Medical Microbiology and Immunology (2019) 208:487–494
https://doi.org/10.1007/s00430-019-00601-0

ORIGINAL INVESTIGATION

CD4 T cells are required for maintenance of CD8 T_{RM} cells and virus control in the brain of MCMV-infected newborn mice

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Received: 1 March 2019 / Accepted: 19 March 2019 / Published online: 28 March 2019

Abstract

Cytomegalovirus (CMV) infection is a significant public health problem. Congenital CMV infection is a leading infectious cause of long-term neurodevelopmental sequelae, including mental retardation and sensorineural hearing loss. Immune protection against mouse cytomegalovirus (MCMV) is primarily mediated by NK cells and $CD8^+$ T cells, while $CD4^+$ T cells are not needed for control of MCMV in majority of organs in immunocompetent adult mice. Here, we set out to determine the role of $CD4^+$ T cells upon MCMV infection of newborn mice. We provide evidence that $CD4^+$ T cells are essential for clearance of MCMV infection in brain of neonatal mice and for prevention of recurrence of latent MCMV. In addition, we provide evidence that $CD4^+$ T cells are required for induction and maintenance of tissue-resident memory $CD8^+$ T cells in the brain of mice perinatally infected with MCMV.

Keywords Mouse cytomegalovirus · Tissue-resident memory T cells · Brain pathology · CD4 T cells · Congenital CMV infection

Introduction

Cytomegalovirus (CMV) infection is a significant public health problem. In immunocompetent individuals, infection with human CMV (HCMV) is usually asymptomatic. However, it is a major health concern in immunocompromised and immunologically immature individuals. Since cytomegaloviruses are species specific, mouse CMV (MCMV) infection of mice is the most commonly used model to study the pathogenesis of CMV infection [1]. Immune protection against CMV is primarily mediated by NK cells and $CD8^+$ T cells [2, 3]. $CD4^+$ T cells are not needed for control of MCMV in the majority of organs in immunocompetent adult mice, but they mediate IFN-γ dependent clearance of MCMV from salivary glands where viral immunoevasion prevents control by $CD8^+$ T cells [4–7].

Congenital CMV infection is a leading infectious cause of long-term neurodevelopmental sequelae, including mental retardation and sensorineural hearing loss [8]. In contrast to HCMV, MCMV does not cross the placenta. However, the central nervous system in newborn mice is developmentally equivalent to the human fetus at 15 weeks of gestation, a period when HCMV infection in humans is most frequently acquired during pregnancy [9]. Therefore, we have been using MCMV-infected newborn mice to model congenital CMV infection [10]. Using this model, we have previously shown that inflammation in the brain is a major determinant of neurodevelopmental disorders [11, 12]. However, the immune response is required in brain for viral clearance and, therefore, a delicate balance of immune response is necessary to provide virus control and reduce pathology [12]. We have previously shown that in perinatally infected mice, $CD8^+$ T cells are essential for control of MCMV [13]. Furthermore, upon resolution of acute MCMV infection,

Edited by: Matthias J. Reddehase.

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This article is part of the Special Issue on Immunological Imprinting during Chronic Viral Infection.

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$CD8^+$ T cells persist in the brain as tissue-resident memory ($CD8^+$ T_{RM}) cells and prevent reactivation of latent virus [14]. In addition to $CD8^+$ T cells, $CD4^+$ T cells infiltrate the brain of MCMV-infected newborn mice. However, whether they provide protection against acute or latent MCMV upon infection of neonatal mice remained unknown.

Here, we set out to determine the role of $CD4^+$ T cells upon MCMV infection of newborn mice. We provide evidence that $CD4^+$ T cells contribute to the control of acute MCMV infection in newborn mice in several organs. $CD4^+$ T cells were required for clearance of MCMV in salivary glands and surprisingly also in the brain. Furthermore, upon depletion of $CD4^+$ T cells during MCMV latency, the reactivated virus was readily detected in the brain indicating their contribution in the prevention of virus reactivation. In addition, we provide evidence that $CD4^+$ T cells are required for induction and maintenance of $CD8^+$ T_{RM} cells in the brain of mice perinatally infected with MCMV. Further studies are needed to elucidate the mechanisms of $CD4^+$ T cell control of MCMV in newborn mice and their involvement in virus pathogenesis in the central nervous system.

Materials and methods

Mice and viruses

C57BL/6 and C57BL/$6^{JHT/JHT}$ mice were housed and bred under specific pathogen-free conditions at the Central Animal Facility, Faculty of Medicine, University of Rijeka in accordance with the guidelines contained in the International Guiding Principles for Biomedical Research Involving Animals. The Animal Welfare Committee at the University of Rijeka, Faculty of Medicine and National ethics committee approved all animal experiments (525-10/0255-17-4). Newborn mice were infected intraperitoneally (i.p.) with 200 PFU of MCMV. Adult, 8-week-old mice were infected i.p. with 200,000 PFU of MCMV. Tissue culture-derived MCMV reconstituted from BAC pSM3fr-MCK-2fl [15] was used in all experiments. Virus stocks for infection of newborn and adult mice were aliquoted and frozen at − 80 °C before use. Virus stocks and organs homogenates were titrated on murine embryonic fibroblasts (MEF) using standard procedures [16].

Flow cytometry

Flow cytometry was performed according to the guidelines for the use of flow cytometry and cell sorting in immunological studies [17]. Lymphocytes from the brain were isolated using standard protocols. Briefly, mice were perfused with cold PBS and each brain was collected in RPMI 1640 with 3% FCS and mechanically dissociated. A 30% Percoll/brain homogenate suspension was underlaid with 70% Percoll in PBS and then centrifuged at 1050*g* for 25 min. Cells in the interphase were collected for further analysis. Splenic leukocytes were prepared using standard protocols. Before staining of lymphocytes, Fc receptors were blocked using 2.4G2 antibody. The following antibodies, purchased from ThermoFisher were used: anti-mouse CD8α (clone 53-6.7), anti-mouse CD45.2 (clone 104), anti-mouse CD4 (clone RM4-5), anti-mouse CD69 (clone H1.2F3), anti-mouse CD103 (clone 2E7), anti-mouse IFN-γ (clone XMG1.2), anti-mouse CD11a (clone M17/4), anti-mouse/human T-bet (clone 4B10) and anti-mouse CXCR3 (clone CXCR3-173). Fixable Viability Dye (ThermoFisher) was used to exclude dead cells. IFNγ producing MCMV-specific $CD4^+$ T cells were determined according to the previously described protocol [18]. In short, mice were treated intraperitoneally with 1 ml of 3% Thioglycolate 4 days before harvesting peritoneal macrophages (PECs). PECs were pulsed overnight with 100 µg/ml of uninfected or MCMV-infected MEF lysates. The following day, lymphocytes were isolated from brains and spleens of perfused mice and co-incubated with pulsed PECs in 4:1 ratio overnight in the presence of Brefeldin A and Monensin (ThermoFisher). Production of IFN-γ was determined by intracellular staining. All samples were acquired using FACSAriaIIu and data were analyzed using FlowJo v10 (Tree Star) software.

Depletion of T cells

Depletion of $CD4^+$ and/or $CD8^+$ T cells in newborn mice was performed by i.p. injection of 50 µg of $CD4^+$ T cell-depleting antibody (clone YTS 191.1) and/or $CD8^+$ T cell-depleting antibody (clone YTS 169.4) diluted in PBS every 3 days starting from postnatal day (PND) 2 until the termination of experiment. Depletion of $CD4^+$ T cells in adult mice was performed by i.p. injection of 150 µg of $CD4^+$ T cell-depleting antibody (YTS 191.1) once a week for 4 weeks.

Immunohistochemical analysis

MCMV IE1 staining was performed on formalin-fixed and paraffin-embedded tissue sections (3 µm). After de-paraffinization and rehydration, antigen retrieval was performed in sodium citrate buffer (pH 6.0). Endogenous peroxidases were blocked with peroxidase blocking solution (Dako). Samples were stained with MCMV IE1 antibody (clone IE1.01), biotin goat anti-mouse Ig (BD) and streptavidin-POD (Roche). DAB (Dako) was used as the substrate. Samples were counterstained with hematoxylin. The analysis was performed using the Olympus BX40 microscope and Olympus digital camera (DP71).

Statistical analysis

Statistical analysis was performed using the Prism 5 software (GraphPad Software Inc.). Mann–Whitney *U* test was used to determine differences between groups. $P < 0.05$ was considered to be statistically significant ($*P < 0.05$; $**P < 0.01$). In all figures, only statistically significant differences are indicated.

Results

$CD4^+$ T cells are necessary for control of MCMV in brain of newborn mice

We have previously shown that $CD8^+$ T cells are essential for control of MCMV in newborn mice [13]. However, the role of $CD4^+$ T cells remained elusive. To determine the requirement for $CD4^+$ T cells in control of MCMV in newborn mice, we have depleted $CD4^+$ T cells in MCMV-infected newborn mice (Fig. 1). MCMV titers were significantly higher in all the observed organs in the $CD4^+$ T cell-depleted group compared to undepleted controls. As expected, based on previous studies in adult mice, MCMV persisted at higher levels in salivary glands of $CD4^+$ T cell-depleted mice as compared to the control group. Surprisingly, the similar was observed in the brain of newborn infected mice. These data indicate the important role of $CD4^+$ T cells in control of MCMV infection in the brain of infected newborn mice.

$CD4^+$ T cells control MCMV independently of $CD8^+$ T cells in newborn mice

To understand if the impaired control of MCMV in $CD4^+$ T cell-depleted mice is due to lack of help for $CD8^+$ T cells, we have simultaneously depleted both $CD4^+$ and $CD8^+$ T cells (Fig. 2). Individual depletion of $CD8^+$ T cells or $CD4^+$ T cells resulted in an increase of virus titers in both spleen and brain as compared to the undepleted control group. Simultaneous depletion of both $CD8^+$ and $CD4^+$ T cells resulted in a further increase in viral titers in spleen and brain as compared to individually $CD8^+$ and $CD4^+$ T cell-depleted groups (Fig. 2). Altogether, these data indicate that $CD4^+$ T cells contribute to the control of MCMV infection at least partially independent of $CD8^+$ T cells in newborn mice.

Virus-specific $CD4^+$ T cells persist in the brain of mice perinatally infected with MCMV

We have previously shown that virus-specific $CD8^+$ T lymphocytes infiltrate the brain of newborn mice infected with MCMV, and persist in this organ essentially for a lifetime as tissue-resident cells [13, 14]. $CD4^+$ T cells also infiltrate the brain and persist in this organ for up to 120 days [14]. $CD4^+$ T cells persisting in the brain expressed signature markers

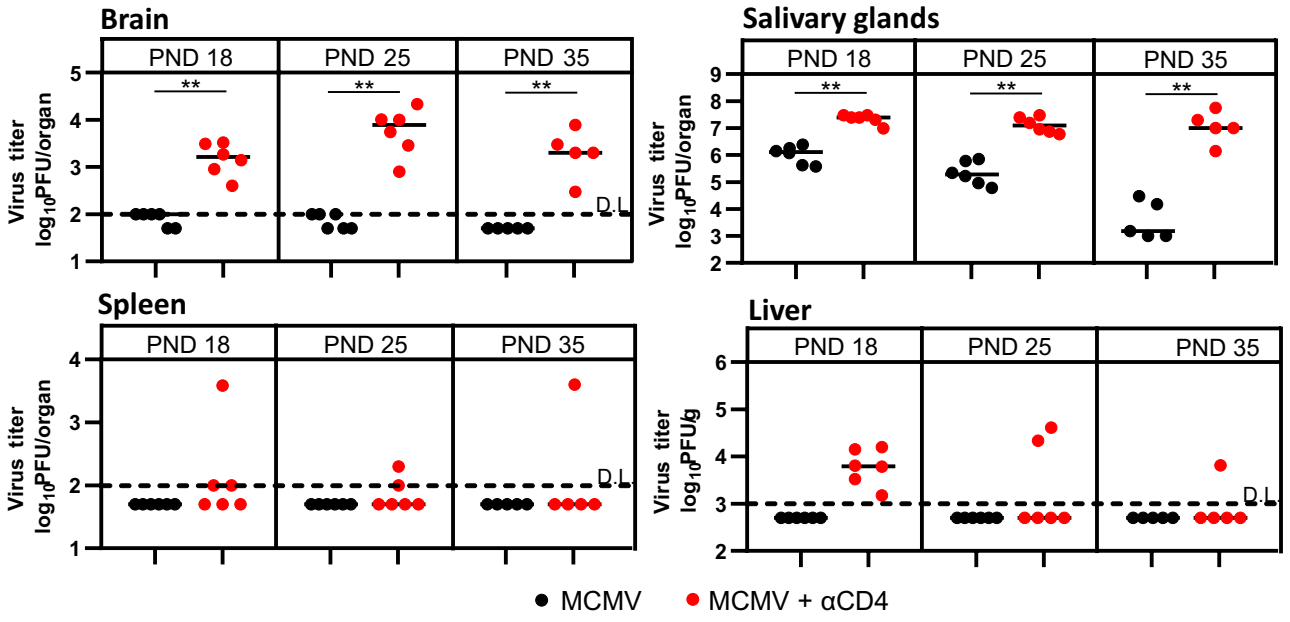

Fig. 1 $CD4^+$ T cells control MCMV in newborn mice. Newborn C57BL/6 mice were injected i.p. with 200 PFU of MCMV on PND 1. Organs were collected at the indicated postnatal days and viral titers were determined by plaque assay. Titers in the organs of individual mice are shown (circles, n = 5–6). Horizontal bars represent medians; data were analyzed using Mann–Whitney *U* test. Asterisks denote significant values: $*P < 0.05$; $**P < 0.01$; *DL* detection limit

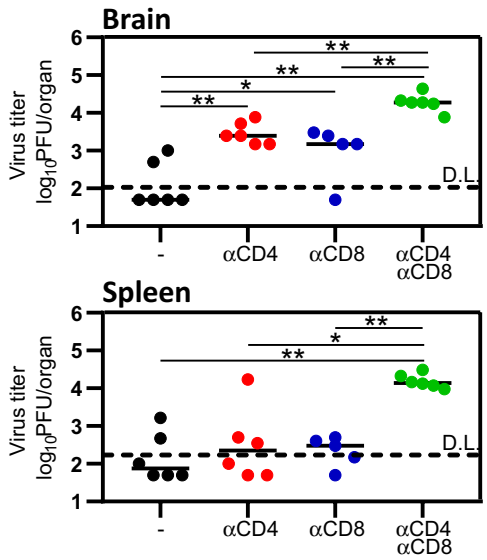

Fig. 2 $CD4^+$ and $CD8^+$ T cell-dependent control of MCMV in newborn mice. Newborn C57BL/6 mice were injected i.p. with 200 PFU of MCMV on PND 1 and depleted for the indicated T cell populations every 3 days starting from PND 2. Organs were collected on PND 14, and viral titers were determined by plaque assay. Titers in the organs of individual mice are shown (circles, n = 6). Horizontal bars represent medians; data were analyzed using Mann–Whitney *U* test. Asterisks denote significant values: $*P < 0.05$; $**P < 0.01$; *DL* detection limit

of T_{RM} cells, such as CD69. Here, we extend this notion and show that $CD69^+CD4^+$ T cells can be found in the brain of mice infected perinatally even 1.5 years after infection, i.e., for the lifetime of mice (Fig. 3a). In addition to CD69, brain $CD4^+$ T cells expressed markers of Th1 $CD4^+$ T cells CD11a, T-bet and CXCR3 (Fig. 3b) [19]. In accordance with their Th1 phenotype, following stimulation of brain-derived lymphocytes with macrophages pulsed with lysates of MCMV-infected MEF, approximately 30% of $CD4^+$ T cells in the brain produced IFN-γ (Fig. 3c, d). At the same time, in spleen less than 5% of $CD4^+$ T cells produced IFN-γ, indicating that virus-specific $CD4^+$ T cells are enriched in brain tissue. The observed frequency of IFN-γ producing $CD4^+$ T cells was similar to the frequency of IFN-γ producing $CD8^+$ T cells in the brain (Fig. 3c). Altogether, these data indicate that virus-specific $CD4^+$ T cells persist in brain for the lifetime of mice perinatally infected with MCMV.

$CD4^+$ T cells are required for generation and maintenance of $CD8^+$ T_{RM} cells

$CD4^+$ T cells are important for the formation of $CD8^+$ T_{RM} cells in some organs [20]. Upon MCMV infection of newborn mice, $CD4^+$ T cells are needed for efficient generation of $CD8^+$ T_{RM} cells in the brain (Fig. 4a and [14]). To understand if $CD4^+$ T cells are continuously required for maintenance of CD103 expression by $CD8^+$ T cells in the brain, we injected $CD4^+$ T cell-depleting antibody for 1 month into 1-year-old mice perinatally infected with MCMV (Fig. 4c). To prevent the development of antibody response to the $CD4^+$ T cell-depleting antibody (of rat origin), we have used B cell-deficient mice ($C57BL76^{JHT/JHT}$). We did not detect $CD4^+$ T cells in brains of mice which received $CD4^+$ T cell-depleting antibodies for 1 month (data not shown). Importantly, $CD8^+$ T cells in the brain of $CD4^+$ T cell-depleted mice did not express CD103, indicating that $CD4^+$ T cells are required for maintenance of $CD8^+$ T_{RM} cells as well.

Depletion of $CD4^+$ T cells during latency in perinatally infected mice results in virus reactivation in the brain

To determine the role of $CD4^+$ T cells in control of latent MCMV in the brain of perinatally infected mice, we applied the depletion regimen of $CD4^+$ T cell as shown in Fig. 5a. We observed significantly more $IE1^+$ cells in $CD4^+$ T cell-depleted mice infected with MCMV perinatally as compared to control mice (Fig. 5b). Expectedly, we did not observe any $IE1^+$ cells in brains of latently infected mice which were infected as adults. These data indicate that $CD4^+$ T cells are not only required for control of primary MCMV infection in brain, but also for prevention of reactivation of latent virus in the brain of perinatally infected mice.

Discussion

In immunocompetent individuals, control of CMV is readily established and the infection is usually asymptomatic, but in immunocompromised and immunologically immature hosts, virus control is inefficient, and can result in morbidity and death. Therefore, understanding the immune response to the infection under such conditions is of great importance. Immune response to CMV in immunocompetent hosts has been studied extensively [1, 2]. Protection against CMV is provided by different immune cell subsets, most importantly $CD8^+$ T cells and NK cells. $CD4^+$ T cells are not needed for control of MCMV in adult immunocompetent mice, with the exception of salivary glands [4]. However, under different immunodeficient conditions, $CD4^+$ T cells can provide a certain level of protection against MCMV infection [21–23]. The role of $CD4^+$ T cells in protection against MCMV in immunologically immature newborn mice was so far unknown. Here, we have determined the role of $CD4^+$ T cells in control of MCMV in newborn mice, with the emphasis on the brain, a prime organ of long-term sequelae following congenital CMV infection.

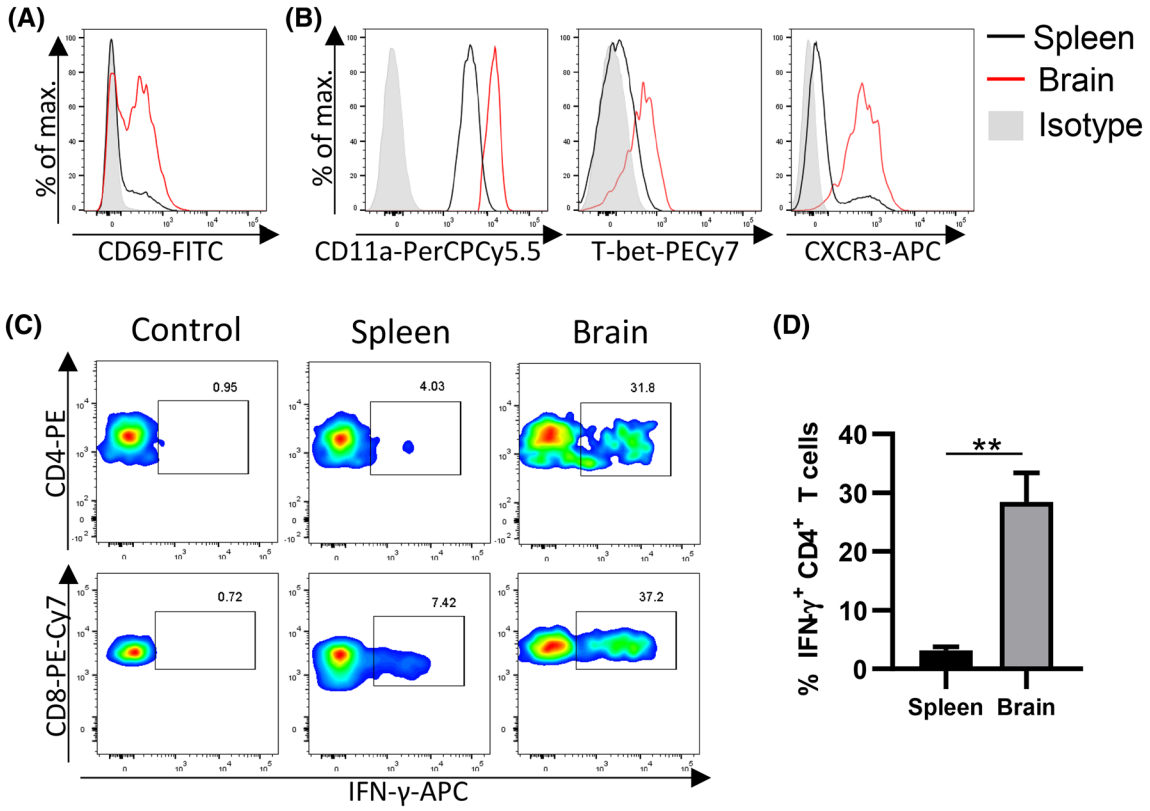

Fig. 3 Virus-specific $CD4^+$ T cells are enriched in the brain of perinatally infected mice and persist as T_{RM} cells. Newborn C57BL/6 mice were injected i.p. with 200 PFU of MCMV on PND 1. **a** Representative expression of CD69 on $CD4^+$ T cells isolated from brain and spleen 1.5 years after infection is shown. **b** Representative expression of CD11a, T-bet and CXCR3 on $CD69^+$ $CD4^+$ T cells isolated from brain and spleen 1.5 years after infection is shown. **c**, **d** One year after infection, splenic and brain lymphocytes were isolated and stimulated with macrophages pulsed with lysates of MCMV-infected MEFs. The frequency of IFN-γ producing $CD4^+$ and $CD8^+$ T cells was determined after 24 h stimulation. Representative flow cytometry plots (**c**) and quantification (**d**) are shown. 3–5 organs were pooled per sample (number of samples = 5)

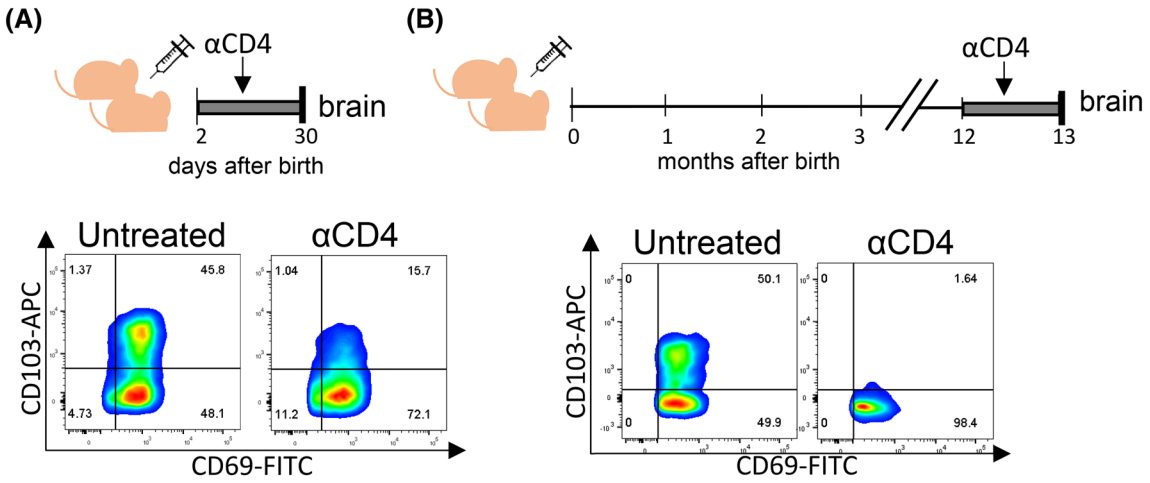

Fig. 4 $CD4^+$ T cells are required for generation and maintenance of $CD8^+$ T_{RM} cells in the MCMV-infected brain. **a** Newborn C57BL/6 mice were injected i.p. with 200 PFU of MCMV on PND 1. Starting with PND 2 mice were depleted of $CD4^+$ T cells for 1 month, followed by lymphocyte isolation and analysis. **b** Newborn C57BL/$6^{JHT/JHT}$ mice were injected i.p. with 200 PFU of MCMV on PND 1. One year after perinatal infection, C57BL/$6^{JHT/JHT}$ mice were $CD4^+$ T cell depleted for 1 month followed by lymphocyte isolation and analysis. Representative expression of T_{RM} markers CD69 and CD103 by $CD8^+$ T cells is shown

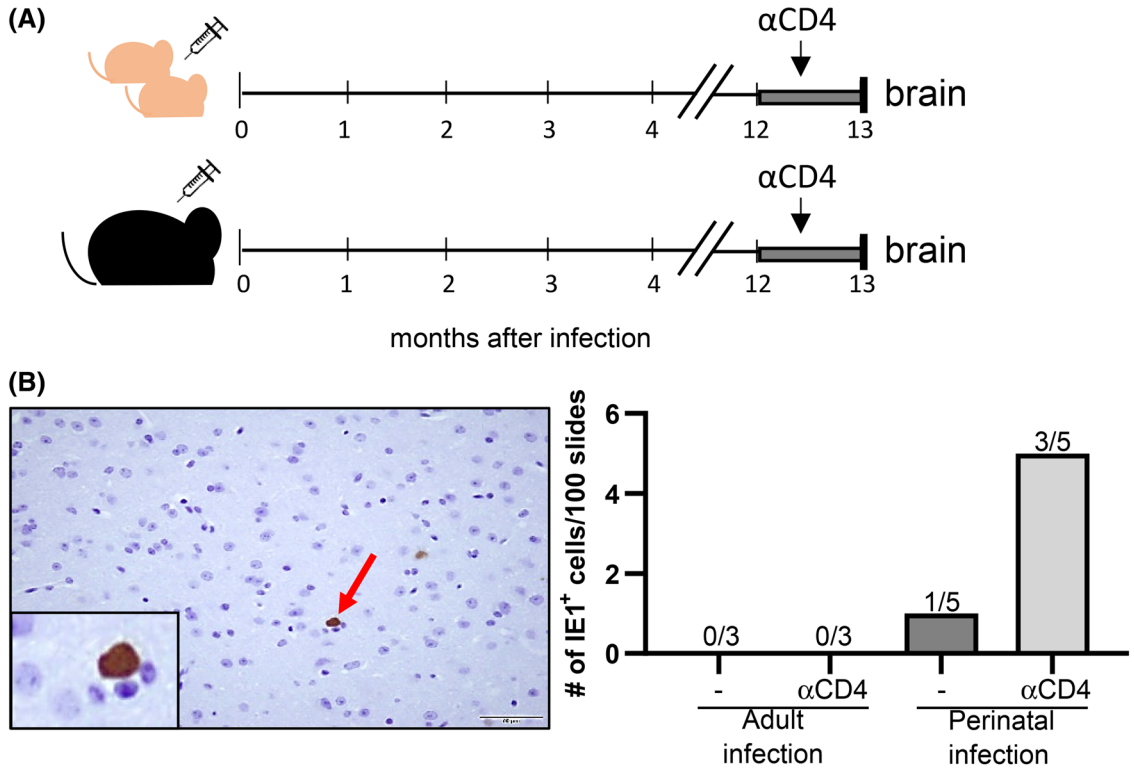

Fig. 5 The absence of $CD4^+$ T cells leads to an increase in virus reactivation in the brain. **a** Newborn C57BL/$6^{JHT/JHT}$ mice were injected i.p. with 200 PFU of MCMV on PND 1. Adult C57BL/$6^{JHT/JHT}$ mice were injected with 200,000 PFU. One year after infection, mice were depleted of $CD4^+$ T cells for 1 month before their brains were harvested. **b** Brains were analyzed for MCMV IE1 expression using immunohistological analysis. A representative analysis of $IE1^+$ cell in the brain is shown (×40; left). Quantification of $IE1^+$ cells is shown on the right. The number of brains in which $IE1^+$ cells were identified is shown above bars

In our study, the absence of $CD4^+$ T cells resulted in impaired control of MCMV in newborn mice, which is not the case for adult mice. Moreover, after perinatal MCMV infection, $CD4^+$ T cells stayed in the brains of infected animals over the lifetime. MCMV does not infect the brain of immunocompetent adult mice; however, following intracerebral MCMV infection $CD4^+$ T cells were not important for virus control, with $CD8^+$ T cells providing protection in a perforin-dependent manner [24]. The explanation why $CD4^+$ T cells are needed for efficient MCMV control in newborn mice, but not in adult mice, remains unknown. Neonatal $CD4^+$ T cell response is skewed towards Th2 response rather than to Th1 [25, 26], though it was shown that Th1-promoting inflammatory treatments, such as certain live viruses or DNA vaccines can overcome this bias [27]. Little is known about the $CD4^+$ T cell-mediated control of HCMV during congenital infection. HCMV-specific $CD4^+$ T cells were identified in congenitally infected infants, and they are impaired in frequency and function as compared to $CD4^+$ T cells in CMV-infected adults [28–30]. However, their role in the protection against infection is still unclear.

One of the possible explanations for impaired MCMV control in $CD4^+$ T cell-depleted mice could be lack of help resulting in impaired $CD8^+$ T cell responses. Here, we show that this is probably not the case at least in early days after infection. Namely, simultaneous depletion of $CD4^+$ and $CD8^+$ T cells further increased viral titers in brain and spleen as compared to a single depleted group lacking either $CD4^+$ or $CD8^+$ T cells. Since $CD8^+$ T cells are essential for the survival of newborn mice after MCMV infection [13], the effect of $CD8^+$ T cell deficiency on the viral burden in later days after infection could not be determined. However, this leaves the possibility of multiple roles of $CD4^+$ T cells in different phases of infection. In line with this, we showed that at later times after infection the absence of $CD4^+$ T cells results in impaired generation and maintenance of $CD8^+$ T_{RM} cells in the brain of MCMV-infected newborn animals. Tissue-resident memory T cells are the first line of defense against reinfections and reactivations and they have been identified in mice and men [20]. In MCMV-infected newborn mice, $CD8^+$ T_{RM} cells are generated in the brain after PND 11 [14]. It was shown previously that $CD4^+$ T cell help is required

for $CD8^+$ T_{RM} development in lungs. However, they were not important once $CD8^+$ T_{RM} cells were established [31]. Similarly, $CD4^+$ regulatory T cells (Treg) were required for generation of $CD8^+$ T_{RM} cells in the brain upon intracranial injection of MCMV in adult mice [32], and upon West Nile virus infection where Treg-dependent TGF-β production was required for the expression of CD103 by $CD8^+$ T cells [33]. Recently, it was shown that in the brain of mice persistently infected with polyomavirus, $CD8^+$ T_{RM} cells require $CD4^+$ T cell help for their induction and maintenance [34]. Similarly, in our model, depletion of $CD4^+$ T cells immediately after infection of newborn mice resulted in the diminished formation of $CD8^+$ T_{RM} cells in the brain. In addition, we have also observed that $CD4^+$ T cells are important for the maintenance of $CD8^+$ T_{RM} cells in the brain. Namely, depletion of $CD4^+$ T cells in latently infected mice for 1 month resulted in loss of $CD103^+$ $CD8^+$ T cells in brain. This was associated with increased numbers of MCMV $IE1^+$ cells in brain tissue. Whether impaired generation of $CD8^+$ T_{RM} cells in brain upon depletion of $CD4^+$ T cells is the reason for impaired MCMV control in brain following PND 18 remains to be verified.

Whether $CD4^+$ T cells residing in the brain are important for the observed findings, or peripheral $CD4^+$ T cells are also involved remains beyond the scope of this study. There are still technical challenges to directly discriminate the role of brain-resident T cells. This seems to be even more demanding in the case of $CD4^+$ T cells as at least in the skin they have been shown to be in equilibrium with the circulation in the steady state, and that they can modulate the expression of CD69 [35]. Whether this is the case for brain $CD4^+$ T cells induced by MCMV infection remains to be determined.

In conclusion, in this study, we provided evidence that $CD4^+$ T cells play an important role in the control of acute MCMV infection in brain of newborn mice. Similarly, in the absence of $CD4^+$ T cells during latency, MCMV reactivates in the brain of latently infected mice. Altogether, we show that $CD4^+$ T cells are an important antiviral mediator in MCMV-infected newborn mice.

Acknowledgements We thank Edvard Ražić, Dijana Rumora, Miro Samsa, Edvard Marinović, Ante Miše and Tina Rudančić for the excellent technical support. The study was supported by NIH (1RO1AI089956-01A1) (to W.J.B. and S.J.), by the European Regional Development Fund (KK.01.1.1.01.0006) (to S.J.) and University of Rijeka (uniri-biomed-18-297 to I.B.).

Compliance with ethical standards

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

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