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*J Immunol* 2012; 189:3150-3158; Prepublished online 15 August 2012; doi: 10.4049/jimmunol.1201140

http://www.jimmunol.org/content/189/6/3150

Supplementary Material  http://www.jimmunol.org/content/suppl/2012/08/08/jimmunol.1201140.DC1.html

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Semaphorin 7A Contributes to West Nile Virus Pathogenesis through TGF-β1/Smad6 Signaling

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Semaphorin 7A (Sema7A) is a membrane-associated/secreted protein that plays an essential role in connecting the vertebrate neuronal and immune systems. However, the role of Sema7A has not been elucidated in viral pathogenesis. In this study, we show that abrogation of Sema7A protects mice from lethal West Nile virus (WNV) infection. Mice lacking Sema7A showed increased survival, reduced viral burden, and less blood–brain barrier permeability upon WNV infection. Increased Sema7A levels were evident in murine tissues, as well as in murine cortical neurons and primary human macrophages upon WNV infection. Treatment with Sema7A Ab blocked WNV infection in both of these cell types. Furthermore, Sema7A positively regulates the production of TGF-β1 and Smad6 to facilitate WNV pathogenesis in mice. Collectively, these data elucidate the role of Sema7A in shared signaling pathways used by the immune and nervous systems during viral pathogenesis that may lead to the development of Sema7A-blocking therapies for WNV and possibly other flaviviral infections. The Journal of Immunology, 2012, 189: 3150–3158.

West Nile virus (WNV) is a mosquito-borne flavivirus that can infect humans and cause fever, encephalitis, and death (1, 2). Treatment of WNV is supportive, because vaccines or therapeutics have not been approved for use in humans. A murine model characterized the kinetics of WNV, a neurotropic virus that replicates in peripheral organs, crosses the blood–brain barrier (BBB), and infects the CNS, leading to death (3–6). Studies using genetically modified mice that lack specific immune molecules provided insight into the immunopathogenesis of WNV (7–13). However, studies that characterize molecular interactions in various tissues is poorly understood. Sema7A can induce monocyte chemotaxis and cytokine production (25) and is a negative regulator of T cell responses (23). The effects of Sema7A in both the nervous and immune system are believed to be mediated via at least two receptors: plexin C1 and β1 integrin (17, 20, 24). However, the significance of these interactions in various tissues is poorly understood.

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Received for publication April 20, 2012. Accepted for publication July 19, 2012.

This work was funded by National Institutes of Health Grants AI 50031 and AI 070343.

H.S., G.N., and E.F conceived and designed the experiments; H.S., G.N., and H.G.F. performed the experiments; H.S., G.N., R.A.K., R.M.M., and E.F. analyzed the data; H.S., G.N., R.M.M., J.F.A., and R.R.M. contributed reagents/materials/analysis tools; and H.S. and E.F. wrote the manuscript.

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The online version of this article contains supplemental material.

Abbreviations used in this article: AHV, Alcelaphine herpes virus; BBB, blood–brain barrier; MOI, multiplicity of infection; p.i., postinfection; Q-PCR, quantitative RT-PCR; Sema7A, semaphorin 7A; siRNA, small interfering RNA; WNV, West Nile virus.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1201140
DNA viruses that infect humans, such as vaccinia and alcelaphine herpesvirus, encode secreted semenin A39R and Alcelaphine herpes virus (AHV)-sema. These viral semenophins show high similarity to vertebrate Sema7A (16, 26, 27). Similarly to Sema7A, these semenophins bind plexin-like receptors (32). Upon vaccinia and AHV infection, A39R and AHV-sema are believed to play immunomodulatory roles (5) by mimicking Sema7A (16, 26, 27). Indeed, A39R induces CD54 expression and stimulates production of IL-6 and IL-8 by cultured monocytes (16, 33). No study has reported the presence of Sema7A homologs in RNA viral genomes. Therefore, we postulated that RNA viruses may use vertebrate Sema7A during infection and assessed the role of vertebrate Sema7A using the murine model of WNV infection.

Materials and Methods

Murine infection and survival studies

All WNV-challenge experiments were performed with 6–8-wk-old female mice in a Biosafety level 3 animal facility, according to the regulations of Yale University. C57BL/6 wild-type mice were purchased from Charles River Laboratories, and Sema7A knockout mice (34) were a kind gift from the Dr. Jack Elias laboratory (Yale University) and were bred at the Yale Animal Resources Center, under pathogen-free conditions, to the C57BL/6 background by backcrossing for 10 successive generations. For each infection study, mice groups were rigorously matched for age and sex. Groups of 10–20 mice were inoculated i.p. with a lethal dose of 10^5 PFU WNV strain CT 2741 (provided by J.F.A.) in PBS with 1% gelatin. Upon WNV infection, mice typically died at 6–14 d postinfection (p.i.) as a result of CNS invasion by the virus. Mice were monitored daily for WNV-associated clinical signs (including lethargy, anorexia, and difficulty ambulating), as well as for survival until days 21–25 p.i., as described (35). Mice survival data summarize the results of two independent experiments. Surviving mice were either euthanized to end the experiment or, in selected experiments, mice were euthanized for harvesting of tissues (brain, blood, and spleen). For Ab-protection studies, mice were inoculated i.p. with 100 μg Sema7A mAb or the isotype-matched negative control rat IgG Ab (obtained from R&D Systems) 1 d prior to WNV challenge; a second dose of either of these Abs was given at 2 d p.i. All mice experiments were approved and performed in accordance with regulations of the Institutional Animal Care and Use Committee at Yale University.

Infection of in vitro cell cultures

Human HEK 293 and mouse cerebrospinal microvascular endothelial cell lines were purchased from the American Type Culture Collection and propagated according to their protocols. Primary cultures of cortical neurons were isolated and established from the brains of embryonic day-16 C57BL/6 or Sema7A-deficient mouse embryos (obtained from pregnant female mice). Primary cultures of neurons and other cell types were seeded on 6- or 12-well plates at cell densities of 2 × 10^5 or 10^6 cells/ml, respectively; incubated for 24 h at 37°C; and infected with WNV at multiplicity of infection (MOI) of 2. Cell lysates were collected for analysis of cytokine mRNA levels. The SMAD genes (7, 34, 35). The mRNA levels of these genes were normalized to β-actin mRNA levels. The ratio of WNV E gene copy/β-actin gene copy was used as an index to determine the infection rate of each sample.

Western blotting

Total brain lysates or HEK 293 cells were resuspended in SDS sample buffer containing 2×ME, boiled for 5 min, and resolved on a 4–15% gradient SDS-PAGE gel. Blots were probed with specific Abs (Sema7A Ab used in Fig. 1D was obtained from Abcam [Cambridge, MA] and Abs against SMAD 2 or 3 used in Fig. 6G were obtained from Cell Signaling Technologies), followed by the respective HRP-conjugated secondary Abs (obtained from Sigma, St. Louis, MO) and detected with an ECL system from GE Healthcare. Quantification of Western blots was performed as described (37).

Confocal microscopy

Cortical neurons were isolated from the brains of embryonic day-16 C57BL/6 mice embryos and infected with WNV (MOI 2). Twenty-four hours p.i., neurons were fixed with 4% paraformaldehyde and processed for immunofluorescence using Sema7A Ab (Abcam), followed by treatment with tetramethyl rhodamine isothiocyanate-conjugated secondary Ab (Sigma). Confocal microscopy was performed, as described (35).

ELISA

ELISA was performed to detect Sema7A protein levels in the total lysates prepared from uninfected or WNV-infected wild-type mice brain tissue. Ten micrograms (100 μg) of total lysates from uninfected or WNV-infected wild-type mice brain tissue was coated onto ELISA plates (Nunc, Rochester, NY) and incubated at 4°C overnight. Following incubation, wells were blocked and incubated with Sema7A Ab diluted in PBS-Tween 20 (0.05%) buffer. Wells were then incubated with secondary Ab conjugated to HRP, followed by treatment with TMB Microwell Peroxidase (KPL) for color development. The reactions were stopped after 15 min using TMB Stop Solution (KPL), and OD was read at 450 nm.

Statistical analysis

Error bars represent mean (± SD) values. We used a log-rank test (Prism 4; GraphPad Software) to assess the statistical differences between the survival rates. For calculating statistical significance between two means, nonpaired two-tailed Student t test was used. A p value < 0.05 was considered statistically significant in our analyses.

Results

Sema7A is upregulated upon WNV infection

Sema7A functions in the nervous and immune systems (14, 16–18). Therefore, we analyzed its role during neurotropic WNV infection. We first determined whether WNV influences sema7A expression in mice. RNA samples were extracted from different tissues of mice infected with WNV on days 2, 4, and 7 p.i. Q-PCR showed that sema7A mRNA levels were significantly upregulated at days 6 and 7 p.i. in blood (p < 0.05) and brain (p < 0.05) compared with the uninfected controls (Fig. 1A–C, Supplemental Fig. 1A). Immunoblot further showed that Sema7A protein (∼75 kDa) levels in the brain were induced 2–3-fold at day 7 p.i. (Fig. 1D), the time point at which WNV invades the CNS. Higher levels
of the glycosylated form of Sema7A protein (~100 kDa) were also evident in WNV-infected brain samples in comparison with controls (Fig. 1D). ELISA confirmed a significant increase (p < 0.05, 2–3-fold) in Sema7A protein expression in the brain at day 7 p.i. (Fig. 1E). Together, these data show that Sema7A mRNA and protein levels are induced upon WNV infection.

Sema7A deficiency decreases murine lethality from WNV infection

Because Sema7A expression was dramatically upregulated upon WNV infection, we characterized its role in vivo in the absence of Sema7A by infecting either Sema7A-deficient (sema7A−/−) or Sema7A Ab-treated mice. When animals were infected with 1000 PFU of WNV, survival rates of sema7A−/− mice were considerably higher compared with the wild-type controls (Fig. 2A). sema7A−/− mice were significantly more resistant to death caused by WNV than were wild-type animals (56 versus 20% survival rate, p < 0.05, log-rank test) (Fig. 2A). Similarly, Sema7A Ab-treated mice showed substantial resistance to the lethality caused by WNV compared with IgG isotype-matched negative control Ab-treated mice (60 versus 20% survival rate, p < 0.05) (Fig. 2B). Collectively, these results show that Sema7A is an important factor in murine resistance to WNV infection.

Sema7A influences WNV neuroinvasion

During the initial stage of murine infection (days 2–4 p.i.), WNV replicates in peripheral organs (3–6). By day 5 p.i., WNV crosses the BBB and invades the CNS, leading to death of the animals starting at day 7 p.i. (3–6, 38). Because Sema7A deficiency resulted in enhanced survival after WNV infection in mice, we analyzed the kinetics of WNV infection to determine whether this was due to reduced viral invasion into the brain. Indeed, when we quantified WNV E-gene transcripts normalized to murine β-actin by Q-PCR, the sema7A−/− mice had a significantly reduced viral burden in the blood at day 2 p.i. compared with the control mice (~5-fold, p < 0.05; Fig. 2C). Decreased viral loads were also evident in both spleen (2–3-fold, p < 0.05) and brains (2–3-fold, p < 0.05) of sema7A−/− mice at day 4 p.i. compared with the controls (Fig. 2D). At days 6 and 7 p.i., sema7A−/− mice had a significantly reduced (2–3-fold, p < 0.05) viral burden compared with control mice in all three tissues (Fig. 2E, Supplemental Fig. 1B). These results suggest that the increased survival of sema7A−/− mice is due to both diminished viremia during early infection and decreased viral neuroinvasion.

Sema7A-deficient mice infected with WNV have less inflammation

To identify a mechanism for reduced neuroinvasion of WNV in sema7A−/− mice, we assessed BBB permeability by quantifying IgG levels (indicative of BBB permeability) in the brain tissue. Immunoblots showed that sema7A−/− mice had less IgG in the brain compared with the control mice, indicating less BBB permeability (Fig. 3A, Supplemental Table I). Increased production of inflammatory and antiviral cytokines, such as TNF-α, IL-6, IL-12, IFN-α, and IFN-β, facilitate WNV neuroinvasion into the CNS by altering the BBB permeability (3, 7). Therefore, we measured mRNA levels of these cytokines in sema7A−/− mice compared with the controls. Consistent with the measurement of viral loads (Fig. 2E) and BBB permeability (Fig. 3A), mRNA levels of TNF-α, IL-6, IL-12, IFN-α, and IFN-β were significantly decreased in brain tissue of sema7A−/− mice at day 7 p.i. compared with the control mice (3–8-fold, p < 0.05, Fig. 3B–F). In addition, blood from sema7A−/− mice showed reduced mRNA levels of IFN-α (9-fold, p < 0.05) and IFN-β (3-fold, p < 0.05) compared with controls at day 7 p.i. (Fig. 3E, 3F), suggesting an important role for Sema7A in facilitating the WNV infection from blood to brain. Furthermore, at day 7 p.i., TNF-α and IL-6 levels were significantly diminished (4- and 2-fold, respectively, p < 0.05) in spleens of sema7A−/− mice (Fig. 3B, 3C). Overall, these results show that Sema7A contributes to cytokine...
responses that are associated with inflammation during WNV infection.

**Abrogation of Sema7A blocks WNV infection of cortical neurons in vitro**

To assess the role of Sema7A in viral infection of neural tissue, we assessed viral replication in cortical neurons, which is a pathogenic feature of WNV during CNS invasion (3–6, 39). We analyzed whether abrogation of Sema7A leads to altered viral replication by determining the *sema7A* mRNA levels in cortical neurons infected with WNV (35). Q-PCR results showed that *sema7A* mRNA levels were significantly elevated in WNV-infected cortical neurons compared with uninfected controls at all time points (24, 48, and 72 h) p.i. (2–5-fold, *p* < 0.05, Fig. 4A). Coassociation of Sema7A with WNV was also evident in cultured cortical neurons (Supplemental Fig. 1C). We then determined whether the absence of Sema7A has any impact on viral infection in cortical neurons. Cortical neurons from wild-type and *sema7A*+/− mice were isolated, and WNV infection kinetics were analyzed at different time points (24, 48, and 72 h) p.i. (Fig. 4B). The results showed a significantly reduced viral burden in cortical neurons isolated from *sema7A*+/− mice compared with cortical neurons isolated from wild-type mice at all time points (*p* < 0.05, Fig. 4B). We next determined whether treatment of cortical neurons with Sema7A Ab leads to reduced viral replication. Uninfected cortical neurons isolated from wild-type mice were treated with either Sema7A or isotype-matched negative control Abs for 2 h, followed by infection with WNV. Viral replication was analyzed 48 h p.i. Q-PCR showed that neurons treated with 5–20 μg of Sema7A mAb showed significantly (*p* < 0.05) reduced WNV E-gene transcripts compared with the control (Fig. 4C). Similar results were obtained when we performed experiments with a mouse cerebral microvascular endothelial in vitro cell line (Supplemental Fig. 1D). These results support a role for Sema7A in neuroinvasion and replication of WNV in the CNS.

**Abrogation of Sema7A blocks WNV infection of human macrophages in vitro**

In addition to the role for Sema7A in enhancing WNV replication in neurons, we analyzed whether it is involved in immune-mediated pathways. Therefore, we investigated whether abrogation of Sema7A in human macrophages also influences WNV replication. PBMCs were isolated from healthy human volunteers and allowed to differentiate into macrophages (36). We first analyzed *sema7A* mRNA levels in macrophages infected with WNV and compared the expression levels with uninfected controls. We found that *sema7A* mRNA levels were significantly elevated in WNV-infected human macrophages compared with WNV and compared the expression levels with uninfected controls. We found that *sema7A* mRNA levels were significantly elevated in WNV-infected human macrophages isolated from all four volunteers compared with uninfected controls at all time points (24, 48, and 72 h) p.i. (3–8-fold, *p* < 0.05, Fig. 5A, Supplemental Fig. 2A–C).
We determined whether treatment of human macrophages with Sema7A Ab has any influence on viral replication. Uninfected human macrophages were treated either with 10–20 μg of Sema7A Ab or an isotype-matched negative control Ab for 2 h, followed by infection with WNV. Q-PCR results showed that macrophages from all four volunteers treated with 10–20 μg of Sema7A Ab showed significantly reduced WNV E-gene transcripts compared with controls at 48 h p.i. (p < 0.05). We next determined whether overexpression of Sema7A influences WNV replication in an in vitro human cell line. We used HEK 293 cells because of their extreme transfection efficiency. Constructs carrying Sema7A-Fc and Fc alone, which was used previously, were first transfected into HEK 293 cells and infected with WNV. At 24 h post-WNV infection, HEK 293 cells transfected with Sema7A-Fc showed significantly increased WNV E-gene transcripts compared with cells transfected with Fc control (2–3-fold, p < 0.05). Taken together, these results further validate the role of Sema7A as a host factor that enhances viral replication and supports the development of Sema7A as a therapeutic target for WNV infection.

Sema7A facilitates WNV pathogenesis through TGF-β1/Smad6 signaling

To dissect the mechanisms underlying Sema7A’s enhancement of viral replication, we assessed signaling pathways involved in Sema7A activity. Because Sema7A signaling in both the nervous and immune systems is believed to be mediated via plexin C1 (32), we first analyzed whether infection of WNV induces plexin C1 mRNA expression in wild-type mice. We found no significant increase in plexin C1 mRNA expression upon WNV infection (Supplemental Fig. 3A) and found no differences in plexin C1 levels from sema7A2/2 mice at day 7 p.i. Both groups of mice were infected with 10^3 PFU of WNV. Results are from five mice/group and two independent experiments, performed in triplicate. Statistical significance (p < 0.05) was calculated using the Student t test. Error bars represent + SD from the mean value.
TGF-β1 signals through Smad transcription factors that mediate various cellular events (40, 41). Therefore, we determined mRNA levels of several smads upon WNV infection in cortical neurons. Only smad6 was significantly elevated upon WNV infection in cortical neurons isolated from wild-type mice. Uninfected cortical neurons were used as controls. Levels of sema7A mRNA were normalized to the levels of actin mRNA. (A) Q-PCR analysis showing expression of sema7A mRNA levels at 24, 48, and 72 h.p.i. with WNV in cortical neurons isolated from wild-type mice. Uninfected cortical neurons were used as controls. Levels of sema7A mRNA were normalized to the levels of actin mRNA. (B) Kinetics of WNV infection in cortical neuronal cells isolated from wild-type or sema7A−/− mice at 24, 48, and 72 h.p.i. (C) Q-PCR analysis showing WNV burden in Sema7A mAb-treated or isotype-matched negative control Ab-treated cortical neurons at 48 h.p.i. All results are from three independent experiments, performed in triplicate. Error bars represent + SD from the mean value. Statistical significance was calculated using the Student t test.

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FIGURE 4. Abrogation of Sema7A blocks WNV infection of cortical neurons in vitro. (A) Q-PCR analysis showing expression of sema7A mRNA levels at 24, 48, and 72 h.p.i. with WNV in cortical neurons isolated from wild-type mice. Uninfected cortical neurons were used as controls. Levels of sema7A mRNA were normalized to the levels of actin mRNA. (B) Kinetics of WNV infection in cortical neuronal cells isolated from wild-type or sema7A−/− mice at 24, 48, and 72 h.p.i. (C) Q-PCR analysis showing WNV burden in Sema7A mAb-treated or isotype-matched negative control Ab-treated cortical neurons at 48 h.p.i. All results are from three independent experiments, performed in triplicate. Error bars represent + SD from the mean value. Statistical significance was calculated using the Student t test.

FIGURE 5. Abrogation of Sema7A blocks WNV infection of human macrophages. (A) Q-PCR analysis showing expression of sema7A mRNA levels at 24, 48, and 72 h.p.i. with WNV in macrophages isolated from a representative healthy human volunteer (Subject A). Uninfected macrophages were used as controls. Levels of sema7A mRNA were normalized to the levels of actin mRNA. (B) Q-PCR analysis showing WNV burden in Sema7A mAb-treated or isotype-matched negative control Ab-treated human macrophages from Subject A at 48 h.p.i. (C) Q-PCR analysis showing viral burden in Fc or Fc-Sema7A–transfected HEK 293 cells. Infected, but untransfected, cells were used as controls. Data are representative of results performed in triplicates. Error bars represent + SD from the mean value. Statistical significance was calculated using the Student t test.

To examine whether Smad6 controls viral replication, we analyzed WNV infection upon silencing of smad6 expression in HEK 293 cells. Q-PCR analysis showed a significant reduction in smad6 mRNA levels in smad6 siRNA-transfected cells (Fig. 6F). In addition, we found that HEK 293 cells transfected with tgf-beta1 siRNA showed reduced Smad6 protein levels upon WNV infection compared with the uninfected controls at both 48 and 72 h.p.i. (Fig. 6G, Supplemental Table I). To test whether Sema7A mediates signaling through smad6 during WNV infection, we analyzed smad6 expression in cortical neurons from sema7A−/− mice. Results showed that, in the absence of Sema7A, no induction of smad6 was observed in sema7A−/− cortical neurons infected with WNV at 48 h.p.i. (Fig. 6E). Reduced levels of smad6 mRNA were also evident in HEK 293 cells transfected with tgf-beta1 siRNA at 72 h.p.i. with WNV compared with the control siRNA-transfected cells (Fig. 6F). In addition, we found that HEK 293 cells transfected with tgf-beta1 siRNA showed reduced Smad6 protein levels upon WNV infection compared with the uninfected controls at both 48 and 72 h.p.i. (Fig. 6G, Supplemental Table I). To examine whether Smad6 controls viral replication, we analyzed WNV infection upon silencing of smad6 expression in HEK 293 cells. Q-PCR analysis showed a significant reduction in smad6 mRNA levels in smad6 siRNA-transfected cells (Fig. 6H). A significant reduction in viral burden was also evident at tested time points (48 and 72 h.p.i.) in smad6–silenced cells compared with the control siRNA-transfected cells (Fig. 6I). Taken together, these results suggest that Sema7A contributes to WNV pathogenesis through the TGF-β1/Smad6–signaling pathway.
Discussion

Viruses, such as vaccinia and alcelaphine herpesvirus, encode semaphorin A39R and AHV-sema, which achieve immunomodulatory functions by mimicking vertebrate Sema7A (16, 26, 27, 33). The role of vertebrate Sema7A in viral pathogenesis has not been directly addressed. In this article, we describe a crucial role for Sema7A in WNV pathogenesis. Our finding that abrogation of Sema7A provides resistance to WNV infection in mice is supported by several lines of evidence. First, the upregulation of Sema7A upon WNV infection in blood and brain tissues suggests its role both in the peripheral organs and CNS. Second, mice that lack Sema7A had a reduced viral burden in the periphery during early infection and reduced levels in the brain during late infection, suggesting a role for Sema7A both in the early and late stages of WNV pathogenesis. Third, the level of TNF-α, which is important for WNV crossing the BBB, was significantly reduced in mice that lack Sema7A. Fourth, in the absence of Sema7A, IgG leakage into the brain was reduced upon WNV infection, impli-
cating a role for Sema7A in protecting BBB permeability. Last, in the absence of Sema7A, viral replication in cortical neurons was significantly reduced, suggesting a role for Sema7A in brain cells. Sema7A is a potent stimulator of monocytes and neutrophils (25). Studies demonstrated that concentrations as low as 1 pM of Sema7A can stimulate monocyte cytokine production and chemotaxis. Indeed, the addition of Sema7A influenced monocyte chemotaxis 1000 times more efficiently than did MCP-1, and it influenced neutrophil chemotaxis 100 times better than did IL-8 stimulation (25). Sema7A is abundantly expressed in monocytes, neutrophils, and other lymphoid cells (25, 28, 29). A recent study showed a biphasic response of PMNs to WNV infection; they serve as a reservoir for WNV replication and dissemination in the early stages of infection and later contribute to WNV clearance (42). The reduced viremia in the early stage of WNV infection in Sema7A-deficient mice (Fig. 2) suggests that Sema7A may participate in the replication of WNV at early stages in these cells and at later stages in the dissemination of WNV to the peripheral tissues and brain. It is possible that the absence of Sema7A results in slower recruitment of neutrophils to the site of WNV infection, which may affect PMN-dependent viral replication and dissemination. Peripheral blood monocytes are relatively immature macrophage precursors that can differentiate into macrophage or dendritic cells based on environmental stimuli (43). Sema7A induces the maturation of monocytes toward dendritic cell morphology (43). Studies show that TLR-3 binding to double-stranded viral RNA inside infected dendritic cells leads to the production of inflammatory cytokines, such as TNF-α (7). Our findings that Sema7A-deficient mice infected with WNV have lower production of TNF-α and less BBB permeability (Fig. 3) support our hypothesis that, in the absence of Sema7A, there might be one possible antiviral mechanism that reduces maturation of monocytes to dendritic cells. The reduction in dendritic cell maturation may lead to decreased TLR-3-mediated recognition of viral RNA and production of TNF-α that subsequently leads to less WNV crossing the BBB. We propose that Sema7A acts upstream of the host inflammatory reaction that results from WNV invasion.

TGF-β1 is synthesized as precursor that is complexed with latent TGF-β-binding proteins and is activated by a variety of mechanisms that include integrin binding (41, 44). Activated TGF-β1 binds to heterodimeric receptors containing types I and II receptor components that have tyrosine kinase activity (41, 44). Our studies demonstrate that mice lacking Sema7A exhibited a significant reduction in TGF-β1 mRNA levels. The reduced TGF-β1 mRNA levels correlated with reduced viral infection in cortical neurons isolated from Sema7A-deficient animals (Fig. 6). Kang et al. (34) showed that TGF-β1 induces the expression of Sema7A in a pulmonary fibrosis model. A more conservative interpretation of the possible mechanisms is warranted, because our study proposes that Sema7A contributes to WNV infection through TGF-β1/Smad-signaling pathways. It is possible that Sema7A induces TGF-β1, which, in turn, may induce Sema7A expression during WNV infection. A feedback mechanism was identified for semaphorin 3E signaling (45). We found that sema7A and tgf-β1 mRNA levels are coordinately induced in mouse cortical neurons during WNV infection (Figs. 4A, 6A). Although, we found that Sema7A deficiency abrogates tgf-β1 mRNA levels, we did not observe any changes in sema7A mRNA expression upon TGF-β1 silencing during WNV infection (data not shown). This role of Sema7A in TGF-β1 signaling and in virus infection suggests that these are two mechanisms that can both contribute to WNV pathogenesis. TGF-β1 signals via Smads, where Smad2 and Smad3 are phosphorylated by TGF-βR1 kinase. The phosphorylated Smad2/3 complex binds to Smad4 and translocates Smad4 to the nucleus (40, 41, 46). However, we did not observe any changes in the mRNA expression of either of these Smads upon WNV infection in murine cortical neurons (Supplemental Fig. 3E) or in WNV-infected mice lacking Sema7A (data not shown). There is increased evidence for Smad2/3-independent pathways that mediate the effects of TGF-β1 (34, 40, 41, 46). Overexpression studies in mammalian cells elucidated that Smad6 associates with TGF-β1 type I receptor and inhibits Smad2 and Smad1 phosphorylation (46). This regulatory function of Smad6 may allow more precise regulation of TGF-β1-mediated Smad signaling (46). Based on our findings that WNV induces Smad6 expression and that the absence of Sema7A or TGF-β1 affected this induction, we hypothesize that Sema7A functions via TGF-β1/Smad6 signaling to contribute to WNV pathogenesis. The reduced WNV infection in Smad6-silenced cells further supports this hypothesis. Collectively, we propose that Sema7A deficiency may affect the ability of the TGF-β1/Smad6 pathway to influence cytokine production, antiproteases, transcription factors, and receptor components that contribute to WNV pathogenesis.

Several GPI-anchored proteins perform diverse roles in immunoregulation (47). GPI-anchored proteins occur in microdomains or “rafts” on the cell surface that can also be cleaved off from the cell surface by proteolytic cleavage, resulting in a secreted form of the proteins (48). Sema7A is a GPI-anchored protein that is released from the cell surface by proteolysis, suggesting its role in autocrine function (30, 49). Sema7A functions both as a cell surface and secreted molecule. Also, lipid rafts have been implicated in flavivirus entry into cells by the activation of PI3K/Akt signaling during the early stages of Japanese encephalitis virus infection in neural stem/progenitor cells (50). Sema7A plays a central role in a PI3K/PKB/AKT-dependent pathway that contributes to TGF-β1–induced fibrosis and modeling (34, 50). It is possible that initiation and propagation of the signaling events taking place during WNV pathogenesis occur in these specialized Sema7A-containing membrane regions. We postulate that, in its GPI-anchored form, Sema7A may facilitate viral entry on the cell surface (when it is linked to lipid rafts) either by directly binding to the virus or indirectly by forming a complex with other cell surface receptors. The fact that WNV infection in vitro is inhibited by the blockage of Sema7A further supports that it may be a receptor or coreceptor for WNV. In its secreted form, Sema7A may trigger downstream signaling in modulating the expression of TGF-β1/Smad6 and other inflammatory cytokines that contribute to WNV pathogenesis. With any of these models, the finding that abrogation of Sema7A results in increased resistance to WNV infection supports the role of Sema7A as a potential therapeutic target for disease caused by WNV.

Collectively, these data provide direct evidence for the involvement of Sema7A in WNV pathogenesis. Our results emphasize molecular mechanisms that WNV uses to establish and invade the brain, leading to the cause of lethal encephalitis. Because treatment with Sema7A Ab is efficacious in mice and provides protection in microvascular endothelial cells, murine cortical neurons, and human macrophages, our study opens up a promising area for the development of Sema7A-blocking therapies for WNV and possibly other flaviviral infections.

Acknowledgments
We thank Drs. Jack Elias and Bing Ma for providing sema7A−/− mice. We also thank Deborah Beck and Lida Yuan for technical assistance.

Disclosures
The authors have no financial conflicts of interest.
References


