Review Article

Indian J Med Res 138, November 2013, pp 632-647

Antiviral macrophage responses in flavivirus encephalitis

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Received October 18, 2012

Mosquito-borne flaviviruses are a major current and emerging threat, affecting millions of people worldwide. Global climate change, combined with increasing proximity of humans to animals and mosquito vectors by expansion into natural habitats, coupled with the increase in international travel, have resulted in significant spread and concomitant increase in the incidence of infection and severe disease. Although neuroinvasive disease has been well described for some viral infections such as Japanese Encephalitis virus (JEV) and West Nile virus (WNV), others such as dengue virus (DENV) have recently displayed an emerging pattern of neuroinvasive disease, distinct from the previously observed, systemically-induced encephalomyelopathy. In this setting, the immune response is a crucial component of host defence, in preventing viral dissemination and invasion of the central nervous system (CNS). However, subversion of the anti-viral activities of macrophages by flaviviruses can facilitate viral replication and spread, enhancing the intensity of immune responses, leading to severe immune-mediated disease which may be further exacerbated during the subsequent infection with some flaviviruses. Furthermore, in the CNS myeloid cells may be responsible for inducing specific inflammatory changes, which can lead to significant pathological damage during encephalitis. The interaction of virus and cells of the myeloid lineage is complex, and this interaction is likely responsible at least in part, for crucial differences between viral clearance and pathology. Recent studies on the role of myeloid cells in innate immunity and viral control, and the mechanisms of evasion and subversion used by flaviviruses are rapidly advancing our understanding of the immunopathological mechanisms involved in flavivirus encephalitis and will lead to the development of therapeutic strategies previously not considered.

Key words Cellular infiltration - dengue virus - flavivirus encephalitis - Japanese encephalitis virus - monocytes - macrophages - myeloid - West Nile virus

The flaviviruses: virology, epidemiology, and human disease

***Virology and ecology*:** Flaviviruses are single-stranded positive-sense RNA viruses, the genome of which consists of three structural, and seven non-structural (NS) proteins[1]. Almost all members are maintained via arthropod vector transmission between vertebrate hosts, usually mosquitoes, where high viraemias facilitate transmission to new vectors[2]. Perhaps the most important flaviviruses are the neurotropic West Nile (WNV), Japanese encephalitis (JEV) and Tick-borne encephalitis (TBEV) viruses and the viscerotropic dengue (DENV) and yellow fever viruses (YFV). JEV is the most significant cause of mosquito-borne encephalitis worldwide, with WNV now the leading cause of viral encephalitis in the USA[3,4], and DENV is the most important arboviral disease worldwide, with an emerging capacity for neuroinvasiveness[4]. WNV and JEV belong to the JE serogroup, which includes other neurotropic flaviviruses, such as the North American St Louis encephalitis (SLEV) and the Australian Kunjin (KUNV) and Murray Valley encephalitis viruses (MVEV). WNV and JEV are maintained in enzootic life cycles, through transmission between amplifying hosts (birds and pigs, respectively) by mosquito vectors[5], but infect a variety of host and mosquito species[6]. For neuroinvasive flaviviruses such as WNV and JEV, human infection typically occurs via Culicine mosquitoes. *Aede*s (*Stegomyia*) aegypti is the primary vector species for DENV, with the secondary vector being *Aedes (Stegomyia) albopictus*[7,8].

***Epidemiology*:** Flaviviruses are found on all continents but Antarctica. JEV is endemic throughout Southeastern and Central Asia[4], while WNV is endemic to many parts of Africa, Asia and the Middle East[9]. WNV emerged in the Americas subsequent to an outbreak in New York City in 1999[10], where there were 62 confirmed cases, with 37 cases of encephalitis and 7 deaths (case fatality rate of 12%)[10]. Following this, WNV spread throughout the USA within 5 years, and is now the leading cause of arboviral encephalitis in North America[11]. DENV is endemic throughout the tropics and sub-tropics, where approximately 2.5 billion people are at risk of infection in over 100 different countries[12]. Additionally, the co-circulation of all four serotypes of DENV (DENV-1-4) has resulted in the 'hyperendemic' occurrence of DENV in many countries. There are approximately 50 million infections with DENV per year, whereas JEV results in approximately 30,000 - 50,000 cases per year[4].

***Clinical disease progression*:** Flaviviral infections have a broad range of clinical presentations, often with unpredictable outcomes. Approximately 20 per cent of WNV-infected individuals will develop a febrile illness termed West Nile Fever[13], and febrile illness also occurs in up to 4 per cent of individuals infected with JEV[4]. It is estimated that approximately 1 in 150 WNV infections will result in neuroinvasive disease[14], which typically manifests as meningitis, encephalitis, or acute flaccid paralysis. It is likely that 30-40 per cent of such cases present as encephalitis[15,16], although these figures may be underestimated due to diagnostic overlap between clinical syndromes[17]. Approximately 20 per cent of WNV neuroinvasive cases result in death, and approximately 70 per cent of the survivors have permanent neurological sequelae[17]. Similar to WNV in that less that 1 per cent of infections are symptomatic, some 70-80 per cent of symptomatic JEV infections result in neuroinvasive disease[4]. In contrast, approximately 50 per cent of DENV infections result in significant clinical syndromes, including dengue fever and severe dengue[4]. It is thought that neurological involvement of DENV infection occurs in up to 5 per cent of cases, principally related to infection with DENV serotypes 2 and 3[18], while DENV infection is thought to be involved in 4-13 per cent of CNS infections in some settings in tropical countries[19].

The myeloid lineage

The myeloid lineage plays an important role in flavivirus pathogenesis. In the CNS infected neurons become surrounded by activated migratory microglia in microglial "nodules", while obvious perivascular cuffing is indicative of both lymphoid and myeloid infiltration into the CNS. In DENV, macrophages may be pivotal in viral spread, but are also involved in promoting capillary permeability associated with dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS)[20]. Recent advances in understanding of myeloid lineage development and differentiation highlight the inherent heterogeneity of macrophage and dendritic cell (DC) populations, and the difficulties this presents in accurately identifying and differentiating these populations. Detailed understanding of these cells is critical in informing possible interventional approaches that may ameliorate disease pathogenesis.

***Myeloid lineage and the mononuclear phagocyte system*:** Monocytes, and the majority of macrophage and DC populations are part of the myeloid lineage, originating from the bone marrow. Residing in the bone marrow, lymphoid, and peripheral tissues and

circulating in the blood stream, these cells constitute the mononuclear phagocyte system[21]. Recent research using *in vivo* models has helped to understand the developmental pathways and lineage relationships in this system[22], with intricate developmental pathways leading to heterogeneity in phenotype, functionality, and differentiation. Thus, haematopoietic stem cells (HSC) differentiate into macrophage/DC progenitors (MDP), which in turn differentiate in to a recently described intermediate population known as the common monocyte progenitor (cMoP)[23] before differentiation in to monocyte in the bone marrow (BM). These monocytes can emigrate out into circulation in response to chemokine (C-C motif) ligand 2 (CCL2) also known as monocyte chemotactic protein-1 (MCP-1)[24,25]. While many steady-state DC populations were thought to arise from BM-derived monocytes, and some do, the MDP has also been shown experimentally to differentiate into the common DC precursor (CDP), which differentiates in the BM into pre-conventional DC (pre-cDC) and plasmacytoid DC (pDC)[26,27]. This occurs without differentiation in to cMoP, as this population is restricted to the monocyte lineage[23]. The pre-DC may then mature into cDC, following migration to lymphoid tissues[28]. Interestingly, microglia and Langerhans cell (LC) populations appear to be renewed independently of the BM during homeostasis[22]. However, this may be different under inflammatory or infectious conditions[22,29]. The mechanisms of replenishment and differentiation for tissue macrophages and DC are still poorly understood[22,30].

***Research challenges and heterogeneity in cellular populations*:** Although monocyte/macrophages and DC were previously considered to be the distinct cellular populations, a great deal of confusion and difficulty has arisen in distinguishing these subtypes[22,30]. While differentiation may commit a cell to a monocyte/macrophage or DC sublineage, the adaptability, heterogeneity, and capacity for differentiation beyond their nominally terminal identity have made their accurate study difficult. This difficulty arises for three main reasons: heterogeneity between anatomical locations, differentiation and migration responses following inflammation, and the use of non-specific markers in flow cytometry[22,30,31]. These are considered below.

Monocytes: Monocytes and macrophages are significant contributors to, and regulators of, innate immunity. In response to infection, monocyte production in the bone marrow may rapidly be increased with recruitment of new cells to the sites of inflammation[22,30,32-34]. Pre-differentiated monocytes detect pathogens via a number of pathogen recognition receptors (PRR), producing various soluble cytokine and antimicrobial toxic mediators in response. The key contributions of monocytes to innate and adaptive immunity are a result of the activities of these differentiated populations. In humans two populations of circulating blood monocytes have been identified. These have been traditionally termed the $CD14^{+}/CD16^{-}$ classical (or inflammatory) and $CD16^{+}/CD14^{-/lo}$ inflammatory monocytes. More recently it has become clear that the $CD16^{+}$ population is composed of two sub-populations, including a unique $CD16^{+}/CD64^{+}$ population that exhibited characteristics of both macrophages and DC[35].

Murine monocytes have analogous subsets, making them useful for understanding human monocyte biology[22,30,36]. These are identified primarily by forward and side scatter (FSC/SSC) characteristics by flow cytometry and their $CD45^{+}/CD115^{+}/CD11b^{+}$ expression[33,36]. However, expression of CD11b on monocytes in the spleen can be low or negative, while other non-specific markers like major histocompatibility complex (MHC)-II may be negative on monocytes/macrophages in non-inflammatory scenarios[22,30,31]. Additionally, $F4/80^{+}$ and $CD11c^{-}$ expressions, also considered specific for monocytes, are dependent on their anatomical location and macrophage differentiation status[22,30,31,37,38]. Two principle subsets of murine monocytes are identified, based on the expression of lymphocyte antigen 6C (Ly6C). Inflammatory/classical monocytes are $Ly6C^{hi}/CCR2^{hi}/CXCR31^{lo/int}$, whereas patrolling/non-classical monocytes are $Ly6C^{lo/-}/CCR2^{-}/CX3CR1^{hi}$ [33,36]. $Ly6C^{hi}$ monocytes develop in the bone marrow and migrate out via a CCR2-dependent mechanism to replenish $Ly6C^{hi}$ monocytes in circulation[33,39]. Additionally, $Ly6C^{hi}$ monocytes may downregulate Ly6C expression both in the circulation and in inflamed tissues, giving rise to $Ly6C^{lo}$ populations[24,40]. However, this relationship is still unclear, as depletion of $Ly6C^{hi}$ monocytes did not reduce the number of $Ly6C^{lo}$ monocytes[22], while $Ly6C^{lo}$ BM monocytes can clearly give rise to $Ly6C^{hi}$ monocytes[40]. During inflammation or infection, $Ly6C^{hi}$ monocyte production in the bone marrow can increase substantially. Monocytes recruited to the site of infection or inflammation can differentiate into a variety of macrophage and DC subtypes (including in the brain) to carry out their principle anti-microbial functions[22,29,30,32-34].

Macrophages: The term 'macrophage' is traditionally assigned to resident phagocytic cells in lymphoid and non-lymphoid tissues and also inflammatory monocytes that have entered inflamed tissue, and undergone defined phenotypic changes, presumably related to *in situ* function. In addition to CD11b, F4/80 is the marker most commonly associated with macrophages in the mouse. However, as with monocytes, DC in the spleen and other tissues have also been shown to express F4/80, making this distinction moot[22,30,31]. The typical changes associated with macrophage activation, or monocyte differentiation may include the downregulation of Ly6C, and the upregulation of MHC-II, which, as alluded to above, is not generally expressed on resting macrophages[22,30,31]. The interferon gamma (IFN-γ)-induced activation of macrophages by pathogen-specific $CD4^+$ T helper (Th) cells results in enhanced recognition and phagocytosis of pathogens and the release of toxic-mediators, such as nitric oxide (NO) and reactive oxygen species (ROS) to eliminate these. While macrophages can re-stimulate T-cell effector responses, more recent research has revealed a broader capacity for immune stimulation by macrophages[41-43], with some monocyte subsets displaying functionality of both macrophage and DC, to enable initiation of naïve T cell responses, also[44].

Dendritic cells: Dendritic cells (DC) are a type of mononuclear phagocyte of either myeloid or lymphoid origin, which most efficiently initiate pathogen-specific adaptive immune responses. This activity is central to the eradication of foreign pathogens and the establishment of immune memory and tolerance. DCs express high levels of CD11c and MHC-II simultaneously[30,45]. The two major populations of DC are the conventional DC (cDC) and Plasmacytoid DC (PDC)[22]. The cDC are believed to be of myeloid origin, and function primarily in immune stimulation. The PDC, on the other hand, produce large quantities of IFN-α/β in response to viral infection, and are traditionally identified as $CD11c^+$/$B220^+$, although CD11c is expressed at a lower level than cDC[22,30,45-47]. While PDC are not usually associated with adaptive immune stimulation, these are capable of stimulating adaptive immune responses[47] and are recruited in large numbers to the draining lymph node during viral infection[40]. Studies have also described the differentiation of inflammatory monocytes into inflammatory DC, including into the tumour necrosis factor (TNF)/NO-producing dendritic cell (Tip-DC) during some bacterial infections, such as *Listeria monocytogenes*, but not others[47,48], as well as in WNV infection in the skin[40]. These cells are believed to contribute to the innate immune response by preventing pathogen dissemination[48,49]. Recently, TipDC have been shown to produce IFN-β, and subsets have been shown to contribute to adaptive immune responses[50,51]. The use of non-specific markers in distinguishing heterogenous macrophage populations from DC has lead to some confusion in their identification. While it is clear that the CD11c-expressing cells in some lymphoid tissues (such as the spleen and lymph nodes) are DC, macrophages in tissues such as the peritoneal cavity can express relatively high levels of CD11c[22,30,37,41-43]. These findings have led to the notion that populations such as Tip-DC are analogous to inflammatory macrophages[30]. Interestingly, the clone of antibody used to detect levels of CD11c on peritoneal macrophages is important; clone N418 shows much higher levels of CD11c expression than HL3[37]. These potentially confounding factors suggest that CD11c expression alone is insufficient for accurate identification of DC. Thus, while co-expression of CD11c and MHC-II generally provides good resolution of DC populations, confirmation of their identification relies on functional studies of flow-sorted cells.

Macrophages and the pathogenesis of flavivirus infection

The recruitment of the monocyte/macrophage lineage is a key component in the first line of defence in controlling viral spread, among a variety of cellular processes to detect, respond to, and eliminate flaviviral pathogens. The innate response to viruses broadly consists of virus recognition and cellular activation; induction of the antiviral state; and cell-mediated viral eradication. However, co-evolution of the flaviviruses with the vertebrate host has resulted in a number of mechanisms that enable evasion and even subversion of the innate immune system. As such, this response may enhance viral replication and dissemination, exacerbating systemic disease through cytokine manipulation, in turn enabling neuroinvasion, with activation of immune responses in the brain that can lead to immune-mediated CNS pathology.

Early events after peripheral inoculation: Virus is inoculated into the dermis during the bite of an infected mosquito via its saliva[52] which may be locally immunosuppressive, thus contributing to enhanced virus survival[53]. It is thought that Langerhans and dermal dendritic cells become infected[54,55], potentially via [DC specific intracellular adhesion molecule-3 (ICAM-3) grabbling non-integrin] (DC-SIGN), a C type lactin expressed on the surface of DC, or DC-SIGN

receptor (DC-SIGNR) binding. Certainly WNV has been shown to infect a variety of peripheral cell types, including monocytes and macrophages[56,57], DC[55], myoblasts[58], trophoblasts[59], fibroblasts[60,61], and endothelial cells[62,63], in addition to CNS-associated cells such as Schwann cells[64], astrocytes[65], and neurons[29]. Following the initial infection, migration of WNV-infected LC and dermal DC to the draining lymph node (DLN) may facilitate viral spread. While many studies have demonstrated LC migration[55,66,67], WNV replication within LC *in vivo* has yet to be demonstrated. Interestingly, DENV has been shown to replicate within DC, followed by migration to the DLN in humans[68]. During the infectious cycle in the skin, an inflammatory response is initiated which results in a vigorous recruitment of inflammatory BM monocytes to the site of infection. Differentiation of these monocytes into inflammatory DC in the skin and DLN, may limit viral dissemination early during infection[40].

Viraemia: Following the establishment of initial infection, the dissemination of virus via the bloodstream is temporally associated with febrile illness and systemic infection. Work showing antibody-dependent enhancement (ADE) of macrophage infection by various flaviviruses[69-71], has led to the notion that macrophages are the principle cells that facilitate viral replication during this phase of the disease[72]. Febrile illnesses such as West Nile fever are usually self-limiting, and though these have the potential for organ involvement and severe disease, the medically important consequences of WNV and JEV are primarily associated with neuroinvasive disease[4,12]. In contrast, the usual progression from non-severe to severe dengue is associated with substantial morbidity and mortality independent of CNS involvement[4,73].

Antiviral response by macrophages

***Cellular infection: viral entry receptors*:** The earliest event in the establishment of cellular infection is the recognition of molecular targets by flaviviruses, which facilitates receptor-mediated endocytosis. The understanding of the cellular targets required for cellular entry has improved dramatically, although the molecules necessary for this event are still unclear. Current research in flavivirus entry and membrane fusion has been reviewed in detail elsewhere[74]. The flaviviral envelope (E) glycoprotein has been to shown to be critical for cellular infectivity, as it mediates attachment of the virus to cellular receptors[75]. The variety of cell types that may be infected suggests that flavivirus E glycoprotein recognizes either a ubiquitous molecular target present on all cell types, or a variety of molecular targets to facilitate infection.

A variety of surface molecules can facilitate flavivirus attachment and entry, in particular, the greatest focus is perhaps on the C-type lectin, DC-SIGN[76]. This molecule is expressed at high levels on monocyte-derived DC *in vitro*, and at lower levels on macrophage and DC subsets *in vivo*[77]. DC-SIGN-mediated binding enhances cellular infection by strains of lineage I WNV, through interaction with a glycosylated E-glycoprotein, in contrast to the less prevalent lineage II in which this molecule is mostly non-glycosylated[78]. DC-SIGN binding is thought to mediate DENV entry into DC[67]. Interestingly, other studies have shown that WNV preferentially binds to the DC-SIGNR, due to the location of glycosylation sites[77]. This occurs through the glycosylation of the WNV pre-membrane protein (prM) or glycoprotein-E, where the cleavage of the prM protein influences viral tropism[77,79]. Microvascular endothelial cells express DC-SIGNR, particularly in the lymph node. The contribution of other molecules, such as heat shock protein (hsp)-90 and -70, mannose receptor, and CD14 have been reviewed elsewhere[74].

***Viral recognition by macrophages*:** A key group of PRR molecules responsible for the detection of flaviviruses, are the toll-like receptors (TLR)-3 and TLR-7, which recognize intracellular dsRNA and ssRNA, respectively, and are important in WNV recognition[80,82]. Other molecules such as retinoic acid inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA-5) both detect cytoplasmic dsRNA, and have also been shown to play a role in WNV recognition[83-85]. Viral recognition leads to an antiviral response, which includes the phagocytosis of virus. Flaviviruses are able to evade and/or subvert the macrophage response to favour survival and replication[72,86].

***Macrophage antiviral responses and effector molecules*:** Inflammatory cells, including microglia and macrophages entering the CNS during a flaviviral infection produce various cytokines, chemokines and antiviral effector molecules. Inflammatory mediators released by immigrating monocytes are likely to exert both a protective and pathological action, depending on the concentration and the timing of these effector molecules. Among these, TNF and nitric oxide (NO), catalyzed by inducible nitric oxide synthase (iNOS, NOS2), feature prominently[40,57,87,88]. Elevated TNF has been shown in several flaviviral models of infection, including JE[89], DHF[90-92] and WNV. In the case of WNV,

this may be directly or indirectly protective[40,57,87]. There is however, evidence for a positive correlation between the severity of DHF and levels of TNF and IL-6[90,92,93]. Both human and murine monocytes infected with dengue virus produce TNF[92,94-96]. Several observations have been made, indicating a correlation between the release of TNF by infected cells and the development of severe haemorrhage associated with this disease[91,97]. This is hypothesized to occur via the TNF-induced production of reactive oxygen species (ROS) and reactive nitrogen species (RNS)[91].

Increased NOS2 expression has been documented in murine models of MVE, JE and WNV[88,89,98,99]. Nitric oxide is clearly antiviral. *In vitro* experiments with peripheral blood mononuclear cell (PBMC) derived from dengue-infected patients indicate that NO inhibits DENV-1 replication[100] and NO production in IFN-γ-treated macrophage cell lines reduced intracellular replication of JE. Experiments in neuroblastoma cultures suggest that NO-mediated inhibition blocks RNA synthesis and inhibits intracellular viral protein production[101]. *In vivo* studies showed that treatment with *N*-nitro-L-arginine methyl ester (L-NAME), a competitive inhibitor of NOS, to BALB/c mice increased mortality rates[101]. The efficacy of NO as an antiviral molecule can also be observed in the better survival of WNV-infected virus-resistant wild-type 129/SvEvxC57BL/6 strain than NOS2$^{-/-}$ mice of the same background strain[102]. Moreover, NOS2 deficiency reduces the ability of BMDC to induce a TH1 immune response by suppressing the production of Ly6C^{hi} type inflammatory cells[103], which has been implicated in the immunopathogenesis seen during flaviviral infection[29]. Although evidence suggests a neuroprotective[104] and/or antiviral role[101] for NO[89], it clearly also contributes to immunopathology. Both the protective and pathological effects of NO are likely due to oxidative damage caused by the interaction of NO with oxygen radicals such as the superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2) and the hypochlorite anion (ClO^-)[105]. Nitric oxide is produced in *in vitro* cultures of activated monocytes isolated from human patients with dengue[100], as well as in WNV-infected murine macrophage cultures[56]. *In vivo*, immune-mediated damage is argued to be NO-mediated in MVE, where aminoguanidine-mediated inhibition of NOS in a murine model of MVE resulted in prolonged survival[98], and WNV[80], although virus-induced neuronal apoptosis is not a significant feature of disease pathogenesis in either of these models, suggesting that death of the animal is indirectly mediated by the immune system and not by the virus itself[98]. Dysregulation of NO production may also cause immunopathology in tick borne encephalitis[106]. Indeed, NO has been argued to be a determinant of disease severity in animal models[106-108]. In JE-infected BALB/c mice also, minocycline treatment links reduced NOS levels to increased blood-brain barrier (BBB) integrity[99], in this case emphasizing the likely role of the endothelium in disease pathogenesis, a feature strongly borne out in dengue, where inhibition of reactive oxygen species and reactive nitrogen species reverses the apoptotic effect of dengue haemorrhagic fever on endothelial cells[91].

These apparent discrepancies between antiviral and immunopathological effects can be reconciled if, as we propose, NO is important in viral control at a critical time point(s) in infection, while at others, particularly later time points, it may become dysregulated and thus pathogenic[88]. We have shown that experimentally abrogating NO activity in WNV encephalitis in NO-competent mice at a specific, relatively late time point prolongs survival, while pharmacological inactivation throughout disease does not[88]. How the antiviral or immunopathogenic role of NO during flaviviral infection is regulated remains to be determined. However, IFN-γ is involved in its robust induction via NOS2[109]. IFN-γ production is in turn dependent on the joint action of interleukin (IL)-12 and IL-18[110-112]. Fagundes and colleagues[112,113] demonstrated that IFN-γ is crucial for survival in both DENV-2 and DENV-3 *in vivo*. The complete ablation of IFN-γ in mice inoculated with these serotypes was associated with reduced NO levels and resulted in an increased disease severity and higher viral titres when compared to WT controls. NOS2$^{-/-}$ mice showed much higher susceptibility to dengue infection. In human patients infected with DENV, increased IFN-γ levels were associated with increased disease severity and clinical manifestations[114]. Although it is clear that during DENV infection, IFN-γ-induced NO production has a role in antiviral defence, it is likely that dysregulation of the IL-IFN-NO axis leads to the immune-mediated damage in certain flaviviruses.

IFN-γ is also responsible for the induction of another antiviral but immune-suppressive enzyme, indolamine-2-3 dioxygenase (IDO), a key enzyme in the kynurenine pathway responsible for the depletion of the rate-limiting essential amino acid, L-tryptophan. This pathway can result in the replication inhibition of certain pathogens and also functions in suppressing

the immune response. Infection of numerous cell types, including myeloid lineage cells, results in the production of IDO[57,115]. During dengue infection patient serum exhibits a significant increase in IDO levels. Competitive inhibition of L-tryptophan in dengue-infected DC with 1-methyl-tryptophan (1-MT) resulted in a reduced antiviral effect from IFN-γ, indicating that IDO may be partially involved with IFN-γ-mediated antiviral protection[116]. Recently, we have shown that both replicating WNV and JEV induce IDO expression in cultured human monocyte-derived macrophages (MDM) in a time and dose-dependent manner[57]. Interestingly, the cytokine critically involved in inducing IDO expression was TNF and not IFN-γ, via nuclear factor-kappa B (NF-κB). Moreover, while IDO-mediated tryptophan starvation was clearly antiviral for WNV in epithelial cells, it did not play a significant role in controlling virus in infected human macrophages, but was highly induced in neighbouring uninfected cells, indicating a more likely role in inhibiting viral spread[57].

Both type I and II interferons are crucial for protection against dengue infection, with IFN α/β being critical in early antiviral defences and IFN-γ mediating virus elimination during the later stages of this disease[117, 118]. Blocking type I IFN responses during the early stages of WNV infection with an IFN-α receptor (IFNAR) monoclonal antibody resulted in uncontrolled viral replication. Conversely type I IFN is not as crucial to antiviral responses during the late stages of the infection but does however, regulate the maturation of WNV-specific $CD8^+$ T cells[119]. Recent studies with IFN-$\beta^{-/-}$ mice have implicated this cytokine in modulating $CD4^+CD25^+FoxP3^+$ regulatory T cells during WNV infection, where regulatory T cells increase in the knockout mice[120]. Thus, manipulating IFN levels may be a key factor in mitigating immune-mediated injury to tissues through the regulatory action of T cells[121].

Myeloid cells and flavivirus encephalitis

The point at which flavivirus gains access to the CNS marks a critical step that differentiates peripheral disease with classic febrile illness where recovery is likely, from severe encephalitic disease associated with a poor clinical outcome. In order to cause CNS disease, a virus must possess two properties: neuroinvasiveness, or the ability to infect cells of the CNS, and neurovirulence, or the ability to cause disease in the CNS. Following neuroinvasion, WNV preferentially infects neurons in the brainstem, anterior horn neurons of the spinal cord[122], variably involving midbrain, cortical, and cerebellar neurons[54,123], with infection demonstrated by the presence of viral antigen[124]. Although *in vitro* findings have shown a variety of CNS cells to be supportive of infection in WNV and DENV infection[125], *in vivo* findings indicate that neurons are the only cells infected in WNV infection[123]. The case for DENV infected cells in the CNS is still unclear, as different groups have reported DENV RNA or DENV antigen in neurons, astrocytes, microglia, endothelial cells, and perivascular cells[126-131]. In contrast to some findings in WNV, DENV tropism for neurons has been found to be associated with DENV-triggered cellular apoptosis[132-137].

***Immune effects on the blood-brain barrier and neuroinvasion*:** Several mechanisms underlying flavivirus neuroinvasion of the CNS have been suggested, including replication within endothelial cells, passive transfer through the BBB, breakdown of the BBB, retrograde axonal transport through the peripheral nervous system, or via a 'Trojan Horse' scenario involving infected infiltrating leukocytes[102]. Numerous studies have shown that peripheral flavivirus infection can affect the BBB. In particular, the activation of TLR-3 during peripheral WNV infection, by inducing TNF, was argued to break down the BBB, which in turn, enabled WNV neuroinvasion[138]. However, BBB breakdown in WNV infection is not consistent, despite demonstrable neuronal infection[29,139]. Additionally, the release of IFN-γ in response to peripheral infection, although crucial to antiviral defence[140], has been shown to increase the expression of the adhesion molecules, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), on endothelial cells[88], which may facilitate leukocyte entry, although upregulation of ICAM-1 in WNV encephalitis is evidently not dependent on IFN-γ[141].

***The contribution of myeloid cells to neuroinvasion*:** The 'Trojan-horse' hypothesis is commonly used to explain the neuroinvasive mechanism *in vivo*. As monocytes are susceptible to WNV infection *in vitro*, these are implicated here[56,57,61], and it is argued that upregulated adhesion molecules facilitate migration of infected leukocytes into the brain. This mechanism has also been suggested in neuroinvasive JEV infection[143], and DENV encephalitis[144] and is potentially consistent with the notion that DENV can subvert macrophage function to enhance replication. Significant numbers of monocytes are recruited to the CNS during WNV

encephalitis[29,88]. However, there is no direct evidence of a Trojan-horse mechanism. In particular, there has been no convincing immunohistochemical demonstration of flavivirus-infected macrophages in the CNS and studies examining the brain using electron microscopy in MVE did not indicate virions present in infiltrating leukocytes[145]. Furthermore, alteration of adhesive properties and permeability of the BBB are unlikely to be sufficient to enable leukocyte ingress into the brain, without prior CNS infection[146] to generate the crucial recruiting gradient of CCL2 and CCL5[29,147], *inter alia*. The TLR-3 mediated effect on the BBB does not occur in a range of viral infections, and may be specific to flavivirus infections[148]. Moreover, significant variability in BBB stability occurs between different flavivirus infections. While the Trojan-horse mechanism may not be responsible for initial CNS infection, enhancement of CNS infection following neuroinvasion may possibly occur via recruitment of infected monocytes to the infected brain. This is consistent with the findings that viral RNA fluctuates between days 3 and 4 after peripheral infection, suggesting early viral clearance and possible later re-introduction[149]. It is not clear whether infected monocytes are functionally capable of infiltration and trafficking into the CNS. Irrespective, since virus has already entered the brain, this could not be regarded as a Trojan-horse mechanism, as such.

Flavivirus may gain access to the CNS through the BBB directly from the endothelium and this may occur through the direct infection of endothelial cells, passive transport through endothelial cells, or paracellular migration through the endothelial tight junctions following endothelial damage[150]. Histological studies have demonstrated that JEV virions may bind to the endothelial surface of the BBB, and are then internalized[151], although it is not clear whether this process results in the productive infection. Infection or passage of virus may cause endothelial damage and increased vascular permeability, which may correlate with enhanced pathology, especially in combination with the systemic cytokine effects of DSS or DHF[152]. A further more likely mechanism of neuroinvasion involves retrograde axonal transport of virus following infection in the periphery. Studies have previously demonstrated WNV in the dorsal root ganglion neurons[149]. This notion is supported by experimental models of infection, where intranasal inoculation of WNV results in rostral to caudal spread in the brain[29], while peripheral inoculation shows initial CNS infection to occur in the cervical spinal cord one day prior to infection in the midbrain, with the rest of the brain becoming infected in a caudal-to-rostral fashion (unpublished data).

These potential mechanisms are not mutually exclusive. These may be combinatorial[150], as suggested by early CNS viral clearance and later re-emergence of virus[149]. It thus seems most likely that virus initially gains access to the CNS through axonal transport. The subsequent chemokine release associated with the neuronal infection and the antiviral immune response may facilitate further entry of the virus into the brain, via infected endothelium or possibly via infected leukocytes. It seems likely that alternate pathways of neuroinvasion may lead to a differential progression of disease. The timing of CNS invasion in respect to the generation of the adaptive immune response would presumably influence this, but the specific contributions that lead to immunopathology or viral eradication are still unclear.

***Recruitment of monocytes to the brain: viral clearance vs. immunopathology*:** Monocytes have shown distinct migratory and functional properties based on their cell surface expression of various receptors. $CD14^+$ $CD16^+$ monocytes produce predominantly inflammatory cytokines such as IL-1β, IL-6 and TNF and low levels of anti-inflammatory IL-10[153-155]. CCL2, CCL3 (MIP-1α) and CCL4 (MIP-1β) levels are significantly increased in $CD14^+$ $CD16^+$ monocytes and it is likely that these $CD14^+$ $CD16^+$ or "inflammatory" monocytes play a deleterious role in dengue virus infection[154].

In the murine model, circulating/resident monocytes are classified as $CX3CR1^{hi}$/$CCR2^-$/$GR1^-$ and are involved in surveillance and patrolling during homeostatic conditions. In contrast, $CCR2^{hi}$/$GR1^+$/$CX3CR1^{lo}$ inflammatory monocytes become activated in response to infection[36]. Monocytes and macrophages are major sources of CCL2, which is a potent chemotactic factor for various inflammatory leukocytes[156]. Flaviviral infection of mice is associated with a significant elevation of the ligands for CCR1, CCR2, CCR4[114,157,160], and CCR5[147], although in the case of WNV infection, it is likely that the high levels of CCL2 come principally from the infected neurons[29]. Similar amplification of CCL2, CCL4, IL-1Ra and CXCL10 expression has been documented in the serum of human patients infected with dengue[161]. The extravasation of monocytes from the bone marrow to blood and subsequently to inflamed tissues rely on the signalling pathways produced by CCR2 and CX3CR1 and their ligands. Despite the production of normal levels of CCL2 and CCL7, $CCR2^{-/-}$ mice have

reduced numbers of Ly6C^{hi} inflammatory monocytes during WNV and HSV-1 infection. Adoptive transfer experiments indicate that this deficiency of Ly6C^{hi} cells might be caused by the inability of CCR2$^{-/-}$ mice to produce monocytes during the early stages of WNV infection[159,162]. Various studies suggest that CCR2 is mainly involved in the egress of monocytes from the bone marrow to the blood[25,39]. This is further supported by the fact that CCR2$^{-/-}$ mice are monocytopaenic prior to infection with WNV[159].

The regulation of Ly6C^{hi} inflammatory monocyte recruitment by chemokine receptor CX3CR1 is crucial for the survival of mice during herpes simplex virus (HSV)-1 infection. CX3CR1$^{-/-}$ mice have much higher levels of TNF and Ly6C^{hi} inflammatory macrophages[162]. Temporal studies where CCL2 is neutralized during the course of WNV infection, resulted in an increased survival and decreased inflammatory microglia and monocytes in the CNS. Interestingly, although viral titres in these mice were comparable to untreated mice, CCL2 neutralization resulted in prolonged, although not permanent, survival, indicating that CCL2-dependant migration of Ly6C^{hi} inflammatory monocytes may ultimately be responsible for the immunopathology seen in these animals[29]. On the other hand, the high mortality rates found in both CCR2$^{-/-}$ and CX3CR1$^{-/-}$ mice infected with WNV or HSV-1 suggest that Ly6C^{hi} inflammatory monocytes may be crucial for survival during certain time-points of infection, but that it is the dysregulation of this cell type that leads to severe immunopathology[88,159,162]. Paradoxically, the absence of CCR2 and CCR4 leads to higher survival rates and reduced liver damage due to leukocyte activation in experimental dengue infection[163]. Thus, the virus-specific involvement of these receptors may depend on where the focus of inflammatory damage occurs. CCR1 levels are also increased in DENV patients but this does not contribute to the lethality of the virus[114,163]. There is evidence for a neuroprotective and antiviral role for CCR5 during WNV encephalitis in mice[147] and in human patients with dengue, elevated serum CCL4, its ligand, was associated with a good prognosis[114].

As implied by the above, the promiscuous nature of chemokine interactions may make it difficult to target specific subsets even temporally, to produce long-term survival. The focus on elucidating and inactivating the multifarious chemokine networks responsible for leukocyte recruitment in encephalitis has mostly ignored the mechanistic aspects of cell surface molecular interactions necessary for leukocyte immigration into the brain. The interaction of leukocyte integrins, such as leukocyte function antigen-1 (LFA-1) and very-late antigen-4 (VLA-4), with upregulated endothelial ICAM-1 and VCAM-1, respectively, is just as crucial to immigration as the chemokine milieu. We have recently shown that targeted antibody blockade of VLA-4, but not LFA-1, reduced the infiltration of Ly6C^{hi} monocytes into the brain by ~66 per cent, resulting in long-term survival in 60 per cent of WNV-infected mice with viral clearance and sterilising immunity to rechallenge, despite a concurrent reduction in immigrating T cells. As this blockade had no effect on the viral titres, it clearly highlights the specific temporal pathogenic role of the infiltrating monocytes in flavivirus encephalitis and moreover argues against the idea of a Trojan Horse scenario in the brain in this model. However, it illustrates the possibility of targeting the inflammatory cell subset without interfering with adaptive immune outcomes[88].

***Macrophages response to flavivirus infection in the eye*:** While most research aimed at the effects of flaviviral infection is geared towards encephalitis and meningitis, a substantial number of patients suffer from ocular complications. These include chorioretinitis, occlusive retinal vasculitis and retinal haemorrhages[164-166]. Some case reports of dengue-infected patients, report similar symptoms[167-169]. Interestingly, the route of ocular infection by WNV appears to be from the brain, via the optic nerve, as suggested by the linear patterns of WNV-associated chorioretinitis originating from the optic nerve[170]. The majority of literature concerning flaviviral infection in the eye is limited to case reports by ophthalmologists, two studies have reported cell culture models of WNV infection in retinal pigment epithelial cells (RPE)[171,172], which form the outer blood retinal barrier. This barrier restricts the passage of molecules from the choroidal blood supply into the retina, and is one of the first barriers that leukocytes encounter when migrating from the choroid in response to an intraocular infection. These two studies have shown that a variety of immune-related factors are upregulated by WNV-infected retinal pigmented epithelium (RPE), including CSF1, CCL5 and CXCL10. CSF1 induces the differentiation of haematopoietic stems cells into macrophages[173]. CXCL10 is chemotactic for macrophages, while CCL5 has been shown to promote their recruitment and survival in other tissues[174,175]. From these results, and from studies investigating the effects of these factors on macrophages in other infection models, it seems likely that the resident microglia and DC in the retina

would be the first to respond to flaviviral infection in the eye. Additionally, factors released by infected RPE at least, would recruit additional monocytes and DC, and facilitate differentiation of monocytes into macrophages.

Much of what is known about macrophage activity in the eye comes from studies looking at experimental autoimmune uveitis (EAU) models. These studies show that microglia and recruited macrophages are important during various phases throughout the course of inflammation, and during the post inflammatory clean up stage[176]. Zinkernagel *et al*[176] discussed some of the effects that viral infections in the eye could have on macrophages. They refer to unpublished data showing a peak increase in MHC-II$^+$ F4/80$^+$ cells between 7-12 days post-infection, and suggest that macrophages and microglia may sustain inflammatory responses during viral retinitis. Furthermore, in WNV-infected RPE, upregulation of the *TNF* gene[172] may predispose macrophages down a pro-inflammatory pathway[177,178]. Additionally, TNF induces breakdown of the blood retinal barrier when injected into the murine eyes[179]. However, an immunosuppressive function for TNF can be seen in certain situations. In one example, pre-treatment of macrophages with transforming growth factor (TGF)-β results in an anti-inflammatory effect of TNF on these same macrophages, mediated via TNFR2[180]. This effect is mediated the by TGF-β induced increase of TNFR2 rather than a direct effect of TGF-β itself and is shown by the failure of TGF-β to induce this same effect in TNFR2-deficient macrophages. Under normal circumstances TGF-β is present in significant amounts in the eye[181], and is also produced by RPE[182], which maintains resident and any infiltrating macrophages in a tolerogenic state. In autoimmune uveitis, macrophages can inhibit *in vitro* proliferation of T-cells re-stimulated with target antigen[183]. On the other hand, WNV-infected RPE also show downregulated TGFB2. This means that the WNV infection of the eye inhibits the formation of tolerogenic macrophages via TNF and thus TNF would be acting in a more traditionally pro-inflammatory manner[184]. Given the pathology in individuals with flaviviral ocular manifestations, it is likely that damage is being caused at least in part by infiltration and cytotoxicity of both resident and infiltrating macrophages and this may be mediated by NO and ROS[185]. While currently there has been no research looking into tolerogenic macrophages in the eye during viral infection, the impairment of these macrophages may contribute to ocular pathology in flavivirus-infected patients.

Conclusions

Recent advances in understanding the role of myeloid lineage cells in the immune response have shown that the network of myeloid cells is more complex than previously realized. Considering the role of these cells in flavivirus encephalitis, a careful investigation of the myeloid lineage response to infection is of crucial importance to its understanding. Improved methodologies to accurately distinguish myeloid cell subsets, and their immunological functions will better elucidate how these cells contribute to immunopathological damage, as opposed to their critical role in antiviral defence, at various stages of peripheral infection and subsequent encephalitis. Since the immigration of inflammatory monocyte subsets into the infected brain can be temporally abrogated, reducing pathology and enabling long-term survival with viral clearance and robust immunity, defining the timing of the factors that predispose to antiviral immunity or immunopathology will likely further inform novel possible interventional approaches to neurotropic encephalitides in human patients.

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