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Resistance to Dengue Virus Infection in Mice Is Potentiated by CXCL10 and Is Independent of CXCL10-Mediated Leukocyte Recruitment

Peng-Peng Ip* and Fang Liao*†

CXCL10 is an IFN-inducible chemokine ligand that binds CXCR3, a receptor that is expressed on lymphocytes; CXCL10 shares the CXCR3 receptor with another two ligands, CXCL9 and CXCL11. Previously, we found that CXCL10−/− mice were more susceptible than wild-type (WT) mice to dengue virus (DENV) infection. In this study, we explored the mechanisms underlying this enhanced susceptibility. We found that viral loads were higher in the brains of CXCL10−/− mice than in WT mice. Unexpectedly, compared with WT, CXCL10−/− mice had comparable numbers of total infiltrating T cells, higher numbers of CXCR3+ T cells, and higher numbers of Ab-secreting cells in the brain. Additionally, we found that CXCL10 was induced in neurons following DENV infection and that CXCL10 competed with DENV for binding to cell surface heparan sulfate, a coreceptor for DENV entry, thus inhibiting binding of DENV to neuronal cells. These results demonstrate that the enhanced susceptibility of CXCL10−/− mice to DENV infection is not due to a defect in recruitment of effector lymphocytes but rather to an antiviral activity that promotes viral clearance. The Journal of Immunology, 2010, 184: 000–000.

Dengue virus (DENV), a positive single-stranded RNA virus belonging to the family Flaviviridae, is a mosquito-borne human pathogen. DENV infection is frequently found in tropical and subtropical areas of the world, with ~50–100 million individuals infected each year (1–3). Individuals infected with DENV may develop dengue fever, an acute febrile illness; however, a life-threatening syndrome, dengue hemorrhagic fever/dengue shock syndrome, frequently occurs after a second DENV infection (4). In addition, neurologic manifestations of DENV infection have also been reported in a number of cases, including encephalitis (5, 6), encephalopathy (6–8), and myelitis (9, 10). Although the mechanism of DENV-induced disease is not well understood, chemokines and chemokine receptors are thought to play important roles in DENV infection. The induction of several chemokines by DENV infection has been reported in a variety of cell lines (11–16), and we have reported the requirement of CXCR3 and CXCL10 in host defense against DENV infection (17). Previously, we showed that both CXCR3-deficient (CXCR3−/−) and CXCL10-deficient (CXCL10−/−) mice had higher mortality than wild-type (WT) mice following DENV infection (17). We further demonstrated that CXCR3−/− mice had increased mortality accompanied by increased viral titers and reduced T cell infiltration within infected brain tissue compared with WT mice, suggesting that CXCR3 plays a critical role in host defense by mobilizing T cells into DENV-infected brain tissue for viral clearance (17). However, the mechanism leading to the high mortality of CXCL10−/− mice following DENV infection remains unclear. CXCL10, also known as IFN-γ-inducible protein-10, is a chemoattractant for activated T cells and NK cells bearing CXCR3, the chemokine receptor for CXCL9, CXCL10, and CXCL11 (18). CXCL10 has been reported to play an important role in host defense following a variety of viral infections. Using CXCL10−/− mice, the major function for CXCL10 in host defense against viral infection has been shown to be recruitment of effector T cells and NK cells to sites of infection/inflammation (19–25). However, CXCL10 function specific to DENV infection has not been examined in vivo. Recent studies have shown that CXCL10 is the most highly upregulated gene in dengue patient blood samples (26), in muscle satellite cells exposed to DENV in vitro (27), and in primary human dendritic cells infected by DENV (28), supporting the suggestion that CXCL10 is critical for defense against DENV infection. In this study, we investigated the antiviral activity of CXCL10 in DENV infection. Unexpectedly, our results revealed that recruitment of effector cells to sites of infection was not impaired in CXCL10−/− mice. Furthermore, primary neuronal cells infected with DENV produced CXCL10 but not CXCL9, and CXCL10 inhibited viral infection in neuronal cells by competing with DENV for binding to heparan sulfate, a coreceptor for DENV entry. The data presented support an unappreciated role for CXCL10 in innate host defense against DENV infection, thus reducing mortality during DENV infection.

Materials and Methods

Mice

CXCL10−/− bred mice were provided by Dr. A. Luster (Massachusetts General Hospital, Charlestown, MA). WT bred mice were purchased from BioLASCO Taiwan (Taipei, Taiwan). Mice were maintained under specific pathogen-free conditions at the Institute of Biomedical Sciences, Academia Sinica (Taipei, Taiwan), and were used at 6–8 wk old. All animal experiments were approved by the Institutional Animal Care and

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Abbreviations used in this paper: ASC, Ab-secreting cell; DENV, dengue virus; MFI, mean fluorescence intensity; MOI, multiplicity of infection; NGS, normal goat serum; WT, wild-type.

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Utilization Committee at Academia Sinica and were performed in accordance with institutional guidelines.

Cell culture

Baby hamster kidney cells (BHK21) and N18 neuroblastoma cells were maintained in RPMI 1640 medium (Life Technologies, Rockville, MD) supplemented with 10% FBS (Life Technologies). All cell lines were maintained at 37°C in a humidified atmosphere of 5% CO₂. The C6/36 mosquito cell line established from Aedes albopictus were cultured in RPMI 1640 supplemented with 10% FBS at 28°C in the presence of 5% CO₂ (29).

Dengue virus preparation

Mouse-adapted, neurovirulent dengue type 2 virus (strain New Guinea C-N) was provided by Dr. C.-I. Lai (National Institutes of Health, Bethesda, MD) and was propagated in a C6/36 mosquito cell line. C6/36 cells were incubated with DENV at a multiplicity of infection (MOI) of 0.1 at 28°C for 2 h under 5% CO₂ with periodic agitation. Supernatants were removed, and fresh RPMI 1640 medium supplemented with 5% FBS was added for further culture. Culture supernatants containing DENV were collected on days 3, 5, and 7 postinfection, filtered through a 0.22-μm filter, and stored at −80°C for subsequent infection.

Viral infection

Mice were infected intracerebrally with 2.1×10⁹ PFU DENV in a total volume of 30 μl after anesthetization with 30 s with ether. Mice were examined daily, and mortality rates were recorded for 3 wk postinfection. For secondary infection, mice were injected with 2.1×10⁹ PFU DENV 3 wk after primary infection.

Plaque assays and real-time PCR analysis of viral load in mouse brain tissues

For plaque assays, mice were sacrificed, and brains were homogenized in ice-cold PBS containing protease inhibitors using a 5-ml Dounce tissue homogenizer. For plaque assays, total RNA was extracted from brain tissues using TRIzol reagent (Invitrogen Life Technologies), according to the manufacturer’s instructions. Total RNA was extracted from mouse brain tissue using TRIzol reagent (Invitrogen Life Technologies), according to the manufacturer’s instructions. Reverse transcription reaction was performed on cDNA equivalent to 9.2 μg total RNA was reverse transcribed in the presence of 25 U murine reverse transcriptase (Applied Biosystems, Foster City, CA). Reverse transcription reaction was performed according to the manufacturer’s instructions. Real-time PCR amplification were performed as described previously (30). In brief, 2 μg total RNA was reverse transcribed in the presence of 25 U murine leukemia virus reverse transcriptase and 20 μM reverse primer (5'-CAT TTC ATT TTC TGG CTT C-3') using the TaqMan Gold RT-PCR kit (Applied Biosystems, Foster City, CA). Reverse transcription reaction mixtures were incubated at 25°C for 10 min, then at 48°C for 30 min, and finally at 95°C for 5 min to stop the reaction. PCR amplification reactions (20 μl total) contained 2 μl reverse transcription reaction mixture, 3 μM reverse primer, 10 μM forward primer (5'-AAG GTG AGA TGA AGC TGT AGT CTC-3'), and 1 μl TaqMan probe (5'-CTG CCT CTT CAG CAT CAT TCC AGG CA-3'). The latter contained a 5' reporter dye (FAM) and a downstream 3' quencher dye (TAMRA). Real-time PCR amplification conditions were 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 s and 60°C for 1 min.

Viral RNA was isolated from DENV-infected C6/36 cell culture supernatants (2×10⁵ PFU/ml) by QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA), according to the manufacturer’s protocol. The viral RNA was then reverse transcribed into cDNA as described above. The amount of cDNA equivalent to 9.2×10⁹ PFU was subjected to serial dilution (seven 5-fold dilutions) to prepare a standard curve.

Plaque assay

Tissue lysates or supernatants from cell culture containing DENV were serially diluted (2-fold dilutions) in RPMI 1640 medium, and 200 μl of each dilution was added to BHK21 monolayer cells in 6-well plates. Cells were then incubated for 2 h at 37°C under 5% CO₂, and supernatants were removed and replaced with 4 ml 1% SeaPlaque Agarose (Cambrex, East Rutherford, NJ) in RPMI 1640 medium supplemented with 2% FBS. Cells were further cultured for 5 d to allow plaques to develop.

Quantitative real-time PCR analysis of cytokine and effector molecules in mouse brain tissues

Total RNA was extracted from mouse brain tissue using TRIzol reagent (Invitrogen Life Technologies), according to the manufacturer’s instructions. Total RNA (1 μg) was reverse transcribed using SuperScript III reverse transcriptase (Invitrogen Life Technologies) and oligo(dT) primers according to the manufacturer’s instructions. Real-time PCRs for CXCL9, perforin, granzyme A, granzyme B, and GAPDH cDNA were conducted using Assays-on-Demand gene expression products (Applied Biosystems) containing a 20× mix of unlabeled PCR primers and TaqMan MGB probe (FAM dye labeled) specific for each gene. The PCR amplification cycles were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. The resultant PCR products were measured using an ABI Prism 7500 Sequence Detection System (Applied Biosystems). All amplification reactions were conducted in duplicate. All quantifications were normalized to the level of GAPDH gene expression. Quantification of changes in gene expression was performed using the 2−ΔΔCT method as described previously (31).

Isolation of infiltrating leukocytes from mouse brain

Whole mouse brains were harvested, cut into small pieces, placed into RPMI 1640 medium supplemented with 10% FBS, and homogenized using a 70-μm strainer (BD Biosciences, San Jose, CA) equipped with a 1-ml syringe plunger. Cells were pelleted by centrifugation at 400×g for 5 min at 4°C. Cell pellets were resuspended in 10 ml 30% Percoll (Amersham Biosciences, Piscataway, NJ), overlaid onto 1 ml 70% Percoll, and centrifuged at 13,000×g for 30 min at 4°C. Infiltrating leukocytes were isolated from the interface of the Percoll gradients, washed three times with RPMI 1640 medium supplemented with 10% FBS, and subjected to FACS analysis or ELISPOT assay. For FACS staining, PE–anti-CD3 (clone RM4.5), FITC–anti-CD4 (clone RM4.5), PE–Cy7–anti-CD4 (clone RM4.5), allopurinocyanin–anti-CD8 (clone 53-6.7), FITC–anti-CD8 (clone 145-2C11), and PE–anti-IFN-γ (clone XMG1.2) were purchased from Ebioscience (San Diego, CA); PerCp–anti-CD8 (clone 53-6.7) was purchased from BD Biosciences; allopurinocyanin–Cy7–anti-CD4 (clone GK1.5) and PE–Cy7–anti-IFN-γ (clone XMG1.2) were purchased from BioLegend (San Diego, CA); and PE–anti-CXCR3 was from R&D Systems (Minneapolis, MN). FACS analysis was conducted using either a FACSCalibur or FACS Canto system (BD Biosciences), and data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Lymphocyte culture

Splenocytes were isolated from both WT and CXCL10−/− mice and stained with FITC–anti-CD3. The CD3+ cells were then depleted using anti-FITC microbeads (Miltenyi Biotec, Auburn, CA), according to the manufacturer’s instructions. The CD3-depleted splenocytes were used as APCs. Infiltrating leukocytes were isolated from DENV-infected brains on day 6 postinfection, washed once with 0.1% BSA in PBS, and labeled with 2.5 μM CFSE at 37°C for 8 min. The labeled brain leukocytes (2.5×10⁷/well) were cultured with CD3-depleted splenocytes (1.25×10⁶/well) in 96-well plates in RPMI 1640 medium supplemented with 10% FBS, 50 μM 2-ME, 2 mM l-glutamine, 100 U/ml penicillin, and 50 μg/ml streptomycin in the presence or absence of DENV. After 60 h culture, cells were further stimulated with PMA (20 ng/ml), ionomycin (1 μM), and brefeldin A (10 μg/ml) for 4 h. Cells were then harvested, washed, and stained with allopurinocyanin-Cy7–anti-CD4 (clone GK1.5) and allopurinocyanin–anti-CD8 (clone 53-6.7), followed by intracellular staining with PE–Cy7–anti-IFN-γ (clone XMG1.2). The CFSE− cells were gated for the analysis of IFN-γ expression in CD4 or CD8 using a FACScanto, and data were analyzed using FlowJo software.

ELISA

Uninfected and DENV-infected C6/36 cells were freeze-thawed five times using dry ice/ethanol and a 37°C heat block. Each well of a 96-well plate (Costar, Cambridge, MA) was coated with 5 μg cell lysate at 4°C overnight. Nonspecific binding was blocked using PBS containing 10% FBS and 0.05% sodium azide at room temperature. Serial dilutions of mouse serum (100 μl/well) were then applied to the 96-well plates and incubated at room temperature for 3 h. DENV-specific IgG in serum was detected using HRP-conjugated goat anti-mouse IgG (Thermo Fisher Scientific, Waltham, MA) with tetramethylbenzidine (R&D Systems) as a substrate. Background was defined as the signal from uninfected cells and was subtracted from each sample. All signals were normalized to signal generated by serum from a WT mouse at day 7 postinfection, which was set as 1 to normalize samples.

ELISPOT assay

MultiScreen 96-well plates containing HA-cellulose ester membranes (Millipore, Billerica, MA) were coated with 5 μg DENV-infected or uninfected C6/36 cell lysates in 50 μl PBS and incubated at 4°C overnight. Lysates were removed, and the plates were washed and blocked with PBS containing 10% FBS and 0.05% sodium azide. Serial dilutions of infiltrating mononuclear cells isolated from mouse brains were prepared in RPMI 1640 medium containing 10% FBS and antibiotics. Each diluted suspension was added to coated plates (100 μl/well) and incubated for 16 h at 37°C under 5% CO₂. After incubation, HRP-labeled goat anti-mouse
IgG was added to each well and incubated for 2 h at room temperature, followed by three washes. HRP substrate solution (50 μL) containing 38 mM 3-amin-9-ethylcarbazole, 0.51 M dimethylformamide, and 0.015% H2O2 in 50 mM acetate buffer (pH 5.0) was added to each well and incubated for 20 min at room temperature with gentle agitation for the development of spots. The reaction was stopped by rinsing the wells with running tap water, and spots were counted using an AID ELISPOT plate reader system (Autoimmun Diagnostika, Strassberg, Germany).

Analysis of virus binding by flow cytometry
N18 cells (10⁵) were suspended in 50 μL binding buffer (HBSS/1% FBS/10 mM HEPS) and incubated with DENV (MOI = 1) on ice for 2 h in the presence of various concentrations of CXCL10 (PeproTech, Rocky Hill, NJ) or the CXCL10 mutant CR22A, provided by Dr. A. Luster (Massachusetts General Hospital) (32). Unbound DENV was removed by washing three times with binding buffer, and bound DENV particles were detected using rabbit anti-DENV envelope protein (lanti-DENV E; 1/1000) provided by Dr. W. Chang, Institute of Molecular Biology, Academia Sinica), followed by three washes and then PE-conjugated goat anti-rabbit IgG for 1 h at 4°C. After a final three washes, cells were subjected to FACS analysis using a FACScalibur flow cytometer (BD Biosciences). The mean fluorescence intensity (MFI) of specific DENV binding in the absence of chemokines was set to 100%.

Isolation and culture of primary hippocampal neurons
Hippocampal neurons were isolated from C57BL6 mouse brains at embryonic day 14.5 as described previously (33). In brief, mouse embryos were isolated from pregnant females after CO2 anesthetization. The brains were isolated and stored in ice-cold HBSS solution. Meninges were removed, and hippocampi were dissected under a dissecting microscope. The tissues were washed with ice-cold HBSS twice and trypsinized at 37°C in 1 ml prewarmed trypsin (0.25%) for 15 min. The digested tissues were washed twice with HBSS and resuspended in 1 ml plating medium containing MEM supplemented with 10% horse serum and antibiotic-antimycotic (Life Technologies). The tissues were dissociated by pipetting 60 times with a Pasteur Pippet. Cells were seeded at 5 × 10⁴ cells/ml in poly-L-lysine-coated plates (0.1 mg/ml) for 1 h at 37°C in a CO2 incubator. After incubation, plating medium was replaced with HBSS, and neuronal basal medium supplemented with B-27 (Life Technologies), and antibiotic-antimycotic (Life Technologies) and cells were cultured for 5 d before DENV infection.

Immunohistochemistry
For staining of tissue sections, brain tissues were embedded in OCT (DakoCytomation, Carpinteria, CA) and solidified on dry ice before storage at −80°C. Cryosections (10 μm) were air-dried at room temperature for 10 min and fixed with ice-cold 5% paraformaldehyde for 10 min. The sections were then permeabilized with 0.25% Triton X-100 for 5 min, followed by blocking with 5% normal goat serum (NGS) for 30 min. After blocking, sections were stained with mouse anti-DENV E (1/1000; provided by Dr. Y.-L. Lin, Institute of Biomedical Sciences, Academia Sinica) or mouse IgG isotype control Ab in 2% NGS for 2 h at room temperature. The sections were then washed three times with PBS containing 0.1% Tween 20, followed by incubation for 1 h at room temperature in a mixture of Cy3-conjugated goat anti-mouse IgG (1/200; Chemicon International, Temecula, CA), FITC-conjugated anti-NeuN (1/250; Chemicon International), and DAPI (Molecular Probes, Eugene, OR). Sections were washed three times with PBS/0.1% Tween 20, mounted with DakoFluor solution (DakoCytomation), and analyzed by fluorescence microscopy.

For staining of isolated primary neurons, cells were washed once with PBS and fixed in PBS containing 4% sucrose and 4% paraformaldehyde for 15 min at room temperature. Fixed cells were washed once with PBS/0.1% Tween 20 and three times with PBS and blocked in blocking buffer (PBS containing 2% BSA and 2% NGS) for 1 h at room temperature. Cells were then incubated with mouse anti-β-III-tubulin (1/1000; Promega, Madison, WI) in blocking buffer at 4°C overnight. Cells were washed three times with PBS and incubated with rabbit anti-DENV E in blocking buffer at room temperature for 2 h. After incubation, cells were washed with PBS and stained with a mixture of Cy3-conjugated goat anti-mouse IgG, FITC-conjugated goat anti-rabbit IgG, and DAPI in blocking buffer. After washing three times with PBS, sections were mounted using DakoFluor solution and analyzed under a fluorescence microscope.

H&E and Nissl staining
Brain and spinal cord tissues were fixed in formalin and embedded in paraffin. Tissue sections (10 μm) were dewaxed and rehydrated prior to H&E staining or Nissl staining. For Nissl staining, tissue sections were incubated in 1% thionin acetate (Sigma-Aldrich, St. Louis, MO) buffered with 200 mM acetic acid and 36 mM sodium hydroxide at pH 4.0 for 5 min. Stained sections were washed with distilled water, dehydrated, and mounted.

Statistical analysis
Differences between two groups were calculated using the Mann-Whitney U test, and p < 0.05 was considered statistically significant.

Results
CXCL10−/− mice exhibit increased neural impairment compared with WT mice following DENV infection
We previously demonstrated that survival of CXCL10−/− mice was significantly lower than WT mice following intracerebral infection with DENV (17). Additionally, we found that surviving CXCL10−/− mice developed weakness and paralysis in one or both limbs (17). The increases in mortality and paralysis in CXCL10−/− mice following DENV infection led us to examine whether DENV targeted neurons. Immunofluorescence staining of brain sections from CXCL10−/− mice infected with DENV revealed that DENV-positive cells are also positive for NeuN, a marker for neurons, indicating that DENV targets neurons (Fig. 1A). We further performed H&E and Nissl (Fig. 1C) staining to examine the neuronal pathology in DENV-infected mice. By H&E staining, although we found neuronal damage only infrequently in DENV-infected WT mice, neuronal damage was easily detected in DENV-infected CXCL10−/− mice (Fig. 1B, right panel). Neurons in the CA1 area of the hippocampus and motor neurons in spinal cord were significantly affected in CXCL10−/− mice but not in WT mice (Fig. 1B). Notably, neuronal eosinophilia (an increased uptake of eosin in neurons, which is an indicator of neuronal degeneration) and apoptotic bodies (an indicator of microglial phagocytosis of neurons, which is an indicator of neuronal apoptosis) were clearly observed in the hippocampal neurons of the DENV-infected CXCL10−/− mice (Fig. 1B, right upper panel, inset). In addition, neuronophagia and apoptosis of motor neurons were also found in the anterior horn of the spinal cord of DENV-infected CXCL10−/− mice (Fig. 1B, lower right panel, insets). We further analyzed the neuronal pathology in DENV-infected brain by Nissl staining and found that CXCL10−/− mice infected with DENV exhibited increased loss of neurons in the hippocampus (Fig. 1C) as well as spinal cord (motor neurons) data (not shown) Together, these results reveal that DENV infection results in significant and severe neuronal damage/loss in CXCL10−/− as compared with WT mice.

CXCL10−/− mice infected with DENV have higher viral loads in the brain than WT mice
We next examined whether the increases in neural impairment, mortality, and paralysis among CXCL10−/− mice were due to a failure of viral clearance in CXCL10−/− mice by using a plaque assay and real-time PCR to assess viral load. DENV was detectable in the brains of both WT and CXCL10−/− mice on day 3 postinfection, and the viral load peaked on day 5 postinfection in both groups. The viral load in WT mice declined significantly on day 6 postinfection and became nearly undetectable on day 7 postinfection (Fig. 2A). In contrast, the viral load in CXCL10−/− mice declined more slowly, and the virus was still detectable on day 7 postinfection (p < 0.02) (Fig. 2A). These results indicate that viral clearance among CXCL10−/− mice was significantly reduced (Fig. 2A). Consistent with these results, real-time PCR analysis of total RNA from brain tissues using virus-specific primers revealed a significantly higher viral load in CXCL10−/− mice than in WT mice on day 6 postinfection (p < 0.02) (Fig. 2B).

CXCL9 expression is not impaired in CXCL10−/− mice
Given that the CXCR3 ligands CXCL9, CXCL10, and CXCL11 are potent chemotacticants for activated T cells and that CXCL10
FIGURE 1. DENV infection of CXCL10−/− mice results in neuronal loss in both brain and spinal cord. A, Cryosections of brains from DENV-infected CXCL10−/− mice were collected on day 6 postinfection and immunostained using anti-DENV E, followed by Cy3-conjugated goat anti-mouse IgG (red) and FITC-conjugated anti-NeuN (a marker for neurons; green) and counterstained with DAPI nuclear stain (blue). An illustrative section from a CXCL10−/− mouse is shown (original magnification ×400). The immunofluorescence staining was representative of four DENV-infected CXCL10−/− mice. B, Brain (upper panels) (original magnification ×100) and spinal cord (lower panels) (original magnification ×100) tissues were collected from WT and CXCL10−/− mice on day 10 postinfection. Sections were fixed with paraformaldehyde and subjected to H&E staining. Upper panel. Magnified inset (original magnification ×1000) highlights neuronal eosinophilia (filled arrowheads) and apoptotic bodies (empty arrowheads) in brain sections from DENV-infected CXCL10−/− mice. Lower panel. Magnified insets (original magnification ×1000) highlight different stages of neuronophagia of the motor neurons in the spinal cord from DENV-infected CXCL10−/− mice. 1, Early stage; 2 and 3, midway stage; and 4, late stage. C, Brain tissue sections were fixed with paraformaldehyde and subjected to Nissl staining (original magnification ×1000). Arrows indicate the loss of hippocampal neurons.
indirectly regulates the expression of CXCL9 and CXCL11 (34, 35), we investigated whether CXCL10 deficiency results in impaired expression of CXCL9 following DENV infection; such impairment would result in failure of effector cell recruitment to sites of infection and thus inefficient clearance of virus. Because it has been reported that C57BL/6 mice do not produce functional CXCL11 protein because of a stop codon in the CXCL11 leader sequences (36, 37), we did not analyze CXCL11 further. Using real-time PCR, we found that, although CXCL9 was induced in CXCL10−/− mice following DENV infection, CXCL9 expression was somewhat lower than in WT mice (Fig. 3A). Decreased CXCL9 expression in CXCL10−/− mice compared with WT mice on day 7 postinfection was confirmed by ELISA ($p < 0.01$) (Fig. 3B).

CXCL10−/− and WT mice exhibit comparable levels of infiltrating CD4+ and CD8+ T cells in cerebral tissue following DENV infection

Because recruitment of CXCR3-bearing effector T cells is required for resistance to DENV infection (17), we examined recruitment of effector T cells to brain tissue in WT and CXCL10−/− mice following DENV infection. Mononuclear cells isolated from the brains of infected mice were stained with anti-CD4 and anti-CD8 and subjected to flow cytometry to quantify the subpopulations of T cells. The numbers and the percentage of CD4+ and CD8+ T cells in the brains of DENV-infected CXCL10−/− mice and WT mice were not significantly different (Fig. 4), suggesting that deficiency of CXCL10 did not affect the recruitment of effector T cells to DENV-infected brain tissue.

Because viral clearance was impaired in CXCL10−/− mice (Fig. 2), but recruitment of CD4+ and CD8+ T cells was not (Fig. 4), we examined whether the recruited T cells in CXCL10−/− mice were functionally defective by analyzing the expression of the effector molecules IFN-γ, perforin, and granzymes A and B following DENV infection. Infiltrating mononuclear cells isolated from five-pooled
brains of DENV-infected WT and CXCL10−/− mice were subjected to intracellular IFN-γ staining after stimulation with PMA and ionomycin. Slightly higher levels of IFN-γ were observed in both CD4+ (WT versus CXCL10−/−, 1.86 versus 3.81%) and CD8+ (WT versus CXCL10−/−, 6.21 versus 6.45%) T cells from CXCL10−/− mice than in those from WT mice (Fig. 5A), indicating that the infiltrating T cells in CXCL10−/− mice are functionally active following DENV infection. To demonstrate further that the infiltrating T cells in DENV-infected brains were DENV specific, we cultured infiltrating leukocytes isolated from infected brains with splenic APCs in the presence or absence of DENV and analyzed IFN-γ expression in infiltrating T cells. The number of IFN-γ–expressing CD4 or CD8 T cells cultured in the presence of DENV was increased as compared with those cultured in the absence of the virus (Fig. 5B), indicating that there were DENV-specific T cells in infected brains. However, IFN-γ–producing T cells were also observed even in the culture without DENV. This may be as a result of some infiltrating T cells being fully activated T cells, which produce IFN-γ constitutively in the absence of DENV stimulation. Alternatively, these T cells may represent bystander-activated T cells, which are driven by cytokines instead of the Ag stimulation during massive proliferation of T cells in viral infection. Furthermore, the expression of perforin and granzyme A/B, which play roles in viral infection, was also examined using real-time PCR; comparable expression in CXCL10−/− and WT mice was demonstrated (Fig. 5C).

Analysis of CXCR3 expression in infiltrating T cells from DENV-infected brains revealed higher levels of CXCR3 expression in CD8+ T cells (Fig. 6A, upper panel; MFI for CD8, WT versus CXCL10−/−, 61.7 versus 69.5; MFI for CD8, WT versus CXCL10−/−, 52.2 versus 91.3) and significantly increased numbers of CXCR3high-bearing CD8+ T cells in DENV-infected brains of CXCL10−/− mice compared with WT mice (p < 0.005) (Fig. 6A, lower panel). We therefore examined whether the number of CXCR3-bearing cells is intrinsically higher in CXCL10−/− mice than WT mice. As shown in Fig. 6B, increased populations of CXCR3-bearing T cells were found in lymph nodes and spleens from naive (uninfected) CXCL10−/− mice compared with WT mice (p < 0.02) (Fig. 6B), suggesting that the increased number of CXCR3-bearing T cells in DENV-infected brains is likely intrinsic to the CXCL10 deficiency.
Brains were somewhat higher in CXCL10, interestingly, the levels of dengue-specific ASCs in DENV-infected (38–40), we examined whether the recruitment of ASCs to DENV-(ASCs) and is critical for ASC trafficking to sites of infection because CXCR3 is expressed on the surface of Ab-secreting cells. Abs were the same in CXCL10 mice. WT and CXCL10 mice were infected with DENV (primary infection). Surviving mice were subjected to a second infection 3 wk after primary infection (secondary infection). Sera were collected at the indicated day postinfection, and DENV-specific Ab levels were determined by ELISA using serum from a WT mouse at day 7 postinfection for normalization. Data represent means ± SEM from four to seven mice and reflect two independent experiments. Brains from DENV-infected WT and CXCL10 mice were collected and homogenized on day 7 and day 10 postinfection. Infiltrating leukocytes were isolated by Percoll gradient centrifugation, and the number of DENV-specific ASCs per brain was determined by ELISPOT assay. Data are representative of two independent experiments.

**CXCL10 inhibits DENV binding to neurons by competing for heparan sulfate on the cell surface**

Because the high mortality rate and viral loads found in DENV-infected CXCL10 mice could not be explained by impaired recruitment of effector cells or by impaired effector cell function, it appeared that an undefined function of CXCL10 may be important for host defense against DENV infection. Previously, we demonstrated that CXCL10 inhibits DENV binding to Hepa1-6 cells via competition for heparan sulfate, a coreceptor for DENV entry (31). We therefore examined whether CXCL10 also inhibits DENV binding to neuronal cells. We used a mouse neuronal cell line (N18) as an in vitro model for examining the effect of CXCL10 on interference of DENV binding to neuronal cells. N18 cells were incubated with DENV (MOI = 1) in the presence of various concentrations of CXCL10 for 2 h at 4°C. After incubation, cells were washed and stained with goat anti-DENV E, followed by PE-conjugated anti-goat secondary Ab. CXCL10 inhibited DENV binding to N18 in a dose-dependent manner with an IC50 of ~5.7 μg/ml (Fig. 8A, upper panel). Consistent with the inhibitory effect of CXCL10 on DENV binding to neurons, we also observed a dose-dependent inhibitory effect on viral replication, as determined by plaque assay (Fig. 8B).

We then examined whether CXCL10 inhibition of DENV binding to neuronal cells resulted from competition for heparan sulfate binding on the neuronal cell surface. It is known that Arg22 and the basic residues in the C-terminal helix (Lys71, Arg72, Lys74, and Arg75) of CXCL10 are critical for heparan binding (32). We therefore examined whether CXCL10 also inhibits DENV binding to neuronal cells. We used a mouse neuronal cell line (N18) as an in vitro model for examining the effect of CXCL10 on interference of DENV binding to neuronal cells. N18 cells were incubated with DENV (MOI = 1) in the presence of various concentrations of CXCL10 for 2 h at 4°C. After incubation, cells were washed and stained with goat anti-DENV E, followed by PE-conjugated anti-goat secondary Ab. CXCL10 inhibited DENV binding to N18 in a dose-dependent manner with an IC50 of ~5.7 μg/ml (Fig. 8A, upper panel). Consistent with the inhibitory effect of CXCL10 on DENV binding to neurons, we also observed a dose-dependent inhibitory effect on viral replication, as determined by plaque assay (Fig. 8B). Because cellular immunity to DENV infection was not impaired in DENV-infected CXCL10 mice (Figs. 4, 5), we examined whether humoral immunity was affected. Following both primary and secondary infection (Fig. 7A), serum levels of DENV-specific Abs were the same in CXCL10 mice as in WT mice over time. Because CXCR3 is expressed on the surface of Ab-secreting cells (ASCs) and is critical for ASC trafficking to sites of infection (38–40), we examined whether the recruitment of ASCs to DENV-infected brain tissue was affected using ELISPOT analysis. Interestingly, the levels of dengue-specific ASCs in DENV-infected brains were somewhat higher in CXCL10 mice than in WT mice on days 7 and 10 following secondary infection (Fig. 7B). Taken together, these results demonstrate that, like cellular immunity (Figs. 4, 5), humoral immunity (Fig. 7) to DENV infection is not impaired in CXCL10 mice.

**FIGURE 6.** Comparison of CXCR3 expression in WT and CXCL10−/− mice. WT and CXCL10−/− mice were infected with DENV. A, WT and CXCL10−/− mice were infected with DENV. On day 6 postinfection, infiltrating leukocytes were isolated from brain tissue by Percoll gradient centrifugation and then subjected to surface staining using PE-anti-CXCR3 together with FITC-anti-CD4 or allophycocyanin-anti-CD8. The representative FACS plot shows the percentage of infiltrating CXCR3lowCD4+ and CXCR3highCD8+ T cells (left panels) as well as CXCR3lowCD8+ and CXCR3highCD8+ T cells (right panels) in infected brains (upper panel), and the bar graph (lower panels) shows means ± SEM from six mice. B, Lymphocytes isolated from spleens or cervical lymph nodes of naive mice were subjected to surface staining using allophycocyanin-anti–CD4, PerCP–anti–CXCR3, and PE-anti–CD8. The representative FACS plot shows the percentage of infiltrating CXCR3lowCD4+ T cells (upper panel) and CXCR3highCD8+ T cells (lower panel) in infected brains (left panels) and in the same percentage of infiltrating CXCR3highCD8+ T cells (right panels) in infected brains (upper panel), and the bar graph (lower panels) shows means ± SEM from six mice. *p < 0.02, **p < 0.005.

**FIGURE 7.** Comparison of DENV-specific Ab production and recruitment of DENV-specific ASCs in CXCL10−/− and WT mice. A, WT and CXCL10−/− mice were infected with DENV (primary infection). Surviving mice were subjected to a second infection 3 wk after primary infection (secondary infection). Sera were collected at the indicated day postinfection, and DENV-specific Ab levels were determined by ELISA using serum from a WT mouse at day 7 postinfection for normalization. Data represent means ± SEM from four to seven mice and reflect two independent experiments. B, Brains from DENV-infected WT and CXCL10−/− mice were collected and homogenized on day 7 and day 10 postinfection. Infiltrating leukocytes were isolated by Percoll gradient centrifugation, and the number of DENV-specific ASCs per brain was determined by ELISPOT assay. Data are representative of two independent experiments.
CXCL10 inhibits DENV infection via competition with DENV for binding to heparan sulfate on the surface of neuronal cells.

**Primary neurons produce CXCL10**

Given that DENV targets neurons (Fig. 1) and that CXCL10 inhibition of DENV binding to neurons (Fig. 8), CXCL10 may be directly produced by neurons and provide immediate protection for neurons following DENV infection. We then examined whether CXCL10 was directly produced by neurons. Hippocampal neurons were isolated from the brains of C57BL/6 mice at embryonic day 14.5 and grown as primary cultures. Primary neurons were infected with DENV (MOI = 1), and the secretion of CXCL10 and CXCL9 into culture supernatants was measured by ELISA. As shown in Fig. 9, interestingly, primary neurons infected with DENV produced CXCL10 but not CXCL9.
taken up by peripheral APCs. If DENV has low binding affinity for heparan sulfate, DENV entry into APCs would be reduced. Given that APCs are crucial for the initiation of immune response to pathogens and for the elimination of pathogens, the reduction of DENV entry into APCs could result in a decreased immune response to DENV and poor elimination of virus, leading to the increase of virus half-life in serum and allowing more virus to reach and infect visceral organs. In our study, we used intracerebral injection to deliver DENV into the mouse brain, which allows DENV to directly encounter neuronal cells in which DENV can replicate and from which virus can disseminate in the CNS. In this regard, inhibiting DENV binding to heparan sulfate should reduce DENV entry into neuronal cells, thereby decreasing the DENV infection specifically of these cells in the CNS.

Interestingly, we found that DENV-infected neurons produce CXCL10 but not CXCL9, indicating that CXCL10 specifically induced by DENV-infected neurons should have its unique role in mediating DENV infection. It has been shown that microglia that constitutively express CXCR3 (49, 50) are responsible for innate immunity in brains and are involved in neuronal remodeling as well as brain repair and that CXCR3 is required for microglial migration within brains (51). Thus, it is possible that DENV-infected neurons produce CXCL10 to attract microglia, which promote clearance of infected neurons, and thereby restrict the dissemination of DENV within the brain. According to this model, impaired microglial recruitment in CXCL10−/− mice reduces clearance of infected neurons in the brain, resulting in viral dissemination and neuronal impairment leading to mortality and paralysis. We therefore propose that CXCL10 has at least two functions in resistance to DENV infection: competition with DENV for neuronal binding to inhibit entry and recruiting microglia to promote clearance of infected neurons. These antiviral functions would provide for the early control of viral dissemination, protecting neurons until DENV-specific effector cells reach the site of infection. The importance of CXCL10 during the early phase of DENV infection is supported by the observation that sera from patients in the early stage of DENV infection (1–2 d after fever onset) have significantly higher levels of CXCL10 than sera from convalescent patients (26).

Our study demonstrates that CXCL10−/− mice can mount both cellular and humoral immunity responses following DENV infection. Recruitment of effector T cells to the brain in CXCL10−/− mice and WT mice was similar following DENV infection, and the effector T cells from CXCL10−/− mice produced somewhat higher levels of IFN-γ. These results indicate that both the quantity and quality of effector cells induced by DENV infection were unimpaired by CXCL10 deficiency. Intriguingly, recruitment of CD4+CXCR3high and CD8+CXCR3high cells was higher in DENV-infected brains from CXCL10−/− mice compared with WT mice, likely resulting from the higher number of CXCR3+ T cells intrinsic to the lymphoid organs of naive CXCL10−/− mice compared with naive WT mice. The underlying mechanism of CXCL10 deficiency that leads to increased numbers of CXCR3+ T cells in lymphoid organs remains to be investigated. In Th1 cells, IFN-γ positively regulates T-bet expression (52), which in turn regulates CXCR3 expression (53). IFN-γ levels are elevated in both CD4+ and CD8+ T cell in brain tissue from CXCL10−/− mice following DENV infection, and this likely drives the observed increase in CXCR3 expression in these cells. The mechanism driving the increased production of IFN-γ in DENV-infected CXCL10−/− mice also requires further examination.

Following DENV infection, the rate of Ab production and the recruitment of ASCs were somewhat higher in the brains of CXCL10−/− mice compared with WT mice, indicating that CXCL10 deficiency did not affect humoral immunity. Several reports have demonstrated that plasmablasts express CXCR3 and are attracted to sites of inflammation, where they differentiate into long-lived plasma cells (38–40). This again suggests that CXCR3 ligands play a role in recruitment of effector cells to infection sites. Because both CXCL9 and CXCL10 are important for recruitment of ASCs to inflamed tissues (54), it is possible that CXCL9 is able to compensate for the CXCL10 deficiency by recruiting ASCs in CXCL10−/− mice following DENV infection. Other chemokines, such as CXCL12, may also be involved in ASC recruitment (38).

As previously discussed, it is clear from our current study that CXCL9 cannot compensate for all antiviral functions of CXCL10 in response to DENV infection. The unique antiviral function of CXCL10 seems critical and indispensable for host defense during the early phase of DENV infection. Interestingly, this early role may not be restricted to DENV infection, because the production of CXCL10 by neurons has been reported for other infections of the CNS (21, 36). Therefore, it is plausible that CXCL10 provides unique innate host defense to viral diseases that target neurons by providing an important means of protecting neurons from further infection before the adaptive immune response (recruitment of effector lymphocytes) is mounted.

We have used a mouse model to demonstrate that CXCL10 plays a novel role in early resistance to DENV infection by promoting viral clearance in addition to its previously identified function in effector lymphocyte recruitment. Although we have shown the important role of CXCL10 in DENV infection using intracerebral challenge model in mice, it is noted that neuronal manifestation in DENV infection only represents the small number of dengue cases reported. Because mice are not the natural host for DENV, mice only can become susceptible to DENV infection via intracerebral infection. There may be limitations of this intracerebral challenge model for reflecting human dengue infection, which may argue the importance of CXCL10 in DENV infection in humans. However, of particular interest, recent human studies have demonstrated that CXCL10 expression is substantially induced upon DENV infection in a variety of human primary cells (28) and that serum levels of CXCL10 are significantly increased in patients with early DENV infection (26), indicating the importance of CXCL10 in human DENV infection. Altogether, it is possible that CXCL10 may play a critical role in DENV infection independent of the route of infection.

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Disclosures

The authors have no financial conflicts of interest.

References


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