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Both CXCR3 and CXCL10/IFN-Inducible Protein 10 Are Required for Resistance to Primary Infection by Dengue Virus[1]

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We examined the extent to which CXCR3 mediates resistance to dengue infection. Following intracerebral infection with dengue virus, CXCR3-deficient (CXCR3$^{-/-}$) mice showed significantly higher mortality rates than wild-type (WT) mice; moreover, surviving CXCR3$^{-/-}$ mice, but not WT mice, often developed severe hind-limb paralysis. The brains of CXCR3$^{-/-}$ mice showed higher viral loads than those of WT mice, and quantitative analysis using real-time PCR, flow cytometry, and immunohistochemistry revealed fewer T cells, CD8$^+$ T cells in particular, in the brains of CXCR3$^{-/-}$ mice. This suggests that recruitment of effector T cells to sites of dengue infection was diminished in CXCR3$^{-/-}$ mice, which impaired elimination of the virus from the brain and thus increased the likelihood of paralysis and/or death. These results indicate that CXCR3 plays a protective rather than an immunopathological role in dengue virus infection. In studies to identify critical CXCR3 ligands, CXCL10/IFN-inducible protein 10-deficient (CXCL10/IP-10$^{-/-}$) mice infected with dengue virus showed a higher mortality rate than that of the CXCR3$^{-/-}$ mice. Although CXCL10/IP-10, CXCL9/monokine induced by IFN-γ, and CXCL11/IFN-inducible T cell α chemoattractant share a single receptor and all three of these chemokines are induced by dengue virus infection, the latter two could not compensate for the absence of CXCL10/IP-10 in this in vivo model. Our results suggest that both CXCR3 and CXCL10/IP-10 contribute to resistance against primary dengue virus infection and that chemokines that are indistinguishable in in vitro assays differ in their activities in vivo. *The Journal of Immunology,* 2006, 177: 1855–1863.

The mosquito-borne human pathogen dengue virus is a single-stranded, positive RNA flavivirus prevalent in tropical and subtropical areas of the world. Approximately 50–100 million individuals are infected with dengue each year, and there is a high mortality rate among children (1–3). Individuals with primary infections usually develop dengue fever, an acute febrile illness that is accompanied by debilitating headache and myalgias but is not life-threatening (4). In contrast, a life-threatening syndrome, dengue hemorrhagic fever/dengue shock syndrome, can occur after a second infection due to virus-specific serotype-cross-reactive immune responses that result in Ab-mediated enhancement of infection (5–8) and inappropriate T cell activation and death (9). Although neurological complications during dengue infection are not well-characterized, a number of cases in which dengue fever was accompanied by neurological symptoms have been reported (10–12), including dengue encephalitis (13).

Recruitment of effector T cells, CD8$^+$ T cells in particular, to sites of infection plays an essential role in the host defense against viral infection (14–16). Chemokines are the principal chemotactic factors that mediate leukocyte migration to sites of inflammation and/or infection (17, 18). Among these, the IFN-γ-inducible CXC chemokines, CXCL10/IFN-inducible protein 10 (IP-10),[4] CXCL9/monokine induced by IFN-γ (Mig), and CXCL11/IFN-inducible T cell α chemoattractant (I-TAC), mainly attract effector T cells and NK cells that express the receptor CXCR3 (19, 20), and several studies have shown that viral infection leads to up-regulation of their expression (21–25). Of these chemokines, CXCL10/IP-10 has been the most widely studied in the context of mouse models of CNS infection (22, 23), and has been shown to play a key role in the trafficking and recruitment of effector T cells (26). In humans, expression of CXCL10 has been shown to be elevated in the cerebral spinal fluid of patients suffering from viral meningitis (27).

The activity of IFN-γ-inducible CXC chemokines mediated via CXCR3 during dengue virus infections has never been investigated. In the present study, therefore, we used a model of dengue virus-induced neurological disease in wild-type (WT) and CXCR3-deficient (CXCR3$^{-/-}$) mice to evaluate the contributions of IFN-γ-inducible CXC chemokines and CXCR3 to the host defense against dengue infection. Our results suggest that both CXCR3 and CXCL10/IP-10 play crucial roles in mediating resistance to primary dengue infection and that CXCL9/Mig and CXCL11/I-TAC cannot compensate for the loss of CXCL10/IP-10 in this in vivo model.

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[4] Abbreviations used in this paper: IP-10, IFN-inducible protein 10; Mig, monokine induced by IFN-γ; I-TAC, IFN-inducible T cell α chemoattractant; WT, wild type; MOI, multiplicity of infection; C_T, threshold cycle; DAB, diaminobenzidine; FSC, forward scatter; SSC, side scatter; MHV, mouse hepatitis virus.

Materials and Methods

Mice

Breeder pairs of CXCR3$^{-/-}$ and CXCL10/IP-10$^{-/-}$ mice were provided by Drs. G. Gerard (Children's Hospital, Harvard Medical School, Boston, MA) and A. Luster (Massachusetts General Hospital and Harvard Medical School, Charlestown, MA), respectively. WT C57BL/6 mice were purchased from the National Laboratory Animal Center (Taipei, Taiwan). This study was conducted using age- and sex-matched groups of WT and CXCR3$^{-/-}$ or CXCL10/IP-10$^{-/-}$ mice. The mice used were 6–8 wk old and were housed under specific pathogen-free conditions at the Institute of Biomedical Sciences (Academia Sinica, Taipei, Taiwan). All animal experiments were approved by the Institutional Animal Care and Utilization Committee at Academia Sinica and were performed in accordance with institutional guidelines.

Virus preparation

Mouse-adapted, neurovirulent dengue virus type 2 (strain New Guinea C-N), provided by Dr. C.-J. Lai (National Institutes of Health, Bethesda, MD), was propagated in the C6/36 mosquito cell line established from *Aedes albopictus*. The cells were maintained in DMEM supplemented with 10% FBS until they were harvested, resuspended in medium containing 2% FBS in a centrifuge tube, and infected at a multiplicity of infection (MOI) of 0.1 at 28°C. After exposing cells to the virus for 2 h, the cells were centrifuged and the virus-containing supernatant was removed. The cells were then resuspended in medium containing 5% FBS and cultured at 28°C. Virus-containing culture supernatants were harvested every 2–3 days, and fresh medium was added until the infected cells fully expressed the cytopathic effect. After removing the cell debris by centrifugation, the supernatants were frozen and stored at −80°C until used. Virus titers were determined using plaque assays with BHK21 cells, which were routinely maintained in RPMI 1640 supplemented with 10% FBS. Dengue virus was used for infection of mice at an MOI of 5.

Virus infection

Mice were infected intracerebrally with dengue virus at a dose of 2×10^5 PFU in a volume of 30 μl. After infection, the mice were checked daily for 3 wk, and the mortality rate was determined. Deaths caused by dengue virus usually occurred within 6–7 days after infection in both WT and CXCR3$^{-/-}$ or CXCL10/IP-10$^{-/-}$ mice; deaths occurring within <5 days postinfection were excluded from our analysis.

Viral load in brain

Brain tissues from infected mice were collected, weighed, and homogenized in PBS containing antibiotics using a Dounce homogenizer. The resultant homogenates were centrifuged, after which the supernatants were collected and viral loads were determined with plaque assays, as described below.

Plaque assay

Virus-containing supernatants from cells or tissue homogenates were diluted serially, and 200-μl samples from each dilution were placed onto BHK-21 monolayers in 6-well culture plates. After incubating for 2 h at 37°C in a 5% CO$_2$ incubator, the supernatants were removed, and 5 ml of 1% SeaPlaque Agarose (Cambres) in RPMI 1640 containing 2% FBS was overlaid on the BHK-21 monolayer. The cultures were then incubated at 37°C in a 5% CO$_2$ incubator for 5 days to develop the plaques. The amount of infectious virus in tissues was reported as PFU/tissue.

RNase protection assays

Total RNA was isolated from tissues using TRIzol (Invitrogen Life Technologies) according to the manufacturer's instructions, after which the levels of various chemokine mRNAs were analyzed using multiprobe RNase protection assays. Multiprobe templates were purchased from BD Biosciences, and the assay was conducted according to the manufacturer's instructions. Briefly, radiolabeled RNA probes were generated from multiprobe templates using T7 RNA polymerase and a mixture of pooled unlabeled nucleotides and α-[^{32}P]UTP. The probes were hybridized overnight with 5–20 μg of total RNA and then digested first with an RNase mixture and then with proteinase K. RNase-resistant duplex RNAs were extracted with phenol, precipitated with ammonium acetate, solubilized, and resolved on 5% sequencing gels, which were then dried and subjected to autoradiography and analysis by phosphor imager (Typhoon 9410, Amersham Biosciences).

Measurement of CXCL9/Mig and CXCL10/IP-10 protein in the brain

Mouse brains were weighed and then homogenized on ice in PBS buffer (PBS containing 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM DTT, and 1 mM PMSF). The resultant homogenates were centrifuged, after which the supernatants were collected and assayed for CXCL9/Mig and CXCL10/IP-10 using a DuoSet ELISA Development kit purchased from R&D Systems. Background signals were obtained from brains of uninfected mice in assays done without capture Ab. Background signals were subtracted from all experimental values.

Quantitative real-time PCR

Total RNA was isolated using TRIzol (Invitrogen Life Technologies) according to the manufacturer's instructions, after which 5 μg of total RNA were reverse transcribed using an oligo(dT) primer and SuperScript II reverse transcriptase (Invitrogen Life Technologies) according to the manufacturer's instructions. For real-time PCR analysis, the $2^{-\Delta\Delta C_T}$ method was used to quantify the relative changes in gene expression (28), where C_T is the threshold cycle. Real-time PCR for CD4, CD8, CXCR3, CXCL10/IP-10, CXCL9/Mig, CXCL11/I-TAC, perforin, granzyme A, granzyme B, and GAPDH was conducted using Assays-on-Demand gene expression products (Applied Biosystems), which included a 20× mix of unlabeled PCR primers and TaqMan MGB probe (FAM dye labeled). The PCR cycling protocol entailed 1 cycle at 50°C for 2 min and then 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 and elaborated using an ABI Prism 7700 Sequence Detection System (Applied Biosystems). All samples were run in duplicate. All quantifications were normalized to the level of *GAPDH* gene expression. Analysis of the relative changes in gene expression required calculations based on the threshold cycle (C_T: the fractional cycle number at which the amount of amplified target reaches a fixed threshold) as follows: ΔC_T was calculated as the difference between the mean C_T values of the samples evaluated with CD4-, CD8-, CXCL9/Mig-, CXCL10/IP-10-, CXCL11/I-TAC-, CXCR3-, perforin-, granzyme A-, and granzyme B-specific primers and the mean C_T values of the same samples evaluated with GAPDH-specific primers; $\Delta\Delta C_T$ was calculated as the difference between the ΔC_T values of the samples and the ΔC_T value of a calibrator sample; $2^{-\Delta\Delta C_T}$ was the relative mRNA unit representing the fold induction over control.

Isolation of infiltrating leukocytes from the brain

Brains were harvested and placed in buffer containing RPMI 1640 with 1% FBS. They were then cut into pieces, and the tissue was disrupted by pressing it through a nylon mesh cell strainer. The resultant cell suspensions were centrifuged at $400 \times g$ for 5 min at 4°C. The cell pellets were resuspended in 10 ml of 30% Percoll (Amersham Biosciences), which were then overlaid onto 1 ml of 70% Percoll, and centrifuged at $1300 \times g$ for 30 min at 4°C. After centrifugation, the cells were removed from the interface, washed three times with RPMI 1640 containing 1% FBS, and subjected to FACS analysis. Abs used for flow cytometry were FITC-CD4 (clone GK1.5), PE-CD8α (clone 53-6.7), and allophycocyanin-CD44 (clone IM-7) from BD Biosciences; FITC-CD19 (MB19-1) from eBioscience; PE-NK1.1 from BioLegend; and biotinylated CXCR3 from R&D Systems. FACS analysis was conducted on a FACSCalibur (BD Bioscience), and the data were analyzed using FlowJo (Tree Star).

Immunohistochemistry

Brain tissues were embedded in OCT (DakoCytomation) and frozen in liquid nitrogen. Cryosections (10 μm) were air dried for 10 min, fixed with acetone for 10 min, washed with PBS, and blocked with 20% FBS in PBS for 20 min. The sections were then incubated overnight at 4°C with anti-mouse CD8 (1/1 dilution, clone 53-6.7; hybridoma supernatant) and anti-mouse CD4 (1/500 dilution, clone RM4.5; eBioscience), washed twice with PBS and incubated for 5 h at 37°C with peroxidase-conjugated goat anti-rat IgG (Jackson ImmunoResearch Laboratories). After two additional washes, the color was developed using diaminobenzidine (DAB) substrate (Vector Laboratories).

Splenocyte culture

Mice were intracerebrally infected with dengue virus for 6 days, after which splenocytes were isolated and labeled with 5 μM CFSE (Molecular Probes). The CFSE-labeled splenocytes were cultured in the presence or absence of dengue virus (MOI = 0.5) that had been irradiated with UV for 5 min at an intensity of 772 mJ/cm^2 in a Stratalinker 2400 (Stratagene). Three days later, the cells were harvested for FACS analysis.

Measurement of IFN-γ production

Mice were intracerebrally infected with dengue virus for 6 days, after which splenocytes were isolated and cultured in the presence or absence of UV-irradiated dengue virus (MOI = 0.5). Three days later, the culture supernatants were collected and assayed for IFN-γ using a purified unconjugated capture mAb (clone R4-6A2) and a biotinylated detecting Ab (clone XMG1.2). Both Abs were purchased from BD Biosciences.

Statistical analysis

Statistical significance was performed using the nonparametric two-sample Wilcoxon rank-sum (Mann-Whitney U) test. Kaplan-Meier survival curves were analyzed using the log-rank test. Values of $p < 0.05$ were considered significant.

Results

Mice intracerebrally infected with dengue virus show prominent cerebral expression of CXCL10/IP-10

Using RNase protection assays, we examined the expression of chemokines in the brains of mice intracerebrally infected with dengue virus. We found that dengue infection induced expression of mRNAs encoding multiple chemokines, including CXCL10/IP-10, CCL5/RANTES, CCL3/MIP-1β, CCL4/MIP-1α, and CCL1/T cell activation 3 (TCA3), among which expression of CXCL10/IP-10 mRNA was the most pronounced, although substantial levels of CXCL5/RANTES mRNA were also detected (Fig. 1A). This induction of CXCL10/IP-10 mRNA was independent of the route of infection, as CXCL10/IP-10 was also highly induced in livers from mice infected either i.v. (Fig. 1B) or i.p. (data not shown).

Like CXCL10/IP-10, CXCL9/Mig and CXCL11/I-TAC are IFN-γ-inducible CXC chemokines and show similar in vitro functional activity mediated by CXCR3. Therefore, we tested whether CXCL9/Mig and CXCL11/I-TAC also were induced by infection with dengue virus. Using real-time PCR, we found that cerebral mRNA expression of all three CXC chemokines was significantly up-regulated by dengue infection (Fig. 2A). Consistent with those findings, cerebral levels of both CXCL10/IP-10 and CXCL9/Mig protein were increased following dengue infection, though, as with its transcript, levels of CXCL10/IP-10 proteins were substantially higher than those of CXCL9/Mig (Fig. 2B). We then examined the effect of cerebral infection with dengue virus on CXCR3 expression and found that it, too, was significantly up-regulated in the brains of infected of mice (Fig. 2C).

CXCR3$^{-/-}$ mice are more susceptible to dengue infection

We next sought to assess the impact of the up-regulating IFN-γ-inducible CXC chemokines on dengue infection. To address that question, we used CXCR3-deficient (CXCR3$^{-/-}$) rather than ligand-deficient mice, reasoning that, given the redundancy of the effects of these ligands, mice deficient in any one of them might still behave like WT mice. We found that both WT and CXCR3$^{-/-}$ mice began to die on day 6 following intracerebral infection with dengue virus, but that 3 wk postinfection, the survival rate among WT mice was 76%, while that among CXCR3$^{-/-}$ mice was only 34% ($p = 0.0014$) (Fig. 3). Moreover, ~50% of surviving CXCR3$^{-/-}$ mice, but none of the surviving WT mice, developed severe hind-limb paralysis. Thus, CXCR3-mediated signaling appears to contribute significantly to resistance against primary dengue infection.

CXCR3$^{-/-}$ mice infected by dengue virus show higher viral loads in brains than WT mice

We used plaque assays to test whether the increased rates of dengue-induced mortality and paralysis among CXCR3$^{-/-}$ mice reflected higher viral loads in the brain. The virus became detectable in the brains of both WT and CXCR3$^{-/-}$ mice on day 3 postinfection. The viral loads peaked on day 5 or 6 postinfection and were significantly higher in CXCR3$^{-/-}$ than WT mice ($p < 0.02$) (Fig. 4). Similar results were obtained when the brain viral load was evaluated using real-time PCR of the viral genome (data not shown). These results suggest that WT mice were better able to clear dengue virus from infected tissues than CXCR3$^{-/-}$ mice and that the persistent presence of the virus in the brains of CXCR3$^{-/-}$ mice led to CNS damage, resulting in paralysis and/or death.

CXCR3$^{-/-}$ mice show diminished cerebral infiltration by CD4$^+$ and CD8$^+$ T cells following dengue infection

Given that effector T cells, particularly CD8$^+$ T cells, play a key role in host defense against viral infection (14, 15), that CXCR3 is mainly expressed on activated/effector T cells and NK cells (19), and that CXCR3 ligands are strongly induced in nervous tissues during dengue infection (Figs. 1 and 2), we tested whether diminished recruitment of effector T cells to sites of infection contributed to the higher viral loads observed in the brains of CXCR3$^{-/-}$ mice. We first used real-time PCR to determine cerebral levels of
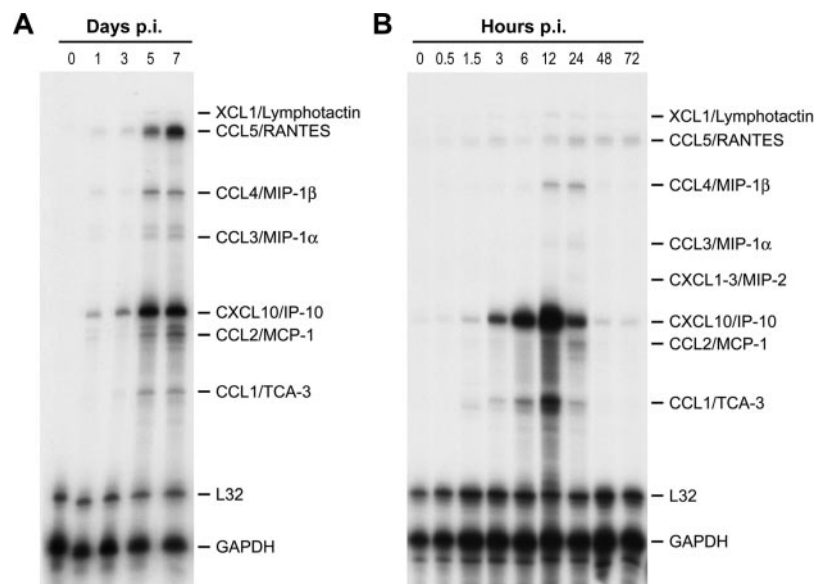
FIGURE 1. CXCL10/IP-10 is strongly up-regulated in mice following infection with dengue virus. *A*, Total RNA was extracted from brains collected at the indicated times after intracerebral infection with dengue virus (2×10^5 PFU). RNase protection assays were conducted with 20-μg samples to assess chemokine gene expression. *B*, Total RNA was extracted from livers collected at the indicated times after mice were i.v. infected with dengue virus, and RNase protection assays were conducted as in *A*. The data shown are representative of two independent experiments in *A* and four in *B*. The gel was exposed for 2 days at −70°C.

A

B

C

FIGURE 2. IFN-γ-inducible CXC chemokines and CXCR3 are dramatically upregulated in the brains of mice infected with dengue virus intracerebrally. *A*, Total RNA was extracted from brains collected at the indicated times after intracerebral infection with dengue virus (2×10^5 PFU), after which 5-µg samples were used for first-strand cDNA synthesis. The first-strand cDNAs were then subjected to real-time PCR using specific primer pairs for CXCL10/IP-10, CXCL9/Mig, and CXCL11/I-TAC. The $2^{-\Delta\Delta C_T}$ from uninfected mice (day 0) was set at 1, and the relative chemokine mRNA units ($2^{-\Delta\Delta C_T}$) represent the fold induction over uninfected mice. Data shown are representative of three independent experiments. *B*, Brains from mice infected with dengue virus for 5 days were homogenized in lysis buffer, after which levels of CXCL9/Mig and CXCL10/IP-10 in the homogenates were assayed using specific ELISAs. The data are presented as means ± SEM from two independent experiments, three to four mice per time point. *C*, CXCR3 expression was detected using real-time PCR as in *A*.

CD4 and CD8, which served as an index of CD4$^+$ and CD8$^+$ T cell infiltration. We found that the time courses of the increases in CD4 and CD8 were similar, and that the increase in CD8 was more pronounced than that of CD4, peaking on day 7 postinfection (Fig. 5*A*). Notably, cerebral levels of CD4 and CD8 were substantially lower in CXCR3$^{-/-}$ than WT mice.

We also isolated infiltrating leukocytes from the brains of dengue-infected and uninfected mice on day 7 postinfection and evaluated the leukocyte population infiltrating the brain. Consistent with the results obtained from real-time PCR, numbers of infiltrating CD4$^+$ and, in particular, CD8$^+$ T cells were significantly lower in the brains of CXCR3$^{-/-}$ mice than WT mice (Fig. 5*B*). This finding was confirmed by immunohistochemical analysis, which also showed the presence of lower numbers of CD4$^+$ and CD8$^+$ T cells in the brains of CXCR3$^{-/-}$ than WT mice on day 7 following dengue infection (Fig. 5*C*). All infiltrating T cells were activated/effector cells, as they all expressed CD44^{high} (data not shown). Interestingly, only ~12% of infiltrating CD8$^+$ and ~16% of infiltrating CD4$^+$ T cells expressed CXCR3 (data not shown). In addition, infiltration of NK cells was also somewhat diminished in CXCR3$^{-/-}$ mice, although levels had not reached statistical significance ($p = 0.28$) (Fig. 5*B*). Taken together, these results clearly demonstrate that T cell recruitment, particularly recruitment of CD8$^+$ T cells, is impaired in CXCR3$^{-/-}$ mice.

To demonstrate the greatly increased numbers of CD8$^+$ T cells were responsible for effector function during dengue infection, we determined the levels of perforin, granzyme A, and granzyme B, all of which are important effector molecules in CD8$^+$ T cells. Using real-time PCR, we found that there was less cerebral expression of effector molecules in CXCR3$^{-/-}$ mice than WT mice (Fig. 5*D*).

T cells responding to dengue virus show up-regulated CXCR3 expression

We have shown that CXCR3 expression is important for recruiting T cells to sites of dengue infection, where they exert effector function (Figs. 3–5). We then asked whether CXCR3 expression is up-regulated in T cells specific for dengue viral Ag. Mice were intracerebrally infected dengue virus or left uninfected, and splenocytes were harvested 6 days later. The splenocytes were then labeled with CFSE to track cell division, and cultured in the presence or absence of UV-irradiated dengue virus. After 3 days in culture, cells were harvested and stained with anti-CD4 or anti-CD8 along with anti-CXCR3. We found that the CFSE^{low} cells were only present within the forward scatter (FSC)^{high} side scatter (SSC)^{high} population and that T cells within the CFSE^{low} population were presumably proliferating T cells resulting from the response to the UV-irradiated dengue virus. These CFSE^{low} T cells
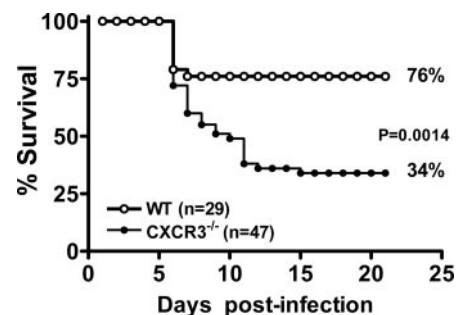

FIGURE 3. CXCR3$^{-/-}$ mice show increased susceptibility to dengue infection. WT (○) and CXCR3$^{-/-}$ (●) mice were intracerebrally infected with 2×10^5 PFU of dengue virus in a volume of 30 µl. Deaths were recorded daily for 3 wk.
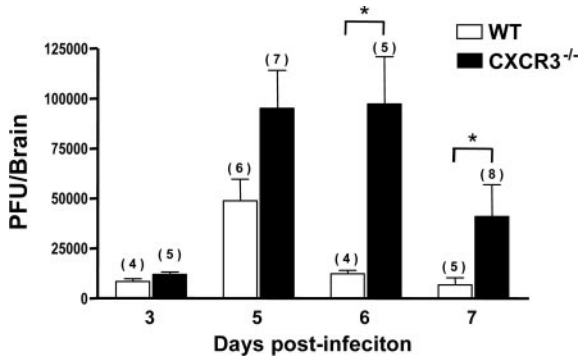
FIGURE 4. The brains of CXCR3$^{-/-}$ mice show higher viral loads than those of WT mice following dengue infection. Mice were intracerebrally infected with 2×10^5 PFU of dengue virus and then sacrificed on indicated days postinfection. Viral titers in brain homogenates were determined using plaque assays. The numbers in parenthesis indicate the numbers of mice used at each time point. Data are presented as means ± SEM. *, $p < 0.02$ (vs WT mice).

were larger than resting T cells, indicating they were activated/effector cells likely specific for dengue viral Ag. The number of both CFSE^{low}CD4$^+$ and CFSE^{low}CD8$^+$ T cells were significantly increased among splenocytes cultured in the presence of UV-irra-

diated dengue virus, as compared with those cultured in the absence of the virus (Fig. 6A). We then compared CXCR3 expression in dengue virus-responding T cells (CFSE^{low}/large) with that in unstimulated, resting T cells (CFSE^{high}/small) and found that CXCR3 expression was up-regulated in both dengue viral Ag-specific CD4$^+$ and CD8$^+$ T cells (Fig. 6B).

We also analyzed the IFN-γ production to assess the effector function of dengue viral Ag-specific T cells. Only culture supernatants from splenocytes isolated from dengue-infected mice and cultured with UV-irradiated dengue virus produced significant levels of IFN-γ. No IFN-γ was produced in splenocytes cultured in the absence of UV-irradiated dengue virus (data not shown).

CXCR3$^{-/-}$ and WT mice express similar levels of inflammatory chemokine receptors

In addition to CXCR3, we also tested whether the expression of other chemokine receptors was also enhanced during dengue infection. RNase protection assays revealed that CXCR3$^{-/-}$ and WT mice expressed similar levels of CCR1, CCR2, and CCR5 following dengue infection (Fig. 7). Induction of these chemokine receptors is associated with Th1-mediated immune responses (29, 30), which is consistent with the idea that dengue infection elicits a Th1-mediated immune response (31). As such, CCR3 and CCR4,
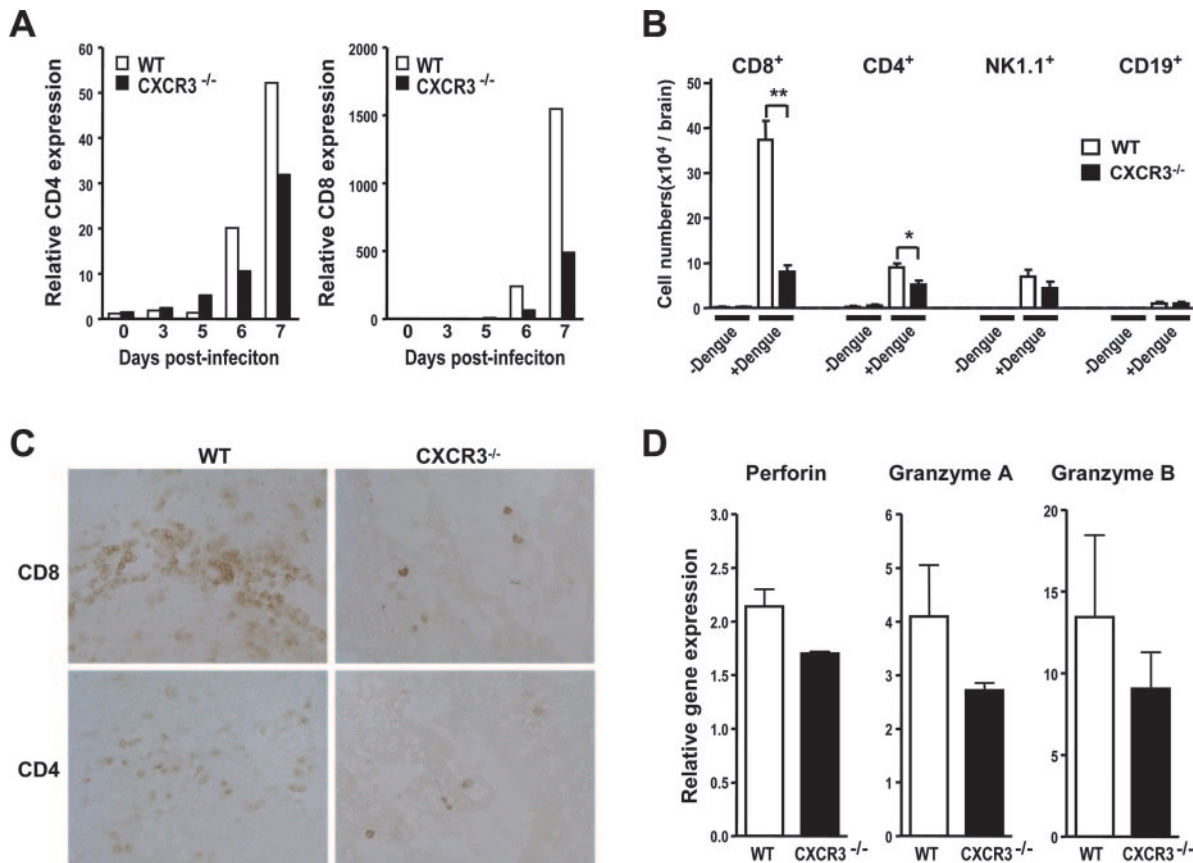

FIGURE 5. Brains from CXCR3$^{-/-}$ mice show diminished CD4$^+$ and CD8$^+$ T cell infiltration following dengue infection. *A*, Total RNA was extracted from brains collected at the indicated times after intracerebral infection with dengue virus (2×10^5 PFU), and 5-μg samples were used for first-strand cDNA synthesis. The first-strand cDNA together with specific primer pairs for CD4$^+$ or CD8$^+$ T cells were then subjected to real-time PCR. The relative CD4 and CD8 mRNA expression ($2^{-\Delta\Delta C_T}$) represents the fold induction over control (uninfected WT mice). Data shown are representative of three independent experiments. *B*, Mice were either left untreated or intracerebrally infected with dengue virus. On day 6 postinfection, brain tissues were collected, and infiltrating leukocytes were isolated by Percoll gradient centrifugation. Actual numbers of infiltrating CD4$^+$ T cells, CD8$^+$ T cells, NK cells, and B cells per brain are shown. The total number of Percoll-enriched cells was multiplied by the percent for each population obtained by FACS analysis. Data are presented as means ± SEM from two independent experiments, five mice per group; *, $p < 0.01$, **, $p < 0.001$. *C*, Brain sections from WT and CXCR3$^{-/-}$ mice were stained with anti-mouse CD8 and anti-mouse CD4 Abs followed by HRP-conjugated goat anti-rat Ab, after which the color was developed using DAB substrate. *D*, Expression of perforin, granzyme A, and granzyme B in brain tissues was measured using real-time PCR as in *A*. Data are presented as means ± SEM of two mice.
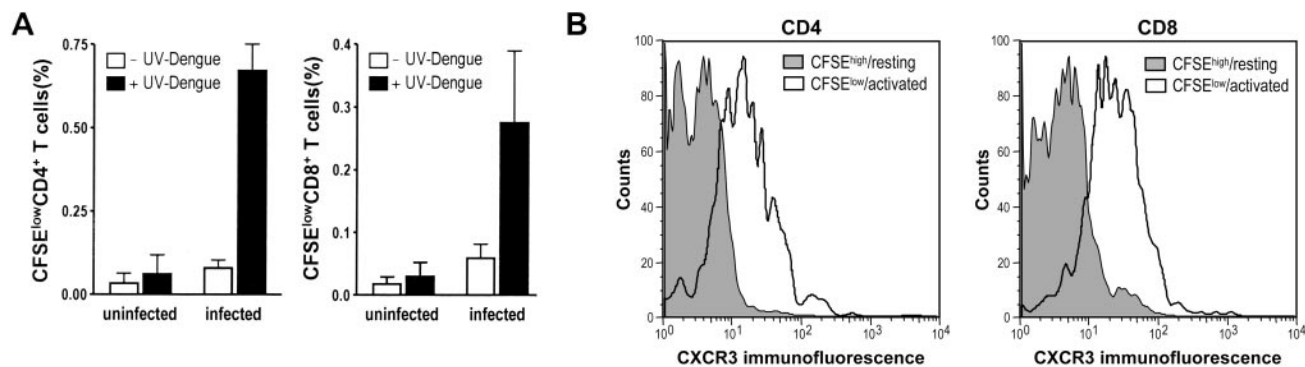
FIGURE 6. Dengue viral Ag-specific T cells up-regulate CXCR3 expression. *A*, Mice were intracerebrally infected with 2×10^5 PFU of dengue virus or left uninfected, and 6 days later splenocytes were isolated and labeled with CFSE. The CFSE-labeled splenocytes were then cultured with or without UV-irradiated dengue virus. After 3 days in culture, the cells were harvested and subjected to FACS analysis. CFSE^low cells were only found within the population of cells that showed FSC^high SSC^high. This population was then gated to determine the percentage of CFSE^low CD4$^+$ and CFSE^low CD8$^+$ T cells, which were presumably proliferating T cells as a result of responding to the UV-irradiated dengue virus. Data shown are representative of two experiments and presented as means ± SEM, three mice per group. *B*, CXCR3 expression in CD4$^+$ or CD8$^+$ T cells within the CFSE^low population was compared with that of CD4$^+$ or CD8$^+$ T cells within the resting population.

which are associated with Th2-type immune responses (30, 32), were not induced during dengue infection. The fact that, like WT mice, CXCR3$^{-/-}$ mice may recruit effector T cells bearing CCR1, CCR2, and CCR5 receptors could explain, at least in part, why infiltrating lymphocytes were also detected in the brains of CXCR3$^{-/-}$ mice.

CXCL10/IP-10$^{-/-}$ mice are susceptible to dengue infection

To investigate whether the prominent induction of CXCL10/IP-10 in this model might be indicative of CXCL10/IP-10-specific function during dengue infection, we examined the susceptibility of CXCL10/IP-10$^{-/-}$ mice to the virus. We found that CXCL10/IP-10$^{-/-}$ mice were significantly ($p < 0.0001$) more susceptible to dengue infection than WT mice (Fig. 8), despite their ability to express comparable levels of CXCL9/Mig and CXCL11/I-TAC (data not shown). Apparently, despite the similarity of their properties in vitro, CXCL9/Mig and CXCL11/I-TAC are not able to substitute for CXCL10/IP-10 during viral infection in vivo. Notably, when we compared the susceptibilities of CXCR3$^{-/-}$ and CXCL10/IP-10$^{-/-}$ mice to dengue virus, CXCL10/IP-10$^{-/-}$ mice tended to be more susceptible than of CXCR3$^{-/-}$ mice ($p = 0.056$), highlighting the critical role played by CXCL10/IP-10 during dengue infection.

Discussion

In the present study, we investigated the activity of CXCR3 during dengue infection and found that CXCR3$^{-/-}$ mice had an impaired ability to recruit effector T cells to sites of infection, resulting in inefficient clearance of virus, which in turn led to neuronal damage and paralysis and/or death. To assess the importance of CXCR3 ligands in dengue virus infection, we also tested the susceptibility of CXCL10/IP-10$^{-/-}$ mice to dengue virus and found that the mortality rate among CXCL10/IP-10$^{-/-}$ mice was even higher than among CXCR3$^{-/-}$ mice. The increased susceptibility of CXCR3$^{-/-}$ and CXCL10/IP-10$^{-/-}$ mice to dengue virus reveals that both CXCR3 and CXCL10/IP-10 are crucial molecules governing the protective response against infection.

Chemokine-mediated recruitment of effector T cells, CD8$^+$ T cells in particular, into sites of viral infection is crucial for efficient clearance of virus from the CNS (33, 34). The receptor for the IFN-γ-inducible CXC chemokines is CXCR3, which is mainly expressed on activated CD4$^+$, memory/activated CD8$^+$ and NK cells (19, 20), so that CXCR3 is thought to play a key role during

viral infections. As such, the CXCR3$^{-/-}$ mouse has been used as an animal model to investigate the function of CXCR3 during such infections. Somewhat surprisingly, those studies found that CXCR3 often either causes immunopathology (35, 36) or does not exert any significant effect (37). For instance, most CXCR3$^{-/-}$ mice survived infection with lymphocyte choriomeningitis virus, whereas WT mice invariably died as a result of CD8$^+$ T cell-mediated immunopathology (35). The survival rate among CXCR3$^{-/-}$ mice was also significantly higher than among WT mice following infection with HSV type 1 (36). Leukocyte recruitment and viral replication were identical in CXCR3$^{-/-}$ and WT mice following influenza infection (37). And although CXCR3$^{-/-}$ mice infected with gamma herpesvirus 68 showed delayed clearance of replicating virus from the lungs, which correlated with delayed T cell recruitment, no mortality was seen (38). In contrast to those reports, our results showing that the mortality rate was higher among CXCR3$^{-/-}$ than WT mice following dengue infection, and that surviving CXCR3$^{-/-}$ mice, but not WT mice, often were severely paralyzed. This clearly suggests that CXCR3 mediates a crucial protective response against dengue virus and does not trigger a prominent T cell-mediated immunopathological response. To our knowledge, this is the first study showing that the absence of CXCR3 significantly damages host defense against viral infection.

The greater infiltration by T cells seen in WT mice was accompanied by lower cerebral viral loads than were seen in CXCR3$^{-/-}$ mice, which is indicative of the importance of CXCR3-bearing effector T cells for clearance of dengue virus and inhibition of viral replication. We found that mice infected with dengue virus showed much greater infiltration by CD8$^+$ T cells, which was associated with increased expression of effector molecules. This likely means that CD8$^+$ T cells are the predominant effector cells recruited to infected tissues during dengue infection. In addition, given that the increased numbers of Th1 cells seen in the peritoneum in the presence of Ag is at least partially attributable to local proliferation (39), that CXCR3-mediated signaling stimulates the activation and proliferation of CD8$^+$ T cells (40), and that expression of both CXCL10/IP-10 and CXCR3 are significantly increased following dengue virus infection (Figs. 1 and 2), we propose that dengue-induced expression of high levels of CXCL10/IP-10 might lead to the activation and proliferation of CD8$^+$ T cells recruited to infected tissues.
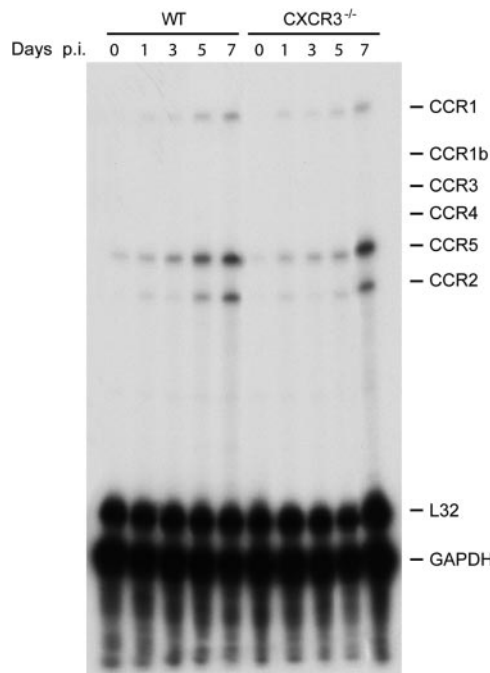
FIGURE 7. WT and CXCR3$^{-/-}$ mice infected with dengue virus express similar levels of CCR1, CCR2, and CCR5 in brain. Total RNA was extracted from brains collected at the indicated times after intracerebral infection with 2×10^5 PFU of dengue virus. RNase protection assays were conducted with 20-μg samples to assess chemokine gene expression. The gel was exposed for 2 days at $-70°C$.

FIGURE 8. CXCL10/IP-10$^{-/-}$ mice show increased susceptibility to dengue virus infection. WT ($\bigcirc$) and CXCL10/IP-10$^{-/-}$ ($\bullet$) mice were intracerebrally infected with 2×10^5 PFU of dengue virus in a volume of 30 μl. Deaths were recorded daily for 3 wk.

Analysis of CXCR3 expression in T cells infiltrating the brains of dengue-infected mice revealed that only about ~12% of CD8$^+$ and ~16% of CD4$^+$ T cells express CXCR3. Whether these percentages reflect the percentages of CXCR3-bearing T cells originally migrating to the inflamed brain is not clear at this point. However, CXCR3 expression is reportedly up-regulated in Th1-mediated inflammation during T cell activation in peripheral lymphoid organs, while CXCR3 expression is diminished in the inflammatory organs (41). CXCR3 expression in T cells also is reportedly down-regulated via internalization upon interaction with its ligands expressed on endothelial cells (42). It is thus plausible that many of the infiltrating T cells detected might express CXCR3 before re-exposure to the dengue viral Ag in the infected brains. Alternatively, it may be that despite the small size of the CXCR3-bearing T cell population, the numbers are sufficient to mediate the response to dengue infection, which further highlights the importance of CXCR3 for resistance to dengue infection

Following dengue infection, expression of Th1-type chemokine receptors (CCR1, CCR2, and CCR5) was similar in CXCR3$^{-/-}$ and WT mice. CCR2- and CCR5-bearing CD8$^+$ T cells have been shown to migrate to sites of mouse hepatitis virus (MHV) infection, where they clear the virus (43, 44). It is noteworthy that although induction of CCR2, CCL5/RANTES (data not shown) and their receptors (CCR1 and CCR5) was perfectly normal in CXCR3$^{-/-}$ mice, they were apparently not sufficient to mediate effective host defense against dengue infection. Alternatively, given that CCR5, CCR2, and CCR1 are highly expressed on macrophages (45, 46), it is also possible that most of the receptors detected were expressed on macrophages rather than T cells.

It is well-established that infecting cell lines with dengue virus in vitro induces expression of various chemokines, including CXCL1/IL-8 (47–50), CCL3/MIP-1α (47, 51, 52), CCL5/RANTES (47, 50, 53), and CCL4/MIP-1β (51, 52). In contrast, ours is
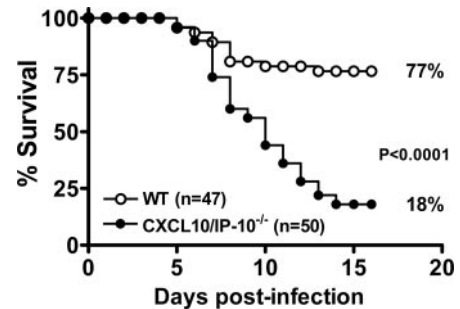
the first study to show in vivo induction of chemokines following infection with dengue virus. Most prominent was CXCL10/IP-10, expression of which was independent of both the tissue type infected and the route of infection. Moreover, the susceptibility of CXCL10/IP-10$^{-/-}$ mice to dengue virus highlights the indispensable role played by this chemokine during dengue infection. The effect of CXCL10/IP-10 during infection by a variety of viruses, including mouse hepatitis virus (26, 54–56), HSV type 1 (36, 57), lymphocytic choriomeningitis virus (35), murine gamma herpesvirus 68 (38, 58), vaccinia virus (24, 59–61), Newcastle disease virus (25), West Nile virus (62, 63), and severe acute respiratory syndrome-coronavirus (64), has been investigated. Of particular interest to us was the finding that CXCL10/IP-10$^{-/-}$ mice are more susceptible to dengue infection than CXCR3$^{-/-}$ mice. Given that CXCL9/Mig, CXCL10/IP-10 and CXCL11/I-TAC all share the same receptor (CXCR3) and show similar functional properties in vitro, and that CXCL9/Mig and CXCL11/I-TAC, in particular, were dramatically induced in CXCL10/IP-10$^{-/-}$ mice, one might expect that CXCL9/Mig and CXCL11/I-TAC would exert a compensatory effect in the absence of CXCL10/IP-10. This was not the case, however, which implies that CXCL10/IP-10 functions in ways other than via regulation of leukocyte recruitment and that these other functions cannot be replicated by CXCL9/Mig and CXCL11/I-TAC during dengue virus infection. It has been suggested that CXCL10/IP-10 is involved in leukocyte activation and generation of Ag-specific effector T cells in the periphery (26, 40) and that induce apoptosis via a p53-dependent pathway during viral infection (65). The function of CXCL10/IP-10 other than recruitment of effector cells in dengue virus infection remains to be identified. Differences in the in vivo activities of IFN-γ-inducible CXC chemokines also have been observed in other systems. For instance, treating MHV-infected mice with anti-CXCL10/IP-10 (55) or anti-CXCL9/Mig (54) had similar adverse effects on T cell infiltration, viral clearance, and mortality, suggesting both CXCL10/IP-10 and CXCL9/Mig are equally important in the host defense against cerebral MHV infection and so may not compensate for one another. Likewise, MHV-infected CXCL10/IP-10$^{-/-}$ mice showed reduced T lymphocyte infiltration and impaired control of viral replication in the brain, suggesting that CXCL9/Mig and CXCL11/I-TAC cannot adequately compensate for the loss of CXCL10/IP-10 activity (26) in that model. We are currently using CXCL9/Mig$^{-/-}$ mice to investigate whether CXCL9/Mig also plays an important role following dengue infection.

Shresta et al. (66) demonstrated that mice deficient in IFNs are more susceptible to dengue infection. They proposed that IFN-$\alpha\beta$ is critical for the early immune response to dengue and that IFN-γ-mediated immune responses are necessary for both the early and

late clearance of the virus. Considered together with the earlier reports that CXCL10/IP-10 is induced by both IFN-$\alpha\beta$ and IFN-γ (20, 24, 67–69), our findings that both CXCR3$^{-/-}$ and CXCL10/IP-10$^{-/-}$ mice are more susceptible to dengue virus underscores the importance of characterizing the mediators situated downstream of IFNs in the host defense against primary dengue infection.

In summary, our results demonstrate the protective role played by CXCR3 against primary dengue infection of the CNS, mediating migration of effector T cells that participate in clearing the virus from sites of infection. In addition, our finding that mortality rates are higher among CXCL10/IP-10$^{-/-}$ than CXCR3$^{-/-}$ mice strongly suggests that CXCL10/IP-10 is critical for host defense against dengue virus and that other IFN-γ-inducible CXC chemokines (CXCL9/Mig and CXCL11/I-TAC) do not adequately compensate for the loss of CXCL10/IP-10. Apparently, these chemokines exert distinct effects in vivo, despite the apparent redundancy of their effects in vitro.

Disclosures

The authors have no financial conflict of interest.

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