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Of Mice and Men: Protective and Pathogenic Immune Responses to West Nile Virus Infection

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Abstract West Nile virus, a mosquito-borne flavivirus, first emerged in the Western Hemisphere in 1999. Although the majority of infections are asymptomatic, West Nile virus (WNV) causes significant morbidity and mortality in a minority of individuals who develop neuroinvasive disease, in particular the elderly and immunocompromised. Research in animal models has demonstrated interactions between WNV and the innate and adaptive immune system, some of which protect the host and others which are deleterious. Studies of disease pathogenesis in humans are less numerous, largely due to the complexities of WNV epidemiology. Human studies that have been done support the notion that innate and adaptive immune responses are delicately balanced and may help or harm the host. Further human investigations are needed to characterize beneficial responses to WNV with the goal of such research leading to therapeutics and effective vaccines in order to control this emerging viral disease.

Keywords West Nile virus (WNV) · Flavivirus · Encephalitis · Innate immunity · T cell · CD8 · Emerging virus

Introduction

West Nile virus (WNV) is a mosquito-borne virus of the *Flaviviridae* family. First discovered in Uganda in 1937, WNV subsequently caused intermittent epidemics of mild febrile illness in Africa, Middle East, Asia, and Australia. The epidemiology of WNV changed in the mid-1990s with recognition of outbreaks associated with high frequencies of severe neurologic disease in Eastern Europe [1••]. WNV emerged in New York City in 1999 and has spread throughout the continental USA as well as to Canada, Mexico, and parts of Central and South America [2]. Over 39,000 cases of WNV with 1668 fatalities have been reported in the USA between 1999 and 2013, with the highest numbers of reported cases in 2003 and 2012. Neuroinvasive disease (WNND) accounted for 44 % of all reported cases [3]. WNV exists in nature as an enzootic between *Culex* mosquitoes and avian hosts. Man and horses are believed to serve as dead-end hosts in the virus life cycle. Human-to-human transmission has occurred primarily via blood transfusion and organ transplantation [4••].

In humans, WNV is asymptomatic in 80 % of infected individuals. Symptomatic cases may present with an acute self-limited febrile illness characterized by acute onset of fever and may be associated with myalgia, fatigue, headache, gastrointestinal symptoms, and rash, also known as West Nile fever (WNF). Less than 1 % of WNV infections result in WNND, which may manifest as meningitis, meningoencephalitis, or acute flaccid paralysis. WNND can occur in all age groups, but the elderly and immunocompromised are at higher risk for severe disease and long-term neurologic sequelae resulting in 10 % mortality. Additional co-morbid conditions that are associated with increased risk for WNND include diabetes, hypertension, and chronic kidney disease [1••]. There are currently no specific therapeutic for WNV illness or vaccine licensed for use in humans.

Data are limited on the mechanisms of protection and pathogenesis in humans, largely due to the sporadic nature of WNV epidemics leading to significant challenges to performance of clinical research. This review will focus on human studies and will compare and contrast findings in humans to those in animal model systems.

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Virus Tropism

WNV is a positive sense RNA flavivirus related to Japanese encephalitis virus, dengue virus, and yellow fever virus. The 11-kb genome encodes a single open-reading frame of ten polyproteins—three structural proteins (C, prM, and E) and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) [5••]. Following the bite of an infected mosquito, tissue resident dendritic cells are believed to be the primary cellular target in humans [6]. In murine models, footpad inoculation with WNV results in infection of keratinocytes, spread to local lymph nodes, followed by a period of viremia, and subsequent spread to secondary lymphoid and other peripheral organs (e.g., kidneys) followed by neuroinvasion [7, 8]. In humans, viremia tends to occur prior to the onset of symptoms and has been identified in pre-symptomatic blood donors [4••]. At autopsy, WNV is commonly detected in the mid-brain, brainstem, and spinal cord but only occasionally in cerebral cortex, hippocampus, and cerebellum [9–11]. Peripheral spread of WNV has been seen predominantly in severely immunocompromised individuals [9].

Innate Immune Responses to West Nile Virus

The innate immune response is the first line of defense against WNV infection and is essential in limiting virus dissemination to peripheral tissues. Mouse models have been essential to understanding the fundamental aspects of the innate immune response to WNV. Following virus infection, recognition of WNV by cytoplasmic and endosomal RNA sensors leads to the induction of IFN-β and interferon-stimulated genes (ISGs). Interferon regulatory factor 3 (IRF3) and IRF7 coordinate IFN-β and IFN-α production helping to establish a positive feedback loop [5••]. In macrophages, constitutively expressed IRF7 allows for rapid production of systemic IFN-α/β in WNV-infected mice [12]. In non-myeloid cells such as neurons, IRF3 upregulation is required for production of IFN-α and IFN-β [13]. IRF5 can also participate in the induction of IFN in myeloid dendritic cells [14•]. In one study, single nucleotide polymorphisms (SNPs) in human IRF3 have been associated with symptomatic infections; however, no correlation has yet been found with IRF5 or IRF7 and symptomatic WNV infection [15•].

Cell-specific regulation of IFN-α/β production occurs during WNV infection. TLR 3 signaling in neurons restricts WNV replication in the CNS without affecting peripheral viral replication [16]. A similar phenotype is seen in mice deficient in a downstream TLR signaling molecule, MyD88 [17, 18]. Mice deficient in MyD88 also had reduced trafficking of leukocytes to the CNS due to reduced production of CCL5, CXCL9, and CXCL10, which contributed to higher viral burdens in the CNS [17].

Recently, research has shifted to understanding how cellular ISGs restrict WNV viral replication and how WNV evades this host mechanism. 2′-*O* Methylation of the viral mRNA cap evades IFN-induced proteins with tetratricopeptide repeats (IFIT) protein-mediated viral restriction [19]. Interferon-inducible transmembrane proteins (IFITM) restrict early WNV viral replication, while viperin restricts WNV only in the CNS [20, 21]. In humans, SNPs in the antiviral protein Mx1 are a risk factor for symptomatic WNV infections [15•]. Protein kinase R and RNAse L can also contribute to IFN-mediated signaling in the periphery and CNS and are both required for WNV clearance [22]. Genetic variation in the 2′-5′ oligoadenylate synthetase 1 (OAS1) gene, which contributes to RNase L-mediated degradation of viral RNA, has been found to contribute to increased susceptibility in humans in two of three studies [15•, 23, 24]. Inbred mouse strains are susceptible to WNV due to a single point mutation encoding a stop codon in the mouse ortholog, oas1b [25]. Recent genetic analyses using systems biology approaches have identified additional tissue-specific ISGs and previously unknown ISGs that may also play a role in restricting WNV replication [26, 27••].

Downstream effects of other cytokines may lead to beneficial or detrimental outcomes in WNV infection. Early IFN-γ production by γ/δ T cells in the periphery of WNV-infected mice limits viral dissemination into the CNS [28, 29]. Tumor necrosis factor (TNF)-α production aids in the recruitment of leukocytes to the CNS and also helps to mediate neuronal survival by preventing caspase-3 upregulation [30, 31]. Individuals receiving anti-TNF-α therapeutic antibodies have been found to be at high risk for developing WNND, which is consistent with a protective role for TNF-α in WNV infection [32]. However, TNF-α and macrophage migration inhibitory factor (MIF) can also increase the permeability of the blood brain barrier (BBB), thereby increasing susceptibility to WNV disease [33, 34•]. Age-specific defects in cytokine production may result in a decreased ability to restrict WNV infection and dissemination. In humans, dendritic cells infected with WNV have impaired IFN signaling when obtained from older individuals compared to younger individuals [35]. WNV infection of macrophages from elderly individuals has been shown to lead to increased TLR3 expression and increased cytokine and chemokine production leading to potential downstream effects on the immune response [36].

Adaptive Immune Responses to West Nile Virus

After initial WNV infection and subsequent virus restriction by the innate immune system, the adaptive immune response kicks in to help to prevent virus dissemination and eliminate virus-infected tissues. Long-term memory T and B cell responses help protect against a secondary infection and are important for the design of an effective WNV vaccine [37].

Anti-WNV IgM and IgG antibody responses are required for limiting viral spread and neuroinvasion [38, 39]. WNV antibody responses are primarily directed towards the structural envelope (E) protein but can also be directed against the secreted non-structural NS1 protein [40••]. Anti-WNV E therapeutic and prophylactic antibodies that are directed against domain III (DIII) of the E protein neutralize WNV particles in mice [41]. However, in humans, responses against the DII region predominated, with only a small fraction of the total antibody response directed against DIII [42]. Although passive immunotherapy with anti-E antibodies following WNV infection protected mice, recent findings showed no benefit from high titer WNV immunoglobulin in individuals who presented with WNND [43]. In mice, anti-NS1 antibodies also provide protection against WNV infection through non-neutralizing mechanisms [44].

Elimination of $CD4^+$ T cells, $CD8^+$ T cells, or B cells from mice result in increased susceptibility to WNV infection [38, 45, 46]. Murine and human WNV-specific $CD4^+$ and $CD8^+$ T cell epitopes have been identified to assess WNV-specific T cell responses in infected hosts [47–51, 52•]. WNV-specific $CD4^+$ and $CD8^+$ T cells in mice are polyfunctional cytokine producers that have cytolytic activity and help to reduce virus-infected cells in tissues [47–49, 53]. WNV-specific $CD4^+$ and $CD8^+$ T cells can protect mice against a WNV challenge when adoptively transferred into naïve mice or immunodeficient mice [46–48]. WNV-specific $CD8^+$ T cells also contribute to vaccine-induced protective immunity in mice [54]. In humans, a chimeric live-attenuated WNV vaccine was able to induce E-specific T cell responses and generated a WNV-specific $CD8^+$ T cell response to an immunodominant WNV human leukocyte antigen (HLA)-A*2-restricted epitope [55, 56]. Enhanced protection and reduced viral burden in the CNS were seen in HLA-A*2 transgenic mice after immunization with this immunodominant WNV HLA-A*2 $CD8^+$ T cell epitope [57]. HLA class I and class II alleles have been associated with asymptomatic and symptomatic WNV infections; however, more work is needed to understand how protective and susceptibility alleles contribute to WNV disease in elderly vs. younger individuals [58].

Since advanced age in humans is associated with increased WNND, researchers have sought to determine whether defects in the adaptive immune responses in the elderly contribute to symptomatic WNV disease. In aged mice, dysfunctional $CD4^+$ and $CD8^+$ T cell cytokine production, expansion, and trafficking contributed to increased WN viral titers in the brain [59]. Contrary to what was seen in mice, there is little functional evidence to suggest that adaptive immune responses to WNV in the elderly contribute to disease severity. WNV-specific memory T cell numbers and the breadth of the responses to WNV epitopes in the elderly were similar to those seen in younger individuals [51, 60]. Monofunctional and polyfunctional WNV-specific memory $CD8^+$ T cells were present in all age groups and exhibited similar phenotypes, except a terminally differentiated phenotype was seen in WNND [60, 61••]. This data suggests that T cell dysfunction in the elderly may not be the sole mechanism contributing to increased susceptibility, and other dysfunctional immune responses may contribute to disease outcomes.

$CD4^+$ regulatory T cells (Tregs) are important immune mediators as they blunt effector T cell responses to pathogens, thereby preventing unintentional immunopathology [62]. Tregs also play a critical role in modulating T cell effector function and memory differentiation in lymph nodes and CNS [63]. Although frequencies of Tregs in PBMCs from WNV-infected blood donors increased over time, a lower frequency of Tregs was detected in symptomatic vs. asymptomatic WNV^+ blood donors up to 90 days after infection in an age-independent fashion. In mice, the frequency of Treg cells decreased over time, but similar to humans, those with more severe disease had lower Treg frequencies. Mice lacking Treg cells had increased mortality rates, suggesting a protective role for Treg during WNV infection in mice [64]. Blocking interleukin (IL)-10 signaling, a negative regulator of immune responses secreted by Tregs and $CD4^+$ T cells, increased survival after WNV infection [65]. These results suggest that regulation of the adaptive immune responses is critical in determining outcomes upon WNV infection.

Innate Immune Responses to WNV Influence Adaptive Immune Responses

Interferon (IFN)-α/β and the IRF transcription factors are also important for the induction of adaptive immune responses to WNV. Pinto et al. determined that inhibiting type I IFN signaling early after infection greatly increased WNV-specific $CD8^+$ T cells due to increased virus replication. Blocking IFN signaling later during infection reduced cytokine production by WNV-specific $CD8^+$ T cells but did not alter their frequencies [66]. IFN-α/β can also induce non-specific activation of B cells in draining lymph nodes during WNV infection, demonstrating that IFN can have widespread effects on the immune response [67]. IFN-α/β can also help stabilize the BBB through upregulation of tight junction proteins, thereby preventing viral dissemination into the CNS as well as leukocyte trafficking which may contribute to immunopathology [34•].

As described earlier, the production of IFN-α/β is regulated by IRFs. IRF1 has both intrinsic effects through regulation of T cell receptor signaling and proliferation of $CD8^+$ T cells and extrinsic effects through shaping the environment of expanding $CD8^+$ T cells [68]. IRFs can also have cell-specific effects on adaptive immune responses as IRF5 signaling in the draining lymph node in WNV-infected mice led to upregulation of chemokine and cytokine production, enhancing antibody generation but with no effect on T cell responses [69].

Chemokine gradients help in the trafficking of immune cells to the CNS during WNV infection. WNV infection of CNS resident macrophages induces TLR7 activation and production of IL-23, which recruits peripheral macrophages to the CNS [18]. These infiltrating macrophages then produce IL-1β, which promotes expression of CXCL12 and further recruitment of $CXCR4^+$ T cells to the endothelial barrier [70]. Later in infection, a decline in CXCL12 expression permits trafficking of T cells from the endothelial barrier into the brain parenchyma leading to reduced viral loads and enhanced recovery from infection [70, 71]. Secretion of CXCL10 by WNV-infected neurons recruits $CXCR3^+$ $CD8^+$ T cells preferentially to the cerebellum [72]. Cortical neuronal production of IL-1β enhances effector $CD8^+$ T cell function and further promotes antiviral activity in neurons [73•]. In humans, decreased IL-1β production was seen in WNV-infected convalescent macrophages isolated from patients recovered from symptomatic WNV infection which is consistent with the in vivo findings in mice [74].

A fine balance exists for the interplay between innate immune responses and $CD4^+$ and $CD8^+$ T cells in mediating immunoprotection or immunopathology. Recruitment of $CD4^+$ and $CD8^+$ T cells to the CNS through chemokine-mediated mechanisms is required for the elimination of virus-infected cells [34•, 71, 72, 75]. Deletion of chemokine receptor 5 (CCR5) reduces trafficking of leukocytes to the CNS and increased virus replication in mice [76]. Individuals encoding a deletion of 32 nucleotides in CCR5 were shown to be at risk for symptomatic WNV infections and increased mortality [77]. However, this association was not found in other WNV cohorts [15•, 24]. An overactive WNV $CD8^+$ T cell response can result in immunopathology in the CNS, suggesting that regulation of the adaptive immune response to WNV is important for recovery from infection [78, 79].

Diabetes in humans is identified as a risk factor for severe WNV disease and persistent WNV symptoms [1••]. Individuals with diabetes have impaired T regulatory cell function and altered expression of co-stimulatory molecules that help regulate adaptive immune responses [80, 81]. Increased susceptibility to WNV in diabetic mice (*db/db)* is due to reduced IFN-α/β production and delayed antibody and T cell responses [82]. Reduced trafficking of $CD45^+$ lymphocytes and $CD8^+$ T cells, even in the presence of increased inflammatory molecules in the periphery and the brain, leads to increased viral replication in the CNS in these mice [82, 83•].

As described above, IFN-α/β signaling during the early stages of infection can influence the quality of the adaptive immune response. Cell-specific differences in IFN responses in older individuals could lead to tissue-specific differences in antiviral protein responses as is seen in mice [21, 27••]. These differences early during infection could also lead to qualitatively different antibody and T cell responses at later stages of infection in the CNS. Due to limitations in study design, human studies focus on isolated peripheral blood mononuclear cells (PBMCs) from convalescent WNV individuals [35, 84]. Circulating PBMCs may not accurately reflect responses that may be seen in tissues. These tissue-specific responses may be playing an important role in resolving WNV infection in the elderly as is seen in mouse models of WNV infection.

Controversies in Virus Persistence and Immune Responses

Hamster and murine models of WNV have demonstrated virus persistence in kidney and other tissues [85, 86]. WNV-specific antibody and $CD8^+$ T cell responses persist in the CNS of WNV-infected mice for up to 16 weeks [85]. In a non-human primate model, persistence of WNV was detected in the CNS, kidneys, and lymph nodes [87]. Long-term follow-up studies of WNV-infected individuals have rarely been done due to the relatively short interval since WNV emergence. Several clinical studies have demonstrated persistent symptoms, especially fatigue and cognitive disorders, following WNV infection, regardless of disease severity [1••, 88•]. The persistence of WNV in humans and its potential role in immunoregulation and symptoms remain controversial. In one study, WNV RNA was detected in a subset of patients with persistent symptoms 1–7 years post-WNV infection [89]. However, in a separate study of WNV patients >6 years post-infection, WNV RNA was not detected in the urine [90, 91]. A more recent study showed that high levels of WNV RNA could be amplified in urine during acute infection and up to 30 days after presentation [92••]. Interestingly, a high prevalence of chronic kidney disease was reported in WNV-infected individuals after long-term follow-up of acute illness, similar to what is seen in the Syrian hamster model, suggesting antigen persistence and immunopathology from WNV-specific immune cells [86, 93]. Another recent study showed that RNA can be detected in whole blood up to 4 months after acute infection [4••].

Several studies of symptomatic vs. asymptomatic individuals after WNV infection have demonstrated altered immune responses between these groups. An increased frequency of negative inhibitory receptors $Tim3^+$ and PD-1 was found on $CD4^+$ and $CD8^+$ T cells in symptomatic vs. asymptomatic WNV^+ blood donors, suggesting continual WNV antigen exposure [94••]. An increased frequency of terminally differentiated $CD8^+$ T cells was seen in patients with WNND, which also may be seen in continual antigen exposure during chronic viral infection [60, 95]. In a more recent study, patients who experienced chronic fatigue for prolonged periods of time after WNV infection, regardless of disease severity, were found to have elevations in serum proinflammatory cytokines, again suggesting potential antigen persistence [88•].

In humans and mice infected with WNV, an initial IgM antibody response is generated early in infection followed

by a conversion to an IgG antibody response [38, 39, 96]. IgG antibody levels either remained constant or declined over time in WNV seropositive individuals; however, there was no difference between symptomatic and asymptomatic individuals [84, 97, 98]. Multiple reports have described persistence of IgM antibody in humans for a period of up to 8 years post-infection, which again suggests persistence of WNV antigen [97–99]. Although there are many tantalizing indicators for viral persistence in a subset of individuals after WNV infection, further studies are warranted.

Questions To Be Answered Regarding the Pathogenesis of WNV in Humans

Human clinical studies can be challenging under any circumstance, and prospective cohort studies of acute WNV illness are relatively rare. The distribution of WNV is sporadic and focal in nature from year to year with clustering of cases in different counties and hospitals, making subject recruitment difficult. Approval of human studies by ethical review boards can take months, at which point, the outbreak has ceased and cases may not occur in the same location the following season. Such quandaries have limited not only studies of disease pathogenesis but also studies of therapeutics and phase III clinical studies of candidate WNV vaccines as well.

What has been done, what should be done, and what can be done in order to better understand WNV pathogenesis in humans? It would be optimal to obtain blood samples as early as possible following infection so that virus and innate and adaptive immune responses can be studied. As viremia occurs prior to onset of clinical symptoms, blood banks in WNV endemic regions provide the best potential access to asymptomatic viremic blood donors [4••]. While such cohorts that have thus far been studied yielded samples for characterization of antibody and T cell responses, limitations include low numbers of individuals who develop subsequent WNND as well as a lack of well-defined clinical follow-up and evaluations from which to draw conclusions about disease pathogenesis [43]. Large cohort studies of acutely ill subjects are challenging due to the sporadic nature of outbreaks and IRB issues outlined above. Studies of hospitalized cases alone skew towards late events in WNND, such as what has recently been reported [43]. Most cases of WNF occur in outpatients, a subset of subjects exceedingly difficult to identify and recruit. A series of studies were enabled by the centralized health system in Canada across several regions under a single IRB. Even under these cirucmstances blood samples for research studies on disease pathogenesis could often not be obtained until a week or two after illness onset [51, 61••]. Studies of innate immunity in humans thus far have utilized in vitro infection of human cells or late convalescent clinical samples for identification of biomarkers or risk factors of disease severity [35, 36, 84]. Interpretation of in vitro results to in vivo pathogenesis should be interpreted with caution. Similarly, interpretation of studies on late convalescent clinical samples from WNV-immune individuals has the possible confounder of virus persistence and/or other underlying health issues, which may significantly alter immune responses. Lastly, the issue of virus persistence, while enticing, is still controversial. Autopsy studies of individuals enrolled in long-term WNV cohorts would play a key role in determining the role of persistent antigen in disease pathogenesis. Collaborative studies among scientists and clinicians will be vital to furthering knowledge of WNV pathogenesis in humans.

Conclusions

In vitro and animal studies of disease pathogenesis have greatly added to our understanding of WNV biology and immunology. By comparison, there is a relative paucity of immunologic data in well-defined clinical cohorts of WNV-infected humans. Despite the challenges, by comparing results in mice and humans, some preliminary conclusions can be drawn: (1) Individuals with defects in immune responses (elderly and immunocompromised) are at high risk for WNND. (2) While no obvious defects in antibody and $CD8^+$ T cell responses are seen at convalescent time points following WNV infection, it is unknown whether altered kinetics of these responses early in infection might still play a role in disease pathogenesis. (3) Animal data point to a key role in innate immunity in protection from severe disease, but well-controlled clinical studies on innate immunity during the viremic/early illness stage in humans have yet to be done. (4) The role of virus persistence in disease pathogenesis remains controversial, but alterations in several immune markers suggest persistent WNV antigen. Further clinical studies of well-characterized patients with clinical and asymptomatic WNV infection are desperately needed to better understand disease pathogenesis, as well as to enable development of therapeutics and vaccines to combat this disease.

Compliance with Ethics Guidelines

Conflict of Interest Derek Trobaugh and Sharone Green declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent Among cited articles where at least one of the authors of the current report were authors, animal and human study protocols were approved by the University of Massachusetts Medical School Institutional Animal Care and Use Committee or Institutional Review Board respectively.

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