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West Nile Virus Envelope Protein Inhibits dsRNA-Induced Innate Immune Responses

Alvaro Arjona,* Michel Ledizet,† Karen Anthony,‡ Nathalie Bonafe,† Yorgo Modis,§ Terrence Town,§ and Erol Fikrig2*

The immune response against viral infection relies on the early production of cytokines that induce an antiviral state and trigger the activation of immune cells. This response is initiated by the recognition of virus-associated molecular patterns such as dsRNA, a viral replication intermediate recognized by TLR3 and certain RNA helicases. Infection with West Nile virus (WNV) can lead to lethal encephalitis in susceptible individuals and constitutes an emerging health threat. In this study, we report that WNV envelope protein (WNV-E) specifically blocks the production of antiviral and proinflammatory cytokines induced by dsRNA in murine macrophages. This immunosuppressive effect was not dependent on TLR3 or its adaptor molecule Trif. Instead, our experiments show that WNV-E acts at the level of receptor-interacting protein 1. Our results also indicate that WNV-E requires a certain glycosylation pattern, specifically that of dipteran cells, to inhibit dsRNA-induced cytokine production. In conclusion, these data show that the major structural protein of WNV impairs the innate immune response and suggest that WNV exploits differential vector/host E glycosylation profiles to evade antiviral mechanisms. The Journal of Immunology, 2007, 179: 8403–8409.

West Nile virus (WNV), a mosquito-borne flavivirus, is an emerging pathogen with the ability to cause severe encephalitis that may lead to long-term neurological sequelae and even death in susceptible individuals (5). WNV is endemic to the Middle East, Europe, and Africa. Since its introduction into North America in 1999, WNV has been rapidly disseminating across the Western Hemisphere. Annual outbreaks of WNV fever and neuroinvasive disease occur in the U.S. The envelope protein of WNV (WNV-E) is the outermost component of the virion, and plays a crucial role in cellular attachment and membrane fusion (6). Previous studies have demonstrated that innate immunity against WNV relies partially on dsRNA-sensing PRRs (7, 8). As WNV-E is the first viral molecule to interact with the host, we hypothesized that WNV exploits its major structural protein to impair host antiviral mechanisms during the early phase of infection.

Materials and Methods

Cells

Primary macrophages from C57BL/6J (Jackson ImmunoResearch Laboratories), Tlr3−/− (9), or Trif−/− (10) mice of the same genetic background were obtained 3–4 days after i.p. injection with 5% thioglycollate broth (REMEL) by peritoneal lavage. Macrophages were purified by adherence following standard procedures and cultured overnight in RPMI 1640 containing 10% FBS plus penicillin-streptomycin (Invitrogen Life Technologies). We followed Yale University institutional guidelines for care and use of laboratory animals.

Virus

WNV isolate CT2741 and the mutant strain with nonglycosylated envelope protein NY99/E154 (11) were passaged once in mammalian (Vero) or mosquito (C6/36) cells. Viral titers were determined from cleared culture supernatants by standard plaque assay in Vero cells. Studies with WNV were conducted in a Biosafety Level 3 facility, following the regulations of the State of Connecticut and the Office of Environmental Health and Safety of Yale University.

Recombinant proteins

Recombinant WNV-E comprising the first 406 amino acids of the viral envelope protein was expressed in S2 and Sf9 cells as described (12, 13). A soluble recombinant protein consisting of the first 400 amino acids of
dengue-2 virus envelope protein was expressed in Drosophila S2 cells and purified by cation-exchange and size exclusion chromatography.

**Treatments**

Cells were incubated with recombinant protein or with WNV at the indicated multiplicity of infection for 1 h unless indicated otherwise and then challenged with poly(I:C) (Amersham Biosciences), Pam3Cys, lipoteichoic acid, LPS, flagellin, imiquimod (R837), or unmethylated CpG-DNA (InvivoGen) at the indicated concentrations (see Fig. 1 legend). For experiments with recombinant protein, treatments were conducted in the presence 10 μg/ml polymyxin B (Fluka). For experiments with whole WNV, cells were pretreated with 1.5 μM bafilomycin A1 (Fluka) for 30 min to inhibit endosomal acidification and viral fusion (14). Cells and culture supernatants were harvested after 3 or 24 h, respectively, unless indicated otherwise.

**Determination of cytokine levels**

Cytokine mRNA levels were determined by quantitative real-time PCR as described (15). Actin mRNA levels were employed as normalizing factor. To determine cytokine protein levels in culture supernatants, we employed commercially available ELISAs (R&D Systems). The detection limits for TNF-α and IFN-β ELISAs were 5.1 and 15.6 pg/ml, respectively.

**Coimmunoprecipitation and immunoblotting**

Cell lysates were immunoprecipitated with anti-RIP1 Ab (BD Biosciences) followed by capture with Protein G Sepharose beads (Sigma-Aldrich). Immunocomplexes were dissociated from the beads by boiling in 2× SDS-PAGE sample buffer. Associated ubiquitin was detected by Western blot using anti-ubiquitin Ab (Santa Cruz Biotechnology). Anti-IκBα Ab (Cell

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**FIGURE 1.** WNV-E specifically blocks poly(I:C)-induced cytokine production. *A*, Mouse peritoneal macrophages were treated with increasing concentrations of recombinant WNV-E expressed in Drosophila S2 cells or the same volume of medium (mock) and then challenged with poly(I:C) (100 μg/ml). Cytokine levels in culture supernatants were determined by ELISA 16 h after challenge. *B*, Mouse peritoneal macrophages were treated with recombinant WNV-E expressed in Drosophila S2 cells (0.3 μg/ml) and then challenged with poly(I:C) (100 μg/ml). Cytokine mRNA levels were determined by Q-PCR 3 h after challenge. *C*, Mouse peritoneal macrophages were treated with recombinant WNV-E expressed in Drosophila S2 cells (0.3 μg/ml) and then challenged with poly(I:C) (100 μg/ml). TNF-α levels in culture supernatants were determined by ELISA 16 h after challenge. *D*, Mouse peritoneal macrophages were incubated with recombinant WNV-E or dengue-2 virus envelope protein expressed in Drosophila S2 cells (1 μg/ml) for 1 h and then challenged with Pam3Cys (500 ng/ml), lipoteichoic acid (LTA, 10 μg/ml), poly(I:C) (100 μg/ml), LPS (100 ng/ml), flagellin (5 μg/ml), imiquimod (10 μg/ml), or unmethylated CpG-DNA (10 μg/ml). TNF-α levels in culture supernatants were determined by ELISA 16 h after challenge. NS, No stimulation. UD, Undetectable. *, Significantly different from mock (p < 0.05). #, Significantly different from the previous (to the left) treatment group (p < 0.05). Bars represent mean ± SD. Data shown are representative results obtained in at least three independent experiments.
Signaling Technology) was employed to determine total IκBα levels in whole cell lysates following standard immunoblotting procedures. Anti-actin Ab (Sigma-Aldrich) was used to monitor protein input.

Statistical analyses

Data are presented as mean ± SD. We analyzed differences between means using the unpaired Student’s t test or one-way ANOVA followed by the Newman-Keuls Multiple Comparison Test. A p value <0.05 was considered significant.

Results

WNV-E specifically blocks poly(I:C)-induced cytokine production

A critical step during the immune response against viral infection is the recognition of virus-associated molecular patterns by the host immune system. This phenomenon leads to the production of cytokines that activate immune cells, promote an antiviral state, and orchestrate subsequent adaptive immune mechanisms (16). We hypothesized that WNV would benefit from impairing the host innate response during the very early stages of infection. Hence, we tested whether WNV-E, the first WNV molecule to interact with host cells, interferes with antiviral responses in primary macrophages. Depletion studies have demonstrated the importance of myeloid cells in WNV infection (17). We therefore treated mouse peritoneal macrophages with recombinant WNV-E expressed in Drosophila S2 cells for 1 h before challenging them with the dsRNA mimetic poly(I:C), a prototypic RNA virus pathogen-associated molecular pattern (PAMP). As shown in Fig. 1A, WNV-E significantly blocked, in a dose-dependent manner, the poly(I:C)-induced production of TNF-α, IL-6, and IFN-β in culture supernatants. Similarly, the poly(I:C)-induced increase in the mRNA levels of TNF-α and IFN-β was significantly inhibited by WNV-E treatment (Fig. 1B), which points toward the impairment of pleiotropic transcriptional activation factors such as NF-κB. Time course experiments revealed that 30 min of treatment with WNV-E was sufficient to induce a strong blockade of cytokine production (over 25-fold reduction), and this effect persisted up to 48 h after WNV-E treatment (Fig. 1C). Additionally, to examine whether WNV-E was able to compromise the production of cytokines induced by other immune stimuli, we challenged WNV-E-pretreated macrophages with multiple PAMPs targeting different TLRs. As shown in Fig. 1D, WNV-E blocked TNF-α production when cells were challenged with poly(I:C), but not when they were challenged with pam3Cys (TLR1/2), lipopolysaccharide (TLR2/6), LPS (TLR4), flagellin (TLR5), imiquimod (TLR7), or unmethylated CpG-DNA (TLR9). These results indicate that WNV-E selectively interferes with dsRNA pattern recognition pathways. Moreover, this effect appears to be specific for WNV, because recombinant dengue-2 virus envelope protein did not have any effect on the cytokine production induced by any of the PAMPs, including poly(I:C) (Fig. 1D).

WNV-E blocks poly(I:C)-induced cytokine production in Tlr3−/− and Trif−/− macrophages

Our experiments show that WNV-E specifically interferes with dsRNA recognition pathways. Double-stranded RNA is detected by TLR3 and by the cytoplasmic RNA helicases RIG-I and MDA5 (4). TLR3 uses Trif as an adaptor molecule to initiate its signaling, whereas RNA helicases use IFN-β promoter stimulator (also known as Cardif, virus-induced signaling adaptor, and mitochondrial antiviral signaling protein). These two signaling pathways converge at the level of downstream signaling molecules such as RIP1 and TBK1 to activate NF-κB and IRFs, which ultimately regulate the expression of proinflammatory cytokines and type I IFN (4). We took advantage of this dual dsRNA recognition mechanism to analyze the effect of WNV-E on poly(I:C)-induced cytokine production in TLR3- and Trif-deficient macrophages. Fig. 2 shows that poly(I:C)-induced TNF-α production was significantly diminished (but not abolished) in Tlr3−/− and Trif−/− macrophages, which is in accordance with previous reports (9). The residual poly(I:C)-induced cytokine production in Tlr3−/− and Trif−/− macrophages depends, at least in part, on the activation of RIG-I/MDA5 signaling (18). Interestingly, we found that WNV-E treatment reduced the residual poly(I:C)-induced cytokine production to undetectable levels in Tlr3−/− and Trif−/− macrophages. These results indicate that WNV-E blocks both TLR3/Trif-dependent and independent signals, and suggest that WNV-E affects downstream signaling events shared by both the TLR3 and the RNA helicases pathways.

WNV-E interferes with poly(I:C)-induced RIP1 polyubiquitination and subsequent NF-κB activation

RIP1 is a serine-threonine kinase that is recruited by both Trif and IPS-1 to mediate NF-κB activation after challenge with dsRNA (19, 20). Of note, absence of RIP1 results in a loss of NF-κB activation when cells are challenged with poly(I:C), but not when they are challenged with LPS (19, 20). Hence, the consequences of RIP1 deficiency mimic the effect that we report here for WNV-E. We therefore hypothesized that WNV-E interferes with poly(I:C)-triggered signaling at the level of RIP1. We tested this hypothesis by examining whether the cytokine blocking effect of WNV-E was affected by resveratrol, a polyphenol that inhibits MyD88-independent signaling by targeting RIP1 and TBK1 (21). As expected, resveratrol-treated macrophages produced less cytokine mRNA in response to poly(I:C) when compared with macrophages treated with vehicle. Interestingly, WNV-E failed to inhibit the poly(I:C)-induced mRNA production of either TNF-α or IFN-β when macrophages were pretreated with resveratrol (Fig. 3A). To assess the...
pretreated with resveratrol, WNV-E significantly inhibited the poly(I:C)-induced mRNA production of both TNF-α and IFN-β in macrophages pretreated with EGCG (Fig. 3B). Thus, these results support the notion that WNV-E mediates its cytokine blocking effect mainly through RIP1.

Ubiquitination of RIP1, but not its kinase activity, has been shown to regulate NF-κB activation upon TNF-R1 stimulation (23), and recent studies showed that RIP1 uses similar ubiquitin-dependent mechanisms to mediate poly(I:C)-dependent NF-κB activation (24). To assess whether WNV-E interferes with RIP1 ubiquitination, we immunoprecipitated RIP1 and analyzed its degree of ubiquitination by immunoblotting. Poly(I:C) treatment induced heavy polyubiquitination of RIP1 in macrophages, as shown previously in mouse embryonic fibroblasts (24). However, treatment with WNV-E dramatically inhibited poly(I:C)-induced RIP1 ubiquitination (Fig. 3C). This defect in RIP1 polyubiquitination in macrophages treated with WNV-E was correlated with a lack of IκBα degradation (Fig. 3D). These data collectively indicate that WNV-E blocks the poly(I:C)-induced production of type I IFN and proinflammatory cytokines in macrophages by interfering with RIP1 polyubiquitination and subsequent NF-κB activation. WNV-E-mediated impairment of NF-κB activation could account for a defective induction of both proinflammatory cytokines and IFN-β (Fig. 1), as NF-κB also contributes to IFN-β gene transcription (25). Nevertheless, given the strong blockade that WNV-E also exerts on IFN-β production, we cannot rule out the existence of adjuvant inhibitory mechanisms acting upon the activation pathways of IRFs.

WNV-E requires a specific glycosylation pattern to block poly(I:C)-induced cytokine production

WNV-E contains a single N-linked glycosylation site at residue 154, and glycosylation of the E protein has been shown to influence WNV pathogenesis (11). Thus, we investigated whether glycosylation of WNV-E was necessary for its cytokine-blocking effect. Interestingly, removal of N-linked glycans from WNV-E expressed in Drosophila (dipteran) S2 cells with peptide N-glycosidase F rendered the E protein unable to inhibit poly(I:C)-induced cytokine production. In sharp contrast, neither glycosylated nor deglycosylated WNV-E expressed in Spodoptera frugiperda (lep- idopteran) Sf9 cells were able to impair poly(I:C)-induced cytokine production (Fig. 4A). As expected, peptide N-glycosidase F treatment produced a mobility shift in both S2 and Sf9-expressed WNV-E. The latter showed a larger shift, consistent with the notion that glycoproteins expressed in Sf9 cells have a higher content of total N-linked glycosylation (26). N-glycosylation heterogeneity between these two insect cell expression systems has been previously reported (26, 27). Drosophila N-linked glycosylation is less complex and tends to have higher mannose content (27, 28). These results therefore suggest that WNV-E requires a specific glycosylation profile to block poly(I:C)-induced cytokine production. Along this line, glycosylation of the closely related flavivirus Japanese encephalitis virus E protein has been shown to differ between Vero (mammalian) and mosquito (dipteran) cells (29). One of the major differences between the mosquito- and mammalian-cell-derived viruses is the exclusive presence of mannose glycans (high mannose and paucimannose) on mosquito-derived viruses and complex/hybrid/high-mannose glycans on mammalian-cell-derived virus (30). To examine whether glycosylation of WNV-E in the host (mammalian) or in the vector (mosquito) would affect the WNV-E cytokine blocking properties, we exposed macrophages to WNV passaged in either Vero or C6/36 cells and then challenged them with poly(I:C). We observed that C6/36-passaged WNV, but not Vero-passaged WNV, significantly impaired poly(I:C-
C)-induced mRNA production of TNF-α and IFN-β, and therefore mimicked the results obtained with recombinant WNV expressed in Drosophila S2 cells (Fig. 1). Moreover, a C6/36-passaged WNV strain that lacks the glycosylation site on E (WNV-C6/36-E154) failed to impair poly(I:C)-induced cytokine production (Fig. 4, B and C), which confirms that this effect is due to E glycosylation. We further examined whether exposure of macrophages to WNV grown in C6/36 cells hampers poly(I:C)-induced cytokine production at the protein level. In these experiments, we pretreated macrophages with bafilomycin A1 to prevent viral fusion (14). In accordance with the mRNA data, cells exposed to C6/36-passaged WNV (0.1–1 multiplicity of infection) produced significantly reduced protein levels of TNF-α and IFN-β in the supernatant after poly(I:C) challenge (Fig. 5A). Furthermore, both Vero-derived WNV and WNV-C6/36-E154 failed to impair poly(I:C)-induced cytokine protein production (Fig. 5B). These data collectively indicate that a specific glycosylation profile, specifically that of dipteran cells, allows WNV-E to block poly(I:C)-induced cytokine production.

Discussion

The host antiviral response relies on the prompt recognition of viral PAMPs by innate immune cells. This phenomenon leads to the production of cytokines that activate immune cells, promote an antiviral state, and orchestrate subsequent adaptive immune mechanisms (16). Viruses are in the constant need of acquiring immune-evasive mechanisms to ensure productive infection and replication. Flaviviruses, because of genomic size constraints, have evolved multifunctional genes that regulate both the viral life cycle and the host immune response (31). In this study, we report that WNV-E, the first WNV molecule to interact with the host, specifically inhibits the production of proinflammatory and antiviral cytokines induced by dsRNA. Of note, macrophages remained unresponsive to poly(I:C) more than 24 h after exposure to WNV-E. This observation supports the relevance of WNV-E immunosuppressive properties during WNV immunopathogenesis, as WNV dsRNA would be produced in the infected cells several hours after the initial interaction with the E protein. The biological relevance
of WNV-E-mediated inhibition of cytokine production is supported by our experiments with whole WNV. However, the magnitude of cytokine inhibition with infectious WNV was not as dramatic as with recombinant WNV-E. One possibility is that E-containing subviral particles, which have been shown to be produced by WNV-infected cells (32), but not virion-associated E, are responsible for the cytokine-blocking effect. In contrast, we consider more likely that the challenge with whole virus dampens the net inhibitory effects of WNV-E due to the exposure to other structural components of the virion and/or the activation of cellular processes (not inhibited by baflomycin A1) that may have an immunostimulatory effect themselves. In any case, the results obtained with the nonglycosylated WNV-E154 strain firmly support that the inhibitory effect seen in experiments with whole WNV is because of the E protein.

Both TLR3 and the RNA helicases RIG-I and MDA5 sense dsRNA (16). The different signaling pathways activated by these PRRs converge at the level of the downstream kinases RIP1 and TBK1 (4). Our experiments indicate that WNV-E cytokine-blocking effect is independent of TLR3 or its adaptor molecule Trif. Instead, WNV-E inhibited dsRNA-induced cytokine production by interfering with RIP1 ubiquitination and subsequent NF-κB activation. Indeed, from an evolutionary perspective, it would seem more efficient for viruses with limited genetic information such as WNV to have evolved proteins that block critical distal processes where multiple antiviral mechanisms converge rather than attempting to block each proximal recognition pathway. Although it has been shown that MDA5 does not play a dominant role over the TLR3 pathway for cytokine responses to naked poly(I:C) in mouse peritoneal macrophages (18), our experiments do not completely rule out the possibility of WNV-E acting partly through MDA5 as well. In contrast, WNV-E could mediate its inhibitory effect on RIP1 either directly or indirectly. Supporting the latter, we did not detect WNV-E associated with RIP1 during our immunoprecipitation experiments (data not shown). Other flaviviruses, however, have been shown to produce nonstructural proteins that directly inactivate proximal components of dsRNA-induced signaling pathways (33, 34).

Our data reveal that WNV-E requires a certain glycosylation profile, specifically that obtained in dipteran cells, to exert its cytokine-blocking effect. Only recombinant WNV-E expressed in S2 cells (but not in Sf9 cells) and WNV grown in mosquito cells (but not in mammalian cells) were able to inhibit dsRNA-induced cytokine production. In line with our findings, Shabman et al. (35) have shown recently that, due to differential N-linked glycosylation, mosquito cell-derived alphaviruses induce less type I IFN than those derived from mammalian cells. In contrast, Shirato et al. (36), using mosquito-derived WNV, found that only glycosylated WNV induced TNF-α production. The fact that in the mentioned study the cytokine read-outs in WNV-infected macrophages were not induced by poly(I:C) challenge, together with differences in mouse strains (BALB/c vs C57BL/6j) as well as in time courses, may account for the discrepancy with our results. The requirement of a specific glycosylation pattern also supports the notion that WNV-E mediates its immunosuppressive effect via the interaction with PRRs specialized in carbohydrate discrimination such as C-type lectins (37). Interestingly, there is evidence showing that pathogens may exploit recognition by C-type lectins to inhibit TLR signaling and cytokine production (38, 39). Nevertheless, the specific differences in WNV-E glycosylation patterns between mammalian and mosquito cells are yet to be characterized.

After the initial round of mosquito-derived WNV replication in the host, the newly produced WNV-E (now carrying a mammalian glycosylation profile) will no longer inhibit dsRNA-induced cytokine production. Interestingly, we have shown that WNV partially depends on the action of inflammatory cytokines (chiefly TNF-α) to open the blood-brain barrier and invade the CNS (8). Thus, it appears that WNV takes advantage of the different vector/host E glycosylation profiles to modulate the immune response in a manner that better suits its pathogenic time course, i.e., inhibition of cytokine production during the first replication round to evade the initial antiviral response (mosquito-derived WNV-E) followed by lack of cytokine inhibition after the first replication round (mammalian-derived WNV-E) so that blood-brain barrier breaching is facilitated. However, the exact contribution of WNV-E-mediated cytokine inhibition to overall WNV immunopathogenesis is yet to be assessed. Impairment of innate immune responses by mosquito-derived WNV during the first replication round may significantly enhance infection. Along this line, mice infected with mosquito-cell-propagated WNV strains lacking glycosylation of the E protein showed reduced peripheral viral titers when compared with those infected with E-glycosylated WNV (40) (which would inhibit dsRNA-induced cytokine production). Nevertheless, glycosylation of WNV-E has been shown to influence other parameters such as particle assembly that would also affect WNV infection levels (41).

In summary, our findings provide novel evidence for the existence of immune evasion mechanisms mediated by the major structural protein of WNV. Further research is warranted to better understand the devices that WNV uses to subvert the host antiviral response and how these immune evasion strategies shape viral pathogenesis. These studies will likely yield valuable information for therapy and prophylaxis. It would be pertinent also to assess whether the E protein of dengue virus and other flaviviruses have immunomodulatory properties similar to that of WNV-E. Although the general arrangement of WNV-E glycoprotein is similar to that of dengue virus envelope protein, several features unique to each virus and visible structural differences corresponding to different numbers of glycosylation sites as well as positional shifts have been described by cryo-EM and density maps (42). Hence, it would not be surprising to find that WNV-E and dengue virus envelope protein have different immunomodulatory properties.

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Disclosures

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References
