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The Immunopathogenesis of Neurotropic Flavivirus Infection

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1. Introduction

The flavivirus genus in the *Flaviviridae* family comprises over 70 species, most of which are tick- or mosquito-borne. They are single-stranded, plus-sense RNA viruses, responsible for significant human and animal morbidity and mortality on all inhabited continents. Medically important viscerotropic flaviviruses include dengue, found equatorially across the world, and the prototypic yellow fever virus, found in Africa and South America. Neurotropic members include tick-borne encephalitis virus (TBEV) in Europe, West Nile virus (WNV) in Africa, parts of Europe and the Indian subcontinent, as well as the USA, St Louis encephalitis virus in the USA, and Japanese encephalitis (JEV) and Murray Valley encephalitis (MVE) viruses in Australasia. Many of these have a history of emergence and re-emergence; indeed, following a novel outbreak in 1999 in New York (Lanciotti et al., 1999; CDC, 2010), WNV spread virtually throughout the Americas in less than 10 years and is the most common cause of meningoencephalitis in North America. WNV is now perhaps the most widely spread of all flaviviruses and may comprise 4 or more lineages, based on isolate homologies (C.G. Hayes, 2001; Bakonyi et al., 2005; Vazquez et al., 2010), with Lineage I and II well-defined and of obvious clinical importance in animals and humans (E.B. Hayes et al., 2005; Venter et al., 2009). WNV was first isolated in 1937 in the West Nile region of Uganda (Smithburn et al., 1940). It is a member of the Japanese encephalitis serogroup, together with JEV, Murray Valley and Saint Louis encephalitis viruses (Poidinger et al., 1996). These viruses are usually propagated in a zoonotic cycle between mosquitoes and amplifying hosts, particularly birds (or pigs in the case of JEV), with humans being incidental, since they may not develop high enough virus titres to infect arthropod vectors (C.G. Hayes, 2001). Rare cases of human-to-human WNV transmission have been documented via organ transplants and blood transfusion, as well as vertical transmission to the foetus *in utero* (Iwamoto et al., 2003; Lindsey et al., 2009). Although less than one percent of WNV infections develop neuroinvasive disease, in some 60% of patients presenting with central nervous system (CNS) symptoms denoting neuroinvasive disease, life-threatening encephalitis supervenes (Samuel & Diamond, 2009). The young,

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immunocompromised and elderly are at highest risk of developing encephalitis (Weiss et al., 2001; Guarner et al., 2004; Lindsey et al., 2009). Climate change, geographic factors and international travel, as well as local factors, such as mosquito rates and land clearing (E.A. Gould & Higgs, 2009) are cited as influencing the spread and prevalence of arbovirus infections across the globe. Despite considerable effort, the only successful live attenuated vaccine for neurotropic flaviviruses is for JEV (X. Liu et al., 2011), although considerable success has been achieved with a killed vaccine for TBEV (Barrett et al., 2003; Heinz et al., 2007). Treatment for all flavivirus encephalitides remains palliative. Indeed, one of the cardinal features of flavivirus infection complicating treatment approaches is immunopathology, evident in human cases and demonstrable in various animal models. In this review, we analyse the principal elements involved in the immunopathogenesis of flavivirus disease, with a view to novel constructive approaches to disease intervention.

2. Local flavivirus infection - responses of infected cells

Live WNV infection of various cell types leads to an upregulation of various cellular adhesion molecules, either directly or co-modulated by cytokines (Shen et al., 1995b; Shen et al., 1997; Arnold et al., 2004). These molecules include major histocompatibility complex (MHC) class I and II, intracellular adhesion molecule-1 (ICAM-1), vascular cellular adhesion molecule-1 (VCAM-1) and E-selectin (King & Kesson, 1988; Argall et al., 1991; Shen et al., 1997; Verma et al., 2009). The increase in MHC-I in mouse embryonic fibroblasts is independent of tumour necrosis factor (TNF) (Cheng et al., 2004b), although many cell types produce it in response to infection. In both mouse embryonic fibroblasts and human skin fibroblasts it can be induced by type I interferon (IFN)-dependent and -independent pathways (King & Kesson, 1988; Kesson & King, 2001; Cheng et al., 2004a), with the IFN-independent pathway relying on the activation of the transcription factor nuclear factor-κB (Kesson & King, 2001). This seemingly paradoxical increase in immune recognition molecule expression may enable evasion of early NK responses *in vivo* (Lobigs et al., 2003).

3. Earliest local changes in dermis and draining lymph nodes

Flaviviruses, transmitted by the bite of an infected mosquito, are injected with saliva during feeding. While several structural cell types are likely to come into contact with injected virus, the first leukocyte subset that virus is likely to encounter is the Langerhans cell (LC). LC are dendritic cells (DC) in the epidermis replenished by myeloid precursors in the bone marrow (BM) (Steinman, 1991; Merad et al., 2002). Following cutaneous inoculation, virus may infect LC, which increases cell surface expression of MHC-I, MHC-II, adhesion molecules, as well as costimulatory molecules, such as CD80, differentiating into a migratory phenotype in the process (Johnston et al., 1996). LC migrate to draining lymph nodes (DLN) in an accelerated manner in response to live virus, due to the induced soluble factor milieu (Johnston et al., 2000). *In vivo* experiments in mice show that the cytokine IL-1β is crucial for the emigration of LC from the epidermis to DLN (Byrne et al., 2001). It is presumed that these DC pass antigen to CD8α+ DC in the DLN, since LC alone evidently do not initiate antiviral immune responses (Allan et al., 2003; Kissenpfennig et al., 2005; Hildner et al., 2008), but direct evidence of WNV antigen transfer has yet to be shown. While mosquito saliva may skew adaptive responses towards a more Th2-like response profile

(Schneider et al., 2007; Schneider et al., 2010), it is still unclear how its early presence influences disease outcome, since WNV infection in the genetic absence of IFN-γ still results in the generation of neutralising immunity (King et al., 2003). Arrival of virus in the DLN is accompanied by significant induction of CCL2 expression there that recruits monocyte-derived macrophages (MDM) from the bone marrow. The bone marrow undergoes a significant myeloid response to infection, releasing monocytes with a qualitatively different phenotype from the homeostatic state. Monocytes rapidly become TipDC (producing TNF and nitric oxide) in the DLN and likely contribute to the initiation of type 1 immune responses by inducing more IFN-γ from responding T cells than other DC subtypes in the DLN (Davison & King, 2011). Since flaviviruses significantly upregulate MHC expression on infected cells (King & Kesson, 1988; Shen et al., 1997), it is of interest how this influences the outcomes of innate immune interactions, such as with NK cells, and/or bears on the ultimate affinities of later adaptive cytotoxic T cell responses, reviewed in detail elsewhere (King & Kesson, 2003; Lobigs et al., 2003; King et al., 2009).

At the same time, at the dermal site of infection, CCL2, produced by infected cells, also recruits considerable numbers of MDM, which surround the infected focus, becoming DC within 24h of arrival. While it would appear that the DLN and dermis, by both producing CCL2, compete for MDM from the bone marrow, it seems likely that a subtly different cytokine milieu at each site selects for specific MDM subsets. This is suggested by adoptive transfer studies of flow cytometrically-sorted BM-derived monocyte subsets during infection. Thus, although similar numbers of inflammatory (Ly6C^{hi}) monocytes migrated to each site, 5-fold more Ly6C^{lo} monocytes migrated to the DLN than Ly6C^{hi}, while some 2-fold more Ly6C^{hi} monocytes migrated to the focus of infection in the skin than Ly6C^{lo} monocytes. Perhaps consonant with these proportions, immigrant Ly6C^{lo} monocytes in the DLN were 3-fold more likely to become DC, while in the dermis, immigrant Ly6C^{hi} cells were 2-fold more likely to become DC. This suggests that other chemokines may differentially contribute to the recruitment of these cells to the relevant site, but more importantly, it suggests that relevant precursors to specific cells are already ordained before they reach their target site, rather than necessarily being randomly and non-specifically recruited (*e.g.,* by CCL2) and induced to differentiate once they have arrived. Irrespective, it is evident that Ly6C expression on immigrating monocytes quickly changes at both sites, with downregulated Ly6C expression being the end point in both subsets. Thus, as shown in ours and other studies, Ly6C^{hi} monocytes arriving in response to infection quickly downregulate expression of this molecule (Sunderkotter et al., 2004; Leon et al., 2007; Getts et al., 2008; Davison & King, 2011). In contrast, at both sites, within 24h, immigrating Ly6C^{lo} monocytes become Ly6C^{hi} at the site, only to downregulate expression of this molecule again within a further 24h, while also upregulating CD11c to become DC. Furthermore, in the dermis, it is exclusively these cells originally entering the dermis as Ly6C^{lo} monocytes, that then migrate from the dermis to the DLN, presumably carrying viral antigen with them (Davison & King, 2011). Since so many Ly6C^{lo} cells also migrate from the BM to the DLN to become DC, it is tempting to speculate that these cells may differentiate into the final migratory couriers of antigen between the subcapsular sinus of the DLN, where DC arrive from the dermis via the afferent lymphatics, to the CD8α$^+$ DC in the deep paracortex.

Early antiviral activity by Ly6C^{hi} monocyte-derived TipDC in the dermis may influence the outcome of infection by reducing virus titres locally; indeed, antiviral activity may account

for the high survival rates observed in a mucosal model of flavivirus infection after intravaginal inoculation of a viral dose that is lethal by all other routes. In this model, large numbers of DC accumulate just beneath the epidermal site of infection, essentially separating infected epithelium from uninfected stroma below (Burke et al., 2004). Interestingly, CCL2 is also strongly expressed at this site (King, Unpublished observations).

4. Entry into the CNS

Virus undoubtedly spreads via the bloodstream at some point to further infect peripheral organs, including the CNS, where neurons are the main target (Xiao et al., 2001; Shrestha et al., 2003; Cheeran et al., 2005; Samuel & Diamond, 2009). However, the factors controlling these phases of virus spread are virtually unknown. The blood of acutely viraemic human donors shows substantial increases in IFN-γ and α as well as IFN-stimulated CXCL10 and CCL2. This upregulation of cytokines and chemokines occurs before IgM seroconversion and is therefore argued to be involved in the initial response to acute viraemia. On the other hand, this could be the result of chemokine gradients from the WNV-infected brain, since both chemokines are increased in the WNV-infected brain (Klein et al., 2005; Getts et al., 2008). Upregulated IL-4 was also detected in the same time frame, implicating IL-4 in early modulation of immune responses (Tobler et al., 2008).

How virus gains entry into the CNS is still unclear. In some models it is argued that breakdown of the blood-brain barrier (BBB) enables virus entry into the CNS. Toll-like receptors, TLR3, 7 and 8, are responsible for detecting RNA products of viral replication and although TLR3 and 7 are protective against WNV (Daffis et al., 2008; Town et al., 2009; (Daffis et al., 2008), TLR3 has also been implicated in aiding entry of WNV into the brain. TLR3 knockout mice have less BBB leakiness and significantly lower viral levels in the brain than wild type mice, with higher viral loads in the periphery, thought to be due to reduced production of TNF, a cytokine strongly implicated in BBB breakdown (T. Wang et al., 2004). Interestingly, macrophages isolated from young humans show a marked reduction in TLR3 levels during WNV infection, while those from elderly donors do not. TLR3 levels are controlled by a signal transducer and activator of transcription 1 (STAT1)-mediated pathway, which is downregulated by WNV E-protein in cells from young, but not elderly donors. Cells from elderly donors produced increased levels of inflammatory cytokines, including TNF (Kong et al., 2008), supporting the notion that TLR3 may play a role in immunopathology in the elderly, ironically via an overly aggressive immune response to WNV. Endothelial tight junction proteins (TJP), such as ZO-1 and claudin-1, are crucial components of BBB integrity and this seal is maintained, *inter alia*, by astrocytes, non-neuronal support cells of the CNS that are susceptible to WNV infection *in vitro* (Y. Liu et al., 1989; Cheeran et al., 2005; Verma et al., 2010). WNV-infected astrocytes increase matrix metalloproteinase (MMP) production to degrade TJP (Verma et al., 2010). However, astrocyte infection *in vivo* has not been reported and furthermore, BBB breakdown is quite variable in flavivirus infection (T.H. Liu et al., 2008; Verma et al., 2010). Alternative ways in which virus could cross the BBB include endothelial transcytosis and/or endothelial infection (German et al., 2006). However, there are few if any reports of endothelial infection itself *in vivo* by flaviviruses. Moreover, in 167 mice examined at various timepoints after intraperitoneal infection, where significant viraemia occurs within 6 h of inoculation, we saw unequivocal endothelial infection only once (King, Unpublished).

Trojan horse transmission into the brain by infected monocytes remains a possibility. Macrophages are susceptible to WNV infection (Cardosa et al., 1986; Shen et al., 1995a; Rios et al., 2006) and could be a crucial cell type contributing to transmission of virus into the brain. However, this has not been shown, and in our hands, while both human and mouse monocytes are readily infectable *in vitro*, they also very effectively control flavivirus infection, a point further supported *in vivo* by the lack of infected microglia (or for that matter, infiltrating macrophages) in flavivirus encephalitis (Getts et al., 2007; Getts et al., 2008). A recent study has shown that neutrophils, recruited to the intraperitoneal infection site in large numbers by CXCL-1 and -2 produced by WNV-infected macrophages, exhibited much higher viral loads than macrophages. The removal of these chemokines or depletion of neutrophils prior to WNV infection resulted in delayed death of animals and reduced viral levels, suggesting a possible role for neutrophils as reservoirs for viral replication and dissemination during the early stages of WNV infection (Bai et al., 2010). However, of the leukocytes attracted to the brain after intranasal infection, less than 3% are neutrophils (King, Unpublished). The intranasal route of infection enables the separation of systemic anti-viral responses from those mediated by the CNS. Thus, insignificant numbers are in and of themselves attracted to the brain during infection, making it unlikely that they are a primary source of virus in neuroinvasion, although they may be a source of spread to other organs. Notwithstanding the susceptibility of most cells to *in vitro* infection with flaviviruses, it has yet to be convincingly demonstrated that cells in the brain parenchyma other than neurons become infected in *in vivo* infection. This begs the question of how infected macrophages and neutrophils specifically contribute to neuronal infection. Furthermore, infection via a Trojan horse scenario would presumably occur only if infected leukocytes or monocytes were attracted by chemotactic signals originating in the parenchyma of the brain, whether or not the BBB was intact. This assumes a response to infection, either directly, *e.g.*, via infected neurons producing CCL2 (Getts et al., 2008), or indirectly via other leukocytes already attracted by infection producing chemokines that in turn attract infected neutrophils and monocytes, *i.e.*, once the immune response is well and truly under way. Increases in virus in the brain temporally consistent with this scenario have not to our knowledge been reported.

In vitro cellular adhesion molecules like E-selectin, VCAM-1 and ICAM-1 are upregulated in WNV infection and could support the extravasation of leukocytes into the brain (Shen et al., 1997). Expression of ICAM-1 and VCAM-1 is clearly increased on cerebrovascular endothelium in WNV-infected mice *in vivo* and this is also associated with infiltration of large numbers of macrophages and microglia into the brain (Figure 1). In primary human brain microvascular endothelial cells, an increase in VCAM-1 and E-selectin is induced when viral replication is at its highest (Verma et al., 2009). Together with TLR3-mediated BBB breakdown, upregulated adhesion molecules could facilitate the infiltration of infected leukocytes into the CNS. However, it is worth noting that WNV-infected IFN-γ knockout mice upregulate cerebrovascular endothelial ICAM-1 and VCAM-1 expression significantly, but have substantially fewer immigrant leukocytes than wild type mice (King, Unpublished). Moreover, although WNV induces BBB permeability in some models, it may not be the primary route of entry into the CNS. Mice and hamsters infected with WNV exhibited signs of BBB leakiness at the same timepoint they succumbed to virus, indicating that infection of the brain began prior to the BBB becoming compromised (Morrey et al., 2006; Morrey et al., 2008) (King, Unpublished). Similarly, in TBEV infection in mice, BBB breakdown occurs 2-3 days after virus is detectable in the brain (Růžek et al., 2011).

There is however, good evidence of centripetal nerve spread from the periphery (Engle & Diamond, 2003; Hunsperger & Roehrig, 2006; Getts et al., 2007). In the footpad model, viral infection occurs in the dermal layer of the epithelium in the footpad, which is enervated by sensory fibres of the dorsal root ganglion (DRG) of the peripheral nervous system (PNS). *In vitro*, PNS neurons support WNV infection and in mice the DRG is highly susceptible to WNV infection, suggesting that it may be involved in retrograde axonal transport of virus from the PNS to CNS (Hunsperger & Roehrig, 2005, 2006). This is consistent with our own studies using intraperitoneal inoculation of WNV in mice, where WNV is seen in the cervical spinal cord 1-2 days before it is seen in the brain parenchyma, spreading in a caudal to rostral manner. In contrast, intranasal infection results in rostral to caudal spread through the brain, with spread to various anatomical regions via known neural connections (King, Unpublished). If the sciatic nerve of golden hamsters is transected above the infection site, a systemic infection with less severe clinical symptoms of CNS invasion occurs (Samuel et al., 2007), denoting a contribution to spread by intact axons. Treatment of mice with nocodazole, a microtubule inhibitor that prevents retrograde axonal transport, prior to inoculation, delays virus spread to the CNS by 6 days (Hunsperger & Roehrig, 2009). However, taken together, it is likely that more than one pathway is involved in WNV spread to the CNS, with inoculation site, dose, age and genetics all influencing this.
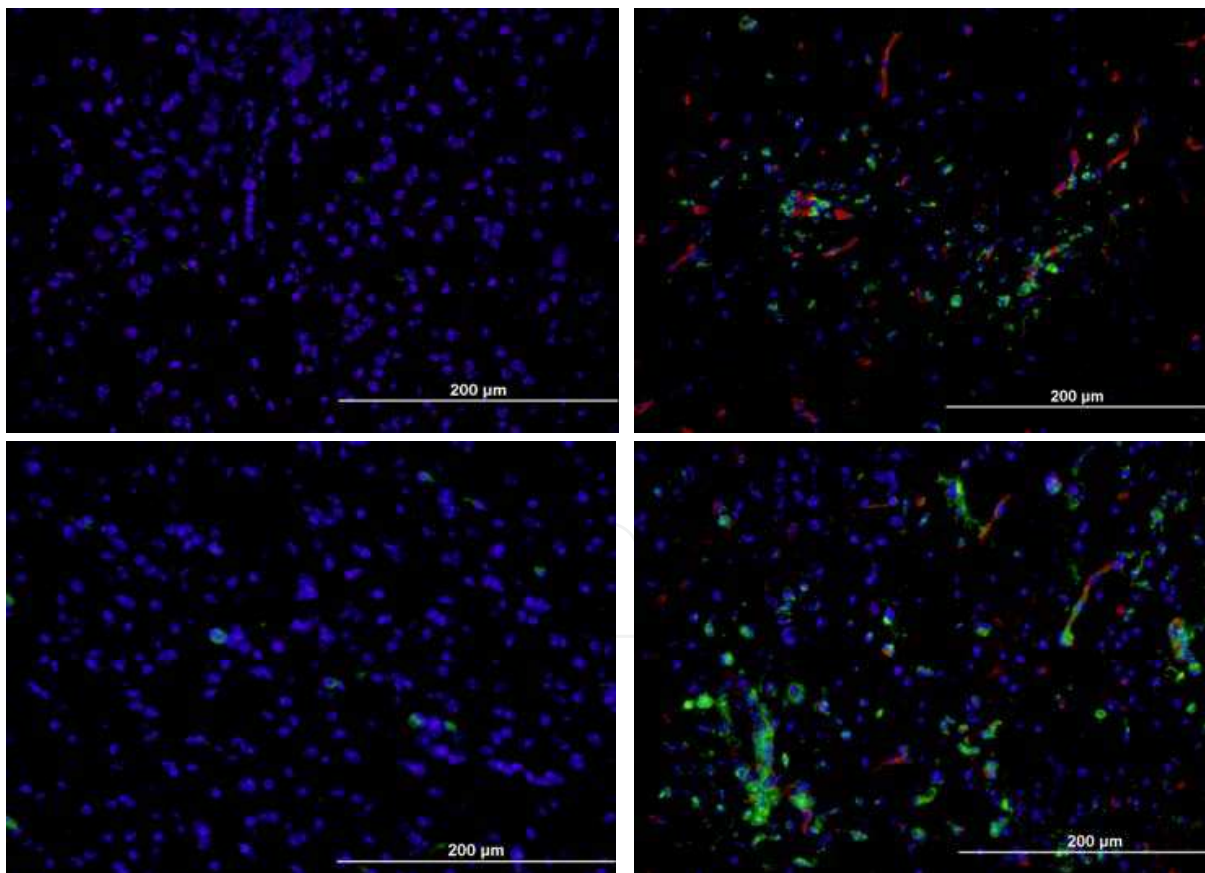

Fig. 1. WNV infection results in the upregulation of ICAM-1 and VCAM-1 on brain vasculature in WNV encephalitis. Green = macrophages and microglia (lectin staining), Red = ICAM-1 (top right), VCAM-1 (bottom right) Blue = DAPI.
Left panels = mock-infected, Right panels = d7 WNV-infected

5. CNS infection

In vitro, virions attaching to the plasma membrane enter cells via clathrin-mediated endocytosis to form endosomes. A lysosomal, pH-dependent fusion mechanism expels the nucleocapsid into the cytoplasm in close proximity to the ER where the virus is thought to replicate (Chu & Ng, 2004). All cells of the CNS are readily susceptible to WNV infection *in vitro* within 16-24 h, including neurons, Schwann cells and astrocytes, although microglia are more resistant (Y. Liu et al., 1989; Argall et al., 1991; Shrestha et al., 2003; Cheeran et al., 2005). In contrast, in animal models, detectable infection is seen in the brain between d3 and 6 post infection (p.i.), depending on the model, with virus in all cases evidently replicating exclusively within neurons, a behaviour distinguishing it from the encephalitic DNA viruses (Steele et al., 2000; Xiao et al., 2001; King et al., 2003; Shrestha et al., 2003; Getts et al., 2007; Brehin et al., 2008). Antibody labelling of monkey brain sections for neuron-specific endose, glial fibrillary acidic protein and WNV were argued to indicate WNV infection of both glia and neurons, but this interpretation is not well supported by the examples provided (He et al., 2009). In humans, viral antigen is found in neuronal cytoplasm and processes, usually at the centre of microglial nodules (Sampson et al., 2000; Guarner et al., 2004).
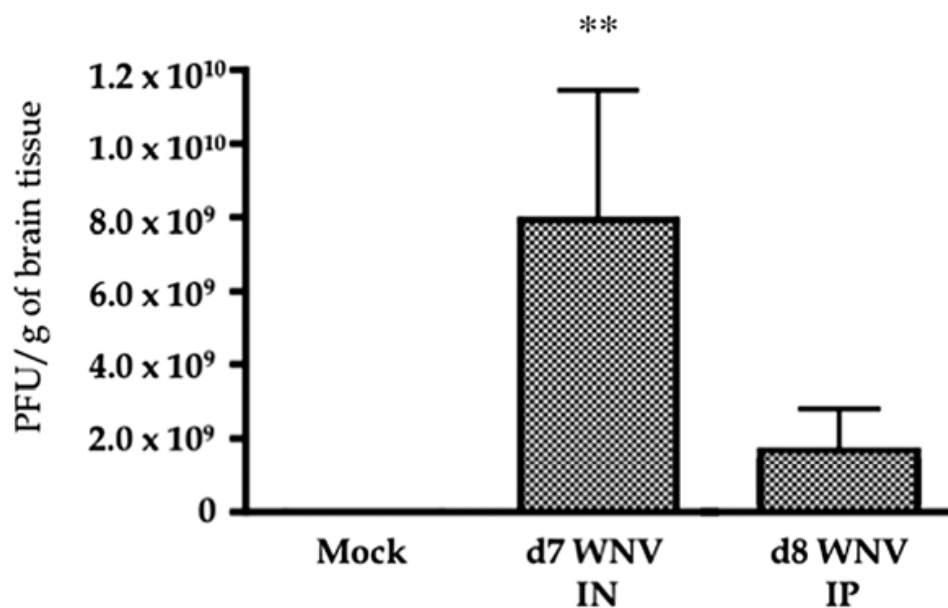

Fig. 2. Virus titres in the WNV-infected brain. IN = intranasal, IP = intraperitoneal infection

Following inoculation at the periphery, WNV replicates in lymphoid tissues, but once in the CNS, titres increase until the onset of encephalitis (Brehin et al., 2008). Plaque assays of brain tissue from mice intranasally infected with WNV show much higher viral titres at the time of death on d7 p.i. than peripherally inoculated animals (Figure 2). After footpad inoculation, virus RNA can be isolated from parts of the nervous system like the brainstem, as early as 24 h p.i., long before the peak of viraemia and symptoms are present. There are also indications that the virus may be cleared, only to be followed by re-emergence in that area (Hunsperger & Roehrig, 2006). In the murine model of WNV infection the brain structures most susceptible and showing the highest viral titres appear to be the cortex, hippocampus, brainstem and spinal cord (Xiao et al., 2001; Hunsperger & Roehrig, 2006). In

humans, WNV seems to affect the brainstem and anterior horn of the spinal cord (Sejvar et al., 2003; Guarner et al., 2004). Neuroinvasion also occurs in animals that do not succumb to infection (Hunsperger & Roehrig, 2006; Appler et al., 2010). Damage to neurons can result from either excessive virus replication or an over-exuberant immune response, or both, with death ensuing in either eventuality. Increases in CD45^{+} leukocyte numbers as well as viral antigen are associated with areas of neuronal injury, degeneration by apoptosis and onset of encephalitis, making it difficult to distinguish between viral and immune-mediated injury (Xiao et al., 2001; Shrestha et al., 2003; Brehin et al., 2008). In general, mice die soon after seizures become evident; if seizures can be suppressed, then survival may be extended several days and virus growth in the brain increases by a further 10-fold or more (Getts et al., 2007). This indicates that the neurons are capable of supporting much higher levels of virus replication than is usually evident at death. Microglial nodules, perivascular cuffing, principally comprised of infiltrating macrophages and T cells, and in advanced disease, neuronal loss, are prominent among the neurohistopathological and immunohistochemical findings (Steele et al., 2000; Guarner et al., 2004; Petzold et al., 2010). Significant leukocyte infiltration seems to occur around d5 p.i., irrespective of the model used, associated with full activation of the adaptive immune response.

Cells of the CNS each respond differently towards infiltrating virus. *In vitro* one of the responses to viral infection of astrocytes and microglia is the production of chemoattractants like CCL5 and CXCL10 (Cheeran et al., 2005), which recruit T cells and possibly monocytes; both astrocytes and microglia can produce CXCL9, which similarly attracts T cells (Muller et al., 2007) (Getts, Unpublished). Infected neurons produce copious amounts of CCL2, which recruit macrophages and microglia from the bone marrow (Getts et al., 2008). IFN-γ plays an important role in the formation of the seizure cascade during the development of the CNS, as IFN-γ knockout mice do not develop seizures during WNV infection. Seizures cannot be recapitulated by IFN-γ-producing T cells infiltrating into the brain in WNV-infected IFN-γ knockout chimeras previously reconstituted with wild type bone marrow. The role of the glutamate receptor, N-methyl-D-aspartate in the development of these seizures has also been shown (Getts et al., 2007). While it is clear that many soluble factors are produced in the brain, it is not so obvious which cells produce them *in vivo*. Much work will be required to fully characterise the sequence of inductive and inhibitory interactions between local cells, infected cells and immigrant leukocytes.

6. Microglial activation

Microglial activation is clearly associated with virus infection of neurons rather than immune leukocyte infiltration. After intraperitoneal inoculation, WNV can be seen in the brain by d6 p.i., when activated microglia are first observed, one day after leukocyte infiltration is first evident. However, in the intranasal model, microglial activation is seen at d3 p.i. when virus is first observed, while leukocyte infiltration is still seen first at d5 p.i., as in the intraperitoneal model (Getts et al., 2008) (King, Unpublished). Microglia form nodules around infected neurons soon after infection (King et al., 2003; Guarner et al., 2004; Petzold et al., 2010), presumably recruited by the soluble factor outputs from infected neurons, including CCL2. Consistent with this, microglia, at first dendritic, quickly lose these normal projections to assume a more motile amoeboid phenotype

(Getts et al., 2008). Interestingly, as the number of infected neurons increases, these nodules become less apparent, perhaps because the concentrations of these soluble factors reach a virtual equilibrium within the brain, thus abrogating the concentration gradients likely required for nodule formation (King, Unpublished). Whether the formation of nodules is protective or damaging to neurons is not known, but relatively few infected neurons at the centre of microglial nodules are TUNEL-positive in WNV encephalitis (Getts et al., 2007). There is a significant increase in microglial numbers in the brain during infection, with a decrease in resting microglial numbers. This increase is accounted for by bone marrow-derived monocyte immigration and is not due to *in situ* proliferation of microglia (Getts et al., 2008). Whether immigrant microglia subsequently return to a resting state in the brain if the host survives and/or whether these cells have a role in the causation of any subsequent neurological sequelae, remains to be elucidated.
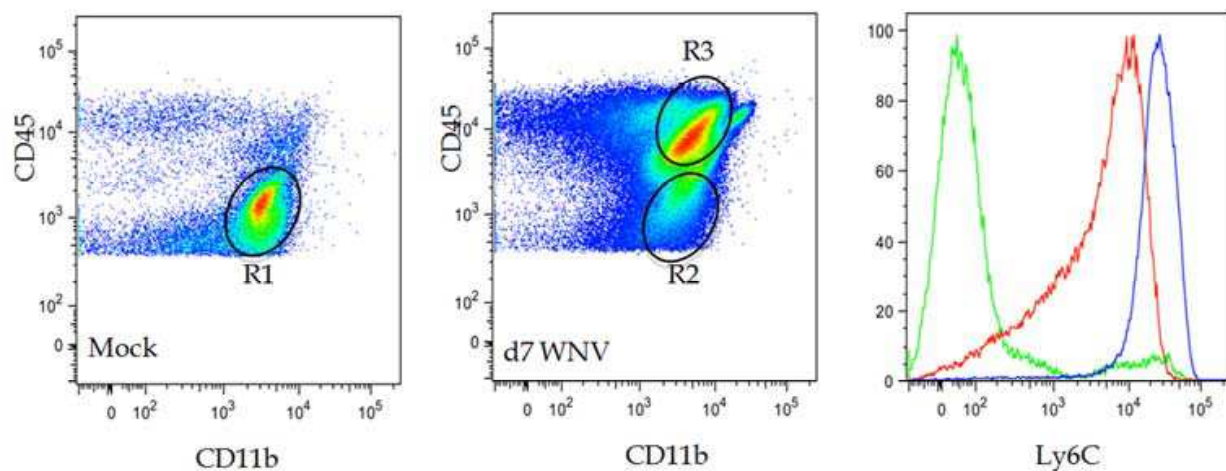

Fig. 3. Flow cytometry showing infiltration of macrophages and microglia in the WNV-infected and mock-infected mouse brain. R1 (green profile at right) = CD45^{lo-int} microglia (mock-infected), R2 (red profile at right) = CD45^{lo-int} microglia (WNV-infected), R3 (blue profile at right) = CD45^{hi} macrophages (WNV-infected)

Usually by d6 p.i. the whole microglial population in the brain is activated and this can be detected as an increase in CD45 expression by flow cytometry (Figure 3) and by their amoeboid morphology using lectin immunohistochemistry (Brehin et al., 2008; Getts et al., 2008). Similar to infiltrating macrophages, activated microglia in the WNV-infected brain also express higher levels of Ly6C, compared to mock-infected mice. In Rhesus monkeys infected intracerebrally with three antigenically divergent flaviviruses, activated microglia are identified by their enlarged morphology, short thicker processes and CD68 expression. In this model, microglia are found next to dying neurons and neuronal debris where they perform a phagocytic function (Maximova et al., 2009). They also secrete various proinflammatory mediators and cytokines as part of the response to local and systemic infection. The increased microglial production of leukocyte-recruiting chemokines, CXCL10 and CCL2, is triggered by the activation of the p53-mitogen-activated protein kinase and extracellular signal-regulated kinase intracellular signaling pathways by WNV infection (Cheeran et al., 2005). Neutralisation of CCL2 by antibody on d6 p.i., results in prolonged survival, strongly implicating microglia as contributors to fatal neuropathology (Getts et al.,

2008). In Japanese encephalitis, bystander damage caused by activated microglia is a major contributor to the severity of the disease. Microglial activation is associated with elevation of proinflammatory mediators like TNF, IL-1β, IL-6, (inducible) nitric oxide synthase-2 (NOS2) and cyclooxygenase-2 (Cox-2), all of which have the potential to be neurotoxic (Ghoshal et al., 2007; Chen et al., 2010). Nitric oxide (NO), formed from the catalysis of arginine by sustained high levels of induced NOS2, can cause significant oxidative damage to neurons, while the production of Cox-2 results in reactive oxygen species as a byproduct that is also capable of inflicting irreversible damage on neurons (Ghoshal et al., 2007). TNF-induced endothelial expression of NOS2 and the formation of reactive nitrogen- and oxygen species is also implicated in the apoptosis of cells and development of hemorrhage in the murine model of dengue virus infection (Yen et al., 2008).

7. Macrophage infiltration

In mice infected with WNV by intranasal inoculation, virus is detectable only in the brain, making this model ideal to study brain responses to neuronal infection. In this model, the predominant leukocyte subtype immigrating into the brain in flavivirus encephalitis is the MDM (Figure 4, 5). By d7 p.i. large numbers of infiltrating macrophages can be seen surrounding infected neurons in the brains of WNV-infected mice. Neutralisation of CCL2 reduces macrophage immigration into the brain, indicating that CCL2, produced by infected neurons, is a major driver of MDM immigration into the CNS (Getts et al., 2008; Lim et al., 2011), however, this may later be supplemented with IL-23, which may intensify macrophage infiltration, or result in the recruitment of different functional macrophage subsets (Town et al., 2009). IL-23 is argued to originate from microglia, but this has not been shown. Some 35-50% of infiltrating leukocytes in the brain are Ly6C^{hi} macrophages, with NK cells and Ly6C$^+$ immigrant microglia in each case making up approximately half these numbers (Figure 5).
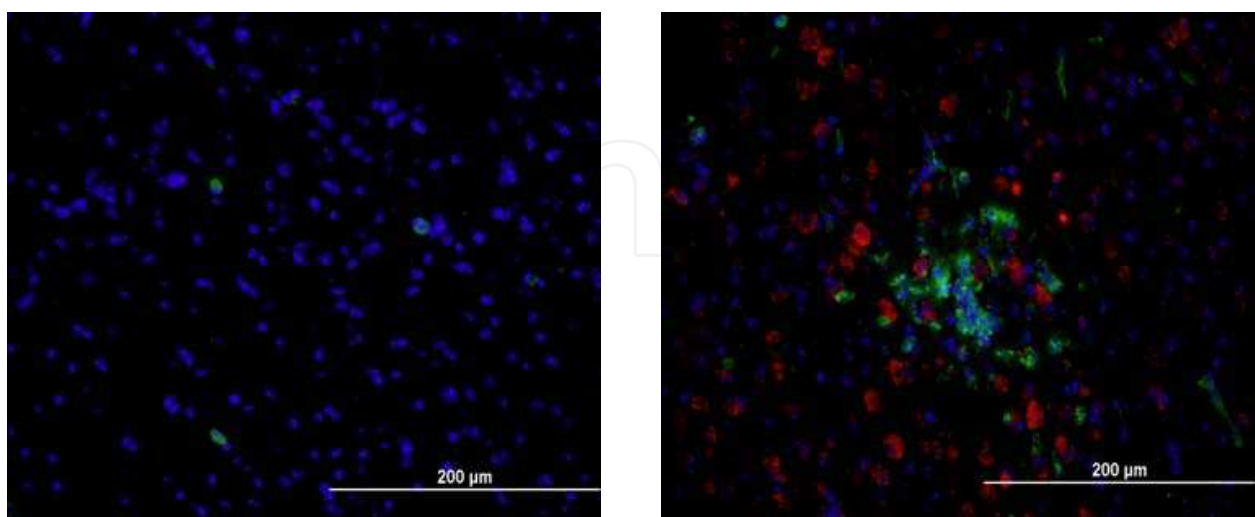

Fig. 4. Immunohistochemistry showing myeloid lineage cells infiltrating the WNV-infected brain. Green = macrophages and microglia (lectin staining); Red = anti-NS-1 (WNV-infected neurons); Blue = DAPI. Left = Mock-infected, Right = d7 p.i. WNV-infected

Once in the brain, both MDM and activated microglia can process antigen and reactivate WNV-specific memory T cells (Ford et al., 1996; Olson & Miller, 2004), but cannot initiate responses in naïve T cells (Getts, Unpublished). Both cell types produce significant amounts of NO, presumably via NOS2 induced by IFN-γ from infiltrating T cells (Schoneboom et al., 2000; Jana et al., 2001) (Figure 6). Pharmacological inhibition of NO at specific time points in an MVE model results in extended survival (Andrews et al., 1999). This is different from models in which NO is abrogated throughout infection, where survival is reduced (Fenyk-Melody et al., 1998; King et al., 2007). This highlights a crucial point about models used to determine susceptibility to lethal flavivirus infection. While the absence or presence of a particular gene may dictate susceptibility, the involvement of this gene in the disease process may vary significantly during the course of infection. It may be critical for virus control at one point, but be responsible for immunopathological damage at another. This is further emphasised by the fact that depletion of the macrophage population throughout the disease course results in accelerated death (Ben-Nathan et al., 1996), while temporally targeted inhibition of macrophage immigration into the brain in WNV encephalitis results in long term survival and immunity (Getts et al., 2008).
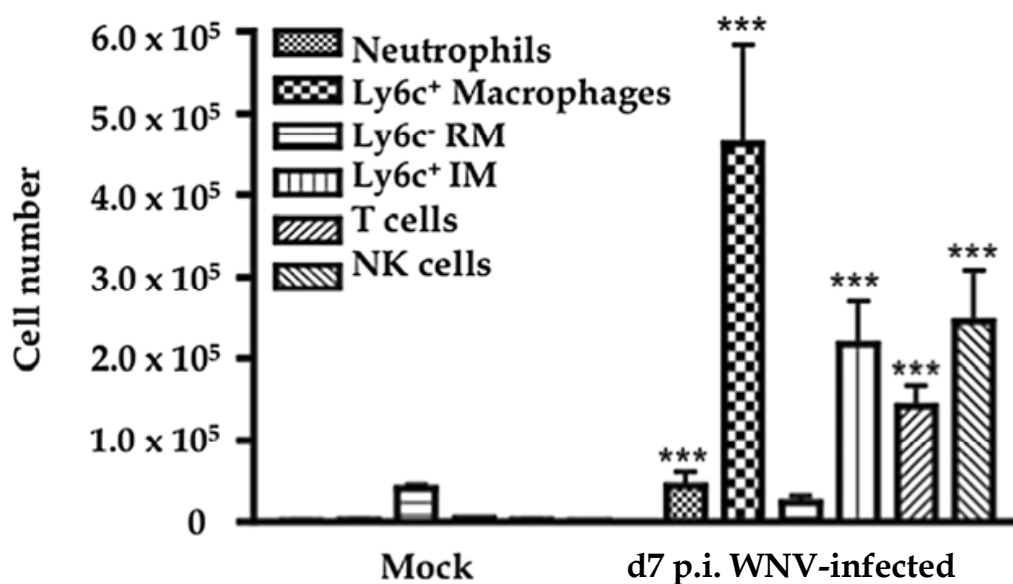

Fig. 5. Leukocyte subsets in the WNV-infected brain on d7 p.i. RM= resident microglia, IM= immigrant microglia

Peripheral production of NO is also involved in DC-programmed T cell responses. Bone marrow-derived DC from NOS2 knockout mice elicited a much stronger Th1 immune response with increased chemokine expression. Interestingly, lack of NO-mediated modulation resulted in expansion of a Ly6C^{hi} type inflammatory DC subset, implicating NO as a potential negative regulator of immune-mediated pathology (Giordano et al., 2010). On the other hand, in influenza infection, increased Th1 responses in the absence of NOS2 may result in higher antiviral antibody titres and more effective virus clearance with less immunopathology (Jayasekera et al., 2006). As with WNV encephalitis, neuroinflammation plays a significant role in the severity of experimental autoimmune encephalomyelitis (EAE). NOS2 knockout mice are much more susceptible to EAE than wild type mice (Fenyk-

Melody et al., 1998). This raises the question of the role of Ly6C^{hi} inflammatory cells in the severity of neuropathology in WNV infection (Giordano et al., 2010).
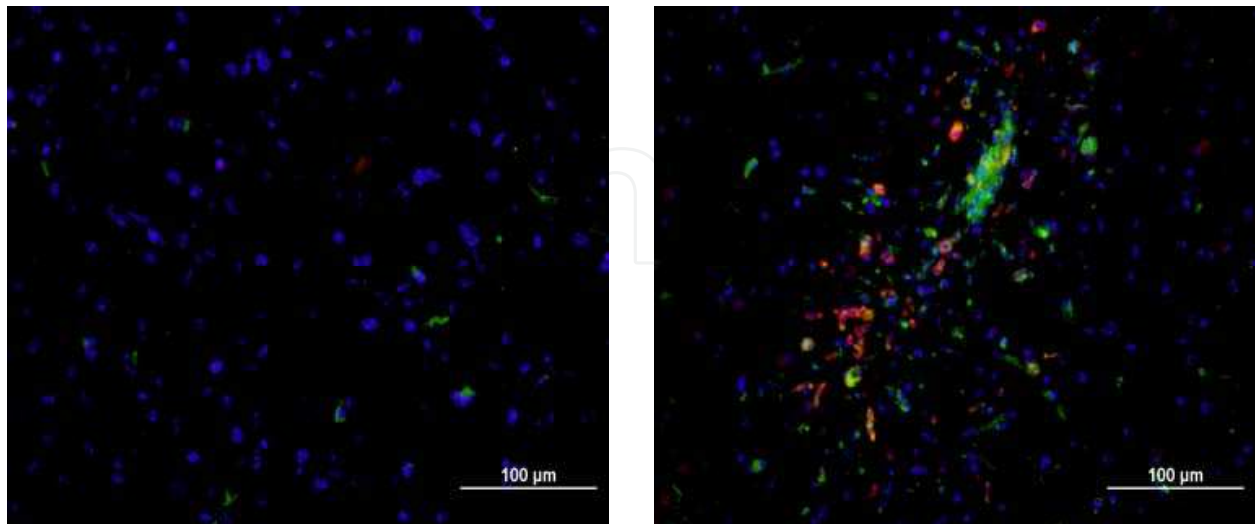

Fig. 6. Macrophages and microglia produce NO in response to WNV infection. Green = macrophages and microglia (lectin staining); Red = iNOS/NOS2; Blue = DAPI. Left = Mock infected, Right = d7 WNV

Mice and human peripheral blood monocytes are divided into circulating and inflammatory subsets. As mentioned above, inflammatory monocytes are recruited to inflamed tissue by chemokines like CCL2, while circulating monocytes may replace resident macrophages in the steady state. In the murine model, circulating monocytes have Gr1$^-$(Ly6C^{lo})/CX3CR1$^+$ phenotype; inflammatory monocytes have a Gr1^{hi}(Ly6C^{hi})/CCR2$^+$ phenotype (Geissmann et al., 2003; Tacke & Randolph, 2006). As mentioned, Ly6C^{hi} monocytes can differentiate into DC in the spleen and lung as well as into LC and macrophages under inflammatory conditions, where they are capable of stimulating naïve T-cells (Geissmann et al., 2003; Leon et al., 2004; Ginhoux et al., 2006; Landsman et al., 2007). During WNV infection there is a substantial increase in CD45^{int}/CD11b$^+$/CD11c$^-$ activated microglia in the brain. Depletion of blood monocytes reduces numbers of activated microglia in the CNS and adoptive transfer of Ly6C^{hi} inflammatory monocytes from the bone marrow confirms that activated microglia in the CNS are derived from inflammatory monocytes in WNV infection (Getts et al., 2008).

CCR2 and its ligand, CCL2, are important in the emigration of inflammatory monocytes from the bone marrow and migration to inflamed sites, including the CNS (Serbina & Pamer, 2006; Tsou et al., 2007; Getts et al., 2008; Lim et al., 2011). Immunohistochemical staining shows CCL2 expression by WNV-infected neurons, often with macrophages and microglia in close attendance (Figure 7). Recent findings however, indicate that CCR2 does not control the accumulation of Ly6C^{hi} monocytes from blood to CNS directly, but rather by regulating the levels of monocytes in the blood (Lim et al., 2011). Interestingly the neutralisation of CCL2 leads to increased survival rates in WNV-infected mice, indicating the possible role that inflammatory cells play in the immunopathology of WNV encephalitis (Getts et al., 2008). CCR2 and CCR4 also seem to be involved in the pathogenesis of dengue virus disease. Dengue-infected CCR2 and CCR4 knockout mice show increased survival

rates and have decreased levels of systemic cytokines like IL-6 and IFN-γ, as well as lower numbers of activated NKT, CD4⁺, CD8⁺ T cells and macrophages in the spleen compared to wild type mice. Although the viral load in the CCR4 knockout and wild type mice was similar, it was suggested that the reduced mortality rates in these mice is due to reduced damage to organs and tissues associated with the abrogated inflammatory response (Guabiraba et al., 2010).
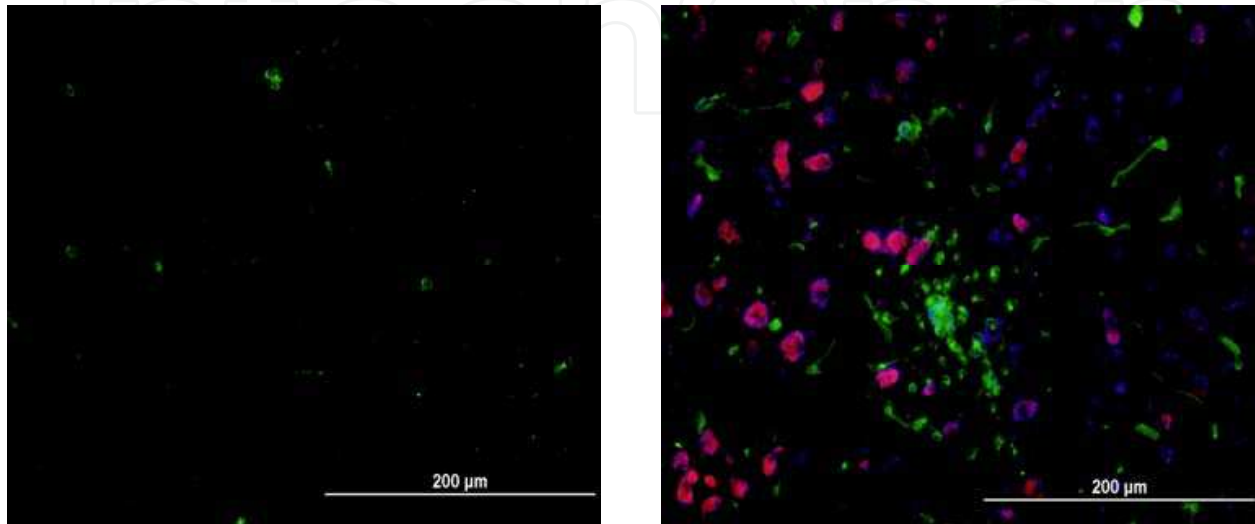

Fig. 7. Infected neurons produce CCL2 which recruits monocytes to the brain.
Green = macrophages and microglia (lectin staining); Red = NS-1 (actively infected neurons); Blue = CCL2. Left = Mock-infected, Right = d7 p.i. WNV-infected

8. Infiltration of other leukocyte subtypes

While up to 50% of leukocytes infiltrating into the CNS following infection are MDM, significant numbers of CD4⁺, CD8⁺ T cells (10-15%) and NK cells (10-20%) also immigrate into the brain, although there are relative few neutrophils (1-3%) (Figure 5, 8). T cells are recruited by increased astrocyte and microglial CXCL10 and CXCL9 expression, as well as CCL5 (Glass et al., 2005).

As for mice, T cell effector subsets migrate into the perivascular space and brain parenchyma during flavivirus infection of monkeys. T cells are seen spread evenly throughout the parenchyma and perivascular space, whereas labelling for CD4⁺ and CD8⁺ subsets shows a more compartmentalised distribution, with CD4⁺ cells located mainly in the perivascular space. Either the CD4⁺ or CD8⁺ T cell subset may be the dominant lymphocyte present in CNS in infection (Kelley et al., 2003; Maximova et al., 2009). Whether they contribute to immunopathology or not, T cells are clearly crucial to virus clearance; mice deficient in this subpopulation exhibit higher mortality rates (Y. Wang et al., 2003; Y. Wang et al., 2006; Brien et al., 2008). Further highlighting these points, despite significantly lower myeloid cell infiltration in WNV infection in RAG knockout mice and prolonged survival, these animals die with high titres of virus in the CNS (King, Unpublished). In contrast to WNV, in TBEV infection in mice, immunopathology is evidently mediated by CD8⁺ T-cells, although these cells are not responsible for BBB breakdown (Růžek et al., 2011). CD4⁺ T cells evidently play a protective role in TBEV, possibly via secretion of IFN-γ and

other pro-inflammatory cytokines and/or stimulation of 'macrophage-like' cells (Růžek et al., 2009).

The chemokine receptor CXCR3 is a key regulator of T-cell recruitment and thus plays a pivotal role in the pathogenesis of many diseases (Muller et al., 2007). During WNV encephalitis in mice the chemokine receptor CXCR3 enables recruitment of antiviral CD8+ T cells to the CNS in a region-specific manner. Loss of this receptor leads to increased mortality rates in these animals (B. Zhang et al., 2008). TNF is also essential in the recruitment of antiviral CD8+ T cells to the CNS in WNV infection (Shrestha et al., 2008).
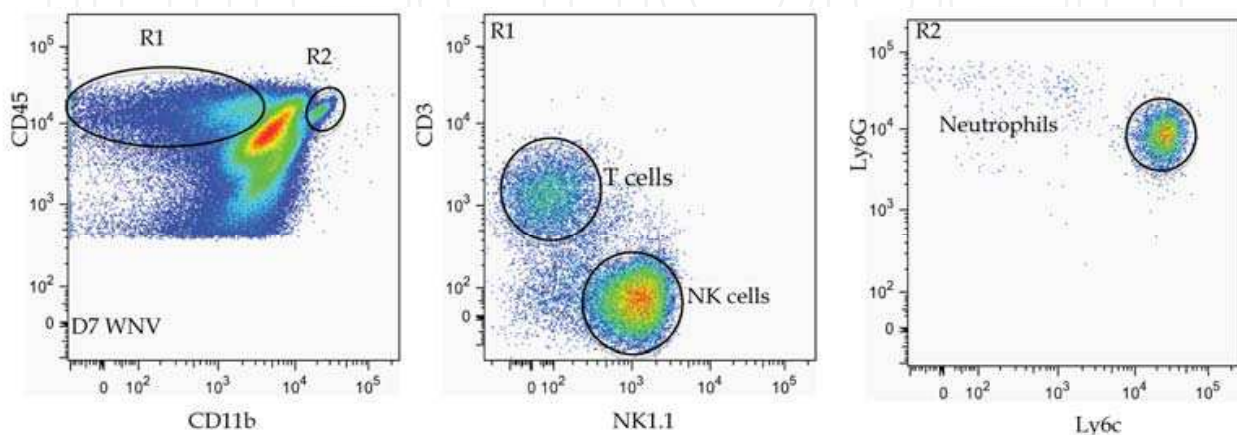

Fig. 8. Flow cytometry showing regional analysis of infiltrating leukocytes in murine brain. R1 denotes CD45hi, CD11blo lymphocytes, which can be further divided into T cells and NK cells; R2 denotes CD45hi, CD11bhi neutrophils, which are Ly6G+, Ly6C+

Although B cells are also observed in the brain in some studies (Brehin et al., 2008), CD20+ B cells are predominantly found in the perivascular space surrounding neurons. Proportions of B and T cells in the brain vary depending on the infecting flavivirus (Maximova et al., 2009). By flow cytometry, we find few B cells in the brain parenchyma and while the BBB is intact it seems unlikely that antibodies have a significant effect against WNV within the brain, although antibody is clearly important systemically in protection against WNV (Diamond et al., 2003). NK cells, while present in significant numbers in the brain parenchyma, have not been studied in any detail. Indeed, to date they have been regarded as redundant in flavivirus infection, since upregulated MHC expression on infected cells reduces NK cell recognition. However, *in vitro*, under artificially-stimulated conditions, NK cells are cytotoxic for WNV-infected cells (M. Zhang et al., 2010).

The lack of neutrophil infiltration is perhaps not surprising as there is little or no CXCL1 expression in the brain during infection. However, in MVE, infiltration of NO-producing neutrophils, attracted by TNF and N51/KC, are a significant part of the inflammatory leukocyte infiltration and contribute to the severity of disease. Neutrophil depletion and inhibition of NOS prolongs survival of infected animals and reduces the severity of pathology in the CNS (Andrews et al., 1999). Since the neutrophil-depleting antibody used also depletes monocytes, however, it is not clear to what extent immigrating macrophages may have contributed to lethal pathology. Nevertheless, this again highlights the point that temporally selective depletion may reduce pathology, while genetic deletion may exacerbate it.

9. Immunomodulating therapies

Despite the large amount of research in the field, there is currently no approved antiviral therapy against WNV in humans and treatment remains symptomatic. Currently, the only live flavivirus vaccines with a relatively good record of safety are against yellow fever (Monath, 2010) and JEV (X. Liu et al., 2011) and the only commercially available live vaccines against WNV are for horses. Two highly purified formalin-inactivated whole virus vaccines against TBEV have been used in Europe since 1976 (FSME Immun, Baxter) and 1999 (Encepur, Novartis) and an efficacy of 99% has been reported in Austria, with no difference between age groups, and no major safety issues reported (Barrett et al., 2003; Heinz et al., 2007). Another avenue that is currently being investigated is antivector vaccines that target mosquitoes or ticks directly (Kortekaas et al., 2010). Recently, stable infection with the bacterium, *Wolbachia*, has been achieved, with significant potential for limiting the transmission of dengue (McMeniman et al., 2009; Bian et al., 2010). Antivirals like Ribavirin can successfully act against WNV *in vitro* (Jordan et al., 2000; Anderson & Rahal, 2002), but have no notable antiviral effect when used to treat infected humans (Chowers et al., 2001). Although initial studies with antiviral medication appeared promising, increased mortality rates led to several candidates being disqualified. This and the fact that a significant amount of neuropathology is induced by the immune system itself has led to more research being conducted into modulation of the immune system. Indeed, general immunomodulators that inhibit inflammatory mediator synthesis and can cross the BBB are potential candidates. Patients infected with TBEV receiving the anti-inflammatory antibiotic, tetracycline, had reduced levels of IL-6, IL-1β, TNF, quicker improvement of symptoms and faster clinical recovery than untreated patients (Atrasheuskaya et al., 2003).

Because interferons play such a prominent role in mitigating viral infection and modulating the adaptive immune system, they have been prime candidates for immunomodulating therapies (Samuel & Diamond, 2005; Jiang et al., 2010). IFN-α2b has protective and therapeutic properties in Vero cells (Anderson & Rahal, 2002). IFN-γ and β play a role in inhibiting viral spread and replication in the periphery as well as the CNS and are also argued to reduce WNV-induced neuronal death (Samuel & Diamond, 2005). RNA for interferon-stimulated genes (ISG) and their protein expression are upregulated during viral infections (Wacher et al., 2007). Five of the IFN-α-induced ISG, namely Viperin, ISG20, protein kinase R (PKR) and IFN-induced transmembrane proteins 2 and 3 (IFITM2, IFITM3) have been shown to disrupt multiple steps in the dengue and WNV life cycle. IFITM2 and 3 in particular serve to inhibit viral entry into host cells (Jiang et al., 2010). The matrix metalloproteinase (MMP) inhibitor, GM6001 has been shown to reverse the damage to BBB integrity induced by MMPs with WNV infection (Verma et al., 2010). Cytotoxic T cells can eradicate WNV by lysing infected cells and this could potentially be used in developing treatment against WNV. The vaccination of individuals with a chimeric WNV/yellow fever vaccine, coding a WNV envelope antigen, results in the production of CD8+ T cells that persist for up to a year with similar qualities to memory T cells (Smith et al., 2010). Cytotoxic T lymphocytes generated against WNV-specific epitopes are able to eradicate infected cells *in vitro* as well as increase survival rates when transferred into mice. This approach may thus result in a more targeted immune response and optimised eradication of virus (Purtha et al., 2007).

Neutralising antibodies have also been successful in treating and preventing early infection in animal models and humanised antibodies could provide the basis for treatment after known exposure to infection (Throsby et al., 2006; Vogt et al., 2009). The use of certain human single chain variable fragments fused with an IgG1 Fc region is able to confer some protection against lethal WNV in mice. The addition of Fc region to this protein is critical for the effectiveness of its neutralising function and suggests whole monoclonal antibodies (mAbs) may more effectively neutralise WNV (L.H. Gould et al., 2005). Further supporting this, the neutralising potential of mAb E16 is less effective in mice lacking the Fc receptor (Oliphant et al., 2005). It is tempting to speculate also, that NK cell-mediated antibody-dependent cell-mediated cytotoxicity may contribute to viral clearance in the periphery.

Of the myriad antibodies induced by WNV infection only a few neutralise WNV and have a possible therapeutic role. Neutralising antibodies are mainly directed against domain III of the WNV envelope-protein where they prevent the conformational change in the virus required to fuse to cellular membranes, consequently inhibiting it from entering the cell (Oliphant et al., 2005; Throsby et al., 2006; Morrey et al., 2007; S. Zhang et al., 2009). Polyclonal antibodies promote survival in B- and T cell-deficient mice, indicating their therapeutic potential (Engle & Diamond, 2003). The axonal spread of virus can also be blocked by neutralising antibodies *in vitro* and *in vivo* (Samuel et al., 2007). Two neutralising human monoclonal antibodies with therapeutic potential are CR4348 and CR4354, both capable of inhibiting WNV *in vitro* and *in vivo*. They bind to epitopes on the E-protein of the virus and neutralise the virus at the postattachment step, it is thought by preventing fusion of the virus to the endosomal membrane. Prophylactic treatment with these antibodies protects mice against lethal WNV infection (Vogt et al., 2009). On the other hand, humanised mAb E16 is able to increase survival of both mice and hamsters, even when administered after the CNS is infected (Oliphant et al., 2005; Morrey et al., 2007). The treatment of hamsters 5 days after WNV inoculation with this E16 antibody also results in decreased viral burden in the CNS and increased survival (Morrey et al., 2006). The affinity of neutralising antibodies has also been associated with neutralising efficacy. Affinity alone, however, does not ensure the efficacy of inhibiting WNV activity, as some non-neutralising antibodies bind with high affinity to their epitopes. Thus, not surprisingly, both the specific epitope and binding affinity play a role (Sanchez et al., 2005).

Other factors limiting antibody-mediated treatment include the poor movement of antibodies across an intact BBB to enable them to act on virus-infected neurons (Cheeran et al., 2005; Morrey et al., 2007) and the possibility that mutant virus strains might arise through antibody treatment. For example, mutations of the E16 epitope of the virus can abrogate the neutralising activity of monoclonal antibodies (Li et al., 2005; Sanchez et al., 2005). Thus, although resistant strains to specific mAbs are currently rare, they need to be considered in the design of antibody therapy (S. Zhang et al., 2009). Time of therapeutic administration and viable administration routes for human patients are also important considerations because of the possibility that antibodies could further exacerbate disease via antibody-dependent enhancement of infection. This classically occurs in a second dengue infection. Here, virus-specific antibodies from a first dengue infection with a low affinity for the second strain of dengue do not neutralise, but complex with virus. This enables virus to gain access to monocytes, macrophages and mature DC via Fcγ receptors typically borne by the myeloid lineage, resulting in increased viral loads in individual cells, as well as

increasing numbers of productively-infected cells (Halstead & O'Rourke, 1977; Boonnak et al., 2011).

This currently leaves cell subset targeting as an immunomodulating therapy, but issues about which subset(s) to target, how to target them effectively and when to target them during disease remain to be resolved. However, the temporal targeting of immunopathological subsets and/or the enhancement of potentially anti-inflammatory subsets in viral encephalitis, with the idea of enabling successful virus clearance by the remaining elements of the adaptive immune response, remain a legitimate goal, since, unlike the long-term requirement for subset blockers in *e.g.*, multiple sclerosis, which may be associated with progressive multifocal leukoencephalopathy, short-term use is much less likely to have permanent sequelae.

10. Conclusion

Research on WNV and flaviviruses in general has increased dramatically in the last 10 years, since the advent of WNV in New York in August 1999. It has become clear that different flaviviruses have different interactions with the immune system and what may be pathogenic in one infection may prolong survival in another. This is undoubtedly further complicated by the variability of human (and mouse) genetics, as well as the variety of disease models. While it may eventually be possible to personalise treatments ranging from cancer to infectious disease, at this stage we do not know enough about the pathogenesis of these infections to inform the kind of generic approach required to intervene to prevent lethal disease in a meaningful number of patients successfully. While it seems likely that an effective vaccine will eventually be available, many issues have to be addressed before general immunisation is a viable proposition. Notwithstanding the success of this endeavour, there will always be individuals in which it will be necessary to intervene clinically, due to poor vaccine compliance, failure of vaccination, as well as other uncontrollable factors. Unlike species-specific viruses like smallpox, the pleotropic nature of flaviviruses, their carriage by migratory wild birds capable of amplifying them, the difficulty of eradicating their arthropod vectors, in addition to other factors, many of our own making, that continue to extend vector habitats, will make them virtually impossible to eradicate by vaccination.

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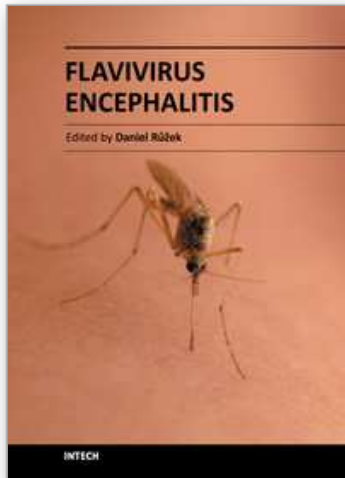
Flavivirus Encephalitis

Edited by Dr. Daniel Ruzek

Encephalitis is an inflammation of the brain tissue associated with clinical evidence of brain dysfunction. The disease is of high public health importance worldwide due to its high morbidity and mortality. Flaviviruses, such as tick-borne encephalitis virus, Japanese encephalitis virus, Murray Valley encephalitis virus, or St. Louis encephalitis virus, represent important causative agents of encephalitis in humans in various parts of the world. The book Flavivirus Encephalitis provides the most recent information about selected aspects associated with encephalitic flaviviruses. The book contains chapters that cover a wide spectrum of subjects including flavivirus biology, virus-host interactions, role of vectors in disease epidemiology, neurological dengue, and West Nile encephalitis. Special attention is paid to tick-borne encephalitis and Japanese encephalitis viruses. The book uniquely combines up-to-date reviews with cutting-edge original research data, and provides a condensed source of information for clinicians, virologists, pathologists, immunologists, as well as for students of medicine or life sciences.

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