+}\) cytotoxic T (Tc) lymphocytes play an important role in the immune response against intracellular pathogens, particularly viruses (Blanden, 1970). Tc cells exert their effector function by at least two mechanisms, one being cytotoxicity and the other release of cytokines such as gamma interferon (IFN-\(\gamma\)) (Boehm et al., 1997), tumour necrosis factor alpha (Vassalli, 1992) and interleukins (Biron, 1994). To date, two major pathways of target-cell killing by cytolytic leukocytes [natural killer (NK) cells and Tc cells] have been described. Killing of target cells by effector Tc cells occurs primarily via either the granule exocytosis pathway (Henkart, 1994; Kagi et al., 1995; Podack et al., 1991) or the Fas–Fas ligand mechanism (Nagata, 1997; Rouvier et al., 1993). Antigen-specific killing by CD8\(^{+}\) T cells requires direct cell-to-cell interaction with infected target cells and depends on the ability of lymphocytes to migrate to the site of infection.

IFN-\(\gamma\), a pleiotropic cytokine secreted by activated T (CD8\(^{+}\) and CD4\(^{+}\) T helper 1 cells) and NK cells (Boehm et al., 1997), is an important component of the cytokine-mediated immune response to viral infections. Although originally defined as a cytokine with direct antiviral activity (Wheelock, 1965; Wong & Goeddel, 1986), IFN-\(\gamma\) is qualitatively different from type I IFN and is principally not an antiviral (Landolfo et al., 1988) but an immunoregulatory cytokine, affecting a plethora of immune mechanisms, such as T-cell homeostasis, upregulation of Fas on target cells (Müllbacher et al., 2002) and the class I and II antigen-presentation pathways, including upregulation of cell-surface expression of major histocompatibility complex (MHC) class I and class II molecules (Boehm et al., 1997; Dalton et al., 1993; Huang et al., 1993; Refaeli et al., 2002; Sobek et al., 2002). A protective role of IFN-\(\gamma\) has been demonstrated in several viral infections (Bartholdy et al., 2000; Cantin et al., 1999; Guidotti et al., 2000; Karupiah et al., 1998b; Nansen et al., 1999; Ramshaw et al., 1997), but not in others (Mo et al., 1997; Sarawar et al., 1997). Thus, the requirement for IFN-\(\gamma\) in recovery from virus infection is variable between different viruses. Recent studies with flaviviruses indicate that the deficiency of IFN-\(\gamma\) does not cause significant changes in mouse susceptibility to Yellow fever virus or Murray Valley encephalitis virus (MVEV) (Liu & Chambers, 2001; Lobigs et al., 2003b), but resulted in increased viral load and mortality in infections with the
highly virulent lineage I New York strain of West Nile virus (WNV-NY) (Shrestha et al., 2006b; Wang et al., 2003a).

WNV and Kunjin virus (KUN), two flaviviruses of the Japanese encephalitis virus antigenic group, can induce fatal encephalitis in humans and mice (Chambers & Diamond, 2003; Halevy et al., 1994; Müllbacher et al., 2003; Smithburn et al., 1940; Weiner et al., 1970). WNV has been found within neurons throughout the central nervous system (CNS) (Chambers & Diamond, 2003) and astrocytes have been shown to be susceptible to WNV infection in vitro (Liu et al., 1989b). It is not known whether other cells of the CNS are susceptible to WNV infections. We have recently established a model of WNV encephalitis in C57BL/6j mice (Wang et al., 2003b). This model, using high- and low-dose infection regimes with the lineage II WNV-Sarafend strain, established that CD8+ T cells are the principal leukocyte population infiltrating the CNS after WNV has breached the blood–brain barrier and are involved in both protection and immunopathology (Wang et al., 2003b). Furthermore, using mice deficient in granule exocytosis- and/or Fas-mediated cytolytic effector function of CD8+ T and NK cells, we established that survival of WNV infection was at least partially dependent on these pathways (Wang et al., 2004). Studies by others also found an involvement of CD8+ T cells in the clearance and control of the WNV-NY strain (Shrestha & Diamond, 2003). However, in contrast to our observations with WNV-Sarafend, which expressed a null phenotype in the perforin knock-out mouse (Wang et al., 2004), Shrestra et al. (2006a) found increased viral load and mortality in perforin-deficient mice in response to WNV-NY infections. Here, we analyse further the role of WNV-immune CD8+ T cells in recovery from and/or immunopathology of WNV-Sarafend encephalitis, with an emphasis on the requirement of IFN-γ in these processes.

METHODS

Mice and virus. C57BL/6j (B6, H-2b) wild-type (wt) mice and IFN-γ−/− mice bred onto the B6 background (Dalton et al., 1993) were supplied by the Animal Breeding Facility, The John Curtin School of Medical Research, ANU, Canberra, Australia. The presence of the IFN-γ−/− mutation was verified by PCR screening of DNA extracted from mouse tissue (35 cycles of 94 °C for 20 s, 55 °C for 60 s and 72 °C for 60 s) using primer pairs specific for the neomycin gene and undisrupted IFN-γ second exon, respectively (5′-TTGAAAACAGATGATTGCACGGAG-3′, 5′-GGCTGGCGCGAGCCCTT GATTGCT-3′; and 5′-AGAACCTTGAAGGAGCCCGACAGG-3′, 5′-AGGGGAACTTTGGGAGGAGAAAAT-3′). All animals were housed in specific pathogen-free conditions and given access to standard mouse chews and distilled water ad libitum. Female mice were used at 6–8 weeks of age and were age-matched in all experiments. All animal experiments were conducted with approval from the ANU Animal Ethics Committee.

WNV-Sarafend and KUN (Kesson et al., 1987) stocks were prepared by passage in suckling mouse brain followed by a single passage in C6/36 mosquito cells. Semliki Forest virus (SFV) avirulent strain (A-7) was prepared by passage in BHK cells from mouse-brain stock (Mullbacher & King, 1989). Virus titres were estimated by plaque assay on Vero cell monolayers as described previously (Wang et al., 2003b). Aliquots of virus stocks were stored at −70 °C.

Mouse inoculation and tissue processing. Mice were infected via the tail vein with a single injection of either 106 or 105 p.f.u. WNV or 105 p.f.u. KUN in 100 μl Hanks’ balanced salt solution containing 0.2% BSA and 20 mM HEPES (pH 8.0) (HBSS/BSA). Animals were monitored twice daily and moribund mice were killed to limit suffering. Previous studies showed that moribund WNV-infected mice would die within 24 h.

For tissue processing, mice were anaesthetized deeply with Rhodia Halothane (Merial Australia Pty Ltd). After cardiac puncture for blood-sample collection and exsanguination, animals were perfused with 10 ml sterile ice-cold PBS. The brain was excised intact and then bisected. Half of the brain was then cut at midsagittal or coronal planes for virus titration and histological and fluorescent staining. For virus titration, tissue was snap-frozen in liquid nitrogen. For histology, tissue was placed in 10% neutral-buffered formalin fixative at room temperature (RT). For fluorescent staining, tissue was immersed in 22-oxycalcirol (OCT compound) (Tissue-Tek; Sakura Finetek Inc.) and snap-frozen in liquid nitrogen. The other half of the brain was kept in culture medium and then homogenized for lymphocyte isolation.

Virus titration. Vero cells were cultured under standard tissue-culture conditions at 37 °C in a humidified atmosphere of 5% CO2 in air and were grown in minimum essential medium (MEM; Gibco Invitrogen) with non-essential amino acids, PSN (penicillin G, 0.1 g l−1; streptomycin, 0.16 g l−1; neomycin, 0.16 g l−1) and 5% heat-inactivated fetal calf serum (FCS; Trace Biosciences Pty Ltd). Samples of brain tissue were homogenized in HBSS/BSA (10% w/v), serially diluted 10-fold on ice and inoculated onto Vero cell monolayers grown in six-well plastic plates (tissue-culture grade; ICN Biomedicals Inc.). After 1 h adsorption of virus under standard tissue-culture conditions with occasional shaking, monolayers were overlaid with 1% Bacto-Agar (Difco) in MEM with 2% FCS. After 72 h at 37 °C/5% CO2, monolayers were stained with 0.02% neutral red in HBBS (for up to 16 h). Plaques were counted and virus titres were estimated as p.f.u. (g tissue)−1.

Histology and fluorescent staining of tissue sections. Midsagittal- or coronal plane-bisected brains were fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections (6 μm) were stained with haematoxylin and eosin (HE) to examine cell morphology. For WNV antigen staining, brain and draining lymph nodes (DLNs) were removed from recipients at various time points. Frozen brain and lymph nodes were cut into 6 μm sections and fixed in acetone at −20 °C. The sections were incubated with mouse anti-WNV mAb (2B2) (Hall et al., 1991) for 1 h at RT. Phycoerythrin (PE)-conjugated goat anti-mouse IgG (Sigma-Aldrich) was applied for 1 h as the second antibody (Ab). The slides were counterstained with Harris haematoxylin (Sigma-Aldrich) and were viewed using a Nikon TE300 confocal microscope. For a negative control, normal mouse IgG (Sigma-Aldrich) was used as the primary Ab.

Cytotoxic assay and limiting-dilution analysis. Cytotoxic activity of splenocytes from B6 and IFN-γ−/− mice was measured by using a 51Cr-release assay as described previously (Kesson et al., 1987). Briefly, mice were infected intravenously (i.v.) with 105 p.f.u. WNV. Six days later, at the height of the primary Tc-cell response to WNV (Kesson et al., 1987), spleens were harvested and single-cell suspensions were prepared in MEM with 10% FCS and tested for lytic activity on WNV-infected targets. All samples were diluted in threefold steps in triplicate in 96-well plates (Becton Dickinson). MC57G target cells were infected for 16 h with WNV at an m.o.i. of 5 p.f.u. per cell, then labelled with Na251CrO4 [100 μCi (3.7 MBq) per well].
per 10^6 cells; Amersham Biosciences). Target cells (2 x 10^6) in 100 μl MEM with 10% FCS were seeded into each well and incubated with effector Tc cells for 6 h under standard tissue-culture conditions. Release of ^51Cr from the targets was measured in a Packard Auto-Gamma counter. Percentage specific lysis was calculated as ([experimental release − spontaneous release]/[maximal release − spontaneous release]) x 100%.

Limiting-dilution analysis was performed as described previously (Regner et al., 2001). Briefly, B6 and IFN-γ−/− mice were infected i.v. with 10^3 p.f.u. WNV and spleens were harvested at day 8 post-infection (p.i.). Splenocyte suspensions were diluted serially (starting from 2 x 10^6 cells per well in 100 μl medium, twofold steps) in 96-well plates and stimulated with 2 x 10^5 cells per well of mitomycin C-treated, WNV-infected (5 p.f.u. per cell) syngeneic stimulator splenocytes in culture medium supplemented with 10% FCS and 10% supernatant of the interleukin-2-secreting cell line 6310 (ATCC). Forty-eight wells were used per dilution. Five days later, cultures were replenished with medium and 10% 6310 cell supernatant. At day 7, all wells were split into two fractions and incubated with ^51Cr-labelled MC57G target cells that were mock-infected or infected with WNV. Those wells giving specific lysis of WNV-infected target cells of 3 SD above background (mock-infected targets) were considered to contain WNV-reactive Tc splenocytes (background lysis was 21.9±2.5%). The frequency of WNV-reactive Tc cells can be derived from the number of seeded splenocytes per well required to give 37% negative wells, according to the method described by Taswell (1981).

**Lymphocyte isolation from the brain.** Lymphocytes in the brains of wt and IFN-γ−/− mice that had been infected with 10^6 p.f.u. WNV per mouse were isolated by density-gradient centrifugation as described previously (Wang et al., 2003b). Briefly, after in situ perfusion with PBS, one half of the brain was harvested and homogenized. Homogenates were digested with 2 mg collagenase type I ml−1 (Gibco-Life Technologies) in MEM/5% FCS for 30 min at 37°C with shaking, then centrifuged at 400 g for 10 min. The pellet corresponding to cells obtained from one half brain derived from a single animal was resuspended in 2 ml 90% Percoll (Sigma) in MEM and transferred to a 15 ml tube. The suspension was then overlaid gently with 60, 40 and 10% Percoll in MEM. The gradients were centrifuged at 800 g for 45 min at 22°C. The lymphocytes were collected from the 40–60% interface and washed twice with MEM/5% FCS. Numbers of CD8+ T cells were determined by fluorescence-activated cell-sorting (FACS) analysis.

**CD8+ T-cell enrichment, labelling and adoptive transfer.** Donor animals were infected i.v. with 10^6 p.f.u. WNV or SFV in 100 μl HBSS/BSA, or mock-infected with 100 μl HBSS/BSA. At day 5 p.i., spleens were removed and single-cell suspensions were prepared. After the erythrocytes were lysed, spleen-cell suspensions were loaded onto nylon wool columns at a density of 5 x 10^6 cells ml−1 in MEM/5% FCS and incubated for 1 h at 37°C. The cells were washed with three column volumes of warm (37°C) MEM/5% FCS and cells in the effluent were pelleted at 400 g for 10 min at 4°C. The cells were then labelled with PE-conjugated rat anti-mouse CD45R/B220 (RA3-6B2; PharMingen) and anti-CD4 (GK1.5; PharMingen) mAb cocktail (25 and 50 μl per 10^6 total cells, respectively) for 30 min at 4°C. The cells were washed and incubated with magnetic anti-PE Ab (Miltenyi Biotec) at a concentration of 100 μl per 10^6 total cells for 30 min at 4°C. After washing, the cells were resuspended and applied to a MACS LS separation column (Miltenyi Biotec). The cells in the effluent were collected and washed. Cells (2 x 10^7) were then injected into the tail vein of recipients, followed by an i.v. infection with either 10^6 or 10^3 p.f.u. WNV 2 h after transfer. For the non-CD8+ T-cell control group, the CD8+ T cell-enriched cell suspensions were incubated with anti-mouse CD8 mAb (53-6.7; PharMingen) for 30 min at 4°C, followed by a 45 min incubation with rabbit complement (Cedarlane Laboratories Ltd) at 37°C. Cells (1 x 10^6; the approximate aliquot from one donor) were then injected into the tail vein of recipients, followed after 2 h by i.v. infection of either 10^6 or 10^3 p.f.u. WNV.

For cell-tracking experiments, CD8+ T cell-enriched cell suspensions (5 x 10^6 cells ml−1 in warm, protein-free MEM) from mock- or virus-infected animals were labelled with 5-(and 6)-carboxyfluorescein diacetate succinimimid ester (CFSE; Molecular Probes) at a final concentration of 2 μM. After 15 min incubation at 37°C, labelling was stopped by adding an equal volume of ice-cold PBS. The cells were washed and resuspended in PBS. CFSE-labelled CD8+ T cells (2 x 10^6) were injected i.v. into recipients, followed after 2 h by an infection with WNV using either 10^6 p.f.u. i.v. or 10^3 p.f.u. subcutaneously (s.c.) into each footpad of the hindlimbs.

**Statistical analysis.** Experimental results were analysed for statistical significance by using Student’s t-test for the mean survival time (MST) and χ^2 analysis for survival rates. All statistical analyses were performed using Microsoft Excel software. A value of P<0.05 was considered statistically significant. P values between 0.05 and 0.1 were considered to indicate a trend.

**RESULTS**

**Transfer of activated WNV-immune CD8+ splenocytes reduces WNV-mediated mortality**

Previous studies have shown that CD8+ T cells become activated rapidly after WNV infection (Wang et al., 2003b) and that peak cytolytic activity is reached in the spleen at days 5–6 p.i. (Kesson et al., 1987). Furthermore, a lack of CD8+ T cells caused an increase in mortality and infectious virus after i.v. infection of mice with 10^3 p.f.u. WNV (Wang et al., 2003b). This suggests an involvement of these immune effectors in virus clearance. To obtain more specific information regarding CD8+ T cells and their role in viral clearance, activated WNV-immune CD8+ T cells from B6 mice infected i.v. with 10^3 p.f.u. WNV 5 days previously were transferred into B6 mice, and the recipients were infected with WNV 2 h later. The donor splenocyte population consisted of >95% CD8+ cells, as estimated by FACS analysis. Transfer of WNV-immune CD8+ T cells reduced the mortality significantly and prolonged the MST of mice infected with a high dose (10^6 p.f.u.) of WNV (Table 1). Three mice recovered despite showing clinical signs, such as wasting, hunching and ruffling, and one mouse recovered even after the development of hindlimb paralysis. Recipient mice infected with a low dose (10^3 p.f.u.) of WNV did not show reduced mortality, but the MST was increased relative to that of control mice, although not statistically significantly (P=0.08), but this may indicate a trend. WNV reactivity of the transferred CD8+ T-cell population was necessary to obtain reduced mortality and increased MST, as transfer of CD8+ T cells from neither naive nor SFV-immune mice conferred protection (Table 1). SFV-immune Tc cells are not cross-reactive on flavivirus-infected target cells (Müllbacher et al., 1986).

To eliminate the possibility that residual (≤5%) non-CD8+ cells were fully or partially responsible for the observed protective effect of transferred WNV-immune
splenocytes, aliquots of enriched CD8+ T-cell population were treated with anti-mouse CD8 mAb and complement (see Methods). FACs analysis showed that, after anti-CD8 Ab treatment, the residual cell population contained 54% NK cells, 15% macrophages, 14% B cells, 1% CD4+ T cells and 3.5% CD8+ T cells. Transfer of about 10^6 of these cells did not confer protection when mice were subsequently infected with either 10^8 p.f.u. (mortality, 100%; MST, 6.1 ± 1.0) or 10^7 p.f.u. (mortality, 25%; MST, 10.5 ± 2.1) WNV.

### Table 1. Consequences of WNV-immune CD8+ T-cell adoptive transfer between wt mice

<table>
<thead>
<tr>
<th>Donor* (virus prime)</th>
<th>Recipient†</th>
<th>WNV infection (i.v., p.f.u. per mouse)</th>
<th>No. per group</th>
<th>No. dead mice</th>
<th>Time to death (days)</th>
<th>Mortality (%)</th>
<th>MST (days)</th>
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<tbody>
<tr>
<td>WNV 1 × 10^8</td>
<td>10</td>
<td>2</td>
<td>5</td>
<td>40‡</td>
<td>7.8 ± 3.2‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SFV 5</td>
<td>3</td>
<td>5</td>
<td>100</td>
<td>5.4 ± 0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mock 10</td>
<td>6</td>
<td>3</td>
<td>6</td>
<td>5.5 ± 0.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WNV 1 × 10^3</td>
<td>10</td>
<td>1</td>
<td>12</td>
<td>20</td>
<td>13.5 ± 2.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SFV 5</td>
<td>1</td>
<td>9</td>
<td>20</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mock 10</td>
<td>2</td>
<td>10</td>
<td>30</td>
<td>10.3 ± 0.6</td>
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</table>

*Donors were B6 wt mice immunized i.v. with 10^7 p.f.u. of either WNV or SFV in 100 μl HBSS/BSA, or mock-immunized i.v. with 100 μl HBSS/BSA 5 days prior to adoptive transfer.
†Recipients were B6 wt mice infected i.v. with 10^8 or 10^9 p.f.u. WNV 2 h after i.v. transfer of 2 × 10^7 CD8+ T cells from donor animals.
‡Statistically significant (P < 0.05) compared with mice in other two groups infected with 10^8 p.f.u. WNV (see text for details).

### WNV-immune CD8+ T-cell migration to the brain and DLNs after WNV infection

CD8+ T cells are the dominant leukocyte population in infiltrates in the brain after WNV infection (Liu et al., 1989a; Wang et al., 2003b). To investigate the recruitment and antigen specificity of those cells, 5 day WNV-immune splenocytes were enriched for CD8+ T cells, labelled with CFSE and 2 × 10^7 cells were transferred i.v. into B6 mice. Recipients were infected with 10^5 p.f.u. WNV s.c. into their hindlimb footpad and with 10^8 p.f.u. WNV into the tail vein 2 h after cell transfer. A high-dose i.v. infection of WNV was chosen, as it causes rapid viral invasion into the brain (within 24 h) and allows assessment of early migration of antigen-reactive CD8+ T cells into the brain and other sites of infection. The same animals were inoculated with a low dose s.c. to examine CD8+ T-cell migration to the DLNs. Popliteal DLNs and brains were removed at 3, 6, 12, 24, 48 and 72 h p.i. By using fluorescence microscopy, CFSE+ donor CD8+ T cells, which were WNV-immune, were readily detectable in the DLNs by 6 h p.i. The number of CFSE+ cells increased until 24 h post-transfer, then decreased [Fig. 1a(i–iii)]. In the brain, labelled cells were first detected at 48 h p.i., and increased for the next 24 h [Fig. 1a(iv–vi)]. In contrast, the number of CD8+ T cells derived from either naïve or SFV-infected B6 donors and labelled with CFSE was low or absent at any time point (see above) in the DLNs and brains of WNV-infected recipients. These observations indicate that activated WNV-immune CD8+ T cells migrate rapidly into the CNS and facilitate either viral clearance or immunopathology.

### Histology of brain after transfer of WNV-immune CD8+ T cells

To determine tissue damage and cell infiltration in the brain after WNV-immune CD8+ T-cell transfer and subsequent WNV infection, brains of moribund mice (for time points, see Table 1) were examined. Mice that received naïve or SFV-immune CD8+ T cells and were infected i.v. with either 10^8 or 10^3 p.f.u. WNV showed histological changes comparable to those of naïve B6 mice infected with the same doses of WNV [Fig. 1b(ii), (v); Wang et al., 2003b]. In contrast, the number of CD8+ T cells in brains of WNV-immune CD8+ T-cell recipients that recovered after infection with 10^8 p.f.u. WNV decreased [Fig. 1b(i–iii)]. In the brain, labelled cells were first detected at 48 h p.i., and increased for the next 24 h [Fig. 1b(iv–vi)]. In contrast, the number of CD8+ T cells derived from either naïve or SFV-infected B6 donors and labelled with CFSE was low or absent at any time point (see above) in the DLNs and brains of WNV-infected recipients. These observations indicate that activated WNV-immune CD8+ T cells migrate rapidly into the CNS and facilitate either viral clearance or immunopathology.
i.v., only moderate inflammation in their brain was evident at 21 days p.i., despite manifestation of clinical signs at 7–14 days p.i. (data not shown). This suggests that, in most of the animals that received WNV-immune CD8$^+$ T cells, infectious virus could be cleared efficiently in the periphery and/or CNS at the early stage of infection, therefore limiting viral spread and subsequent inflammation.

Lack of IFN-γ affects viral load and mortality only marginally in WNV-Sarafend- and KUN-infected mice

To determine the role of IFN-γ in the aetiology of WNV encephalitis, IFN-γ-deficient (IFN-γ$^{-/-}$) mice were infected i.v. with either $10^8$ or $10^5$ pf.u. WNV and the mortality and kinetics of virus replication in brains were investigated. Infection with the high virus dose resulted in reduced mortality of IFN-γ$^{-/-}$ mice (80%) and a slightly prolonged MST (6.9 ± 2.2) compared with wt mice [100% mortality, not statistically significant ($P>0.05$); MST, 5.8 ± 0.8 ($P=0.08$)] (Fig. 2a). Infection with the low virus dose produced no differences in mortality of IFN-γ$^{-/-}$ mice (75%) compared with wt mice (75%) (Fig. 2b), although the MST for IFN-γ$^{-/-}$ mice was decreased significantly (IFN-γ$^{-/-}$, 7.2 ± 3.2; wt, 10.6 ± 0.8; $P<0.01$).

Brain virus titres after infections with either high- or low-dose virus inocula (Table 2) were estimated in two separate...
experiments. After a high virus dose, virus titres in brains from 5 days p.i. onwards were very high (10^6–10^9 p.f.u.) in both wt and IFN-γ−/− mice. There was no statistically significant difference between these two strains 5 and 6 days p.i., nor was there any statistically significant difference between the two independent experiments (Table 2). With the low-dose inoculum, low virus titres were detected in brains of IFN-γ−/− mice 1–2 days earlier than in wt mice. Shrestha et al. (2006b) reported similar observations with WNV-NY, and this may reflect some marginal early effects of IFN-γ. However, virus doses were not statistically significantly different at any time point between these two strains, nor did we observe differences between the two separate experiments (see footnotes to Table 2).

In view of the surprising differences in susceptibility of IFN-γ−/− mice to WNV infection between our study and those of others who used the highly virulent lineage I WNV-NY (Shrestha et al., 2006b) and found a significantly beneficial effect of IFN-γ−/− in disease outcome, we used KUN in mortality studies to investigate this discrepancy further. KUN is an Australian WNV isolate closely related genetically to the WNV-NY isolate, although relatively avirulent in mice and humans (Scherret et al., 2002). KUN (10^6 p.f.u., i.v.) caused one death in a group of six wt B6 mice (with typical hindleg paralysis prior to death), but no IFN-γ−/− mice showed any sign of encephalitis (Fig. 2c), demonstrating that the absence of IFN-γ−/− also had no deleterious effect on recovery from a lineage I, albeit relatively avirulent, isolate of WNV.

**Absence of IFN-γ−/− does not affect precursor frequency or cytolytic potential of WNV-immune splenocytes**

To evaluate whether the WNV-immune Tc-cell response in IFN-γ−/− mice is compromised due to the lack of IFN-γ, we compared the precursor frequency as well as the potency of the primary in vivo Tc-cell response to WNV in IFN-γ−/− and wt B6 mice. To assess the strength of the primary ex vivo WNV-immune Tc-cell response, two mice of both strains were immunized i.v. with a single dose of WNV (10^7 p.f.u.). Six days later, individual spleen-cell suspensions were prepared and tested on 51Cr-labelled, H-2b-matched, MC57G target cells that were WNV- or mock-infected in a 6 h 51Cr-release assay. The Tc-cell responses from both wt and IFN-γ−/− mice were comparable over the range of effector:target ratios (Fig. 3a). The cytotoxicity was Tc cell-mediated, as the fibroblast target cells are poor NK-cell targets and flavivirus-infected targets are far less susceptible to lysis than uninfected targets, due to MHC class I upregulation (Momburg et al., 2001).

We also compared the frequencies of WNV-immune Tc-cell precursors in the spleens of IFN-γ−/− and wt mice after WNV infection. Mice were immunized with 10^5 p.f.u. WNV i.v. and, 8 days later, spleens were removed and set up for limiting-dilution analysis as described in Methods. The results (Fig. 3b) show that wt and IFN-γ−/− mice have similar numbers of WNV-immune Tc-cell precursors (1:10,800 and 1:12,000, respectively). These values are comparable to those of MVEV-immune Tc-cell precursors in MVEV-infected CBA (H-2b) mice (Regner et al., 2001).

**IFN-γ involvement in brain inflammation**

To investigate further the role of IFN-γ in WNV infection of the CNS, groups of wt and IFN-γ−/− mice were infected with 10^7 p.f.u. WNV i.v. and leukocytes in the brain were isolated by density-gradient centrifugation. Leukocytes in brains from randomly selected infected wt and IFN-γ−/− mice for each time point were measured (Fig. 4). In both wt and IFN-γ−/− mice, CD8+ T cells were first observed in the brain at day 5 p.i., with peak numbers on day 9 p.i. (Fig. 4), which was consistent with our previous study (Wang et al.,...
Table 2. Virus growth in the brain of B6 wt and IFN-γ−/− mice infected with a high dose (10^8 p.f.u.) or low dose (10^3 p.f.u.) of WNV

<table>
<thead>
<tr>
<th>Time p.i. (days)</th>
<th>Wild-type</th>
<th>IFN-γ−/−</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Exp I</td>
<td>Exp II</td>
</tr>
<tr>
<td>High dose</td>
<td></td>
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<tr>
<td>5</td>
<td>3.2 × 10^3</td>
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<tr>
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<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>Low dose</td>
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</tr>
<tr>
<td>5</td>
<td>&lt;10^7</td>
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<tr>
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<td>3.5 × 10^9</td>
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<tr>
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<td>&lt;10^7</td>
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<tr>
<td>9</td>
<td>&lt;10^7</td>
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<td>2.9 × 10^7</td>
</tr>
<tr>
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<td>5.0 × 10^7</td>
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<tr>
<td>12</td>
<td>2.9 × 10^8</td>
<td>1.3 × 10^6</td>
</tr>
</tbody>
</table>

*In experiment I, brains were harvested from moribund or dead mice as part of the mortality study shown in Fig. 2(a, b).
†In experiment II, mice were selected randomly from groups of wt or IFN-γ−/− mice infected with 10^3 or 10^8 p.f.u. WNV-Sarafend and virus titre in the brains was detected by plaque assay.
‡Cohorts with the same letter were compared for statistical significance by using Student’s t-test. None was significant.
§Brain harvested from a dead mouse where death had occurred over a period of <16 h.
‖Only one out of three wt mice survived for 8 days p.i. Note that 10^8 p.f.u. WNV kills 100% of wt mice at ≤8 days p.i.
¶Only one out of three IFN-γ−/− mice survived for 12 days p.i.
#Limit of detection: ≥10^7 p.f.u. (g tissue)^{-1}.
assessed by limiting-dilution analysis.

To compare the in vivo antiviral effects of CD8$^+$ T cells from wt and IFN-γ$^{-/-}$ mice, WNV-immune CD8$^+$ T cells from IFN-γ$^{-/-}$ mice were transferred into WNV-infected wt recipients. IFN-γ$^{-/-}$ donors were immunized i.v. with $10^8$ p.f.u. WNV. After 5 days, splenocytes were harvested and CD8$^+$ T cells were selected negatively. The enriched WNV-immune CD8$^+$ T cells were then transferred i.v. to wt recipients, followed by an i.v. infection of either $10^8$ or $10^3$ p.f.u. WNV after 2 h. Results of the experiment are shown in Table 3. WNV-immune CD8$^+$ T cells from IFN-γ$^{-/-}$ mice showed an in vivo antiviral effect comparable to that seen with wt WNV-immune CD8$^+$ T cells (Table 1). This can be concluded because transfer of WNV-immune CD8$^+$ T cells derived from IFN-γ$^{-/-}$ mice did not change the mortality rate or MST significantly in the groups of recipients compared with those of mice that received WNV-immune CD8$^+$ T cells from wt donors. The fact that no statistically significant differences in mortality and MST between IFN-γ$^{-/-}$ and wt donors were observed indicates that IFN-γ does not play a significant role in the host response to WNV-Sarafend infections, especially given that the effector function of CD8$^+$ T cells is not impaired by an IFN-γ deficiency.

**DISCUSSION**

The prevalence of CD8$^+$ T cells in the CNS of WNV-infected mice (Liu et al., 1989a; Wang et al., 2003b) is indicative of their participation in recovery and/or immunopathology in WNV encephalitis. By using an adoptive-transfer model, we could show clearly that WNV-immune CD8$^+$ T cells exert a protective effect in an antigen-specific manner. In particular, mortality was reduced sharply when high doses of WNV were used for infection, and mice with severe signs of encephalitis, such as hindlimb paralysis, were able to recover from infection. By using a low dose of WNV, no change in mortality rate was evident, but the survival time of moribund mice was prolonged significantly as a result of WNV-immune CD8$^+$ T-cell transfer. This indicates that a vigorous and rapid
Tc-cell response is beneficial in WNV encephalitis. Our previous finding that defects in either exocytosis itself (perforin plus granzymes) or perforin- plus Fas-mediated cytolytic mechanisms, but not perforin deficiency alone, increase the severity of WNV encephalitis (Wang et al., 2004) is consistent with a recovery role for WNV-immune Tc cells. These observations and interpretation contrast sharply with disease outcomes observed with the closely related encephalitic flavivirus MVEV (Licon Luna et al., 2002), where defects in the cytolytic machinery of NK and Tc cells enhance the survival rate of infected mice. It is also at variance with results obtained by Shresta et al. (2006a) using the highly virulent WNV-NY, who showed that perforin deficiency influences virus load and disease outcome greatly. Given the similarity of the pathophysiology of these viruses in mice, these differences are at present not readily explainable, but could be the consequence of a differential capacity for extraneural growth of these encephalitic flaviviruses. This is in fact observed when virus titres are compared (Wang et al., 2003b; Shrestha et al., 2006a).

Besides cytolytic function, CD8+ T cells also exert an effector function via the secretion of cytokines, in particular IFN-γ (Boehm et al., 1997). To evaluate the role, if any, of IFN-γ in WNV encephalitis, we used IFN-γ−/− mice and determined their sensitivity to WNV infection. Similar to MVEV infection (Lobigs et al., 2003b), lack of IFN-γ had only marginal effects on disease outcome, with a slight increase in mortality (30–60%), but not statistically significant increases in MST. We observed a slight reduction in mortality at the high dose of infection and a slightly shortened, but not statistically significant, MST at the low dose of infection in IFN-γ−/− compared with wt mice (Fig. 2). However, both the lytic potential and the precursor frequency of WNV-immune Tc cells were unaffected in the absence of IFN-γ. Similar observations of the strength of the virus-immune Tc-cell response in the absence of IFN-γ have been made in response to Sendai virus (Mo et al., 1997) and murine gammaherpesvirus 68 (Sarawar et al., 1997) infections. Adoptive transfer of activated WNV-immune Tc cells from IFN-γ−/− mice (Table 3) did give outcomes similar to that when WNV-immune Tc cells from wt mice were given (Table 1). Thus, lack of IFN-γ seems to have little impact on WNV infection in the mouse when strains Sarafend or KUN are used. This contrasts with most other viral infections in mouse models, where IFN-γ is either essential for host survival, such as in infections with poxvirus (Ramshaw et al., 1997) and SFV (alphavirus) (Alsharifi et al., 2006), or is required for virus clearance, as is the case with Lymphohytic choriomeningitis virus (Bartholdy et al., 2000; Ou et al., 2001), influenzavirus (Karupiah et al., 1998a), mouse hepatitis virus (Parra et al., 1999) and mouse cytomegalovirus (Lucin et al., 1992). During the course of this study, Wang et al. (2003a) and Shrestha et al. (2006b) reported that IFN-γ-deficient mice were more susceptible to the highly virulent NY isolates of WNV than wt B6 mice and that γδ T cells were the principal producers of IFN-γ early during infection. The most likely explanation for this discrepancy in IFN-γ-dependent resistance to WNV infection is the use of WNV strains of different virulence. Our study used the only moderately virulent Sarafend and even less virulent KUN isolates, whilst Wang et al. (2003a) used a recent isolate from the New World, which has been shown to have increased neuroinvasiveness and is more efficient in extraneural replication (Beasley et al., 2002). This may also explain the differences found in the requirement of perforin in controlling WNV-NY versus WNV-Sarafend (Shrestha et al., 2006a; Wang et al., 2004). The discrepancy between the outcome of our study and those of the other two studies is another example that illustrates that host–virus interactions, dependent on immune responses, cannot be generalized even between the most closely related viruses (Müllbacher et al., 2004). In addition, the different routes of infection chosen in the study by Wang et al. (2003a) (s.c.) versus i.v. in the study here, together with possible variability in background genes in the ‘inbred’ knock-out strains, may have contributed to the differing outcomes.

The role of IFN-γ in recovery from human WNV infections remains elusive. With few exceptions (Komatsu et al., 1996), there is little evidence that IFN-γ has direct antiviral activity, but rather that it is a potent immune regulator that

<table>
<thead>
<tr>
<th>Donor*</th>
<th>WNV infection (i.v., p.f.u. per mouse)</th>
<th>No. per group</th>
<th>No. dead mice</th>
<th>Time to death (days)</th>
<th>Mortality (%)†</th>
<th>MST‡ (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ−/−</td>
<td>1 × 10⁸</td>
<td>8</td>
<td>1</td>
<td>6</td>
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<td>8.5 ± 1.7</td>
</tr>
<tr>
<td>IFN-γ−/−</td>
<td>1 × 10⁸</td>
<td>8</td>
<td>1</td>
<td>10</td>
<td>25</td>
<td>11.0 ± 1.0</td>
</tr>
</tbody>
</table>

*Donors were immunized i.v. with 10⁵ p.f.u. WNV in 100 μl HBSS/BSA 5 days prior to adoptive transfer.
†Recipients were B6 wt mice infected i.v. with 10⁸ or 10⁷ p.f.u. WNV 2 h after i.v. transfer of 2 × 10⁷ CD8+ T cells from donor animals.
‡Mortality and MST values are comparable to those obtained following transfer of CD8+ T cells from WNV-immunized wt mice (Table 1).
facilitates antiviral outcomes (Boehm et al., 1997; Landolfo et al., 1988). Upregulation of antigen presentation via the MHC class I and class II pathways is one of the major consequences of IFN-γ signalling and has been suggested to be key in the IFN-γ-induced response necessary to clear adenovirus infections (Yang et al., 1995). It is well documented from our work (Liu et al., 1988; Lobigs et al., 2003a; Momburg et al., 2001; Müllerbach & Lobigs, 1995; Müllerbach et al., 2003), as well as from that of others (King & Kesson, 2003; King et al., 2003), that infection with flaviviruses upregulates MHC class I and II cell-surface expression, mimicking the effect of IFN-γ treatment. Thus, we speculate that the absence of a prominent phenotype in several models of flavivirus infection of IFN-γ−/− mice is due to the ability of flaviviruses to mimic IFN-γ-induced outcomes. Support for this notion comes from two recent studies on the control of coronavirus infections of the CNS, which require perforin and IFN-γ in the absence of CD4+ T cells (Bergmann et al., 2004), and the latter is required for upregulation of MHC class I (Bergmann et al., 2003). It would be of interest to compare the differentially IFN-γ-sensitive WNV isolates (WNV-NY versus WNV-Sarafend and/or KUN) in their ability to influence class I MHC cell-surface expression.

REFERENCES


CD8^+ T cells in WNV encephalitis


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