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The host immunologic response to West Nile encephalitis virus

Michael S. Diamond[1,2,3], Erin Mehlhop[1], Theodore Oliphant[3], Melanie A. Samuel[3]

[1]Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO, [2]Department of Medicine,, Washington University School of Medicine, St. Louis, MO, [3]Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, MO

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1. ABSTRACT

West Nile encephalitis virus (WNV) is a small, enveloped, mosquito-transmitted, positive-polarity RNA virus of the *Flaviviridae* family. This virus is closely related to other arthropod-borne viruses that cause human disease including Dengue, Yellow fever, and Japanese encephalitis viruses. WNV cycles in nature between mosquitoes and birds, but also infects human, horses, and other vertebrates. In humans, WNV disseminates to the central nervous system (CNS) and causes severe disease primarily in the immunocompromised and elderly. Experimental studies have made significant progress in dissecting the viral and host factors that determine the pathogenesis and outcome of WNV infection. This review will focus on the interactions between WNV and the protective and pathogenic host immune responses.

2. INTRODUCTION

West Nile virus (WNV) is a neurotropic flavivirus that has emerged globally as a significant cause of viral encephalitis. WNV is maintained in an enzootic cycle between mosquitoes and birds (reviewed in reference (1)), but can also infect and cause disease in other vertebrate animals. Infection of humans is associated with a febrile illness that can progress to the neuroinvasive forms of WNV infection, which include acute flaccid paralysis, meningitis, and encephalitis (2). Overall, about 1 of 150 WNV infections, result in the most severe and potentially lethal form of the disease. The mortality rate following neuroinvasive infection is approximately 5 to 10% (2-4), and long term neurological sequelae are common (>50%) (2, 5, 6). Neuronal damage is most prevalent in the brain stem and anterior horn neurons of the spinal cord, although

in immunosuppressed individuals infection can disseminate throughout the CNS (7).

WNV historically caused sporadic outbreaks of a mild febrile illness in regions of Africa, the Middle East, Asia, and Australia (8). However, in the 1990's, the epidemiology of infection appeared to change. New outbreaks in parts of Eastern Europe were associated with higher rates of severe neurological disease. In 1999, WNV entered North America, and caused seven human fatalities in the New York area as well the deaths of a large number of birds and horses. Since then, WNV has spread to all 48 of the lower United States as well as to parts of Canada, Mexico, South America and the Caribbean (9). Because of the increased range, virulence, and amplification in birds (10), the number of human cases that have presented to clinical attention continues to rise: in the United States between 1999 and 2007, 26,000 clinical cases were diagnosed and associated with ~1,000 deaths (http://www.cdc.gov/ncidod/dvbid/westnile/surv&control.htm). Based on seroprevalence screening of human blood donations, it is now estimated that at least 2 million people in the United States have been infected with WNV (11, 12). No vaccines or specific therapies for WNV are currently approved for humans.

WNV is a member of the *Flaviviridae* family of RNA viruses and is related to other important human pathogens, including dengue (DENV), yellow fever (YFV), Japanese encephalitis (JEV), and tick-borne encephalitis (TBEV) viruses. Similar to other flaviviruses, WNV is an enveloped virus with a single-stranded, positive sense, ~11 kilobase RNA genome. The flavivirus genome is transcribed as a single polyprotein that is cleaved by host and viral proteases into three structural and seven non-structural proteins (13, 14). The structural proteins include a capsid protein (C) that binds viral RNA, a pre-membrane (prM) protein that blocks premature viral fusion and may chaperone E protein folding, and an envelope (E) protein that mediates viral attachment, membrane fusion, and viral assembly (15). The flavivirus non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A NS4B, and NS5) regulate viral transcription, translation, and replication and attenuate host antiviral responses. NS1 has co-factor activity for the viral replicase (16, 17), is secreted from infected cells (18, 19), and attenuates complement activation (20). NS2A inhibits IFN responses (21, 22) and may participate in virus assembly (23), and NS3 has protease, NTPase, and helicase activities (23, 24). NS2B is a co-factor required for NS3 proteolytic activity (25) and contributes to antagonism of IFN responses (26). NS3 has protease, NTPase, and helicase activities (24). NS4A and NS4B modulate IFN signaling (27, 28), and NS5 encodes the RNA-dependent RNA polymerase and a methyltransferase (29, 30) and also inhibits IFN responses (31-33).

WNV infection occurs following cellular attachment and receptor-mediated endocytosis. Although both DC-SIGN-R and the $alpha_v beta_3$ integrin have been suggested as WNV attachment ligands (34, 35), the cellular receptors for WNV on physiologically relevant cell types such as neurons remain uncharacterized. Cellular entry of WNV requires the formation of clathrin-coated pits (36, 37). Following a pH-dependent conformational change in the E protein (38, 39), the viral and endosomal membranes fuse, releasing the viral nucleocapsid into the cytoplasm (40, 41). Upon nucleocapsid release, viral RNA associates with endoplasmic reticulum (ER) membranes and is translated. Translation is a prerequisite for generating a negative-strand RNA intermediate that serves as a template for nascent positive-strand genomic RNA synthesis (42). Flavivirus RNA synthesis is semi-conservative and asymmetric, as positive-strand RNA genome production is about ten times more efficient than negative-strand synthesis (14). Positive strand RNA is either packaged within progeny virions or used to translate additional viral proteins. WNV assembles and buds into the ER to form enveloped immature particles containing the prM protein. Following transport through the trans-Golgi network, furin-mediated cleavage of prM to M generates mature, infectious virions that are released by exocytosis (43-45).

Intensive study of WNV pathogenesis and the nature of the protective immune system response have accompanied the current epidemic. Host factors clearly influence the expression of WNV disease in humans (46-49). Infants, the elderly, and those with impaired immune systems are at greatest risk for severe neurological disease (8, 50, 51). Similarly, in animals, the maturation and integrity of the immune system correlates with resistance to WNV infection (reviewed in (52)).

3. PATHOGENESIS OF WNV INFECTION

Rodent models have provided insight into the mechanisms of WNV dissemination and pathogenesis. Following peripheral inoculation, initial WNV replication is thought to occur in skin dendritic cells (53). These cells migrate to and seed draining lymph nodes, resulting in a primary viremia and subsequent infection of peripheral tissues such as the spleen and kidney. By the end of the first week, WNV is largely cleared from the serum and peripheral organs, and infection in the different regions of the CNS is observed in a subset of immunocompetent animals. Rodents that succumb to infection develop CNS pathology similar to that observed in human WNV cases, including infection and injury of cerebellar, basal ganglia, brain stem, hippocampal, and spinal cord neurons (4, 7, 50, 54-61). WNV infection is not significantly detected in non-neuronal CNS cell populations in humans or animals. In most surviving rodents, WNV is cleared from all tissue compartments within two to three weeks after infection. However, persistent viral infection in the brains of CD4 or $CD8^+$ T cell (62, 63) or perforin deficient mice (64) and in brains and kidneys of infected hamsters has been reported (58, 59). Persistent infection has also been documented in a WNV-infected immunosuppressed patient in which viremia was detected for over 60 days (65).

The mechanisms by which WNV crosses the blood-brain-barrier (BBB) and enters the CNS remain largely uncharacterized, although TNF-alpha-mediated changes in endothelial cell permeability may facilitate CNS entry (66, 67). It is likely that WNV infects the CNS at least in part via hematogenous spread, as increased viral

burden in the serum correlates with earlier viral entry into the brain (54, 68). Additional mechanisms may contribute to WNV CNS infection, including: (i) infection or passive transport through the endothelium or choroid plexus epithelial cells (69), (ii) infection of olfactory neurons and spread to the olfactory bulb (70), (iii) a "Trojan horse" mechanism in which WNV-infected immune cells or WNV-adsorbed to the surface of erythrocytes traffics to the CNS (68, 71, 72), and (iv) direct axonal transport from infected peripheral neurons (56, 73, 74). In support of this latter entry mechanism, recent studies have established that WNV undergoes retrograde and anterograde spread in neurons and that axonal transport promotes viral entry into the spinal cord and acute limb paralysis in hamsters (75). Although the precise mechanism (s) of WNV entry into the brain requires additional study, changes in cytokine levels that modulate BBB permeability and infection of blood monocytes and choroid plexus cells have been documented in animal models (61, 76, 77).

4. INNATE IMMUNE RESPONSES TO WNV INFECTION

4.1. Interferon (IFN)

Type I (IFN-alpha and IFN-beta), type II (IFN-γ), and type III (IFN-λ) IFN are important host response cytokines that enable control of infections of most RNA and DNA viruses (reviewed in references (78-80)). IFN-alpha and beta are produced by many cell types following virus infection and induce an antiviral state by upregulating genes with direct and indirect antiviral functions. IFN-alpha and -beta also link innate and adaptive immune responses through stimulation of dendritic cell maturation (81), direct activation of B and T cells (82, 83), and by promoting survival of recently activated T cells (84). Pretreatment of cells with IFN-alpha or -beta inhibits WNV replication *in vitro*, but treatment after infection is much less effective (68, 85, 86). Although WNV can directly antagonize IFN induced responses after infection (22, 26-28, 87), type I IFN is still required to restrict WNV replication and spread *in vivo* (22, 68, 88). Mice lacking the IFN-alpha and -beta receptors (IFN-alpha/betaR$^{-/-}$) show uncontrolled viral replication, rapid dissemination to the CNS, and enhanced lethality. Altered viral tropism in IFN-alpha/betaR$^{-/-}$ mice was also observed with enhanced infection in normally resistant cell populations and peripheral tissues.

IFN-gamma is produced primarily by γδ T cells, CD8^{+} T cells and natural killer (NK) cells and limits infection through several mechanisms. IFN-gamma restricts viral replication directly by inducing an antiviral state, or indirectly by modulating the adaptive immune response through the activation of myeloid-derived cells, inducing CD4^{+} T-cell activation and T_H1/T_H2 polarization, and increasing cell surface expression of MHC class I molecules (89, 90). Although WNV is also resistant to the antiviral effects of IFN-gamma after infection *in vitro* (68), *in vivo* IFN-gamma limits early viral dissemination to the CNS: mice deficient in either IFN-gamma or the IFN-gammaR showed higher peripheral viral burden, earlier entry into the CNS, and increased lethality (91, 92). Interestingly, no major deficits in adaptive immune responses were observed in these studies, suggesting that the dominant function of IFN-gamma in controlling WNV infection is innate and antiviral. Additional experiments demonstrated a cell-specific requirement for IFN-gamma, as γδ T cells utilized IFN-γ to limit WNV dissemination whereas CD8^{+} T cells did not (64, 92, 93). Finally, recent studies suggest that IFN-□amma may also have an immunopathological effect in the CNS as seizure incidence was decreased after WNV infection in IFN-□amma$^{-/-}$ mice (94).

4.2. Virus Recognition

Cells recognize and respond to RNA virus infection through several nucleic acid sensors, including Toll-like receptor 3 (TLR-3) and 7 (TLR-7), and the cytoplasmic dsRNA sensors retinoic acid-inducible gene I (RIG-I) and melanoma-differentiation-associated gene 5 (MDA-5) (95, 96). Binding of RNA to these pathogen recognition receptors results in downstream activation of transcription factors, such as interferon regulatory factors 3 and 7 (IRF-3 and IRF-7), and the expression of IFN stimulated genes. An emerging literature suggests that RIG-I, MDA5, and TLR-3 have essential functions in responding to WNV infection. Murine embryonic fibroblasts (MEF) deficient in RIG-I, MDA5, and IPS-1 demonstrated delayed induction of host responses, decreased IRF-3 activation, and augmented viral replication (97-99). Nonetheless, MDA5 may be less essential for cellular recognition and host response to WNV in some myeloid cell types, as IFN production by MDA-5$^{-/-}$ myeloid dendritic cells remains largely intact after WNV infection (100). Unlike RIG-I and MDA5, TLR-3 is expressed primarily in endosomes and activates IRF-3 downstream of the kinases TBK1 and IKKε (101, 102). Although WNV appears to interfere with poly I:C induced interferon responses (103), initial studies suggest that TLR-3 may be dispensable for recognition of WNV *in vitro* (98). TLR-3$^{-/-}$ mice injected by an intraperitoneal route paradoxically showed decreased lethality despite higher peripheral viral titers, presumably because of blunted cytokine responses (e.g., TNF-alpha) that normally facilitate WNV entry into the CNS (66). However, when TLR-3$^{-/-}$ mice are infected with WNV via a subcutaneous route, increased viral burden in the spleen, early entry into the brain, and enhanced lethality are observed (104), as might be expected for a pathogen recognition molecule that triggers a protective host immune response.

Activation of pathogen recognition receptors stimulates IFN production and feedback amplification of the IFN stimulated gene response. Despite data from MEF suggesting that RIG-I and MDA5 are critical for recognition of WNV and induction of IFN responses (99), IFN-alpha and beta production in mice appears largely independent of the downstream transcription factor, IRF-3 (105, 106). Recent studies suggest individual cell types (myeloid, fibroblast, and neuronal) use distinct IRF-3 responses to protect against WNV infection through both IFN-dependent and independent pathways (106). In cells that generate robust IFN responses after WNV infection in the absence of IRF-3, it is likely that alternate sets of pathogen recognition and transcription regulators are used,

including TLR-7 and IRF-7. Recent studies also suggest a role for dsRNA-dependent protein kinase R (PKR) in the early induction of IFN in fibroblasts after WNV infection (107).

Studies have begun to elucidate the specific antiviral effector molecules that control WNV infection. PKR and 2'-5'-oligoadenylate synthase (OAS) proteins independently mediate intracellular resistance to WNV (108). PKR is activated by binding dsRNA and phosphorylates the eukaryotic translation initiation factor 2 (eIF2-alpha) resulting in attenuation of protein synthesis (109). RNase L is activated by 2'-5'-linked oligoadenylates synthesized by OAS enzymes and functions as an endoribonuclease that cleaves viral and host RNA (110). RNase $L^{-/-}$ MEFs and $PKR^{-/-}$ x RNase $L^{-/-}$ bone marrow derived macrophages supported increased WNV replication *in vitro* (111, 112). Moreover, mice deficient in both PKR and RNase L showed increased lethality following WNV infection, with higher viral loads in peripheral tissues at early time points after infection (112). Flavivirus susceptibility in mice has been mapped to a mutation in the *Oas* gene 1b that results in the expression of a truncated Oas isoform (113, 114). However, the mechanisms by which *Oas* gene alleles affect flavivirus pathogenesis remain uncertain; the *Oas1b* gene effects on WNV replication are independent of RNase L and IFN (111).

4.3. Complement

The complement system is a family of serum proteins and cell surface molecules that participate in pathogen recognition and clearance. Complement activation occurs through the classical, lectin, and alternative pathways, which are initiated by binding of C1q or mannan-binding lectins, or through the spontaneous hydrolysis of C3, respectively. Complement contributes to host protection through direct opsonization and/or cytolysis, chemotaxis, immune clearance, and modulation of B and T cell functions (115). Complement is required for protection from lethal WNV infection in mice. WNV activates complement *in vivo*, and mice lacking in the central complement component C3 or complement receptors 1 and 2 showed enhanced lethality after WNV infection (116, 117). All three pathways of complement activation are important for controlling WNV, as mice deficient in alternative, classical, or lectin pathway molecules exhibited increased mortality. Interestingly, the activation pathways modulated WNV infection through distinct mechanisms. Alternative pathway deficient mice demonstrated normal B cell function but impaired $CD8^+$ T cell responses, whereas classical and lectin pathway deficient mice had defects both in WNV-specific antibody production and T cell responsiveness (116).

Complement also augments the protective efficacy of IgG antibodies against WNV. Whereas initial studies with anti-WNV IgM antibodies suggested that complement could efficiently enhance WNV infection in macrophages *in vitro* (118, 119), more recent investigations indicate that the complement component C1q augments the potency of neutralizing antibody against WNV in an IgG subclass-specific manner (E. Mehlhop, T.Pierson, and M. Diamond, manuscript in preparation), analogous to that observed for other viruses including measles (120), influenza (121, 122), vesicular stomatitis (123), hepatitis C (124) and human immunodeficiency (125, 126) viruses. C1q also restricts antibody-dependent enhancement of WNV infection *in vitro* and *in vivo* (127).

4.4. Cellular innate immunity

While few studies have directly addressed the function of cellular innate immunity in WNV infection, limited data suggests that macrophages and dendritic cells likely inhibit WNV though direct viral clearance, enhanced antigen presentation, and cytokine and chemokine secretion. Consistent with this, depletion of myeloid cells in mice enhanced lethality after WNV infection (128). Macrophages basally express key host defense molecules, including RIG-I, MDA5, ISG54, and ISG56, and thus, restrict WNV infection by induction of IFN-alpha and -beta (106). Macrophages may also control flaviviruses through the production of nitric oxide (NO) intermediates (129, 130), although the role of NO in WNV infection has not been established. Less is known about the specific protective roles of DCs in WNV infection, although it is likely that they produce IFN-alpha and beta soon after infection and function as antigen presenting cells to prime the adaptive immune response (131).

γδ T cells also function in early immune responses and directly limit WNV infection. As they lack MHC restriction, γδ T cells can react with viral antigens in the absence of conventional antigen processing (132). γδ T cells expand following WNV infection in wild type mice, and increased viral burden and mortality and delayed priming of adaptive immune responses were observed in mice deficient in γδ T cells (92, 133). Bone marrow chimera reconstitution experiments demonstrated that γδ T cells require IFN-gamma to limit WNV infection (91). Natural killer (NK) cells also have the potential to control WNV infection through recognition and elimination of virus-infected cells. NK cell activity was transiently activated and then suppressed following flavivirus infection in mice (134). As WNV infection *in vitro* increases surface expression of class I MHC molecules by enhancing the transport activity of TAP and by NF-□B-dependent transcriptional activation of MHC class I genes (135-137), natural killing may be inhibited (138-140). Notably, antibody depletion of NK cells in mice did not alter morbidity or mortality after WNV infection (64, 141), and similar results were seen using Ly49A transgenic mice (142) that lack functional circulating NK cells (M. Engle and M. Diamond unpublished results).

5. ADAPTIVE IMMUNE RESPONSES TO WNV INFECTION

5.1. Humoral responses

Humoral immunity is an essential aspect of immune mediated protection from WNV (46, 54, 143-151). B cell deficient mice uniformly died after WNV infection, but were protected by passive transfer of immune sera (54). IgM is required, as $IgM^{-/-}$ mice developed high viral loads in all tested tissues and demonstrated complete lethality

after WNV infection (143). In prospective studies the level of WNV-specific IgM at day 4 after infection in mice predicted disease outcome. While it is apparent from passive transfer studies that immune IgG can protect against flavivirus infection, the function of IgG during primary infection is less clear. In mice, WNV-specific IgG is not produced until somewhat late in infection (day 6 to 8), after both viral seeding of the CNS and clearance from peripheral tissues have occurred (54, 152). Thus, while it is possible that WNV-specific IgG alters WNV infection in the CNS, current data suggests that by the time IgG is produced the survival of the animal may already have been largely determined (152). Additional studies are required to more clearly establish how IgG modulates WNV pathogenesis during primary infection.

The E glycoprotein is the major surface protein on the flavivirus virion and is the principal antigen that elicits protective neutralizing antibodies (153). However, a subset of neutralizing antibodies to *Flaviviruses* may also recognize the prM protein on the virion (154, 155). Interestingly, antibodies to the non-structural protein NS1, which is absent from the virion, also are protective against WNV *in vivo* (141, 156). Antibody responses to NS3 and NS5 have also been observed during WNV infection (154, 157, 158), although their functional significance remains uncertain.

Recent studies have elucidated critical structural determinants of antibody-mediated protection against WNV E protein (159-163). The E protein has three structural domains that mediate viral attachment, entry, and viral assembly Domain I (DI), the central structural domain, is an 8-stranded beta-barrel that contributes to the conformational changes that occur after exposure to acid pH in the endosome. Domain II (DII) is a 12-stranded beta-barrel that is involved in dimerization (164) and contains a highly conserved hydrophobic fusion-peptide that mediates the class II acid-catalyzed fusion event (38, 165, 166). Domain III (DIII) adopts an immunoglobulin-like fold and contains a putative receptor-binding domain (164, 167, 168). Short, flexible linkers connect the E protein domains and allow for the conformational changes associated with virus maturation and membrane fusion. Several unique characteristics of WNV E protein relative to other flaviviruses have been identified by x-ray crystallography. Differences in the angle observed at the DI-DII hinge between WNV and DENV suggest critical WNV E dimer contact residues may be weaker or more easily disrupted. Additionally, the single N-linked glycosylation site on E, which is essential for DC-SIGN-R recognition (35) and neurovirulence of WNV (169, 170) is located on a unique alpha-helical segment in DI of WNV E in comparison to other *Flaviviruses*. Differences in the location of the glycosylation site may contribute to differences in viral tropism and pathogenesis among flaviviruses (171-173).

Understanding E protein structure in the context of WNV virion assembly has provided fundamental insights into the potential mechanisms of antibody-mediated neutralization. The glycoproteins on the surface of the 600 Å immature virion are organized into 60 asymmetric trimeric spikes of prM-E heterodimers (174, 175). At the apices of the spikes, prM caps the fusion loop of E (176), presumably to prevent premature fusion as the virus passes through the acidic secretory pathway (177, 178). A membrane proximal furin-catalyzed cleavage releases the N-terminal pre-peptide from prM (45, 179) allowing the transition from trimeric prM-E heterodimers to E homodimers found in the mature 500 Å enveloped virion (39, 175, 180). Cryoelectron microscopy (cryoEM) has shown that in the mature virus head-to-tail homodimers of E form a smooth icosahedral protein shell over the lipid bilayer in a “herringbone” pattern that defines three repeating environments. The 2-fold, 3-fold, and 5-fold axes of symmetry are defined by the dimerization of E, radial arrangement of DI, and DIII, respectively (180, 181). Antibody recognition or receptor binding may occur in different symmetry environments, resulting in differential occupancy of the virion (182, 183). This likely has functional consequences for the recognition of viral particles by different cell types and the immune system.

The antigenic domains of E proteins were initially characterized by mapping and competition experiments with mAbs (reviewed in (153)). These studies identified three antigenic domains (C, A, and B), which were later correlated with the structural domains DI, DII, and DIII on the E protein (164, 184-186). Many of the B domain epitopes of DENV-2, JEV, and TBE elicited neutralizing virus-specific antibody responses (184, 185, 187-190). However, not all E-glycoprotein-reactive antibodies neutralize virus infectivity. Indeed, some virus-specific non-neutralizing mAbs were found to recognize C domain epitopes of DENV recombinant proteins (191). Additionally, mAb recognition of TBE A domain epitopes was inhibited by low pH treatment, but recognition of B domain was unaffected (184, 192). These studies suggest that distinct domains of WNV E protein elicit antibodies with distinct functional activities.

Sequencing of neutralization escape mutants identified DIII as a major target of mAb-mediated flavivirus neutralization (162, 185, 187, 193-195). More recent studies have confirmed the epitopes on DIII responsible for eliciting potently neutralizing antibodies. Using both forward and reverse genetic strategies, several groups have established the most potent WNV neutralizing mAbs bind to the distal lateral ridge of DIII with key contacts to residues K307, T330, and T332 (144, 162, 163, 195, 196). Neutralizing mAbs that recognize the same residues were also characterized by NMR (163, 197) and x-ray crystallography (161). The latter structural studies demonstrated that one DIII-specific neutralizing antibody engaged 16 residues in four discontinuous regions that localize to the amino terminus (residues 302-309) and three strand connecting loops (residues 330-333, 365-368, and 389-391). Antibody binding at this epitope correlated with potent *in vitro* neutralization and strong *in vivo* protection (144, 183) suggesting this site in DIII may be an important neutralizing epitope. Both Fab fragments and single chain Fv that recognize the DIII lateral ridge epitope neutralize

infection, indicating that bivalent cross-linking is not required for DIII-directed antibody-mediated inhibition of WNV infection (147). Although DIII has been suggested to contribute to virus attachment, at least some DIII-directed neutralizing mAbs appear to block at a post-attachment step. Potent neutralization was still observed following pre-incubation of cells with WNV prior to the addition of DIII-directed mAb (161). In contrast, the activity of a neutralizing mAb directed at the fusion peptide in DII was completely lost if virus was bound to cells prior to mAb addition.

Cross-reactive, neutralizing mAbs against *Flaviviruses* generally map to the fusion peptide (amino acids 98-110) in DII (145, 198, 199). In one study 45% (40 of 89) of the DI-DII-specific mAbs showed markedly reduced binding to WNV E protein with mutations at the W101 residue in the fusion peptide, and 85% of these (34 of 40) cross-reacted with the distantly related DENV (145). Other groups also have established that mutations at either G106 or L107 in the fusion peptide eliminate mAb recognition of *Flavivirus* group-specific epitopes (198, 199). Additionally, ~30% of the cross-reactive antibodies in DENV patient sera mapped to a single amino acid (L107) in the fusion loop (199). Preliminary studies with human mAbs and serum suggest that the cross-reactive fusion-peptide epitope may be immunodominant, whereas the DIII-specific neutralizing epitope appears less dominant (147, 148, 152).

Less strongly neutralizing WNV-specific mAbs mapped to six additional sites in DI and DII outside of the fusion loop: the lateral ridge of DI, the linker region between DI and DIII, the hinge interface between DI and DII, the lateral ridge, the central interface, and the dimer interface of DII (145). These mAbs exhibited little neutralization activity by classical plaque reduction assays, but inhibited infection on cells expressing alternate WNV attachment receptors, such as DC-SIGN-R. Interestingly, most DI-DII-specific mAbs still protected mice from lethal WNV challenge (145), although they were less effective than that observed with DIII-specific neutralizing mAbs. This data suggests DIII- and DI-DII-specific mAbs neutralize and protect against WNV infection through independent mechanisms.

5.2. T cell responses during primary infection

Experiments in small animal models demonstrate that T lymphocytes are an essential component of protection against WNV (62-64, 93, 200-202). Consistent with this, individuals with hematologic malignancies and impaired T cell function have an increased risk of neuroinvasive WNV infection (203, 204). Upon recognition of a WNV-infected cell that expresses class I MHC molecules, antigen-restricted cytotoxic T lymphocytes (CTL) proliferate, release proinflamatory cytokines (136, 201, 202, 205, 206), and lyse cells directly through the delivery of perforin and granzymes A and B, or via Fas-Fas ligand interactions. Mice deficient in $CD8^+$ T cells or class I MHC molecules had normal humoral responses but higher and sustained WNV burdens in the spleen and CNS and increased mortality (62, 200). Granzymes appear important for control of the lineage II isolate Sarafend, with perforin, Fas and Fas ligand having a more limited role in modulating infection (207). In contrast, $CD8^+$ T cells require perforin and Fas ligand interactions to control lineage I WNV as mice deficient in these molecules had increased CNS viral burdens and lethality (64, 208). Moreover, adoptive transfer of wild type but not perforin or Fas-ligand deficient $CD8^+$ T cells decreased CNS viral burden and enhanced survival. $CD4^+$ T cells also restrict WNV pathogenesis *in vivo*. A genetic or acquired deficiency of $CD4^+$ T cells resulted in a protracted WNV infection in the CNS that culminated in uniform lethality by 50 days after infection. Virologic and immunologic experiments indicate that the dominant protective role of $CD4^+$ T cells during primary WNV infection is to provide help for antibody responses and sustain WNV-specific $CD8^+$ T cell responses in the CNS that enable viral clearance (63).

T cell-mediated immunity is essential for controlling WNV infection in the CNS. $CD8^+$ T cells traffic to the brain after WNV infection in mice, and their presence correlates temporally with viral clearance (62, 116, 200). $CD8^+$ T $cell^{-/-}$, MHC class $I^{-/-}$, MHC class $II^{-/-}$, and $perforin^{-/-}$ mice all showed WNV persistence in the brain, with detectable infectious virus up through 1 to 2 months after infection (62-64). Thus, the absence of functional $CD8^+$ or $CD4^+$ T cells results in a failure to clear WNV from infected neurons in the CNS. Since the CNS experiences limited immune surveillance in the absence of inflammation, chemokine-dependent T cell recruitment to infected CNS tissues modulates viral pathogenesis. Following viral infection in the CNS, inflammatory chemokines (e.g. CCL5) are expressed by trafficking leukocytes and resident astrocytes and microglia (209, 210). Surprisingly, WNV infection also induced expression of the chemokine CXCL10 in neurons, which recruited effector $CD8^+$ T cells through its cognate ligand CXCR3 (76, 77). A genetic deficiency in CXCL10 resulted in reduced T cell trafficking to the CNS, higher viral loads in the brain, and enhanced mortality. The chemokine receptor CCR5 also regulates T cell trafficking to the brain during WNV infection; its absence resulted in depressed CNS leukocyte migration and increased lethality in mice (211), and may be associated with more severe WNV disease in humans (47). More recently, we have observed that CD40-CD40L interactions also facilitate T cell migration across the blood-brain barrier and perivascular space to control WNV infection (212).

6. IMMUNOPATHOGENESIS AFTER WNV INFECTION

Injury to neurons after WNV infection is believed to occur because of both viral and host immune-mediated effects. *In vitro* studies have begun to elucidate the pathways involved in WNV-induced cell death. WNV infection triggers apoptosis in different transformed cell lines, resulting in caspase-3 activation, cytochrome C release, and exposure of phosphatidylserine on the outer leaflet of the plasma membrane (213, 214). Primary cortical neurons, mouse embryonic stem cell-derived neurons and neuroblastoma cells rapidly undergo apoptosis within 2 to 3

days after WNV infection (57, 214, 215). Several other encephalitic flaviviruses also induce apoptosis: St. Louis encephalitis virus (SLEV) triggered apoptosis in neuroblastoma cells, and Japanese encephalitis virus (JEV) induced apoptosis in cell lines via the endoplasmic reticulum stress pathway (216, 217). These results suggest that induction of programmed cell death may be a common feature of flavivirus replication.

The cellular outcome of WNV replication depends on interactions between host and viral factors. Viral replication is required to trigger apoptosis (214) and several WNV proteins may contribute directly to this process. Ectopic expression of the WNV NS3 protein or its helicase or protease domains induces apoptosis and activation of caspase-3 and -8 (218). Expression of WNV caspid protein either *in vitro* or in the striatum of mouse brains also triggers apoptosis downstream of caspase-3 and caspase-9 activation (219). Transcriptional profiling analysis of WNV infected cells and mice demonstrated upregulation of several apoptosis related genes, although the physiologic relevance of these observations is unclear (220, 221).

Caspase-3-dependent apoptotic cell death of WNV-infected neurons could be a protective or pathologic host response. Apoptosis can act as an innate defense that restricts viral spread by eliminating infected cells and triggering pathogen recognition pathways (222). Alternatively, cell death could directly contribute to the spread and replication of WNV. Recent studies suggests that caspase-3 dependent apoptosis has an immunopathologic effect after WNV infection. Mice that were genetically deficient in caspase-3 mice were more resistant to lethal WNV infection, although there were no significant differences in tissue viral burden or the kinetics of viral spread (215). Instead, decreased neuronal death was observed in the cerebral cortex, brain stem, and cerebellum of caspase-$3^{-/-}$ mice. Analogously, primary central nervous system (CNS)-derived neurons demonstrated caspase-3 activation and apoptosis after WNV infection, and treatment with caspase inhibitors or a genetic deficiency in caspase-3 significantly decreased virus-induced death (215, 223). These experiments establish that caspase-3 dependent apoptosis contributes to the pathogenesis of lethal WNV encephalitis. Recent studies also indicate that WNV infection activates the pro-apoptotic cAMP response element-binding transcription factor homologous protein (CHOP); this pathway also leads to virus-induced cell death and may contribute to the wide-spread neuronal loss observed in infected animals (224).

7. PERSPECTIVES

The use of animal and cell culture models has fostered an improved understanding of the balance between WNV pathogenesis and immune control. These studies establish that innate, humoral, and T cell-mediated immunity are all required to orchestrate effective control of WNV. To modulate these host defenses, WNV has developed mechanisms that attenuate immune responses and facilitate infectivity *in vivo*. Several questions regarding WNV infection and the host interaction remain unanswered. Although WNV induces lethal encephalitis, the exact mechanisms by which WNV crosses the BBB, and causes neuronal dysfunction and death require further study. Few studies have characterized which aspects of the memory response to WNV mediate protection, obviously an area that is central to the development of effective vaccines. Enhanced investigation of the immunologic basis of protection and injury may also facilitate the design of targeted immunotherapies. Such efforts also will likely enhance our understanding of the pathogenesis of related flaviviruses that cause human disease.

8. ACKWNOLEDGEMENTS

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Send correspondence to: Michael S. Diamond, Departments of Medicine, Molecular Microbiology, Pathology and Immunology, Washington University School of Medicine, 660 South Euclid Avenue, Box 8051, St. Louis, MO 63110, Tel: 314-362-2842, Fax: 314-362-9230, E-mail: diamond@borcim.wustl.edu