13. CD4$^+$ and CD8$^+$ T-Cell Immune Responses in West Nile Virus Infection

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Abstract

CD4+ and CD8+ T cells, in combination with the innate and humoral immune responses, are critical for recovery of mice from infection with West Nile virus. Mouse models of West Nile virus encephalitis are therefore excellently suited to investigate the role of T cells in the balance between viral clearance and CNS disease following infection with a cytopathic and neurotropic virus. Here we review the in vitro properties of West Nile virus-immune T cell responses, their in vivo disease ameliorating and potentiating effects, and the contribution of the different T cell effector functions to disease outcome.

Keywords

flavivirus; viral encephalitis; cellular immunity

1 Role of T Cells in Viral Infection: An Overview

A role for T cells in recovery from viral infection in humans has been suspected from the empirical observation that individuals with genetically impaired T-cell development are highly susceptible to viral infections, while B-cell deficiency generally leads to uncontrollable bacterial infections (Fulginiti et al., 1968). Thus, an understanding of the antiviral T-cell response in humans is vital to advance our ability to prophylactically and therapeutically intervene in clinical infections.

The mouse, *Mus musculus*, has become over the past 100 years the animal model of choice for biomedical research (Fenner, 1982). As for viral disease models in mice, two have been the most extensively studied and have provided fundamental insights into host/viral pathogen interactions: Mouse pox, infection with the natural mouse pathogen ectromelia

virus, proved an excellent model for an acute viral infection and its pathogenesis was elegantly described (Fenner, 1949). The role of cytolytic T cells in this infection model as essential mediators in recovery was established by Blanden in the early 1970s (Blanden, 1974). Infection of mice with lymphocytic choriomeningitis virus is the second model, which has provided enormous basic understanding on the role of T cells in persistent viral infections and has become the principal model for elucidating virus-induced immunopathology (Doherty and Zinkernagel, 1974; Lehmann-Grube, 1982). Recent studies into the role of immune T cells in flavivirus infection showed that both beneficial (recovery) as well as detrimental aspects (immunopathology) are observed as a result of T-cell function during encephalitic episodes (Camenga et al., 1974; Licon Luna et al., 2002; Müllbacher et al., 2003; Wang et al., 2003b, 2004b; Samuel et al., 2007) Accordingly, both classical mouse models are of relevance to our appreciation of T-cell-mediated immunity in flavivirus infection.

Phenotypically, T cells have been subdivided on the basis of their antigen receptor usage (α/β vs. γ/δ), their co-receptor expression (CD4 vs. CD8) or their functionality (cytotoxic vs. helper). Functionally, T cells can belong to either of the antigen receptor usage or co-receptor expressing subsets, although cytolytic T (Tc) cells are predominantly of $CD8^+$ and helper T (Th) of $CD4^+$ phenotype. Both α/β and γ/δ T cells can express cytolytic activity as well as release cytokines (Carding and Egan, 2002). While γ/δ T cells recognize non-classical MHC antigens in the mouse as part of the innate immune response, α/β T cells recognize MHC class I or II plus peptide antigen as mediators of adaptive immunity (Rudolph et al., 2006). Members of all the above subsets of T cells have been reported to play a role in the immune response to WNV, albeit not always in a protective capacity (see below).

Early research on T-cell immunity in flavivirus infection focused on the Tc cell responses. Gajdosova et al. (1980, 1981) first obtained cytotoxicity of ex vivo derived splenocytes from mice immunized with one of several flaviviruses, including a European isolate of WNV. This early report already demonstrated a broad cross-reactivity of effector cells on targets infected with distantly related members of the genus, flavivirus. A more detailed analysis of the Tc cell response to flavivirus infection was undertaken by Kesson et al. (1987, 1988) using WNV, strain *Sarafend*. One of the reasons for the choice of WNV *Sarafend* as the preferred experimental model for investigating Tc cell responses in flavivirus infection over other related viruses, such as Kunjin virus (now classified as a strain of WNV; Scherret et al., 2001), Japanese encephalitis

virus (JEV) or Murray Valley encephalitis virus (MVEV), was that it induced a substantially stronger Tc cell response in most mouse strains as measured by in vitro target cell lysis using the ^{51}Cr release assay, than the other viruses (Hill et al., 1992, 1993) for reasons as yet poorly understood.

More recent advances in our understanding of T-cell immunity of both helper and cytotoxic phenotype against WNV became possible with the use of vaccinia virus expression vectors encoding regions of the WNV polyprotein for the mapping and characterization of T-cell determinants (Parrish et al., 1991) and the use of knock-out mouse strains for the evaluation of the contribution of effector functions of the cellular immune responses in recovery from infection or disease exacerbation (see below).

2 WNV-Immune $CD4^+$ and $CD8^+$ T-Cell Responses, In Vitro

2.1 The $CD4^+$ T-Cell Response

Early evidence from clones of L3T4 T cells ($CD4^+$) derived from splenocytes of mice immunized with either WNV *Sarafend*, Kunjin virus or MVEV showed that they were highly cross-reactive (Uren et al., 1987) and responded to heterologous virus stimulation with a similar levels of proliferation and cytokine release as in response to the homologous virus. This indicated that some of the viral peptide determinants presented by MHC class II are conserved between these viruses. Subsequent studies showed that synthetic peptides selected from the E protein sequences of WNV, JEV and dengue virus (Kutubuddin et al., 1991) or from that of MVEV (Mathews et al., 1991) based on predictive algorithms, elicited virus-specific as well as flavivirus cross-reactive proliferative responses of virus-immune splenocytes. WNV cross-reactivity of human JEV-immune $CD4^+$ T cells has also been observed and, comparable to that found in mice, extends to distantly related flaviviruses (Aihara et al., 1998). The strong cross-reactivity in the $CD4^+$ T-cell response between members of the JEV serocomplex, which includes WNV (Kuno et al., 1998), could be one of the critical immunological correlates for the cross-protection noted between these viruses following live virus infection with one of the members of the serocomplex (for instance, Goverdhan et al., 1992; Monath, 2002; Tesh et al., 2002).

Assays for in vitro $CD4^+$ T-cell proliferative responses were optimized and applied to the mapping of the antigenic determinant(s)

recognized by WNV-immune $CD4^+$ T cells in the context of a suite of mouse MHC class II alleles by Kulkarni et al. (1991a–c, 1992), using a panel of vaccinia virus recombinants encoding together the entire Kunjin virus polyprotein. The MHC class II molecules of most mouse haplotypes presented determinants derived from the NH_2-terminal one-third of the polyprotein, which encodes the viral structural (C, prM and E) and the non-structural NS1 proteins. This was not unexpected, given that $CD4^+$ T cells provide "help" to B cells responsible for the humoral antiviral immune response, which is primarily directed against the E and, to a lesser degree, the NS1 proteins in flavivirus infections. The precise peptide determinants recognized by WNV-immune $CD4^+$ T cells in the context of different mouse and human MHC class II alleles remain to be identified. $CD4^+$ T-cell clones from human PBL donors immunized with an inactivated JEV vaccine also exhibited cross-reactivity with WNV and predominantly recognized peptides derived from the E protein (Aihara et al., 1998). Intriguingly, these $CD4^+$ T-cell clones exhibited cytolytic function, in vitro, the relevance of which during live virus infection is uncertain; the phenomenon has been discussed in an earlier review from our group (Müllbacher et al., 2003).

2.2 The $CD8^+$ T-Cell Response

More is known in regard to the $CD8^+$ T-cell response against WNV, in vitro, than the $CD4^+$ T-cell response, in part due to the availability of a sensitive and robust in vitro assay with functional read-out, the ^{51}Cr release assay. The first detailed analysis of conditions required for the generation of flavivirus-immune secondary, in vitro stimulated, Tc cells was by Kesson et al. (1988) using the WNV *Sarafend* model. One consistent observation made in this and subsequent studies was the high cytolytic activity of secondary flavivirus-immune Tc cells against uninfected target cells following in vitro re-stimulation of primed splenocytes with virus-infected stimulator cells (Lobigs et al., 1996). Interestingly, this high anti-self cytolytic activity was much reduced or not apparent when virus-derived peptides were used to modify stimulator cells (Regner et al., 2001c; Müllbacher et al., 2003). We have suggested previously (Lobigs et al., 1996) that the up-regulation of MHC class I on flavivirus-infected cells is one possible explanation for this phenomenon, given that the associated increased presentation of self peptides via MHC class I may exceed the activation threshold of self-reactive Tc cells. Analysis of the mechanism for flavivirus-mediated up-regulation of MHC class I revealed that WNV and other flavivirus infections

increase the supply of peptides into the lumen of the endoplasmic reticulum, thereby augmenting the rate of assembly and level of cell surface expression of the class I restriction elements (Müllbacher and Lobigs, 1995; Momburg et al., 2001; Lobigs et al., 2003a).

Mapping of the peptide determinants recognized by WNV-immune Tc cells, in the context of MHC class I of various haplotypes, was first undertaken with the use of vaccinia virus recombinants encoding polyprotein regions from Kunjin virus (Hill et al., 1992, 1993) or MVEV (Lobigs et al., 1994, 1997). The dominant peptide determinants for most class I MHC alleles mapped to the non-structural region of the polyprotein with the NS3 protein as the major source. A similar non-random distribution of immunodominant Tc cell determinants on the viral polyprotein has been noted for other flaviviruses (for instance, van der Most et al., 2002; Rothman, 2003; Kumar et al., 2004). Given that all flaviviral proteins are synthesized as part of a single polyprotein in equimolar ratio, it is uncertain why this should be the case. One explanation is that the polyprotein region, which is the source of most Tc cell determinants, is subject to rapid proteolytic degradation, or forms part of short-lived alternative cleavage products. Only in the H-2^b haplotype was a significant WNV (Kunjin strain) peptide determinant mapped one-third of the polyprotein to the NH_2-terminal, in addition to another determinant found in the NS4B protein (Hill et al., 1992). This has recently been confirmed by identification of peptides from these regions of WNV (North-American isolates) with the strongest response directed against a D^b-restricted peptide derived from the NS4B protein: SSVWNATTA (single letter amino acid code; Brien et al., 2007; Purtha et al., 2007). Unprecedented $CD8^+$ T-cell cross-reactivity between the flaviviruses at large and not only between closely related viruses became apparent from this research yet again. Most surprisingly, this cross-reactivity could not simply be explained by amino acid sequence conservation, but was dependent on homologous location of the determinants on the polyprotein of the different flaviviruses (Regner et al., 2001a). This led us to propose that highly conserved structural imprints of the NS3 protein on peptide determinants recognized by the class I restriction element, but not primary amino acid sequence of the peptides, can account for cross-recognition in the Tc cell response between distantly related members of the flavivirus genus (Regner et al., 2001a).

$CD8^+$ T cells express their effector function via two distinct pathways: one by the release of cytokines such as interferon-γ (IFN-γ), a potent immune regulator, and the other by cellular cytotoxicity either via the

death ligand/death receptor (e.g. FasL/Fas) pathway or alternatively via the exocytosis pathway mediated by perforin and granzymes (see below). $CD8^+$ T cells generated as a result of WNV *Sarafend* infection are able to lyse target cells in vitro via either of the cytolytic pathways (our unpublished observations), which corroborates the in vivo phenotypes observed when mice with deficiencies of one or the other or both cytolytic pathways are used as infection models for WNV (see below). Little is known in regard to IFN-γ release by WNV-immune $CD8^+$ T cells; however, it has been observed that MVE-immune Tc cells, when stimulated in vitro with virus-infected stimulator cells, express cytolytic effector function in the absence of IFN-γ production (Regner et al., 2001b). It appears that the generation of cytolytic activity is independent of the presence of IFN-γ during priming in vivo or boosting in vitro with WNV *Sarafend* (Wang et al., 2006).

3 Role of $CD4^+$ T Cells in Recovery from WNV Infection

$CD4^+$ T lymphocytes provide helper function to B cells to orchestrate the humoral immune response (Bishop and Hostager, 2001; Mills and Cambier, 2003) and, in some instances, to $CD8^+$ T cells for elicitation of sustained Tc cell effector and/or memory responses (Bevan, 2004; Castellino and Germain, 2006). They also can restrict viral replication by secreting cytokines (e.g. IFN-γ) (Ramshaw et al., 1997; Binder and Griffin, 2001) and can directly kill virus-infected cells by Fas- and granule exocytosis-mediated cytotoxic mechanisms (Heller et al., 2006). There is ample evidence for the vital role of antibody in the control of WNV infection (for instance, Camenga et al., 1974; Diamond et al., 2003a, c); hence, $CD4^+$ T-cell help, required for IgM-to-IgG antibody isotype switching, is expected to be a critical component of the adaptive immune response against the virus, given that IgM antibody has a much shorter half-life in serum than IgG antibody (2 and 6–8 days, respectively) (Vieira and Rajewsky, 1988) and the improved ability of IgG to diffuse into inflamed lesions (Hangartner et al., 2006). However, one report (Halevy et al., 1994) alludes to a comparable resistance of BALB/c nude mice (which are deficient in functional T cells) to intraperitoneal WNV infection (Israel 1952 strain) relative to immunocompetent ICR mice, suggesting a significant contribution of T-cell-independent (IgM) antibody in recovery. Viruses which display a highly organized, repetitive, antigenic structure can induce B-cell responses in the absence of T-cell help due to the ability to extensively cross-linked B-cell receptors,

thereby delivering strong activation signals to B cells (Bachmann et al., 1993; Hangartner et al., 2006); the WNV surface envelope (E) protein conforms with this requirement (Nybakken et al., 2006). T-cell-independent IgM production at levels sufficient to clear extraneural virus replication with a kinetics identical to that in immunocompetent mice was also found in $CD4^+$ T-cell-depleted or T-cell-deficient ($CD4^{-/-}$ and MHC class $II^{-/-}$) C57Bl/6 mice following infection with a virulent strain (New York isolated from 2000) of WNV by the subcutaneous route (Sitati and Diamond, 2006). However, in the absence of functional $CD4^+$ T-cell responses, IgM antibody titres were not maintained; a precipitous decline of virus-reactive serum IgM occurred at 15 days post-infection in the absence of a vigorous IgG response (100–1,000-fold lower than in wild-type mice) and despite high virus titres in brain and spinal cord. All $CD4^+$ T-cell-deficient or T-cell-depleted mice succumbed to the infection, although with different kinetics: groups of $CD4^{-/-}$ mice showed an average survival time of 11 days, which was similar to the fraction (~30%) of wild-type mice that died, while $CD4^+$ T-cell-depleted and MHC class $II^{-/-}$ mice displayed a protracted time to death of up to 50 days post-infection (Sitati and Diamond, 2006). CD40 ligand-deficient mice, which are defective in a co-stimulatory membrane protein required for producing $CD4^+$ T-cell help for B-cell responses, produced a similar picture to that of $CD4^{-/-}$ mice when infected with WNV: virus clearance from extraneural tissues and $CD8^+$ T-cell priming were not affected; however, $CD8^+$ T-cell trafficking into the CNS was impaired, allowing the virus to grow to high titres in brain and spinal cord; all mice succumbed to infection in contrast to 70% survival in groups of wild-type mice (Sitati et al., 2007). Collectively, the data show that in the absence of functional $CD4^+$ T-cell responses a persistent and uniformly lethal WNV infection is established, which is cleared from extraneural sites, most likely by IgM antibody (Diamond et al., 2003c) and $CD8^+$ T cells (Wang et al., 2003b; Shrestha and Diamond, 2004), but not from the CNS. Sitati and Diamond (2006) also showed that $CD4^+$ T cells are important for sustaining the $CD8^+$ T-cell response against WNV, and that a marked decline in WNV-immune $CD8^+$ T cells at 15 days post-infection accounts, at least in part, for the inability of $CD4^+$ T-cell-depleted and MHC class $II^{-/-}$ mice to clear the virus from the CNS. It is not clear if the reported resistance of nude (athymic) mice against WNV infection (Halevy et al., 1994), which contrasts with the uniform susceptibility of mice lacking functional $CD4^+$ T cells (Sitati and Diamond, 2006), is reflected in complete elimination of the virus or whether delayed mortality would occur, as has been described for infections of

nude mice with JEV (Lad et al., 1993); alternatively, WNV isolated with different virulence properties (Beasley et al., 2002) may have been used in the two studies.

4 Role of CD8$^+$ T Cells in Recovery from WNV Infection

The role of CD8$^+$ T cells in recovery from WNV infection has been investigated in two mouse models of WNV encephalitis: 6-week-old C57Bl/6 mice infected with 10^3 PFU of the *Sarafend* strain by intravenous injection (Wang et al., 2003b) and 8–10-week-old C57Bl/6 mice infected with 10^2 PFU of the New York 2000 strain by the subcutaneous route into the foot pad (Shrestha and Diamond, 2004). In both models mortality was approximately 30% with a mean time to death of 10–12 days. Growth in extraneural tissues was more readily detectable in infections with the New York 2000 WNV strain, which produced viraemia in the first 4 days of infection and a viral burden in spleen on days 4 and 6, post-infection, while the *Sarafend* strain failed to give measurable viraemia but produced low virus titres in spleen and lymph nodes on days 4 and 5 post-infection in about half of the infected animals. Virus entry into the brain occurred slightly faster in infections with the more virulent New York 2000 strain (at 4–6 days, post-infection) (Diamond et al., 2003a) than infections with the *Sarafend* strain (at 6–8 days post-infection) (Wang et al., 2003b) and the frequency of CNS invasion was lower for the latter.

CD8$^+$ T cells were essential in the control of WNV infection in both mouse models. Depletion of CD8$^+$ T cells resulted in increased mortality and 100–1,000-fold greater viral burden in the brains of WNV *Sarafend*-infected mice (Wang et al., 2003b). A similar outcome was observed in congenic CD8$^{-/-}$ mice and mice defective in MHC class I-restricted antigen presentation due to deletion of β2-microglobulin (β2-m$^{-/-}$) or that of the classical MHC class I restriction elements (MHC-Ia$^{-/-}$). In β2-m$^{-/-}$ mice infected with WNV *Sarafend*, earlier CNS invasion and greatly increased virus load in the brain and mortality were found relative to wild-type mice. WNV infection of CD8$^{-/-}$ mice with the New York 2000 strain gave 80–90% mortality with virus persistence in spleen for ≥11 days and up to 1,000-fold increased viral load in the CNS with concomitant increased number of infected neurons and cytopathology (Shrestha and Diamond, 2004). Interestingly, the small number of CD8$^{-/-}$ and MHC-Ia$^{-/-}$ mice that survived infection with WNV (New York 2000 strain) showed residual virus in the brain even at 35 days, post-infection (Shrestha and Diamond, 2004). Finally, transfer

of WNV-primed CD8+ T cells also conferred disease amelioration to WNV-challenged recipient mice (Shrestha et al., 2006a; Wang et al., 2006). Accordingly, for both lineage I and lineage II WNV infections, CD8+ T cells are essential (1) for the elimination of virus from extraneural tissues to reduce the incidence and delay the kinetics of virus entry into the CNS and (2) for suppression of virus replication and clearance of virus-infected cells from the CNS by immune effector mechanisms, which allow recovery of the animals.

5 Effector Mechanisms of WNV-Immune T Cells: Cytokines and Cytolytic Pathways

The effector functions of antiviral T cells can be divided into those mediated by soluble molecules (cytokines) and those that are mediated via direct contact between effector and antigen-presenting target cell. CD4+ T cells produce, depending on their polarization during activation, predominantly Th1 cytokines (IFN-γ, IL-2) or Th2 cytokines (IL-4, IL-5, IL-13), or Th17 (IL-17), of which the former are considered beneficial effector cytokines to intracellular pathogens such as viruses (Boehm et al., 1997). Effector CD8+ T cells and γδ T cells, as well as natural killer cells produce IFN-γ and TNF-α in response to cognate antigen. Furthermore, CD8+ T cells destroy cells that they recognize as virally infected (or transformed) via two distinct mechanisms: (1) ligation of the death receptor Fas (CD95) on the target cell by FasL (CD178) on the T cell or (2) via exocytosis of specialized granules that contain cytotoxic proteins, the pore-forming protein perforin and the pro-apoptotic serine proteinases, granzymes amongst others (Trapani and Smyth, 2002).

5.1 Cytokines

Historically, there is little evidence for a direct antiviral effect of IFN-γ, i.e. through inhibition of virus replication in an infected cell, although IFN-γ has recently been reported to inhibit growth of WNV in bone marrow-derived dendritic cells, in vitro (Shrestha et al., 2006b). The main effects contributing to a successful antiviral immune response are indirect. IFN-γ promotes differentiation of CD4+ T cells into Th1-type cytokine producing cells, thereby ensuring production of immunoglobulin isotypes most beneficial in antiviral immune responses, activation of macrophages and natural killer cells as well as cytolytic function in

$CD8^+$ T cells and antigen presentation via the MHC class I and II pathways (Boehm et al., 1997).

IFN-γ-deficient mice succumb to infection with the virulent, North-American lineage I (Wang et al., 2003a; Shrestha et al., 2006b), but not the less virulent Kunjin or lineage II *Sarafend* (Wang et al., 2006) WNV strains. Interestingly, the main IFN-γ-producing cell type mediating this cytokine's protective effect are γδ T cells (Wang et al., 2003a; Shrestha et al., 2006b). They proliferate early in response to WNV infection (Wang et al., 2003a), and limit WNV replication in peripheral lymphoid organs, apparently delaying CNS invasion by the virus (Shrestha et al., 2006b).

$CD8^+$ T cells do not seem to employ IFN-γ against WNV, since transfer of WNV-primed IFN-γ-deficient $CD8^+$ T cells efficiently controls lineage I (Shrestha et al., 2006a) and lineage II (Wang et al., 2006) WNV infections. IFN-γ also does not seem to play a major role in the induction of anti-WNV $CD8^+$ T cells, as the cytolytic potential as well as the precursor frequency of antiviral $CD8^+$ T cells are the same in IFN-$\gamma^{-/-}$ as in wild-type mice (Wang et al., 2006). A marginally improved survival was observed when WNV-primed, IFN-γ-sufficient $CD4^+$ cells were adoptively transferred into challenged recipients, compared to IFN-$\gamma^{-/-}$ $CD4^+$ cells, although the data did not reach statistical significance (Sitati and Diamond, 2006).

TNF-α is a second pleiotropic cytokine that may be secreted by effector T cells. It activates neurovascular endothelium and attracts neutrophils (King et al., 2003), can cause cell death in susceptible cells and is a potent regulator of endothelial permeability (Tracey and Cerami, 1994).While the immunological role of TNF-α in WNV infection remains unclear, TNF-mediated inflammation has been shown to trigger, in response to Toll-like receptor-3 activation, increased permeability of the blood-brain barrier, resulting in CNS invasion by the virus (Wang et al., 2004a).

5.2 Cytotoxicity

FasL-mediated cytotoxicity contributes to the control of the New York 2000 (Shrestha and Diamond, 2007) but not *Sarafend* (Wang et al., 2004b) strains of WNV. Lack of FasL (*gld* mice) increased the viral burden and delayed clearance from the CNS compared with wild-type mice infected with the former strain. Interestingly, Fas expression is augmented on WNV-infected neurons (Shrestha and Diamond, 2007), suggesting they may become more vulnerable to attack by this pathway.

The cytotoxic granules of effector CD8+ T cells contain a variety of proteins, but the pore-forming protein, perforin, is considered essential in mediating target cell death by granule exocytosis (although human CD8+ cells also contain another pore-forming protein, granulysin) (Krensky and Clayberger, 2005). Perforin is able to form lytic pores in target cell membranes at high concentrations in vitro, leading to osmotic failure and necrotic death. However, its in vivo function is likely to deliver pro-apoptotic enzymes such as granzymes into the target cell cytosol (Voskoboinik et al., 2006). No significant difference in susceptibility to infection with WNV *Sarafend* was observed between wild-type and perforin-deficient mice (Wang et al., 2004b). In contrast, infection of mice with the New York 2000 isolate demonstrated a significant effect of perforin in recovery from the infection (Shrestha et al., 2006a). Perforin-deficiency led to an increase in mortality, despite similar kinetics and magnitude of viraemia as well as similar kinetics of CNS invasion relative to wild-type mice, although viral load in the brain was increased, and increased neuronal infection evident. Furthermore, perforin deficiency led to viral persistence in those mice that survived. Provided they were perforin sufficient, primed anti-WNV CD8+ T cells could, when transferred into challenged recipients, reduce peripheral but not CNS viral load.

The discrepancy between two strains of WNV as well as a second JEV serocomplex flavivirus, MVEV (Licon Luna et al., 2002), in the role of CD8+ T-cell effector molecules (perforin, FasL, IFN-γ) in recovery from infection (Table 1) suggests that extrapolation on the importance of specific immune mechanisms even between closely related viruses must be considered with caution (Müllbacher et al., 2004). The complex interplay between beneficial and immunopathological effects of cytolytic effector molecules may not be revealed in sufficient detail when only survival and viral load are investigated. Thus, one contributing factor to the substantial difference in disease outcome between infections of perforin$^{-/-}$, *gld* and IFN-γ$^{-/-}$ mice vs. wild-type mice with North-American, Kunjin or *Sarafend* strains of WNV may be their different virulence properties (Beasley et al., 2002): whereas CD8+ T-cell cytotoxicity may lead to net immunopathology in the less virulent *Sarafend* and Kunjin strains, the greater virulence of the North-American WNV isolates may render removal of infected cells in the CNS obligatory, despite the destructive nature of Tc cell-mediated cytotoxicity.

The role of granzymes have only been investigated in the mouse model of infection with WNV *Sarafend*, where low-dose infection of granzyme A × B$^{-/-}$ and perforin × granzymes A × B$^{-/-}$ mice increased

Table 1. Contribution of CD4⁺ and CD8⁺ T-cell immunity to recovery from infection in mouse models of WNV and that of a closely related flavivirus

Knock-out mouse or treatment	Impact of cell-mediated immunity on pathogenesis		
	WNV (lineage I)	WNV (lineage II)	MVEV
$CD4^+$ T cell deficient or depleted	↑ (Sitati and Diamond, 2006)	nt	nt
MHC class II deficient	↑ (Sitati and Diamond, 2006)	nt	nt
CD40 deficient	↑ (Sitati et al., 2007)	nt	nt
$CD8^+$ T cell deficient or depleted	↑ (Shrestha and Diamond, 2004)	↑ (Wang et al., 2003b)	nt
β2m deficient	nt	↑ (Wang et al., 2003b)	↓ (Licon Luna and Lobigs, unpublished)
Perforin deficient	↑ (Shrestha et al., 2006a)	No effect (Wang et al., 2004b)	No effect (Licon Luna et al., 2002)
Granzyme A × B deficient	Nt	[a] (Wang et al., 2004b)	No effect (Licon Luna et al., 2002)
Perforin × granzyme A × B deficient	Nt	[a] (Wang et al., 2004b)	No effect (Licon Luna et al., 2002)
Fas ligand deficient (*gld*)	↑ (Shrestha and Diamond, 2007)	No effect (Wang et al., 2004b)	↓ (Licon Luna et al., 2002)
Perforin × Fas ligand (*gld*) deficient	Nt	[a] (Wang et al., 2004b)	↓ (Licon Luna et al., 2002)
Caspase 3 deficient	↓ (Samuel et al., 2007)	Nt	nt
IFN-γ deficient	↑ (Shrestha et al., 2006b)	No effect[b] (Wang et al., 2006) or ↓ (King and Kesson, 2003)	↑ (Lobigs et al., 2003a)
CCR5 deficient	↑ (Glass et al., 2005)	nt	nt
CXCL10 deficient	↑ (Klein et al., 2005)	nt	nt

(↑) increased, (↓) decreased disease severity, *nt* not tested
[a] Increased mortality accompanied by later time to death
[b] Earlier virus entry into the CNS

mortality, but with longer average survival times (Wang et al., 2004b). This suggests that without granzyme A and/or B mice less efficiently control WNV infection, and that the granzymes ultimately mediate immunopathological effects. Interestingly, granzyme B is able to inhibit WNV growth in a human cell line by >10-fold (Fig. 1), raising the possibility that the protective effect of granzyme B observed in vivo may be, at least partially, due to a direct influence on viral replication, rather than to a T-cell-mediated clearance of infected cells. Given that neurons are a predominant target of WNV, non-lytic viral clearance would be particularly beneficial.

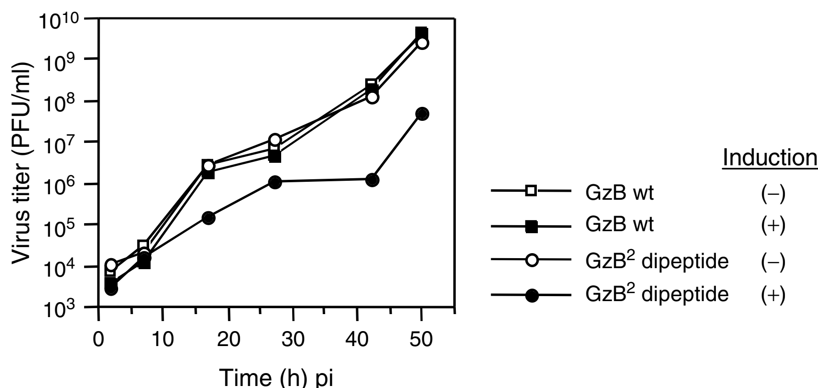

Figure 1. Intracellular granzymes B inhibits WNV growth. For stable, tetracycline-inducible expression of inactive (GzB wt; *squares*) or active, NH_2-terminal dipeptide-deleted (Smyth et al., 1995) granzymes B (GzB Δ dipeptide; *circles*) in 293 cells, mouse granzymes B cDNA with or without a NH_2-terminal dipeptide was cloned into vector pcDNA5/FRT/TO (Invitrogen, Carlsbad, CA) and transfected into Flp-In T-REx-293 cells (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Cells were induced with tetracycline (+) for 6 h, or left untreated (−), followed by infection with WNV *Sarafend* at a multiplicity of 1 PFU/cell for 1 h. Cells were washed and growth medium with or without tetracycline (1 μg/ml final concentration) was added. Growth samples were titrated by plaque assay on Vero cells (Wang et al., 2003b).

6 T-Cell Trafficking into the CNS

Lymphocyte trafficking into the CNS is governed by a multi-step process in specialized post-capillary venules, the high-endothelial venules (Engelhardt and Ransohoff, 2005). Contact with the vascular endothelium leads to initial slowing down of the leucocytes, followed by rolling of the cell along the venule wall. This deceleration allows sensing of chemotactic factors, which, when present, trigger activation of adhesion molecules on the leucocyte surface and diapedesis through the inter-endothelial cell junctions or through the endothelial cells themselves. The uninflamed brain is an immune-privileged site, meaning that access to leucocyte and inflammatory processes are more tightly regulated than in other organs. However, while naïve T cells do not circulate through the CNS, activated T cells do traffic even to the non-inflamed CNS (Hickey et al., 1991).

In WNV infection of mice, significant leucocyte numbers have only been shown to enter the brain at the time of, or after, viral entry to the CNS, following virus replication in extraneural tissues for 4–6 days (Diamond et al., 2003b; Wang et al., 2003b). CNS infiltrating leucocytes during WNV infection of mice were predominantly T cells, NK cells

and macrophages (Liu et al., 1989; Wang et al., 2003b; Glass et al., 2005; Klein et al., 2005), although the precise composition of the infiltrate has varied between studies and virus strains. Mouse infection with lineage I WNV strains led to a similar infiltration by $CD8^+$ and $CD4^+$ T cells (Glass et al., 2005; Klein et al., 2005), whereas that with WNV *Sarafend* induced a remarkable bias in T-cell infiltration, with almost total predominance of $CD8^+$ over $CD4^+$ T cells (Liu et al., 1989; Wang et al., 2003b), suggesting that these T-cell types may be governed by distinct trafficking mechanisms. Limited clinical data suggest that a $CD8^+$ T-cell predominance in the brain infiltrate also occurs during human infection with lineage I WNV strains (Sampson et al., 2000; Kelley et al., 2003; Omalu et al., 2003).

A wide range of chemokines are induced in the WNV-infected mouse brain of which CXCL10, CCL12 and CXCL12 have been detected early enough to play a role in initiating T-cell trafficking into the CNS (Klein et al., 2005; Garcia-Tapia et al., 2007). Two chemokine/chemokine-receptor pairs have been shown to exert an important, albeit not exclusive, influence on T-cell migration and outcome of viral infection with lineage I WNV strains. Remarkably, WNV-infected neurons are able to produce, in vivo and in vitro, the chemokine CXCL10 (IP-10), a chemokine attracting monocytes and T cells (Dufour et al., 2002). Although infiltrating macrophages, glial cells and some astrocytes and endothelial cells also produce CXCL10 after WNV invasion (Cheeran et al., 2005), neurons appear to be the predominant and earliest producers (Klein et al., 2005), suggesting that infected neurons may play a significant role in the establishment of an immune reaction in the CNS. CXCL10-deficient mice infected with WNV experienced slower T-cell infiltration of the CNS, and higher viral titres; infiltration of $CD8^+$ T cells bearing the receptor for CXCL10 (CXCR3) was reduced to a large extent, but not exclusively, suggesting that CXCL10 is not the sole chemoattractant in this system (Klein et al., 2005; Garcia-Tapia et al., 2007). Whereas peripheral virus titres were independent of CXCL10, CXCL10-deficient mice had higher viral loads in the brain and higher mortality than wild-type mice (Klein et al., 2005). Interestingly, CXCR3-deficiency also led to reduced $CD8^+$ T-cell infiltration and higher viral loads in dengue virus-infected brains (Hsieh et al., 2006) and was suggested to decrease resistance in tick-borne encephalitis virus infection (Lepej et al., 2007).

The second chemokine/chemokine-receptor pair involved in recruitment of WNV-immune T cells into the CNS is the CCL5-CCR5 pair. CCR5-deficient animals revealed reduced T-cell infiltration of

the brain associated with increased mortality following WNV infection The maximal effect of this reduction was seen at the later stages of the infection that coincide with viral clearance from the brain by CCR5-sufficient, but not CCR5-deficient mice (Glass et al., 2005). As noted, other chemokines are likely to play additional roles in immune cell recruitment, as in these studies, deficiency in a particular chemokine or chemokine receptor only led to a relative difference in T-cell infiltration.

Clearly, much still remains to be resolved in this area. This is particularly highlighted in the recent discovery of a role for CD40, a molecule hitherto implicated in the development of B and T-cell responses, in $CD8^+$ T-cell trafficking into the WNV-infected brain (Sitati et al., 2007), as egress of $CD8^+$ T cells from the perivascular space into the parenchyma appears to be blocked in $CD40^{-/-}$ mice. Consistent with the role of $CD8^+$ T cells in recovery, this reduced CNS infiltration was associated with increased viral load and mortality.

7 T-Cell-Mediated CNS Pathology in WNV Infection

Neurons are the primary target cells in WNV infection of the CNS of mice (for instance, Eldadah and Nathanson, 1967; Diamond et al., 2003a; Shrestha et al., 2003; Garcia-Tapia et al., 2007) and humans (for instance, Sampson et al., 2000; Sejvar and Marfin, 2006). The virus is cytopathic in neuronal cell cultures, inducing cell death by apoptosis (Parquet et al., 2001; Yang et al., 2002; Shrestha et al., 2003; Diniz et al., 2006). It is the role of the immune system to control viral infections of the CNS, but the response may be pathological and life-threatening, if it results in destruction of neuronal tissue, given its limited capacity for renewal. The relative contributions of virus-induced and immune-mediated neuronal injury in WNV infection of the CNS cannot be clearly defined, other than that a correlation between severity of signs of neuronal injury, number of infected foci and number of inflammatory $CD45^+$ cells in the vicinity of infected neurons exists (Shrestha et al., 2003; Wang et al., 2006). Despite the morphological changes and damage to neuronal tissue as a consequence of WNV infection of the CNS, human patients with WNV meningitis or encephalitis can have good long-term outcomes (Sejvar and Marfin, 2006). Similarly, a significant number of mice showing signs of WNV encephalitis survive and can clear the infection (Diamond et al., 2003a; Wang et al., 2003b), in some cases even after

development of hind limb paralysis (Wang et al., 2006). As discussed above, WNV-reactive $CD8^+$ T cells with cytolytic effector function traffic into and clonally expand in the CNS parenchyma and are critical for recovery. The $CD8^+$ T cells kill WNV-infected neurons mainly by cytolytic effector pathways, in contrast to other viral infections of the CNS for which non-cytolytic mechanisms of virus clearance from neurons involving antibody and IFN-γ have been observed (Griffin, 2003). Accumulation of activated Tc cells in the brain and lysis of infected neurons is dependent on antigen presentation via MHC class I; functionally intact neurons suppress expression of the restriction elements and cell adhesion molecules. However, in the event of cell damage (for instance that associated with a viral infection) MHC class I-restricted antigen presentation is induced and dysfunctional neurons become targets for lysis by Tc cells (Neumann et al., 1995, 2002; Wekerle, 2002). The observation that the protective effect of WNV-primed $CD8^+$ T-cell transfer against WNV CNS infection is lost in recipient mice lacking expression of classical MHC class I molecules (Shrestha et al., 2006a) supports the requirement for MHC class I-restricted antigen presentation on neurons for Tc cell lysis. The immune response may be potentiated by up-regulation of MHC class I expression as a result of flavivirus infection (Liu et al., 1988; Lobigs et al., 1996, 2003a; King and Kesson, 2003).

Immune-mediated destruction of infected neurons is key to resolution of WNV infection; however, there is evidence that the immune response in the brain can produces pathology greater than the minimal damage required for recovery. Groups of mice defective in MHC class I antigen presentation ($\beta 2\text{-m}^{-/-}$) or lacking IFN-γ showed a slight reduction in mortality and survived longer relative to wild-type mice, when infected with a high dose (10^8 PFU) of the WNV *Sarafend* strain, a condition, which results in rapid virus entry into the brain (Wang et al., 2003b, 2006). Intriguingly, in the absence of a key apoptotic regulator, caspase 3, mice acquired increased resistance to lethal infection with WNV, New York 2000 strain (Samuel et al., 2007). This phenotype was the result of reduced neuronal damage, despite comparable virus spread into and burden in the CNS relative to infected wild-type mice. WNV infection induced caspase 3 activation in neurons, thus the reduced apoptosis in the CNS of infected caspase $3^{-/-}$ mice could have been due to reduced virus- and/or Tc cell-mediated cell death. Taken together, the data from the mouse models of WNV encephalitis indicate that treatments targeting death or inflammatory pathways could ameliorate disease outcome.

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