Enterovirus 71 infection increases expression of interferon-gamma-inducible protein 10 which protects mice by reducing viral burden in multiple tissues

Fang-Hsiu Shen,1 Chia-Chun Tsai,2 Li-Chiu Wang,1 Kung-Chao Chang,3 Yuk-Ying Tung,4 Ih-Jen Su5 and Shun-Hua Chen1,2,6

Correspondence
Shun-Hua Chen
shunhua@mail.ncku.edu.tw
Ih-Jen Su
suihjen@nhri.org.tw

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Enterovirus 71 (EV71) infection has induced fatal encephalitis in thousands of young children in the Asia-Pacific region over the last decade. EV71 infection continues to cause serious problems in areas with outbreaks, because vaccines and antiviral therapies are not available. Lymphocytes are present in the brains of infected patients and mice, and they protect mice from infection by decreasing the viral burden. The chemokines responsible for recruiting lymphocytes to infected organs are yet to be identified. Among the lymphocyte chemokines detected, high levels of interferon-gamma-inducible protein-10 (IP-10) are found in the plasma and cerebral spinal fluid of patients with brainstem encephalitis as compared with the levels of a monokine induced by gamma interferon (Mig). Using a murine model to investigate the induction of IP-10 by EV71 infection, we observed that EV71 infection significantly enhanced IP-10 protein expression in the serum and brain, with kinetics similar to viral titres in the blood and brain. Brain neurons of infected mice expressed IP-10. Using wild-type mice and IP-10 gene knockout mice to investigate the role of IP-10 in EV71 infection, we found that IP-10 deficiency significantly reduced levels of Mig in serum, and levels of gamma interferon and the number of CDB T cells in the mouse brain. Absence of IP-10 significantly increased the mortality of infected mice by 45%, with slow virus clearance in several vital tissues. Our observations are consistent with a model where EV71 infection boosts IP-10 expression to increase gamma interferon and Mig levels, infiltration of CDB T cells, virus clearance in tissues and the survival of mice.

INTRODUCTION

Enterovirus 71 (EV71) can infect the human central nervous system (CNS) to induce fatal neurological manifestations, aseptic meningitis, encephalomyelitis and brainstem encephalitis (Chang et al., 2007; Ho et al., 1999; Huang et al., 1999). Severe symptoms, such as brainstem encephalitis combined with pulmonary oedema complications, often cause death or long-term neurological sequelae, especially in young children (Chang et al., 2007). Over the last decade, widespread and deadly EV71 outbreaks have been reported, particularly in the Asia-Pacific region, which are estimated to have infected millions of children and have caused death
and severe neurological sequelae in thousands of children (Ho et al., 1999; Huang et al., 1999; Lee & Chang, 2010; Qiu, 2008). EV71 infection is becoming endemic in areas with outbreaks and has massive potential for explosive epidemics because vaccines and antiviral therapies are not available.

The pathogenesis of EV71-induced mortality is controversial. Lymphocyte and antibody responses are suspected to contribute to the pathogenesis of neurological symptoms, so anti-inflammatory agents have been used to treat patients with neurological symptoms (McMinn et al., 2001; Nolan et al., 2003). Our previous report detected the influx of B cells, CD4 T cells and CD8 T cells in the CNS of an infected patient who died (Lin et al., 2009). Using the murine model, we observed that EV71 infection recruited these three types of lymphocytes into the brain (Lin et al., 2009). Moreover, lymphocytes function to reduce the viral burden as indicated by the finding that mice deficient in B cells, CD4 T cells or CD8 T cells were highly susceptible to infection with elevated organ viral loads (Lin et al., 2009).

There are few reports regarding lymphocyte chemokines in EV71 infection. One in vitro report has found the enhanced expression of lymphocyte chemokines, such as interferon-gamma-inducible protein-10 (IP-10/CXCL10), in virus antigen-stimulated PBMCs isolated from infected patients (Chang et al., 2006). Another clinical report detected three lymphocyte chemokines, IP-10, a monokine induced by gamma interferon (Mig) and monocyte chemotactic protein-1 (MCP-1), in the plasma and cerebral spinal fluid of EV71-infected patients with brainstem encephalitis (Wang et al., 2008). Among these three lymphocyte chemokines, high levels of IP-10 are present in the plasma and cerebral spinal fluid of patients with brainstem encephalitis.

IP-10 is a CXC chemokine secreted by several cell types, including macrophages, microglia and astrocytes in response to stimuli, such as interferon (IFN)-α, IFN-β, IFN-γ or viruses (Lane et al., 1998; Marques et al., 2006; Vanguri & Farber, 1990; 1994). It is a potent chemottractant for NK cells and particularly T cells during viral infection (Thapa et al., 2008; Trifilo et al., 2004; Yuan et al., 2009). It is also a fascinating chemokine because of its controversial roles in viral infection. It has been implicated to aggravate diseases in mice infected with mouse hepatitis virus, herpes simplex virus or lymphocytic choriomeningitis virus, and in humans infected with human immunodeficiency virus, hepatitis C virus or severe acute respiratory syndrome coronavirus (Carr et al., 2003; Christensen et al., 2009; Dufour et al., 2002; Harvey et al., 2003; Kolb et al., 1999; Tang et al., 2005). However, endogenous IP-10 has been shown to protect mice infected with coxsackievirus B3, dengue virus, herpes simplex virus or mouse hepatitis virus by promoting viral clearance in tissues (Chen et al., 2006; Thapa et al., 2008; Walsh et al., 2007; Yuan et al., 2009). Interestingly, the mechanisms mediated by IP-10 to reduce the viral burden in mouse tissues vary among viruses. IP-10 can suppress viral infection by recruiting mainly leukocytes (Lindell et al., 2008; Thapa et al., 2008; Trifilo et al., 2004; Walsh et al., 2007) or directly by inhibiting viral replication (Chen et al., 2006; Lokensgard et al., 2001). Moreover, leukocyte recruitment mediated by IP-10 varies with the infecting virus and infected organ (Lindell et al., 2008; Thapa et al., 2008; Trifilo et al., 2004; Walsh et al., 2007).

Because of the paucity of studies on the induction and role of IP-10 in EV71 infection, we addressed these issues using a murine infection model that can reproduce neurological symptoms, as well as death, similar to those seen in humans (Li et al., 2008). We found that EV71 infection significantly increased serum and brain IP-10 levels. Moreover, the absence of IP-10 due to gene knockout significantly decreased the survival of infected mice.

RESULTS

**EV71 infection significantly enhances IP-10 protein expression in mice**

EV71 infection has been reported to increase IP-10 levels in the plasma and cerebral spinal fluid of patients (Wang et al., 2008). To investigate the induction of IP-10 by EV71 infection, we used a murine infection model that can reproduce neurological symptoms, as well as death, analogous to those of infected patients (Li et al., 2008). C57BL/6J mice were mock infected or infected with 3 × 10^5 p.f.u. per mouse of EV71 by intraperitoneal injection. At this viral dose, mice displayed signs of encephalitis manifested by hunched posture, lethargy, hind limb paralysis and ataxia, with a death rate of 37%. Mouse blood and brain specimens were harvested and processed to measure viral titres and the IP-10 protein. In the mouse blood, viral titres were high at day 1 post-infection (p.i.) and were reduced at days 4–6 p.i. (Fig. 1a). Mouse blood was processed to obtain serum for measuring the IP-10

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Fig. 1. Kinetics of blood EV71 titres and serum IP-10 levels of mice. Levels of (a) blood viral titres and (b) serum IP-10 in mice mock infected (Mock) or infected with EV71 at the indicated days p.i. are shown. Data are the means ± SEM of greater than or equal to three samples per group, with samples derived from individual mice. *P<0.05; **P<0.01, via the ANOVA test followed by the Newman–Keuls test compared with mock-infected mice.
protein. Results of the ELISA showed low IP-10 levels (<35 pg ml\(^{-1}\)) in the serum of mock-infected mice (Fig. 1b). In infected mice, serum IP-10 levels dramatically increased at day 2 p.i., with an average concentration of 3052 pg ml\(^{-1}\), and then declined. The serum IP-10 levels of infected mice were 138- to 19-fold greater than those of mock-infected mice from days 2–10 p.i., with significant differences found from days 2–6 p.i. (\(P<0.05\)).

The kinetics of blood viral titres and serum IP-10 expression were similar. We also analysed viral growth and antigen expression as well as IP-10 levels in the mouse brain. As sample processing procedures for these three analyses were different, separate samples were used in the assays. In the mouse brain, viral titres appeared to increase from 2–5 days p.i. and then declined (Fig. 2a). Immunofluorescence staining detected the EV71 capsid protein in the brainstem of infected mice, but not in mock-infected mice at day 6 p.i. (Fig. 2c). Most of the EV71 antigen-positive cells expressed NeuN, a neuronal cell marker. ELISA results showed minimal IP-10 (<35 pg ml\(^{-1}\)) in the brains of mock-infected mice (Fig. 2b). In infected mice, brain IP-10 levels dramatically increased from day 2 p.i., reached a peak at day 6 p.i., with a mean concentration of 8368 pg ml\(^{-1}\), and declined to a low level at day 10 p.i. (Fig. 2b). The brain IP-10 levels of infected mice were 239- to 11-fold greater than those of mock-infected mice, with significant differences found from days 2–6 p.i. (\(P<0.01\)). The kinetics of viral titres and IP-10 expression in the mouse brain were similar. Collectively, EV71 infection increases the serum and brain IP-10 levels in mice, as is the case for patients.

We then determined the cell type that expresses IP-10 in the brains of infected mice, as a high level of IP-10 was detected. Immunofluorescence staining detected IP-10-positive cells in the brainstem of infected mice, but not in mock-infected mice at day 6 p.i. (Fig. 2d). The majority (93%) of IP-10-positive cells were also positive for NeuN. However, in sections of brainstems from infected mice costained for IP-10 and the EV71 capsid protein, few cells were dually positive (Fig. S1, available in JGV Online).

![Fig. 2. EV71 infection enhances IP-10 protein expression in the mouse brain. Levels of (a) viral titres and (b) IP-10 protein in the brains of mice mock infected (Mock) or infected with EV71 at the indicated days p.i. are shown. Data are the means ± SEM of greater than or equal to three samples per group, with samples derived from individual mice. **P<0.01; ***P<0.001, via the ANOVA test followed by the Newman–Keuls test compared with mock-infected mice. The brain samples from mice mock infected (Mock) or infected with EV71 were harvested 6 days p.i. and processed for staining with Hoechst and antibodies against (c) mouse neurons (NeuN) and EV71 antigen or (d) mouse IP-10 and NeuN in the brainstem region. Data are representative of at least three experiments. Bars, 50 μm (c, d).](http://vir.sgmjournals.org)
Fig. 3. Type I IFNs enhance IP-10 protein expression in the mouse neuronal cell line. (a) EV71 titres in the mouse neuronal cell line (Neuro-2a) at the indicated hours p.i. (m.o.i.=0.1) are shown. (b) Neuro-2a cells were infected with or without EV71 (m.o.i.=0.1) and treated with or without type I IFNs (α/β; 200 and 100 units ml⁻¹, respectively) or type II IFN (γ; 100 units ml⁻¹). Twenty-four hours after infection or treatment, cultures were harvested to assay for IP-10 by ELISA. Data are the means ± SEM of greater than or equal to three samples per group. NS, Not significant; *P<0.05; ***P<0.001, via the ANOVA test followed by the Newman–Keuls test compared with samples without infection and treatment or compared between groups as indicated. (c) IFN-β levels in the brains of mice mock infected (Mock) or infected with EV71 at the indicated days p.i. are shown. Data are the means ± SEM of greater than or equal to three samples per group with samples derived from individual mice. ***P<0.001, via the ANOVA test followed by the Newman–Keuls test compared with mock-infected mice.

Collectively, uninfected neurons produce IP-10 in the brains of EV71-infected mice.

Type I IFNs enhance IP-10 protein expression in the mouse neuronal cell line

The neuron is not a well-known IP-10 producer. Therefore, we studied how EV71 infection induces neurons to express IP-10 using a mouse neuron cell line (Neuro-2a), which supported virus replication (Fig. 3a). ELISA results showed that mock-infected Neuro-2a cells produced a low level of IP-10 protein (Fig. 3b). EV71 infection (at an m.o.i. of 0.1 or 1) failed to increase IP-10 production in cells (Fig. 3b and data not shown).

Both type I and II IFNs can induce IP-10 expression in leukocytes (Vanguri & Farber, 1990; 1994). We also found that Neuro-2a cells responded to type I and II IFNs, as both IFNs reduced EV71 replication in the cells (Figs S2 and S3). We therefore tested whether IFNs could induce IP-10 production in Neuro-2a cells, as few reports have investigated this issue. Treatment with type I IFNs (α/β; 200 and 100 units ml⁻¹, respectively) significantly increased IP-10 production by about sixfold (P<0.001) when compared with that of no treatment (Fig. 3b). Combined EV71 infection (m.o.i.=0.1) and type I IFNs modestly enhanced IP-10 production by 10 % when compared with that of type I IFNs alone. Treatment with type II (γ) IFN at concentrations of 50, 100 or 500 units ml⁻¹ failed to boost IP-10 expression (Fig. 3b and data not shown). Combined EV71 infection and type II IFN also failed to augment IP-10 expression. Combined treatments with type I and II IFNs were unable to further upregulate IP-10 expression, but combined EV71 infection and treatments with type I and II IFNs significantly increased IP-10 production by 40 % (P<0.05) when compared with treatments with type I and II IFNs.

As type I IFN, but not EV71 infection or type II IFN, induces IP-10 expression in Neuro-2a cells, we monitored type I IFN expression and detected IFN-β in the brains of EV71-infected mice (Fig. 3c). In addition, CD45⁺ leukocytes, the major IFN-α producer, were detected in the brain of EV71-infected mice (Fig. S4). These results suggest that type I IFNs may induce IP-10 expression in mouse brain neurons.

Absence of IP-10 significantly increases the mortality of EV71-infected mice, with slow virus clearance in tissues

To determine the role of IP-10 in EV71 infection, we used C57BL/6J mice and C57BL/6J-derived mice with a targeted disruption of the gene encoding IP-10. Mice were infected with 3 × 10⁶ p.f.u. per mouse of EV71 and were monitored for survival and tissue viral loads. The final survival rate of infected IP-10 gene knockout (IP-10⁻/⁻) mice was significantly lower than that of infected wild-type mice (P<0.05) by 45 % (Fig. 4). We harvested mouse CNS (brain without the brainstem region, brainstem and spinal cord), peripheral organs (lung, liver and spleen) and blood to measure tissue viral loads. At day 7 p.i., the average viral titres in the lung, liver and blood specimens from IP-10⁻/⁻ mice were slightly higher than those from wild-type mice.

Fig. 4. Absence of IP-10 significantly increases the mortality of EV71-infected mice. The survival rates of wild-type mice (●; n=19) and IP-10⁻/⁻ mice (○; n=11) infected with EV71 at the indicated days p.i. are shown. *P<0.05, via the log-rank test.
(Fig. 5). The average viral titres in the other four tissues of wild-type and IP-10−/− mice were comparable. At day 9 p.i., the average viral titres in all seven tissues of IP-10−/− mice were all higher than those of wild-type mice, with significant differences found in several vital tissues, the brain without the brainstem region, brainstem, lung and liver (P<0.05).

**IP-10 treatment fails to inhibit EV71 replication in the mouse neuronal cell line**

We further investigated how IP-10 could contribute to viral clearance. Treatment with IP-10 is reported to inhibit the replication of dengue virus and herpes simplex virus *in vitro* (Chen *et al.*, 2006; Lokensgard *et al.*, 2001). We therefore examined the anti-EV71 activity of IP-10 by treating Neuro-2a cells with recombinant murine IP-10 protein at the concentration previously shown to inhibit dengue virus binding to cells (0.1 mg ml−1). Pretreatment of the cells 30 min before and also during infection with EV71 (m.o.i.=0.1) failed to reduce viral yields 8–48 h p.i. (Fig. S5).

**Absence of IP-10 significantly decreases the number of CD8 T cells in the infected mouse brain**

Since IP-10 failed to directly inhibit EV71 replication, we investigated whether the absence of this chemokine would influence leukocyte influx in infected tissues. IP-10 is a potent chemoattractant for T cells and NK cells during viral infection (Thapa *et al.*, 2008; Wuest & Carr, 2008; Yuan *et al.*, 2009), and B cells have been shown to express the IP-10 receptor CXCR3 (Komura *et al.*, 2008; Park *et al.*, 2002). We previously showed that EV71 infection recruited lymphocytes to reduce the viral burden in mouse tissues (Lin *et al.*, 2009). Hence, we quantified lymphocytes and NK cells in infected tissues, the brain and spleen at day 9 p.i., when high viral loads were detected in IP-10−/− mouse tissues. Flow cytometric analyses performed on the mouse brain showed that EV71 infection increased the numbers of CD4+ T cells, CD8+ T cells and NK1.1+ NK cells in both wild-type and IP-10−/− mice (Fig. 6a–c). Moreover, the average numbers of CD4+ and CD8+ T cells in infected IP-10−/− mice were lower than those in infected wild-type mice, with a significant difference found in the numbers of CD8+ T cells (P<0.001). However, the average number of NK1.1+ NK cells in infected IP-10−/− mice was 20 % higher than that in infected wild-type mice.

In the spleen, EV71 infection decreased the numbers of CD4+ T cells, CD8+ T cells, CD19+ B cells and NK1.1+ NK cells in both wild-type and IP-10−/− mice (Fig. 6d–g). This finding is consistent with previous reports showing that the spleen of EV71-infected mice was greatly atrophied with reduced numbers of leukocytes due to the migration of cells to other infected tissues (Lin *et al.*, 2009; Wang *et al.*, 2004). Notably, the average numbers of CD4+ T cells, CD8+ T cells and CD19+ B cells in infected IP-10−/− mice were lower than those in infected wild-type mice by about twofold. The average numbers of NK1.1+ NK cells in the spleens of infected wild-type and IP-10−/− mice were comparable.

**Absence of IP-10 significantly reduces the expression of Mig and IFN-γ in infected mice**

In addition to IP-10, two other lymphocyte chemokines, Mig and MCP-1, are highly expressed, especially in EV71-infected patients with brainstem encephalitis (Wang *et al.*, 2008). We monitored the effect of IP-10 on the expression of these two chemokines in infected mice. The serum and brain Mig levels of IP-10−/− mice were 93- to 3-fold lower than those of wild-type mice at days 6 and 8 p.i., with a significant difference found in serum levels at day 6 p.i. (Fig. 7a–b; P<0.05). The serum and brain MCP-1 levels of IP-10−/− mice were 2- to 1.3-fold lower than those of wild-type mice.
at days 6 and 8 p.i., except the serum MCP-1 level at day 6 p.i., which was 1.2-fold higher than that of wild-type mice (Fig. 7c–d). We monitored IFN-γ expression, as IP-10 has been shown to increase the expression of IFN-γ (Gangur et al., 1998), which is the major inducer of Mig (Müller et al., 2010). In infected wild-type mice, IFN-γ was detected in the brain (Fig. 7e), but not in the serum. In infected IP-10−/− mice, both serum and brain IFN-γ levels were below detection.

**DISCUSSION**

Our report is the first, to the best of our knowledge, to show that EV71 infection increases IP-10 expression to enhance resistance in mice. In our study, uninfected neurons express IP-10 in the EV71-infected mouse brain. This observation is similar to a study with humans that showed that uninfected neurons in ganglia were the main source of IP-10 during natural and experimental varicella-zoster virus infection (Steain et al., 2011). As type I and II IFNs are potent IP-10 inducers for leukocytes (Vanguri & Farber, 1990; 1994), we investigated and found that type I IFNs (α/β), but not type II IFNs, increased IP-10 production in the mouse neuronal cell line Neuro-2a (Fig. 3b). In the brain of EV71-infected mice, the major IFN-α producer (CD45+ leukocytes) and IFN-β were detected (Figs S4 and 3c). In addition, we found that Neuro-2a cells were competent for IFN-β expression upon poly(I:C) transfection (Fig. S6). Future study is needed to investigate how EV71 infection induces the expression of type I IFNs in the mouse brain.

Our finding that type I IFNs, but not EV71 infection, enhance IP-10 expression in Neuro-2a cells (Fig. 3b) differs from those of other investigations that show that other neurotropic viruses, West Nile virus and measles virus can directly induce neurons to express IP-10 (Klein et al., 2005; Patterson et al., 2003). Hence, neurotropic viruses can use several methods to boost neuronal IP-10 expression. They can induce type I IFNs to stimulate uninfected neurons. Additionally, some neurotropic viruses per se can induce neuronal IP-10 expression by mechanisms which remain unclear. The issue regarding the lack of IP-10 induction by the mouse brain neuron and Neuro-2a cells upon EV71 infection remains to be clarified.

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CD8 T cells were detected in the brains of EV71-infected mice. This was shown to promote T cell and antibody responses to EV71. The importance of IP-10 in recruiting lymphocytes to suppress EV71 infection has been demonstrated.

**Methods**

**Cells, virus and mice.** Human rhabdomyosarcoma (RD) and Neuro-2a cell lines were maintained in media according to the instructions of the ATCC. EV71 strain M2 was propagated and titrated on RD cell monolayers and was used to infect mice as previously described (Li et al., 2008; Lin et al., 2009). C57BL/6J mice and C57BL/6J-derived IP-10−/− mice (B6.129S4-Cxcl10tm1Adl/J) (Dufour et al., 2002) purchased from The Jackson Laboratory were bred and maintained under specific pathogen-free conditions in the animal centre of our college. All mouse experiment protocols were approved by the Laboratory Animal Committee of National Cheng Kung University.

**Infection of mice.** Fourteen-day-old male and female mice were infected with 3 x 10⁵ p.f.u. per mouse of EV71 strain M2 or mock infected with lysates of uninfected RD cells by intraperitoneal inoculation. The survival of infected mice was monitored. In separate experiments, mice were anaesthetized before mouse blood was collected. Then the mice were perfused by intracardial injection of ice-cold PBS, and mouse tissues were harvested. Mouse blood was frozen and sonicated. Mouse tissues were frozen and homogenized, and then were frozen and sonicated. The resulting samples were assayed for viral titres by plaque assay using RD cell monolayers.

**Chemokine measurement.** Mouse blood was processed to extract the serum. Brains harvested from mice perfused with PBS were frozen at −80 °C for more than 2 h, thawed and homogenized in PBS containing a protease inhibitor cocktail (Sigma-Aldrich). The brain homogenates were centrifuged to obtain supernatants. The supernatants and serum samples were subjected to ELISA to measure mouse IP-10, Mig, MCP-1, IFN-γ (R&D Systems) and IFN-β (PBL InterferonSource) using commercially available kits, according to the manufacturer’s instructions.

**Immunofluorescence staining.** Brains harvested from mice perfused with PBS and 4 % paraformaldehyde were stained for the EV71 capsid protein, NeuN and IP-10. Briefly, samples were fixed in 10 % neutral buffered formalin, embedded in paraffin and sectioned. Sections (5 μm) were deparaffinized and treated with 1 % FBS to block non-specific binding before incubating with antibodies against the EV71 capsid protein (clone 422-8D-4C-4D; Chemicon), mouse NeuN (clone A60; Millipore) or IP-10 (R&D Systems) with isotype-matched control antibodies overnight at 4 °C. Subsequently, bound antibody was detected by donkey anti-goat immunoglobulin G Alexa Flour 488, donkey anti-mouse immunoglobulin G Alexa Flour 594 or donkey anti-rabbit immunoglobulin G Alexa Flour 488 (Invitrogen). Images were photographed using OLYMPUS Cell (Olympus).

**IP-10 production in the mouse neuronal cell line infected with EV71 or treated with IFNs.** Neuro-2a cells were plated on 24-well plates to a density of 2 x 10⁵ cells per well and incubated overnight at 37 °C. Cells were then mock infected or infected with EV71 at an m.o.i. of 0.1 or 1 for 45 min and cultured in medium with or without mouse IFN-α (200 units ml⁻¹; National Institutes of Health) plus recombinant IFN-β (100 units ml⁻¹; PBL InterferonSource) or recombinant IFN-γ (50, 100 or 500 units ml⁻¹; BD Pharmingen). Cultures were harvested 24 h post-infection, frozen at −80 °C for future analysis.
more than 2 h, thawed and centrifuged. Culture supernatants were subjected to ELISA to measure mouse IP-10.

**Flow cytometry.** Splenocytes and brains were harvested from mice perfused with PBS. Mouse spleens were homogenized, treated twice with buffer to lyse red blood cells, and washed with RPMI medium containing 10% FBS. Mouse brains were harvested and homogenized in PBS containing 0.01 M EDTA and 0.2% BSA. The brain homogenates were passed through strainer filters (BD Biosciences). Brain leukocytes were purified by Percoll (GE Healthcare) gradient centrifugation. Freshly isolated leukocytes from mouse brains and spleens were stained with fluorescein isothiocyanate- or phycoerythrin-conjugated control antibodies or antibodies against mouse leukocyte antigens, CD4 (clone GK 1.5; eBioscience), CD8a (clone 53-67; eBioscience), CD19 (clone 6D5; eBioscience) or NK1.1 (clone PK136; eBioscience) for 45 min on ice. The stained cells were analysed by a FACSCalibur (BD Biosciences) using WinMDI software.

**Statistical analyses.** Data are expressed as means ± SEM values. For statistical comparison, all data were analysed by the ANOVA test followed by the Newman–Keuls test, except that the Kaplan–Meier survival curves were analysed by the log-rank test and that tissue viral titres were analysed by the Mann–Whitney U test. All P-values are for two-tailed significance tests. A P-value <0.05 was considered statistically significant.

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