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Murine cytomegalovirus infection in mice results in an acute inflammatory reaction in peripheral nerves

Oladayo Oladiran[a], Mu Yang[a], Vladimir Andrey Gimenez Rivera[b], XiangQun Shi[a], JiaMin Huang[a], Sylvie Fournier[b], Ji Zhang[a,b,c,d,*]

[a] The Alan Edwards Centre for Research on Pain, McGill Univeristy, Montreal, QC, Canada
[b] Department of Microbiology and Immunology, Faculty of Medicine, McGill University, Montreal, QC, Canada
[c] Department of Neurology and Neurosurgery, Faculty of Medicine, McGill University, Montreal, QC, Canada
[d] Faculty of Dentistry, McGill University, Montreal, QC, Canada

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ABSTRACT

Human cytomegalovirus (CMV) infection is asymptomatic in immunocompetent individuals. However, it can lead to disease in immunodeficient population. Little is known of the mechanisms underlying the pathogenicity of the virus. We investigated the impact of CMV infection on mouse nervous system. Peripheral nerves but not spinal cord was permissive to MCMV during acute infection. Activated CD8$^+$ T cells, monocytes/macrophages and cytokine expression were increased in the blood and sciatic nerves of infected mice, which exhibited transient sensory dysfunction. This study indicates that systemic MCMV infection leads to a dissemination of MCMV into peripheral nerves, which is associated with a local inflammation but not nerve tissue damage in the acute phase.

1. Introduction

Human cytomegalovirus (HCMV) is a widespread pathogen that affects most of the world's population. Subsequent to initial virus entry and replication, CMV infects many organs and tissues in vivo, but only causes disease at some of these and then only in certain patient populations. It is largely asymptomatic in majority of healthy individuals. However, CMV infection can lead to a significant disease in immunocompromised individuals. HCMV has been considered as a prime viral candidate in the etiology of several immune mediated diseases including, autoimmune peripheral neuropathy such as Guillain-Barre syndrome (GBS), atherosclerosis and inflammatory bowel disease (Chan et al., 2012; Rahbar et al., 2003; Lunn and Hughes, 2011). The mechanisms underlying these diseases are elusive. Many factors influence the pathogenesis of infection and the expression of symptomatic disease. It could be due to direct viral replication in specific organs, arising from either a primary infection or a reactivation of latent endogenous virus. It could also be attributed to the modulating effect of the virus on the host immune response.

CMV has different tissue/organ tropisms. Following acute infection, HCMV and MCMV were found in many types of cells/organs, such as macrophages, spleen, lung, liver and salivary glands where the latter secrete higher levels of virus than others (Cousins et al., 2012; Sweet, 1999). However, no data is available regarding the consequence of a systemic CMV infection on peripheral nerves. It is particularly important, since GBS, a prototypic autoimmune disorder that affects peripheral nerves and roots has been highly associated with CMV infection (10–22%) (Winer et al., 1988). Up to date, no immunologic target molecule from CMV infection has been identified for autoimmune response in peripheral nerves. Underlying mechanisms for CMV-GBS are basically unknown.

Although it is too early to mechanistically link acute CMV infection to GBS, it is nevertheless important to unravel whether MCMV attacks the nervous system following a systemic infection, how the immune system reacts, and whether there is any nervous tissue damage immediately after infection. In this study, we infected C57BL/6 mice via intraperitoneal injection of wild type MCMV and explored nervous tissue damage and systemic immune response. We have focused mainly on CD8$^+$ T cells and macrophages, since our previous animal studies and human data from literature demonstrated the accumulation of activated CD8$^+$ T cells and macrophages in inflammatory demyelinating peripheral nerves (Yang et al., 2014; Kiefer et al., 2000; Sommer et al., 2005; Schmidt et al., 1996). Some evidence of their functional contribution has been documented (kiefer et al., 2000; Yang et al., 2015). Our results revealed that in addition to systemic viral immunity, MCMV attacks peripheral nerves shortly after the infection, leading to a local

* Corresponding author at: The Alan Edwards Centre for Research on Pain, McGill University, 740, Dr. Penfield Avenue, Montreal H3A 0G1, QC, Canada.
E-mail address: Ji.Zhang@McGill.ca (J. Zhang).

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inflammatory response with minimal impact in the spinal cord. More specifically, the data demonstrated an accumulation of activated $CD8^+$ T cells and macrophages in peripheral nerves, which corresponds to the invasion of the virus in the peripheral nervous system (PNS). However, peripheral nerves are not damaged at this point, mice exhibit transient sensory dysfunction.

2. Material and methods

2.1. Mice

C57BL/6 were purchased from Jackson laboratory. They were housed and bred under specific pathogen free conditions. Male mice, at the age of 8–10 week-old, were used in this study. All procedures were in accordance with the guidelines of the Canadian Council on Animal Care and approved by the animal care committee of McGill University.

2.2. Viral infection

Smith strain of MCMV (kindly provided by Dr. Sylvia Vidal) has been described and used in this study (Fodil et al., 2014). Infections were performed using 5×10^4 PFU and the intraperitoneal (i.p.) route (100 µl per injection) in all experiments. Mice in vehicle group received the same volume of PBS.

2.3. Flow cytometry

Blood, sciatic nerve and lumbar spinal cord samples were collected at day 5, 8 and 14 post-infection (n = 3–6/group) for flow cytometry analysis. Single cell suspension from blood, sciatic nerve and spinal cord was prepared as described previously (Yang et al., 2014). In brief, 50 µl whole blood was collected from sub-mandibular venous plexus of mice and kept in pre-cold Alsevier's solution (Gibco) to prevent coagulation. After a brief spin down and removal of the supernatant, erythrocytes were lysed by incubating samples with ACK lysing buffer (Thermo Fisher Scientific) at room temperature for 5 min. Approximately 2 cm-long segments of sciatic nerve and 1 cm of lumbar spinal cord were collected following a quick perfusion with 50 µl cold saline. Samples were diced into small pieces and digested by collagenase IV (1.6 mg/ml, Sigma-Aldrich) in 1 × HBSS, then passed through a 70 µm cell strainer to obtain the single cell suspension. Following several washes with 1xHBSS, Fc receptors were blocked with 2.4 G2 blocking buffer for 30 min at 4 °C. Samples were then stained with specific fluorochrome-conjugated antibodies for 30 min at 4 °C. For intracellular staining, cells were suspended in RPMI-1640 complete medium (RPMI-1640 medium + 1% penicillin/streptomycin + 10% deactivated fetal bovine serum, Gibco), incubated for 4 h at 37 °C, 5%CO_2 in the presence of 1 µg/mL ionomycin calcium salt (Sigma-Aldrich), 100 ng/mL PMA (Sigma-Aldrich) and 1 µL/mL Golgi plug (BD). Samples were then blocked and stained for surface markers. After fixation and permeabilization (BD cytofix/cytoperm plus kit) for 60 min, intracellular proteins were labeled with specific fluochrome-conjugated antibodies. Data was acquired with FACS Canto II (BD), and analyzed by using flowjo software. Detailed information of antibodies used in the study is listed in Table 1.

2.4. Behavior tests

Motor and sensory behavior tests were used to monitor functional disability in mice infected with MCMV (n = 4–7/group).

Rotarod test was used to assess motor coordination in mice following infection. The speed was set and fixed at 30 rpm. Mice were placed on the rod and allowed to walk for up to 180 s. The latency to fall from the rod was recorded. A decrease of the latency is suggestive of motor impairment.

Von Frey test was performed to test paw sensitivity to mechanical stimuli. Mice were placed on a metal mesh floor with small Plexiglas cubicles for at least an hour for habituation before testing. Calibrated monofilaments were applied to the plantar surface of the hind paw, the 50% threshold to withdraw was determined by an average of two tests separated by at least 1 h. An increase in threshold suggests mechanical hyposensitivity.

Acetone test was performed to evaluate sensitivity to cold stimulation. A drop of acetone, approx. 25 µL, was applied to the plantar surface of the hind paw, the duration of acetone evoked behaviors (flinching, licking or biting) within 1 min observation was recorded. An increase in the duration of above-mentioned pain like behavior indicates cold hypersensitivity.

Table 1
List of antibodies used for FACS.

Antibody	Dilution	Source	Catalog number	Clone
CD8	1:50	eBioscience	17-0081-83, 46-0081-82	53-6.7
CD11b	1:50	BD	552850	M1/70
CD45	1:50	Biolegend	103116	30-F11
CD62L	1:50	eBioscience	11-0621-85	MEL-14
CD86	1:50	eBioscience	12-0862-82	GL1
CD115	1:50	eBioscience	53-1152-82	AFS98
CD127	1:50	eBioscience	12-1271-81	A7R34
CCR2	1:50	Biolegend	150604	SA203G11
CX3CR1	1:50	Biolegend	149005	SA011F11
F4/80	1:50	eBioscience	45-4801-82	BM8
IFNγ	1:50	eBioscience	12-7311-82	XMG1.2
KLRG1	1:50	eBioscience	17-5893-81	2F1
Ki67	1:50	Biolegend	652404	SOIA15
MHCI	1:50	Biolegend	116517	AF6-88.5

2.5. Histological analyses

Mice (n = 3–7/group) were perfused with a fixative solution (0.5% PFA + 2.5% glutaraldehyde + 0.1 M phosphate buffer) at 14 d after infection for semi-thin section preparation. Sciatic nerves were removed and post-fixed in the same fixative overnight. Nerve tissue samples were processed with osmium tetroxide, dehydrated and embedded in epon kit. Sciatic nerves were sectioned at 0.5 µm using an ultramicrotome (Leica-Reichert) with a diamond knife (Diatome Switzerland). Sections were then stained with toluidine blue for 1 min at 60 °C. Images were obtained by using an Olympus BX51 microscope equipped with a color digital camera. G ratios (Chomiak and Hu, 2009) of 100 myelinated axons were measured using ImageJ software (NIH).

For hematoxylin and eosin (HE) staining, mice (n = 3/group) were perfused with 50 µl cold saline at day 14 post infection. Sciatic nerves were removed and frozen in −80 °C, then sectioned at 12 µm using a cryostat (Leica). Nerve sections were (1) stained with hematoxylin 560 (Leica SelectTech) for 8 min; (2) rinsed in running tap water for 15 min; (3) incubated in blue buffer 8 (Leica SelectTech) for 1 min (4) washed in 80% alcohol; (5) counterstained in eosin Y515 (Leica SelectTech) for 30 s; (6) dehydrated, cleared and mounted with DPX (Sigma-Aldrich). Images were obtained using an Olympus BX51 microscope equipped with a color digital camera.

2.6. Total RNA extraction and RT-PCR

At day 4, 8 and 14 post-infection, mice (n = 3–5/group) were sacrificed to harvest sciatic nerve, spinal cord, salivary gland and spleen. Total RNA was extracted by using TRIzol reagent (Ambion Life Technologies). In brief, samples were homogenized in TRIzol with 0.2 mm glass beads (Sigma-Aldrich) by using Precellys 24 tissue homogenizer (Bertin technologies) at 6500 rpm for 30 s. Chloroform and isopropanol were then added to remove total protein and genomic DNA, respectively. After washing by 75% ethanol, total RNA was resolved in DEPC treated RNase-free H_2O. The purity and concentration

Table 2
List of primers used for RT-PCR.

Primers	Forward	Reverse
GAPDH	GTGAAGGTCGGTGTGAAC	AATCTCCACTTTGCCACTG
IE1	TGTAGTTGCTAAGTTACATAAGC	TACTGTTTCCCCCACTGTGC
IFNγ	TCCACATCTATGCCACTTGAG	CTGAGACAATGAACGCTACACA
IL-1β	CTATACCTGTCCTGTGTA	GCTCTTGACTTCTATCTTG
IL-6	CTGAAACTTCCAGAGATAC	TTCATGTACTCCAGGTAG
TNFα	TTCTGTCTACTGAACTTC	CCATAGAACTGATGAG

of RNA was assessed using Nanodrop 2000 (Thermofisher Scientific). 1 μg of total RNA was added into each reverse transcription system, which contains superscript IV reverse transcriptase (Invitrogen), Oligo-dt (18mer) and dNTP. Real-time quantitative PCR (qPCR) reactions were processed with a Rotor-Gene Q real-time PCR cycler (Qiagen) using SYBR Green mix from Qiagen (RT2 SYBR Green FAST Mastermix). The levels of target genes were normalized against the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and interpreted using the comparative Ct method. qPCR primers were designed based on gene sequence from GeneBank database on NCBI and synthesized by Integrated DNA Technologies. Primer sequences are listed in Table 2.

2.7. Statistical analysis

Data are presented as mean ± SEM and analyzed using the Graph Pad Prism software. Flow cytometry results, behavior test and RT-PCR results were analyzed using two-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test. The 2-tailed unpaired *t*-test was used to analyze histological data. Differences were considered significant at $p < 0.05$.

3. Results

3.1. MCMV immediate early (IE1) mRNA was detected in peripheral nerves following an intraperitoneal inoculation

MCMV IE1 has a critical role in viral replication (Tang and Maul, 2003). Alongside other immediate-early (IE) genes/proteins, IE1 is expressed at the beginning of infection. It is also a prerequisite for progression into the late phase of infectious cycle and the subsequent replication of viral DNA, which is in turn required for entry into the late phase, and virion assembly and release (Nevels et al., 2004; Gawn and Greaves, 2002). To first confirm whether an intraperitoneal injection of MCMV (5×10^4 PFU) in C57/BL6 mice could result in a successful infection, we assessed the mRNA expression of IE1 by quantitative RT-PCR in different organs at days 5, 8 and 14 post-infection. As expected, a robust expression of MCMV IE1 was readily detected in the salivary gland of infected mice at day 4, which persisted up until day 14 (Fig. 1A). IE1 expression was also found in the spleen at day 8 (Fig. 1B). Interestingly, IE1 expression, a sign of active viral replication, was detected in sciatic nerve at day 8 post-infection, which remained at high level until at least day14 (Fig. 1C), whilst IE1 was barely detected in the spinal cord (Fig. 1D). These data demonstrated an efficient MCMV dissemination in peripheral organs, not only in salivary glands and the spleen, but also in peripheral nerves, although we did not attempt to identify what types of cells were directly infected by the virus.

3.2. MCMV infection led to an activation of $CD8^+$ T cells and monocytes in the blood

$CD8^+$ T cells and monocytes are important players in viral infection. Patients with recent CMV infection have abnormally high numbers of $CD8^+$ T cells in the blood (Sindern et al., 1997; Mausberg et al., 2013). Monocytes have been implicated in the delivery of MCMV to tissues and shown to cause tissue damage following infection (Noda et al., 2006; Henson and Strano, 1972). We explored the response of $CD8^+$ T cells and monocytes in the blood at day 5, 8 and 14 following intraperitoneal infection. Flow cytometric analysis revealed a significant ($p < 0.05$) increase in the number and the percentage of $CD8^+$ T cells in the blood (Fig. 2A). The majority of these $CD8^+$ T cells exhibited an activated phenotype which were $CD62L^-$ and $CD127^-/KLRG1^+$ (indicating short lived effector cells) (Fig. 2B, C). Almost all $CD8^+$ T cells were $Ki67^+$ while half of them were able to secrete $IFN\gamma^+$ (Fig. 2D). The reaction of circulating $CD8^+$ T cells to MCMV infection peaked at day 8. This reaction did remain higher than that in the vehicle group until day 14 post-infection, although statistically not significant.

In parallel, an increase in the number and the percentage of total monocytes ($CD115^+CD11b^+$) (Fig. 2E), and the subset of $CCR2^+$ ($CCR2^+CX3CR1^-$ and $CCR2^+CX3CR1^+$) inflammatory monocytes (Fig. 2F) was found in the blood of MCMV infected mice. The increase of CCR2 single positive monocytes ($CCR2^+CX3CR1^-$) preceded the increase of total $CD115^+CD11b^+$ monocytes (Fig. 2F), suggesting that MCMV triggered an active emigration of monocytes from the bone marrow. The reaction of circulating monocytes to systemic viral infection also peaked at day 8 post-infection.

3.3. MCMV infection resulted in an accumulation of activated $CD8^+$ T cells and macrophages in peripheral nerves

Having observed an increased number of $CD8^+$ T cells and inflammatory monocytes in the blood following infection, it was imperative to know whether these activated immune cells were recruited to peripheral nerves, especially where active MCMV infection was detected. To answer this question, we performed flow cytometric analysis of $CD45^+$ cells in the sciatic nerve at day 5, 8 and 14 post-infection. Three major cell types were monitored for their recruitment into the nerve: $CD8^+$ T cells ($CD45^+CD8^+$), macrophages ($CD45^+CD11b^+F4/80^+$), and NK cells ($CD45^+NK1.1^+$). Whereas NK cells infiltration was seemingly absent at days 8 and 14 (data not shown), $CD8^+$ T cells and macrophages were consistently found in high numbers at day 8 and 14 (Fig. 3). Among $CD8^+$ T cells present in the nerve (day 8), the majority displayed $CD62L^-$, $KLRG1^+/CD127^-$, the phenotype of short living effector cells (Fig. 3A–C). As seen in the blood, $CD8^+$ T cells in peripheral nerves were also able to proliferate and secrete IFN-γ (Fig. 3D). Although there was a decrease in the activation of $CD8^+$ T cells observed at day 14, it was still significantly higher than vehicle group (Fig. 3A–D).

Macrophages are cells of the innate immune system with versatile function in host defense against foreign microorganisms, including viruses, bacteria, fungi and parasites (Murray and Wynn, 2011). We were expecting an increase in macrophages since their precursor monocytes were activated and increased in the blood of MCMV-infected mice. Not surprisingly, an increase in the number of macrophages at day 8, with a further augmentation at day 14 was found in the sciatic nerve of MCMV infected mice (Fig. 3E). Among them, the number of $MHC\text{-}I^+/B7.2^+$ macrophages increased at day 8 and 14 following infection. In addition, compared with constitutive expression of MHC-I in nerve macrophages in the vehicle group, MHC-I expression in nerve macrophages of MCMV infected mice were significantly upregulated (Fig. 3F), indicating activation status for macrophages and suggesting a potential role in antigen presentation. As a further confirmation, we performed H & E staining on sciatic nerves, as depicted in Fig. 3G, although mice were thoroughly perfused with 50 μl cold saline before tissue sample collection, there still had much more hematoxylin stained cells in nerves of MCMV infected mice than those of control mice. More specifically, while cells in PBS treated nerves were in elongated shape, most likely nerve resident macrophages or Schwann's cells, those in viral infected nerves were roundish (Fig. 3G, inserts), suggesting infiltrated immune cells. They were either dispersed or clustered in endoneurieum or tightly adhered to nerve vascular structures or nerve

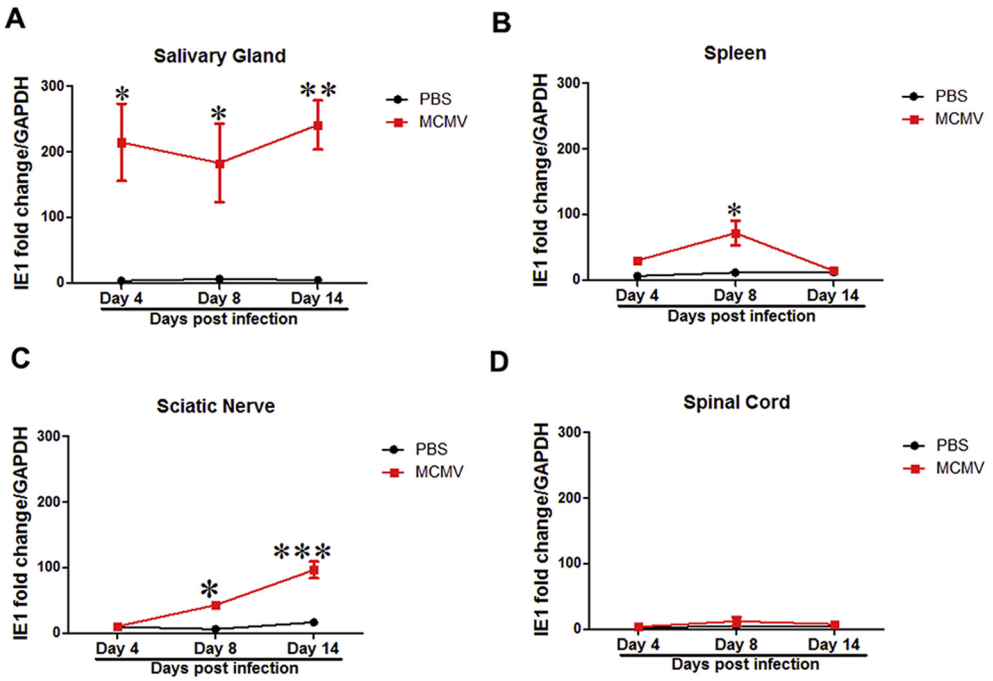


Fig. 1. Detection of MCMV IE1 mRNA expression in salivary gland, spleen, sciatic nerve and spinal cord. MCMV IE1 expression in salivary gland, spleen, sciatic nerve and spinal cord was assessed by quantitative RT-PCR. (A): High level of MCMV IE1 expression was detected in the salivary gland as early as day 4 and persisted till day 14. (B): MCMV IE1 was detected in the spleen, peaked at day 8. (C): IE1 mRNA was found in the sciatic nerve, which increased progressively until day 14. (D): MCMV IE1 was undetectable in the spinal cord across all time points. n = 3–5/group; $p < 0.05$.

perineurium. Interestingly, we also noticed that MCMV infected nerve was thicker, almost double sized compared to sciatic nerve in PBS treated mice (Fig. 3G). Increased accumulation of immune cells in peripheral nerve micro-environment could contribute to local inflammation.

3.4. MCMV infection triggered an inflammatory response in peripheral nerves but did not result in neuropathy

Cytokines play an important role in inflammation and infection by coordinating and activating the immune cell response. To investigate their involvement in viral infection induced-inflammation in peripheral nerves, the mRNA expression of IFNγ, TNFα, IL-1β and IL-6 was quantified. As shown in Fig. 4, sciatic nerve of infected mice exhibited increased levels of all examined cytokines at day 8. While IFNγ was maintained at a high level until at least day 14 (Fig. 4A), TNFα, IL-1β and IL-6 were reduced afterwards (Fig. 4B–D), and a sharp drop for IL-1β was noticed (Fig. 4C). The increased levels of pro-inflammatory cytokines coincided with the elevated number of immune cells in the nerves.

We next examined whether local inflammatory reaction led to peripheral nerve damage. We performed histological analyses of myelin and axons in sciatic nerve of mice with or without MCMV infection. The ratio of the inner axonal diameter to the total outer diameter or G ratio is widely utilized as a functional and structural index of optimal axonal myelination (Chomiak and Hu, 2009). We measured the size of axons and the thickness of myelin sheath on Toluidine stained semi-thin sciatic nerve cross sections, as depicted in Fig. 5A, and calculated G ratio from 100 myelinated axons per mouse. In infected mice, the average G ratio was 0.71 which is very similar to the 0.69 observed in control mice (Fig. 5B). The myelin area and axon area were also similar in both groups (Fig. 5C & D). Altogether, it demonstrated that 14 days post-MCMV infection, the integrity of nerve structure was preserved. There was no overt sign of axonal damage, nor loss of myelin in MCMV infected mice at the acute phase (Fig. 5). However, we observed an increase in the number and the size of capillaries in the sciatic nerve of infected mice (Fig. 5E), which is a typical feature of inflammation, contributing to leukocyte adhesion and migration (Aurora et al., 2005; Baluk et al., 2009).

3.5. MCMV infection had less of an impact on the spinal cord than on peripheral nerves

The robust response of $CD8^+$ T cells and macrophages observed in the peripheral nerve led us to question whether such phenomena is also present in the CNS. Using flow cytometric analysis, we quantified the number of $CD8^+$ T cells and microglia ($CD45^{low}$ $CD11b^+$) in the spinal cord. As shown in Fig. 6, only few $CD8^+$ T cells were observed in the spinal cord at day 14 post infection (Fig. 6A). Although we observed an increase in the number of spinal microglia at days 8 and 14 post-infection (Fig. 6B), the impact seems much less important as compared with what was seen in peripheral nerves. Together with the fact that MCMV IE1 was not detected in the spinal cord, we assume that the effect of MCMV infection in the spinal cord was minor. As reported in the literature (Raghavendra et al., 2004; Qin et al., 2007), observed spinal microglia activation could be derived from systemic inflammation associated with viral infection.

3.6. MCMV infection led to a transient abnormal sensory behavior

To further understand the impact of MCMV infection in the function of the nervous system, we monitored mouse motor and sensory behavior by using rotarod and von Frey/acetone tests, respectively. In rotarod test, latency of fall was similar in both infected and vehicle mice at day 8 and comparable to the day before infection (Fig. 7A), suggesting no apparent motor deficits. However, infected mice developed sensory abnormalities as early as 3–4 days post-infection (Fig. 7B & C). Infected mice became hyposensitive to mechanical stimulation from days 3 to 7 post-viral infection. While mice in vehicle group responded to von Frey filaments at 0.6940 ± 0.0469 g, the paw withdrawal thresholds in MCMV infected mice were increased to 0.9100 ± 0.1553 g at day 3, which were reversed to normal at day 11 (Fig. 7B). In acetone tests, cold hypersensitivity was found in MCMV infected mice. The increase on the duration of pain-like behavior was significant at day 4 to day 8 post-infection, after which a small decline was observed, but remained high with a *P* value of 0.059 at day 12 post infection (Fig. 7C).

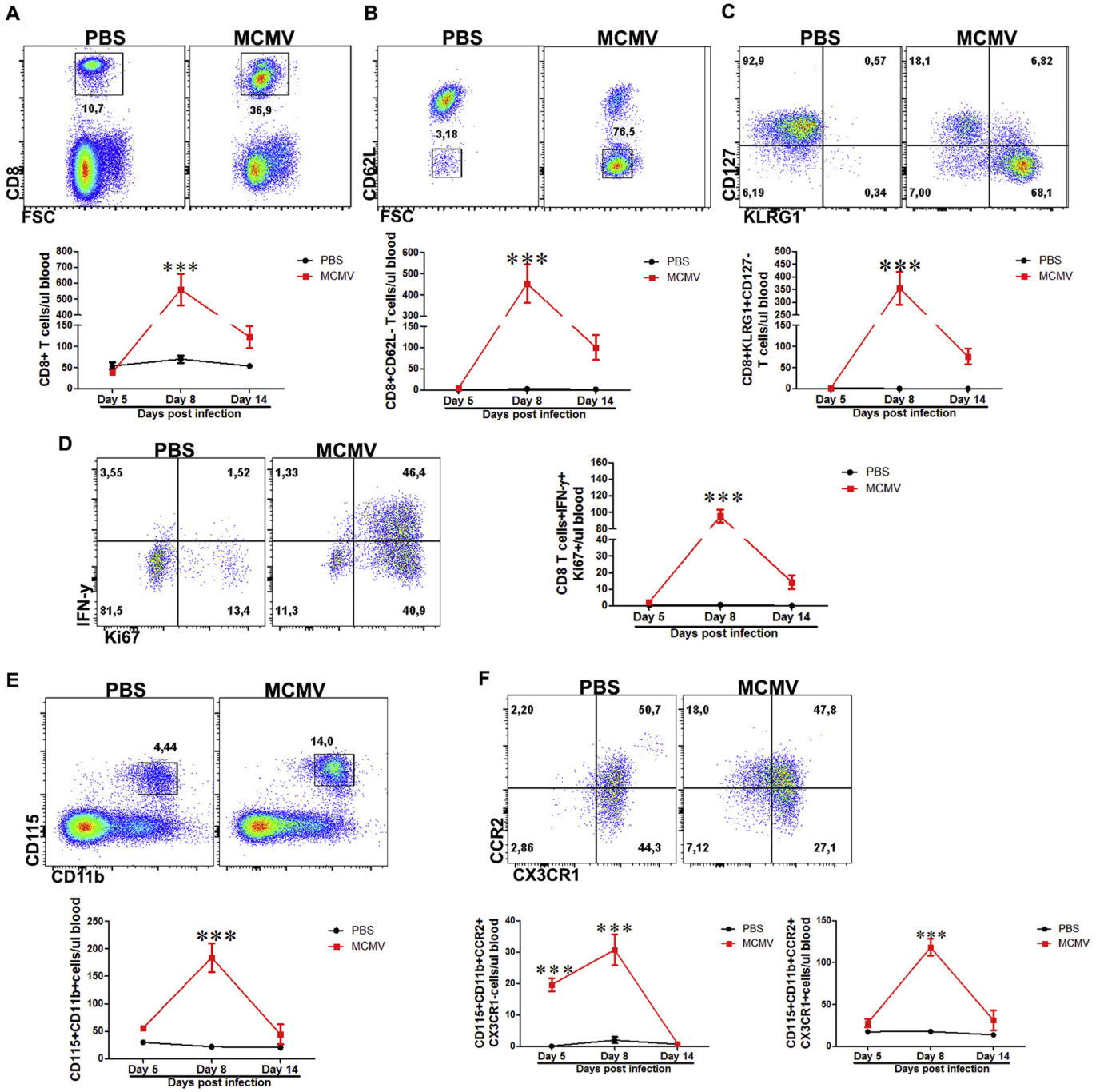
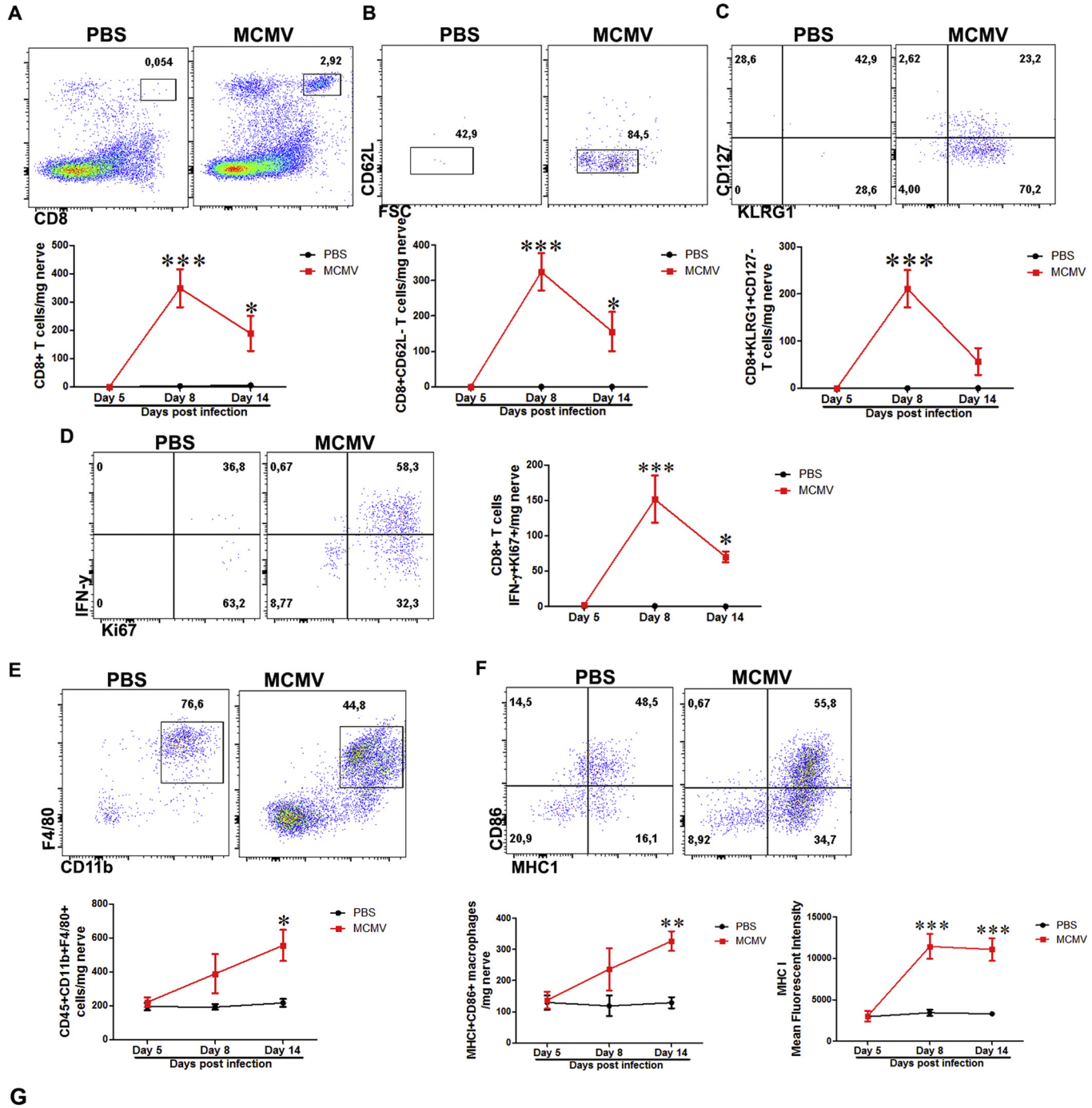


Fig. 2. Activation of $CD8^+$ T cells and monocytes in the blood following acute MCMV infection. (A): $CD8^+$ T cells increased in the blood following infection, which peaked at day 8, (B-C): these $CD8^+$ T cells showed an effector phenotype shown as $CD8^+CD62L^-$, $CD8^+CD127^-KLRG1^+$ cells, (D): $CD8^+$ T cells in MCMV infected mice displayed an increase in Ki67 and IFNγ expression. (E): Total number of monocytes identified by CD115 and CD11b expression was increased after infection. The highest number of monocytes was seen at day 8. (F): $CCR2^+$ monocytes (pro-inflammatory) subset which includes the $CCR2^+/CX3CR1^-$ and $CCR2/CX3CR1^{++}$ groups were significantly increased in MCMV group. The increase of $CCR2^+/CX3CR1^-$ monocytes started at day 5, prior to the increase of the total monocytes. n = 4–6/group; $p < 0.05$.

4. Discussion

In this study, we investigated the immediate impact (within 2 weeks) of systemic MCMV infection in the blood and in the nervous system of C57BL6 mice. Our results provided clear evidence that in addition to systemic viral immune response, MCMV infection precipitates an acute and transient inflammatory response in the nervous system, with a preference for the PNS. In this acute phase, however, no axonal damage nor demyelination was found in peripheral nerves.

In rodent experimental models of MCMV infection, immune responses vary based on the strain-specific differences in NK and $CD8^+$ T cell responses. In susceptible BALB/c mice, which lack the Ly49h NK receptor, host defense is primarily $CD8^+$ T cell dependent (Robbins et al., 2007). In C57BL/6 mice (used in the current study), both cell populations are important in viral immunity (Krmpotic et al., 2003). NK cells play a crucial role in the early stage of MCMV infection, prior to the induction of the adaptive immune response (Robbins et al., 2007; Krmpotic et al., 2003). $CD8^+$ T cells are essential in controlling



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pathogens that establish both acute and persistent infections, which directed our focus on CD8$^+$ T cell response in this study. In coincidence with previous reports (Klenerman and Oxenius, 2016; Mitrovic et al., 2012), our results demonstrated a significant expansion of CD8$^+$ T cells in the blood, peaking at 8 days following i.p. inoculation of MCMV in C57BL/6 mice. Most of these cells are activated effectors as they

Fig. 3. Accumulation of CD8$^+$ T cells and macrophages in sciatic nerve of MCMV infected mice. (A): CD8$^+$ T cells were found to infiltrate the sciatic nerve of infected mice at day 8 and day 14 following infection. (B-C): Vast majority of these CD8$^+$ T cells showed an effector phenotype evidenced by CD62L$^-$ and CD127$^-$KLRG1$^+$. (D): The number of Ki67 + and IFN-γ$^+$ was increased in sciatic nerves of MCMV infected mice. (E): The number of macrophages (CD45/CD11b/F4/80) was increased in the sciatic nerves of MCMV infected mice at day 8 and 14. (F): MHCI expressions were upregulated on macrophages at day8-day14 post-infection, indicative of an activation status for macrophages. n = 4–6/group; $p < 0.05$. (G): H&E staining demonstrated an increase of hematoxylin stained cells in MCMV infected sciatic nerves, in both endoneurieum and perineurieum. Inserts illustrated that in PBS group, cells were in elongated shape, while in MCMV infected mice, many of them were roundish, suggesting newly infiltrated immune cells. Scale bar: 50 μm.

produce IFN-γ. Strikingly, MCMV triggered-CD8$^+$ T cell expansion/activation was found not only in the blood, but also in the peripheral nervous system. An accumulation of CD8$^+$ T cells was detected in sciatic nerves, but not in the spinal cord. The recruitment of CD8$^+$ T cells into the peripheral nerve peaked at day 8 and was maintained at least until day 14 post-infection, in parallel to the presence of MCMV IE1 expression in the nervous system. Similar to what we observed in the blood, CD8$^+$ T cells in peripheral nerves also displayed an activated, effector phenotype.

In addition to CD8$^+$ T cells, a significant increase of monocytes was detected in the blood following MCMV infection. More specifically, the number of CCR2$^+$ subset was higher than the vehicle group, indicating active monocyte emigration from the bone marrow. This is in line with previous studies that have reported an increased number of CCR2$^+$ monocytes in the blood following MCMV infection (Shi and Pamer, 2011). It is known that MCMV encodes the pro-inflammatory factor (MCK-2) (Saederup et al., 2001), analog of chemokine CCL2 which enhances monocyte recruitment and viral dissemination (Noda et al., 2006). Thus, we assumed that CCR2$^+$ monocytes could be attracted by MCK-2 derived from MCMV in the circulation, emigrating from bone marrow to the blood. Same as seen in CD8$^+$ T cells, the number of macrophages was increased, between days 8 and 14 post-infection, in the nerves of infected mice which could be derived, at least in part, from an enhanced recruitment from blood monocytes. Nerve macrophages not only contribute to the innate immunity of the nervous system, they are also key player in acquired immune responses through their interactions with T cells which enter the nervous system during infection or inflammation (Nguyen et al., 2002). Particularly, nerve macrophages in these MCMV infected mice have an upregulation of MHC-I expression. They also express T cell co-stimulation factor, CD86. Surrounded by MCMV activated-CD8$^+$ T cells, in the presence of several pro-inflammatory cytokines, IFN-γ, TNFα, IL-1β, and IL-6, these nerve macrophages are equipped to serve as a source of antigen presentation and cross presentation.

Thus, several lines of evidence clearly indicated that following primary systemic MCMV infection, in the acute phase (within 2 weeks post-infection), the virus was disseminated into the PNS, but not the CNS. Peripheral nerves were inflamed with immune cell infiltration, increased expression of cytokines and increased number of capillaries. Although it remains elusive how MCMV invades peripheral nerves and from where local inflammation is initiated, and the possibility that CMV has specific tropism towards nerves cannot be excluded, we assume that dorsal root ganglion (DRG) could eventually serve as the entry point. While direct entry of circulating components into the nerves could be restricted by blood nerve barrier, this structure is virtually absent in DRG. In other words, DRG are physiologically permeable to various immune cells/pathogens in the circulation. This assumption was endorsed by the fact that at acute phase, only transient sensory but not motor dysfunction was observed, seeing that sensory neuron cell bodies are in the DRG while motor neurons are in the spinal cord ventral horns. Indeed, MCMV infection and associated inflammation are important contributor to observed cold hypersensitivity. Various pro-inflammatory cytokines are known to increase sensory neuron excitability and enhance pain response (Schafers and Sorkin, 2008). We have reported similar phenomenon in our previous study of autoimmune peripheral neuropathy. In B7.2 transgenic mice, infiltration of CD8$^+$ T cells and macrophages in DRG occurred prior to their entry into the nerves and preceded disease onset (Yang et al., 2014). Blocking inflammatory reaction rescued abnormal sensory response (Yang et al., 2018). In addition, Inflammation and angiogenesis are two processes dependent on each other and are mutually regulated (Szade et al., 2015). Cytokine IFNγ can polarize macrophages and boost angiogenesis (Li et al., 2018). In addition, TNFα has pro-angiogenic function in post-ischemic neovascularization (Goukassian et al., 2007). Increased

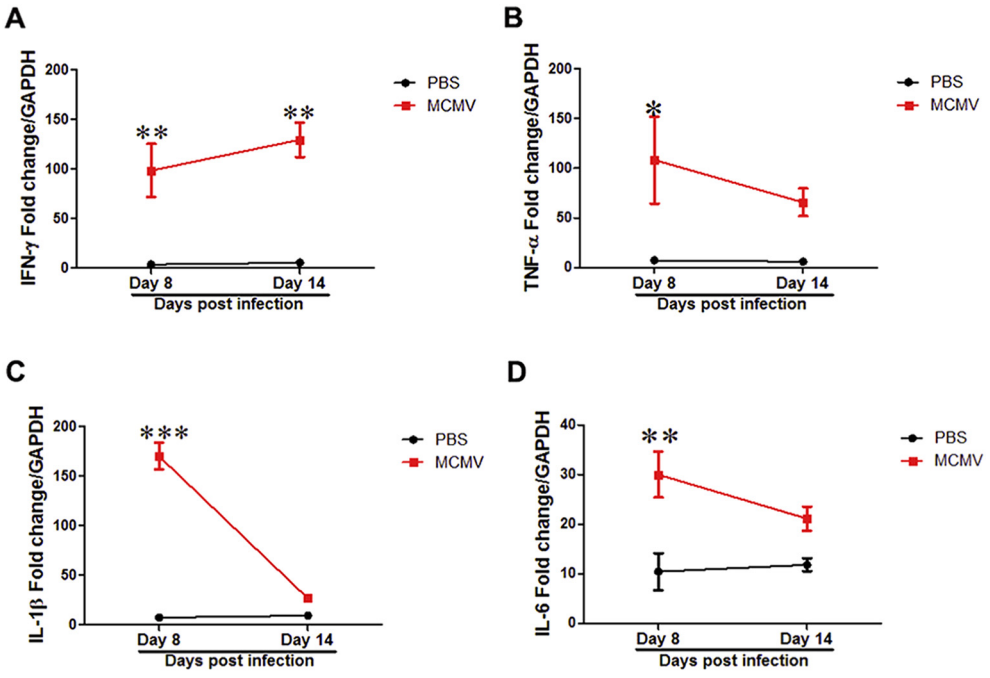

Fig. 4. Enhanced secretion of proinflammatory cytokines in the sciatic nerve following MCMV infection. Plots of cytokine mRNA expression of IFNγ (A), TNFα (B), IL-Iβ (C), and IL-6 (D) at days 8 and 14 following infection. Increased mRNA levels of all cytokines were observed at day 8. IFNγ mRNA level further increased at day 14. TNFα and IL-6 mRNA decreased at day 14, although still higher than in control mice at day 14. IL-Iβ mRNA level in infected group dropped to the level of vehicle group at day 14. n = 3–5/group; $p < 0.05$.

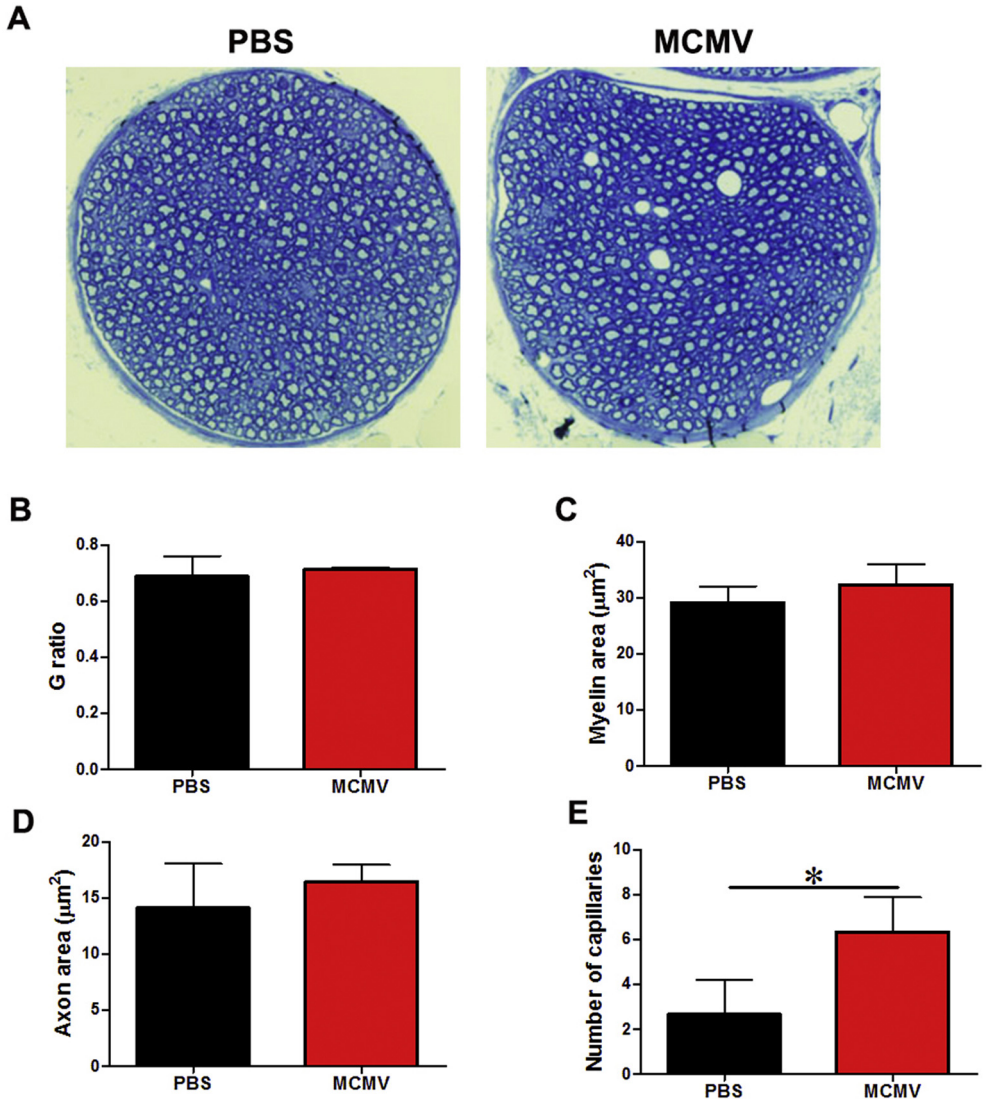

Fig. 5. Preserved axonal and myelin integrity in MCMV infected mice. (A): Representative micrographs of the sciatic nerve cross-sections of 14 days post-MCMV infected mice and control mice. (B): G ratio which reflects nerve conduction properties remained same in both study groups. (C): Quantification of myelin area revealed similar areas in both groups. (D): There was no difference of axon area in MCMV-infected and vehicle groups. (E): The number and size of capillaries in sciatic nerve of infected mice were significantly higher than those in vehicle group. n = 3/group; $p < 0.05$.

number of capillaries could in turn facilitate immune cell recruitment and amplify local inflammation. Nevertheless, histology analysis confirmed no apparent nerve tissue damage at this stage. Structures of both axons and myelin are not really affected by viral infection. Although the dynamic of MCMV in peripheral nerves, e.g., when the virus will be cleared from the PNS and what the $CD8^+$ T cell/macrophage response will be at later stage, remains to be determined, to the best of our knowledge, this is the first report showing that following systemic infection, MCMV attacks peripheral nerves and triggers a local inflammatory reaction whilst the CNS remains minimally affected.

Epidemiological study reported a close association of CMV infection with GBS (Kuwabara, 2004), however, underlying mechanisms are poorly understood, since no direct link between PNS and viral antigen has been established. Few clinical studies have explored CMV-GBS cases and reported the potential roles of anti-ganglioside antibodies and cellular immune responses. Anti-GM_2 antibodies have been detected in approximately 1/3rd of CMV-GBS cases (Orlikowski et al., 2011; Khalili-Shirazi et al., 1999). We may speculate that antibodies to ganglioside-like molecules on virus-infected cells could cross-react with axonal or Schwann cell membranes and play an important role in CMV-GBS pathogenesis. However, these antibodies were also found positive in CMV infections without GBS (Lunn and Hughes, 2011). Although largely overlooked, cellular immune response, especially $CD8^+$ T cell-mediated viral immunity, inevitably has a role in the pathogenesis of CMV-GBS. $CD8^+$ T cells were found to out-number CD4 T^+ cells at lesion sites of GBS patients (Wanschitz et al., 2003). Abnormally high percentages of circulating $CD8^+$ lymphocytes have also been reported in CMV-GBS cases (Eckhart et al., 1997). Similarly, a higher concentration of adhesion molecules associated with T cell activation and migration have been reported in CMV-GBS patients when compared with C. jejuni-GBS (Hadden et al., 2001). It has been well established that in response to HCMV and MCMV infection, CMV-specific $CD8^+$ T cells undergo “memory inflation”, accumulating at high frequencies in the memory phase with a characteristic effector/memory phenotype (Klenerman and Oxenius, 2016; Snyder et al., 2008, 2011). Although to this day no clinical evidence is available to support the functional role of these cells in autoimmune peripheral neuropathy, our previous preclinical study (Yang et al., 2018) clearly indicated the crucial roles of effector/memory $CD8^+$ T cells in initiating inflammatory peripheral neuropathy. We have demonstrated that $CD8^+$ T cells in B7.2 transgenic mice which develop spontaneous autoimmune peripheral neuropathy exhibit effector/memory phenotype (Yang et al., 2018), which

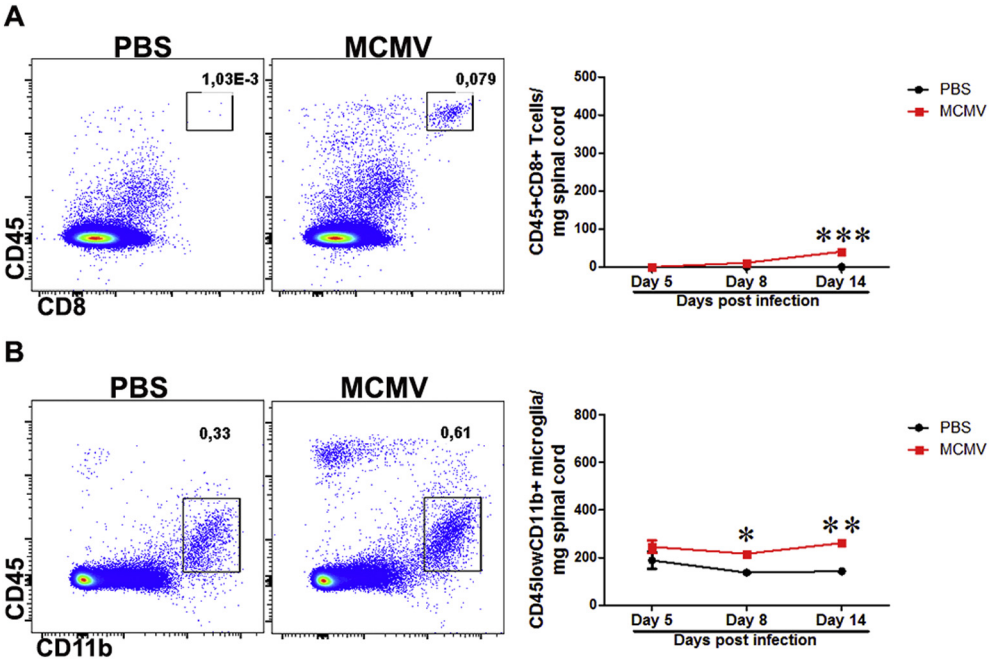

Fig. 6. Response of CD8$^+$ T cells and microglia in the spinal cord following acute MCMV infection. (A) No CD8$^+$ T cells were detected in the spinal cord of MCMV infected mice at day 5–8 post-infection, only very few CD8$^+$ T cells were found at day 14. (B) There was a slight, but significant increase on the number of spinal microglia in MCMV infected mice at day 8–14 post-infection. n = 3–4/group; $p < 0.05$.

bears resemblance to CMV infection triggered memory inflation. These cells were found accumulating abundantly in diseased peripheral nerves. Inhibiting CD8$^+$ T cell activation prevented the disease onset. Together with activated B7.2 expressing nerve macrophages, these CD8$^+$ T cells are required and pathogenic in developing autoimmune peripheral neuropathy in mice (Yang et al., 2018). Due to some technical limitation, the current study did not identify whether these activated CD8$^+$ T cells are MCMV specific, whether viral infection triggered nerve inflammation is autoimmune. At this point, the data is not sufficient to mechanistically link this acute inflammatory neuropathy to GBS. However, it provides important message on the immediate response of PNS to MCMV infection. It also paves the path for future investigation to determine whether such MCMV triggered unspecific inflammatory peripheral neuropathy contributes to the development of GBS at chronic stage.

In summing up, we report here that MCMV infection attacks peripheral nerves and triggers local inflammatory reaction in the acute phase, resulting in transient sensory dysfunction in mice without however damaging nerve structure. We expect that our findings will open avenues for further investigation on whether CD8$^+$ T cells could have the potential to act as a functional link between viral infection and autoimmune peripheral neuropathy through an interaction with co-stimulatory competent macrophages.

Author's contributions

OO, MY, VAGR, XQS and JMH performed experiments; OO, MY, SF and JZ designed the study; OO and JZ wrote the manuscript. All authors read and approved the manuscript.

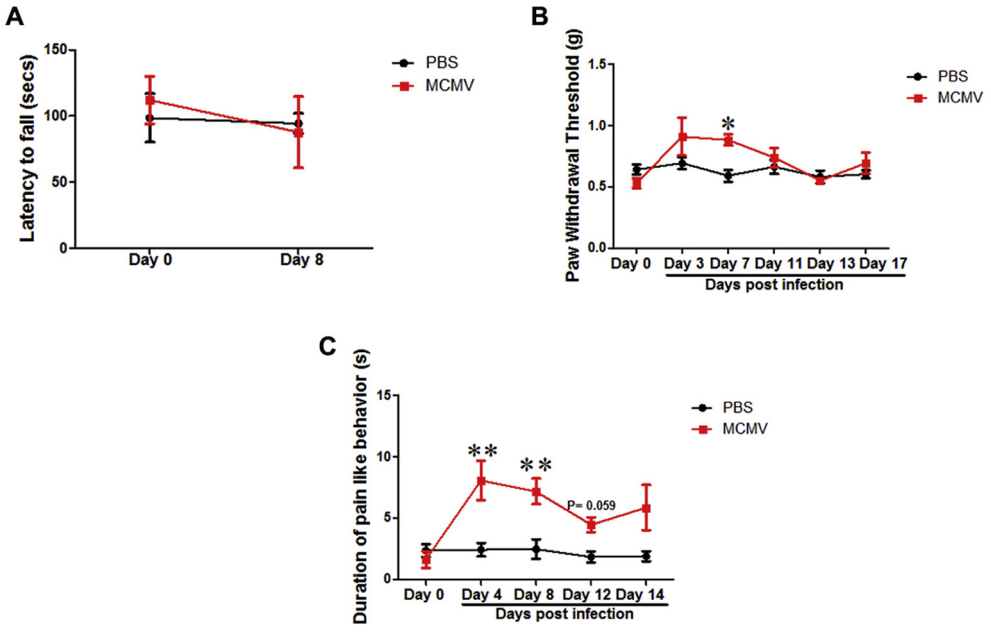

Fig. 7. Transient abnormal sensory behavior in MCMV infected mice. (A): Mice from MCMV infected and vehicle group exhibited same latency on rotarod test (day 8), indicating no overt motor deficits. (B): MCMV infected mice showed an increased paw withdrawal threshold in von Frey test at day 3–7 post-infection, which was reversed to normal afterwards. (C): MCMV infected mice were more sensitive to cold stimuli, as acetone-induced pain like behavior lasted longer in MCMV infected mice than in vehicle mice, from day 4-day 12 post-infection. n = 4–7/group; $p < 0.05$.

Declaration of Competing Interest

The authors declare no competing interests.

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