Drak2 Contributes to West Nile Virus Entry into the Brain and Lethal Encephalitis[1]

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Death-associated protein kinase-related apoptosis-inducing kinase-2 (Drak2), a member of the death-associated protein family of serine/threonine kinases, is specifically expressed in T and B cells. In the absence of Drak2, mice are resistant to experimental autoimmune encephalomyelitis due to a decrease in the number of cells infiltrating the CNS. In the present study, we investigated the role of Drak2 in West Nile virus (WNV)-induced encephalitis and found that $Drak2^{-/-}$ mice were also more resistant to lethal WNV infection than wild-type mice. Although $Drak2^{-/-}$ mice had an increase in the number of IFN-γ-producing T cells in the spleen after infection, viral levels in the peripheral tissues were not significantly different between these two groups of mice. In contrast, there was a reduced viral load in the brains of $Drak2^{-/-}$ mice, which was accompanied by a decrease in the number of $Drak2^{-/-}$ CD4$^+$ and CD8$^+$ T cells in the brain following WNV infection. Moreover, we detected viral Ags in T cells isolated from the spleen or brain of WNV-infected mice. These results suggest that following a systemic infection, WNV might cross the blood brain barrier and enter the CNS by being carried by infected infiltrating T cells. *The Journal of Immunology,* **2008, 181: 2084–2091.**

West Nile virus (WNV),[5] a vector-borne neurotropic flavivirus, has caused annual outbreaks of viral encephalitis in North America since its arrival here in 1999 (1, 2). Following initial virus replication at a site peripheral to the CNS, WNV can enter the brain either via a hematogenous pathway or by retrograde axonal transport from a peripheral site (3–5). Severe neurological disease (including encephalitis) and death have been observed in >30% of the confirmed WNV cases, with a higher frequency in the elderly and immunocompromised (1, 2). Currently, no vaccines are available for humans, and treatment is mostly nonspecific and supportive.

The murine model is an effective in vivo experimental model to investigate WNV pathogenesis and host immune response to human infections (6–8). Work from animal models has demonstrated that type 1 IFNs, $\gamma\delta$ T cells, and humoral immunity are critical in controlling dissemination of WNV (9–15). In addition, CD4$^+$ (16) and CD8$^+$ $\alpha\beta$ T cells (17, 18) contribute to host survival following WNV infection. WNV can gain access to the CNS after a brief viremia in the periphery, a process called neuroinvasion, that may turn a mild viral infection into severe lethal encephalitis (6, 19, 20). Recent studies have shown that a WNV replication induces a proinflammatory response that modulates the blood brain barrier (BBB) permeability, which in turn may enable viral entry into the brain and induce lethal encephalitis (5, 21). Nevertheless, the underlying mechanism(s) in which WNV crosses the BBB and enters the CNS is not yet clearly understood. Once inside the brain, WNV-induced CNS disease might be caused by neuronal degeneration, a direct result of viral infection, and/or by bystander damage from the immune response to the pathogen, including lymphocyte and microglial cell responses (18, 22–24). The contributions of direct cell damage by WNV and damage induced by the host immune response in the development of disease are still under investigation.

Drak2 is specifically expressed in T and B cells (25, 26). Drak2 functions to negatively regulate signals involved in T cell activation (27). T cells from $Drak2^{-/-}$ mice exhibited enhanced sensitivity to TCR-mediated stimulation with a reduced requirement for costimulation (26). However, $Drak2^{-/-}$ mice were shown to be remarkably resistant to experimental autoimmune encephalomyelitis (EAE), and this resistance was due in part to a reduction of infiltrating cells into the CNS (26). In the present study, we investigated the role of Drak2 in WNV-induced encephalitis.

Materials and Methods

Mice

$Drak2^{-/-}$ mice were bred on the C57BL/6 (B6) background and had been fully backcrossed (26). Littermates were used as controls. Experiments were performed with 6- to 14-wk-old animals. Groups were age and sex

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[5] Abbreviations used in this paper: WNV, West Nile virus; BBB, blood brain barrier; EAE, experimental autoimmune encephalomyelitis; i.c., intracranial; Q-PCR, quantitative PCR.

matched for each experiment and were housed under identical conditions. All animal experiments were approved by the Animal Care and Use Committee at Colorado State University.

Infection in mice

We inoculated mice i.p. with 800 PFU (a dose close to LD_{100}) of WNV isolate 2741 in 200 μl of PBS with 5% gelatin. For intracranial (i.c.) infection, we anesthetized mice and infected them with 160 PFU of WNV isolate 2741 in 20 μl of PBS with 5% gelatin. Infected mice were monitored twice daily for morbidity, including lethargy, anorexia, and ataxia.

Quantitative PCR (Q-PCR) for determining viral load, T cell levels, and cytokine production

Blood, spleen, and brain tissues from control and experimental mice were collected at indicated days postinfection. H36.12j cells (a mouse macrophage cell line) were obtained from the American Type Culture Collection, incubated on 12-well plates (1×10^5 cells/well) for 24 h at 37°C, and then infected with WNV (multiplicity of infection = 1). Cells were harvested at day 1 postinfection. Viral RNA was extracted using RNeasy extraction (Qiagen). The extracted RNA was eluted in a total volume of 50 μl of RNase-free water. A total of 250 ng of each extracted RNA sample was used to synthesize cDNA using the ProSTAR first-strand RT-PCR kit (Stratagene). The sequences of the primer-probe sets for WNV envelope gene (*WNE*) and PCR conditions were described previously (5, 28). Probes contained a 5' reporter, FAM, and a 3' quencher, TAMRA (Applied Biosystems). The assay was performed on an iCycler (Bio-Rad). The sequences of the primer sets for CD4, CD8, and CXCL10 genes were described in previous publications (10, 29). Q-PCR analysis for these genes was performed using RT Real-Time SYBR Green/Fluorescein PCR master mix (Superarray). To normalize the samples, the same amount of cDNA was used in a Q-PCR for β-actin. The ratio of the amount of amplified gene compared with the amount of *β-actin cDNA* represented the relative levels in each sample.

ELISA

Microtiter plates were coated with rWNV-E protein expressed in *Drosophila melanogaster* S2 cells (30) overnight at 4°C at 100 ng/well in coating buffer (0.015 M Na_2CO_3, 0.03 M $NaHCO_3$, and 0.003 M NaN_3 (pH 9.6)). Sera from infected mice were diluted from 1/40 or 1/100 in PBS with 2% BSA, added to the duplicate wells, and incubated for 1 h at room temperature. Plates were washed three times with PBS-Tween 20 (PBS-T). Alkaline phosphatase-conjugated goat anti-mouse IgG or IgM (Sigma-Aldrich) at a dilution of 1/1000 in PBS-T was added for 1 h at room temperature. After washing three times with PBS-T, color was developed with *p*-nitrophenyl phosphate (Sigma-Aldrich) for 10 min, and intensity was determined at an absorbance of 405 nm using a spectrophotometer.

Isolation of brain leukocytes

Brain leukocytes were isolated at day 11 postinfection based on a previously described method (31). Before harvest, extensive cardiac perfusion was done using PBS to deplete intravascular leukocytes. Brains were collected and homogenized. The cell homogenates were centrifuged and resuspended in 7 ml of PBS with 2% FBS mixed with 3 ml of 90% Percoll (Sigma-Aldrich) in PBS. The suspension was next underlaid with 1 ml of 70% Percoll in RPMI 1640 and centrifuged at 800 × *g* for 20 min at 22°C. Leukocytes at the interface were harvested and counted.

T cell purification from spleen and brain

Single-cell suspensions of T cells were made from spleens of WNV-infected mice or brain leukocytes by a positive selection method, using anti-CD90 magnetic beads (Miltenyi Biotec), according to the manufacturer's instructions.

In vitro T cell infection and virus titration

Purified splenic T cells were incubated on 96-well plates (2×10^5 cells/well) overnight at 37°C and infected with WNV (multiplicity of infection = 0.5) for 1 h. Cells were washed after infection and incubated at 37°C. Supernatant was harvested at days 2, 3, and 5 postinfection. Virus titers were determined by plaque assay. Briefly, Vero cells were seeded in 6-well plates in DMEM (Invitrogen) supplemented with 10% FBS (Sigma-Aldrich) 24 h before infection. Serial dilutions of supernatant from infected T cell culture were added and incubated for 1 h. Subsequently, DMEM containing 1% low-melting-point agarose was added and the plates were incubated for 4 days. A second overlay of 2.5 ml of 1% agarose-medium containing 0.01% neutral red was added to visualize plaques. Virus concentrations were determined as PFU/ml.
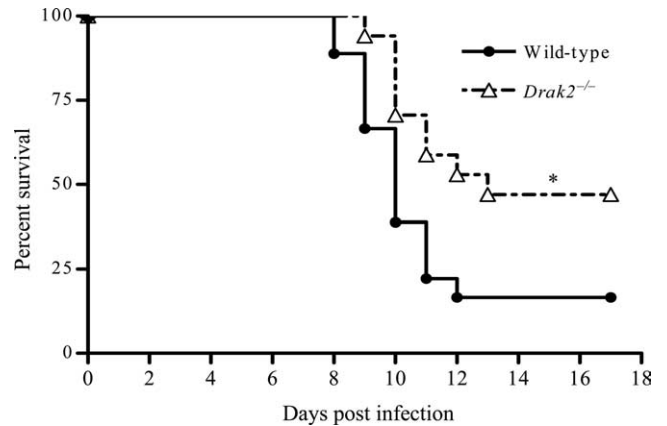

FIGURE 1. $Drak2^{-/-}$ mice are more resistant to lethal WNV infection. Wild-type and $Drak2^{-/-}$ mice were infected with a dose close to an LD_{100} of WNV and monitored twice daily for mortality. Data shown are pooled from three independent experiments. Value of $p = 0.02 < 0.05$ for wild-type ($n = 18$) vs $Drak2^{-/-}$ mice ($n = 17$).

Flow cytometry

Freshly isolated splenocytes were stained with Abs specific for CD4, CD8 (BD Biosciences), and CXCR3 (R&D Systems). Isolated brain leukocytes were stained with Abs for cell surface markers, including CD3, CD4, CD8, and CD45 (BD Biosciences). After staining, cells were fixed with 0.5% paraformaldehyde in PBS and examined using a Coulter XL flow cytometer (Beckman Coulter). Dead cells were excluded on the basis of forward and side light scatter. Data were analyzed using FCS express 2 (De Novo Software).

Intracellular cytokine staining

To measure cytokine production, splenocytes from WNV-infected mice were isolated and then stimulated at 2.5×10^6 cells/ml with 50 ng/ml PMA (Sigma-Aldrich) and 500 ng/ml ionomycin (Sigma-Aldrich) for 5 h at 37°C. Golgi-plug (BD Biosciences) was added during the final 3.5 h. The cells were then harvested, stained with Abs (BD Biosciences) for TCR$\alpha\beta$ and CD8α, and fixed in 2% paraformaldehyde. The cells were then permeabilized with 0.5% saponin before adding PE-conjugated anti-IFN-γ (clone XMG 1.2) or control PE-conjugated rat IgG1 (BD Biosciences). Cells were analyzed using a Coulter XL instrument, as described above.

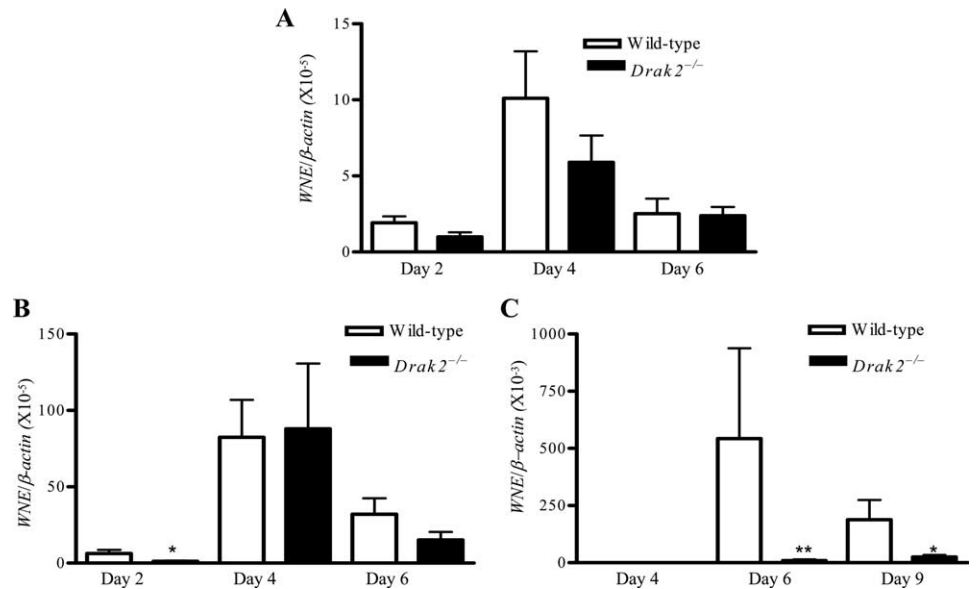### Cytometric bead array

At days 2 and 4 postinfection, sera were collected from mice and measured for IFN-γ production using a mouse Th1/Th2 cytokine kit (BD Biosciences; which includes assays for IL-2, IL-4, IL-5, IFN-γ, and TNF-α) using a FACSArray analyzer (BD Biosciences).

Immunofluorescence microscopy

Mice were transcardially perfused with PBS, and brains were placed in 4% paraformaldehyde in PBS overnight at 4°C, and, in the case of cryoimmunohistochemistry, cryoprotected with 10% (w/v), followed by 20% (w/v) and then 30% (w/v) sucrose in PBS for 24 h each before embedding in Optimal Cutting Temperature compound. Subsequently, specimens were processed and histological slides were prepared for staining with various Abs. For paraffin sections, we processed Ag retrieval at 90°C for 30 min in 10% (v/v) target retrieval solution (DakoCytomation) or by treatment with 88% formic acid for 8 min. The endogenous peroxidase activity was quenched in 0.3% H_2O_2 for 30 min at room temperature. We performed the staining using the Vectastain Elite ABC kit coupled to the 3'-3 diaminobenzidine substrate (Vector Laboratories). Rat Ab specific for mouse CD45 (clone YW 62.3; Serotec), or mouse ascitic fluid WNV Abs were used. Sections were digitized with Kodak scientific imaging software (Eastman Kodak). For splenic and brain T cell staining, 80,000 cells were fixed by submerging them in acetone at −20°C for 30 min. Cells were stained and Ags were detected with PE-Cy5-conjugated Abs to CD8 or CD4 and the flavivirus E protein-specific mAb 4G2 (32) (1/150 dilution) for 1.5 h at 37°C, followed by biotinylated anti-mouse IgG (1/200 dilution) for 1.5 h and streptavidin-FITC (GE Healthcare Bio-Sciences) for 30 min at 37°C.

FIGURE 2. Viral load analysis in wild-type and $Drak2^{-/-}$ mice. Viral load was determined in blood (*A*), spleen (*B*), and brain (*C*) of wild-type and $Drak2^{-/-}$ mice at the indicated days following WNV infection using Q-PCR. The *y*-axis depicts the ratio of the amplified *WNV-E* cDNA to *β-actin* cDNA of each sample (unit-less ratio ± SEM). Five to eight samples were collected at each condition. Value of $p < 0.05$ compared with wild-type mice. Data shown were representative of two independent experiments.

Statistical analysis

Survival curve comparisons were performed using Prism software (Graph-Pad) statistical analysis, which uses the log rank test (equivalent to the Mantel-Haenszel test). Values of *p* for viral burden, cytokine production, Ab titer, and T cell number experiments were calculated with a nonpaired Student's *t* test or Mann-Whitney *U* test.

Results

Drak2$^{-/-}$ mice are more resistant to WNV infection

$Drak2^{-/-}$ mice were previously shown to be more resistant to EAE than wild-type mice due in part to a decrease in the number of cells infiltrating the CNS (26). WNV infection also induces the migration of lymphocytes into the CNS (10, 18). To examine whether Drak2 is involved in WNV-induced encephalitis, we challenged $Drak2^{-/-}$ mice and wild-type mice i.p. with 800 PFU of WNV and monitored the mice twice daily for survival. $Drak2^{-/-}$ mice were more resistant (47% survival) to lethal WNV infection than wild-type controls (16.7% survival) (Fig. 1; $p < 0.05$). Throughout infection, the kinetics and magnitude of viremia in $Drak2^{-/-}$ mice were not significantly different from that of wild-type controls (Fig. 2*A*; $p > 0.05$). Although viral load was slightly reduced in spleens of $Drak2^{-/-}$ mice at day 2 (Fig. 2*B*; $p < 0.05$), no difference was noted between these two groups at the later stage of viral infection (days 4 and 6; $p > 0.05$). Nevertheless, there was a nearly 60-fold reduction in viral load in the brains of $Drak2^{-/-}$ mice at day 6 (peak of infection in the brain) and 7- to 8-fold reduction at day 9 following WNV challenge (Fig. 2*C*; $p < 0.05$). These data suggest that Drak2 plays a role in the lethality and affects the virus levels in the CNS following WNV challenge.

Humoral responses remain normal in Drak2$^{-/-}$ mice upon WNV infection

Drak2 is specifically expressed in T and B cells (26). B cell-mediated humoral immune responses are critical for the host defense against disseminated infection by WNV (14, 33). Therefore, we next measured WNV-specific IgM and IgG levels in the sera from $Drak2^{-/-}$ and wild-type mice following primary WNV infection. At days 5 and 9 postinfection, we did not detect significant differences between the two groups in regard to either the IgM or IgG response (Fig. 3; $p > 0.05$). These data suggest that the absence of Drak2 does not influence the development of Ab following WNV infection.

There are more IFN-γ-producing splenocytes from Drak2$^{-/-}$ mice after ex vivo stimulation

Cellular immunity plays an important role in host survival during WNV infection (8, 13). To understand the role of Drak2 in regulating peripheral T cell responses during WNV infection, we analyzed IFN-γ production of splenic $CD4^+$ and $CD8^+$ T cells from WNV-infected mice using an ex vivo intracellular cytokine assay. As shown in Fig. 4*A*, at day 5 postinfection, more splenic $CD4^+$ T cells in $Drak2^{-/-}$ mice produced IFN-γ upon ex vivo stimulation with PMA and ionomycin than those of wild-type mice, whereas there was not a difference in the number of IFN-γ-producing $CD8^+$ T cells at this time. However, at day 9 postinfection, there
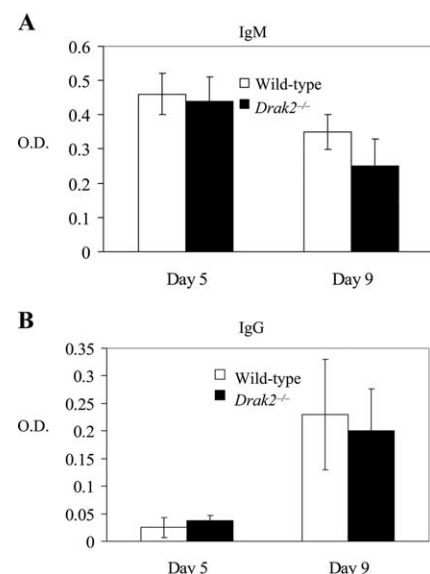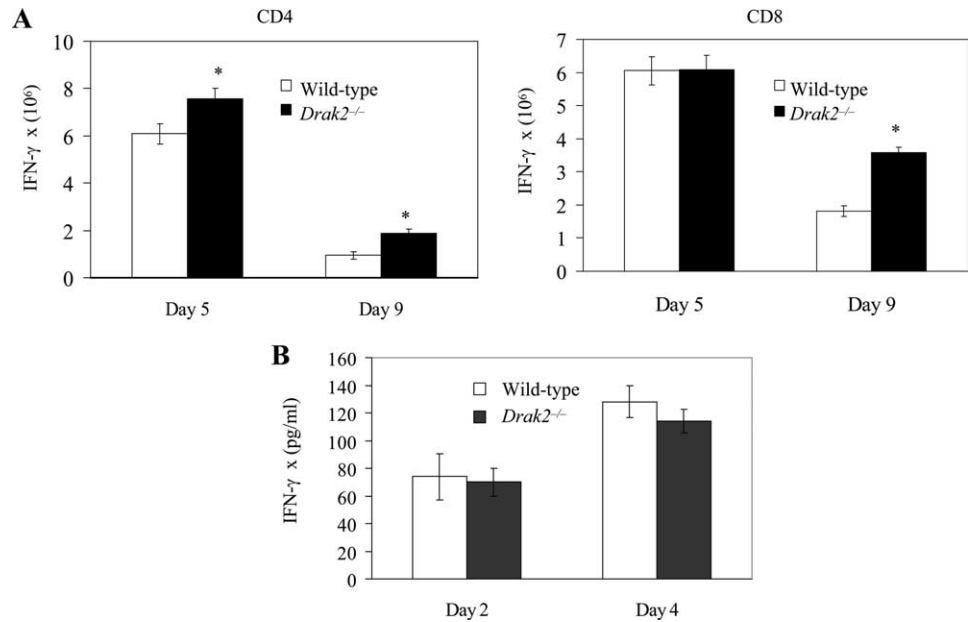
FIGURE 3. Humoral responses remain normal in $Drak2^{-/-}$ mice upon WNV infection. Sera were collected from wild-type or $Drak2^{-/-}$ mice at the indicated days during WNV infection. The development of specific IgM (*A*) or IgG (*B*) Abs to WNV was determined after incubating sera with absorbed purified rWNV-E protein. Data shown are representative of three similar experiments and are the average of four mice in each experiment per time point, performed in duplicate.

FIGURE 4. IFN-γ production in wild-type and *Drak2*$^{-/-}$ mice following WNV infection. *A*, Splenocytes were isolated from WNV-infected wild-type and *Drak2*$^{-/-}$ mice (four samples per condition) at days 5 and 9 postinfection and were cultured ex vivo with PMA plus ionomycin, as described in *Materials and Methods*, and stained for IFN-γ, CD4, or CD8. Total number of IFN-γ$^+$CD4$^+$ (*left panel*) and IFN-γ$^+$CD8$^+$ (*right panel*) is shown. Data presented are representative of two similar experiments. Value of $p < 0.05$ compared with wild-type mice. *B*, Sera were collected from wild-type or *Drak2*$^{-/-}$ mice at the indicated days during WNV infection. IFN-γ production was measured using mouse Th1/Th2 cytokine kit. Data shown are representative of two independent experiments and are the average of four mice in each experiment per time point.

was a 2-fold increase in the number of CD4$^+$ and CD8$^+$ T cells that produced IFN-γ in *Drak2*$^{-/-}$ mice compared with wild-type mice (Fig. 4*A*). These results suggest that *Drak2*$^{-/-}$ T cells re-

spond to WNV infection; in fact, there is an increase in the number of cells that produce IFN-γ upon ex vivo stimulation compared with wild-type mice during WNV infection. Surprisingly, we noted
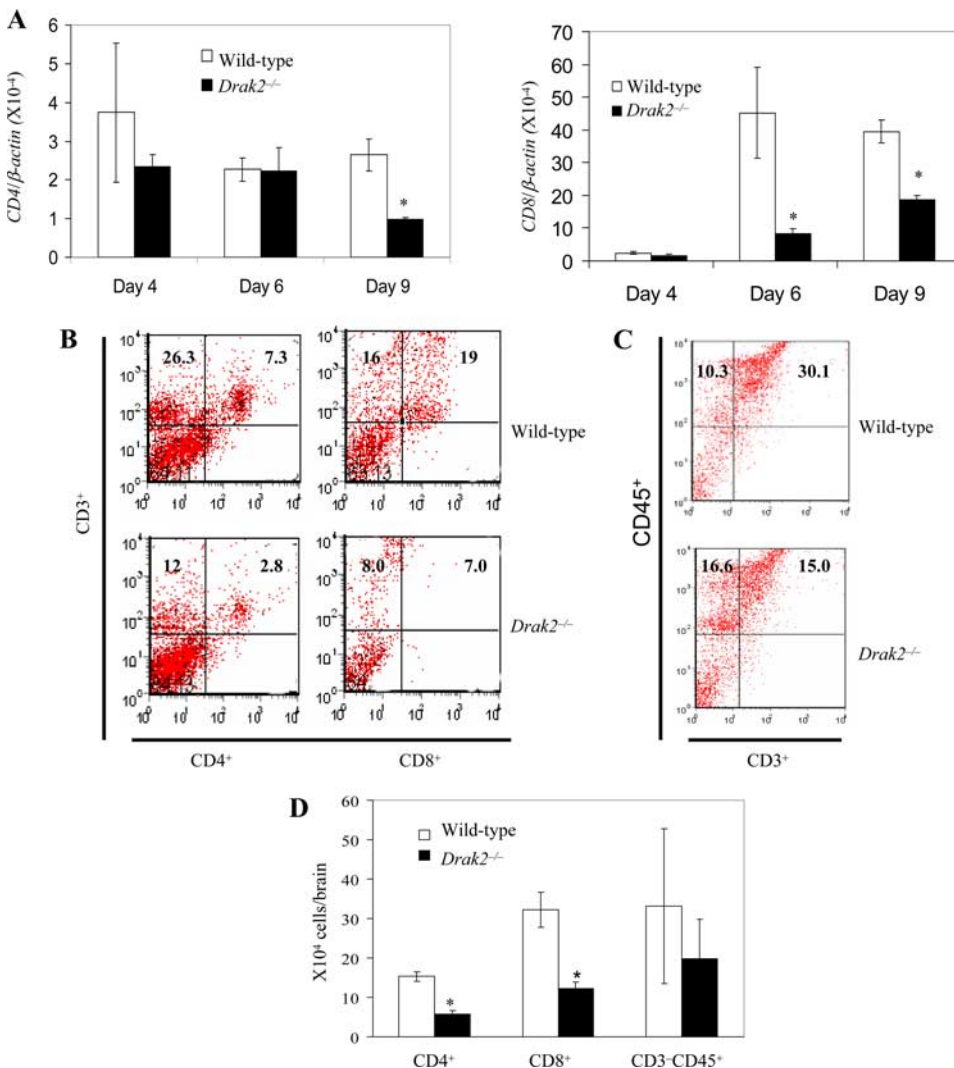
FIGURE 5. There are fewer *Drak2*$^{-/-}$ T cells in the CNS following systemic WNV infection. *A*, CD4 (*left panel*) and CD8 levels (*right panel*) in WNV-infected mice brains were measured by Q-PCR at indicated days. The *y*-axis depicts the ratio of the amplified *CD4* or *CD8*-cDNA to *β-actin* cDNA of each sample (unitless ratio ± SEM). Value of $p < 0.05$ compared with wild-type mice. Data shown are one representative of three independent experiments. Four samples were collected from each condition. *B* and *D*, Numeric reduction of CD4$^+$ and CD8$^+$ T cells in the brains of wild-type and *Drak2*$^{-/-}$ mice at day 11 postinfection. *C* and *D*, Number of CD45$^+$CD3$^-$ cells in the brains of *Drak2*$^{-/-}$ mice and wild-type mice was not different. At least three samples were collected at each condition. Experiments were repeated four times.
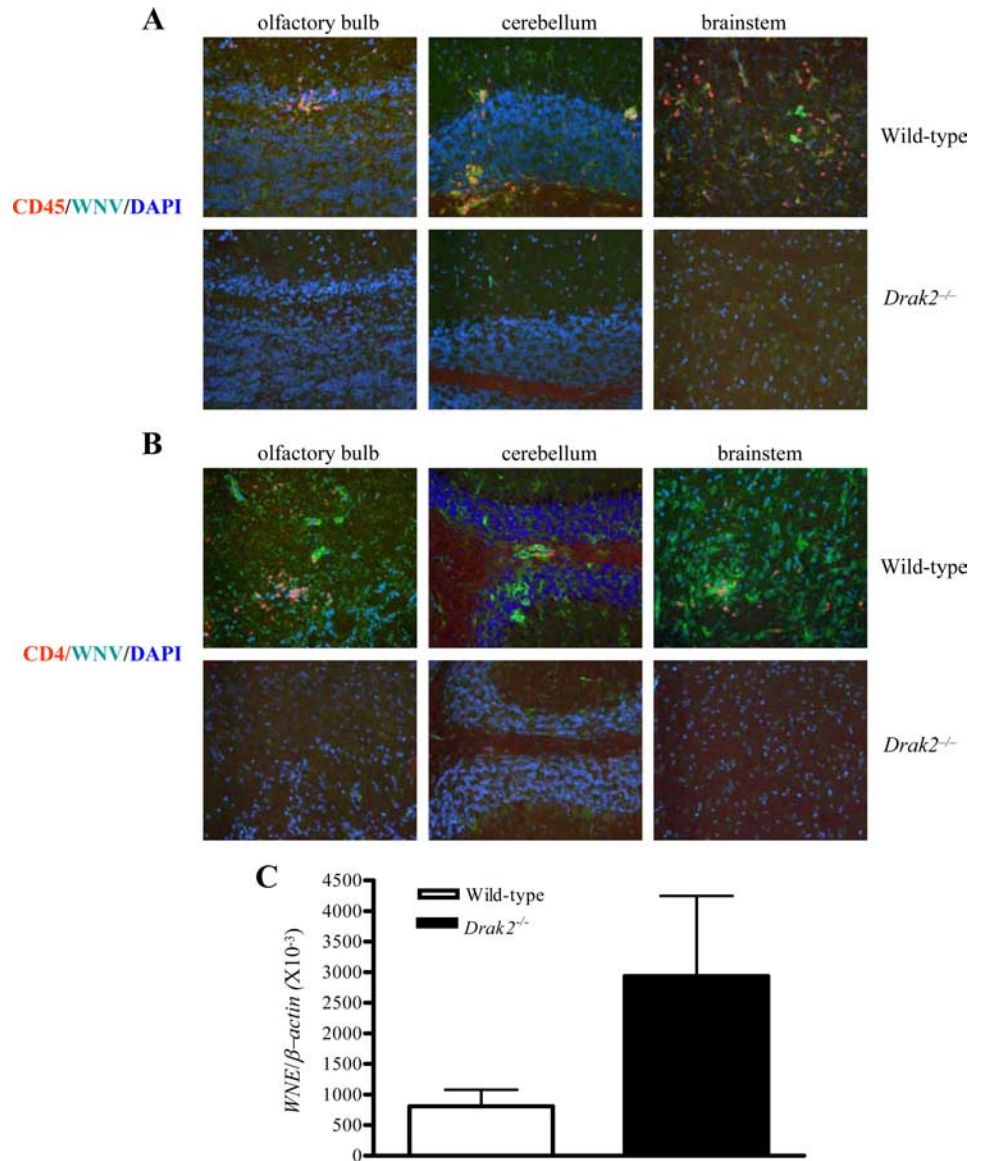
FIGURE 6. T cell migration into the CNS is correlated with enhanced viral load in the brains following systemic WNV infection. *A* and *B*, Wild-type ($n = 4$) and $Drak2^{-/-}$ ($n = 4$) mice were infected with WNV, and brains were isolated at day 13 postinfection and immunostained with Abs against CD45 (*A*, red signal) or CD4 (*B*, red signal) and WNV Ag (green signal) to reveal infiltrating leukocytes or T cells in WNV-infected brain regions (olfactory bulbs, cerebellum, and brain stem sections). The 4′,6′-diamidino-2-phenylindole was used as a nuclear counterstain (blue signal), and merged images are shown. *C*, Viral load in wild-type and $Drak2^{-/-}$ mice. Brains were harvested at day 5 following i.c. infection. Value of $p = 0.84 > 0.05$ for wild-type ($n = 12$) vs $Drak2^{-/-}$ ($n = 12$) mice.

no significant differences in production of IFN-γ (Fig. 4*B*; $p < 0.05$) or other Th1/Th2 cytokines (data not shown in this study) in sera of these two groups of mice at days 2 and 4 postinfection.

There are fewer Drak2$^{-/-}$ *T cells in the brain following WNV infection*

In the EAE model, $Drak2^{-/-}$ mice exhibited a reduced number of infiltrating cells in the spinal cord (26), suggesting a role for Drak2 in T cell infiltration into the CNS. A hallmark of WNV encephalitis is the accumulation of inflammatory infiltrates, which reportedly varies between brain regions and consists predominantly of lymphocytes and macrophages (10). Thus, to study T cell infiltration into the CNS, we first measured CD4$^+$ and CD8$^+$ T cell levels in the brain by Q-PCR analysis. In the brains of $Drak2^{-/-}$ mice, we observed >2-fold reduction of CD4$^+$ T cells (Fig. 5*A*, *left panel*) at day 9 postinfection and at least a 2-fold reduction of CD8$^+$ T cells (Fig. 5*A*, *right panel*) at days 6 and 9 postinfection, compared with wild-type mice. To verify these results, we isolated brain leukocytes at day 11 postinfection. As shown in Fig. 5*B*, the percentage of CD3$^+$CD4$^+$ T cells in the brains of $Drak2^{-/-}$ mice was lower than in those of wild-type mice (2.8 vs 7.3%, $Drak2^{-/-}$ vs wild type). The percentage of CD3$^+$CD8$^+$ among $Drak2^{-/-}$

mice brain leukocytes was also reduced (7.0 vs 19%, $Drak2^{-/-}$ vs wild type). The total number of CD4$^+$ and CD8$^+$ T cells in the brains of $Drak2^{-/-}$ mice was also >2-fold lower compared with wild-type mice (Fig. 5*D*; $p < 0.05$). Consistent with these results, the percentage of CD45$^+$CD3$^+$ leukocytes in $Drak2^{-/-}$ mouse brains was lower than in those of wild-type mice (Fig. 5*C*). Interestingly, no differences were noted when we compared the number of CD45$^+$CD3$^-$ cells in the brains of $Drak2^{-/-}$ mice and wild-type mice (Fig. 5*D*). These results further suggest that Drak2 is important for T cell infiltration into the CNS upon WNV infection.

T cells are potential carriers for WNV entry into the CNS

Earlier Q-PCR analyses revealed a significantly reduced viral load in $Drak2^{-/-}$ mice brains at days 6 and 9 following WNV challenge (Fig. 2*C*). At day 13 postinfection, immunofluorescence double staining of wild-type mouse brains (olfactory bulbs, cerebellum, and brain stem) showed either numerous CD45$^+$ leukocytes (Fig. 6*A*) or CD4$^+$ T cells (Fig. 6*B*) in the vicinity of WNV-positive cells, but in the same regions of $Drak2^{-/-}$ mouse brains the staining of both WNV Ag and T cells was less obvious. These data indicate that reduced T cell migration into the CNS is correlated with decreased viral levels in the brains. To test whether
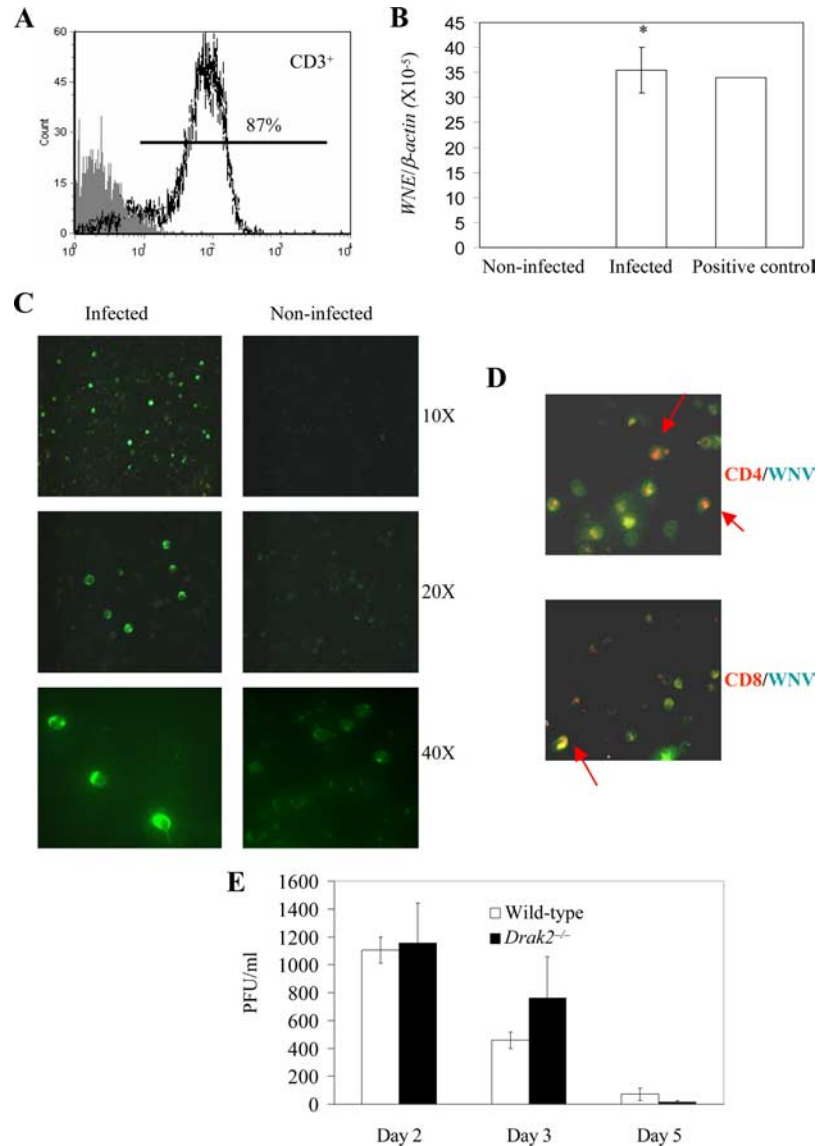
FIGURE 7. T cells are permissive to WNV infection. *A*, Splenocytes from WNV-infected mice were harvested at day 6 postinfection. Splenic T cells were purified with CD90 microbeads, followed by positive selection with an autoMACS separator (Miltenyi Biotec). These T cells were stained with Ab to CD3 and analyzed by flow cytometer. Open area represents purified T cells stained with anti-CD3; gray area, unstained purified T cells. *B*, WNV infection in purified splenic T cells of infected mice as measured by Q-PCR. Negative and positive controls represent splenic T cells isolated from naive mice and WNV-infected H36.12j cells (multiplicity of infection = 1, day 1 postinfection), respectively. The *y*-axis depicts the ratio of the amplified *WNV-E* cDNA to *β-actin* cDNA of each sample (unitless ratio ± SEM). *C*, Immunofluorescence photomicrographs of purified splenic T cells stained for WNV Ag with various magnifications. *D*, Immunofluorescence photomicrographs of purified brain T cells isolated at day 10 postinfection. T cells were isolated by positive selection and double stained for WNV Ag and CD4 (*top panel*) or CD8 (*bottom panel*). Arrows point to CD4$^+$WNV$^+$ and CD8$^+$WNV$^+$ populations. *E*, In vitro WNV infection in purified splenic T cells isolated from wild-type ($n = 4$) and *Drak2*$^{-/-}$ ($n = 4$) mice, as measured by plaque assay.

non-T cells in the CNS contribute to difference of susceptibility to WNV infection between wild-type and *Drak2*$^{-/-}$ mice, we next infected mice i.c. with 160 PFU of WNV isolate 2741. As shown in Fig. 6C, viral load in the brains of *Drak2*$^{-/-}$ mice was not significantly different from that of wild-type controls around the peak of infection (day 5; $p > 0.05$). Therefore, it is possible that WNV enters the CNS through infiltrating T cells. To test this possibility, we determined whether peripheral T cells were permissive to WNV infection. Following i.p. challenge, splenic T cells were isolated at day 6 postinfection. The purity of these cells was close to 90% as analyzed by flow cytometry (Fig. 7A). Q-PCR analysis for viral load of the purified T cells revealed a similar level of infection as the day 1 WNV-infected H36.12j cells (Fig. 7B). Immunofluorescence staining (Fig. 7C) also demonstrated WNV Ags in these cells. Furthermore, we isolated brain-infiltrating T cells from wild-type mice at day 10 postinfection and stained for WNV Ag and CD4 or CD8 T cell markers. Immunofluorescence double staining shows CD4$^+$WNV$^+$ and CD8$^+$WNV$^+$ populations in these purified brain T cells (Fig. 7D). Plaque assay showed WNV replicated productively in purified splenic T cells from both groups of mice at days 2 and 3 postinfection, but decreased at day 5, suggesting *Drak2*$^{-/-}$ T cells supporting a short-term productive virus replication as efficiently as wild-type T cells (Fig. 7E). Over-

all, these results indicate that infected T cells might carry WNV as they migrate into the brains, thereby serving as one of the pathways for WNV entry into the CNS.

Drak2 affects chemokine expression during WNV infection

Interactions between chemokines and their receptors is an important step in the control of leukocyte migration into sites of inflammation. Neurons are the primary target for WNV in the CNS (23). Infected neurons secrete chemokines, such as CXCL10, which recruits effector T cells via the chemokine receptor CXCR3 (10, 31). To investigate whether Drak2 plays a role in chemokine signaling, we analyzed CXCL10 levels in the brain following WNV infection. At day 4 postinfection, there was a 2-fold reduction of CXCL10 mRNA in the brain of *Drak2*$^{-/-}$ mice as measured by Q-PCR. This difference was more dramatic at day 6 postinfection (Fig. 8A). In addition, we found that the percentage of splenic CD8$^+$ T cells expressing CXCR3 was slightly lower in *Drak2*$^{-/-}$ mice than wild-type mice at day 5 postinfection, whereas the percentage of CD4$^+$ T cells expressing CXCR3 did not differ in mice of these two groups (Fig. 8B, *left panel*). Furthermore, the total numbers of CD4$^+$ T cells ($20.7 \pm 1.2 \times 10^6$ vs $13.9 \pm 0.5 \times 10^6$, wild-type vs *Drak2*$^{-/-}$; $p < 0.05$) and CD8$^+$ T cells ($15.6 \pm 0.8 \times 10^6$ vs $9.8 \pm 0.8 \times 10^6$, wild-type vs *Drak2*$^{-/-}$; $p < 0.05$) were higher in wild-type mice than those of
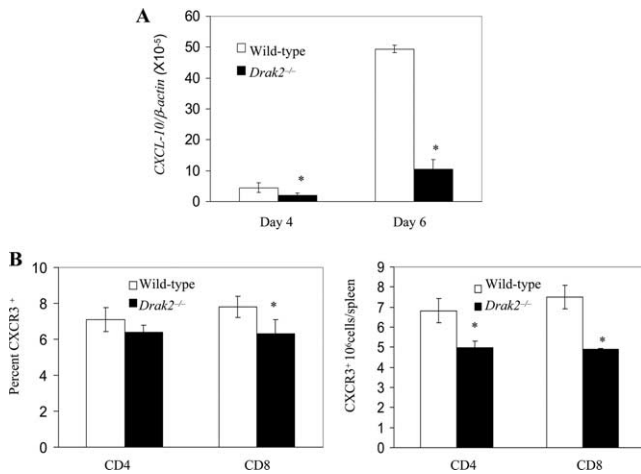
FIGURE 8. Drak2 affects chemokine expression during WNV infection. *A*, CXCL10 levels in the brains of infected mice at indicated time points, as measured by Q-PCR. The *y*-axis depicts the ratio of the amplified *CXCL10* cDNA to *β-actin* cDNA of each sample (unitless ratio ± SEM). Value of $p < 0.05$ compared with wild-type mice. Data shown are representative of three independent experiments. $n = 4$ for each group. *B*, Peripheral CXCR3 expression was reduced on peripheral T cell surface in $Drak2^{-/-}$ mice as compared with wild-type mice. Splenocytes were stained with CD4, CXCR3, or CD8. Percentage (*left panel*) and total number (*right panel*) of $CD4^{+}CXCR3^{+}$ or $CD8^{+}CXCR3^{+}$ are shown. In each experiment, three to four mice per group were analyzed. Similar experiments were repeated twice. ∗, A value of $p < 0.05$ compared with wild-type mice.

$Drak2^{-/-}$ mice. The total numbers of $CD4^{+}$ and $CD8^{+}$ spleen T cells expressing CXCR3 were also higher in wild-type mice than in $Drak2^{-/-}$ mice (Fig. 8*B*, *right panel*).

Discussion

The mechanism(s) by which WNV evades the immune system in susceptible organisms and enters the CNS is not completely understood. In this study, we have investigated the role of Drak2 in WNV-induced encephalitis. We demonstrated that $Drak2^{-/-}$ mice were more resistant to lethal WNV infection than wild-type controls. In the peripheral tissues, virus infection was not significantly different between wild-type and $Drak2^{-/-}$ mice. A defect in Drak2 signaling did not affect the development of Abs following WNV infection. Although more $Drak2^{-/-}$ T cells isolated from WNV-infected mice produced IFN-γ upon ex vivo stimulation, no significant difference in IFN-γ production was found in the sera of mice in these two groups at days 2 and 4 postinfection. These data indicate that Drak2 is not involved in the control of virus dissemination in the peripheral tissues. Nevertheless, we noted a drastically reduced viral load in the brains of $Drak2^{-/-}$ mice, compared with that in wild-type controls. Interestingly, there was also a reduction in $CD4^{+}$ and $CD8^{+}$ T cell infiltrates in the brains of $Drak2^{-/-}$ mice. Together, these results indicate a correlation between the number of T cells in the brain and virus dissemination in this organ. Moreover, we demonstrated that upon WNV challenge, T cells from either a peripheral organ or brain infiltrates contained WNV Ags. Overall, our data suggest that Drak2 is important for maintaining $CD4^{+}$ and $CD8^{+}$ T cells in the CNS following a systemic WNV challenge, and may also be involved in WNV entry into the CNS.

There are several possible explanations for the reduction of T cells in the brain of $Drak2^{-/-}$ mice. One possibility is that Drak2 is required for T cell migration into the CNS. However, a recent report by Ramos et al. (34) revealed that $Drak2^{-/-}$ T cells migrate to the brain following mouse hepatitis virus infection. However, in

this experiment, the virus was injected directly into the brain, and the migration of T cells into the CNS may be mediated by different mechanisms when physical trauma is induced. Nevertheless, when $Drak2^{-/-}$ mice were infected i.p. with lymphocytic choriomeningitis virus, a similar number of T cells was present in the brain compared with wild-type mice early after infection. However, by 13 days postinfection, there were fewer T cells in the brain of $Drak2^{-/-}$ mice, and of the T cells that were present, a higher percentage were undergoing apoptosis (M. McGargill, C. Choy, B. Wen, and S. Hedrick, manuscript submitted). Likewise, during EAE, there were similar numbers of $Drak2^{-/-}$ and wild-type T cells early after the induction of disease; however, the $Drak2^{-/-}$ T cells were more sensitive to apoptosis, and therefore did not accumulate and induce disease similar to the wild-type T cells. These data suggest that T cells can migrate to the CNS in the absence of Drak2; however, the cells are more sensitive to death, and therefore do not remain in this organ. Although we did detect a difference in chemokine expression in the brain and a slight reduction in chemokine receptor on T cells, this may be secondary to the fact that the T cells do not remain in the brain in the absence of $Drak2$. It is possible that after systemic WNV infection, T cells carry virus into the brain, which results in the infection of neurons and up-regulation of CXCL10, and in turn, amplifies the recruitment of T cells to the CNS. However, in $Drak2^{-/-}$ mice, T cells do not remain in the CNS, which may limit the opportunity of virus to spread to the neurons, and therefore there is not an up-regulation of chemokines. It is interesting to speculate whether there are fewer T cells in the CNS of $Drak2^{-/-}$ mice due to a decrease in viral Ag in the brain, or whether there is a decrease in viral Ag in the CNS as a result of the reduction of T cells in this organ. Regardless, there is a decrease in WNV-induced lethality in the absence of Drak2, which is most likely due to both the decrease of T cells and viral load in the CNS.

Recent studies have shown that WNV-induced proinflammatory responses could modulate BBB permeability, which might enable viral entry into and infection of the brain, leading to induction of lethal encephalitis (5, 21). As a result, mice that are deficient in proinflammatory cytokine signaling, including macrophage migration inhibitory factor and TNF-α receptor 1, have a reduced viral load in the brain and are subsequently more resistant to lethal WNV infection (5, 21). Following BBB compromise, WNV may enter the CNS via one or more pathways. The virus may invade the CNS by crossing the BBB directly (20, 35) or by being carried into the brain by infected infiltrating myeloid cells (36, 37) and inducing encephalitis. In this study, we have shown that the absence of Drak2 attenuates lethality from WNV encephalitis by 30%, and this is accompanied by a decrease of T cells in the CNS.

The role of T cells in protecting the host against WNV infection has been the subject of recent investigations. Both αβ and γδ T cells have been shown to contribute to host survival during WNV infection (8, 13). Among αβ T cells, $CD4^{+}$ T cells respond vigorously in the periphery (16), whereas $CD8^{+}$ T cell responses have been observed in both the spleen and brain following WNV infection (38). $CD4^{+}$ T cells are known to provide help for Ab responses and to sustain WNV-specific $CD8^{+}$ T cell responses in the CNS, enabling viral clearance (39). $CD8^{+}$ T cells have important functions in clearing infection from peripheral tissues and CNS, and in preventing viral persistence (40, 41). Furthermore, Fas ligand effector or perforin-dependent mechanisms (17, 42) are critical for $CD8^{+}$ T cells to clear WNV infection in the CNS. CD40-CD40L interactions have also been demonstrated to be important for T cell trafficking into the CNS and for protection of the host from a low-dose WNV challenge (43).

Immune responses are also involved in viral pathogenesis. For example, one study has shown that CD8^{+} T cells contribute to immunopathology upon high-dose WNV challenge (18). Nevertheless, little is known about the role of T cell-mediated pathology in WNV-related brain damage. Our data now provide the first evidence that Drak2-mediated T cell survival in the CNS plays an important role in WNV pathogenesis. These results may prompt the development of new pharmacotherapeutic treatment using Drak2 signaling inhibitors, alone or in combination with currently existing antiviral agents, in treating encephalitis caused by WNV and perhaps other viral encephalitides.

Acknowledgments

Disclosures

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