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TNF- α -dependent regulation of CXCR3 expression modulates neuronal survival during West Nile virus encephalitis

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Abstract

The chemokine CXCL10 exerts antiviral effects within the central nervous system (CNS) through the recruitment of virus-specific T cells. However, elevated levels of CXCL10 may induce neuronal apoptosis given its receptor, CXCR3, is expressed by neurons. Using a murine model of West Nile virus (WNV) encephalitis, we determined that WNV-infected neurons express TNF- α , which down-regulates neuronal CXCR3 expression via signaling through TNFR1. Down-regulation of neuronal CXCR3 decreased CXCL10-mediated calcium transients and delayed Caspase 3 activation. Loss of CXCR3 activation, via CXCR3-deficiency or pretreatment with TNF- α prevented neuronal apoptosis during *in vitro* WNV infection. These results suggest that neuronal TNF- α expression during WNV encephalitis may be an adaptive response to diminish CXCL10-induced death.

Keywords

CXCL10; CXCR3; neurons; West Nile virus; CNS; TNF- α

Introduction

West Nile virus (WNV), an encephalitic flavivirus that infects mosquitoes, birds and mammals, directly infects neurons and may induce potentially severe neurologic sequelae in humans including encephalitis, paralysis and anterior myelitis (Campbell et al., 2002). These neuroinvasive forms of WNV infection are characterized both by neuronal death and the infiltration of mononuclear effector cells, whose activities promote both viral clearance and neuronal injury. Thus, neurons may be destroyed by a variety of direct and indirect

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mechanisms including virus- or cytokine-mediated apoptosis (Ramanathan et al., 2006; Samuel et al., 2007; Yang et al., 2002) and T cell-mediated cytolysis (Bergmann et al., 2004; Lavi and Wang, 1995; Liu et al., 1989; Parra et al., 2000; Rossi et al., 1998). Yet, in humans and in animals, recovery from neurologic deficits can and does occur (Binder and Griffin, 2001; Komatsu et al., 1999; Matsubara et al., 2007), suggesting that neurons must possess adaptive mechanisms to survive during leukocyte-mediated viral clearance so that virus is eliminated without inducing excessive damage. Consistent with this, altered neuronal expression of pro- or anti-apoptotic proteins has been observed to occur in neurotropic viral infections including those caused by herpes simplex and rabies viruses (Carpenter et al., 2008; Lafon, 2008). Few studies, however, have defined cellular mechanisms that promote neuronal survival during WNV infection despite the demonstration that WNV-infected neurons can be effectively cleared of virus *in vivo* (Hunsperger and Roehrig, 2006; Shrestha et al., 2006a).

Although the mechanisms that direct and regulate the inflammatory responses that lead to clearance of WNV from the central nervous system (CNS) are incompletely understood, prior studies have demonstrated that expression of TNF- α (Shrestha et al., 2008), CD40 (Sitati et al., 2007), chemokine receptors CCR5 and CXCR3 (Glass et al., 2005; Zhang et al., 2008) and the CXCR3 chemokine ligand, CXCL10 (Klein et al., 2005) are all essential for the antiviral activities of virus-specific CD8⁺ T cells that lead to viral clearance within the CNS. Of interest, both TNF- α and CXCL10 have also been implicated in pathways involved in neuronal apoptosis. TNF- α is a proinflammatory cytokine produced by macrophages, T and NK cells that exert both neurotoxic and neuroprotective effects in the CNS. TNF-induced neurotoxicity has been observed in models of ischemia (Hallenbeck, 2002) whereas direct neuroprotective effects have been observed via signaling through either of its two receptors, TNFR1 and TNFR2 (Bruce et al., 1996; Cheng et al., 1994; Marchetti et al., 2004). TNFR1 and TNFR2 signaling leads to activation of the antiapoptotic transcription factor NF- κ B and the Akt-mediated cellular survival pathway, respectively (Fontaine et al., 2002; Furukawa and Mattson, 1998; Tamatani et al., 1999).

CXCL10 is a member of a subfamily of non-Glu-Leu-Arg (non-ELR) T cell chemokines that recruit activated, CD45RO⁺ memory T cells and NK cells via binding to CXCR3, a G_i-coupled receptor, whose three isoforms: CXCR3A, CXCR3B and CXCR3-alt exhibit distinct affinities to the chemokine ligands CXCL4, CXCL9, CXCL10 and CXCL11 (Ehlert et al., 2004; Lasagni et al., 2003). Although the early expression of CXCL10 by WNV-infected neurons is essential for the recruitment of virus-specific T cells, for certain neurons there is the added complexity that these cells also express CXCR3 at baseline. Postmortem examination of human brain specimens determined that CXCR3 is expressed by subpopulations of neurons within the hippocampus, basal ganglia and the cerebellum (Xia et al., 2000), regions that are specifically targeted by WNV. Although few studies have evaluated the role of neuronal CXCR3 during viral infections, CSF levels of CXCL10 in patients with HIV-1 encephalitis were positively correlated with the progression of neuropsychiatric impairment, suggesting that this chemokine may directly affect neuronal functioning (Sui et al., 2004). Moreover, *in vitro* studies showed that treatment of human CXCR3-expressing cortical neurons with CXCL10 induces a dose-dependent increase in caspase-3-dependent apoptotic cell death via elevations in intracellular calcium (Sui et al., 2006). Thus, neurons face an essential dilemma in that the chemoattractant required to recruit effector immune cells may promote death of the very cells that the antiviral immune response is attempting to save.

In the present study, we evaluated the role of neuronal CXCR3 in WNV-mediated apoptosis. We demonstrate that in response to WNV infection, neurons express TNF- α , which, via activation of TNFR1, down-regulates CXCR3 expression in both infected and uninfected

neurons. Moreover, down-regulation of neuronal CXCR3 expression was observed *in vivo* in WNV-infected CNS tissues of wild-type but not TNFR1^{-/-} mice. TNF- α -mediated loss of CXCR3 prevented calcium transients in response to CXCL10 and delayed caspase 3 activation and death during WNV infection *in vitro*. These studies identify an adaptive mechanism whereby neurons can both facilitate an appropriate antiviral inflammatory response and prevent injury from the pro-apoptotic effects of the required inflammatory chemokine.

Methods

Mice

8-wk-old male and female C57BL/6J (H-2KbDb) mice (Jackson Laboratories) and their backcrossed (six to seven generations) CXCR3^{-/-} counterparts were used for all studies. The congenic backcrossed TNF-R1^{-/-} mice were a gift from J. Russell (Washington University School of Medicine, St. Louis, MO). All mice were bred at the Washington University School of Medicine, and all studies were performed in compliance with the guidelines of the Washington University School of Medicine Animal Safety Committee.

Mouse model of WNV infection

The WNV strain 3000.0259 was isolated in New York in 2000, passaged once in C6/36 *Aedes albopictus* insect cells, and was used for all studies. BHK21-15 cells were cultured as previously described. 8-wk-old age-matched mice were inoculated subcutaneously via footpad injection with 10² PFU of WNV, which was diluted in HBSS and 1% heat-inactivated FBS. For Real-time quantitative RT-PCR, CNS tissues (frontal cortex and cerebellum) were harvested after intracardiac perfusion with 20 mls phosphate-buffered saline (PBS), frozen in dry ice, and stored in -80°C until use.

Antibodies

Rat Anti-MAP-2 (Sigma-Aldrich), anti-GFAP (Dako) Abs, and unconjugated rabbit polyclonal Abs against CXCR3 (Zymed Laboratories), mouse monoclonal antibodies against WNV E protein (E16) have been described in detail elsewhere (Oliphant et al., 2005), and Alexa Fluor-conjugated secondary Abs were purchased commercially (goat anti-mouse Alexa 488, goat antirabbit Alexa 555, and donkey anti-rat Alexa 647 (Molecular Probes, Inc.).

Primary neuronal and microglial cultures

Primary cultures of purified cortical and granule cell neurons were prepared as previously described (Zhang et al., 2008). Purification via Percoll step-gradient centrifugation of granule cell neurons yields cultures containing ~97% granule cells and ~3% Purkinje neurons (52). Purity of cortical neurons prepared from embryonic day 15 (E15) mouse embryos was determined via staining with anti-MAP-2 (Sigma-Aldrich) and anti-GFAP (Dako) Abs (~95%). All experiments were performed on neurons cultured for 4–6 days. Microglial cells were obtained from mixed cell cultures of the forebrains as described previously (Zhang et al., 2002). Briefly, the forebrains of newborn C57BL/6J mice were dissected and the dissociated brain cells were seeded in a poly-L-lysine-coated 80 cm²-culture flask (Becton Dickinson Labware, Franklin Lakes, NJ). They were cultured for 8–10 days until confluence was achieved, then shaken for 1 hour at 37°C at 150 rpm. The supernatants were collected and microglia cells were obtained via differential adherence during a 10 minutes incubation period on plastic dishes. Microglia were then dislodged via cell-scraping and replated at 1 × 10⁵ cells/ml. Purity of the microglial cultures is determined by CD11b staining (>99%).

Neuronal survival studies

Cortical neurons were harvested from wild-type and congenic CXCR3^{-/-} embryonic day 15 mouse embryos and seeded at a density of 1×10^5 cells per well in poly-D-lysine/laminin-coated cover slips in 24-well plates. Cerebellar granule cell neurons were harvested from wild-type and CXCR3 mice at post-natal day 6 as previously described (Zhang et al., 2008). Primary neurons were infected over a range of virus concentrations. After a 1-h incubation at 37°C, free virus was removed by serial washing with neuron medium. At 24, 48, and 72 hours after infection, cells were stained with 7-Amino-actinomycin D, BD pharmingen (7-AAD) and then fixed with 4% PFA and scored using a Zeiss Axiovert 200 microscope. For TNF- α studies, neurons were treated with 50 ng/ml for 20 hours. TUNEL staining was performed with the Fluorescein In Situ Cell Death Detection Kit (Roche) according to the manufacturer's instructions, and the cells were counterstained with DAPI. Neurons were visualized using a Zeiss Axiovert 200 microscope. The number of TUNEL-positive cells relative to the total number of cells was determined from five to seven randomly selected fields high-power fields from coverslips from two or three independent experiments.

Viral infection of neuronal cultures

Primary neurons or microglia were infected over a range of virus doses (0.03, 0.3 and 3.0 MOI). After a 1-h incubation at 37°C, free virus was removed by serial washing with DMEM media, and cells were incubated for an additional 24 h. The production of infectious virus was measured in collected supernatants by plaque assays on BHK21 cells as previously described (Klein et al., 2005). The cellular RNA from infected neurons was harvested and purified using the RNEasy kit according to the manufacturer's instructions (Qiagen).

Immunohistochemistry

Uninfected and WNV-infected primary neurons (see above) were fixed with 2% paraformaldehyde for 20 min, washed for 4 times with 1X PBS and kept at 4 °C until use. Cells were permeabilized with 0.1% Triton X-100 (Sigma) and nonspecific antibody was blocked with 10% normal goat serum antibodies for 1 h at room temperature. Monoclonal antibodies specific for CXCR3, were added at 2 μ g/ml in PBS containing 10% goat serum and 0.1% Triton X-100 overnight at 4°C. Antibodies specific for MAP-2 or WNV antigen were then added at 2 μ g/ml for 1 hours at room temperature. Primary antibodies were detected with secondary goat antirabbit or mouse IgG conjugated to Alexa 555, Alexa 647 or Alexa 488 (Molecular Probes, Inc.) for immunofluorescence. Sections were analyzed using a Zeiss LSM 510 laser scanning confocal microscope and accompanying software (Zeiss). Volocity image analysis software (Improvision) was used to generate and analyze confocal images. Stained regions were identified by applying a classifier to exclude objects smaller than 0.1 cubic μ m and pixels of intensity less than 45 (scale 0–225).

TNF- α ELISA

TNF- α protein within the supernatants of primary neuronal cultures were assayed in duplicate with the Mouse TNF- α ELISA Ready-SET-Go kit (eBioscience) according to the manufacturer's instructions, which has a sensitivity of 8 pg/ml. The protein concentration of each supernatant was determined using a BCA protein assay (Sigma, St Louis).

Calcium flux analysis

Primary neuronal cells were seeded onto 35 mm poly-D-lysine, pre-coated microwell dishes (MatTek cultureware). Cells were washed with PBS and loaded with 5 μ M Fura-2 (Molecular Probes, Eugene, OR) for 1 hour in a dark chamber at 37°C. Cells were then washed with PBS and DMEM/N2 was added to the culture, Cells were kept at 37°C until

analyzed for calcium flux response. Loaded cells were washed twice and calcium flux buffer were added to the culture as previously described (Klein et al., 2001). For calcium flux analysis, culture dish were placed in to a 37 C warming chamber and examined under TILL microscope in a dual-wavelength excitation source fluorimeter. Group of three to eight neurons were analyzed for their responses to stimulation with chemokine CXCL10 at a concentration of 3 ug/ml. Changes of cytosolic free calcium were determined after addition of CXCL10 by monitoring the excitation fluorescence intensity in response to sequential excitation at 340nm and 380nm. The data are presented as the relative ratio of fluorescence at 340 and 380nm.. Additional experiments were performed after pretreatment of neurons with TNF- α at concentration of 50 ng/ml for 20 hours.

Real-time quantitative RT-PCR

Total RNA was prepared from WNV-infected tissues or cultured neurons as previously described (Zhang 2008). Following DNase I treatment (Invitrogen), RiboGreen (Invitrogen: Molecular Probes) was used to quantitate total RNA, and cDNA was synthesized using random hexamers, oligodT15, and MultiScribe reverse transcriptase (Applied Biosystems). A single reverse transcription master mix was used to reverse transcribe all samples to minimize differences in reverse transcription efficiency. The following conditions were used for reverse transcription: 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min. All oligonucleotide primers used for quantitative PCR were designed using Primer Express v2.0 (Applied Biosystems). CXCR3 mRNA levels were detected via quantitative RT-PCR using previously published forward and reverse primer sets and protocols (44). Calculated copies were normalized against copies of the housekeeping gene GAPDH.

In situ hybridization

Sense and antisense digoxigenin (DIG)-labeled riboprobes were synthesized using a linearized riboprobe plasmid containing a 1 kb fragment of CXCR3 cDNA (kindly provided by Dr. Richard Miller, Chicago, IL) (Tran et al., 2007), according to the manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany). This probe detects a CXCR3 mRNA form that generates all CXCR3 isoforms. The paraformaldehyde fixed frozen tissue sections were digested with 20 μ g/ml proteinase K for 5 minutes at room temperature. Sections were refixed in 4% paraformaldehyde, washed in PBS and then in situ hybridization was conducted for 20 hours at 65°C using DIG-labeled cRNA probes in hybridization buffer (formamide, 5X SSC, 200 μ g/ml yeast tRNA, 100 μ g/ml heparin, 1 Denhardt's, 0.1% Tween 20, 1% CHAPS, and 5 mM EDTA). The sections were washed with 0.2X SSC, 0.1% Tween 20 at 65°C and then treated with blocking reagent (20% sheep serum in buffer). DIG-labeled cRNA probe:mRNA hybrids were detected with anti-DIG antibody followed by antibody detection according to the manufacturer's protocol (Boehringer Mannheim, Mannheim, Germany).

Statistical analysis

In vitro experiments with neurons were assessed by an unpaired, two-tailed Student's t test. All data were evaluated using Prism software (GraphPad, San Diego, CA) with values of $p < 0.05$ considered to be statistically significant.

Results

WNV infection leads to down-regulation of CXCR3 in neurons

CXCR3 is a chemokine receptor normally expressed by NK and activated T cells that regulates the trafficking of virus-specific CD8⁺ T cells into the brain during WNV encephalitis (Zhang et al., 2008). Studies examining alternative sites of CXCR3 expression

have demonstrated constitutive expression on subpopulations of neurons in the neocortex, hippocampus, striatum, cerebellum and spinal cord (Goldberg et al., 2001; Xia et al., 2000), all targets of WNV infection (Shrestha et al., 2003). We previously determined that CXCL10, a ligand for CXCR3, is highly up-regulated by WNV-infected neurons, leading to viral clearance and improved survival (Klein et al., 2005). CXCR3 expression by virus-specific T cells was also essential for virologic control within the CNS, especially within the cerebellum (Zhang et al., 2008). Because overexpression of CXCL10 during viral encephalitis has been linked to neuronal apoptosis via CXCR3 activation (Sui et al., 2004), we wondered whether CXCR3 expression and/or signaling were altered during WNV encephalitis. To address this, we first evaluated the kinetics of expression of CXCR3 mRNA in eight week-old, wild-type B6 mice infected with WNV via quantitative RT-PCR (QPCR). Significant down-regulation of CXCR3 mRNA expression within the cerebral cortex was observed at day 3 post-infection (Figure 1A). To determine the cellular site of CXCR3 down-regulation during WNV encephalitis, *in situ* hybridization analysis was performed on brain tissue from 8-week-old mice at 3 days after WNV infection compared with age-matched, uninfected brain tissues. Consistent with our QPCR analyses, CXCR3 mRNA was detectable within hippocampal and cerebellar neurons in uninfected animals, but was decreased within neurons of these brain regions in WNV-infected animals at days 3 and 5 post-infection (Figure 1B). In the cerebella, loss of CXCR3 expression was first evident in Purkinje neurons at day 3 and then extended to granule cell neurons by day 5 post-infection. Hybridization experiments with control sense-strand probes were negative in all tissue specimens (Figure 1B). Prior studies using this murine model have demonstrated that infectious WNV may be detected within the CNS at approximately day 4 post-infection (Diamond et al., 2003) and that infiltrating CD8 T cells are not detected within the CNS until approximately one week post-infection (Shrestha and Diamond, 2004). Thus, our data suggest that CXCR3 expression is down regulated at the mRNA level in neuronal targets of WNV during early phases of viral infection. The increased levels of CXCR3 observed at day 7 post-infection have previously been correlated with CD8 T cell entry (Klein et al., 2005).

To further evaluate this phenomenon, we utilized an *in vitro* system of purified primary cortical and cerebellar granule cell (GC) neurons. These cells had previously been shown to produce infectious WNV over a wide multiplicity of infection (MOI) range (Klein et al., 2005). In comparative studies we also examined CXCR3 expression in primary microglial cultures, which also express CXCR3 and may be infected with WNV *in vitro* (Cheeran et al., 2005). Examination of CXCR3 mRNA by QPCR revealed significant down-regulation of expression in neurons at most MOIs tested with no change in CXCR3 mRNA expression in WNV-infected microglia (Figure 2A). Examination of neurons and microglia treated with various doses of CXCL10, which is highly up regulated by WNV-infection did not induce any changes in CXCR3 mRNA levels (Figure 2B). Immunohistochemical localization of CXCR3 within WNV-infected primary neurons revealed significant decreases in receptor expression in both perikarya and neurites compared with uninfected primary neurons (Figure 2C and 2D). WNV-infected neurons exhibited similar levels of expression of microtubule associated protein (MAP)-2, a neuronal cell marker, as uninfected neurons, suggesting that the decreased expression of CXCR3 in WNV-infected neurons was not due to neuronal injury or death (Figure 2D and 2E). Examination of WNV-infected cultures also revealed that uninfected neurons within WNV-infected cultures exhibited reduced expression of CXCR3, suggesting CXCR3 down-regulation might be induced via a cell-non-autonomous mechanism (Figure 2F).

Loss of CXCR3 is associated with increased survival of WNV-infected neurons

While the role of CXCR3 in neuronal function is poorly understood, several studies suggest its activation induces injury (Sui et al., 2004; Sui et al., 2006; Zhu et al., 2009). As WNV-

infected neurons express ligands for CXCR3 (Klein et al., 2005), we speculated that down-regulation of CXCR3 during infection might improve neuronal survival and thereby influence viral replication. To address this, GC and cortical neurons were generated from wild-type and CXCR3^{-/-} mice, infected with WNV and evaluated via multi-step viral growth curves. No differences in viral growth were observed between wild-type and CXCR3^{-/-} GC neurons infected at MOIs of 3.0 (Figure 3A), 0.3 or 0.03 (data not shown). Similar results were observed with WNV-infected cortical neurons (data not shown). Despite the lack of effect of CXCR3-deficiency on viral replication, WNV-infected CXCR3^{-/-} GC neurons had significantly higher survival rates, as assessed by 7-Aminoactinomycin D (7-AAD) viability staining, at 24, 48 and 72 hours post-infection (Figure 3B, *p < 0.05). These data suggest that CXCR3 signaling leads to cell death at early time-points after WNV infection of neurons, presumably via WNV-mediated expression of CXCL10.

WNV-mediated expression of TNF- α by infected neurons down-regulates CXCR3

CXCL10 expression is highly induced in a variety of cells by stimulation with type I and II interferons, which may be augmented by treatment with TNF- α in certain cell types (Luster et al., 1988; Ohmori et al., 1993). Recent studies indicate that WNV-infected neurons express low levels of type I interferons during the first 24 hours post-infection (Daffis et al., 2007). Because neurons express TNF- α in murine models of acute and chronic brain injury (Janelins et al., 2008; Knoblich et al., 1999), we hypothesized that WNV-induced expression of this cytokine in neurons might regulate the expression of CXCL10 and CXCR3. To test this, we assessed TNF- α expression in cultured GC and cortical neurons. Primary cultures of purified GC neurons exhibited significant up-regulation of TNF- α mRNA and protein that positively correlated with ranges of MOI (Figure 4A and 4B). Similar results were observed using primary cultures of cortical neurons (data not shown). Analysis of neuronal expression of the two TNF- α receptors, TNFR1 and TNFR2, revealed that uninfected and WNV-infected GC neurons expressed uniform levels of TNFR1 mRNA, but very little TNFR2 mRNA (Figure 4C). Immunocytochemical studies of TNF- α receptor expression in uninfected, cultured GC neurons detected only TNFR1 (Figure 4D). Uninfected microglia constitutively expressed TNFR2, as previously described (Dopp et al., 1997), but this receptor was not detected on neurons. As microglia are therefore not a target for WNV or TNF- α , our data demonstrating that CXCR3 expression levels in this cell type are unchanged during WNV infection is not surprising. As expected, pretreatment of primary cultures of GC neurons for 24 hours with TNF- α led to a dose-dependent increase in CXCL10 mRNA levels (Figure 5A). Analysis of CXCR3 mRNA levels expressed by the TNF- α -treated GC neurons, however, revealed a dose-dependent decrease in this receptor (Figure 5B).

Prior studies have demonstrated that CXCR3 activation in neurons leads to transient increases in intracellular calcium, as seen in leukocytes (Coughlan et al., 2000; Nelson and Gruol, 2004; Sui et al., 2006). To determine whether TNF- α -mediated down-regulation of CXCR3 had functional consequences, we evaluated calcium flux responses of GC neurons to CXCL10 treatment. While untreated GC neurons exhibit a robust calcium flux response after brief treatment with CXCL10 (Figure 5C), GC neurons pretreated for 24 hours with TNF- α exhibited a complete loss of response to CXCL10 and only responded to the nonspecific ionophore ionomycin (Figure 5D). Consistent with our immunocytochemical results, neutralizing antibodies against TNFR1 but not TNFR2 blocked the TNF- α -mediated down-regulation of CXCR3 expression (Figure 5E). Finally, in situ hybridization studies of CXCR3 mRNA expression in the cerebella of WNV-infected TNFR1^{-/-} mice revealed persistent expression of this message on GC neurons (Figure 5F). Taken altogether, these data indicate that WNV-infected neurons express TNF- α , which leads to down-regulation of

CXCR3 expression on neurons via TNFR1 signaling. As CXCR3-deficient neurons exhibit enhanced survival during WNV infection, loss of CXCR3 expression *in vivo*, may protect neurons from CXCL10-mediated apoptotic cell death.

TNF- α promotes neuronal survival early after WNV infection

Although TNF- α treatment down-regulates CXCR3, it was unclear whether this would directly affect neuronal survival during WNV infection. Because prior studies have demonstrated antiviral effects of proinflammatory cytokines on WNV replication within permissive cells (Samuel and Diamond, 2005; Shrestha et al., 2006b), we first evaluated whether pretreatment with TNF- α modulated WNV replication. GC neurons treated with increasing doses of TNF- α for 24 hours prior to infection with WNV at MOIs of 3.0 (Figure 6A), 0.3 or 0.03 (data not shown) exhibited identical replication kinetics as untreated neurons. In contrast, TNF- α pretreated neurons exhibited a significant decrease in death during the first 48 hours of WNV infection at low MOI (0.03) (Figure 6B). By 72 hours post-infection, however, there were no differences in survival observed between TNF- α pretreated and untreated cultures. Given that CXCR3 down-regulation can be observed in uninfected neurons within infected cultures (Figure 1F), the enhanced survival observed at early time-points during TNF- α pretreatment supports the notion that this cytokine might promote survival via bystander effects.

Discussion

Viral clearance without immunopathologic insult is critical for resolution of infections within the CNS, which has little reserve for injury. Although a variety of studies have implicated proinflammatory cytokines in bystander injury of neurons during viral encephalitides (Ghoshal et al., 2007; Kimura and Griffin, 2003), our data suggest that TNF- α may exert neuroprotective effects via down-regulation of neuronal CXCR3, whose activation triggers death-signaling pathways in neurons (Sui et al., 2004; Sui et al., 2006). Decreased expression of CXCR3 was observed in neurons both *in vitro* and *in vivo* during WNV infection. In addition, down-regulation of CXCR3 was observed in uninfected neurons within WNV-infected primary cultures, suggesting that WNV infection can modulate CXCR3 down-regulation via expression of a secreted factor. Our data suggests that this factor is TNF- α , whose expression is also induced in neurons by WNV-infection and leads to the expression of CXCL10. Loss of neuronal CXCR3, via targeted deletion or pretreatment with TNF- α , was associated with loss of calcium flux responses to CXCL10, delayed caspase 3 activation and increased survival of neurons at early time-points after *in vitro* infection with low inoculum of WNV. Studies using neutralizing antibodies against receptors for TNF- α suggest that it mediated effects on neurons occurred via activation of TNFR1. Although similar studies were performed with cultured microglia, these cells did not exhibit CXCR3 down-regulation, suggesting specificity for this effect within neurons. This is consistent with prior studies that detect WNV antigen and markers of cell death only in neurons during WNV encephalitis (Shrestha et al., 2003). These data provide evidence that neuronal expression of TNF- α during WNV infection of neurons induces at least two coordinated neuroprotective mechanisms involving chemokines: the expression of CXCL10, which recruits virus-specific lymphocytes (Klein et al., 2005) and down-regulation of CXCR3, which prevents the activation of apoptotic pathways in response to CXCL10. As this latter effect also occurs in uninfected neurons, our data suggest that the CNS has evolved mechanisms for preventing potentially deleterious bystander effects of inflammatory molecules whose expression is required for pathogen clearance.

CXCR3 is a pleiotropic chemokine receptor whose activation triggers both cell survival and apoptosis pathways that depend on the cell type (Feldman et al., 2006; Giuliani et al., 2006; Hirota et al., 2006; Li et al., 2006; Schwarz et al., 2009; Sui et al., 2004). The role of

CXCR3 in the recruitment of activated T and NK cells is well-established and prior studies have shown that CXCR3, via binding CXCL10, is essential for clearance of WNV within the CNS (Klein et al., 2005; Zhang et al., 2008). Prior studies have also demonstrated CXCR3 expression within subpopulations of neurons in CNS regions targeted by WNV, including the cortex, hippocampus, cerebellum and spinal cord (Tran et al., 2007; Xia et al., 2000). Although the physiologic role of CXCR3 in neuronal function is unknown, numerous studies suggest its activation by CXCL10 leads to neuronal injury and or death via mechanisms that may involve calcium dysregulation, activation of ERK1/2 or cleavage of caspase-3 (Nelson and Gruol, 2004; Sui et al., 2004; Sui et al., 2006; Xia et al., 2000). Indeed, prior studies indicate that flaviviruses induce cell death by triggering apoptosis via caspase 3-dependent pathways (Prikhod'ko et al., 2002; Ramanathan et al., 2006; Samuel et al., 2007; Shafee and AbuBakar, 2003; Su et al., 2002). Thus, the elevated CNS levels of CXCL10 required for WNV clearance within the CNS pose a conundrum for CXCR3-expressing neurons. Given that wild-type mice that successfully clear WNV do not exhibit undue neuronal apoptosis, neuronal CXCR3 down-regulation may allow virus-specific T cell recruitment to proceed without incurring excessive bystander neuronal injury. The loss of calcium flux responses to CXCL10 in TNF- α -treated neurons suggests that this cytokine might also prevent excitotoxic injury in neurons during WNV encephalitis. However, as down-regulation of CXCR3 only delays caspase 3 activation and death, this process may only protect early in the course of infection.

Studies examining CNS CXCR3 expression also detected the receptor within neurogenic areas of the CNS including the subventricular and subgranular zones (Tran et al., 2007). Thus, it is also possible that WNV-mediated upregulation in CXCR3 ligands plays a role in recruitment of neural progenitor cells for repair. In this context, loss of CXCR3 expression might inhibit this process, leading to abrogated repair mechanisms. Indeed, reports of the longterm outcome of WNV infections indicate poor recovery with persistent neurologic sequelae including movement disorders, cognitive complaints and functional disability (Sejvar et al., 2003). Thus, it is possible that down-regulation of CXCR3 also occurs in neural progenitor cells, preventing them from migrating to areas with neuronal damage. Alternatively, these cells may be targets of WNV infection, as has been reported for Japanese encephalitis virus (JEV) (Das and Basu, 2008), a related flavivirus that is a significant cause of encephalitis-related morbidity and mortality in Asia (Oya and Kurane, 2007). Further studies are needed to determine whether CXCR3 expression is altered in neural progenitors during WNV encephalitis or if CXCR3 ligands play roles in their recruitment in response to cortical damage.

A neuroprotective role for TNF- α is not a novel concept; TNF- α signaling has been observed to prevent neuronal damage following ischemic injury, excitotoxic damage and to stimulate antioxidant pathways (Bruce et al., 1996; Bruce-Keller et al., 1999; Dolga et al., 2008). Studies in mice with targeted deletions of TNFR1 or TNFR2 suggest that the former mediates neuroprotective actions of TNF- α in models of acute brain injury, similar to our observations during WNV infection (Dolga et al., 2008). However, there are data suggesting that TNFR2 may also exert neuroprotective effects, depending on the neuronal subtype (Fontaine et al., 2002; Marchetti et al., 2004; Tezel et al., 2004). Although its precise mechanisms of neuroprotection is not known, TNF- α activates NF- κ B, which induces the expression of anti-apoptotic proteins, including Bcl-2 and Bcl-x (Mattson et al., 1997; Tamatani et al., 1999). In addition, recent studies suggest that TNFR1 signaling up-regulates the expression of FLIP₁, an inhibitor of caspase-8, in neurons (Taoufik et al., 2007).

The differential effects of TNF- α within neurons may also depend on relative ratios of TNFR1 and TNFR2 expressed by various neuronal subtypes and/or the pathways that influence survival. Thus TNF- α is neuroprotective via TNFR2 on retinal neurons, which

triggers cell survival pathways via Akt/phosphokinase B (Fontaine et al., 2002), and via TNFR1 on cortical neurons by mediating the expression of certain growth factors, such as VEGF and erythropoietin (Taoufik et al., 2008). Neuronal chemokine receptors, which are also differentially expressed by neurons, affect neuronal viability through similar signal transduction pathways (Kaul et al., 2007). Recently, activation of GTPase Ras, which occurs downstream of TNF- α signaling (Auer et al., 1998), was observed to up-regulate expression of CXCL10 and down-regulate CXCR3 in human breast cancer cell lines (Datta et al., 2006), providing a putative signaling pathway for the differential expression of these molecules. Further studies examining TNFR1 signaling during WNV encephalitis are required to determine if this occurs in neurons.

Several other cytokines that are expressed within the CNS during infectious diseases confer neuroprotection against excitotoxic injury. TGF- β , which is expressed within the CNS in response to bacterial lipopolysaccharide (LPS), affords protection to CA3 neurons against kainic acid (Boche et al., 2003) and oncostatin M, a member of the interleukin (IL)-6 cytokine family that is produced by activated T cells, selectively down-regulates NMDA receptor subunit gene expression (Weiss et al., 2006). Finally, neuronal expression of fractalkine promotes neuroprotection within the CNS of individuals with HIV-1-associated neurologic disease via inhibition of viral peptide-induced apoptosis (Tong et al., 2000). Studies addressing endogenous mechanisms of neuroprotection during viral encephalitis have focused on the expression of neurotrophic factors, such as brain derived neurotrophic factor (BDNF), which inhibits caspase 3 activation and protects neurons during HIV-1 encephalitis (Bachis and Mocchetti, 2005; Madeddu et al., 2004). The current study suggests that blockade of receptors upstream of apoptosis pathways could have utility for preventing widespread neuronal injury during recovery from viral encephalitis. Small molecule inhibitors of CXCR3 are currently being evaluated for the treatment of inflammatory diseases and cancer (Cambien et al., 2009; Rosenblum et al., 2009; Walser et al., 2006; Wijtmans et al., 2008). However, given the essential role of CXCR3 in the CNS infiltration of virus-specific T cells during WNV encephalitis (Zhang et al., 2008), CXCR3 antagonism would not be desirable unless neuron-specific targets could be developed. Because TNF- α specifically down-regulated CXCR3 expression in neurons via TNFR1, complete elucidation of this signaling pathway may uncover additional targets to prevent apoptosis in neurons during WNV or other viral encephalitides.

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Figure 1. CXCR3 mRNA expression is down regulated during WNV infection *in vitro*
(A) Q-PCR analysis of CXCR3 mRNA expression in cortical (white bars) and cerebellar (black bars) regions of the CNS of uninfected (day 0) and WNV infected (days 3 and 7 post-infection) mice. Each group represents at least five individual animals and data are expressed as mean foldchange differences compared with uninfected tissues + standard error of the mean, * = $p < 0.05$. **(B)** In situ hybridization analyses of brain sections from hippocampus (HIP) and cerebellum of WNV-infected mouse at days 0, 3 and 7 post-infection (pi) using digoxigenin-labeled antisense and sense riboprobes. Higher magnification images of boxed areas on lower power images are provided. Note decreased CXCR3 expression is first decreased in Purkinje neurons on day 3 post-infection (arrowheads). Scale bars = 25 μ m. Data are representative of three experiments each performed with 2–3 mice.

Figure 2. Neuronal CXCR3 mRNA and protein expression is decreased in both WNV-infected and bystander neurons *in vitro*

(A–B) Primary cultures of cerebellar granule cell neurons (gray bars) or microglia (white bars) were infected with WNV at a multiplicity of infection of 0.03, 0.3, or 3 (A) or treated with various doses of CXCL10 (0–100 ng/ml) (B) and evaluated for expression of CXCR3 via QPCR. Data are derived from two separate experiments with duplicates or triplicates and expressed as mean $\pm$ standard error of the mean, * = $p < 0.05$. (C–D) Triple label immunofluorescence microscopy for expression of WNV antigen (green), CXCR3 protein (red) and MAP-2 (blue) within uninfected (left panels) and WNV-infected (right panels) granule cell neurons. All scale bars = 10 μ m. Note decreased CXCR3 detection (arrows)

within WNV-infected (arrowhead) neurons without loss of MAP-2 (arrows) (C) and in uninfected bystander neuron (arrow) (D). (E) Volocity™ image analysis software was used to quantitate MAP-2 expression and revealed no difference in neurons between uninfected and WNV-infected cultures. (F) Similar quantitation was performed for expression of CXCR3 staining in uninfected neurons (white bars) and in neurons within WNV-infected cultures that stained negative (dark gray bar) and positive (black bar) for WNV antigen. Data are presented as average density of CXCR3 per cell + standard error of the mean for 3–5 replicate culture systems, * = $p < 0.05$.

Figure 3. CXCR3-deficient neurons exhibit enhanced survival during WNV-infection *in vitro*
(A) Cerebellar granule cell neurons were infected with WNV at a multiplicity of infection of 3 and evaluated for production of infectious WNV via plaque assay on Vero cells. Data derived from two separate experiments with duplicates or triplicates and expressed as mean $\pm$ standard error of the mean. **(B)** Neuronal viability was assessed via 7-AAD uptake in granule cell neuronal cultures from wild-type and CXCR3^{-/-} mice at various time-points post-infection with WNV at low MOI (0.03). Data are expressed as mean percentages of dead cells $\pm$ standard error of the mean, * = $p < 0.05$. Data are representative of 3 different experiments with 3–5 replicates per condition.

Figure 4. Cultured WNV-infected neurons express TNF- α and TNFR1

(A–C) Primary cultures of cerebellar granule cell neurons were left uninfected or infected with WNV at a multiplicity of infection of 0.03, 0.3, or 3 and evaluated for expression of TNF- α mRNA via QPCR (A) and protein via ELISA (B) and for TNFR1 (white bars) or TNFR2 (black bars) mRNAs via QPCR (C). Data derived from two separate experiments with duplicates or triplicates and expressed as mean $\pm$ standard error of the mean. * = $p < 0.05$. (D) Immunofluorescence microscopy for expression of TNFR1 and TNFR2 (both green) revealed expression of the former in primary cerebellar granule cell neurons (left panel) while TNFR2 could be detected only on cultured microglia (right panel). Data are representative of three experiments performed in triplicate. IC= isotype control and nuclei are counterstained with DAPI (blue).

Figure 5. TNF- α -mediated down regulation of CXCR3 occurs via TNFR1 and has functional consequences

(A–B) Primary cultures of cerebellar granule cell neurons were treated with increasing doses of TNF- α and evaluated for expression of CXCL10 (A) and CXCR3 (B) mRNAs via QPCR. Data derived from two separate experiments with duplicates or triplicates and expressed as mean $\pm$ standard error of the mean. * = $p < 0.05$. (C–D) *In vitro* calcium transients in Fura-2 loaded granule cell neurons in response to CXCL10 treatment (100 μ g/ml) (C) are abolished after pretreatment with TNF- α (10 ng/ml) (D). Data are presented as the relative ratio of fluorescence at emission frequency of 510 nm and excitation frequencies of 340 and 380 nm over time (sec= seconds) and are representative of 3–4 separate experiments with similar

results. (E) Granule cell neurons preincubated with anti-TNFR1 and/or anti-TNFR2 neutralizing antibodies were treated with TNF- α (10 ng/ml) and evaluated for CXCR3 mRNA expression via QPCR. Data are expressed as fold-change in CXCR3 mRNAs compared with levels of untreated neurons and expressed as + standard error of the mean, * = $p < 0.05$; ** = $p < 0.001$. Experiment was performed 2–3 times in triplicate. (F) *In situ* hybridization analyses of brain sections from cerebellum of wild-type (top) and TNFR1 $^{-/-}$ (bottom) WNV-infected mouse at day 3 post-infection (pi) using digoxigenin-labeled antisense and sense riboprobes for CXCR3. Data are representative of three experiments each performed with 2–3 mice.

Figure 6. Pretreatment of neurons with TNF- α delays WNV-induced death

(A) Cerebellar granule cell neurons were pretreated with various doses of TNF- α (ng/ml), infected with WNV at a multiplicity of infection of 3 and evaluated for production of infectious WNV via plaque assay on Vero cells. Data derived from two separate experiments with duplicates or triplicates and expressed as mean $\pm$ standard error of the mean. (B) Neuronal viability was assessed via TUNEL staining in granule cell neuronal cultures that remained untreated (white bars) or treated with TNF- α (10 ng/ml) (gray bars) and infected with WNV at low MOI (0.03). Data are expressed as mean percentages of dead cells + standard error of the mean, * = $p < 0.05$. Data are representative of 2 different experiments with 3–5 replicates per condition.