IMPACT OF TRIGGERING RECEPTOR EXPRESSED ON MYELOID CELLS-1 (TREM-1) ON ANTI-VIRAL IMMUNITY TO WEST NILE VIRUS

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ABSTRACT

West Nile virus (WNV) has emerged as a pathogen of importance in the Western Hemisphere over the past two decades. Its association with severe neurological disease justifies the need to understand mechanisms of immune control in order to promote the development of therapies. One of the earliest steps in controlling WNV infection occurs through the activation of innate immune pathways such as Toll-like receptor-3 (TLR-3), RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs). Typically, binding of WNV-derived replication products to these receptors causes activation of downstream signaling resulting in the production of antiviral type I interferon (IFN) and pro-inflammatory cytokines such as TNF-α and IL-1β. The activation of inflammatory pathways is very tightly regulated at multiple steps to promote efficient clearance of the virus without causing tissue damage. In WNV, the mechanisms associated with regulation of inflammation are not completely characterized as yet. To begin to understand how WNV regulates host inflammatory response, we initiated studies to examine the role of triggering receptor expressed on myeloid cells 1 (TREM-1) in antiviral immunity. TREM-1 signaling is linked with the amplification of inflammation. However, other recently characterized roles of TREM-1 such as activation of antigen presenting cells and recruitment of immune cells to the site of infection, suggest that this receptor might be an important player in immunity to WNV. Here, we investigated the effect of virus infection on the expression of TREMs and the role TREM-1 plays during anti-WNV immunity.
Our results demonstrated that TREM-1 expression increased in immune cells following flavivirus (WNV, dengue virus and Japanese encephalitis virus) and influenza virus infection, and exposure to Ebola virus glycoprotein. In vivo, TREM-1 and TREM-3 transcripts were significantly up regulated in the peritoneal cells from WNV-infected mice, which correlated with high viremia, while anti-inflammatory TREM-2 and TREM-like transcript-1 (TLT-1) expression was reduced. We further document that infiltrating inflammatory monocytes, as well as resident glial cells, contributed to increased TREM-1 in WNV-infected brain. The cellular consequence of TREM-1 activation using an agonist antibody was the amplification of WNV-associated TNF-α and IL-6 production, which was attenuated in the presence of TREM-1 inhibitory peptide. Importantly, significantly higher mortality and viremia in WNV-infected TREM-1/3−/− mice suggested antiviral function of this receptor. TREM-1/3−/− mice exhibited enhanced virus titers in the periphery and brain, and failed to sustain the production of pro-inflammatory cytokines, such as IL-6, in the serum. Although the levels of WNV-specific antibodies were comparable in both groups of mice, TREM-1/3−/− mice had fewer IFN-γ producing CD8 T cells in the WNV-infected spleen following re-stimulation with WNV peptide. Moreover, brains from TREM-1/3−/− mice displayed major hallmarks of neuroinflammation including increased levels of inflammatory cytokines, activation of GFAP and infiltration of leukocytes. Intriguingly, survival of mice following intracranial inoculation of WNV was not significantly different between both groups suggesting that TREM-1/3-dependent immune responses in the periphery contribute to the WNV disease outcome.
Collectively, our results support novel roles of TREM-1 as an amplifier of WNV-associated innate immunity and initiation of adaptive immunity to effectively and timely clear the virus from periphery and prevent neuroinvasion.

These studies provide novel insights into the complex interactions that occur between inflammatory pathways responding to WNV and may allow for the characterization of similar networks of innate immune regulation in other viruses of global importance. Further, these studies may lead to identification of viral and cellular targets for the development of urgently required immunotherapeutic strategies.
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<td>Acute flaccid paralysis</td>
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<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>BMDC</td>
<td>Bone marrow derived dendritic cell</td>
</tr>
<tr>
<td>BMDM</td>
<td>Bone marrow derived macrophage</td>
</tr>
<tr>
<td>CHIKV</td>
<td>Chikungunya virus</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebral spinal fluid</td>
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<tr>
<td>DAMP</td>
<td>Danger associated molecular pattern</td>
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<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>DENV</td>
<td>Dengue virus</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbant assay</td>
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<tr>
<td>GP</td>
<td>Glycoprotein</td>
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<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ISG</td>
<td>Interferon stimulated gene</td>
</tr>
<tr>
<td>JEV</td>
<td>Japanese encephalitis virus</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein 1</td>
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<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblasts</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>NLR</td>
<td>NOD-like receptor</td>
</tr>
<tr>
<td>NY99</td>
<td>New York '99 strain (WNV)</td>
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<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
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<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PFU</td>
<td>Plaque forming unit</td>
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<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
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<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
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<tr>
<td>RLR</td>
<td>RIG-I-like receptor</td>
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<tr>
<td>TBEV</td>
<td>Tick-borne encephalitis virus</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>TLT</td>
<td>TREM-like transcript</td>
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<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor α</td>
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<tr>
<td>TREM</td>
<td>Triggering receptor expressed on myeloid cells</td>
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<tr>
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<td>West Nile virus</td>
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<td>WT</td>
<td>Wild type</td>
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Chapter 1

Triggering receptor expressed on myeloid cells-1 (TREM-1): a new player in antiviral immunity?

Abstract

The triggering receptor expressed on myeloid cells (TREM) family of protein receptors is rapidly emerging as a critical regulator of a diverse array of cellular functions, including amplification of inflammation. Although the ligand(s) for TREM have not yet been fully identified, circumstantial evidence indicates that danger- and pathogen-associated molecular patterns (DAMPs and PAMPs) can induce cytokine production via TREM-1 activation. The discovery of novel functions of TREM, such as regulation of T-cell proliferation and activation of antigen-presenting cells, suggests a larger role of TREM proteins in modulation of host immune responses to microbial pathogens, such as bacteria and fungi. However, the significance of TREM signaling in innate immunity to virus infections and the underlying mechanisms remain largely unclear. The nature and intensity of innate immune responses, specifically production of type I interferon and inflammatory cytokines is a crucial event in dictating recovery versus adverse outcomes from virus infections. In this review, we highlight the emerging roles of TREM-1, including synergy with classical pathogen recognition receptors. Based on the literature using viral PAMPs and other infectious disease models, we further discuss how TREM-1 may influence host-virus interactions and viral pathogenesis. A deeper conceptual understanding of the mechanisms associated with pathogenic and/or protective functions of TREM-1 in antiviral immunity is essential to develop novel therapeutic strategies for the control of virus infection by modulating innate immune signaling.
Introduction

The robust induction of innate immunity is the first line of defense against virus infections and depends on the ability of the host immune cells to detect invading pathogens and alert adaptive immune cells. Viral pathogens and replicative intermediates present pathogen-associated molecular patterns (PAMPs), such as viral double-stranded (ds) RNA, uncapped single-stranded (ss) RNA and viral proteins, which are detected by pattern recognition receptors (PRRs) expressed on immune cells. Typically, binding of virus PAMPs to PRRs namely Toll-like receptors (TLRs), RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs) cause activation of downstream signaling resulting in the production of antiviral type I interferon (IFN) and pro-inflammatory cytokines (1). A timely production of IFN-α/β and pro-inflammatory cytokines is critical for the clearance of the virus during early phase of the infection and fine-tuning the innate-adaptive interface for long-lasting protection. The activation of these PRRs is very tightly regulated at multiple steps to prevent tissue damage due to uncontrolled production of cytokines. Thus, overall virus disease outcome depends on the magnitude and the nature of innate immune events triggered following virus entry. Understanding the host factors that participate in positive or negative regulation of innate immunity to virus infections is currently the focus of intense research to seek out cues for designing therapeutics to modulate inflammation at the molecular level. Several novel innate immune molecules, including members of triggering receptors expressed on myeloid cells (TREM) family of receptors, have
been recently characterized to play an important role in modulating the intensity of innate immune responses.

Since the discovery of TREM in 2000, they have been described as critical immunomodulators in several inflammatory disorders of both infectious and non-infectious etiology (2). TREM-1 is the most well characterized member of the TREM family and plays an important role in the amplification of inflammation, crosstalk with other PRR pathways and activation of antigen-presenting cells (APC). A substantial amount of data supports the link between TREM-1 signaling and several diseases, such as polymicrobial septic shock and inflammatory bowel disease, and in animal models of pneumonia and asthma (2, 3). Although the data on the functions of TREM-1 in virus infections is limited, *in vitro* studies using viral PAMPs suggest the ability of viruses to modulate TREM signaling. In this review, we first discuss the current understanding of the immunoregulatory functions of the TREM family, in particular TREM-1, and then highlight potential roles of TREM-1 in antiviral immunity.

**TREM family of receptors**

Members of the TREM family (designated TREM-1 to TREM-4) belong to the immunoglobulin variable (IgV) domain receptor superfamily of proteins (4). They are cell surface activating receptors with a transmembrane region containing charged lysine residues and a short cytoplasmic tail lacking signaling motifs (4). Signaling through these receptors is facilitated by the adaptor protein DNAX-
activating protein of 12 kDa (DAP12) (4). TREM-1 was designated as CD354 by the Ninth Workshop on Human Leukocyte Differentiation Antigens in 2011 (5).

The human TREM family members, which share low sequence homology with each other, are located on chromosome 6p21.1 (6, 7) and cluster with the related NKp44 receptor (8). Several related receptors, such as the TREM-like transcripts (TLT) 1-5 also map to this region of the human genome and share the V-domain of the Ig superfamily but unlike TREM, they also contain an immunoreceptor tyrosine inhibitory motif in their cytoplasmic tail (8). The TREM genes on the mouse chromosome 17C3 (9–11). TREM-3, which is not found in humans and shares 43% sequence similarity with TREM-1, is located directly next to TREM-1 in the mouse genome, and is predicted to be functionally similar to TREM-1 (6, 11). pDC-TREM (also called TREM-4 and not expressed in human cells) is present on mature mouse plasmacytoid dendritic cells (pDCs) and share ~20% amino acid homology with TREM-1 and TREM-2 (10). Two independent groups have solved the structure of the extracellular IgV domain of TREM-1. Although, the results have been conflicting, both groups have validated that TREM-1 belongs to the Ig superfamily (6, 7, 12).

**Cellular localization**

TREM-1 and TREM-2 were first identified on lipopolysaccharide (LPS)-stimulated monocytes and neutrophils (4), following which the initial search for TREMs was mostly limited to immune cells. The types of human immune cells expressing
high levels of TREM-1 includes monocytes, neutrophils, granulocytes, DCs and natural killer (NK) cells, and low level expression on T cells and all subsets of B cells (5, 8, 13). However, more recent studies have characterized the expression of TREM family members in several human and mouse non-immune cells and tissues. Studies describing the presence of TREMs in different cell types and species are summarized in Table 1. The presence of TREM-1 is now reported in varying human and mouse non-immune cells such as epithelial cells and fibroblasts, and in tissues such as lymph nodes, spinal cord, lung, heart and placenta (14–17). TREM-2 has the widest range of expression so far and includes myeloid cells, osteoclasts and microglia, and tissues such as kidney, liver, heart, brain and lung (18, 19). As seen in Table 1, the literature on the expression patterns of other TREM and TLT family members is limited and mostly reports presence on mouse immune cells. However, this expanding list of cell types and species is likely to continue growing as further research on TREMs is undertaken.

**Soluble TREM as marker of disease severity**

Although TREM family members are typically membrane-bound receptors, multiple members are now reported to exist in a soluble form in the clinical samples of patients from several inflammatory conditions. So far, soluble forms of TREM-1, TREM-2 and TLT-1 have been identified (20). However, it is possible that other members of the family might also be recognized in this form. The soluble form of TREM-1, a 27kDa glycosylated peptide, is most likely produced
by the cleavage of the extracellular ectodomain of the membrane-bound form by matrix metalloproteinases (21). Using *in vitro* time-course analysis, Gómez-Piña and colleagues demonstrated that increased levels of sTREM-1 correlated with decreased cell surface TREM-1 expression after 6 hours of LPS stimulation of CD14⁺ monocytes (21). It remains probable, however, that sTREM-1 may be produced through alternate pathways, such as alternate spicing (17).

In clinical conditions, sTREM was first identified in the plasma of patients with sepsis (22) and in bronchial lavage specimens of pneumonia patients (23). Elevated levels of sTREM-1 have now been found in multiple infectious and chronic inflammatory diseases such as pneumonia, pleural effusion, intra-abdominal infections, inflammatory bowel disorders, inflammatory rheumatoid disorders and lung cancer as reviewed previously (24). In addition to the serum, two studies reported increased sTREM-1 levels in cerebral spinal fluid (CSF) of bacterial meningitis patients implying that it may be a marker of differentiating bacterial versus non-bacterial meningitis (25, 26). Although the sample size of non-bacterial cases was very small as compared to bacterial meningitis cases, nonetheless, these studies emphasize that TREM-1 signaling may be modulated during CNS infections. Initial interpretations of these data led to the proposal of developing sTREM-1 as a biomarker for the diagnosis of acute inflammatory diseases, such as septic shock. However, this observation was not supported by subsequent studies and is now considered as a possible indicator of increased severity of the disease (26, 27). At present, the function of sTREM is unknown,
but based on the data on other soluble forms of membrane receptors such as ICAM-1 and VCAM-1 (28), it is possible that sTREM-1 and sTREM-2 may negatively regulate TREM receptor signaling via neutralization of the respective ligands.

**TREM ligands**

Identification of the ligands for any receptor is a crucial step in establishing a link between a signaling receptor and disease pathogenesis. The search for TREM family ligands has been elusive, although multiple putative ligands have been proposed. Haselmayer and co-workers identified a membrane-bound ligand for TREM-1 on the surface of platelets, which upon interacting with TREM-1, amplified LPS-induced neutrophil activation (29). Evidence for the presence of a soluble ligand for TREM-1 comes from a study showing that exposure of human monocytes to the serum of septic patients and LPS increased production of tumor necrosis factor-α (TNF-α), which was blocked by TREM-1/Fc fusion peptide (30). Moreover, a few DAMPs and PAMP are described as possible TREM-1 ligands, indicating that TREM-1 could act as a PRR in a similar capacity as TLRs. Mohamadzadeh and colleagues used a viral replicon system to demonstrate that the surface glycoprotein, but not the nuclear protein, of Marburg virus was able to bind to a TREM-1/Fc fusion peptide (31). In the list of potential DAMPs, two independent studies have proposed high-mobility group box 1 protein (HMGB1) or heat shock protein 70 (HSP70) as possible ligands for TREM-1 (15, 32). HMGB1 and HSP70 present in the necrotic cell lysates of
myeloid cells were responsible for significant induction of the proinflammatory
cytokine expression, which was reduced by blocking TREM-1, thus confirming
the role of TREM-1 in cytokine expression cascade via responding to these
endogenous DAMPs. Collectively, these data suggest that TREM-1 may not
have a single ligand, as most activating receptors do, but might recognize
multiple epitopes and bind to a range of viral ligands.

**TREM-1 signaling and inflammation**

Activation of TREM-1 signaling is initiated upon binding of the ligand to the
receptor, which triggers the association and phosphorylation of immunoreceptor
tyrosine-based activation motif of the adaptor protein DAP12. The signaling
pathways triggered downstream to DAP12 phosphorylation are very specific to
the TREM family member. *In vitro* studies in relevant cell types such as
neutrophils and macrophages demonstrate that phosphorylation of DAP12 by Src
family kinases results in the recruitment and activation of nonreceptor tyrosine
kinase Syk. The Syk, in turn, activates the downstream signaling molecules
including PI3K, PLCγ, ERK1/2 and MAP kinases that regulate NF-κB activation
and expression of inflammatory genes in a cell-specific manner (2, 4, 33).
Although the exact pathway remains unclear, TREM-1 signaling via
phosphorylation of Syk also regulates calcium influx, which further activates
MAPK/ERK pathway (34). In neutrophils, TREM-1 also regulates neutrophil
degranulation and production of reactive oxygen species in addition to cytokines
and chemokines (29, 35, 36). On the other hand, activation of DAP12 by TREM-2
is shown to promote anti-inflammatory response in a cell-specific manner (9). In contrast to TREM-1, TREM-2 signaling does not involve NF-κB translocation and is shown to induce DAP12-dependent calcium influx followed by activation of ERK and PI3K (37). Although the overlap and cross-talk between TREM-1 and TREM-2 signaling pathway is not yet clear, several studies demonstrating downregulation of cytokines such as TNF-α by TREM-2 emphasize opposing physiological roles of these two receptors (38).

Amplification of inflammation is the best-characterized function attributed to TREM-1. Because of the absence of a well-characterized ligand, an agonist antibody to TREM-1 is routinely used to over activate TREM-1 signalling. Bouchon and colleagues first showed that the key functional outcome of the artificial over-activation of TREM-1 receptor in monocytes was the increased production of cytokines, such as TNF-α, monocyte chemoattractant protein 1 (MCP-1) and interleukin-1β (IL-1β) following LPS treatment (4). Following this observation, several studies demonstrated the ability of TREM-1 to amplify inflammation during septic shock. Pharmacological inhibition of TREM-1 by the use of synthetic peptides or fusion protein repeatedly prevented hyper-responsiveness and death during various experimental septic shock models: endotoxemia in mice or monkeys (35, 39), bacterial pneumonia in rats (40), polymicrobial peritonitis in rodents and pigs (41, 42). By contrast, genetic deletion of TREM-1 leads to contrasting results: while some report a decreased bacterial clearance and survival during Pneumococcal pneumonia and Klebsiella
pneumonia liver abscesses in mice (43, 44), the opposite has been described in the setting of *Leishmania major* infection (45). Further, using siRNA silencing of TREM-1 in the mouse, Gibot and colleagues demonstrated the importance of the balanced activation of TREM-1 signaling during sepsis. This study showed that partial silencing of TREM-1 was protective during peritonitis, while complete silencing was lethal to septic mice (46). This effect of TREM-1-dependent enhancement of inflammatory response is also observed in non-infectious disease models including hemorrhagic shock and pancreatitis (acute inflammation) and chronic inflammatory bowel diseases and inflammatory arthritis (24). TREM-1-deficient mice displayed significantly attenuated disease that was associated with reduced inflammatory infiltrates and diminished expression of pro-inflammatory cytokines, thus representing an attractive target for treatment of chronic inflammatory disorders (45). Such data are significant in suggesting that TREM-1 is not simply an inflammatory amplifier, but also plays a regulatory role in influencing the disease outcome.

It is now established that for the culmination of balanced and effective innate immunity, it is crucial to have crosstalk between multiple innate immune signaling pathways. Many recent studies support a model of synergy between TREM-1 and other PRRs, although the precise mechanisms are yet unclear. Available data indicates that the synergy between TLRs and TREMs might be at two levels. First, is the ability of TLR ligands to increase the mRNA expression of TREM-1, and second, the amplification of TLR-induced inflammatory response
by TREM-1. Exposure of immune cells to several PAMPs, such as LPS (a TLR-4 ligand) and lipoteichoic acid (a TLR-2 ligand), and microbial pathogens such as *Pseudomonas aeruginosa* have shown to increase TREM-1 mRNA (47–49). In a TLR-2 dependent manner, soluble fungal antigens were shown to up-regulate the expression of TREM-1 transcripts in macrophages (50). Similarly, expression of TREM-1 mRNA following activation of macrophages by LPS was dependent on the TLR-4/NF-κB pathway (49). The reverse was not true, TREM-1 signaling had no effect on TLR-4 expression. The mechanisms by which TREM-1 amplifies TLR-initiated inflammation are still being investigated. However, available data suggest that the synergy is at the level of NF-κB and IRAK-1 and appears to be cell-specific (34). Activation of TREM-1 using agonistic monoclonal antibody in combination with the ligands for TLR-4 was shown to synergistically amplify the production of proinflammatory cytokines in monocytes (4). More recent studies by Ornatowska and colleagues used pathway-specific microarray analysis to show that TREM-1 silencing did not alter expression of TLR-4, but reduced the expression of adaptor protein Myd88 and cytokines such as IL-1b and IL-10, thus emphasizing that regulating expression of downstream signaling molecules may be one of the mechanisms of TREM-1/TLR cross talk (51). Likewise, Hu and group further support the role of MyD88 protein as the point of cross talk between TREM-1 and TLR-4 signaling in the infection of corneal epithelial cells with fungi *Aspergillus fumigatus* (52). Additionally, artificial activation of TREM-1 is also shown to down-regulate expression of Tollip and ST2, negative regulators of
TLR-2 and TLR-4 pathways (53, 54) These studies collectively emphasize the fact that cross talk between TLRs and TREM-1 is at multiple levels.

Most of the TREM research so far has focused on non-viral infections and autoimmune diseases. However, characterization of several additional roles of TREM-1 such as modulation of T-cell proliferation and APC activation clearly argues for its crucial immunomodulatory role in virus infections. Below we present essential elements of antiviral immune responses and then discuss how TREM-1 signaling fits into these immune events based on the current literature on TREMs in innate-adaptive interface.

**Antiviral immunity**

Immune responses to virus infections are as diverse and complex as the viruses that induce them. However, there are specific events shared by many viruses, which are important determinants of virus clearance versus immunopathology. An important feature of an efficient innate response to the entry of both DNA viruses, such as herpes simplex virus and cytomegalovirus, and RNA viruses, including Influenza A virus, West Nile virus (WNV) and chikungunya virus (CHIKV), is the rapid detection by PRRs, notably endosomal TLRs (TLR-3, TLR-7/8 and TLR-9), RLRs (RIG-I and MDA5) and the NLRs (NLRP3 and NOD2) (55–60). The production of inflammatory cytokines is one of the hallmarks of PRR activation in innate immune cells including dendritic cells (DCs), and is required for the recruitment and activation of inflammatory cells such as macrophages, NK
cells and neutrophils to the site of infection (61, 62). The profile of cytokines produced by innate immune cells dictates the adaptive immune response and the virus disease outcome. Unlike bacterial infections, another major innate immune response to virus infection is the production of type I IFN. The paracrine and autocrine secretion of IFN renders cells “antiviral” by inducing several interferon-stimulated genes (ISGs). These ISGs confer an antiviral state by blocking virus replication at different levels such as early-stage virus infection, inhibition of post-transcriptional modification and virus maturation, activation of macrophages and DC and stimulation of NK cells to kill virus-infected cells.

As an immediate effect of innate immune activation, effector cells such as NK cells and CD8+ T cells are recruited at the site of infection. These cells act to kill virus-infected cells and macrophages clear the resulting debris. Further, depending on the cytokines induced by the APCs, different types of T helper cell responses are induced. Recruited CD4+ T cells progress towards a T_{H1} phenotype in most viral infections, eventually leading to the induction of several components of adaptive immunity. However, viruses such as human immunodeficiency virus type 1 (HIV-1), HSV and hepatitis C virus (HCV) also drive T_{H17} and T regulatory cells (T_{REG}) cell expansion (63). Influenza virus is another example where T_{H17} cell responses mediate recruitment of neutrophils, which then become responsible for the associated lung pathology (64). Humoral immunity provided by specific neutralizing antibodies is also an essential component of the adaptive immune response to virus infection that inhibits virus
attachment, internalization and protection against subsequent infection. At the later stages of infection, resolution of inflammation and the return to homeostasis is mediated by anti-inflammatory components, classically T\textsubscript{REG} and the cytokines IL-10 and TGF-β, to prevent tissue damage after virus clearance. Viruses, such as HSV, HCV and HIV use the strategy of increased T\textsubscript{REG} functions to facilitate persistent infection (65). Thus, induction of an effective and balanced innate immune response is an important determinant of virus disease outcome and is fine-tuned by multiple immune components. Understanding the specific mechanisms associated with the fine control of innate immune signaling pathways has been greatly enhanced because of the identification of several novel host molecules involved with either blocking or facilitating the synergy between important immune signaling pathways. The TREM family of proteins represents this class of novel innate immune molecules, which can influence the innate immune responses to viruses. The potential roles of TREM-1 in the different arms of viral immunity are discussed in the sections below.

**Activation of TREM-1 signaling by viruses**

In 2006, Mohamadzadeh and colleagues first reported activation of TREM-1 signaling in filovirus-infected neutrophils. Although Marburg and Ebola viruses do not replicate in primary human neutrophils, they increased TREM-1 expression following internalization, which correlated with phosphorylation of DAP12 and ERK1/2. Additionally, this study also suggested that the surface glycoprotein (GP) of filoviruses may act as a ligand for TREM-1 (31). In the clinical scenario,
increase in the neutrophils has been reported during the human Ebola disease (66, 67). Further, in vitro studies have demonstrated that the soluble variant of Ebola GP can interact with neutrophils (68). Therefore, it is possible that the interaction between TREM-1 on neutrophils with the GP protein during infection contributes to the ‘cytokine storm’ associated with lethal filovirus disease (69). Similarly, exposure of PBMC to the gp41 protein of HIV-1 has been shown to up-regulate the mRNA expression of TREM-1 (70). Another recent study by Suthar and co-workers used transcriptional profiling and pathway modeling to show that TREM-1 signaling was enriched in the liver following infection with WNV in mice (71). Although this study implies that TREM-1 signaling might be one of the pathways responsible for restricting tissue tropism, the precise role of TREM-1 in WNV pathogenesis has not been explored.

However, indirect evidence supports the ability of viruses to induce TREMs. Bleharski and colleagues first showed that poly (I:C), a mimetic ligand for TLR-3, can induce the transcription of TREM-1 in primary monocytes (47). Later, studies by Begum and co-workers could not validate the increase of TREM-1 mRNA following stimulation of monocytes with poly (I:C), however the reason for this discrepancy might be the different time points of analysis, 6 hours after stimulation, as compared to 24 hours time point used in the previous study (72). Watarai and colleagues demonstrated that TREM-4 mRNA expression increased in mouse pDCs following TLR-7 or -9 activation and led to DAP12-phosphorylation, activation of ERK1/2 signaling and ultimately IFN-α secretion
(10). Although studies using the analogs of dsRNA and microbial DNA such as poly (I:C) and CpG are limited, they strongly suggest that diverse RNA and DNA viruses that produce nucleic acid PAMPs during replication may be capable of inducing TREMs.

Similarly, our understanding of whether viruses can induce production of sTREM-1 is unclear and so far comes from only one clinical study. Ruiz in-Pacheco and colleagues recently demonstrated increased levels of sTREM-1 in the serum of dengue virus (DENV)-infected patients during the early stages of infection (first 5 days) as compared to healthy individuals (73). At this point, one can only speculate the role of sTREM in pathogenesis of DENV, an important global human pathogen, however, this is an important finding and provides direct evidence of modulation of TREM-1 in response to virus infection. Elevated levels of sTREM1s could signify either a virus-induced compensatory mechanism to counteract inflammatory process, or a host-induced mechanism to control tissue damage by attenuating downstream inflammatory signals. Therefore, such clinical studies will be highly relevant to identify the potential of sTREM-1 as a marker of disease severity in acute virus infections as with influenza virus and CHIKV.
Impact of TREM-1 on virus-associated inflammation

The function of TREM-1 in modulating virus-associated inflammation appears to be supported more by in vitro studies using viral PAMPs, than actual virus infections (Figure 1).

**Figure 1.** The putative interactions between viruses and TREM-1 signaling. During early stages of infection, viral nucleic acids and some proteins are detected by TLR-3, TLR-7/8 and TLR-9, which induces the mRNA production of pro-inflammatory cytokines, chemokines and cell surface receptors including TREM-1. Viruses shown to increase TREM-1 mRNA and/or soluble TREM-1 levels are depicted in red, although the associated pathways are not clear. The TREM-1 receptor responds to yet uncharacterized viral or host ligands and activates signaling via DAP12 and Syk tyrosine kinase. Downstream PI3K and ERL signaling further activate NF-κB and synergizes with the TLR cascade to amplify inflammation. The potential interactions between virus-induced TREM-1 and other cellular pathways such as type I IFN are not yet defined.

Activation of TREM-1 by TLR-9 ligand CpG DNA enhanced TNF-α production in mouse bone marrow-derived dendritic cells (BMDCs) (74). Similarly, Netea and
colleagues noted increased production of TNF-α in TREM-1-activated human PBMC following stimulation with poly (I:C) and CpG (75). This was also the first study to indicate that TREM-1 synergizes with NLR pathways. NLR ligands amplified the production of TNF-α, IL-1β and IL-6 when TREM-1 signaling was activated (75). Similarly, TLR-TREM synergistic activation of neutrophils is not restricted to TLR-2 and TLR-4 but also occurs with TLR-7 and TLR-8, which are common TLRs responding to virus PAMPs (36). Filoviruses are the only viruses, where the function of TREM-1 in regulating production of pro-inflammatory cytokines such as TNF-α and IL-1β is documented (31). The impact of TREM-1 in regulating inflammation in other virus diseases awaits discovery. Comprehensive analysis of virus infections in vitro as well as in TREM-1 deficient mice will be required before we fully understand the cooperation between TREM-1 and other viral PRRs in the context of the virus-associated inflammation.

**Modulation of Type I IFN by TREM-1**

The ability of the innate immune cells to rapidly produce type I IFN is one of the major determinants of the virus disease outcome. However, its role in other non-viral disease models, including bacterial infections, shock, and autoimmunity is less well defined and complicated. With the emphasis of TREM-1 studies mainly in bacterial infection models, the role of TREM-1 in the regulation of type I IFNs has not been investigated so far. However, other TREM members have been shown to positively or negatively regulate type I IFN responses. In plasmacytoid DCs, stimulation of TREM-4 resulted in increased IFN-α secretion, and was
dependent on the phosphorylation of DAP12 and PI3K and ERK1/2 pathways (10). Conversely, mouse BMDCs deficient in TLR-2 had increased transcriptional levels of type I IFN following TLR-9 activation (76). Therefore, the fact that TREM-1 can synergize with TLR-3 and TLR-7 and signal through the ERK1/2 pathway leads us to speculate that TREM-1 might play a role in positively regulating type I IFN levels.

**Role of TREM-1 in APC activation, migration and T-cell priming**

The availability of recently developed TREM-1-deficient mice have led to studies describing several novel functions of TREM family members in addition to amplifying inflammation. Wu and colleagues developed a TREM-1 knockout (KO) mouse and demonstrated that TREM-1 was essential for the activation of Kupffer cells in the diethylnitrosamine model of hepatocellular carcinoma and contributed to chronic liver damage. TREM-1 knockout macrophages were not as responsive as WT to signals from necrotic hepatocytes and exhibited attenuated APC responses including reduced production of IL-1β, IL-6, TNF-α and CCL2 (15). In line with this, the role of TREM-1 in leukocyte recruitment is clearly demonstrated by Klesney-Tait and co-workers who used a double knockout TREM-1/3 mice for Pseudomonas aeruginosa infection (3). TREM-1/3 deficiency increased mortality of infected mice, which correlated with higher local and systemic cytokine production. However, although TREM-1/3-deficient neutrophils had intact bacterial killing and chemotaxis properties, histologic examination of TREM-1/3-deficient lungs revealed decreased neutrophil infiltration of the airways (3).
Another novel function attributed to TREM-1 recently is its ability to influence the differentiation of primary monocytes into immature DCs. TREM-1 governs the upregulation of the surface expression of CD86 and MHC class II, rendering them more efficient at eliciting T-cell proliferative activity (47). Likewise, it has been demonstrated that signaling through TREM-1 on hypoxic iDCs up-regulates T cell co-stimulatory molecules, including CD83, CD86 and HLA-DR. Co-culture of these hypoxic iDCs with T-cells resulted in increased the cell proliferation and IFN-γ and IL-17 production (77). Alternatively, Ito and Hamerman used TREM-2 deficient BMDCs to demonstrate that TREM-2 inhibited TLR-induced DC maturation and antigen presentation to T cells (76) thus supporting the dynamic opposite roles of TREM-1 and TREM-2 at the level of antigen presentation as well.

APC activation is essential during virus infection for the stimulation of T_{H1} responses and subsequent development of neutralizing antibodies vital for viral clearance. In addition to APC activation, TREM signaling also can skew T-cell responses in a T_{H1} or T_{H17} direction. Pierbon and colleagues, for instance showed that TREM-1 signaling in hypoxic iDCs was responsible for T_{H1}/T_{H17} priming (77), while two independent studies showed a reduction in systemic T_{H1} responses following inhibition of TREM-1. In a model of Pseudomonas aeruginosa-induced keratitis, inhibition of TREM-1 reduced IFN-γ responses (T_{H1} phenotype), while increasing T_{H2} cytokines including IL-4 and -5 (53). Similar
results were obtained in a model of cardiac allografts, in which TREM-1 inhibition led to increased allograft survival by dampening the differentiation and proliferation of IFN-γ secreting CD4+ T cells (78). In addition, TREM-1 signaling can be influenced by a T_{H1} environment; treatment of primary human NK cells with the classic T_{H1} cytokines IL-12 and -18 led to the activation of TREM-1 signaling events, as measured by microarray analysis (79). Currently, there is no evidence supporting the hypothesis that TREM-1 may modulate innate-adaptive immune interface in virus infections. However, because viral ligands can activate TREM-1 signaling and cytokines production, there is high likelihood that cytokines governed by TREM-1 may influence activation of APCs, which in turn may impact protective T-cell functions and viral clearance.

**Return to homeostasis**

Anti-inflammatory reactions are important in any immune reaction to return the host to homeostasis. In virus infections, one of the factors that determine the balance between virus clearance and tissue damage is the timely induction of anti-inflammatory molecules. Available data indicate that TGF-β and IL-10 treatment of primary monocytes synergistically down-regulates cell surface expression of TREM-1 (80), but whether TREM-1 signaling can influence production of TGF-β and IL-10 has not been clearly defined. Nonetheless, this study strongly supports the notion that TREM-1 is the target of anti-inflammatory cytokines and that attenuating TREM-1- associated information might be one of the events in the immune homeostasis process. In microglia, TREM-2
participates in the process of tissue debris clearance and resolution of latent inflammatory reactions in Nasu-Hakola disease, a recessively inherited chronic neurodegenerative disease (81). Similar anti-inflammatory and protective functions of TREM-2 have been proposed for other acute neuroinflammatory diseases, such as multiple sclerosis (82) and Alzheimer's disease (83, 84), implying that alterations in the immune homeostasis may be mediated by TREMs in infections with neurotropic viruses, such as HIV, WNV or tick-borne encephalitis virus (TBEV).

Conclusions and future perspectives
The immune functions of the TREM family are broad and diverse (Table 2) and may contribute to antiviral immunity. One of the pertinent questions is, can TREM-1 signaling respond to infection with globally important human viruses? Of particular interest would be the viruses associated with acute inflammation as the major cause of pathology such as orthomyxoviruses (influenza virus), filoviruses (Ebola and Marburg viruses), flaviviruses (DENV, WNV and TBEV) and alphaviruses (CHIKV). More importantly, it is essential to characterize the role of TREM-1-dependent responses in disease outcome, i.e., protective versus pathogenic. For example, it is likely that TREM-1 signaling may promote inflammation and cause substantial tissue damage thereby contributing to disease pathogenesis of acute infections as with influenza virus or CHIKV. In this regard, Weber and colleagues recently demonstrated that TREM-1 deficient mice were protected from severe influenza disease without affecting virus clearance,
although this study did not look into the innate immune markers governed by TREM-1 (45). On the other hand, in virus infections with WNV, TBEV or Japanese encephalitis virus, activation of TREM-1 might be protective and enhance the robustness of innate immune responses in the periphery thereby facilitating efficient virus clearance and reduced neuroinvasion.

The question of how TREM-1 might influence disease outcome differently in acute versus chronic infections with HIV or HCV also remains to be explained. Identification of inflammatory cytokines and chemokines regulated by TREM-1 will provide valuable insights into the role of TREM-1 in APC activation, T-cell responses and anti-viral immunity. Further, given that the type I IFN system is a powerful line of defense, characterization of the function of TREM-1 in modulating IFN levels and development of effective neutralizing antibody responses will further enhance our understanding of the complex network of antiviral immunity and fine-tuning of adaptive immunity.

In conclusion, this review describes the possible roles TREM-1 might play during virus infections. Future research using newly developed TREM-1 knockout mice and clinical samples from infected patients should focus on assigning protective or pathogenic functions for different viruses, examining the underlying cell- and tissue-type specific mechanisms of action, and identification of potential ligands responsible for TREM-1 receptor activation. There is such a large impact of the innate immune responses on the outcome of various viral diseases, therefore
knowledge of the interactions between TREM-1 and viral PRRs (TLRs, NLRs and RLRs) may lead to a better understanding of the pathophysiology of viral diseases. Further advancement in this field is crucial before TREM-1 can be proposed as an immunotherapeutic target to ultimately promote virus clearance with minimum tissue damage (85–89).

Acknowledgements

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Table 1. Cellular expression profiles of the TREM family proteins

<table>
<thead>
<tr>
<th>Cell Type/Tissue</th>
<th>Species</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>TREM-1</strong></td>
<td></td>
<td></td>
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<tr>
<td>Neutrophils, CD14&lt;sup&gt;high&lt;/sup&gt; Monocytes</td>
<td>Human</td>
<td>4</td>
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<tr>
<td>NK cell line, fibrosarcoma (HT1080)</td>
<td>Human</td>
<td>8</td>
</tr>
<tr>
<td>Differentiated U937 cells</td>
<td>Human</td>
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<tr>
<td>Lymph nodes, placenta, spinal cord, lung, spleen and heart tissues</td>
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<td>17</td>
</tr>
<tr>
<td>High expression: Monocytes, granulocytes, DCs, NK cell</td>
<td>Human</td>
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<tr>
<td>Low expression: T cells and all subsets of B cells except plasma cells</td>
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<td>Normal bronchial epithelial cells</td>
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<td>Myofibroblasts and primary hepatic stellate cells</td>
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<td>Liver endothelial cells</td>
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<td>Kupfer cells and neutrophils</td>
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<td>Peritoneal macrophages</td>
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<td>Immature dendritic Cells</td>
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<td><strong>TREM-2</strong></td>
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<td>Monocyte derived dendritic cells</td>
<td>Human</td>
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<td>NK cell line, fibrosarcoma (HT1080), U937 and THP-1</td>
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<td>Microglia</td>
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<td>High expression in CNS, heart and lungs as compared to lymph nodes, kidney, liver and testes</td>
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<td>RAW264, MT2 macrophage cell lines, and T cell lines</td>
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<td><strong>pDC-TREM (TREM-4)</strong></td>
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<td>pDCs (CD11c&lt;sup&gt;dull&lt;/sup&gt; population)</td>
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<td><strong>TLT-1</strong></td>
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<td>Platelets and megakaryocytes</td>
<td>Mouse</td>
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<tr>
<td><strong>TLT-2</strong></td>
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<td></td>
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<tr>
<td>B cells, neutrophils and macrophages</td>
<td>Mouse</td>
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<td>Referenece</td>
<td>Function of TREM-1</td>
<td>Other elements of viral inflammation</td>
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<td>Exposure to Poly(I:C) up-regulates transcription of TREM-1</td>
<td>Human monocytes</td>
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<td>Human macrophages</td>
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<td>Mouse macrophages and endothelial cells</td>
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<td>76</td>
<td>Exposure to Poly(I:C) up-regulates transcription of TREM-1</td>
<td>Mouse BMDC</td>
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**TREM-1 mediated Type I IFN response**

**TREM-2 mediated Type I IFN response**

**APC activation**

**Type I IFN response**

**Other elements of viral inflammation**

**Inflammation/PRR signaling**
Chapter 2

West Nile virus and human disease
Identification and classification

West Nile virus (WNV) is a mosquito-borne flavivirus of the *Flaviviridae* family (90). It derives its name from the West Nile district of Uganda from where it was first isolated in 1937 (91). Together, with Japanese encephalitis virus (JEV), St. Louis encephalitis virus (SLEV) and Murray Valley encephalitis virus, WNV forms the JE serocomplex of viruses (92). All four of these viruses cause central nervous system (CNS) infections. WNV is also closely related to the Dengue serocomplex of viruses, which is responsible for large outbreaks of hemorrhagic fever or septic shock (93).

Based on nucleic acid phylogenetic analysis, there are seven distinct lineages of WNV, although only lineage 1 and 2 are known to cause human illness (94, 95). Lineage 1 strains are widely distributed throughout North America, Europe, Australia, Africa and Asia (96). This lineage can be further broken down into three clades. Clade 1a is the most widely distributed and includes the North American strains, including the New York 99 (NY99) strain used in this study (95). Clade 1b is composed of the Australian strains, usually referred to as Kunjin virus, and is an uncommon source of human disease (95, 97). Lineage 2 strains were restricted to Africa until the early 2000s when they began to spread into central Europe (95). Although previously only associated with mild disease, lineage 2 strains in Russia, Hungary, Italy and Greece have caused neuroinvasive and sometimes fatal, human disease (95).
Structure and replication

The WNV genome is positive-sense (+) single stranded RNA of approximately 11kb. A single copy of this genome is housed in a spherical mature virion, 50nm in diameter, that contains a dense icosahedral core surrounded by a lipid envelope with a smooth spikeless surface (98). The WNV genome consists of a 5’ non-coding region, a single open-reading frame followed by a 3’ non-coding region. The open reading frame encodes a single polyprotein, which is processed by proteolytic activity of various cellular proteases. The resulting ten mature viral proteins include the three structural proteins mentioned above, and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (92). These NS proteins regulate virus transcription and replication in addition to evasion of host antiviral responses (99). The envelope (E) protein, one of three structural proteins, mediates viral attachment, membrane fusion and virus assembly (100). The core is composed of the capsid (C) protein and has been shown to induce apoptosis in host cells during infection, mediated by p53 (101). Another structural protein, the prM protein, acts as a chaperone during E-protein folding by preventing premature folding. The majority of neutralizing antibodies produced during a natural infection target the E protein (102).

The WNV replication cycle closely resembles that of many flaviviruses. The identity of the cellular receptor used by WNV to bind and enter host cells is unclear. However, several putative receptors have been proposed including integrin αvβ3, DC-SIGN and DC-SIGN-R (103, 104). Following receptor binding,
WNV enters its target cell via clatherin-mediated endocytosis. The low pH within the endosome induces a conformational change in the E protein, allowing it to facilitate fusion of the virus with the endosomal membrane. Once in the cytoplasm, the RNA genome serves as an mRNA transcript that binds to ribosomes and is translated into a single polypeptide at the endoplasmic reticulum. Following proteolytic cleavage of the polyprotein into the 10 viral proteins, the NS proteins form a replication complex for the synthesis of full-length negative-sense RNA intermediate, which then serves as a template for the (+)-sense RNA genome. Once sufficient numbers of structural proteins have been formed, they assemble with the newly synthesized RNA genomes to form progeny virions. Following this assembly in the ER lumen, the nascent virions are trafficked within vesicles to the cell surface where they are released by exocytosis.

**Pathogenesis**

Animal models of WNV infection have been used extensively to describe host responses to WNV and pathogenesis. Studies in these models have identified three distinct phases of WNV infection (105). Following peripheral inoculation, WNV replicates in Langerhans dendritic cells (DCs) in the skin. Subsequently, DCs migrate to the nearest draining lymph node, from where the virus enters the blood stream resulting in primary viremia (106). The second stage occurs as the virus disseminates to susceptible organs such as the kidney and spleen (99). Some organs in the mouse, such as liver, do not support productive infections
(71). In the mouse model, virus is detected in the blood 1-2 days after infection and is generally cleared by day 6-7 (107). However, it has been reported that the virus can remain detectable in immunocompromised patients for up to 28-days post infection (108). The third phase of WNV pathogenesis occurs when WNV disseminates into the CNS, which occurs shortly before the virus is completely cleared from the periphery.

**WNV-CNS entry**

Several mechanisms of WNV-CNS entry have been proposed, including the ‘Trojan horse’ in which WNV enters via infected leukocytes, directly infecting the blood-brain barrier, or by retrograde axonal transport (109). Studies from our lab have demonstrated that *in vitro* infection of human brain microvascular endothelial cells (HBMVE) is productive and results in the upregulation of multiple cell adhesion molecules including V-CAM (110). However, we found that direct infection of the HBMVE cells did not directly affect the integrity of an *in vitro* BBB model. We further showed that the transmigration of leukocytes across the infected barrier caused a significant increase in the permeability of the *in vitro* BBB model (111). In addition, blockage of multiple CAMs on the infected HBMVE cells not only reduced leukocyte adhesion and migration across the barrier but also abrogated the loss of barrier integrity, suggesting that leukocyte migration may in itself be a pathological event (111). Further, we have demonstrated that matrix metalloproteinase (MMP-9), produced by astrocytes via COX-2/PGE2 signaling is responsible for the degradation of tight junction proteins between
HBMVE cells, hampering the integrity of the barrier (112, 113). We also validated these observations in the mouse model by showing correlation of BBB disruption with increased MMP-9 levels in the brain rather than in the serum, suggesting that WNV replication in the brain causes altered membrane permeability (107). Other studies also showed that in MMP-9 knockout mouse, WNV titers in the brain were significantly lower than the WT, leading to improved survival (114), suggesting that the MMP-9 induced disruption of the BBB is a critical event in the entrance of WNV into the brain. Other pro-inflammatory factors implicated in the alteration of BBB permeability include tumor necrosis factor α (TNF-α) and migration inhibitory factor (MIF) (58, 115). Interestingly it was recently demonstrated that type I interferons (IFNs) may be key elements to strengthening the BBB (116), although to what extent this response protects from severe neurological disease remains unclear. Once inside the brain, WNV primarily targets neurons, myeloid cells, and to a lesser extent astrocytes, resulting in widespread neuroinflammation (99, 105, 117).

**Host immune responses to WNV**

Both innate and adaptive immunity are crucial in controlling WNV infection (105, 118). In response to WNV infection, both DCs and macrophages are activated via a complex network of pattern recognition receptors (PRRs), inducing the production of both type I IFNs and pro-inflammatory cytokines. A number of PRR have been implicated in contributing to these responses including TLR-3 (58, 59), TLR-7/MyDD88 (119), RIG-I, MDA-5 (120, 121), LGP2 (122) and the NLRP-3
inflammasome (60, 123). RIG-I activation triggers IRF-3-dependent production of type 1 IFN. Meanwhile, TLR ligation triggers both the production of IFN, via IRF signaling, and the secretion of pro-inflammatory cytokines and chemokines via the transcription factor NF-κB (105). In both the mouse model and human patients, high levels of secreted IFN, cytokines and chemokines can be detected during the acute phase of disease. Collectively, these secretory signals are critical for the clearance of WNV virus from the periphery and the stimulation of adaptive immunity. In deed, impairment in innate immune responses results in delayed virus clearance from the periphery and increased neuroinvasion (120, 124, 125). In particular, the induction of IFN stimulated genes (ISGs) plays a critical role in inhibiting WNV replication. Upon binding of IFN-β to the IFN receptor complex (IFNAR) the JAK/STAT pathway is initiated resulting in the transcription of ISGs. These ISGs then directly inhibit WNV replication through a combination of antiviral mechanisms including degrading viral ssRNA and inhibiting RNA translation (105). In addition, activation of the complement system is critical in controlling WNV infection in mice (124). Many of these innate signaling pathways, however, are targeted by WNV in order to evade their actions. For example, WNV establishes replication complexes in vesicles within the ER, effectively hiding dsRNA replication intermediates and avoiding recognition by RLRs (105).

During the adaptive phase, clearance of WNV from periphery and the brain is mediated by antibodies as well as cellular immune responses (105). The early
induction of a neutralizing IgM antibody response is particularly crucial in limiting peripheral viremia and WNV dissemination into the brain (118). Multiple branches of the cellular immune system, including CD4+ T cells (126, 127), CD8+ T cells (128, 129) and IFN-γ-producing gamma delta (γδ) T cells play critical roles in WNV infection control, particularly in mediating virus clearance from the CNS. The inflammatory cells that migrate into the brain have, however, also been implicated in neuropathology, in particular the inflammatory monocyte subset (130, 131).

**Clinical disease**

Clinical disease presentation in human patients infected with WNV ranges from asymptomatic to severe neurological disease. The majority of WNV infections are non apparent, while approximately 20% of human cases result in a mild flu-like illness, termed West Nile fever, while one in every 150 infections progresses to neurological disease (132). Following an incubation period of approximately two to fourteen days (133), West Nile disease develops with the sudden onset of fever (typically above 39°C), nausea, headache, anorexia and myalgia. These typical flu-like symptoms are sometimes accompanied by gastrointestinal symptoms, a maculopapular rash or swollen lymph nodes (134–136).

WNV neurological disease manifests in one of three ways: meningitis, encephalitis or acute flaccid paralysis (AFP)/poliomyelitis. The majority of patients with neuroinvasive disease manifest as encephalitis (60%) while about
40% experience meningitis and a small subset of patients with AFP (137). Muscle weakness is a prominent clinical sign of WNV encephalitis and is often accompanied by fever, weakness/fatigue, headache and a confused or altered mental state (137, 138). Patients who develop AFP tend to do so abruptly and progress very rapidly, displaying signs of asymmetric weakness that typically involves the legs. AFP is believed to arise when WNV invades the lower motor neurons or anterior horn cells of the spinal cord (139). The majority of WNV meningitis patients make a complete recovery, with no associated focal neurological deficits (94). However, case fatality rates of patients with encephalitis or AFP have ranged from 4% to 15%. In addition, individuals with WNV encephalitis or AFP often experience a severe and prolonged recovery, often involving some form of physical therapy or assisted living (140). While recovering, patients report experiencing prolonged muscle weakness and fatigue, insomnia, persistent memory loss, parkinsonism, confusion and depression, which can last up to 18 months after acute disease (141–143).

**Epidemiology**

Since the introduction of WNV into the new world, the virulence of this old world virus has increased dramatically. WNV was first described in Uganda in 1937 and has since been found to be enzootic in many parts of Africa, the Middle East, west Asia and Australia (144). Epidemics in these parts of the world have been intermittent and generally mild, characterized by febrile illness and a few sporadic cases of encephalitis. Israel recorded several minor outbreaks between 1951 to
An epidemic in South Africa in 1974 resulted in thousands of symptomatic infections (94). By the 1990s, however, the virulence changed, resulting in a higher incidence of neuroinvasive disease. In this decade, epidemics in Israel, Russia and Romania witnessed up to 60% of hospitalized patients with neuroinvasive disease and fatality rates ranged from 4 to 7% (146).

WNV emerged into the Western Hemisphere in New York City during the summer of 1999. During this first outbreak, there were 59 symptomatic human patients identified and 7 deaths (94, 144). Analysis of the New York virus (now called NY99) indicated that it most closely resembled the virus strain isolated during an avian outbreak in Israel in 1998. There was >99.8 amino acid homology of the envelope protein between the two strains (96). It remains unclear to this day how the virus crossed the Atlantic to emerge in New York. In the few years that followed, WNV spread along the Eastern seaboard. During the summers of 2003 through 2007 WNV spread rapidly throughout the rest of the contiguous United States, resulting in the largest outbreak of arboviral encephalitis in US history (147). The outbreak in 2003 alone, with almost 10,000 cases reported and 264 deaths, is that largest reported outbreak of meningoencephalitis in the Western Hemisphere (148). Following this period of intense yearly outbreaks, WNV cases decreased for few years. However, 2012 saw a resurgence of cases and was declared as the second largest outbreak in the US, following 2003. Alarmingly, the incidence of neuroinvasive disease reported was very high, at 0.92 per 100,000 as compared to the yearly average
of 0.31 per 100,000 (149). Despite this increase in neuroinvasive disease, the fatality rate remained unchanged compared to previous outbreaks (149). The high rate of neuroinvasive disease may reflect, in part, surveillance or reporting bias. Nonetheless, this outbreak demonstrated the ability of WNV to resurge unexpectedly. Sequence analysis of the 2012 strain did not find any significant changes to explain the sudden resurgence (150). Several other factors have been implicated in the reemergence of WNV including a warm wet winter creating excellent mosquito breeding conditions as well as a decline of public awareness following several years of low activity (151). The incidence of WNV in the tropics increases during the rainy season, when mosquitoes are the most abundant (141, 145). Likewise, in temperate and subtropical zones, human infections coincide with the peak of mosquito season, during the summer and early fall (138, 152).

**Risk factors for WNV disease**

A number of risk factors for the development of WNV encephalitis have been identified. One of the strongest associations is found in persons of advanced age, particularly over the age of 70 (134, 149, 153). Having an underlying condition such as diabetes mellitus or hypertension is also a major risk factor (154, 155). Supporting these epidemiological findings, studies using the db/db mouse model of type 2 diabetes have demonstrated increased morbidity and mortality in diabetic mice, characterized by impaired virus clearance in the periphery and the brain (156). In addition, African-Americans, Hepatitis C positive
individuals and chronic renal disease patients, and other individuals with a compromised immune system have been found to be at increased risk of developing severe disease (155). Several genetic variations have been attributed to developing symptomatic or severe disease as well. These include variants in OAS genes, including OAS1, and gene variations in the interferon pathway, in particular IRF3 and MX1 (157). A further underlying genetic risk factor has been identified as being homozygous for a defective CCR5 allele (CCR5Δ32) in a cohort of WNV-infected Caucasian patients (158). Interestingly, in the mouse model of WNV infection, CCR5 is critical in the regulation of leukocyte trafficking into the brain; CCR5 being a key chemokine receptor (159).

**Vector-host interactions**

WNV is maintained in the wild in an enzootic cycle between mosquitoes and over 200 species of birds (160). The predominant vectors of WNV are the Culex species of mosquitoes, with different species being most important to transmission in different regions of the US. In the Northeast, C. pipiens and C. restuans are the predominant vectors, while in the Southeast C. quinquefasciatus and C. nigripalpus are largely implicated in WNV transmission. Meanwhile, C. tarsalis and C. quinquefasciatus are important to disease transmission in the West (161). Interestingly, although WNV has not been reported in the states of Hawaii and Alaska, both states have susceptible vectors. C. tarsalis has been found to be prevalent in parts of Alaska and Canada, where forest and grasslands meet; indeed, another arbovirus, Western Equine Encephalitis virus was detected in
this population (162). In Hawaii, *C. quinquefasciatus* are widespread. Experimental infections with WNV by mosquito bite in the native bird the Hawaiian Amakihi resulted in high viremia and significant mortality (163). This suggests that an introduction of WNV into Hawaii could result in transmission between local birds and mosquitoes, potentially resulting in endemcity.

Mosquitoes are infected with WNV after taking an infectious blood meal from an amplifying host, primarily birds. Further, WNV can transmit vertically through mosquito populations from female mosquitoes to their eggs by transovarian transmission. Following infection, WNV persists in the mosquito for the duration of its life without causing disease. The virus penetrates the gut of the mosquito and replicates in the salivary glands and nervous system (164).

Birds are the natural reservoir of WNV and develop a high viremia following the bite of an infected mosquito, the length of which is sufficient to infect naïve vectors. It has been documented that this viremia can last more than 100 days (165). For the purposes of public health surveillance, some bird species develop fatal disease and serve as sentinels for the active circulation of WNV in the community, including jays, crows and raptors. Other bird species, including most songbirds, do not develop signs of infection, and are therefore primarily responsible for the spread of the virus over long distances (166). In deed, following it’s introduction in New York, WNV spread down the eastern seaboard along a common migratory route of many bird species (167). Subsequently, WNV spread westward through the continental US following the elliptical migration
patterns of many songbirds (167). Although at least 30 other vertebrate species are susceptible to WNV infection, besides birds, neurological disease is rare in non-human mammals with the exception of horses (168). Transmission to humans, who are secondary or accidental hosts, occurs primarily via the bite of an infectious mosquito. Additionally, human infections have been documented as the result of transfusion of blood or blood products (169). Other routes identified, although very rare, include transplacental route, breast feeding, organ transplants and laboratory exposure (170–173).

**Diagnosis, treatment and prevention**

Diagnosis of WNV infection in humans is typically based on serology of serum and/or cerebral spinal fluid (CSF) by ELISA (174, 175). IgM antibodies, which are the first to appear, are usually measurable by the fourth day of symptom onset and may persist for up to one year following infection (176). Further, the detection of IgM antibodies in the CSF is the diagnostic criteria of neurological disease (175). While the presence of IgM antibodies is indicative of a recent infection, serum IgG antibodies are likely to persist for decades. Diagnosis by serology, although the gold standard, may be complicated because of possible cross-reactivity to other flaviviruses.

Previous vaccination for Yellow Fever or exposure to JE may indicate the possibility of cross-reactive antibodies. If this is suspected, it may be necessary to confirm a positive ELISA result by plaque reduction neutralization test (94). Other diagnostic tools include virus isolation or detection of viral nucleic acids.
(161). However, because of safety concerns, virus isolation is not a preferred method of diagnosis. Further, viremia in human patients peaks 3-4 days before the onset of symptoms. Therefore, the detection of nucleic acids from blood or CSF has a low sensitivity (177). Nonetheless, screening of blood products for potential infection is typically achieved by detecting viral nucleic acids by polymerase chain reaction (PCR) (178).

Treatment of WNV patients is largely supportive, as there are no specific therapies against WNV. Strategies of care include the use of analgesics to combat pain, antiemetics and rehydration for nausea and vomiting, and monitoring for the development of elevated intracranial pressure. Several studies have proposed the use of corticosteroids, IFN-α or WNV-specific immunoglobulin for the management of WNV disease (155). However, the efficacy of these treatment proposals has not been adequately studied. In the cell culture model, Ribavirin, a guanosine analogue, has been shown to inhibit WNV replication and cytopathic effects (179), although there have been no clinical trials in humans (180). Passive transfer of WNV-specific immunoglobulin is protective in multiple animal models (181) and has provided benefit in select human cases (180, 182). The search of novel antivirals and drugs targeting host neuroinflammatory pathways is ongoing and our recent work has demonstrated the protective ability of a novel adenosine analogue, NITD-008, in the mouse model of WNV infection. Mice treated with NITD-008 starting day 1 after infection, were completely protected from WNV-associated mortality. Furthermore, NITD-008 in combination
with the anti-inflammatory histone deactelase inhibitor Vorinostat, administered during the CNS phase of disease significantly reduced neuronal death and inflammation, suggesting a potential synergistic effect of combination therapy of NITD008 with anti-inflammatory drug for the treatment of WNV encephalitis. (Nelson et al. unpublished).

Despite the availability of effective WNV vaccines for horses and geese, there is no licensed vaccine for humans. Currently, there are three equine vaccines commercially available in the U.S. Pfizer markets a formalin-inactivated whole virus vaccine that is reported to show 94% efficacy (183). Another killed virus vaccine is available from Boehringer Ingelheim, while Sanofi Aventis has produced a chimera canarypox vaccine containing WNV prM-E. Another chimera vaccine, containing WNV-prM-E on a yellow fever backbone, was licensed for equines by the USDA in 2006, but later recalled in 2010 because of associated acute adverse events (183).

Many factors have contributed to the lack of an effective human vaccine. Among them, the ability to perform strong efficacy trials in the US is hampered by the low incidence and sporadic nature of WNV outbreaks. Furthermore, cost-benefit analyses have provided little commercial enthusiasm for developing a safe and effective vaccine (184). Nonetheless, there are a number of vaccine candidates in preclinical development and clinical trials. A DNA vaccine candidate, developed by Vical and sponsored by the NIAID, is a closed circular DNA
plasmid that expresses WNV prM and E proteins under the control of the Cytomegalovirus (CMV) promoter (184). This vaccine, VRC-WNVDNA017-00-VP, performed well in a phase I clinical trial, in which 97% of enrolled patients seroconverted (185). Another candidate, ChimeriVax-WN02, is a live, attenuated chimera vaccine in which WNV prM/E has been inserted onto a YFV backbone (186). This vaccine, developed by Acambis and bought by Sanofi Pasteur, progressed through both a phase I and phase II trial, although the program has been suspended (184). Another live, attenuated chimera vaccine also showed promise in a phase I trial, with a seroconversion rate of 89% (184). This vaccine (WN/DEN4Δ) expresses WNV prM/E on an attenuate DENV4 backbone (187). Finally, Hawaii Biotech developed a recombinant subunit vaccine of the WNV E protein that is produced in Drosophila S2 cells. A phase I clinical trial indicated excellent immunogenicity and safety (184).
Chapter 3

Dissertation scope
Background and Research Questions

Viruses in the genus flavivirus such as WNV and Dengue (DENV) represent an expanding threat to global health and economics (93, 105, 188, 189). Although great strides in understanding viral-host interactions contributing to disease pathogenesis have been made in the last decade, there is still no specific antiviral therapy or human vaccine approved for WNV and DENV (190). Studies of dengue pathogenesis have been hampered because of the lack of an appropriate animal model; however the significant advances made in the WNV field has been possible because of an excellent in vivo mouse model. Studies from our lab as well as others have shown that effective and balanced early innate immunity is essential for strong anti-viral responses that block dissemination of WNV into the brain and prevent neuroinvasive disease (60, 105). Detection of WNV by multiple pathogen recognition receptors (PRRs), notably TLR-3, RIG-I, MDA5 and NLRP3 is the first step in the induction of innate immunity, which culminates in the production of antiviral cytokines and type I interferon. To combat anti-viral immunity, both WNV and dengue have evolved strategies of immune evasion by blocking multiple steps of the PRR signaling, mechanisms of which are not well characterized. Several immune molecules including NLRC5, NLRX1, and TRIM21 have been recently identified that can positively or negatively regulate innate immune signaling and facilitate crosstalk between different PRR pathways. Understanding the pivotal role of novel innate immune regulatory molecules in governing early protective responses, programming of the innate-adaptive interface and immune evasion is important.
and may provide new insights into strategies for the control of virus infection as well as vaccine development. One such newly characterized modifier of PRR signaling is the triggering receptor expressed on myeloid cells family proteins (TREM).

TREM-1 is an immune receptor expressed on myeloid, epithelial and endothelial cells and some lymphocytes (4, 5, 8, 14, 86) and is shown to augment pro-inflammatory responses in the context of microbial infections. Signaling through the TREM-1 receptor, triggered by a yet unknown ligand, activates its adaptor protein DAP12 and downstream kinase cascades resulting in the transcription of inflammatory cytokines, chemokines and TREM-1. A soluble form of TREM-1, thought to be the result of matrix metalloproteinase (MMP) cleavage of the ectodomain (21), has been identified in a variety of inflammatory disorders including sepsis and inflammatory bowel disease and proposed as a marker of disease severity (2). Both in vitro and in vivo studies using infection models and microbial PAMPs provide evidence for the participation of TREM-1 signaling in regulating inflammation by synergizing with specific TLRs and NLRs (36, 49–51, 74, 75). More recently, TREM-1 has also been implicated in a broad range of functions including the activation of antigen presenting cells (APCs) and subsequent priming of CD4+ T cells by up-regulating co-stimulatory molecules on the APCs (47, 53, 77, 78).
The role of TREMs, in particular TREM-1, has not been well studied in the context of viral infections. Indirect evidence of involvement of TREM-1 in regulating viral immunity comes from studies using viral PAMPs (poly I:C) or viral proteins (HIV gp40) (47, 70, 75). The only study of virus-TREM interaction is by Weber et al., who developed a TREM-1 knock-out mouse model and showed that a deficiency of TREM-1 resulted in decreased disease severity while not affecting the clearance of influenza virus (45). The dearth of studies relating to TREM immunobiology in virus infections has left a large gap in our continuing understanding of both, complex immunomodulatory signaling pathways associated with pathogenesis of globally important human viruses and the larger contribution of TREMs in the field of host immune responses. On one hand, TREM-1 induced responses may facilitate efficient clearance of the infection, however on the other hand, there is a possibility that TREM-1 signaling may cause substantial tissue damage and contribute to disease pathogenesis. Hence, TREM-1 appears a possible therapeutic target for tempering deleterious host-pathogen interactions. Therefore, the objective of this study is to characterize the activation of TREM-1 by multiple globally significant human RNA viruses and then to utilize a TREM-1 knockout mouse model of WNV infection to investigate the mechanism by which TREM-1 governs the innate immunity and disease outcome in the mammalian host during virus infection.

Hypothesis: We hypothesize that infection with important human RNA viruses will induce TREM-1 expression, which will correlate with peak virus titers. We
further hypothesize that TREM-1 plays a critical regulatory role in the production of inflammatory cytokines during WNV infection and that a deficiency of TREM-1 will result in impaired anti-viral responses leading to increased WNV replication and disease severity in mice.

**Specific Aim 1: To characterize the expression kinetics of multiple TREMs following infection with RNA viruses *in vitro* and *in vivo.*

**Hypothesis:** We hypothesize that infection with important (+)- and (-)-sense RNA viruses will induce TREM-1 and activate TREM signaling.

**Rationale:** Although activation of TREM-1 has been demonstrated by a wide range of microbial PAMPs and bacterial pathogens, it is not known to date whether virus infections can induce the transcription of TREMs. However, based on *in vitro* studies demonstrating increases in TREM-1 expression following stimulation with viral PAMPs, it is likely that active virus infection may induce TREM-1 transcription in immune cells. TREM-1 has been described in a variety of myeloid cell types as well as epithelial and endothelial cells, so we will examine the induction of TREM-1 by various RNA viruses in relevant human myeloid or epithelial cells.

**Justification for using multiple RNA viruses:** In order to determine the broader response of virus infections at the level of TREM-1 induction, we have chosen to
study important RNA viruses of two different families with distinct replication cycles in immune cells. Flaviviruses, (+)-sense RNA viruses that replicate in the cytoplasm; and orthomyxoviruses, (-)-sense RNA viruses that replicate in the nucleus. Finally, since the in vivo studies will use the well-established mouse model of WNV neuroinvasive disease, we also propose to characterize changes in the TREM family members in the immune cells as well as brain of WNV-infected mice.

Objective:

• Determine the induction of multiple TREMs mRNA following infection with (+)-sense viruses (the flaviviruses WNV, DENV and JEV) in human monocytic cells in vitro.

• Determine the induction of TREMs following (-)-sense virus (Influenza A) infection in human epithelial cells.

• Determine the expression profile of multiple TREMs following WNV infection in primary mouse immune cells in vitro and in WT C57BL/6 mice.
Approach:

**Figure 1:** Specific aim 1 approach

**Specific Aim 2:** To determine the role of TREM-1 signaling in the production of anti-viral and inflammatory molecules following West Nile virus infection **in vitro.**

**Hypothesis:** We hypothesize that TREM-1 signaling regulates the production of multiple pro-inflammatory and anti-viral cytokines during WNV infection.

**Rationale:** Previous studies have employed an agonist anti-TREM-1 antibody and inhibitory peptide to demonstrate that TREM-1 signaling amplifies inflammatory signals, including TNF-α and IL-1β (4) following LPS treatment and
bacterial infection. The role of TREM-1 in modulating anti-viral responses, specifically production of inflammatory cytokines and type I IFN has not been categorized so far in flaviviruses.

**Objective:** To analyze the affect of TREM-1 activation on the production of key innate immune markers by:

- Using an agonist antibody to over-activate TREM-1 signaling
- Blocking TREM-1 signaling by using an inhibitory peptide

**Approach:**

![Diagram](image)

**Figure 2:** Specific aim 2 approach
Specific Aim 3: Use a well-established mouse model to determine how TREM-1 deficiency affects anti-viral immunity and overall WNV disease outcome.

Hypothesis: We hypothesize that a deficiency of TREM-1 will decrease the production of IFNs and pro-inflammatory cytokines and impair the innate/adaptive interface thereby restrict virus clearance and enhance WNV-associated morbidity and mortality.

Rationale: In vitro mechanistic studies may provide insights into specific signaling events but are a poor predictor of complex immune interactions and overall disease pathogenesis. Therefore, in vivo studies are critical to ultimately define the role of TREM-1 in flavivirus pathogenesis. TREM-1 knockout models have only been developed recently and are just beginning to shed light on the contributions of TREM-1 in different disease models (15, 45, 191). We have decided to employ the TREM-1/3 double KO mouse model to avoid potential confounding data from TREM-3. In mice TREM-3 is functionally homologous to TREM-1 and is believed to have arisen from a duplication event, while TREM-3 exists in humans as a pseudo-gene.

Objective:

• Analyze the survival pattern, tissue tropism and clinical symptoms of WT and TREM-1/3 KO mice following WNV infection.
• Determine the role of TREM-1 in regulating the innate immune responses.

• Analyze the involvement of TREM-1 in WNV specific antibody production and T-cell responses.

Approach:

![Diagram](image)

**Figure 3:** Specific aim 3 approach

**Significance**

To date, there are no antiviral drugs or vaccine for WNV or Dengue approved for human use. Continuing flavivirus outbreaks, notably the 2012 WNV epidemic on the mainland U.S. (149) and the continued expansion of dengue worldwide (188),
highlight a critical need for expanding our understanding of immune regulatory pathways. The general role of TREM-1 in virus infections is not well characterized. Since a delicate balance exists between protective vs. pathogenic inflammation in WNV pathogenesis, it is possible that TREM-1 signaling may facilitate efficient clearance of the infection or may cause substantial tissue damage and contribute to disease pathogenesis. Therefore, this study is significant, as it will conclusively define the role of TREM-1 in regulating innate immunity to the WNV and its effect on the innate-adaptive interface and disease outcome. Our results will provide the first evidence of a novel role of TREM-1 as an amplifier of WNV-associated innate immunity that may synergize with other virus-detection pathways to elicit protective immunity to WNV. These studies will provide novel insights into the complex interactions that occur between inflammatory pathways responding to WNV and may allow for the characterization of similar network of innate immune regulation in other viruses of global importance. Further, these studies may lead to identification of viral and cellular targets for the development of urgently required immunotherapeutic strategies.
Chapter 4

Modulation of TREM-1 by viruses: Implication in regulating West Nile virus-associated inflammation and survival
Abstract

A rapid and sustained innate immune response is essential for controlling West Nile virus (WNV) infection and requires synergy between multiple virus-recognition pathways. Triggering receptor expressed on myeloid cells-1 (TREM-1) plays an immunoregulatory role in bacterial infections by amplifying Toll-like receptor (TLR)-initiated inflammatory responses. Here, we demonstrate that TREM-1 expression was markedly increased in immune cells following flaviviruses and influenza virus infection, and exposure to Ebola virus glycoprotein. *In vivo*, TREM-1 and TREM-3 transcripts were significantly upregulated in the peritoneal cells from WNV-infected mice, which correlated with high viremia, while anti-inflammatory TREM-2 and TREM-like transcript-1 (TLT-1) expression was reduced. We further document that infiltrating inflammatory monocytes, as well as resident glial cells, contributed to significantly increased TREM-1 in WNV-infected brain. The cellular consequence of TREM-1 activation using an agonist antibody was the amplification of WNV-associated tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) production, which was attenuated in the presence of TREM-1 peptide (LR-12). Importantly, significantly higher mortality and viremia in WNV-infected TREM-1/3−/− mice suggested antiviral function of this receptor. Collectively, our results not only document the induction of TREMs by WNV, but also indicate an important role of TREM-1 in modulating inflammation and WNV disease outcome.
Introduction

The induction of effective and balanced innate immune responses to invading viruses is a complex and challenging task for the mammalian immune system. Detection of virus-derived pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), activates the production of pro-inflammatory cytokines that are critical for early virus clearance and fine-tuning the innate-adaptive interface for long-lasting protection. Although classic virus-induced PRR pathways are well characterized, recent research has recognized the importance of the extensive interdependence and cross talk between different innate immune pathways (192). Several cell- and pathogen-specific positive or negative regulatory molecules control the downstream robustness of these PRR pathways, thus explaining why immune responses can be substantially different even though the inducing trigger may be the same.

The triggering receptor expressed on myeloid cells (TREM) family of proteins are members of the immunoglobulin variable (IgV) domain receptor superfamily (4) and have emerged as important regulators of innate immunity. TREM-1 (CD354) and TREM-2 are the most well characterized receptors of the TREM family, while the cellular localization and functions of other members, including TREM-3 and TREM-like transcripts (TLTs), is still being elucidated. Binding of a yet to be identified ligand to the TREM-1 receptor triggers the phosphorylation of the adaptor protein DNAX-activating protein of 12 kDa (DAP12) (4). The recruitment
of non-receptor tyrosine kinase Syk to the TREM-1/DAP12 complex activates downstream signaling molecules, including PI3K, ERK1/2 and MAP kinases ultimately modulating NF-κB activation and expression of inflammatory genes in a cell-specific manner (2, 4). Because of the ability to integrate with elements of PRR signaling, TREM-1 is considered to act mainly as an amplifier, rather than an initiator of inflammatory innate responses (2). Bouchon and colleagues first showed that artificial over-activation of TREM-1 on monocytes led to increased production of cytokines, such as tumour necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1) and interleukin-1β (IL-1β) following lipopolysaccharide (LPS) treatment (4). Subsequent in vitro studies demonstrated that TREM-1 can synergize with multiple PRR pathways, including TLR-2, TLR-4 and NLRs, to amplify inflammatory signals from monocytes and reactive oxygen species from neutrophils (34).

Limited in vivo studies indicate a pathogen-specific role of TREM-1 in microbial disease outcome. For example, while TREM-1 signaling is protective (191)(190)(189)in the animal model of Pseudomonas aeruginosa (191) and Streptococcus pneumonia infection (43), the receptor contributes to the pathogenesis of polymicrobial sepsis (35) and infection by Leishmania major (45). However, despite the progress in understanding the cellular expression and induction of TREM-1 by several non-viral pathogens, the function of TREM-1 signaling in virus immunity is not yet characterized. It is not known if RNA viruses
can induce the expression of TREM family members and whether this response is virus-specific.

Mosquito-borne flaviviruses, including dengue virus (DENV), Japanese encephalitis virus (JEV) and West Nile virus (WNV), are the causative agents of some of the most globally significant emerging and resurging infectious diseases in humans (188, 193). These viruses induce a complex network of host immune interactions that contribute to disease pathogenesis. WNV replication is detected, via replication byproducts, by intracellular PRRs, including TLR-3, TLR-7 and RIG-I, activating multiple transcription factors and triggering the expression of pro-inflammatory cytokines and type I interferon (IFN) (105). Although pro-inflammatory cytokines and chemokines, such as TNF-α, IL-1β and CCR5, have been implicated in directing WNV clearance, recruitment of immune cells to the CNS and protective T cells responses, the specific mechanisms responsible for fine-tuning inflammatory pathways are still under investigation (105). Given that TREM-1 can be induced by poly(I:C), a dsRNA analog, in vitro (47), we hypothesized that TREM-1 modulates the inflammatory response and disease outcome in WNV infection. Herein, we examined the induction of TREM-1 by different RNA viruses in vitro and then used the mouse model of WNV infection to determine the expression pattern of multiple TREMs and TLTs in the periphery and brain. We further investigated the role of TREM-1 in modulating the inflammatory response and disease outcome following WNV infection.
Results

Multiple human RNA viruses induce the transcription of TREM-1.

We first evaluated the induction of TREM-1 by three positive-sense RNA viruses (DENV, WNV and JEV) from two different serocomplexes of the *Flavivirus* genus. As seen in figure 1A, DENV2 infection of the human monocytic cell line THP-1 cells induced TREM-1 expression at 48 hours, which peaked to 8 fold by 96 hours after infection. Likewise, WNV infection of human PBMCs resulted in increased expression of TREM-1 by 5- to 8-fold at 24 and 48 hours after infection (Fig. 1A). Another neurotropic flavivirus, JEV, also followed the same pattern demonstrating a 9-fold increase in the expression of TREM-1 in mouse BMDCs at 24 hours after infection (Fig. 1A). Although the levels were not comparable to flaviviruses, TREM-1 expression was elevated by 4- to 5-fold over mock at 24 and 48 hours after infection of lung A549 cells with negative-sense influenza virus (Fig. 1B). It has been previously demonstrated activation of TREM-1 signaling in neutrophils following exposure with EBOV (31). However, since neutrophils are not the natural target of EBOV infection, which replicates well in monocytes (194), we measured the ability of the EBOV GP to induce TREM-1 transcripts in THP-1 cells. TREM-1 was significantly induced by 25-fold in just 2 hours following GP treatment and remained high until 12 hours after infection (Fig. 1C). Collectively, these results indicate that multiple RNA viruses and viral proteins can induce TREM-1 in human cells.
Figure 1. TREM-1 expression is induced by multiple RNA viruses. (A) Immune cells were infected with flaviviruses, WNV NY99 (human PBMCs, MOI 1), DENV2 (THP-1 cells, MOI 1 in the presence of the enhancing 4G2 antibody) and JEV (BMDMs, MOI 5) (B) Lung A549 cells were infected with Influenza PR-8 (A/Puerto Rico/8/1934 H1N1, MOI 3) and (C) THP-1 cells were treated with 1µg/mL purified EBOV glycoprotein. Cells were harvested for RNA extraction at different time points after infection and mRNA expression of TREM-1 was determined using qRT-PCR. Data is expressed as relative fold-increase as compared to uninfected controls (M) after normalizing to GAPDH levels. Error bars indicate SEM. *, p<0.05.

TREM-1 expression is induced in multiple mouse cell types.

Since the mouse model is extensively studied to delineate WNV antiviral responses, we next investigated if WNV could induce TREM-1 in multiple primary mouse immune cells. As seen in figure 2A, TREM-1 was rapidly induced by WNV in both MEFs and BMDMs. TREM-1 mRNA levels increased 7- and 13-fold as compared to mock in BMDM and MEFs at 48 and 72 hours after infection respectively. In contrast, there was no significant change in the TREM-1 expression in BMDCs (Fig. 2A). Although TREM-1 expression has been reported
on human DCs (5), WNV infection increased TREM-1 transcripts only by 1.5- to 2-fold by 72 hours after infection, suggesting a cell-type specific induction of TREM-1 by WNV. To further validate the induction of TREM-1 protein by WNV, the cell surface expression of TREM-1 was analyzed by flow cytometry. As seen in figure 2B, the cell surface expression of TREM-1 increased over time and was more than 150% in WNV-infected MEFs as compared to mock–infected cells. WNV-induced TREM-1 transcription may be a direct result of virus replication or caused by soluble viral or host factors secreted by infected cells. To address these different possible mechanisms, MEFs were either infected with UV-inactivated WNV or exposed to UV-inactivated conditioned media from WNV infected cells and analyzed for TREM-1 expression. As expected, UV-inactivated WNV was unable to induce TREM-1 at 48 hours (Fig. 2C). Further, UV-inactivated conditioned media containing infected cell-derived factors also failed to induce TREM-1. These results suggest that active virus replication is required for the induction of TREM-1 by WNV.
Figure 2. WNV replication in primary mouse immune cells induces the expression of TREM-1. (A) Mouse MEFs, BMDCs and BMDMs cells were infected with WNV NY99 and TREM-1 expression was quantified using qRT-PCR. (B) TREM-1 surface expression on MEFs was determined at 48 and 72 hours after WNV infection using flow cytometry. Mean fluorescence of intensity (MFI) showing increase in the TREM-1 surface expression following WNV infection. (C) TREM-1 induction in MEFs was examined 48 hours after infection with UV-inactivated WNV or treatment with UV-inactivated conditioned media from WNV-infected MEFs. n.d. not determined. **, p<0.01; ****, p<0.0001. Graph shows mean +/- SEM from at least two independent experiments.

In vivo WNV infection induces multiple members of the TREM family

To further explore the response of TREM-1 and other TREM family members to WNV infection in vivo, we measured the mRNA levels of TREM-1, -2 and -3 in peritoneal cavity cells from WNV- and mock-infected mice at days 3 and 6 after infection. WNV titers in the periphery peak at day 3 in the serum of mice (107) and as seen in figure 4A, TREM-1 mRNA increased 5- to 7-fold in peritoneal cells from infected mice as compared to mock at day 3 and remained high at day 6.
after infection (p<0.05). Similarly, while TREM-3 was found to be elevated 3- to 5-fold in WNV infected mice, TREM-2, which is shown to exhibit anti-inflammatory properties (2), was down regulated in the peritoneal cells at both days 3 and 6 after infection (p<0.01) (Fig. 3A). In addition to TREM-1 and TREM-2, the TLTs are also involved in cellular activation and inflammation (2). While TLT-1 was significantly down regulated at day 3 and 6 after infection (p<0.01), there was no significant difference in the expression of TLT-2 and TLT-4 as compared to mock-infected cells at these time points (Fig. 3B).

**Figure 3.** Multiple TREMs are induced during WNV infection in vivo. Peritoneal cavity cells were obtained from wild type C57BL/6 mice inoculated with 100 PFU WNV in the footpad at days 3 and 6 after infection and mRNA transcriptional levels of TREM-1 to -3 and (B) TREM-like transcripts (TLT)-1, -2 and -4 were analyzed using qRT-PCR (n=8, from two independent experiments). *, p<0.05; **, p<0.01. Error bars indicate SEM.
Expression of TREMs are induced in the brains of WNV-infected mice

TREM family members are mostly expressed on innate immune cells including macrophages and neutrophils, however, more recent studies have reported the expression of TREM receptors in other cell types such as fibroblasts, Kupffer cells and epithelial cells (195). Since balanced inflammatory responses are critical for virus clearance from the brain, we hypothesized that WNV may induce TREM expression in the brain as well. As depicted in figure 4, the kinetics of TREM-1 induction followed the pattern of virus replication in the brain. TREM-1 was induced at low levels in the brain at day 6 but increased dramatically at day 8 after infection, reaching ~40 fold (p<0.01) (Fig. 4B) and correlated with peak WNV titers (Fig. 4A) and infiltration of immune cells (60). Conversely, WNV did not alter the levels of TREM-2 mRNA. Interestingly, TREM-3 was also induced significantly (p<0.001) at day 6 and 8 after infection (Fig. 4B). Since TREM-1 induction has not been reported in the CNS so far, we first validated the increase of TREM-1 at the protein level and then investigated the cells types contributing to TREM-1 expression in the brain. TREM-1 protein levels, measured in whole brain homogenates using ELISA, increased significantly at day 8 after infection (Fig. 4C). We hypothesized that infiltrating leukocytes, one of the major sources of inflammatory cytokine production in the brain during WNV encephalitis (60, 130), could be contributing to this increased TREM-1 expression in the brain. As shown in figure 4D analysis of TREM-1 expression on the infiltrating leukocytes in the brain using flow cytometry identified TREM-1 expression only on the Ly6c⁺/CD11b⁺ and Ly6c⁺/CD11b⁻ cells in the infected brain. TREM-1 was not
found on CD11c+ or CD3+ cells (data not shown). The influx of the TREM-1+ leukocytes into the brain correlated with the significant induction of TREM-1 transcripts at day 8 after infection (Fig. 4B), however the possibility of TREM-1 induction in resident brain cells, such as microglia and astrocytes could not be ruled out. Interestingly, we found that WNV induced the expression of TREM-1 in mixed glial cultures but not in astrocytes at 72 hours after infection (Fig. 4E), which correlated with the peak of WNV replication in these cells (data not shown). To confirm these results we performed immunostaining on sections of WNV-infected mouse brains 8 days after infection. As seen in figure 4F, TREM-1 was visible only after WNV infection and was found in microglia, as evidenced by the co-localization of TREM-1 and IBA-1, a microglial marker. These results collectively demonstrate that WNV infection in the brain differentially regulates the expression of multiple TREMs and that both infiltrating leukocytes and resident glial cells contribute to TREM-1 expression in the infected brain.
Figure 4. Increased expression of TREMs correlates with high WNV titers in the CNS. Brains harvested from wild type C57BL/6 mice inoculated with 100 PFU WNV in the footpad were used for measuring (A) WNV titers and B) TREM-1 to -3 using qRT-PCR (n=4). (C) TREM-1 protein expression was quantitated in the brain homogenates from mock and WNV-infected mice (day 8) using ELISA. (D) Infiltrating leukocytes were recovered from the brain at day 8 after WNV infection and stained for surface expression of TREM-1. Representative flow cytometry profile of the TREM-1+ population in CD45+ leukocytes. (E) Primary mouse astrocytes and mixed glial cultures were infected with WNV and TREM-1 expression and virus titers were analyzed using qRT-PCR. Data is expressed as relative fold-increase as compared to uninfected controls (M) after normalizing to GAPDH levels. (F) Brain sections from mock- and WNV-infected mice (day 8) were co-stained for TREM-1, a microglia marker (Iba-1) and nuclei using DAPI, and merged to depict co-localization (white arrows). **, p<0.01; ***, p<0.001.

**TREM-1 amplifies the production of WNV-associated pro-inflammatory cytokines**

TREM-1 is the only TREM implicated in regulating pro-inflammatory responses in humans. Therefore, we decided to focus on elucidating the role of TREM-1 in
influencing WNV-associated cytokine production in MEFs. As shown in figure 5A, activation of TREM-1 signaling using a cross-linking agonist anti-TREM-1 antibody (αTREM) (4) resulted in increased phosphorylation of ERK1/2 (pERK), a key kinase in the TREM-1 signaling cascade. While pERK was slightly elevated in MEFs 4 hours after WNV infection, TREM-1 activation via the agonist antibody greatly increased the phosphorylation of ERK1/2, without increasing the overall levels of the ERK1/2 protein (Fig. 5A). Although, the activation of TREM-1 had no effect on WNV replication in MEFs (Fig. 5B), we observed an almost 100% increase in the TNF-α mRNA in TREM-1-activated cells at 48 hours after WNV infection as compared to WNV alone or treatment using a control IgG antibody (Fig. 5C, p<0.001). Similarly, we observed an increase in the transcription of IL-6 in TREM-1 activated cells (Fig. 5C, p<0.05). We next evaluated the protein expression of IL-6 and another key WNV-associated chemokine MIP-1α in the cell supernatant. As seen in figure 5D, WNV-induced IL-6 secretion increased from 2000 pg/mL to 2700 pg/mL in TREM-1 activated MEFs at 72 hours after infection (p=0.0589). Likewise, MIP-1α secretion was amplified significantly in TREM-1-activated MEFs, increasing from 45 pg/mL to 70 pg/mL in the presence of the agonist antibody (p<0.05).
It has been previously demonstrated that a synthetic peptide, LR-12, derived from TLT-1, inhibits TREM-1 in vivo by binding to the TREM-1 ligand (42). When TREM-1 signaling was inhibited in MEFs using LR-12 peptide, we observed a trend of decreased mRNA levels of TNF-α and IL-6, although the reduction was not significant (Fig. 6). Additionally, inhibition of TREM-1 also reduced the secretion of IL-6 and MIP-1α at 72 hours after WNV infection. IL-6 secretion reduced from 1900 pg/mL to 1300 pg/mL (Fig. 6C). Collectively, these
experiments imply that TREM-1 has an important function in regulating key pro-inflammatory cytokines during WNV infection.

Figure 6. Blocking of TREM-1 attenuates inflammatory cytokines produced during WNV infection. MEFs were treated with 50µg/mL of the inhibitory peptide LR-12 for 30 minutes before infection to inhibit TREM-1 signaling and then replaced every 24 hours. A scrambled peptide was used as a control. RNA extracted from WNV-infected MEFs was used to determine (A) WNV copy number and (B) mRNA levels of TNF-α and IL-6, using qRT-PCR. (C) Protein levels of IL-6 and MIP-1α secreted into the supernatant were determined by Luminex assay and expressed as mean concentrations (pg/mL) +/-SEM and are representative of two independent experiments in duplicate. *, p<0.05; **, p<0.01.
TREM-1 and -3 are essential for protection against WNV infection

Finally, to determine whether the immune functions under the control of TREM-1 could affect WNV disease progression and outcome, we examined the survival of C57BL/6 WT and TREM-1/3 deficient mice following sub-lethal challenge with WNV (100 PFU). Since TREM-3 is a pseudogene in humans and functionality similar to TREM-1 in mice, TREM-1/3 double knock out mice have been recently used for delineating TREM-1 immune functions (43, 191). These mice have no observable abnormalities in breeding or development. TREM-1/3−/− mice were highly susceptible to WNV infection and exhibited 85% mortality as compared to WT mice (65% mortality, Fig. 7A, p<0.05). In addition, TREM-1/3−/− mice also demonstrated increased viremia as compared to WT mice. WNV titers in the serum of TREM-1/3−/− mice were significantly elevated, by an average of half a log, over their WT counterparts (Fig. 7B, p<0.05). Collectively, these results indicate that TREM-1/3-dependent signaling is essential for a protective host response against WNV infection.

Figure 7. TREM-1/3 deficient mice exhibit increased morbidity and mortality with increased viremia following WNV infection. Eight-ten week old male and female C57BL/6 WT and TREM-1/3−/− mice were inoculated with 100 PFU WNV in the footpad (n=21/group) and (A) mortality and morbidity was monitored twice daily for 17 days. (B) WNV burden in the WT and TREM-1/3−/− mice sera was analyzed using plaque assay at days 2, 4 and 6 after infection. Data were derived from 5 to 6 mice per time point and horizontal dotted lines denote the detection limit of the plaque assay. *, p<0.05.
Discussion

Induction of effective yet balanced inflammation is a very tightly regulated process mediated by cross talk between multiple inflammatory pathways and can influence virus clearance and pathogenesis. Based on the previously described induction of TREM-1 by viral PAMP poly (I:C), we wanted to more clearly define the induction of TREM-1 and its role in inflammation and host defense during virus infection. Herein, we demonstrate (i) that TREM-1 is induced by multiple RNA viruses in human and mouse immune cells, (ii) WNV infection in mice alters the expression of TREM-1 and other TREM family members in a cell-specific manner and plays a role in amplifying inflammation in vitro and (iii) TREM-1 is protective in the mouse model of WNV encephalitis.

TREM-1 is a highly conserved receptor predominantly expressed on myeloid cells. The promoter region for TREM-1 is under the control of the transcription factors NF-κB and AP-1 (49). Although bacterial pathogens, such as Pseudomonas aeruginosa, and microbial PAMPs including LPS, can induce TREM-1, it is not known if RNA viruses can induce TREM-1 expression and subsequently activate downstream signaling, and whether this response is virus-specific. Thus far, increased TREM-1 expression and activation is only shown in human neutrophils treated with inactivated filoviruses in vitro (31). Our observed substantial increase in the TREM-1 mRNA levels by several RNA viruses (Fig. 1) suggests that common mechanism may be responsible for the induction. As dsRNA is produced during the replication of WNV, DENV and influenza virus
(196, 197), it is likely that this replication by-product contributes to TREM-1 induction via the TLR-3 pathway. This notion agrees with our finding that infection with non-replicating UV-inactivated WNV failed to induce TREM-1 (Fig. 2C). However, the role of other virus-induced mediators, such as TNF-α or viral proteins secreted during infection, cannot be ruled out in inducing TREM-1 and is supported by our results demonstrating TREM-1 induction by purified EBOV GP protein. In line with this, Mohamadzadeh and colleagues previously demonstrated the activation of TREM-1 signaling by live and inactivated EBOV in human neutrophils and that Marburg virus GP directly binds to the TREM-1 receptor (31).

The cell-specific expression of TREM-1 has been well documented (195). Therefore, despite the fact that WNV induces a robust inflammatory response in a wide range of cells, including monocytes, DCs and MEFs (105), it was interesting to note that WNV-induced TREM-1 mRNA was observed in mouse peritoneal cells, BMDMs and MEFs but not in BMDCs (Fig. 2). This WNV-associated cell-specific expression of TREM-1 was further confirmed in the infiltrating leukocyte population in the CNS. Cell-surface TREM-1 was specifically detected on monocytic populations (Ly6c\(^{+}\), CD11b\(^{+/−}\)) in the brain, but not on the dendritic cells (Fig. 4 and data not shown). The Ly6c\(^{hi}\) CD11b\(^{+}\) populations expressing TREM-1 in the brain are inflammatory monocytes, which are recruited in the brains of WNV-infected mice (130). This observation agrees with a recent study by Ruiz-Pacheco and co-workers demonstrating increased TREM-1
expression only on CD14+ human monocytes (corresponding to mouse Ly6c+ monocytes) in PBMCs from DENV-infected individuals (73). These results indicate that TREM-1 expression on “inflammatory” monocyte subsets in both human and mice may be an important phenotype during flavivirus infections.

Inflammation plays a paradoxical role in CNS infections by WNV and other neurotropic viruses. While cytokines and chemokines, such as IL-1β and MCP-1, are important for clearing virus from the periphery and recruiting CD8+ T cells in the brain, they also contribute to bystander neuronal death (117). The cell types involved and the signaling pathways associated with the production of inflammatory mediators in the CNS have been the subjects of recent investigations, but remain incompletely defined. Although the expression of TREM-2 and its role in CNS inflammation has been described in Alzheimer’s disease and experimental autoimmune encephalitis (EAE), so far there are no reports of TREM-1 induction and its role in potentiating inflammation in the brain. Therefore, one of the most intriguing aspects of this study was the observation of high TREM-1 induction in the mouse brain as compared to peripheral cells (Fig. 4 and 5), suggesting the possibility of resident CNS cells promoting TREM-1 signaling in addition to infiltrating monocytes. Our finding of TREM-1 induction only in WNV-infected mixed glial cultures and not in astrocytes cultures ex vivo is significant as it identifies the specific resident CNS cell type contributing to TREM-1 increase in the brain (Fig. 4). Similarly, studies by Sessa and colleagues showed strong expression of TREM-2 in microglia, but not in astrocytes (198).
Since microglia, but not astrocytes, derive from a hematopoietic stem cell lineage (199, 200), this further supports the concept that TREMs are predominantly expressed on myeloid cells of hematopoietic origin. Along this line, another recent study described TREM-1 expression on Kupffer cells, the hematopoietic-derived macrophages of the liver (15).

This study for the first time characterizes the complete profile of TREM family genes in response to virus infection. The cellular expression profile and functions of TREM-3 in the mouse has not been entirely elucidated and is predicted to be functionally homologous to TREM-1 (14). On the other hand, TREM-2 and TLT-1 have both been shown to have anti-inflammatory properties and TREM-2 is neuroprotective in chronic neurodegenerative disease models (2, 201). For example, the upregulation of TREM-2 has been shown in the inflammatory macrophages and microglia in the brain and spinal cord of mice with EAE and is proposed to participate in attenuating neuroinflammation (82). Based on our results demonstrating a downregulation of TREM-2 and TLT-1 expression in WNV-infected peritoneal cells and brain, it appears that WNV infection promotes TREM-1-associated pro-inflammatory signaling while blocking TREM-2 dependent anti-inflammatory responses. However, the specific anti-inflammatory function of these receptors in immunity to WNV remains to be explored.

Since TREM-1 ligands are not well characterized, the agonist antibody and inhibitory peptide (LR-12) serve as ideal tools to understand the function of this
receptor during inflammatory processes. The substantial amplification of TNF-α and IL-6 in the presence of the TREM-1 agonist antibody, as seen in figure 6, provides *in vitro* evidence of the role of TREM-1 in amplifying WNV-associated cytokines. This observation was further supported by our studies using the TREM-1 inhibitory peptide LR-12, which showed a trend of reduced cytokines following WNV infection in MEFs. The data on the immunomodulatory role of TREM-1 is limited in virus infections. Mohamadzadeh and colleagues documented a similar reduction in the production of pro-inflammatory cytokines such as TNF-α and IL-1β by filoviruses in the presence of TREM-1 inhibitory peptide LP-17 *in vitro* (31). Similarly, a deficiency of TREM-1 lead to decreased IL-6 levels in the bronchoalveolar lavage fluid in influenza-infected mice (45). Several other studies provide evidence of amplification of inflammation by TREM-1 in other infection models polymicrobial sepsis and *Streptococcus pneumoniae* infection (43, 46). The inhibition of TREM-1 using synthetic inhibitors (including LR-12) in models of septic shock, limited disease severity and prevented death (42), providing clinical relevance of modulating TREM-1 signaling in infectious diseases.

Both TREM-1 and TREM-1/3 deficient mice have been recently utilized to understand impact of TREM-1 on disease outcome (45, 191). Since TREM-3 is a pseudo-gene in humans and is believed to be functionally homologous to TREM-1 in mice (191), we used TREM-1/3−/− mice for our survival studies rather than mice deficient with TREM-1 alone to avoid potential confounding data from
TREM-3. Our observation of a substantial increase in the WNV-associated mortality in TREM-1/3−/− mice supports our hypothesis that TREM-1 dependent responses can influence WNV disease outcome. This data is significant as it opens new line of investigation to delineate TREM-1 specific responses and underlying mechanisms responsible for protective immunity to WNV and other viruses. Our data is in contrast to a recent study by Weber and colleagues, which demonstrated attenuation of illness without affecting virus clearance in influenza virus-infected TREM-1−/− mice (45). We speculate that while TREM-1-dependent amplification of inflammation in the periphery might mediate lung injury during influenza infection, in WNV infection, fine tuning of innate immune responses in the periphery by TREM-1-signaling most likely facilitates timely virus clearance and reduces neuroinvasion. Accordingly, we observed higher WNV titers in the serum of TREM-1/3−/− mice. Similarly, in non-viral pathogens, the role of TREM-1 in disease outcome is also seen to be highly pathogen-specific. On one hand, while TREM-1 signaling is protective in the mouse model of Pseudomonas aeruginosa (191) and Streptococcus pneumoniae infection (43), this receptor contributes to the pathogenesis in Leishmania major infection (45).

In summary, this study provides direct evidence of the modulation of TREM-1 by WNV and other RNA viruses and the regulatory role this receptor plays in regulating the production of WNV-associated inflammatory cytokines. Our data also highlight a prominent role of TREM-1 in protection against WNV disease and warrants in-depth functional in vivo studies, aimed at uncovering the different
immune responses governed by TREM-1. Our findings collectively suggest that TREM-1 might be one of the key immunoregulatory molecules responsible for amplifying and sustaining inflammatory response induced by classic PRRs such as TLRs during viral infections. Further investigations of TREM-1-dependent immune functions in infection with WNV other viruses will lead to a better understanding of the complicated network of antiviral immune pathways and identify potential immunomodulatory targets for future therapies or vaccine adjuvants.

Materials and Methods

Cell culture. Human THP-1 cells were maintained at 1x10^6 cells/mL in RPMI media supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and β-mercaptoethanol. An Institutional Review Board-approved protocol was used for the isolation of peripheral blood mononuclear cells (PBMCs) from 60-80 mL of anti-coagulated blood from healthy donors, as described previously (111). Primary mouse bone-marrow derived macrophages (BMDM) and dendritic cells (BMDC) were prepared from bone marrow cells from 8- to 10-week-old C57BL/6 mice, as described previously (60). Primary mouse embryonic fibroblasts (MEF) were generated from 13-to 14-day C57BL/6 embryos and maintained in DMEM media supplemented with 10% FBS, 1% L-glutamate, 1% sodium pyruvate, 1% non-essential amino acids, 2.5% HEPES, and 1% penicillin/streptomycin. Brains from 1- to 2-day-old C57BL/6 pups were used to isolate primary mouse astrocytes and mixed glia, as described previously.
Single cell suspensions were generated from the brain using a neural dissociation kit in GentleMACS dissociater (Miltenyi Biotec) and were seeded onto plates coated with poly-D-lysine (Sigma) and Laminin (Sigma). After overnight shaking at 37°C, non-adherent mixed glial cells were re-seeded onto poly-D-lysine/laminin-coated plates and adherent astrocytes were cultured in DMEM F/12 media (Invitrogen) supplemented with 10% FBS, 1% HEPES and 1% penicillin/streptomycin.

**Virus infection.** DENV 2 (New Guinea C strain) was used to infect the human THP-1 cells using the antibody-dependent enhancement model, as described previously (203). DENV2 was incubated with the enhancing monoclonal antibody 4G2 (Millipore) diluted to 1:10,000 for 30 minutes prior to infection of low passage THP-1 cells at MOI 0.5. Cells were infected for 2 hours, washed 3 times, cultured in RPMI media and harvested at different time points after infection. WNV lineage I strain NY99, originally isolated from a crow brain and propagated once in Vero cells to generate a stock virus, was used for infection (110) and UV-inactivated WNV (UV-WNV) was generated using a UVStratalinker 2400 device for 12 minutes. Influenza A/PR/8/34 was propagated in 7-day old chicken eggs and used to infect human airway epithelial cells A549 in the lab of Dr. Adolfo Garcia-Sastre at the Mount Sinai School of Medicine, as previously described (204). THP-1 cells were exposed to Ebola virus (EBOV) glycoprotein (GP) protein, purified from drosophila S2 cells, at the concentration of 1µg/mL.

**Mouse experiments.** Wild-type C57BL/6 mice and TREM-1/3−/− mice on C57BL/6 background were bred in the John A. Burns School of Medicine animal facility
and experiments were performed according to the guidelines and approval of the University of Hawai‘i Institutional Animal Care and Use Committee. Eight to ten week old mice were inoculated with 100 PFU WNV in the left footpad, and serum and brain were harvested, as described previously (107). To collect peritoneal cells, 5 mL PBS was injected into the peritoneal cavity of mice. The resulting extract was centrifuged at 4,000 rpm and cells were frozen at -80°C for future use. To compare the survival, age- and gender-matched WT and TREM-1/3-/- mice were infected with 100 PFU WNV and were observed for mortality for 17 days.

**Quantitation of TREMs.** Total RNA extracted from cells or mouse brains was used to determine mRNA levels of multiple TREM genes using qRT-PCR analysis using specific primers (Table 1). The fold change in virus-infected samples as compared to relevant mock-infected controls was calculated after normalizing to the GAPDH gene, as described previously (60). The levels of TREM-1 protein in mouse brain homogenates was quantitated using the mouse Quantikine TREM-1 ELISA (R&D), according to the manufacturer’s instructions. To quantitate the cell surface expression of TREM-1, MEFs were lifted from plates using 5mM EDTA in PBS, washed, blocked with an anti-CD16/32 antibody and stained for TREM-1-eFluor 660 (eBioscience). Samples were analyzed by flow cytometry using the FACSCalibur. For the analysis of TREM-1 expression in brain, flow cytometry was performed on the infiltrated leukocytes isolated from mouse brain as described previously (60). After isolating the leukocytes over a percoll gradient, the cells were blocked with the anti-CD16/32 antibody and
stained for CD45, CD11b, CD11c, CD3, Ly6c and TREM-1 using directly conjugated antibodies for 30 minutes at 4°C and then fixed with 4% paraformaldehyde (PFA) at 4°C for 15 minutes as described previously (60). Samples were analyzed by multi-color flow cytometry on the FACSARia and data were analyzed with FlowJo software.

**In vitro stimulation and inhibition of TREM-1 signaling.** To activate TREM-1 signaling, an agonist TREM-1 IgG2A antibody (clone 174031, R&D) and the control IgG2A (eBioscience) were prepared to 10µg/mL in 0.5% bovine serum albumin (BSA) in PBS and 24-well polystyrene plates were coated with a total of 1.5µg of antibody/well overnight at 4°C. Wells were washed twice with PBS before plating MEF cells and after 24 hours, infected with WNV (MOI 0.5). Activation of TREM-1 signaling was confirmed by the phosphorylation status of ERK by western blot. Protein extracts (40 µg) were separated by SDS-PAGE, transferred onto a nitrocellulose membrane, blocked and incubated overnight with antibodies against p-ERK, ERK (Cell Signaling) and β-actin (Sigma). Following incubation with secondary antibodies conjugated with IRDye 800 and IRDye 680, the membranes were scanned using the Odyssey infrared imager (Li-Cor Biosciences). Densitometric analysis was determined using Image Studio Lite. Inhibition of TREM-1 was achieved through the small synthetic peptide LR-12 (42), which was designed from the conserved ligand-binding domain of TLT-1 and a scrambled version of the sequence was used as a control. MEFs were treated with LR-12 or the scrambled control (50 µg/mL in PBS) immediately after WNV infection (MOI 0.5) and replenished every 24 hours.
Quantification of virus load. WNV titers from cell culture supernatants, mouse serum or brain homogenates were analyzed using either plaque assay in Vero cells or qRT-PCR, as described previously (107). The data are expressed as WNV PFU per mL supernatant or PFU equivalents per µg RNA.

Quantification of inflammatory cytokines. Total RNA extracted from MEFs 48 and 72 hours after WNV infection was used to quantitate the mRNA expression of TNF-α and IL-6 by qRT-PCR, using specific primers (Table 1). Cytokines and chemokines secreted in the cell culture supernatant were quantified by Luminex assay using a Milliplex Map Mouse Cytokine/Chemokine kit (Millipore) and read on a Luminex200 LiquiChip machine.

Statistical Analysis. All mRNA quantitation data are reported as mean ± SEM and representative of at least two independent experiments in duplicate. Comparison of means was carried out using unpaired Student t test. Survival curves were generated by the Kaplan-Meier method and analyzed using the Gehan-Breslow-Wilcoxon test. All statistics were performed using GraphPad Prism 5.0 and differences with p values of <0.05 were considered significant.

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providing the TREM-1 inhibitory peptide, LR-12 and to Dr. Marco Colonna from Washington University in St. Louis for providing the TREM-1/3 deficient mice.
### Table 1. Primer sequences

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Chapter 5

TREM-1 protects mice from West Nile virus disease by promoting virus clearance and peripheral immunity
Abstract

The triggering receptor expressed on myeloid cells 1 (TREM-1) has emerged as an important and multifaceted immune modulator, specifically as an amplifier of TLR-initiated pro-inflammatory responses. However, its specific functions in regulating immunity to viruses are unclear. West Nile virus (WNV), a neurotropic flavivirus, is a major cause of viral encephalitis in humans. Herein we investigated the role of TREM-1 in antiviral immunity and found that increased susceptibility of TREM-1/3<sup>-/-</sup> mice to WNV infection was marked by enhanced virus titers in the periphery and brain. TREM-1/3<sup>-/-</sup> mice failed to sustain the production of pro-inflammatory cytokines such as IL-6 and TNF-α in the serum. Although the levels of WNV-specific antibodies were comparable in both groups of mice, fewer splenic CD8+ T cells from TREM-1/3<sup>-/-</sup> mice responded to re-stimulation with WNV peptide by producing IFN-γ. Moreover, brains from TREM-1/3<sup>-/-</sup> mice displayed major hallmarks of neuroinflammation, including increased levels of inflammatory cytokines, activation of GFAP and infiltration of leukocytes. Intriguingly, survival of mice following intracranial inoculation with WNV was not significantly different between both groups suggesting that the contributions of TREM-1/3-dependent immune responses to the WNV disease outcome are primarily a result of peripheral immune activation. Collectively, our results provide new insights into the role of TREM-1 as an amplifier of WNV-associated innate immunity that may synergize with other virus-detection pathways to effectively and timely clear the virus from periphery and prevent neuroinvasion.
Introduction

West Nile virus (WNV) is a neurotropic mosquito-borne flavivirus responsible for the large outbreaks of meningoencephalitis in humans in the western hemisphere (147). The most recent outbreak of WNV in 2012 reported over 5,000 clinical cases with more than 2500 neuroinvasive cases and 250 deaths (205). WNV infection remains subclinical in most humans, however 20-30% patients develop symptoms of WNV diseases ranging from mild fever to severe meningitis. The overall case fatality rate in clinical cases is about 4% and many patients experience long-lasting neurological sequelae during recovery, placing a heavy economic burden on the public health system (189). At the present, there is no specific antiviral or therapy approved for use in humans, emphasizing the need for greater understanding of mechanisms of immune control of WNV.

Based on the studies using a well-established mouse model, the pathogenesis of WNV can be divided into three separate phases; the initial virus replication in skin dendritic cells and spread to the draining lymph nodes stage; dissemination to the peripheral organs phase, followed by CNS-replication phase (105). The innate antiviral responses are triggered through the recognition of WNV derived pathogen associated molecular patterns (PAMPs) by specific pattern recognition receptors (PRRs) including toll-like receptor 3 (TLR-3) and RIG-I-like receptors (RLRs) that activate downstream signaling pathways resulting in the production of antiviral type I IFNs and pro-inflammatory cytokines (59, 60, 119, 120). These inflammatory mediators provide a first line of defense against WNV infection and
are critical for direct virus clearance in the periphery, preventing dissemination into the CNS as well as the activation of downstream cellular and humoral responses (124). Once in the CNS, cellular immunity, particularly WNV-specific CD8+ T cells are responsible for virus clearance (105). While several studies highlight the protective role of cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α) in promoting virus clearance from the periphery, other inflammatory mediators such as macrophage inhibitory factor (MIF) and matrix metalloproteinase 9 (MMP-9) contribute to pathogenesis (60, 114, 115, 206). Thus, overall disease outcome depends on the intensity and extent of innate immune response induced by the virus promoting either timely virus-clearance or immune-mediated pathologies. It is now recognized that the regulation of innate immune pathways is through cross-talk between multiple PRR pathways. Recent studies have identified several positive and negative immune regulators that contribute to fine-tuning of innate and adaptive immune responses to virus infection. These regulatory mechanisms are beginning to explain how the same, or very similar PAMPs detected by different cell types can result in markedly different immunological outcomes (192).

The triggering receptor expressed on myeloid cells 1 (TREM-1) has been described as an important modulator of the intensity of inflammatory signals in multiple inflammatory disorders, both of infectious and non-infectious etiology. This receptor belongs to the immunoglobulin IgV superfamily, and is expressed primarily on cells of myeloid lineage (195). The natural ligand of TREM-1 is not
known, although some host derived factors, such as high-mobility group box protein 1 and heat-shock protein 70, have been shown to activate TREM-1 signaling. Activation of TREM-1 signaling is initiated upon binding of the ligand to the TREM receptor, which triggers the association and phosphorylation of immuno-tyrosine activating motif (ITAM) of the adaptor protein DAP12. DAP12 activation by TREM-1 results in the activation multiple kinases, including ERK1/2 and MAP kinases leading to Ca\textsuperscript{2+} mobilization and induction of pro-inflammatory cytokines and the release of reactive oxygen species (ROS) (2, 4, 33).

The most well characterized function of TREM-1 is the amplification of TLR-initiated inflammation. The synergy between TREM-1 and the TLR-2 and -4 pathway is well documented in monocytes and neutrophils, and is shown to be at the level of NF-\kappa B and PI3K activation (34). Exposure of immune cells with multiple PRR ligands, such as LPS (TLR-4) and MDP (NOD2) in concert with TREM-1 activation increases the production of key pro-inflammatory cytokines including TNF-\alpha and IL-1\beta (4, 75). This TREM-1-dependent enhancement of inflammation has been implicated in a poor outcome due to septic shock in rodents and pigs (41, 42). Recently, TREM-1 has been implicated in the response to inflammatory signals controlling cell activation, differentiation and migration. Studies by Pierobon and associates demonstrated that the activation of TREM-1 on immature dendritic cells (DCs) upregulated the surface expression of T cell co-stimulatory molecules including CD86 (77). Subsequent co-culture of these cells with T cells improved cell proliferation and increased the production of
IFN-γ. Further, role of TREM-1 was also shown in activation of Kupffer cells in liver (15) and migration of neutrophils into the lung during *Pseudomonas aeruginosa* infection (191). Thus the signaling events controlled by TREM-1 have implications beyond inducible innate responses and may have long-term effects in pathogenic inflammatory responses. However, the extent to which TREM-1-dependent functions contribute the pathogenesis in virus infection remains unclear.

Our previously studies demonstrated that TREM-1 and TREM-3 transcripts were significantly up regulated in the peritoneal cells from WNV-infected mice, which correlated with high viremia, while anti-inflammatory TREM-2 and TREM-like transcript-1 (TLT-1) expression was reduced. In the brain, infiltrating inflammatory monocytes, as well as resident glial cells, contributed to significantly increased TREM-1 expression following WNV infection. The activation of TREM-1 using an agonist antibody resulted in the amplification of WNV-associated TNF-α and IL-6 production, which was attenuated in the presence of TREM-1 peptide (LR-12). Further, we demonstrated that mice lacking TREM-1 and -3 were more susceptible to WNV infection, however the effects of TREM-1/3 deficiency on WNV-specific immune responses were not characterized. In the present study, we investigated the effect of TREM-1/3 deficiency on antiviral immune parameters in WNV disease.
Results

WNV infected TREM-1/3 deficient mice exhibit increased morbidity and mortality.

We previously demonstrated that WNV infection induces TREM-1 in the brain and peritoneal cavity cells, however the involvement of TREM-1 in regulating disease symptoms and outcome is not clear. To characterize the *in vivo* physiological relevance of increased TREM-1 in immune-mediated protection, we assessed the morbidity and mortality of WT and TREM-1/3 Δ/Δ mice after inoculation with two different infectious doses of WNV. TREM-3 is a pseudogene in humans and is believed to be functionally homologous to TREM-1 (81), therefore we employed the TREM-1/3 Δ/Δ mouse model to avoid potentially confounding results from TREM-3. TREM-1/3 Δ/Δ mice were highly susceptible to WNV infection following subcutaneous challenge with 10 and 100 PFU of virus (Fig. 1). While the infectious dose of 10 PFU resulted in 45% mortality in WT mice, mortality in TREM-1/3 Δ/Δ mice was 70% (*p*=0.0624, Fig. 1A). Similarly TREM-1/3 Δ/Δ mice inoculated with 100 PFU exhibited significantly higher mortality (85%) as compared to WT mice (67% mortality, *p*=0.0398, Fig. 1B). There was no significant difference between the median survival times of infected WT and TREM-1/3 Δ/Δ mice (11 days for 100 PFU dose). Additionally, the TREM-1/3 Δ/Δ mice that survived 100 PFU infection exhibited delayed recovery as evidenced by lingering signs of clinical disease (Fig. 1C, *p*=0.0023) and a failure to regain lost body weight (Fig. 1D, *p*=0.0040). Collectively, these results indicate that TREM-1/3 signaling is essential for recovery of mice from WNV disease.
Figure 1. TREM-1/3-deficient mice exhibit increased morbidity and mortality following WNV infection. Nine-week old male and female C57BL/6 WT and TREM-1/3−/− mice were inoculated with 10 or 100 PFU WNV in the left footpad (n=21 per group). (A) Survival analysis using Kaplan-Meier survival curve. (B) Clinical symptoms of the surviving mice from 100 PFU inoculation. Qualitative clinical symptoms were recorded once a day; 1: hunchback/ruffled fur; 2: paresis; 3: walking disability; 4: moribund; 5: dead. (C) Percent body weight for mice that survived 100 PFU inoculation was calculated as the percent of weight compared to initial weight at day 0. Statistical analysis was performed using 2-way ANOVA with Sidak’s multiple comparisons test. *, p<0.05.

TREM-1 attenuates WNV replication in the periphery and CNS

In order to better understand how TREM-1 provides protection, we first analyzed virus replication kinetics in the serum, peripheral organs and the brains from WT and TREM-1/3−/− mice infected with 100 PFU of WNV. Although WT and TREM-1/3−/− mice had similar WNV titers in the serum at day 2 after infection, the virus was significantly elevated in TREM-1/3−/− mice (4.2x10³ PFU/mL) at day 4 as compared to WT mice (1.1x10³ PFU/mL) (Fig. 2A, p=0.0455). Consistent with the serum, peak WNV titers in the spleens were observed at day 4 after infection,
however there was no difference between WNV titers in WT and TREM-1/3−/− mice at this time point (Fig. 2B). The kidney, on the other hand, is relatively resistant to WNV infection (105, 156). Accordingly, WNV was undetectable in WT mice at day 2 after infection and was detected only in 33% mice (2/6 mice) at day 4 after infection (3x10^2 PFU/g). Conversely, at the same time point, WNV titers were significantly higher and detected in 100% of TREM-1/3−/− mice (5/5 mice, p=0.0108, Fig. 2C). Similarly, a trend of higher number of TREM-1/3−/− mice with detectable WNV titers in the heart was observed as compared to WT mice, although there was no statistical difference in the titer at any time point (Fig. 2D).

To understand the consequence of increased viremia and loss of TREM-1/3 signaling at the level of CNS invasion, we next examined WNV titers in the brains of WT and TREM-1/3−/− mice. Although only 2 out of 5 TREM-1/3−/− mice exhibited WNV titers in the brain at day six after infection, those that did had titers at least one log higher that the WT mice (2.6x10^4 PFU/g vs. 1.1x10^3 PFU/g). By day 8 the WNV titers in the brains of TREM-1/3−/− mice were almost two logs higher than in WT (2.0x10^5 PFU/mL vs. 4.3x10^3 PFU/g). At day 10, WNV was starting to be cleared from TREM-1/3−/− mouse brains, matching WNV replication in WT (Fig. 2F). Collectively these data suggest that the dramatic increase in morbidity and mortality in TREM-1/3−/− mice observed in figure 1 may be due, in part, by increased WNV replication in the periphery and the CNS.
Figure 2. WNV replication is enhanced in TREM-1/3<sup>−/−</sup> mice. Nine-week old C57BL/6 WT and TREM-1/3<sup>−/−</sup> mice were inoculated with 100 PFU WNV in the left footpad (n=5-6 per group). (A-F) WNV titers from serum and homogenates of spleen, kidney, heart, lung and brain were evaluated by plaque assay in vero cells. Horizontal dotted line is the limit of detection. Statistical analysis was performed by Mann-Whitney test. *, p<0.05.

TREM-1 signaling sustains inflammatory cytokine production during WNV infection

TREM-1 has been implicated in the amplification of inflammatory responses in bacterial infections (4, 31). We have demonstrated previously that WNV infection in TREM-1 activated mouse embryonic fibroblasts amplified the production of TNF-α. We therefore examined the levels of circulating cytokines and chemokines in the serum of the WT and TREM-1/3<sup>−/−</sup> mice following WNV infection (Fig. 3). Consistent with previous studies, we observed a robust production of cytokines at day 4 and 6 after infection in WT mice (156). However, TREM-1/3<sup>−/−</sup> mice failed either to sustain early increases in cytokine production or induce a significant increase. We did not observe increases in the levels of
cytokines such as IL-6 and IL-15 in the TREM-1/3⁻/⁻ mice serum at days 4 and 6 after infection as compared to WT mice. However, IL-2 and TNF-α levels did increase in the TREM-1/3⁻/⁻ mice at day 4 after infection, but the increase did not sustain at day 6 after infection. Similarly, levels of the chemokine MIP-1β, which were comparable between WT and TREM-1/3⁻/⁻ at day 4 after the infection, increased further only in WT mice at day 6 after infection. Moreover, there was no significant difference between the levels of other chemokines and cytokines such as IL-10 (Fig.3) and IL-1β (data not shown) in WT and TREM-1/3⁻/⁻ mice serum. These results demonstrate that the absence of TREM-1/3 results in reduced systemic cytokine response to WNV infection in the periphery.

![Graphs showing cytokine levels](image)

**Figure 3. Pro-inflammatory response is impaired in TREM-1/3⁻/⁻ mice.** WT and TREM-1/3⁻/⁻ C57BL/6 were inoculated with 100 PFU WNV in the left footpad. Serum was collected and analyzed for IL-2, IL-6, IL-15, IL-10, MIP-1β and TNF-α by a multiplex luminex assay. Results are presented as mean ± SEM.
**WNV specific antibody responses are not altered in TREM-1/3<sup>−/−</sup> mice**

Virus specific IgM and IgG antibody responses are critical for the clearance of WNV, and recent studies have indicated a role of TREM-1/3<sup>−/−</sup> in adaptive immunity to *Pseudomonas aeruginosa* (53). Therefore, we measured the levels of WNV-specific antibodies in WT and TREM-1/3<sup>−/−</sup> mice to evaluate whether the enhanced virus burden in TREM-1/3<sup>−/−</sup> mice was associated with blunted WNV-specific antibody responses. Notably, serum IgM levels were high in the WT mice at days 6 and increased further at 8 after infection; this response was similar in TREM-1/3<sup>−/−</sup> mice (Fig. 4). Correspondingly, there was no significant difference in the serum IgG responses between WT and TREM-1/3<sup>−/−</sup> mice indicating that downstream signaling to TREM-1/3 does not influence the magnitude of antibody responses to WNV.

**Figure 4. The production of WNV-specific antibodies is independent of TREM-1/3.** Serum was collected at 6 and 8 days after WNV inoculation from WT and TREM-1/3<sup>−/−</sup> mice and evaluated for WNV-specific antibodies by MIA assay using WNV envelope protein. Data represent the mean fluorescent intensity (MFI) of infected mice minus the mean MFI+3 standard deviations of the respective mock group (n=5-7). Data is representative of two independent experiments.
WNV-specific CD8+ T cell responses require TREM-1/3

To characterize the immune parameters that may be associated with impaired virus clearance and inflammatory response in TREM-1/3−/− mice, we analyzed induction of cellular immunity. Several recent studies have implicated TREM-1 in promoting appropriate T cell responses. We therefore hypothesized that during WNV infection TREM-1 may be assisting in the activation of WNV-specific CD8+ T cells. To test this theory, we isolated splenocytes from WT and TREM-1/3−/− mice at 8 days after infection and analyzed CD8+ T cells for the production of IFN-γ and TNF-α following antigen-specific re-stimulation with an immunodominant Db-restricted WNV NS4B peptide (207). As seen in figure 5, we found that splenocytes from TREM-1/3−/− mice were less responsive to re-stimulation as compared to WT mice. Total IFN-γ+ cells in the CD3+/CD8+ population from WT spleens increased to approximately 5% when stimulated as compared to only 3% in the TREM-1/3−/− splenocytes (Fig. 5B). Additionally, TNF-α production was also attenuated in the TREM-1/3−/− CD8+ T cells in response to the WNV peptide. These results indicate that the deficiency of TREM-1/3 impairs the priming of WNV-specific CD8+ T cells in the spleen.
Figure 5. CD8+ T cell responses were attenuated in the absence of TREM-1/3. Splenocytes were isolated from WT and TREM-1/3−/− mice at 8 days after inoculation with 100 PFU WNV and stimulated with an immunodominant Dβ-restricted recombinant WNV NS4B peptide. (A) Representative dot plot of IFN-γ and TNF-α expression in CD45+, CD3+, CD8+ cells from WT and TREM-1/3−/− splenocytes either unstimulated (-NS4B) or stimulated (+NS4B) for 6 hours. Frequency of (B) IFN-γ, (C) TNF-α and (D) IFN-γ-, TNF-α-positive cells of the CD3+, CD8+ population.

TREM-1/3−/− mice exhibit increased neuroinflammation in the CNS

Enhanced WNV replication in the brain is directly linked to increased neuroinflammation, activation of glia and neuronal death. We speculated that increased virus replication in the brains from TREM-1/3−/− mice might lead to elevated inflammatory responses in the brain including enhanced infiltration of leukocytes and activation of brain astrocytes. Therefore, we first evaluated the levels of multiple inflammatory cytokines and chemokines in the brain homogenates from WT and TREM-1/3−/− mice following WNV inoculation via the footpad. We found elevated levels of key inflammatory cytokines in the absence of TREM-1 (Fig. 6A). Although both WT and TREM-1/3−/− mice had comparable levels of cytokine such as IFN-γ and TNF-α at day 6, they were markedly higher
in the brains from TREM-1/3\textsuperscript{+} mice by day 8 after infection. Similarly, IL-6 levels in TREM-1/3\textsuperscript{+} brains at day 8 were almost twice as compared to the WT mice brains (319 pg/mL vs. 137 pg/mL). The chemokine MCP-1 was also elevated in brains from TREM-1/3\textsuperscript{+} mice, at 1207 pg/mL compared to 799 pg/mL in WT mice. Conversely, the anti-inflammatory cytokine IL-10 was reduced in TREM-1/3\textsuperscript{−} mice. These results indicate a shift towards a pro-inflammatory environment in the absence of TREM-1 and explain the increased mortality displayed by these mice.

To assess the correlation of increased cytokines with leukocyte infiltration, brain sections from WT and TREM-1/3\textsuperscript{+} mice at day 10 after infection were evaluated for the presence of CD45 positive cells. WT and TREM-1/3\textsuperscript{+} mice showed infiltration of CD45+ leukocytes both near the blood capillaries as well as in the brain parenchyma. However, the numbers were significantly higher in the brains from TREM-1/3\textsuperscript{−} mice (Fig. 6C). Similarly, compared to the WT mice, increased immunoreactivity to GFAP, a marker of the astrocyte activation, was more pronounced in the brains from the TREM-1/3\textsuperscript{−} mice (Fig. 6B). These results collectively suggest that the absence of TREM-1/3 exacerbates leukocyte trafficking and accumulation in the brain, thereby contributing to increased neuroinflammation.
Figure 6. Inflammatory response and neuropathology markers in the brain of WT and TREM-1/3−/− mice. (A) WT and TREM-1/3−/− C57BL/6 were inoculated with 100 PFU WNV in the left footpad. Whole brains were homogenized in PBS and analyzed for IFN-γ, IL-2, IL-6, IL-10, MCP-1 and TNF-α were analyzed using a multiplex luminex assay. Results are presented as mean ± SEM. (B, C) Brains were harvested 10 days after footpad inoculation of 10 PFU WNV, perfused with paraformaldehyde, sectioned and immunostained for the leukocyte marker CD45 and GFAP, a marker of astrocyte activation. (D) Quantitative representation of total numbers of CD45+ cells from 5 different brain areas per section (total of 2 brain sections per mouse, n=3 mice per group).
Effect of TREM-1/3 on the survival after intracranial WNV inoculation

We next investigated whether the enhanced neuroinflammation observed in the TREM-1/3<sup>−/−</sup> mice was a result of increased neuroinvasion due to improper control of virus replication in the periphery or, alternatively, a direct result of the loss of TREM-1 signaling in resident brain cells. To answer this question, we performed intracranial (i.c.) inoculations of WT and TREM-1/3<sup>−/−</sup> mice to allow direct evaluation of the effect of TREM-1 without potentially confounding effects of the peripheral immune response, which takes 5 to 7 days to develop. I.c. inoculation with WNV causes mortality within 6 to 11 days and as seen in figure 7. 27% mortality was observed in both WT and TREM-1/3<sup>−/−</sup> mice (n=11), with no difference between average survival times (9 days). As an absence of TREM-1/3 failed to promote or attenuate WNV disease after direct inoculation in the brain, it appears that this receptor does not have a dominant direct antiviral or inflammatory effect in the CNS.

Figure 7. Survival analysis after WNV infection by an intracranial route. Nine-week old WT and TREM-1/3<sup>−/−</sup> mice were challenged with 10 PFU WNV via intracranial (i.c.) inoculation (n=11 per group). Mice were observed for 15 days for morbidity and mortality.
Discussion

As a first line of defense, innate immunity is triggered by the detection of a pathogen in phases by the collective action of multiple PRRs that ensures the mounting of an appropriate immune response to clear the pathogen. Based on the previously described relevance in inflammatory amplification *in vitro*, we wanted to more clearly define the *in vivo* role of TREM-1 in innate and acquired mechanisms of host defense to WNV. Herein, we investigated the function of TREM-1 in an experimental model of WNV encephalitis, using Trem-1/3−/− and WT mice. We demonstrate that in the absence of TREM-1/3, mice experienced significantly greater morbidity and mortality, most likely as a consequence of increased WNV replication in the periphery caused by an impaired innate immune response. Importantly, TREM-1/3 deficiency lead to increased WNV titers in the brain that correlated with increased markers of neuroinflammation, including astrocyte activation and leukocyte migration. Further, our results suggest that the beneficial role of TREM-1/3-dependent signaling includes optimal activation of virus-specific CD8+ T cells during WNV infection, the loss of which may contribute to increased virus replication in the CNS and associated inflammation.

In mice, TREM-3 is highly homologous to TREM-1 and reported to have similar cellular distribution. TREM-3 is an activating receptor on mouse macrophages and like TREM-1 signals through DAP12. By contrast, in humans TREM-3 is a pseudogene (191). Thus, to mimic the human situation, for this study, we used
mice deficient for both TREM-1 and TREM-3 to obtain insight into the role of TREM-1 related signaling in WNV disease. The same approach has been recently used to investigate the contribution of TREM-1 in *Pseudomonas aeruginosa* and *Streptococcus pneumoniae* infections (43, 191).

While our previous *in vitro* studies demonstrated that activation of TREM-1, via an agonist antibody, during West Nile infection increased the production of inflammatory mediators including TNF-α and IL-6 (Roe et al. manuscript communicated), how this receptor regulates antiviral immunity to WNV *in vivo* were not characterized. Here, we report the molecular and cellular immune responses in TREM-1/3−/− mice following WNV infection and demonstrate that the expression of TREM-1/3 is crucial for virus clearance and survival in the mouse model of WNV disease. The route of virus inoculation is known to influence the host immune response, thereby affecting WNV replication kinetics. Infection via the subcutaneous route stimulates early host responses in the periphery, resulting in an effective and balanced innate immune response that restricts WNV dissemination. On the other hand, intracranial inoculation induces rapid replication of virus in the CNS before innate and adaptive immune responses are established leading to early mortality between 6-10 days. The fact that there was no significant difference in the mortality in WT and TREM-1/3−/− mice after intracranial inoculation and that subcutaneous route of inoculation led to higher WNV titers in the brain and associated mortality suggest that TREM-1/3 mainly influences peripheral immunity. Our other
experimental observations further support this notion. First, increased virus burden in the peripheral tissues, such as the serum and kidney indicates impaired clearance of WNV from the periphery in TREM-1/3⁻/⁻ mice. Second, reduced cytokine levels (IL-2, -6, and TNF-α) in the serum from TREM-1/3⁻/⁻ mice suggest an impaired innate intracellular immune response in the periphery. Finally, decreased CD8+ T cell function further implicates TREM-1/3⁻ in modulating antiviral immunity during WNV infection. Collectively, our data suggest that TREM-1/3-dependent signaling functions primarily to establish a balanced and protective innate immune response during WNV infection.

Robust inflammation is essential to clear WNV and trigger an effective adaptive immune response. Cytokines such as TNF-α and IL-1β are shown to be critical players in initiating cascades of events, such as amplification of secondary cytokines, migration of protective immune cells into the brain (60, 206). TREM-1 has been implicated as an amplifier of the innate immune response by virtue of its capacity to enhance TLR and NLR signaling upon recognition of microbial components, specifically bacterial PAMPs. Bouchon and colleagues first showed that the key functional outcome of the artificial over-activation of TREM-1 receptor in monocytes was the increased production of cytokines, such as TNF-α, monocyte chemoattractant protein 1 (MCP-1) and IL-1β following LPS treatment (4). Following this observation, several in vitro and in vivo studies demonstrated the ability of TREM-1 to amplify inflammation during septic shock and other microbial infections. It appears that the function of TREM-1 in
influencing disease morbidity and mortality is pathogen specific. While decreased bacterial clearance and survival is observed in during *Pseudomonas aeruginosa* (191), *Klebsiella pneumonia* (44) and *Streptococcus pneumonia* (43) infections in mice, the opposite has been described in *Leishmania major* infection (45). Further, using siRNA silencing of TREM-1 in the mouse, Gibot and colleagues demonstrated the importance of the balanced activation of TREM-1 signaling during sepsis. This study showed that partial silencing of TREM-1 was protective during peritonitis, while complete silencing was lethal to septic mice (46). This effect of TREM-1-dependent enhancement of inflammatory response is also observed in non-infectious disease models including hemorrhagic shock, pancreatitis (acute inflammation), chronic inflammatory bowel diseases and inflammatory arthritis (24).

Most of the TREM research so far has focused on non-viral infections and autoimmune diseases. Although *in vitro* studies previously demonstrated the induction of TREM-1 transcripts and activation of signaling by filoviruses and viral PAMPs such as poly (I:C) and CpG DNA, the *in vivo* effect of this receptor in influencing virus disease outcome has only been shown in influenza virus. Studies by Weber and colleagues recently demonstrated that TREM-1 deficient mice were protected from severe influenza disease without affecting virus clearance, although this study did not look into the complete immune profile governed by TREM-1. These observations are in contrast to our survival data and further support the notion that TREM-1 signaling may have a pathogen
specific role in influencing virus disease outcome. We speculate that the reason for this discrepancy may be that TREM-1 signaling promotes inflammation promoting substantial tissue damage and thereby contributing to disease pathogenesis of acute influenza virus. In contrast, during WNV infection, TREM-1-dependent, protective responses enhance the robustness of innate immune responses in the periphery by facilitating efficient virus clearance and reduced neuroinvasion.

In support of this hypothesis, we observed enhanced virus replication in the periphery of TREM-1/3^−/− mice that correlated with attenuated inflammatory response (Fig. 2,3). Interestingly, serum virus titers and inflammatory cytokines, such as IL-6, were equivalent in WT and TREM-1/3^−/− mice at day two after infection. By day four, however, inflammatory mediators in WT mice continued to increase, correlating with a reduction of WNV titers in the serum. On the other hand, TREM-1/3^−/− mice, failed to amplify much needed inflammation affecting the kinetics of virus clearance in the periphery. These results further support that TREM-1 is not involved in the initial stimulation of inflammation, but rather in sustaining and amplifying inflammatory signals.

Virus-specific antibodies and CD8+ T cell responses are implicated in providing protection against WNV in mice. While we did not observe a noticeable impairment in the levels of WNV-specific IgG/IgM between the two groups, we report that TREM-1/3 plays an important role in initiating CD8+ T cell responses
(Fig. 4). These data are important, as the impact of TREM-1 signaling on T cell responses in microbial infections is not completely clear. Schiechl and associates demonstrated that inhibition of TREM-1 decreased the differentiation and proliferation of IFN-γ producing CD4+ T cells, thereby promoting cardiac allograft survival in mice (78). Indirect evidence of the ability of TREM-1 to control T cell functions comes from studies demonstrating enhanced activation of antigen presentation cells in TREM-1 activated immune cells. Bleharski and colleagues reported enhanced surface expression of CD86 and MHC class II by TREM-1 (47). Secretion of IFN-γ by CD8+ T cells is an important anti-WNV effector mechanism, and impairment in this response may eventually affect virus clearance from the brain. In accordance, our data for the first time substantiates the association of TREM-1 with attenuated CD8 T cell responses to microbial infection and may provide one explanation for increased mortality observed in TREM-1/3−/− mice.

Another intriguing finding of our data was the difference in the inflammatory responses observed in the serum and brain. As compared to the periphery, cytokines such as IL-6 and IFN-γ were increased in the brain, which correlated with increased activation of astrocytes and infiltration of leukocytes in the CNS. Activated astrocytes, along with microglia and infiltrating macrophages, have been associated with WNV neuropathology. The increased inflammation in the brain may be due a differential response to TREM-1/3 signaling in resident CNS cell as compared to peripheral immune cells, or due to enhanced virus replication
as a result of increased infiltration of leukocytes that may activate astrocytes and contribute to neuropathology. Further studies are warranted to clearly delineate the role of TREM-1 signaling in the periphery versus the CNS, however our data demonstrating no difference in the survival following intracranial inoculation strongly suggest that TREM-1/3 mainly regulates peripheral immunity to WNV.

In summary, the results from this as well as other studies suggest that the role of TREM-1 in immunity to microbial pathogens is complex and highly pathogen-specific. While TREM-1 signaling contributed to pathology in a mouse model of influenza virus infection, our data indicates a protective function of this receptor in WNV infection. We propose that TREM-1 coordinates a well-regulated innate immune response to WNV, ultimately restricting entry into the CNS and neuropathogenesis. Based on our data, it is likely that TREM-1 may play an equally profound role in immune protection against WNV in humans. Future studies to characterize TREM-1-dependent functions in other viruses and identification of ligands to artificially activate TREM-1 signaling may have important implications for the development of this receptor as an ideal immunomodulatory target.

**Materials and Methods**

**Animal experiments:** Wild-type C57BL/6 mice and TREM-1/3−/− mice on the C57BL/6 background (obtained from Dr. Marco Colonna) were housed and bred in the animal facility of the John A. Burns School of Medicine at the University of
Hawai‘i at Mānoa. The study was performed according to all guidelines and with the approval of the University of Hawai‘i Institutional Animal Care and Use Committee. All infections were conducted in 8 to 9 week old age- and gender-matched mice by inoculation of 10 or 100 plaque forming units (PFU) of WNV in the left footpad or cranium as described previously (107). The animals were observed twice daily for clinical symptoms (hunchback posture, ruffled fur, paresis, walking difficulty and ataxia), morbidity and mortality for 17 days as described previously (156). All mice were also weighed prior to survival studies and every two days post inoculation. Blood was collected from the tail vein and serum was separated and stored at -80°C. In separate experiments, peripheral organs (spleen, kidney, heart and lung) along with the brain were harvested following PBS perfusion at days 2, 4, 6 and 8 after infection and flash frozen in isopentane.

**Quantitation of viral load in serum and tissues:** WNV titers in the serum or tissue homogenates were determined by plaque assay using Vero cells as described previously (107, 156). The data are expressed as WNV PFU per mL serum or gram tissue.

**Quantification of cytokines and chemokines:** Cytokine and chemokine levels in the serum or brain homogenates were quantified by Luminex assay using a Milliplex MagPix Mouse Cytokine/Chemokine kit (Millipore) and read on a Luminex200 LiquiChip machine.
Measurement of WNV-specific antibodies: The levels of WNV-specific IgM antibodies were measured in the serum using microsphere immunoassay (MIA) for WNV envelope E protein (L2 Diagnostics) as described previously (208). Briefly, serum samples were incubated with the magnetic microspheres (MagPlexTM-C) coated with a recombinant WNV E antigen for 30 min followed by secondary goat anti-mouse IgG or IgM conjugated to red-phycoerythrin (Jackson Immunoresearch) for 45 min. The fluorescence intensity of the microspheres was analyzed with a Luminex 100 instrument (Millipore Corp.).

Immunohistochemistry: At day 8 after infection, mice were transcardially perfused with 10 mL of PBS followed by 10 mL of 4% paraformaldehyde,. Brains were harvested, cryoprotected in 30% sucrose (Sigma), embedded in OCT, frozen and 10µm sections were cut. Tissue preparation and staining with various primary antibodies was performed as previously described (107). Images were acquired using the fluorescent microscope (Zeiss Axiovert 200).

Splenocyte isolation and T cell stimulation: Spleens were harvested from WT and TREM-1/3−/− WNV infected mice at day eight after inoculation, gently homogenized using GentleMACS homogenizer (Miltenyi Biotec) and filtered over a 20µm filter to generate a single cell suspension. Following RBC lysis, splenocytes were plated in a 96-well plate and were either left untreated or were stimulated with the CD8+ T cell restricted WNV NS4B peptide (207, 209) for four
hours at 37°C in the presence of a golgi plug. Following stimulation, splecnocytes were stained for the surface markers CD3 and CD8 (eBioscience), fixed and permeabilized (BD Biosciences) and stained for intracellular IFN-γ and TNF-α. Samples were then analyzed using the FACSFortessa flow cytometer.

**Statistical analysis:** Comparison of means was carried out using the Mann-Whitney test of significance or two-way ANOVA with Sidak’s multiple comparison test. Survival curves were generated by the Kaplan-Meier method and analyzed with the Gehan-Breslow-Wilcoxon test. All statistics were performed using GraphPad Prism 5.0. Differences with p values of <0.05 were considered significant.

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Chapter 6

Summary and future directions
Neurotropic viruses belonging to the genus flavivirus, including WNV, JEV and TBEV, continue to expand and cause human disease in new regions of the world. The rapid spread of WNV across the North American continent following its introduction to New York in 1999 has resulted in a total of 39,400 clinical cases as of 2010 (189). A large multistate outbreak in 2012 resulting in 5,674 cases with 51% of cases representing neuroinvasive disease (132) highlights the continued threat of severe disease in the absence of effective anti-viral therapies or vaccines. Investigations in the last decade have identified the importance of early innate immune responses in determining the overall disease outcome to virus infections, including WNV. The initial detection of WNV by multiple PRR pathways results in the activation of anti-viral mediators such as IFN-α/β and pro-inflammatory cytokines including TNF-α and IL-1β. These mediators are critical for inhibiting virus replication directly as well as activating and fine-tuning the innate-adaptive interphase for long-lasting protection.

It is becoming increasingly evident that individual PRR pathways do not function in a linear manner, but rather interact with each other to create a network of innate signaling (192). Thus, it is through crosstalk between pathways and the actions of regulatory elements, both positive and negative that fine tune and individualize specific immune responses to different pathogens or in different cell types. It is imperative, therefore, to understand the specific host factors that contribute to regulation of the complex web of innate immune signaling during virus infections. The TREM-1 receptor is quickly emerging as a critical regulator
of innate immune signals. While multifaceted immune functions of TREM-1, including inflammation amplification and APC activation, have been well described in the context of model antigens and bacterial infections there remains our understanding of the virus-specific functions of the TREM-1 receptor in severely limited. The objectives of this study, therefore, was to characterize the kinetics of TREM activation during virus infection and begin to parse the specific immune-modulatory functions of TREM-1 in response to WNV in the mouse model. The mouse model of WNV infection is an ideal model to employ for these studies for several reasons. Firstly, WNV infection of mice mimics the development of WNV disease in humans. Further, the specific immune pathways activated by WNV, including TLRs, RLRs and cellular immune responses, are well characterized in the mouse model of WNV infection. Thus, this model is ideal to not only understand WNV-host interactions, but also to characterize the pivotal role of novel innate immune molecules in immune regulation and signaling crosstalk in antiviral immunity.

In the first specific aim, we sought to characterize the activation of TREM-1 after infection by multiple human viruses and to specifically define the kinetics of TREM induction in the mouse model of WNV disease. We found that viruses of distinct families were capable of inducing TREM-1 mRNA in relevant human cells. WNV, DENV and JEV are all positive-sense single-stranded RNA viruses of the genus Flavivirus, while Influenza A is a negative-sense RNA virus of the family Orthomyxovirus. Nonetheless, all four viruses were capable of inducing
TREM-1, suggesting that TREM-1 expression responds to multiple viruses. Further, while EBOV GP alone was capable of inducing TREM-1 mRNA, neither UV-inactivated WNV nor UV-inactivated supernatant from WNV-infected cells was capable of inducing TREM-1. Thus, the mechanism underlying induction of TREM-1 appears to be virus-specific. A particularly intriguing aspect of our data was the demonstration of TREM-1 expression in the brain. To our knowledge, this is the first report of TREM-1 induction in the brain. However, the specific immune-modulatory function of TREM-1 in the brain remains unclear at this point, and will be the subject of future studies.

The objective of specific aim 2 was to characterize the ability of TREM-1 to impact the production of inflammatory mediators during WNV infection. Our model of TREM-1 activation, via an agonist antibody, was very effective at enhancing TREM-1 signaling, as evidenced by enhanced ERK phosphorylation. As expected, key pro-inflammatory cytokines, including TNF-α and IL-6, were amplified by TREM-1 during WNV infection. One of the limitations of using the agonist antibody is that the extent and length of TREM-1 activation is unknown. It may or may not correlate with the actual activation of TREM-1 signaling during WNV infection. However, in the absence of a known ligand, agonist antibodies have been routinely used to define the role of TREM-1 signaling. Additionally, the inhibition of TREM-1, via the inhibitory peptide LR-12, resulted in a reduction of these same cytokines. However, the extent of reduction of these mediators was not comparable to the enhancement observed during TREM-1 activation. We
speculate that this was a result of incomplete inhibition of TREM-1 by the LR-12 peptide in our experimental model. Thus, future studies, utilizing TREM-1/3\(^{-/-}\) immune cells will confirm the trend of reduced inflammation in the absence of TREM-1 signaling.

The last aim of my dissertation was planned to determine the overall effects of TREM-1 on the disease outcome in an *in vivo* model of WNV encephalitis by comparing virus replication and immune activation between WT and TREM-1/3 deficient mice. The survival data supported our hypothesis and showed that the mice lacking TREM-1 signaling were more susceptible to WNV disease, displaying greater overall mortality and disease severity. The lack of sustained inflammation observed in the TREM-1/3\(^{-/-}\) mice correlated with enhanced WNV replication in the serum and peripheral organs and resulted in enhanced neuroinvasion. Although we observed attenuated T cell responses in the TREM-1/3\(^{-/-}\) brain, many questions are yet to be answered regarding T cell functions. In addition, the contribution of TREM-3 towards immune activation during WNV infection cannot be ruled out.

**Where do we go from here?**

Data obtained in this study will open new line of investigation of TREM related functions in WNV and other viruses. Three leading research questions for future studies originate from this study-
i. Mechanism of TREM-1 induction and activation of signaling: It is important to delineate the specific mechanism(s) by which WNV induces TREM-1 signaling. We hypothesize, given that poly (I:C) is capable of inducing TREM-1, that the induction of TREM-1 mRNA and cell surface expression is downstream of TLR-3 signaling, induced upon the binding of WNV dsRNA during viral replication.

ii. Identification of TREM-1 ligands: Viral ligands for TREM-1 are not yet characterized. It will be important to identify the specific viral or host factor that bind directly to TREM-1 and induce TREM-1 signaling. WNV-derived NS1 can be one possible ligand that may activate TREM-1 signaling.

iii. Considering the limitation of knock out mouse and the possible contribution of TREM-3 in our data, additional studies, such as inhibition of TREM-1 alone or use of LR-12 peptide to block TREM-1 will be necessary to confirm the immune modulatory functions of TREM-1.

Collectively, our data provides the first evidence that TREM-1 is an important immune mediator during the host response to virus infections. Given the two-pronged modulatory role of TREM-1, that of enhancing inflammation and cellular immune responses, this novel receptor makes an attractive target for future adjuvant design for the formulation of effective viral vaccines. Tools such as inhibitory peptide LR-12 are available to modulate TREM-1 signaling and are therapeutic candidates for non-viral inflammatory diseases. Since there is a close homology between mouse and human TREM-1, this study will lead to future in-
depth studies to identify its function in other virus diseases and discover viral ligands and agonist small molecules, which could impact the development of this receptor as a ‘novel immunomodulatory target’ to modulate immunity to WNV and other related viruses.
BIBLIOGRAPHY


