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Differential responses of human brain cells to West Nile virus infection

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In recent years, West Nile virus (WNV) has emerged as a major cause of encephalitis in the United States. However, the neuropathogenesis of this flavivirus is poorly understood. In the present study, the authors used primary human brain cell cultures to investigate two neuropathogenic features: viral replication and induction of cytokines. Although neurons and astrocytes were found to support productive WNV infection, viral growth was poorly permissive in microglial cells. Compared to neuronal cultures that sustained viral growth for at least 2 weeks, replication peaked in astrocytes by 72 h post infection. In response to viral infection, astrocytes produced chemokines (CXCL10 and CCL5), but none of the cytokines (tumor necrosis factor [TNF]-α, interleukin [IL]-1β, IL-6, interferon α or γ) tested could be detected. Although microglial cells failed to support viral replication, WNV induced production of the proinflammatory cytokines IL-6 and TNF-α. Microglial cells also released robust amounts of the chemokines CXCL10 and CCL2, as well as lower levels of CCL5, in response to WNV infection. WNV-induced chemokine and cytokine production by microglia was coupled with activation of mitogen-activated protein kinase (MAPK) intracellular signaling pathways. Inhibition of p38 MAPK decreased chemokine production in response to WNV. Taken together, these findings suggest that microglial cell responses may influence the neuropathogenesis of WNV infection. *Journal of NeuroVirology* (2005) **11,** 512–524.

Keywords: chemokines; cytokines; glia; West Nile virus

Introduction

The outcome of central nervous system (CNS) viral infections is dependent upon replication characteristics as well as immune responses to the virus (Carson and Sutcliffe, 1999; Kim *et al*, 2005). Although replication in neurons is a pathogenic feature of neurotropic viruses, the CNS is comprised mainly of glial cells, and it has become increasingly clear that astroglia (Benveniste, 1997; Dong and Benveniste, 2001) and microglia (Rock *et al*, 2004) orchestrate a series of complex immune responses to neurotropic viruses through the production of cytokines/chemokines. These mediators initiate both intrinsic and systemic responses to limit viral spread within the CNS (Cheeran *et al*, 2003). Inflammatory responses that control viral infection within the CNS, however, also have the potential to elicit tissue damage (Becher *et al*, 2000; Poluektova *et al*, 2004). Hence, to understand the pathogenesis of viral encephalitis, it is essential to determine not only the aspects of viral replication but also the temporal and qualitative nature of glial cell responses to viral infection.

At the dawn of the 21st century, the largest outbreak of arboviral encephalitis in the United States was reported. The culprit, West Nile virus (WNV),

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is a positive-sense single-stranded mosquito-borne RNA virus belonging to the genus *Flavivirus* in the *Flaviviridae* family. The emergence of WNV in the western hemisphere appears to be the result of the development of a more neurovirulent virus introduced to a new geographic area of nonimmune hosts (Johnson, 2003). Clinically, WNV infections in humans are manifested as a febrile, paralytic, or encephalitic form, although a majority of infected individuals have subclinical infection. Neurological manifestations of WNV are predominantly seen in elderly and immunocompromised patients, suggesting that an altered immune response plays a critical role in determining the establishment of encephalitis (Garmendia *et al*, 2001). However, little or nothing is known about the replication characteristics of WNV in human neuronal compared to glial cells or the nature of the cytokine/chemokine profiles elicited by WNV-stimulated glial cells.

Not only does the development of WNV encephalitis correlate with host failure to defend against the virus, but also with the production of inflammatory mediators (like tumor necrosis factor [TNF]-α) during the infection. It has been shown in animal models that both humoral and cell-mediated immunity play critical roles in preventing WNV encephalitis. The lack of functional B lymphocytes (Diamond *et al*, 2003a), T lymphocytes (Halevy *et al*, 1994; Shrestha and Diamond, 2004; Wang *et al*, 2003a), and macrophages (Ben-Nathan *et al*, 1996) has been shown to predispose the host to WNV encephalitis. Although immune responses to WNV in the periphery prevent encephalitis, 30% to 50% of immunocompetent animals challenged with a viral inoculum sufficient to establish infection will develop neurological signs (Wang *et al*, 2003b). It was recently demonstrated that production of TNF-α, early during infection, results in disruption of the blood-brain barrier (BBB), thereby facilitating viral entry into the CNS (Wang *et al*, 2004). In addition, recent studies on Japanese encephalitis virus (JEV) infection in humans demonstrated that elevated levels of proinflammatory cytokines and chemokines in the cerebrospinal fluid (CSF) are associated with poor clinical outcomes (Winter *et al*, 2004). A recent long-term convalescence study found that 40% of patients recovering from WNV encephalitis had a protracted convalescence period with long-term physical, cognitive, and functional impairments that lasted over 12 months (Klee *et al*, 2004; Sejvar *et al*, 2003). However, which host factors determine the outcome of WNV infection of the brain is not clear (Granwehr *et al*, 2004). It has been postulated that the early stage of flavivirus encephalitis is associated with the expression of inflammatory mediators by glial cells, which in turn triggers a cascade of neurotoxic molecules, e.g., TNF-α, interleukin (IL)-1β, and nitric oxide (NO), and cellular infiltration within the CNS (Chambers and Diamond, 2003). However, it is not clear whether these inflammatory mediators and infiltrated cells are neuroprotective or cause brain damage. The present study was undertaken to characterize responses of primary human brain cells that could influence the fate of WNV infection in the brain.

Results

WNV infection of human brain cells

Although WNV is a neurotropic virus, its relative ability to replicate in neurons versus glial cells, astrocytes, and microglia, is unknown. Thus, before determining cytokine/chemokine production by WNV-infected glial cells, we investigated the ability of these different human brain cell types to support WNV replication. Highly enriched human neuronal cell (>90% neurons, 5% to 10% astrocytes), purified astrocyte (>99% glial fibrillary acid protein [GFAP] positive), and microglial (~99% CD68 positive) cell cultures were infected with WNV NY99 at multiplicity of infection (MOI) = 1. Productive WNV replication in neuronal cultures began with a distinct eclipse phase that lasted for about 6 to 8 h post infection (p.i.), followed by a rise in production of infectious viral progeny that lasted until about 15 days in culture (Figure 1A), at which time the uninfected cultures began to show signs of deterioration, as evidenced by a 50% increase of cellular lactate dehydrogenase (LDH) release into the culture supernatant (measured using Tox-7 LDH assay kit; Sigma, St. Louis, MO). A 4- to 6-log rise in viral titer was observed by day 10 p.i., which was associated with a 243% increase in LDH released into culture supernatants compared to uninfected cells. Viral replication in astrocytes was characterized by a relatively shorter eclipse period (1 to 3 h), followed by a productive replication phase beginning at 6 to 8 h p.i. Viral titers peaked at 3 to 4 days p.i., increasing by 10^4 fold followed by a rapid decline in infectious viral progeny (Figure 1B). Infectious virus was not detected in astrocyte cultures at 8 to 10 days p.i. WNV replication in astrocytes was not associated with an increase in LDH release into culture supernatants or DNA fragmentation (apoptosis) of the infected cells. In contrast to neurons and astrocytes, microglial cells did not support robust WNV replication. A brief surge of replication was observed by day 2 p.i., which then decreased to below detectable levels (100 PFU/ml) by day 4 p.i. (Figure 1C). On further examination by immunocytochemistry, we found that about 80% of neurons and 30% of astrocytes in the respective cultures express WNV E protein, whereas only a few cells (<1%) in the microglial cell cultures were positive for WNV at 3 days p.i. (Figure 2).

Cytokines and chemokines elicited in response to WNV infection

To investigate whether selected cytokines and chemokines are produced by human glial cells in response to WNV infection, we assayed supernatants from astrocyte and microglial cell cultures

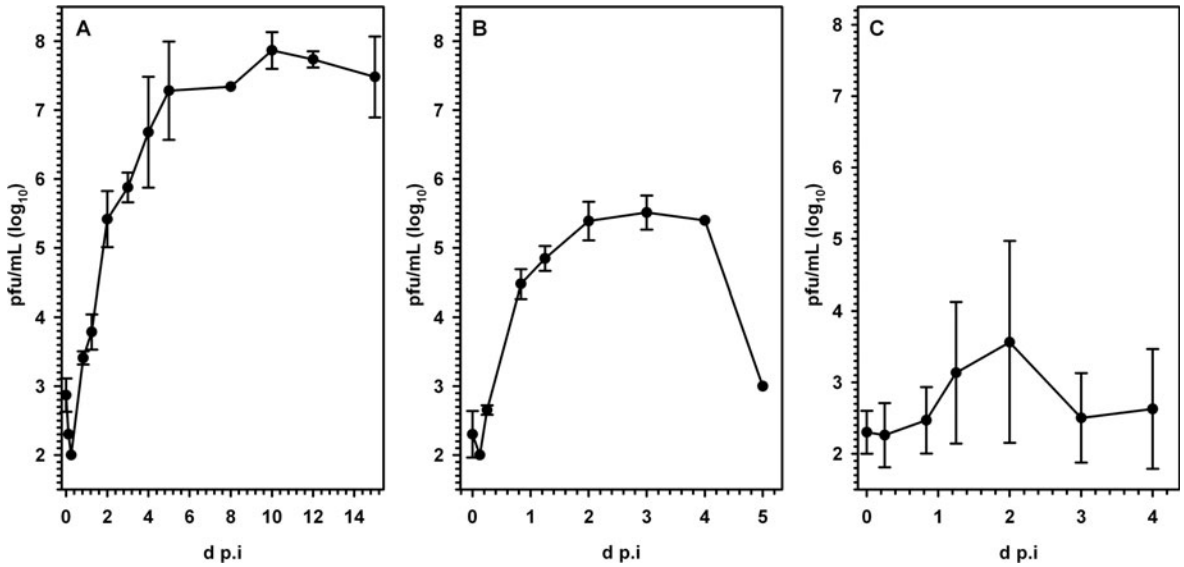

Figure 1 WNV replication in human brain cells. Human fetal (**A**) neuronal, (**B**) astrocyte, and (**C**) microglial cell cultures were infected with WNV (NY-99-35262-11). Viral titers were determined by plaque assay on Vero cell monolayers at various times post infection (p.i.). Viral titers at 0 day indicate the amount of WNV detected after 1 h adsorption and washing. Viral replication is expressed as plaque forming units (PFU)/ml and is plotted against time p.i. Results are presented as mean ± SD of pooled data from three to five different experiments using brain cells from different brain specimens.

for the cytokines TNF-α, IL-1β, IL-6, IL-10, interferon (IFN)-α, IFN-γ, and the chemokines CXCL10 (gamma-interferon–inducible protein-10; IP-10), CCL2 (monocyte chemoattractant protein-1; MCP-1), and CCL5 (regulated upon activation normal T cell expressed and secreted; RANTES). Primary astrocytes were found to respond to WNV infection with robust production of CXCL10, and lower levels of CCL5 were also observed (Table 1). CCL2 was constitutively expressed by cultured astrocytes and WNV infection did not increase its production significantly. Astrocytes did not produce any of the cytokines tested, including IFN-α or -γ, in response to WNV infection. Microglial cells, on the other hand, responded to nonproductive WNV infection by producing robust amounts of chemokines and cytokines. Most notable was the production of TNF-α and IL-6 but the lack of IL-1β and IL-10 in the cell culture supernatants. Production of CXCL10, CCL5, and CCL2 were also detected in WNV-infected microglia (Table 1). Surprisingly, there was a conspicuous lack of detectable IFN-α production by WNV-stimulated microglia.

Time course of chemokine production by WNV-infected astrocytes

To characterize the timing of chemokine production by WNV-infected astrocytes, we analyzed

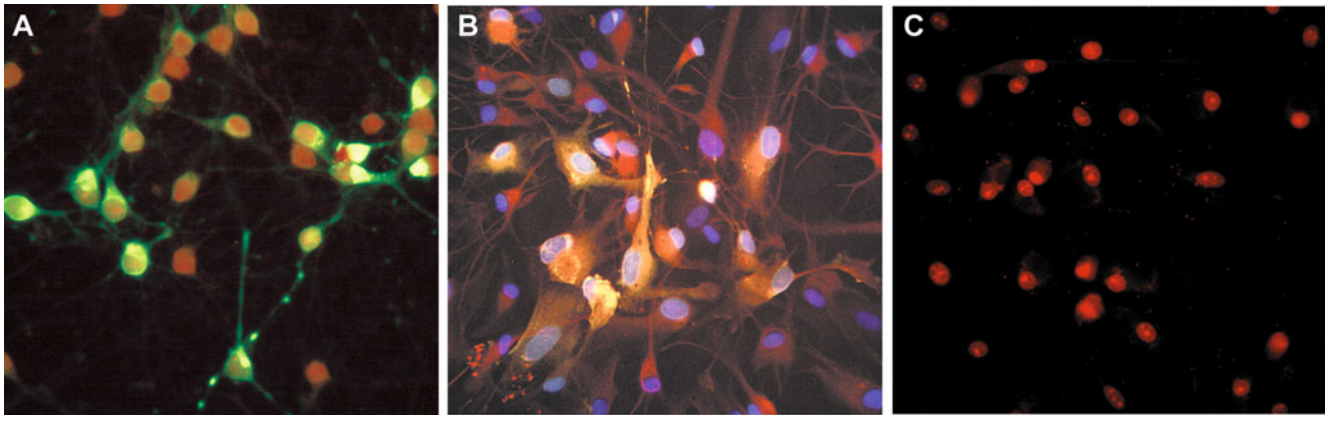

Figure 2 WNV E protein expression in infected brain cell cultures. (**A**) Enriched neuronal, (**B**) astrocyte, and (**C**) microglial cell cultures infected with WNV NY-99 were immunostained for WNV E (envelope) protein at 72 h p.i. (**A**) Double immunostaining for WNV E protein expression (FITC) and neuronal-specific nuclei (NeuN; Texas red) in infected neuronal cultures. (**B**) WNV E protein (FITC), astrocyte-specific GFAP (Texas red), and DAPI counter stain demonstrates WNV expression in infected astrocytes. (**C**) Microglial cultures showing PI stained nuclei (*red*) and lack of WNV antigen.

Table 1 Cytokine and chemokine production from WNV infected glial cells

	Microglia	*Astrocytes*
TNF-α	+++	−
IL-1β	±	−
IL-6	+++	−
IL-10	−	−
IFN-α	−	−
IFN-γ	−	−
CXCL10/IP-10	++++	+++
CCL2/MCP-1	++	±
CCL5/RANTES	+++	+

supernatants of virus-infected cultures for the expressed proteins at 8, 24, 48, and 72 h p.i. For both CXCL10 and CCL5, the highest levels of production were seen at 72 h p.i., the final time point tested. The rise in CXCL10 and CCL5 production began at 24 h p.i., with an 86-fold increase in CXCL10 and a 26-fold increase in CCL5 over mock-infected controls. CXCL10 levels measured at 48 and 72 h p.i. were 3.44 ± 0.44 and 4.54 ± 0.98 ng/ml, which represents 491- and 757-fold increases, respectively, over mock-infected controls. Levels of CCL5 were 73.25 ± 2.78 and 113.22 ± 8.02 pg/ml at 48 and 72 h p.i., respectively, representing 28- and 42-fold increases over control samples at these time points (Figure 3).

Chemokine and cytokine mRNA expression were assessed in WNV-infected cells. Quantitation by densitometry demonstrated that CXCL10 mRNA expression increased by 21.7 ± 2.22 fold, whereas CCL5 expression was found to be 5-fold higher in WNV-infected astrocytes at 24 h p.i. than uninfected controls (Figure 4A). CCL2 was constitutively expressed in astrocytes and an increase in mRNA could not be detected following infection. Consistent with the findings of undetectable cytokine proteins, cytokine mRNA expression was not detected by ribonuclease protection assay (RPA) analysis of WNV-infected astrocytes (Figure 4B).

Chemokine production in astrocytes requires replication competent virus

To determine if replication competent virus was essential for astrocyte chemokine response to infection, the virus was inactivated by exposure to ultraviolet (UV) light. UV inactivation abrogated the ability of the virus to induce chemokine production in astrocytes (Figure 3). Levels of CXCL10 induced by UV-inactivated WNV were 0.04 ± 0.01 ng/ml (compared to 4.54 ± 0.98 ng/ml with replication competent virus) at 72 h p.i. CCL5 production was also reduced by UV inactivation (16.50 ± 4.28 pg/ml versus 113.22 ± 8.02 pg/ml using replication competent virus).

Time course of cytokine and chemokine production by WNV-infected microglial cells

Of the cytokines tested, TNF-α was among the first induced by WNV stimulation of microglial cells. As early as 8 h p.i. TNF-α levels increased 9-fold over mock-infected controls to 189.33 ± 21.39 pg/ml (Figure 5A). TNF-α release from virus-stimulated microglia declined to near that of uninfected cultures by 72 h p.i. Induction of IL-6, on the other hand, increased gradually beginning at 24 h p.i. (16.00 ± 3.86 pg/ml), 48 h p.i. (129.50 ± 26.40 pg/ml), and was the highest at 72 h p.i. (144.60 ± 22.15 pg/ml) (Figure 5B). IL-6 was

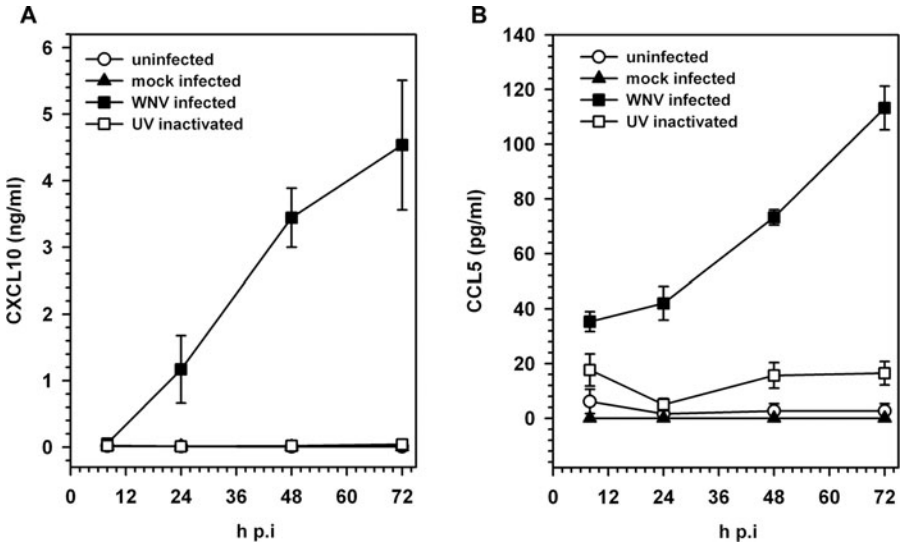

Figure 3 WNV-induced chemokine production in astrocytes. Culture supernatants from WNV-infected, mock-infected, and uninfected astrocyte cultures were collected at various times p.i. (**A**) CXCL10 and (**B**) CCL5 production from infected cell culture supernatants was quantified by ELISA. Data presented are mean ± SEM of pooled data from two or three separate experiments using cells derived from different brain specimens.

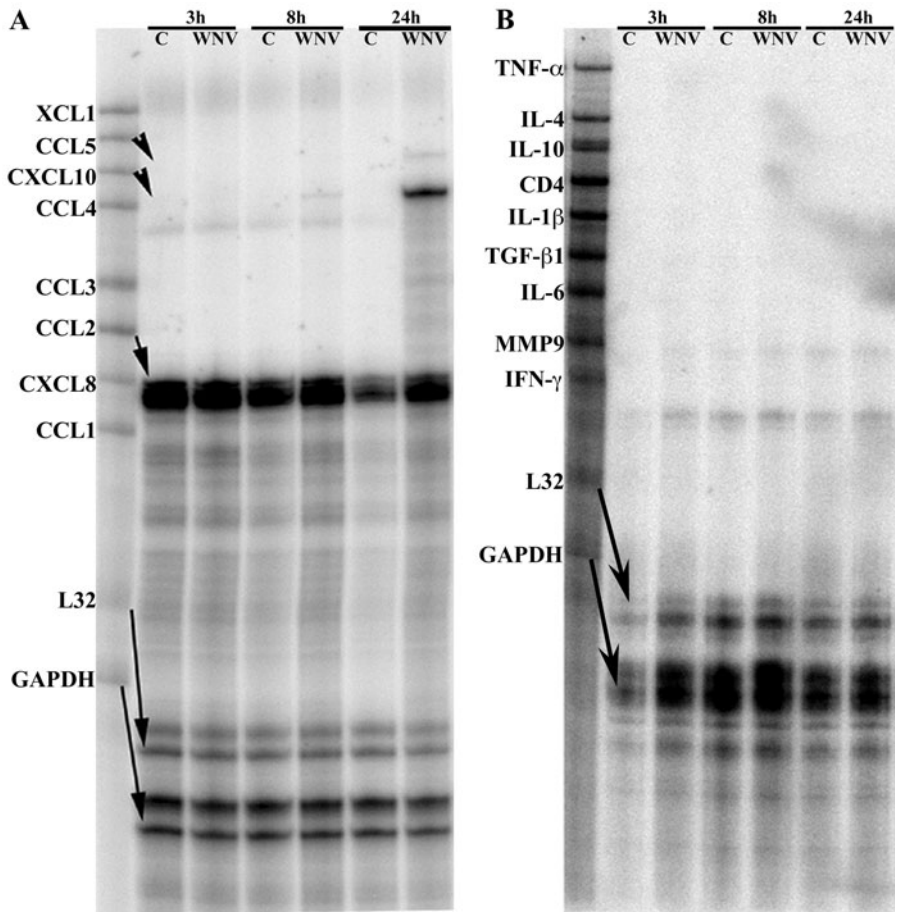

Figure 4 Astroglial responses to WNV infection. Total RNA (2.5 μg) extracted from uninfected (C) and infected (WNV) astrocyte cultures were analyzed by RPA for (**A**) chemokine and (**B**) cytokine gene expression at 3, 8, and 24 h p.i. RPA gels presented are representative of two separate experiments using cells derived from different brain specimens.

undetectable (<10 pg/ml) in supernatants from mock-infected or uninfected microglial cell cultures.

The time course for chemokine production (CXCL10, CCL2, and CCL5) by WNV-stimulated microglial cells was similar for each chemokine tested, rising throughout the period of the study (Figure 6). Levels of CXCL10 released by WNV-stimulated cells were 60-fold greater than mock-infected cultures at 24 and 48 h p.i. (3.92 ± 1.32 versus 0.06 ± 0.019 ng/ml; Figure 6A). CXCL10 production was highest at 72 h p.i., measuring 8.56 ± 1.46 ng/ml (145-fold increase over control). CCL2 (Figure 6B) and CCL5 (Figure 6C) release by WNV-infected microglia followed a pattern similar to CXCL10, reaching levels of 2.79 ± 0.195 ng/ml (4-fold greater than control) and 0.74 ± 0.05 ng/ml (40-fold greater than control) at 72 h p.i., respectively.

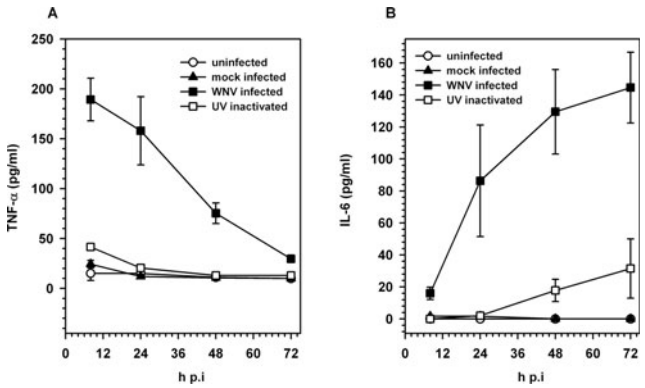

Figure 5 WNV induces cytokine production in microglial cells. (**A**) TNF-α and (**B**) IL-6 levels from WNV-infected and uninfected microglial cultures were measured from the supernatant at various times p.i. using an ELISA assay. Pooled data from three separate experiments using different brain specimens are presented as mean ± SEM.

To confirm the cytokine and chemokine protein production data, RPA analysis of WNV-infected microglial cell cultures was performed. A clear increase (over uninfected controls) in both cytokine (Figure 7A) and chemokine (Figure 7B) RNA expression was observed. TNF-α mRNA expression increased by 6.3 ± 0.47-fold, IL-6 by 2.1 ± 0.60-fold, CCL5 by 5.5 ± 0.76-fold, CXCL10 by 9.0 ± 1.12-fold, CCL3 (macrophage inflammatory protein-1; MIP-1α) by 5.7 ± 0.02-fold, CCL4 (MIP-1β) by 7.0 ± 0.04-fold, CCL2 by 2.6 ± 0.93-fold, and CXCL8 (IL-8) 1.5 ±

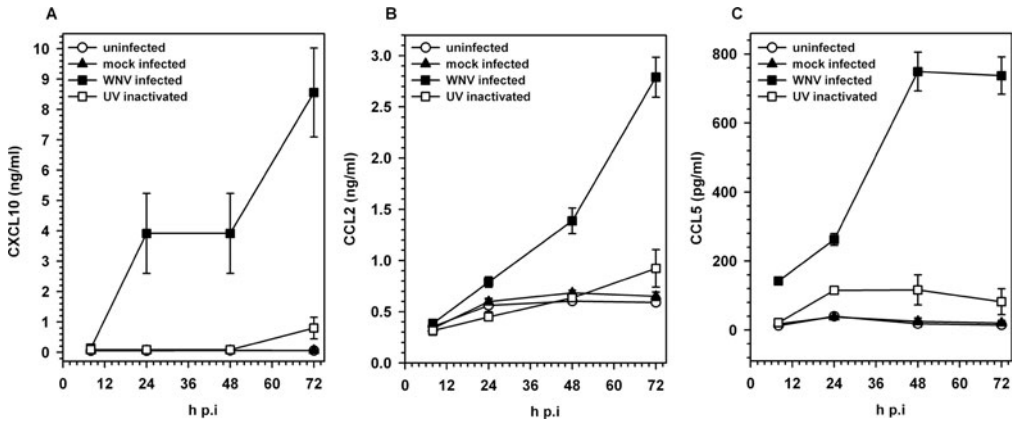

Figure 6 Chemokine production by WNV-infected microglia. Microglial cell culture supernatants were collected at various times p.i. with WNV. (**A**) CXCL10, (**B**) CCL2, and (**C**) CCL5 production from infected cell culture supernatants was quantified by ELISA. Results presented are mean ± SEM of pooled data from two to three separate experiments using cells derived from different brain specimens.

0.68-fold at 24 h p.i. Induction of CCL5 and CXCL10 mRNA were the highest at 24 h p.i., whereas CCL3 (1.4 ± 0.55-fold increase), CCL4 (1.8 ± 0.72-fold increase), CCL2 (2.6 ± 1.6-fold increase), and CXCL8 (1.3 ± 0.13-fold increase) was observed as early as 3 h p.i.

UV inactivation of WNV abrogates microglial cell cytokine/chemokine production

Although microglial cells were not productively infected with WNV, we tested the ability of replication incompetent virus to stimulate cytokine and chemokine production. Exposure of virus to UV light

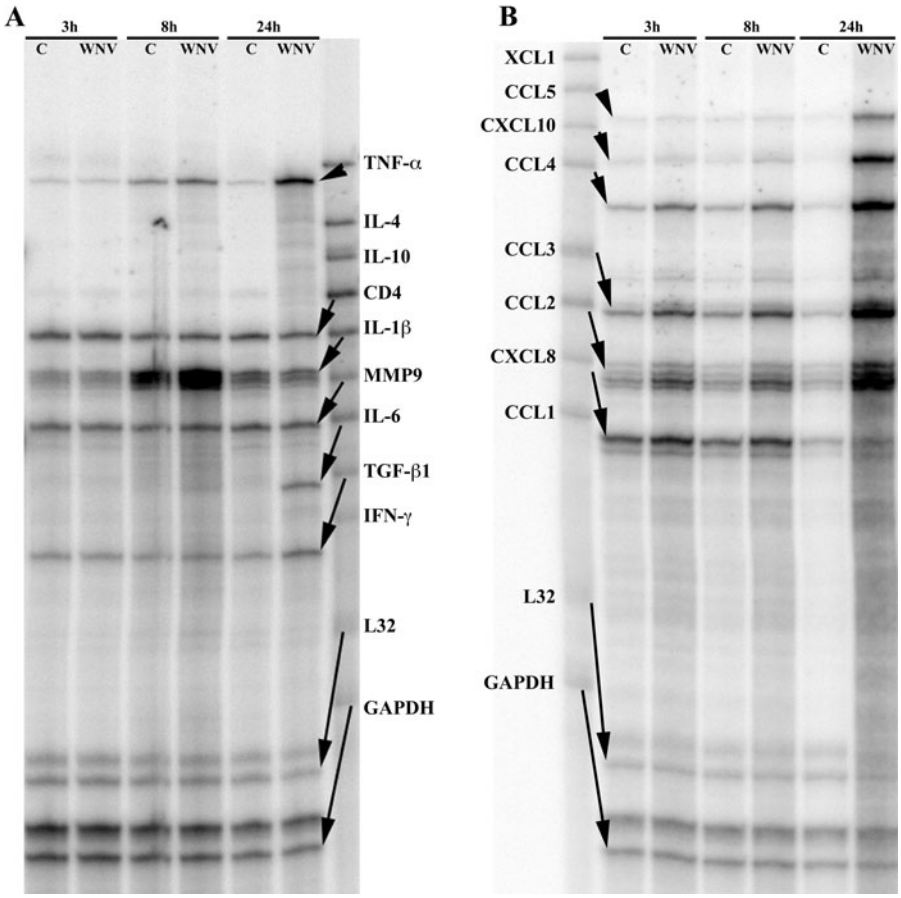

Figure 7 Microglial cell response to WNV infection. Total RNA (2.5 μg) extracted from uninfected (C) and infected (WNV) microglial cell cultures were analyzed by RPA for (**A**) cytokine and (**B**) chemokine gene expression at 3, 8, and 24 h p.i. Gels presented are representative of two separate experiments using cells derived from different brain specimens.

decreased WNV-induced cytokine and chemokine production in microglia. WNV-induced TNF-α production was markedly reduced from 8- to 12-fold to 1.5- to 2-fold with UV-inactivated virus at 8 and 24 h p.i. (Figure 5A). IL-6 production also decreased from 144.60 $\pm$ 22.15 pg/ml with replication competent virus to 31.50 $\pm$ 18.55 pg/ml using UV-inactivated virus (Figure 5B).

Levels of CXCL10 from microglia stimulated with UV-inactivated virus was found to be similar to mock-infected cells at 48 h p.i. (0.09 $\pm$ 0.05 versus 0.06 $\pm$ 0.02 ng/ml), but was 13-fold higher than controls at 72 h p.i. (0.78 $\pm$ 0.34 in stimulated versus 0.06 $\pm$ 0.03 ng/ml; Figure 6A). This contrasts, however, with a 145-fold increase when replication competent virus was used for stimulation. Production of CCL5 was also decreased by UV-inactivated virus resulting in 3- to 5-fold increases in production levels compared to mock controls versus 40-fold increase with replication competent virus (Figure 6C). UV inactivation totally abolished any increase in WNV-induced CCL2 production in microglia (Figure 6B).

Involvement of mitogen-activated protein kinase (MAPK) signals transduction pathways in WNV stimulation of microglial cells

We next investigated if WNV infection of microglial cells would activate MAPK signaling pathways. Levels of phosphorylated p38 MAPK, extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase (JNK) were measured using a cell-based enzyme-linked immunosorbent assay (ELISA) (Fast activated cell-based ELISA, FACE; Active Motif, Carlsbad, CA) in WNV-treated, mock-treated, and untreated microglial cells (30 min p.i.). We observed a significant rise in p38 and ERK MAPK activation with WNV treatment, reaching an average of 2.7-fold increase in levels of p38 MAPK phosphorylation and 3.6-fold increase in ERK phosphorylation over control cells. Phosphorylation of JNK was also increased with WNV stimulation (1.7-fold over control; Figure 8).

To determine if p38 and ERK signaling played a role in the induction of chemokines by WNV, microglial cells were treated for 30 min with SB202190 and PB98059, specific inhibitors for p38 and ERK pathways, respectively, prior to WNV infection. Chemokine and cytokine levels in treated and untreated culture supernatants were measured at 48 h p.i. The p38 MAPK specific inhibitor, SB202190, inhibited CXCL10 production by 28% and 69% at concentrations of 3 and 30 μM, respectively. However, inhibition of the ERK pathway did not have an effect on WNV-induced CXCL10 production (Figure 9A). Both CCL2 (Figure 9B) and CCL5 (Figure 9C) levels were reduced with either inhibitor by 55% at the highest concentration used. Taken together, these findings suggests that although CXCL10 production is mediated through the activation of the p38 MAPK pathway, WNV may induce CCL2 and CCL5 production through the activation of both the p38 and ERK pathways.

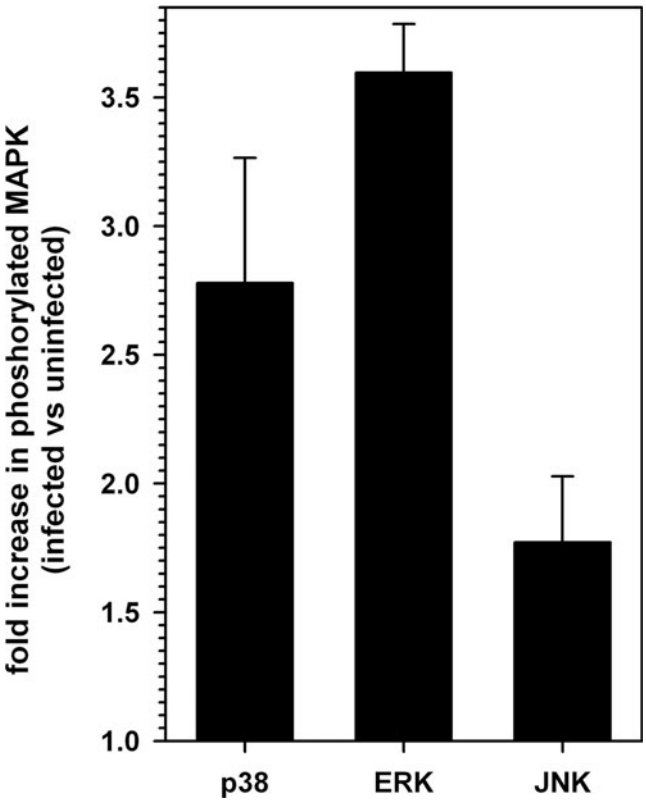

Figure 8 WNV infection activates the MAP kinase pathway in microglial cells. Phosphorylated p38 MAP kinase, ERK, and JNK were quantitated by FACE (a cell-based ELISA) assay. Levels of activated p38 MAPK (p38), ERK, and JNK were measured by ELISA and normalized to the levels of total kinase expression from microglial cell cultures infected with WNV (MOI = 5) at 30 min p.i. Data are presented as fold increase of activated kinase (normalized to total kinase expression) in infected cells over levels from uninfected cells. Data presented are pooled from three separate experiments using cells derived from different brain specimens.

Discussion

Microglia and astrocytes are the principal cells within the CNS responsible for initiating, regulating, and maintaining neuroimmune responses to viral infections. Activated glial cells are known to produce numerous mediators, including cytokines and chemokines, that orchestrate both the defense against and the pathogenesis of CNS viral infections (Benveniste, 1997; Rock *et al*, 2004; Schneider-Schaulies *et al*, 1997). In this study, we characterized for the first time both the susceptibility of these glial cell populations to infection by WNV and their corresponding cytokine/chemokine profiles induced by this virus.

Both *in vitro* and *in vivo* studies have demonstrated that neurons are the primary targets for WNV replication (Ceccaldi *et al*, 2004; Shrestha *et al*, 2003;

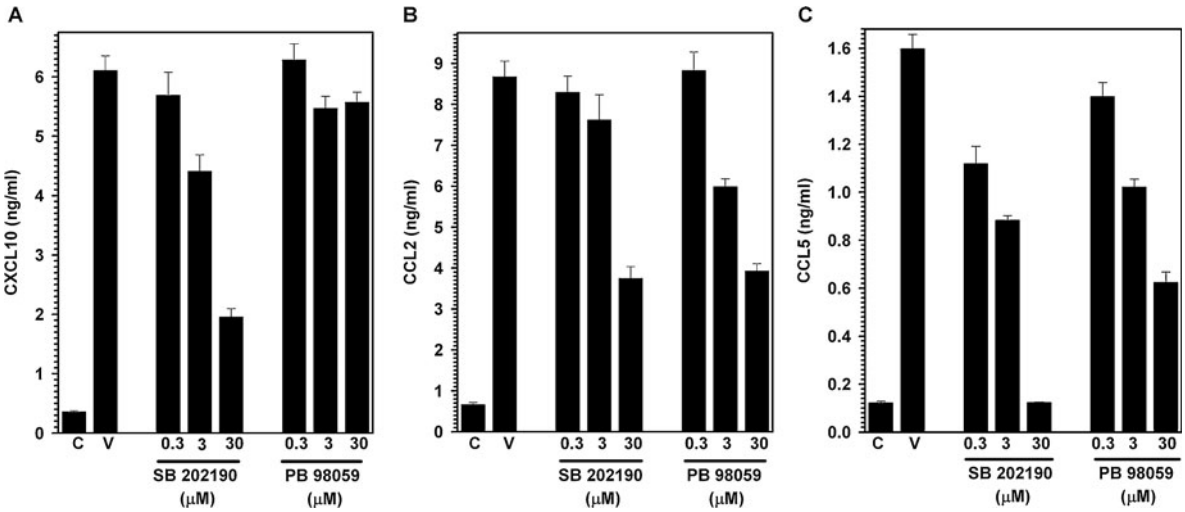

Figure 9 Inhibition of MAP kinase activation abrogates WNV-induced chemokine production in microglia. Microglial cells were treated with specific inhibitors of p38 MAP kinase (SB202190) and ERK (PB98059) 30 min prior to WNV infection. Culture supernatants were tested for (**A**) CXCL10, (**B**) CCL2, and (**C**) CCL5 at 48 h p.i. (C, uninfected cultures; V, WNV infected cultures). Pooled data from two to three separate experiments using cells derived from different brain specimens are presented.

Xiao *et al*, 2001). The results of the present study using highly enriched human neuronal cell cultures demonstrated robust viral replication that was sustained for the 2 weeks of cell culture. In addition to neurons, human astrocytes, the major glial cell type in the brain, supported productive WNV replication. Previous *in vitro* studies have shown that rodent astrocytes (Liu *et al*, 1988, 1989) and human oligodendroglial cell lines (Jordan *et al*, 2000) support WNV replication. Although it is unknown whether astrocytes are also infected by WNV *in vivo*, the results of this *in vitro* study suggest that this may be the case. Microglial cells, the resident macrophages of the brain, on the other hand, were found to poorly support virus replication. Other investigators have shown that WNV replicates in activated rodent peritoneal macrophages (Cardosa *et al*, 1986; Pantelic *et al*, 2005) and dendritic cells (Johnston *et al*, 2000; Pierson *et al*, 2005). The relatively poor replication of WNV witnessed in our study could be explained by a different activation state or source of macrophages (peritoneal cavity versus brain), species differences (rodent versus human), or viral strains. Furthermore, recent studies have shown that WNV uses a specific integrin molecule ($\alpha V\beta 3$) for entry into cells (Chu and Ng, 2004). Little is known about the expression of these receptors on microglial cells. Nevertheless, the central role that macrophages play in WNV dissemination (Johnston *et al*, 2000) and in stimulating protective immune responses (Ben-Nathan *et al*, 1996; Kulkarni *et al*, 1991) is indisputable. In the human brain, WNV-infected neurons are associated with microglial nodule formation and lymphocyte infiltration (Granwehr *et al*, 2004; Guarner *et al*, 2004). This distinctive pathology is indicative of local tissue responses to either viral replication or to virus-induced cellular damage (Xiao *et al*, 2001).

Chemokines play a pivotal role in recruitment of leukocytes to sites of infection within the CNS (Rempel *et al*, 2004). Among the 40 chemokines that have been identified, only CX3CL1 (fractalkine) and CXCL12 (stromal cell derived factor-1α) are constitutively expressed in the CNS, whereas CCL2, CCL3, CCL4, CCL5, CXCL8, CXCL9, and CXCL10 are the predominant chemokines induced during inflammation (for review see Cartier *et al*, 2005; Hesselgesser and Horuk, 1999). It has been well documented that microglia and astrocytes are the major sources of chemokines in the brain (Oh *et al*, 1999). In addition, WNV infection has been shown to induce CCL3, CCL4, and CXCL10 production within the brain (Shirato *et al*, 2004). However, the source of these chemokines during WNV brain infection remains unknown.

We found that WNV induced robust chemokine responses in both astrocytes and microglia, with temporal increases in CXCL10 and CCL5 production. CCL2 production in astrocytes was not detectable above the constitutive levels produced by mock-infected cultures, but increased production of CCL2 was demonstrated in WNV-infected microglial cells. Brains of transplant patients with WNV encephalitis show diffuse macrophage influx in the periventricular white matter (Kleinschmidt-DeMasters *et al*, 2004), indicative of a glial cell–initiated chemotaxic response to infection (Cheeran *et al*, 2001; Lokensgard *et al*, 2002). This infiltration of lymphocytes is clearly protective in some models of viral brain infection (Cheeran *et al*, 2004, 2005), but detrimental in others (Christensen *et al*, 2004). WNV infection in mice spreads into the CNS from a peripheral inoculation site regardless of the immune status of the animal. The CNS viral burden increases dramatically and infection becomes fatal when a protective

immune response is not engendered in the periphery (Diamond *et al*, 2003b; Wang *et al*, 2003a). Although it is clear that outcomes of WNV brain infection are controlled by the peripheral immune response, the production of chemokines by activated glial cells is likely to play a role in attracting lymphocytes and monocytes into the infected brain as well as in directing traffic of these cells and microglia to the sites of WNV infection within the CNS.

Cytokine production by activated microglia early during viral infection results in the amplification of inflammation, ultimately influencing the pathogenesis of infection (Binder and Griffin, 2003). Despite the inability of microglial cells to support productive WNV replication, we found that these glial cells responded rapidly to viral infection by producing cytokines. TNF-α levels from WNV-infected microglial cells peaked at 8 h p.i. and quickly decreased over the next 48 h. Conversely, IL-6 production was delayed, and peaked at 48 to 72 h p.i. The level of TNF-α, along with other proinflammatory cytokines, in the CSF is a key indicator of survival during JEV infection in humans (Winter *et al*, 2004). On one hand, although TNF-α inhibits WNV replication in some cell types (Cheng *et al*, 2004), production of this cytokine *in vivo* results in a leaky BBB (Wang *et al*, 2004), leading to increased neurovirulence. The distinct lack of TNF-α, IL-1β, IL-6, and IFN-α production from WNV-infected astrocytes found in this study was similar to results seen during human cytomegalovirus (CMV) and herpes simplex virus (HSV) infection. Astrocytes, which support productive CMV and HSV replication, were not found to produce cytokines in response to infection by these viruses (Cheeran *et al*, 2001; Marques *et al*, 2004).

A rapid local response to viral infection of the brain is critical to mount an effective defense against the invading pathogen. In the present study, we found that WNV stimulation of microglial cells resulted in rapid activation of p38 MAPK and ERK intracellular signaling pathways. Inhibition of either p38 MAPK or ERK decreased CCL2 and CCL5 production, whereas inhibition of p38 MAPK, but not ERK, abrogated CXCL10 production. Binding of the WNV E protein to cell surface integrin molecules activates FAK (focal adhesion kinase) (Chu and Ng, 2004), which in turn activates a number of docking proteins that mediate signaling to several downstream pathways, including the MAP kinase pathway (Parsons, 2003). Interestingly, UV inactivation of WNV decreased both chemokine and cytokine production by microglial cells indicating that replication competent virus is required for production of these immune mediators. Viral nucleic acids are known to engage Toll-like receptors (TLRs) on macrophages to induce cytokines and chemokines. Recently it has been shown that the presence of TLR3 in mice correlated with an increase in TNF-α production (Wang *et al*, 2004). TLR3, which is expressed on both astrocytes (Farina *et al*, 2005) and microglia (Olson and Miller, 2004), recognizes viral double-stranded RNA (Edelmann *et al*, 2004). Engagement of this receptor occurs within the endosomal compartments of the infected cells (Malmgaard *et al*, 2004), resulting in rapid activation of MAPK signaling pathways (Guillot *et al*, 2005). UV inactivation may destroy the ability of WNV nucleic acids to engage TLR3 efficiently, possibly due to the lack of viral RNA accumulation during infection with replication deficient virus (Malmgaard *et al*, 2004).

The current state of knowledge regarding the pathogenesis of WNV encephalitis is based on studies that have defined the role of systemic immune responses to WNV. Limited studies have been designed to understand the role of glial cells in the defense of or damage to the brain once WNV has gained access to this "immunologically privileged" site. In spite of the limitations of using *in vitro* models, the results of this study using primary human neuronal, astroglial, and microglial cell culture models demonstrate several possible glial cell responses to WNV infection in the human host. These data have now set the stage for further studies, using relevant biological systems, to investigate contributions of glial cells and their mediators to defense and neuropathogenesis of WNV infection, define the cellular specificities of WNV replication, and understand the role of cytokine and chemokine production profiles in different clinical outcomes. Unraveling these processes may yield novel insights into the development of effective therapies for WNV encephalitis.

Materials and methods

Cell lines and virus isolate

Vero cells (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 7.5% fetal bovine serum (FBS). WNV NY-99 (NY-99-35262-11) was kindly provided by Dr. Duane Gubler, the Center for Disease Control and Prevention, Fort Collins, CO (Lanciotti *et al*, 1999). The original viral stock was isolated from an infected Flamingo using Vero cells, passaged once in C6 (mosquito) cells and suckling mice, prior to propagation in our laboratory by a single passage in Vero cells. Clarified cell culture supernatants were titrated by plaque assay on Vero cell monolayers and used in all the experiments described, at a MOI of 1 for viral replication experiments and MOI of 5 to investigate glial cell responses. Mock-infected Vero cell cultures processed in the same manner as viral stocks were used to evaluate nonspecific glial cell stimulation. UV inactivation of WNV was achieved by placing viral stocks on ice at a distance of 8 cm from a 256 nm UV light source for 20 min. Replication competent virus could not be detected from viral stocks inactivated using this method.

Primary human brain cell cultures

Primary human fetal microglial cells, astrocytes and neurons were prepared as described previously (Chao *et al*, 1994; Chao and Hu, 1994; Cheeran *et al*, 2001). Briefly, fetal brain tissues obtained under a protocol approved by the Institutional Human Research Subjects Committee from human abortuses at 16 to 22 weeks of gestation were cleared of meninges and dissociated by passing repeatedly through a pipette under sterile conditions. The triturated tissue was incubated with 0.125% trypsin for 45 min at 37°C to make a single cell suspension. The cell suspension was seeded at 75–100 $\times$ 10^6 cells in 75-cm^2 tissue culture flasks and incubated in a humidified incubator at 37°C with 6% CO_2. Cultures were grown for 2 weeks with weekly changes of medium. Microglial cells floating in the medium and those loosely attached to the monolayer were harvested by gentle shaking. The harvested cells were seeded into tissue culture plates (2×10^5 cells/well in a 48-well culture plate or 2×10^6 cells/well in a 6-well plate). The plated cells were washed after 60 min incubation at 37°C. Microglial cells used in these experiments were $\geq$99% pure, as determined by CD68 antibody staining. Less than 1% of the cells stained with antibodies to glial fibrillary acidic protein (GFAP), an astrocyte marker.

Primary astrocyte cultures were prepared by shaking the flasks, after 21 days in culture, at 180 to 200 rpm for 16 to 18 h. The monolayer was then washed with Hank's buffer to remove any floating, nonastroglial cells. The adherent cells were trypsinized and seeded into fresh flasks with medium change 24 h after plating. This procedure was repeated three to four times at weekly intervals. The final cultures, which contained $\geq$99% astrocytes (GFAP-positive cells), were grown in tissue culture plates for 3 to 5 days prior to use.

To prepare highly purified neuronal cultures, dispersed, single cell suspensions of cortical brain tissue were plated for 24 h as described above. The cultures were extensively washed and maintained in DMEM containing 10% FBS. After 5 days in culture, the medium was changed with DMEM containing 10% FBS, uridine (33.6 mg/ml; Sigma) and fluorodeoxyuridine (13.6 mg/ml; Sigma) for 24 h. By day 12, the cultures were highly enriched for neurons (approximately 90% neurons, 5% astrocytes, and <5% microglia).

Cytokine and chemokine ELISA

A previously described (Peterson *et al*, 1997) sandwich ELISA–based system was used to measure levels of cytokines and chemokines in the supernatants of WNV-infected glial cell cultures. ELISA plates (96-well) were coated with mouse-anti-human cytokine or chemokine antibodies (R&D Systems, Minneapolis MN) at 1 to 2 μg/ml overnight at 4°C. The plates were washed (0.05% Tween-20 in phosphate-buffered saline [PBS]) and blocked with 1% bovine serum albumin (BSA) in PBS for 1 h at 37°C. Serial dilutions of known concentrations of the respective cytokines and chemokines were used to generate standard concentration curves for each assay. Supernatants from WNV-infected, uninfected, or mock-treated cell cultures were incubated in capture-antibody coated wells for 2 h at 37°C. Detection antibody (goat anti-human cytokine or chemokine antibody) was then added (1 to 2 μg/ml; Pharmingen, San Diego, CA), for 90 min at 37°C. This was followed by donkey anti-goat horseradish peroxidase (HRPO) conjugated secondary antibody (1:4000; Jackson Immunoresearch, West Grove, PA) for 45 min. A chromogenic substrate (K-blue; Neogen Corporation, Lexington, KY) was then added for 5 to 10 min at room temperature. Color development was stopped with 1 M H_2SO_4. Levels of cytokines and chemokines in culture supernatants were estimated from the standard concentration curves using absorbance values at 450 nm. The sensitivity of this ELISA assay is 10 to 30 pg/ml depending on the cytokine/chemokine assayed.

Ribonuclease protection assay

To determine the levels of cytokine and chemokine mRNA expression, total RNA was extracted from uninfected and WNV-infected (MOI = 5) astrocyte and microglial cell cultures (Qiagen, Valencia, CA). The RNA was assayed using RiboQuant (Pharmingen, San Diego, CA) a multiprobe ribonuclease protection assay (RPA), following the manufacturer's instructions. Briefly, total RNA was extracted at 3, 8, and 24 h p.i. The RNA was hybridized to ^{32}P-labeled multiprobe templates specific for the indicated chemokines or cytokines and resolved on a 5% denaturing polyacrylamide gel (19:1 40% acrylamide/bis, 10$\times$ Tris-borate-EDTA, and urea) and analyzed using a phosphoimager (Molecular Dynamics, Sunnyvale, CA). Undigested probes, which have a higher molecular weight, were used as markers to identify protected cytokine/chemokine mRNA fragments. Relative expression of cytokine or chemokine mRNA was calculated by normalizing the band density of protected fragments to Glyceraldehyde-3-phosphate dehydrogenase GAPDH from each sample. Increases in RNA expression from WNV-infected cultures (versus uninfected cultures at each time point) were averaged from multiple gels and expressed as mean $\pm$ standard deviation.

Assay to measure MAPK activation

Levels of total and activated MAPK (p38, ERK, and JNK) were measured in microglial cells by a sensitive Fast Activated Cell–based ELISA (FACE; Active Motif, CA) designed to measure proteins activated by phosphorylation (Ajmone-Cat *et al*, 2003). Microglial cells were cultured overnight in 96-well tissue culture plates at a density of 2×10^4 cells per well. WNV-infected (MOI = 5), uninfected, or mock-infected microglia were fixed 30 min p.i. and incubated with antibodies to phosphorylated or total MAPK, as

recommended by the manufacturer. Changes in the ratio of phosphorylated to total MAPK proteins were measured by a colorimetric detection system with secondary HRPO-conjugated antibodies.

Immunocytochemical staining

WNV-infected and uninfected cells on tissue culture chamber slides (LabTec) were fixed with 4% paraformaldehyde and 0.1% gluteraldehyde (in PBS) at room temperature (RT) for 20 min. The cells were permeablized (0.1% Triton X-100, 0.1% bovine serum albumin [BSA], and 10% normal donkey serum in PBS) for 45 min at RT. After washing with PBS (0.1% BSA), the slides were incubated with primary antibody overnight at 4°C. The primary antibodies used in this study were: anti-WNV E protein monoclonal antibody (Mab 8150; Chemicon, Temecula, CA), biotinylated anti-neuronal nuclei antibody (NeuN; Chemicon), rabbit anti-GFAP antibody (Incstar, Stillwater, MN), and anti-CD68 Mab (Dako, Carpinteria CA). After additional washing and incubation with appropriate secondary antibody, the slides were developed with the indicated fluorochrome for 1 h at RT. The slides were washed, mounted with coverslips, and photographed under a fluorescence microscope.

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