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RESEARCH ARTICLE

PD1$^+$CCR2$^+$CD8$^+$ T Cells Infiltrate the Central Nervous System during Acute Japanese Encephalitis Virus Infection

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Abstract

Japanese encephalitis (JE) is a viral encephalitis disease caused by Japanese encephalitis virus (JEV) infection. Uncontrolled inflammatory responses in the central nervous system (CNS) are a hallmark of severe JE. Although the CCR2–CCL2 axis is important for monocytes trafficking during JEV infection, little is known about its role in CNS trafficking of CD8$^+$ T cells. Here, we characterized a mouse model of JEV infection, induced via intravenous injection (*i.v.*) and delineated the chemokines and infiltrating peripheral immune cells in the brains of infected mice. The CNS expression of chemokines, *Ccl2*, *Ccl3*, and *Ccl5*, and their receptors, *Ccr2* or *Ccr5*, was significantly up-regulated after JEV infection and was associated with the degree of JE pathogenesis. Moreover, JEV infection resulted in the migration of a large number of CD8$^+$ T cells into the CNS. In the brains of JEV-infected mice, infiltrating CD8$^+$ T cells expressed CCR2 and CCR5 and were found to comprise mainly effector T cells (CD44$^+$CD62L$^-$). JEV infection dramatically enhanced the expression of programmed death 1 (PD-1) on infiltrating CD8$^+$ T cells in the brain, as compared to that on peripheral CD8$^+$ T cells in the spleen. This effect was more pronounced on infiltrating CCR2$^+$CD8$^+$ T cells than on CCR2$^-$CD8$^+$ T cells. In conclusion, we identified a new subset of CD8$^+$ T cells (PD1$^+$CCR2$^+$CD8$^+$ T cells) present in the CNS of mice during acute JEV infection. These CD8$^+$ T cells might play a role in JE pathogenesis.

Keywords Japanese encephalitis virus (JEV) · CD8$^+$ T cell · CCL2 · CCR2 · PD-1

Introduction

Japanese encephalitis virus (JEV), a neurotropic single stranded RNA virus belonging to the *Flaviviridae* family and the *Flavivirus* genus, is the causative agent of viral encephalitis, most commonly occurring in the Asia–Pacific region, and particularly in southern, eastern, and southeastern Asia, as well as northern Australia (Erlanger *et al.* 2009; Tiwari *et al.* 2012; Wang and Liang 2015). JE is one of the major public health problems not only because of its high case-fatality rate but also due to the occurrence of permanent neuropsychiatric sequelae in survivors (Solomon *et al.* 2000). Although vaccination programs contribute to the prevention of JEV (Campbell *et al.* 2011), there is still no specific curative treatment for individuals developing JE. Therefore, the kinetics and mechanisms underlying JEV dissemination and pathogenesis need to be thoroughly explored and the development of an effective and specific curative treatment for JE is also necessary.

Fang Zhang and Linlin Qi have contributed equally to this work.

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During the progression of viral encephalitis, both the early activated innate immune responses and the subsequently established adaptive immunity play crucial roles in the control of viruses. In JEV-infected mice, numbers of macrophages, inflammatory monocytes, granulocytes and plasmacytoid dendritic cells (pDC) are dramatically increased in peripheral lymphoid organs, such as the spleen and lymph nodes (Han *et al.* 2014; Kim *et al.* 2016). After the peripheral amplification of JEV in DCs and macrophages (Aleyas *et al.* 2009), the virus enter the central nervous system (CNS) through the blood–brain barrier (BBB). Meanwhile, various types of peripheral immune cells infiltrate the CNS. These processes are accompanied by the production of large amounts of related inflammatory cytokines and chemokines including IL-6, IL-1β, TNF-α, CCL2, and CCL5, among others (Gupta and Rao 2011). For example, DCs produce high levels of IL-6, IL-10, IL-12, and TNF-α in response to JEV Beijing-1 strain infection. JEV-infected macrophages showed a similar pattern to that observed in DCs, but failed to up-regulate the anti-inflammatory cytokine IL-10 (Aleyas *et al.* 2009). Additionally, infection with JEV P3 strain enhances the release of IL-10 and CCL2, and suppresses the production of IFN-α and TNF-α by DCs (Cao *et al.* 2011). These contradictory findings regarding TNF-α production in JEV infection might be due to the differences in the infectious specificity of JEV strains. In the CNS, human microglia produces CCL2, CXCL9, and CXCL10 after exposure to JEV (Lannes *et al.* 2017). Rodent microglia also secretes proinflammatory cytokines such as TNF-α, IL-1β, and IL-6, as well as the chemokine CCL2 in response to JEV infection (Chen *et al.* 2012; Kaushik *et al.* 2012). Consequently, JEV-induced inflammation is mainly thought to contribute to disease severity by inducing neuronal cell death, inhibiting the proliferation and differentiation of neural progenitors, and disrupting the BBB.

During the neuroinflammatory stage of JE, the brain-invasion of peripheral immune cells is mediated by a multistep process governed by chemokines and chemokine receptors (Michlmayr and Lim 2014). Previous studies reported that brains of JEV-infected mice exhibit increased levels of CCL2, CCL3, CCL4, CCL5, and CXCL10 (Das *et al.* 2011; Han *et al.* 2014; Li *et al.* 2015). Moreover, in JE patients, the cerebrospinal fluid and serum contain enhanced levels of CXCL8 (IL-8) (Singh *et al.* 2000; Winter *et al.* 2004), and mRNA expression levels of *Ccl2* and *Ccl5* were found to be significantly increased in the peripheral blood mononuclear cells (PBMCs) of severe JEV-infected individuals (Chowdhury and Khan 2018). With regard to chemokine receptors, CCR5 and CCR2 have been extensively studied and regulate the progression of viral encephalitis. CCR5 is involved in the CNS migration of effector leukocytes including macrophages, natural killer cells, and T cells into inflamed tissues (Glass *et al.* 2005; Larena *et al.* 2012). Moreover, CCR5 plays a role in the homing of CD4$^+$Foxp3$^+$ regulatory T cells (Yurchenko *et al.* 2006). CCR2 was observed to be expressed in certain types of cells including monocytes/macrophages, T lymphocytes, B lymphocytes (immature), natural killer cells, basophils and dendritic cells (immature) (Bose and Cho 2013). It is considered critical for CD11b$^+$Ly-6C^{hi} monocyte trafficking during viral encephalitis such as that caused by the West Nile virus (WNV) (Getts *et al.* 2008; Lim *et al.* 2011). CCR2 is the primary and specific receptor for CCL2, but can also bind CCL7, CCL8, CCL12 and CCL13 (Gouwy *et al.* 2004). Based on various studies, the role of the CCR2–CCL2 axis in the progression of viral encephalitis is complicated and depends on the context of viral encephalitis (Bose and Cho 2013; Michlmayr and Lim 2014).

CD8$^+$ T cells frequently function in immune responses to virus infection and several studies have reported their role in viral encephalitis. For example, following Lymphocytic Choriomeningitis Virus (LCMV) infection, CD8$^+$ T cells isolated from the spleens of LCMV-infected mice were found to express high levels of *Ccr2* and *Ccr5* mRNA (Nansen *et al.* 2000). Further, in murine JEV infection and adoptive transfer models, effector CD8$^+$ T cells play a protective role during primary infection possibly by preventing the BBB breach and neuronal damage (Jain *et al.* 2017). In contrast, in Tacaribe virus challenged mice, CD8$^+$ T cells mediate pathology, infiltrate the parenchyma, and induce the apoptotic loss of cerebellar astrocytes, resulting in a loss of balance, paralysis, and death (Ireland *et al.* 2017). Currently, specific subsets of CD8$^+$ T cells were presented for the improved characterization based on the expression of particular molecules. For example, a previous study identified a subset of virus-specific CD8 T cells, namely, Tim3$^+$PD1$^+$CD8$^+$ T cells during chronic LCMV infection (Jin *et al.* 2010). Another study showed that CXCR5$^+$CD8$^+$ T cells regulate autoimmunity in mice (Kim *et al.* 2010). In our study, we try to identify the underlying molecular mechanisms that contribute to the CNS trafficking of CD8$^+$ T cells during JE procession and further study the function and characteristics of the specific subset in these CNS infiltrating CD8$^+$ T cells.

Materials and Methods

Virus, Cells and Mice

The JEV P3 strain was obtained from Professor Shengbo Cao of Huazhong Agricultural University and propagated in the brains of suckling mice. The viral titer was determined by a cytopathic assay using BHK21 cells, which

were grown in Dulbecco's modified Eagle medium (DMEM; Sigma, USA) supplemented with 10% fetal bovine serum (FBS; Gibico, USA) at 37 °C, 5% CO_2. C57BL/6 J mice, 5–6 weeks of age, were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. All *in vivo* experiments were performed in an Animal Biological Safety Level 2 (ABSL2) laboratory of the Wuhan Institute of Virology, CAS.

Antibody and Reagents

The following antibodies were used: rat anti-mouse CD16/32 (mouse BD Fc Block, clone 2.4G2, BD Pharmingen), Fixable Viability Dye eFluor506 (Cat: 65-0866, eBioscience), anti-mouse CD45 Alexa Fluor700 (clone 30-F11, Biolegend), anti-mouse TCR beta APC-eFluor 780® (clone H57-597, eBioscience), anti-mouse CCR2-BV421 (clone 203G11, Biolenged), anti-mouse CCR5 (CD195)-APC (clone HM-CCR5, Biolegend), anti-mouse CD8a-PE/Dazzle (clone 53-6.7, Biolegend), anti-mouse PD1-PE (clone RMP1-30, eBioscience), anti-mouse CD45-AF700 (clone 30-F11, Fluidigm), anti-mouse CD19 (clone 6D5, Fluidigm), anti-mouse CD45R (B220, clone RA3-6B2, Fluidigm), anti-mouse CD3e (clone 145-2C11, Fluidigm), anti-mouse CD4 (clone RM4-5, Fluidigm), anti-mouse CD8a (clone 53-6.7, Fluidigm), anti-mouse CD62L (clone MEL-14, Fluidigm), anti-mouse CD44 (clone IM7, Fluidigm), anti-mouse TNF-α (clone MP6-XT22, Fluidigm), anti-mouse IFN-γ (clone XMG1.2, Fluidigm). Percoll® (P-1644) and collagenase IV (C-5138) were from Sigma. 0.4% (w/v) Trypan Blue was from STEMCELL Technologies. DNase I (11284932001) was from Roche.

Behavior Score of Japanese Encephalitis Virus-infected Mice

Behavior score of JEV-infected mice was conducted as previous study (Lv *et al.* 2018). In brief, Score 0 represents no restriction in movement, no piloerection, no body stiffening and no hind limb paralysis. Score 1 represents that no restriction in movement, no body stiffening, no hind limb paralysis but piloerection, slowless in movement, slight hind limb extension, and stooping posture were observed. Score 2 represents that no restriction in movement, no body stiffening, no hind limb paralysis but piloerection, slowless in movement, slight hind limb extension, and stooping posture were observed. Score 3 represents that restriction in movement, piloerection, mild body stiffening, slight body jerks, slight hind limb extension but no hind limb paralysis were observed. Score 4 represents that restriction in movement, piloerection, body stiffening, hind limb paralysis, occasional tremor. Score 5 represents that restriction in movement, piloerection, body stiffening, hind limb paralysis, tremor, which were followed by the animals succumbing to death.

Histological Analysis

Paraffin-embedded samples were prepared from brains of PBS-and JEV-infected mice. Tissue sections were stained with hematoxylin and eosin (H&E). Stained sections were scanned using a Pannoramic MIDI II (3DHISTECH) and analyzed using Pannoramic Viewer software.

Brain Mononuclear Cells Isolation

To avoid sampling cells from the circulating blood, we perfused the brains before cell isolation. Next, mouse brain tissues were minced finely with surgical scissors and digested with collagenase IV/DNase I solution at 37 °C for 1 h with shaking. Then, tissue samples were homogenized by gently pressing them through tissue sieves. Brain mononuclear cells were isolated by Percoll density gradient centrifugation (300 g for 25 min in room temperature) and washed twice with PBS. Trypan Blue staining was used to determine the numbers of viable cells.

Mass Cytometry Assay

Cell-staining was performed based on protocols provided by Fluidigm. Briefly, cells were stained with 0.5 μmol/L cell-ID cisplatin (Cat. 201064, Fluidigm) for 2 min, which was followed by the addition of 2 mL MaxPar Cell Staining Buffer (Cat. 201068, Fluidigm) to stop the reaction. After centrifugation and removal of the supernatant, cell pellets were resuspended in Fc-receptor blocking solution and incubated for 10 min at room temperature (RT). Then, 50 μL of metal-conjugated surface-marker antibody cocktail was added to each tube, and the samples were incubated for 30 min at RT. For intracellular staining, cells were fixed in 1 mL of 1 × MaxPar Fix I Buffer (Cat. 201065, Fluidigm) at RT for 20 min. After washing twice with 2 mL of MaxPar Perm-S Buffer (Cat. 201066, Fluidigm), the cells were incubated with 50 μL of an intracellular antibody cocktail for 30 min. After washing twice with 2 mL of MaxPar Cell Staining Buffer, the cells were resuspended in 1 mL of the intercalation solution and incubated for 1 h at RT. Then, after washing twice with 2 mL of MaxPar Cell Staining Buffer and twice with 2 mL of MaxPar Water (Cat. 201069, Fluidigm), the cell density was adjusted to 2.5–5 × 10^5/mL with MaxPar Water for the CyTOF assay.

RNA Extraction and Quantitative Real-time PCR (RT-qPCR)

Total mRNA was extracted from mouse brain homogenates using Trizol reagent (Takara). cDNA was generated with M-MLV transcriptase (Takara) and Random Primer 5′-d (NNN NNN)-3′ primers (Sagon). Quantitative RT-PCR was performed on a MX300P machine with SYBR Green Master Mix (TransGen Biotech). Reaction mixture for RT-qPCR consisted of 10 μL SYBR Green Master Mix, 0.8 μL primer mix, 3.2 μL ddH_2O and 6 μL template. PCR conditions were as follows: pre-denaturation at 95 °C for 3 min, denaturation at 95 °C for 20 s, annealing at 60 °C for 20 s, extension at 72 °C for 20 s, repeating this for 40 cycles and then acquiring fluorescence signal between 65 °C and 95 °C. Primers are listed in Supplementary Table S1.

Statistical Analysis

Statistical data analysis was performed with Graphpad Prism 6.0 software. Data represent the mean ± SD. Statistical significance was determined by a two-tailed unpaired Student's *t* test between two groups or a one-way ANOVA with a multiple comparisons test. $P < 0.05$ was considered statistically significant ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$).

Results

JEV Infection Causes CNS Injury in Mice

To study JEV infection with rodent models, several routes of infection *in vivo* have been successfully established

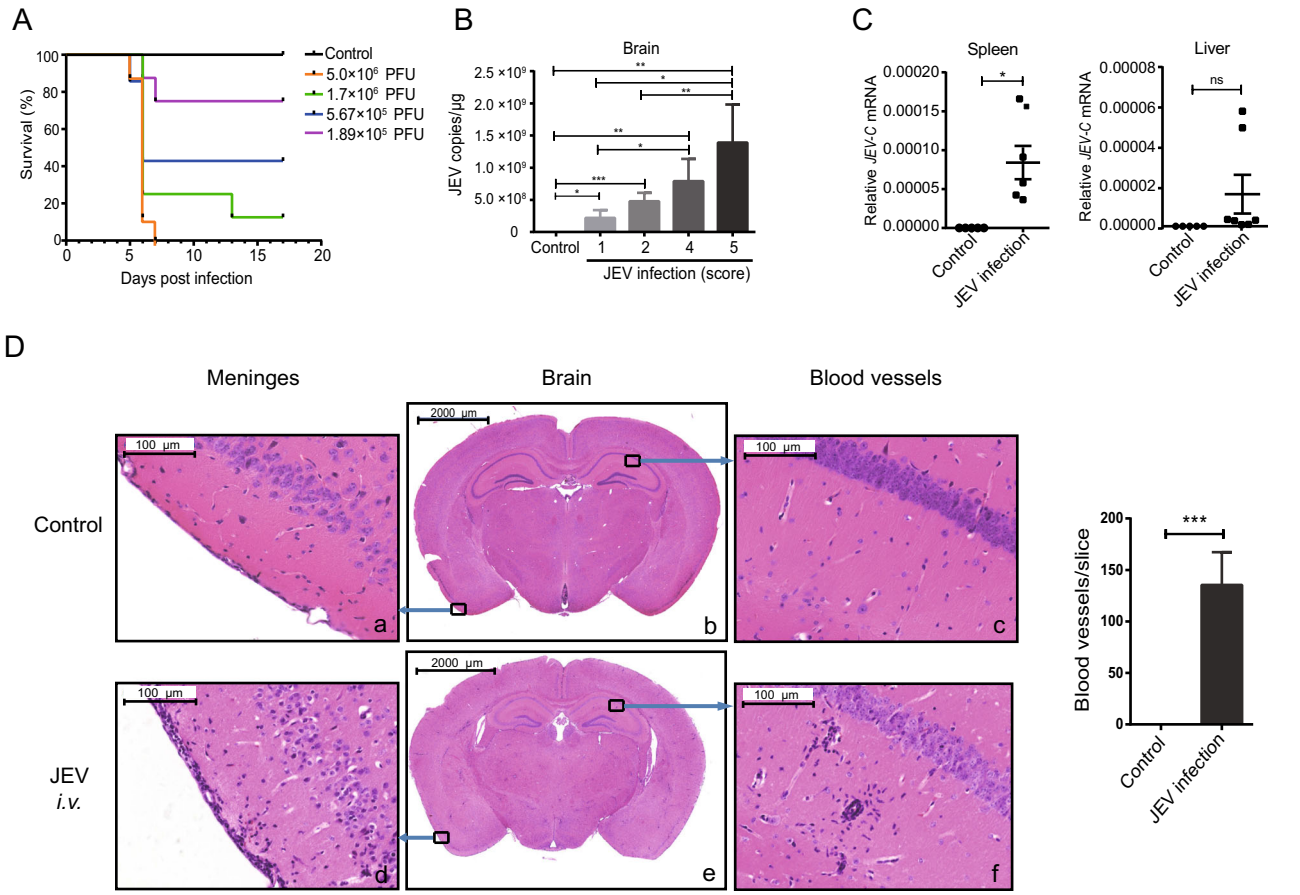
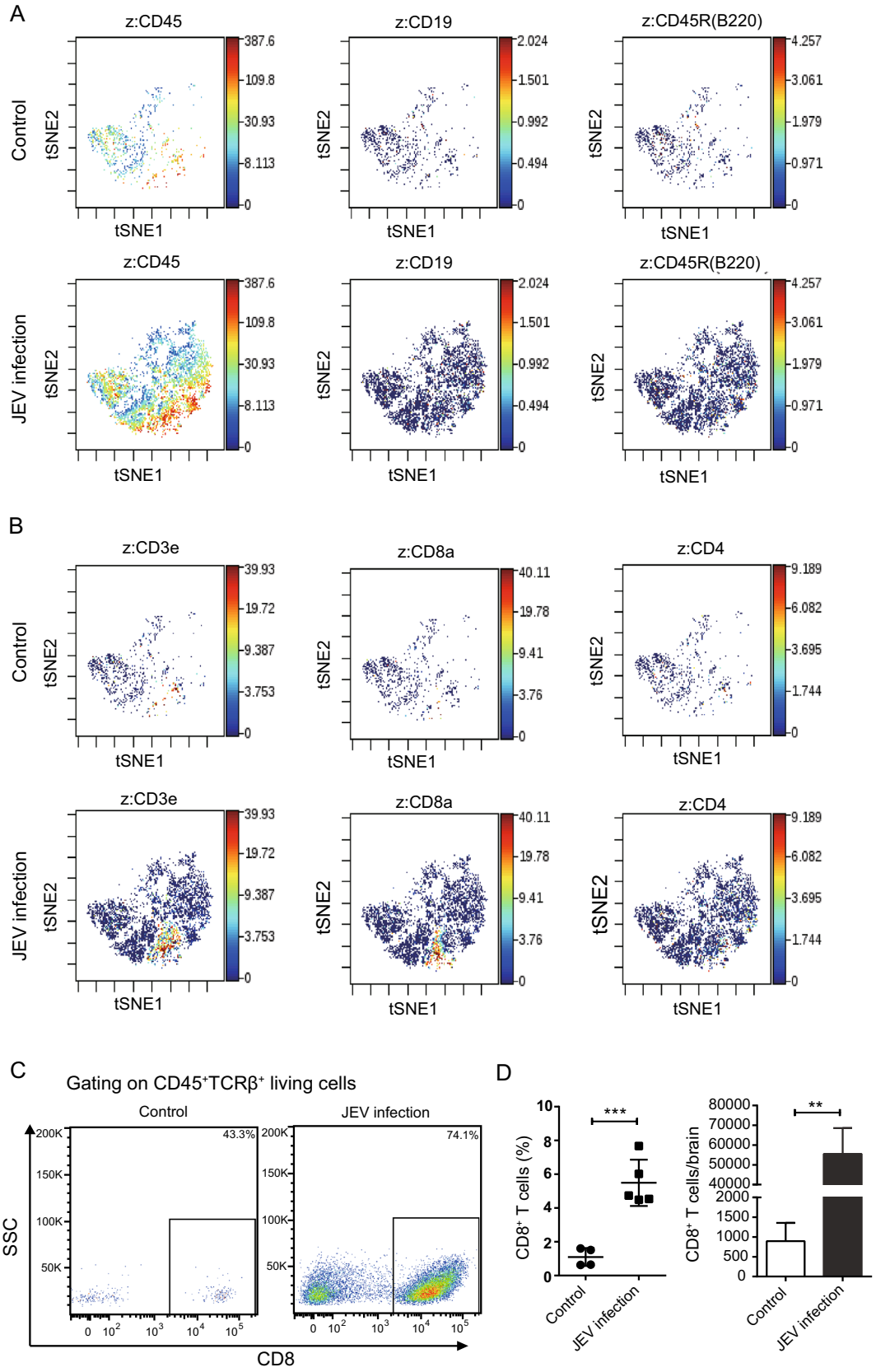


Fig. 1 Japanese encephalitis virus (JEV) infection causes central nervous system injury in mice. **A** Age- and sex-matched wild-type mice were infected intravenously (i.v) with different titers of the JEV P3 strain and survival was monitored for 17 days. **B** Wild-type mice were injected *i.v.* with JEV (5.0×10^6 PFU), and viral copies in the brains of mice with different behavior scores were detected. **C** mRNA levels of the JEV-C gene in spleens and livers of mice after JEV injection. ns no significant difference. Values were normalized for Gapdh mRNA level. **D** Hematoxylin & Eosin (H&E) staining of brain tissues from PBS- and JEV-infected wild-type mice and the statistic of blood vessels in each brain slice. Data are presented as the mean ± SD from three independent experiments.$^{*}P < 0.05$. $^{**}P < 0.01$ and *** $P < 0.001$ based on one-way ANOVA with multiple comparisons test (**B**) or two-tailed, unpaired Student's *t* test (**C**, **D**).


A
z:CD45
z:CD19
z:CD45R(B220)
Control
JEV infection
tSNE1
tSNE2
387.6
109.8
30.93
8.113
0
2.024
1.501
0.992
0.494
4.257
3.061
1.979
0.971
B
z:CD3e
z:CD8a
z:CD4
39.93
19.72
9.387
3.753
40.11
19.78
9.41
3.76
9.189
6.082
3.695
1.744
C
Gating on CD45+TCRβ+ living cells
Control
JEV infection
43.3%
74.1%
SSC
CD8
200K
150K
100K
50K
D
CD8+ T cells (%)
CD8+ T cells/brain
***
**
Control
JEV infection

◀**Fig. 2** Marked CD8$^+$ T cells infiltration into the mouse central nervous system during Japanese encephalitis virus (JEV) infection. **A**, **B** Mass cytometric analyses of infiltrating immune cells isolated from the brains of PBS-and JEV-infected mice. Each marker used for cell characterization, including CD45, CD19, CD45R, CD3e, CD8a and CD4, was overlaid on a viSNE diagram. Each dot represents a single cell, and the color gradient represents the marker's intensity. Dot plots demonstrate the marker labeling on living single cells. Representative images of three independent repeats are shown. **C** Mononuclear cells isolated from the brains of PBS- and JEV- infected mice were stained with indicated antibodies and CD45$^+$TCR-β$^+$ CD8$^+$ T cells were gated. **D** Statistical analyses of the number and proportion of these CD45$^+$TCR-β$^+$ CD8$^+$ T cells among mononuclear cells isolated from the brains. Data are presented as the mean ± SD of two independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$, based on a two-tailed, unpaired Student's *t* test.

previously, such as subcutaneous infection, intracranial infection, intraperitoneal infection and caudal vein infection, individually with diverse JEV strains. Among these, tail vein injection of JEV strains can not only mimic the natural process of infection but is also associated with rapid viral infection with various peripheral immune cells crossing the BBB into the CNS. Therefore, we chose this as our route of infection in the present study. To explore an appropriate titer for JEV P3 strain infection, a series of JEV dilutions were intravenously injected into age- and sex-matched wild type (WT) mice. As shown in Fig. 1A, the mortality rate of mice was elevated with increasing amounts of virus, and injection with 1.7–5.0 × 10^6 PFU of JEV resulted in a mortality rate of greater than 90%. Therefore, for follow-up experiments, all mice were infected via intravenous injection (*i.v.*) with 5.0 × 10^6 PFU of JEV. Upon JEV infection, the virus load in the mouse brains was significantly increased, which was positively correlated with mouse behavior scores (Fig. 1B). Meanwhile, the effective replication of JEV was also observed in mouse spleens (Fig. 1C). Histological analyses of mouse brain tissues showed remarkable CNS injuries with the enhanced infiltration of immune cells in the meninges, as well as increased blood vessels, after JEV infection (Fig. 1D). Together, these data suggest that we generated a successful animal model of JEV infection, which could provide a basic *in vivo* model for follow-up studies.

Marked CD8$^+$ T Cells Infiltration into the Mouse CNS after JEV Infection

To better explore the role of adaptive immunity in the CNS during JEV infection, we performed a high-dimensional characterization of immune cell populations in the brains of PBS- and JEV-injected WT mice using common cell phenotype markers and analysis by mass cytometry. To detect infiltrating immune cell subsets in the brain, we analyzed CD45 expression, which is generally present on all differentiated hematopoietic cells. Among the infiltrating (CD45^{high}) cells, we found no obvious infiltration of B cells, because the expression of CD19 and B220 was too low to be detected (Fig. 2A). Surprisingly, we found that a large number of T cells, and especially CD8$^+$ T cells, exhibited significant infiltration into the mouse CNS after JEV infection (Fig. 2B). To further confirm this observation, mononuclear cells in the brains of mice with or without JEV infection were isolated and CD45^{high}TCR-β$^+$CD8$^+$ T cells were detected by flow cytometry (Fig. 2C). Here, the numbers and proportions of these CD8$^+$ T cells infiltrating the CNS of mice were dramatically enhanced after JEV infection (Fig. 2D).

CCL2 Plays a Role in the Migration of CD8$^+$ T Cells into the Mouse CNS

Considering the remarkable migration of CD8$^+$ T cells into the mouse CNS during JEV infection, we then wanted to determine which molecules have a predominant role in migration process. For this, we first analyzed a previously published microarray dataset of whole brain tissues from PBS-and JEV-infected mice (Yang *et al.* 2011) and placed an emphasis on the different expression profiles of chemokines and their receptors to identify potential molecules that drive CD8$^+$ T cells infiltration. Heatmap analysis of gene expression indicated that JEV-infected mice exhibited the dramatic up-regulation of many chemokines (*Ccl5*, *Ccl3*, *Ccl7*, and *Ccl2*) and corresponding receptors (*Ccr5* and *Ccr2*) in brains compared to levels in mock-infected mice (Fig. 3A). These data were further validated by RT-qPCR analysis. We observed that the transcription of chemokine genes (*Ccl5*, *Ccl3*, and *Ccl2*) and their receptor genes (*Ccr5* and *Ccr2*) in the brains of mice was significantly increased during acute JEV infection, which was correlated with the degree of JE pathogenesis (Fig. 3B, 3C). These observations are in line with previous reports suggesting enhanced *Ccl2* and *Ccl5* expression in mouse brains during the acute phase of JEV infection, as well as in the PBMCs of severely JEV-infected individuals (Gupta and Rao 2011; Chowdhury and Khan 2018). Considering the high expression of chemokines, specifically *Ccl2* and *Ccl5*, in the brains of mice infected with JEV, we speculated that the migration of circulating CD8$^+$ T cells into the CNS might be associated with the chemotactic effect of these chemokines in the brains. To test this hypothesis, we injected WT mice with PBS or JEV and detected the expression of CCR2 and CCR5 on infiltrating CD8$^+$ T cells in the brain by flow cytometry. The same as a previous finding about brain-derived CD8$^+$ T cells with CCR5 high expression (Larena

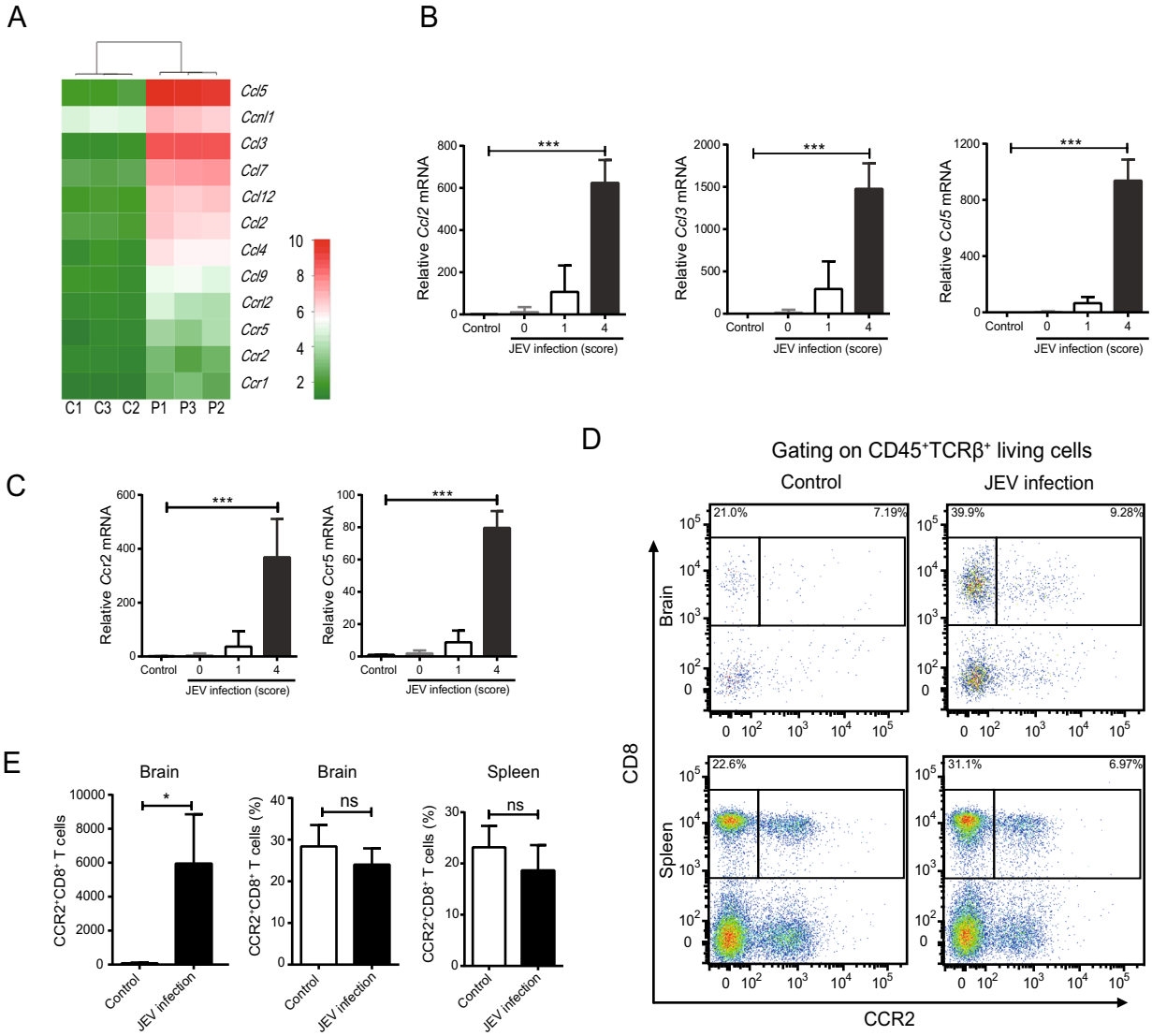


Fig. 3 CCL2 plays a role in the migration of CD8$^+$ T cells into the mouse central nervous system after Japanese encephalitis virus (JEV) infection. **A** Gene expression pattern of chemokines and their co-receptors among severe JE cases in mice, as compared to that in control animals. **B**, **C** mRNA levels of chemokines (*Ccl2*, *Ccl3*, and *Ccl5*) and their receptors (*Ccr2* and *Ccr5*) in mouse brain tissues were assessed by RT-qPCR during mild and severe cases of JE after JEV infection. Values were normalized for Gapdh mRNA level. **D** Mononuclear cells from mouse brain tissues and splenocytes were isolated and CCR2$^+$CD8$^+$ T cells were gated after JEV infection. **E** Statistical analyses of CCR2$^+$CD8$^+$ T cells in the brains and spleens. Data are presented as the mean ± SD of three or more independent experiments. $^*P < 0.05$ and $^{***}P < 0.001$, based on two-tailed, unpaired Student's *t* test.

et al. 2012), we observed that CCR5$^+$CD8$^+$ T cells infiltrating in the brains of mice were notably augmented after JEV infection (Supplementary Figure S1). Additionally, we found that another subpopulation of CD8$^+$ T cells, namely CCR2$^+$CD8$^+$ T cells, was markedly increased in the brains of JEV infected mice, although the proportion of CCR2$^+$CD8$^+$ T cells in the brains and spleens was not associated with significant differences (Fig. 3D, 3E). These observations might suggest that the CCR2-CCL2 axis also has an important role in regulating the migration of circulating CD8$^+$ T cells into the CNS.

Characteristics of Mouse CNS Infiltrating CD8$^+$ T Cells after JEV Infection

As we established the massive CD8$^+$ T cells migrating into the CNS of mice during JEV infection, we then investigated what functions and features those CD8$^+$ T cells have. To this end, we conducted an in-depth analysis of the

infiltrating CD8$^+$ T cell populations in PBS- and JEV-infected brains by mass cytometry. As shown in Fig. 4A, surface markers used for CD8$^+$ T cell characterization, including CD8a, CD44, and CD62L, were overlaid on a viSNE diagram. Consistently, we observed that most infiltrating CD8$^+$ T cells were CD44$^+$ and CD62L$^-$, which is in accordance with the characterization of effector CD8$^+$ T cells (CD44^{high}CD62L^{low}). Meanwhile, we discovered that the expression of TNF-α and IFN-γ in infiltrating CD8$^+$ T cells was enhanced after JEV infection, suggesting that these cells were activated and might function in cell killing (Fig. 4B). The cytotoxic activity of brain infiltrating CD8$^+$ T cells was further proven by the flow cytometric examination of the expression of IFN-γ and granzyme B in CD8$^+$ T cells (Fig. 4C).

Programmed cell death 1 (PD-1) is an immunoinhibitory receptor, and its overexpression on T cells is involved in immune evasion in cancer. It was previously reported that virus-specific CD8$^+$ T cells up-regulate PD-1 expression during acute Friend retrovirus infection (Zelinskyy *et al.* 2011). To assess the expression of PD-1 on CNS infiltrating CD8$^+$ T cells after JEV infection, we isolated peripheral and infiltrating CD8$^+$ T cells from the spleens and brains, respectively, of PBS or JEV-infected mice and compared the surface expression of PD-1 by flow cytometry. Interestingly, based on the method of cell gating, as shown in Supplementary Figure S2, we discovered that PD-1 expression on the infiltrating CD8$^+$ T cells in infected brains was significantly enhanced, whereas no much change occurred in the spleen with respect to CD8$^+$ T cells (Fig. 4D). Of note, PD-1 expression was also dramatically up-regulated both on infiltrating CCR2$^+$CD8$^+$ T cells and CCR2$^-$CD8$^+$ T cells in the brains of virus infected mice (Fig. 4E). Furthermore, the level of PD-1 expression on CNS infiltrating CCR2$^+$CD8$^+$ T cells was much higher than that on CCR2$^-$CD8$^+$ T cells (Fig. 4F). Together, these observations indicate that a specific subpopulation of CD8$^+$ T cells, namely PD1$^+$CCR2$^+$CD8$^+$ T cells, are present in the brains of JEV infected mice during acute infection, which might have a role in the pathogenesis of encephalitis *in vivo*.

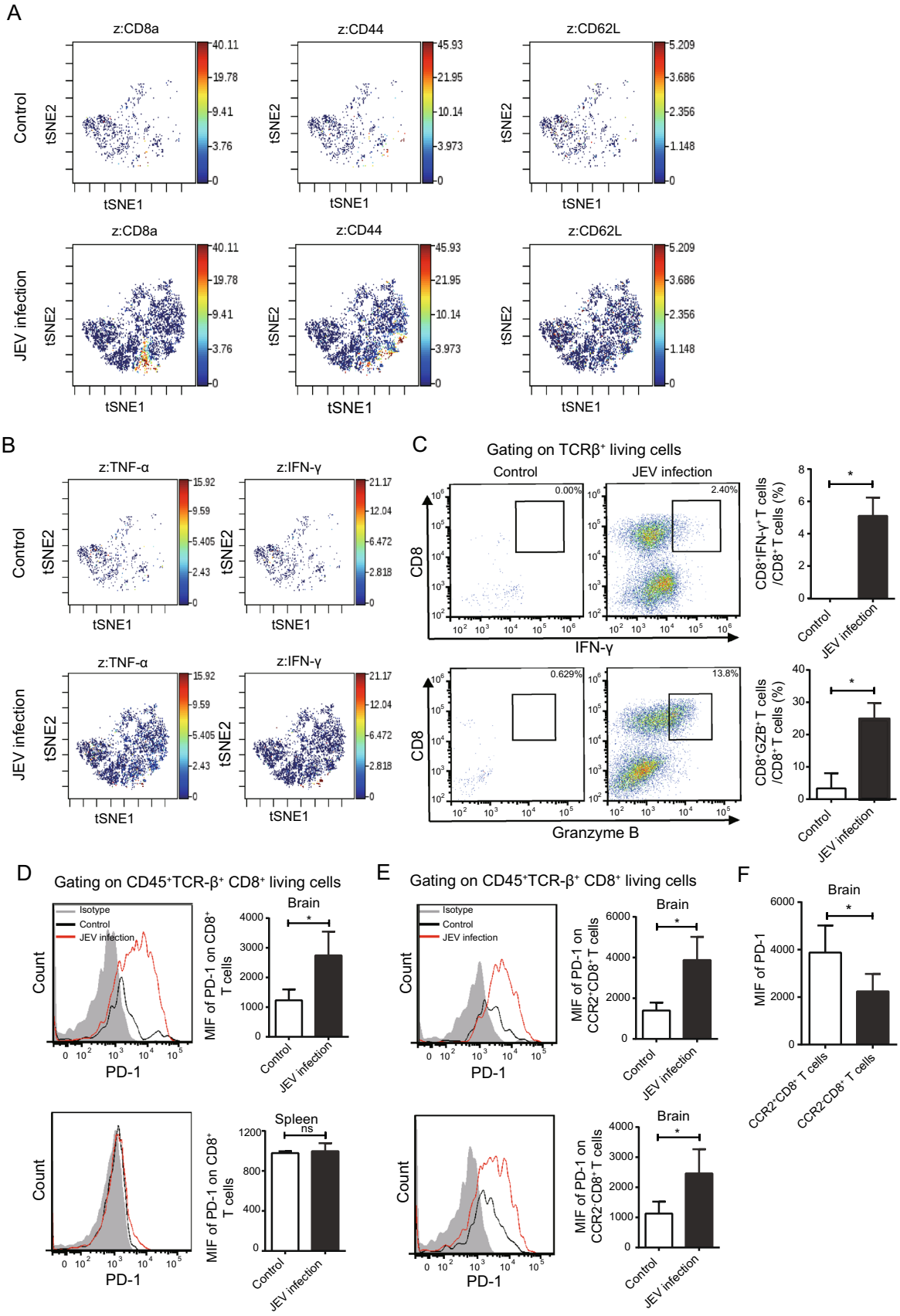
Discussion

JE is one of the major public health issues in Asia. Currently, limits associated with vaccines and the unavailability of antiviral therapy for JE suggest that new therapeutic options are needed. Moreover, the aggravation of CNS inflammation during JE also poses a serious challenge. It is thus necessary to understand the molecular mechanisms underlying disease pathogenesis, as well as the host immune response, to develop an appropriate strategy for JE treatment. In this study, we undertook these studies to understand the relative importance of T cells and specific T cell subsets to both protective immunity and the potential pathogenesis of encephalitis. Our preliminary observations showed that CD8$^+$ T cells, and especially the PD1$^+$CCR2$^+$CD8$^+$ T cells subsets, are closely involved in JEV infection-induced neuroinflammation.

Previous studies have focused on the function of CCR5$^+$CD8$^+$ T cells in viral encephalitis (Glass *et al.* 2005; Larena *et al.* 2012). Regarding CCR2 and its ligand CCL2, although it had been reported that CCL2 expression is significantly increased in the brain during JE, research on the CCR2–CCL2 axis in JEV infection was mainly confined to monocytes/macrophages (Das *et al.* 2009; Kim *et al.* 2016). However, the role of this axis in the regulation of CD8$^+$ T cell migration or function during JE is largely unknown. In our study, we found that CCR2 expression is up-regulated in CNS–infiltrating CD8$^+$ T cells during JEV infection. Therefore, our observation provides solid evidence to support the contribution of the CCR2–CCL2 axis to CD8$^+$ T cell migration and the potent pathogenesis of JE. In addition, CCR2 expression on CD8$^+$ T cells might be related to other regulatory functions during JE, which requires further investigation.

PD-1 is a prototype inhibitory receptor that exists on exhausted CD8$^+$ T cells during chronic infection. However, upon acute JEV infection, we observed that although CNS–infiltrating CD8$^+$ T cells are highly cytotoxic, they up-regulate PD-1 expression, which does not imply an exhausted phenotype. This finding might be explained by a previous study suggesting that the effect of PD-1 signaling varies based on acute immunological responses and chronic antigen exposure (Sharpe *et al.* 2007). In addition, the contradictory role of CD8$^+$ T cells in acute JEV infection in our present study is also consistent with an earlier work on virus-specific CD8$^+$ T cells during acute Friend retrovirus infection (Zelinskyy *et al.* 2011), which suggests that the virus-specific CD8$^+$ T cell up-regulation of PD-1 expression during acute infection could be a universal phenomenon and it could just partially represent the functional state of CD8$^+$ T cells. In addition, many other key functional molecules in CD8$^+$ T also need to be taken into account.

The initiation and termination of neuroinflammation is a complex and comprehensive process. Upon JEV infection, microglia and astrocytes in the brain are substantially activated to modulate brain inflammation. Moreover, large numbers of circulating immune cells such as CD8$^+$ T cells and monocytes/macrophages were also found to invade the CNS and participate in the inflammatory response during JEV infection. Therefore, the combined effect of these different types of immune cells in the brain during JEV infection should also be considered in the future. Using


A
z:CD8a
z:CD44
z:CD62L
Control
JEV infection
tSNE1
tSNE2
B
z:TNF-α
z:IFN-γ
C
Gating on TCRβ+ living cells
Control
JEV infection
CD8
IFN-γ
Granzyme B
CD8+IFN-γ+ T cells /CD8+ T cells (%)
CD8+GZB+ T cells /CD8+ T cells (%)
D
Gating on CD45+TCR-β+ CD8+ living cells
Isotype
Control
JEV infection
Count
PD-1
MIF of PD-1 on CD8+ T cells
Brain
Spleen
E
MIF of PD-1 on CCR2-CD8+ T cells
F
MIF of PD-1
CCR2-CD8+ T cells
CCR2+CD8+ T cells

◀**Fig. 4** Characteristics of mouse central nervous system-infiltrating CD8$^+$ T cells after Japanese encephalitis virus (JEV) infection. **A** Detailed analyses of infiltrating CD8$^+$ T cells by mass cytometry. The expression of CD44 and CD62L overlaid on viSNE diagrams is shown (z: color gradient of expression). **B** Dot plots illustrating the distinction between the expression of TNF-α and IFN-γ on infiltrating CD8$^+$ T cells from JEV-infected brains versus those in control samples. **C** Flow cytometric analysis of the expression of IFN-γ and granzyme B in CD8$^+$ T cells from PBS- and JEV-infected mouse brains. **D** PD-1 expression on CD45$^+$TCR-β$^+$CD8$^+$ T cells isolated from mouse brains and spleens. **E** PD-1 expression on CCR2$^+$CD8$^+$T cells or CCR2$^-$CD8$^+$ T cells in mouse brains after JEV infection. **F** Differential expression of PD-1 on CCR2$^+$CD8$^+$ T cells and CCR2$^-$CD8$^+$ T cells. Data are presented as the mean ± SD of three or more independent experiments. $^*P < 0.05$, based on two-tailed, unpaired Student's *t* test.

mass cytometry, we first tried to identify one specific CD8$^+$ T cell subset that is involved in neuroinflammation process. It can be expected that more solid evidence regarding multiple immune cells that modulates brain inflammation will be provided by this method in the future.

Based on the results of our study, we are still unsure if the infiltration of CCR2$^+$CD8$^+$ T cells in the CNS is associated with beneficial or detrimental outcomes. In view of our finding that CNS–infiltrating CD8$^+$ T cells expressed the chemokine receptor CCR2, and CCR2–CCL2 axis in the progression of viral encephalitis seems to be complicated, and depends on the context of viral encephalitis (Hosking and Lane 2010; Ransohoff and Engelhardt 2012). Therefore, whether the function of CNS–infiltrating CCR2$^+$CD8$^+$ T cells is to protect against or promote the pathogenesis of JE is relatively unclear, which requires more research to identify the properties of CCR2$^+$CD8$^+$ T cells and compare the difference between this subset of CD8$^+$ T cells and the other CD8$^+$ T cells during JEV infection.

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Author Contributions BW and JY conceptualized and designed the study. LLQ, TL and FZ performed the experiments in this study, and analyzed the data. SBC and JY contributed virus strain and virus infection techniques to this study. DY and XJL participated in part of experimental work. FZ wrote the manuscript. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Animal and Human Rights Statement The study was approved by the Animal Ethics Committee of Wuhan Institute of Virology. All institutional and national guidelines for the care and use of laboratory animals were followed.

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