LATENT MURINE CYTOMEGALOVIRUS INFECTION CONTRIBUTES TO EAE PATHOGENESIS

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LATENTNA INFEKCIJA MIŠJIM CITOMEGALOVIRUSOM IMA ULOGU U PATOGENEZI EKSPERIMENTALNOG AUTOIMUNSKOG ENCEFALOMIJELITISA

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ABSTRACT

Viral infection has been identified as the most likely environmental trigger of multiple sclerosis (MS). There are conflicting data regarding the role of cytomegalovirus (CMV) in MS pathogenesis.

We utilised experimental autoimmune encephalomyelitis (EAE)-resistant BALB/c mice and murine cytomegalovirus (MCMV), the murine homolog of CMV, to examine the mechanism by which viral infection enhances autoimmune neuroinflammation. Mice subjected to latent neonatal MCMV infection developed the typical characteristics of EAE. Similar to MS, the MCMV-infected EAE-induced mice developed infiltrates in the central nervous system (CNS) composed of similar percentages of CD4+ and CD8+ T cells. The influx of both Th1 and Th17 cells into the CNS of MCMV-infected EAE-induced mice was observed. Interestingly, the development of autoimmune neuroinflammation after latent MCMV infection was accompanied by a significant influx of Tc17 cells (CD8+IL-17+ and CD8+RoRγt+) but not Tc1, cells. Our results suggest that latent MCMV infection affects the development of inflammatory lymphocytes that exhibit encephalitogenic potential, thereby mediating increased CNS pathology following EAE induction, and that CMV represents a possible environmental factor in the pathogenesis of MS and other autoimmune diseases.

Key words: EAE, viral infection, CMV, BALB/c mice

SAŽETAK

Virusna infekcija se navodi kao najverovatniji faktor okoline koji utiče na razvoj multiple skleroze (MS). Postoje konfliktni podaci o ulozi infekcije citomegalovirusom (CMV) u patogenezi multiple skleroze. Koristili smo BALB/c miševe, rezistentne na indukciju eksperimentalnog autoimunskog encefalomijelitisa (EAE), i mišji citomegalovirus (MCMV), mišji homolog humanom citomegalovirusu da ispitamo kako virusna infekcija može da utiče na razvoj autoimunske neuroinflamacije. Miševi sa latentnom neonatalnom infekcijom mišjim citomegalovirusom su razvili tipičan EAE. Slično kao u MS, MCMV EAE miševi su razvili infiltrate u centralnom nervnom sistemu (CNS) sa sličnom zastupljenošću CD4+ i CD8+ T limfocita. Uočen je influks i Th1 i Th17 ćelija u CNS MCMV EAE miševa. Interesantno je da razvoj autoimunske inflamacije nakon latentne MCMV infekcije prati značajan influks samo Tc17 (CD8+IL-17+ i CD8+RoRγt+), a ne i Tc1 ćelija. Naši rezultati ukazuju da latentna MCMV infekcija verovatno utiče na razvoj inflamatornih limfocita koji mogu da indukuju autoimunski proces u CNS-u, direktno pojačava razvoj patoloških procesa u CNS-u nakon indukcije EAE i ukazuje na CMV kao na mogući faktor okoline koji utiče na razvoj multiple skleroze i drugih autoimunskih bolesti.

Ključne reči: EAE, virusna infekcija, CMV, BALB/c miševi

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory de-myelinating disease of the central nervous system (CNS) that is characterised by varied clinical courses, pathologies, and inflammatory patterns (1). Experimental autoimmune encephalomyelitis (EAE) is an experimental model of multiple sclerosis that is induced in susceptible animals via active immunisation with myelin antigens mixed with an adjuvant. MS is a multifactorial disease that develops in susceptible hosts after introduction to environmental factors which trigger MS by promoting the activation of my-elin-specific T cells that typically circulate in the periphery (2). The most important environmental factor considered to play a causal role in MS pathogenesis is infection (2). It has been postulated that in susceptible individuals, alterations in the mechanisms regulating the immune response to viruses may contribute to MS pathogenesis. Among the various infective agents, Epstein–Barr virus (EBV) has been the most strongly associated with increased MS risk (2). It was shown that EBV infection polarises the adaptive immune response and heightens CNS pathology following EAE induction and likely influences MS pathogenesis (3). Additionally, an elevated CD8+ T cell response to EBV lytic antigens has recently been detected in active MS and during relapses (4).

Cytomegalovirus (CMV), which is classified into the Betherpesvirinae subfamily, is a species-specific herpes virus that establishes a life-long infection in its hosts. The CMV virion contains a double-stranded DNA viral genome whose latent and lytic types of genes encode approximately 230–250 proteins, many of which play immune-regulatory roles (5). CMV initially infects epithelial cells, and after cell-associated viremia, the virus infects different cells, including fibroblasts, epithelial cells, endothelial cells, and smooth muscle cells (6). CMV persists in myeloid precursor cells, from CD34+ pluripotent stem cells to CD14+ monocytes, resulting in the latent infection of these cells (7). When these cells subsequently enter the visceral parenchyma and differentiate into macrophages or myeloid dendritic cells, the latent virus reactivates into the lytic phase, which activates T cell-mediated immunity to suppress the infection, indicating that CMV infection modulates the immune response of the host.

However, studies investigating the association between CMV and MS have been inconclusive due to conflicting findings of both a protective and harmful influence of CMV on MS, likely in part as a consequence of small sample sizes. A recent well-powered meta-analysis found no significant difference in the rate of CMV seropositivity between MS patients and healthy controls based on pooled samples from all studies to date (8). However, some evidence for a protective effect of CMV infection on MS risk was found when only prospective studies were included in the analysis. However, all of these studies contain limitations, as they did not assess the temporal relationship between CMV infection and MS onset or the influence of CMV infection at specific time points on the MS risk. In all of these studies, CMV infection was confirmed only by detecting anti-CMV antibodies without any data demonstrating the presence of CMV DNA in the cells, the expression of lytic or latent viral genes or the temporal changes in their expression during different phases of MS.

We have recently reported that BALB/c mice, which are widely accepted as resistant to EAE induction using the peptide MOG$_{35-55,}$ developed EAE when ST2 signalling was blocked (9, 10, 11, 12). In this study, we used newborn BALB/c mice subjected to infection with MCMV to explore possible effect of latent CMV infection on EAE development.

MATERIALS AND METHODS

Infection, Induction and Scoring of EAE

Female 6- to 8-week-old BALB/c mice were used throughout this study. New-born mice 6 to 12 h post-partum were inoculated i. p. with 200 PFU of wild-type MCMV (MW97. 01 strain) or 200 µl of phosphate buffered saline (PBS) as a control. EAE was induced via sub-cutaneous administration of 200 µL of a suspension at 2 sites above the hind flanks. The suspension consisted of 300 µg of the peptide MOG$_{35-55}$ (Sigma Aldrich, Germany) in 100 µL of PBS emulsified with 100 µL of complete Freund`s adjuvant (Sigma Aldrich, Germany) containing 0.7 mg of heat-inactivated *Mycobacterium tuberculosis* (strain H37 RA; Difco Laboratories, Detroit, MI). Each mouse was immediately injected intraperitoneally and 48 hours later with 300 ng of pertussis toxin (List Biological Laboratories, Campbell, USA) in 100 µL of 0.9% NaCl. Clinical signs of EAE were assessed daily using the following scoring system as previously described: grade 0, no signs; grade 1, paralysed tail; grade 2, ataxic; grade 2.5, one hind leg paralysed; grade 3, both hind legs paralysed; grade 3.5, 3 legs paralysed; grade 4, both hind legs completely paralysed and mild front limb paralysis; and grade 5, moribund (13). The mice were monitored daily and provided with administered fluids and mashed chow at the base of the cage for all mice displaying a clinical score of 3. The mice were maintained at our animal facilities in a temperature-controlled environment under a 12-hour light/12-hour dark cycle and were provided with standard laboratory food and water *ad libitum.* All experiments were approved by and conducted in accordance with the guidelines of the Animal Ethics Committee of the Faculty of Medicine at the University of Kragujevac in Serbia.

Isolation of Mononuclear Cells from the CNS

At day 15 post-EAE induction (mean clinical score of 3 for the MCMV-infected EAE-induced mice), the mice were perfused with PBS, and the brain and the spinal cord were carefully removed. The mononuclear cells from the CNS were isolated as described previously (14). Briefly, the brains and spinal cords were separately homogenised

in RPMI 1640 (Sigma Aldrich) containing 10% FBS and 1 mg/ml collagenase type I (Sigma-Aldrich) and incubated at 37°C for 60 min. After digestion, the tissue was passed through a 70 mm mesh filter, pelleted, resuspended in 10 ml of 30% Percoll (Sigma-Aldrich), overlaid onto 5 ml of 70% Percoll and centrifuged at 390 g for 20 min. The myelin layer was removed, and the mononuclear cells, which accumulated in the intermediate phase, were collected, washed twice in PBS and resuspended in medium. The total cell numbers were determined by counting using a haemocytometer, and cell viability was assessed based on Trypan blue exclusion.

Flow Cytometry

For cytofluorometry, fluorochrome-conjugated antibodies against the following proteins were used: CD4, CD8, CD45, CCR6, CXCR3, T-bet, RoRγt, IL-17, IFN-γ, and TNF-α (BD Biosciences). The cells were incubated in the antibodies in PBS containing 2% FBS for 30 min at 4°C, followed by analysis. For intracellular staining of cytokines, the cells were stimulated for 4 hours in RPMI 1640 containing 10% FBS (Gibco), GolgiPlug (BD Biosciences), 10 ng/ml PMA and 500 ng/ml ionomycin. The antibodies for the cell surface markers were added to the cells in PBS containing 2% FBS for 30 min on ice. After washing, the cells were resuspended in Fix/Perm buffer (eBiosciences) for 30–45 min on ice, washed twice and incubated in the Abs for the intracellular antigens (cytokines) in Perm buffer (for 30 min on ice). For staining of transcription factors, unstimulated cells were used. The data were acquired using a FACSCalibur (BD Biosciences) and were analysed using FlowJo software (Tree Star).

Statistical Analysis

All statistical calculations were performed using SPSS 13. 0 for Windows software. The results were analysed using Student's t and the Mann-Whitney U test. The data in this study were expressed as the means+SD or the means+SEM. Values of p<0.05 were considered to be significant.

RESULTS

MCMV Infection of Neonatal BALB/c Mice Facilitates the Development of EAE

We have previously shown that BALB/c mice, which are resistant to EAE induction using the peptide MOG_{35-55} (11, 12), develop EAE in the absence of ST2 signalling. Because it was shown that altering the immune response modulates the susceptibility to EAE, we aimed to explore the role of latent viral infection in EAE pathogenesis. To study the possible effect of viral infection on EAE, we analysed the clinical characteristics of EAE in BALB/c mice neonatally infected with MCMV (Fig. 1). The absence of clinical signs of EAE in these BALB/c mice was consistent with the minimal total cell number isolated from the
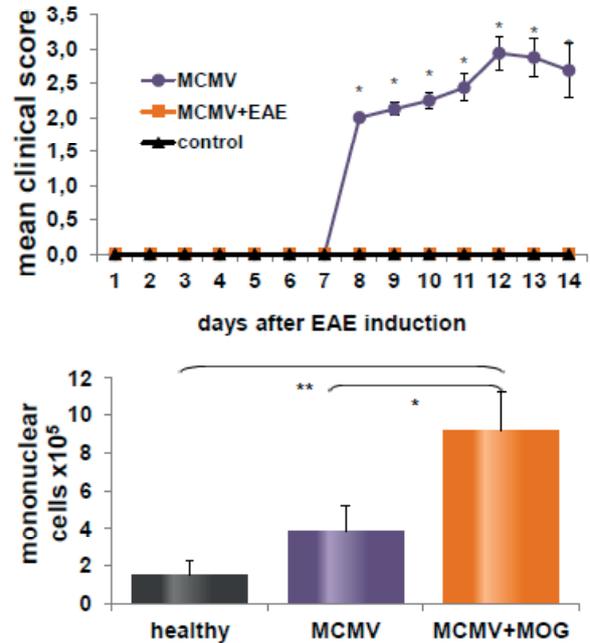

Figure 1. BALB/c mice subjected to neonatal MCMV infection are susceptible to EAE.
BALB/c mice and BALB/c mice subjected to neonatal MCMV infection were immunised with MOG_{35-55}/CFA and were monitored daily for clinical signs of EAE. The data presented were obtained from representative experiment that included eight mice per group (means ± SEM). At the peak of EAE mononuclear cells from the spinal cord and the brain tissues were isolated, and the cells were counted after exclusion of dead cells, which were stained with Trypan blue. The total number of isolated mononuclear cells is presented as the mean ± SD per group, *P<0. 05; **P<0. 005. Significance was assessed using Student's t-test.

CNS of these mice, in contrast to significant cell infiltration into the CNS of MCMV-infected BALB/c mice. The BALB/c mice subjected to neonatal MCMV infection but not to MOG35-55 immunisation did not exhibit any signs of EAE but displayed a higher cell number isolated from the CNS than the BALB/c mice subjected to neonatal MCMV infection without MOG_{35-55} immunisation.

BALB/c Mice Subjected to Neonatal MCMV Infection and EAE Display a Similar Percentage of Infiltrating CD4+ and CD8+ Lymphocytes in the CNS

To characterise the event at the level of the target tissue, we compared the cellular composition of the mononuclear cells in the three groups of mice (Fig. 2). Flow cytometric analysis revealed a significantly higher percentage of infiltrating CD45+CD4+ and CD45+CD8+ T lymphocytes in the CNS of MCMV-infected EAE-induced BALB/c mice compared with untreated BALB/c mice and with BALB/c mice subjected to neonatal CMV infection alone. Analysis of the expression of Th1- and Th17-related chemokine receptors (CXCR3 and CCR6) revealed significantly increased influx of CD4+CXCR3+ and CD4+CCR6+ T lymphocytes in the CNS of MCMV-infected EAE-induced
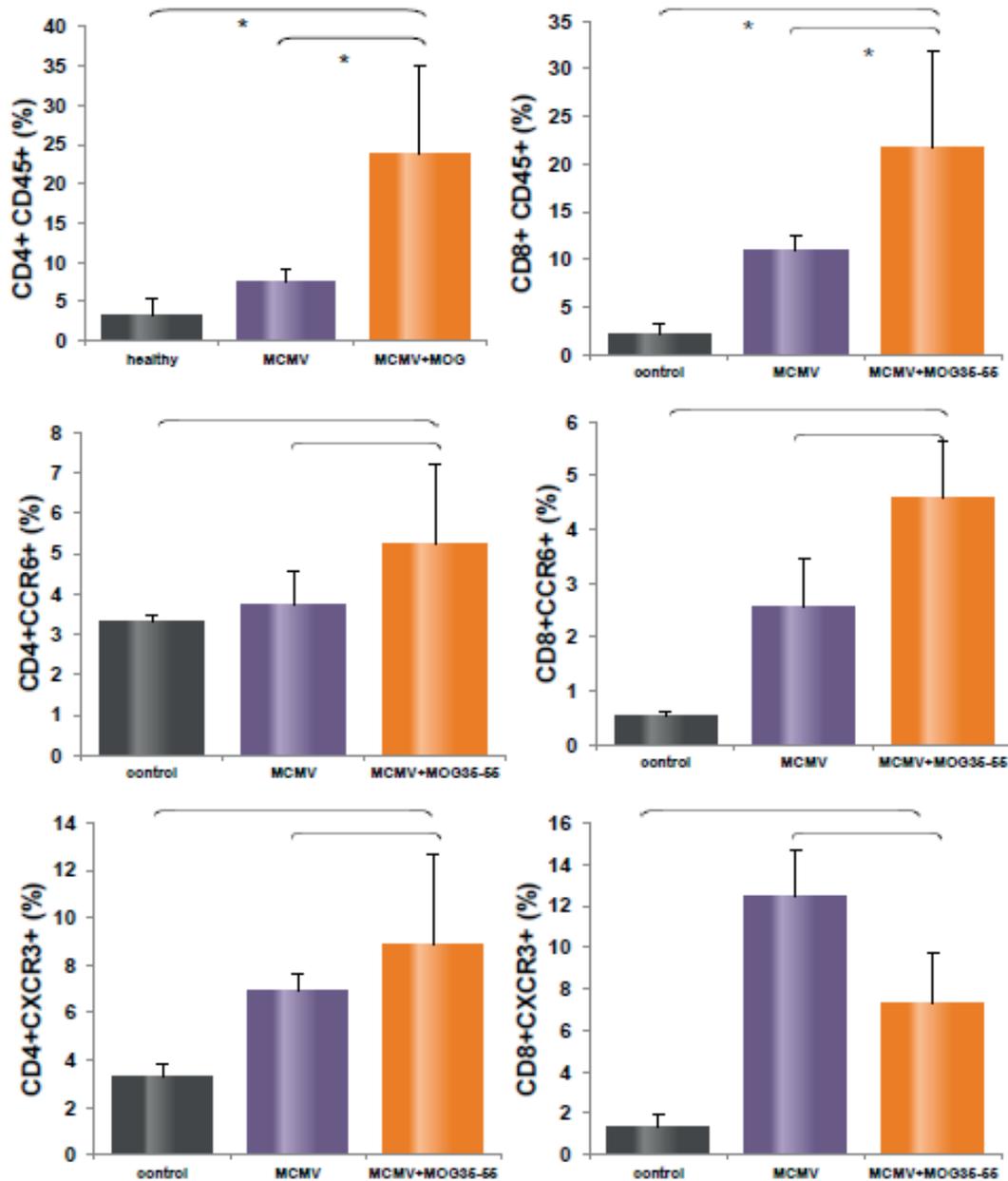
Figure 2. MCMV-infected EAE-induced mice display a similar percentage of CD4+ and CD8+ cells in the CNS.
Fifteen days after immunisation, mononuclear cells were isolated from the spinal cords and the brains and were used for flow cytometric analysis of the percentages of CD45+CD4+, CD45+CD8+, CD4+CCR6+, CD4+CXCR3+, CD8+CCR6+ and CD8+CXCR3+ cells. The data presented were obtained from a representative experiment (means+SD; *P<0. 05; **P<0. 005). Significance was assessed using Student's t-test.

mice compared with untreated and MCMV-infected mice. However, the influx of CD8+ T lymphocytes expressing Th1-related chemokine receptors was significantly higher in the MCMV-infected mice than in the MCMV-infected EAE-induced mice, whereas the percentage of CD8+ T cells expressing the Th17-related chemokine receptors was significantly higher in the EAE-induced MCMV-infected mice (Fig. 2).

EAE Mice Subjected to Neonatal MCMV Infection Display an Increased Percentage of Infiltrating Tc17 Cells in the CNS

Further, we assessed the effects of viral infection on the influx of proinflammatory CD4+ and CD8+ T cells into the nervous tissue. Therefore, we quantified the number of Tbet- and RoRγt-expressing and IL-17-, IFN-γ-, and TNF-α-producing CD4+ T and CD8+ T cells that infiltrated

into the CNS at the peak of the disease. Flow cytometric analysis of the CNS mononuclear cells revealed that inflammatory CD4+ and CD8+ cells were nearly absent from the CNS of the BALB/c mice. Influx of Th1 and Th17 cells was significant only in the EAE-induced MCMV-infected BALB/c mice. The percentage of CD4+ T cells expressing Th1- (IFN-γ and TNF-α) and Th17-related cytokines (IL-17) and expressing Th1- (Tbet) and Th17-related transcription factors (RoRγt) was significantly higher in the MCMV-infected EAE-induced mice compared with the MCMV-infected and untreated mice. The influx of Tc1 cells (CD8+ cells expressing IFN-γ, TNF-α and Tbet) into the CNS was significant in the MCMV-infected mice . The percentages of Tc1 cells were slightly lower in the MCMV-infected EAE-induced mice than in the MCMV-infected mice. However, the percentages of Tc17 cells (CD8+ cells expressing RoRγt and IL-17) in the CNS were significantly higher in the MCMV-infected EAE-induced mice than in the MCMV-infected and untreated mice. Moreover, there were hardly very few Tc17 cells in the CNS of the MCMV-infected mice. Collectively, our results indicate significant influx of CD8+IL-17+ and CD8+RoRγt+ cells into the CNS of new-born MCMV-infected BALB/c mice after MOG$_{35-55}$ immunisation.

DISSCUSION

A very important difference between MS and EAE is the equivalent level of CD8+ and CD4+ T cell infiltration in MS plaques in contrast to the predominance of infiltrating CD4+ T cells in the CNS of mice with EAE (15). Analysis of the antigen receptor expression patterns of CD8+ T cells in the CNS infiltrates in MS suggests the local activation of antigen-induced immune responses (16). There is the lack of experimental models that are suitable to study the role of CD8+ cells in CNS autoimmunity. Recently, it was shown that latent EBV infection exacerbates EAE signs in mice and induces the infiltration of CD8+IFN-γ+granzyme+ cells into the brain parenchyma (3). In this model of EAE, no viral DNA was detected in the CNS (based on PCR) during EAE, and the virus was hypothesised to indirectly influence the autoimmune response. CMV shares homology with EBV and induces latent infections that can subsequently become reactivated, resulting in different consequences.

We demonstrated that latent neonatal infection with MCMV alters the susceptibility of BALB/c mice to EAE, leading to the equivalent infiltration of CD4+ and CD8+ T cells into the CNS after EAE induction, which activates a significant Tc17-mediated immune response in the CNS that is accompanied by typical EAE paralysis.

In our model of neonatal MCMV infection, the mice develop histopathological lesions that are characteristic of meningoencephalitis. Histopathological lesions are composed of infected cells associated with infiltrating mono- and polymorpho-nuclear leukocytes or activated resident microglia (17, 18). This cell infiltration persists in the brain even after the termination of productive viral infection, when the replicating virus is no longer detected in the brain (19). The predominant immune cell population in MCMV-infected new-born brains is CD8+ T lymphocytes, and these cells persist in the CNS after the resolution of acute infection (20). Viral DNA persists in the mononuclear cells outside the CNS.

However, the MCMV-infected EAE-induced mice displayed significantly higher cell infiltration in the CNS than age-matched mice subjected to neonatal MCMV infection alone. EAE induction in the MCMV-infected mice was followed by the significant influx of CD4+ T cells and, importantly, CD8+ T cells. We detected the influx of CD4+ T cells expressing CXCR3, the chemokine receptor for CXCL10, and CD4+ T cells expressing CCR6, a receptor that is known to play a key role in the initial development of autoimmune infiltration into the CNS (21, 22). On the other hand, EAE induction in mice subjected to latent MCMV infection increased the influx of CD8+CCR6+ T cells and decreased the influx CD8+CXCR3+ T cells compared with MCMV infection alone. MCMV infection is known to attract CD8+CXCR3+ cells, and it appears that the newly developing autoimmune process attracts a different population of CD8+ cells (23). This result indicates that latent MCMV infection of BALB/c mice in the periphery affects the immune response to the myelin antigen, driving the immune cells towards an inflammatory phenotype that facilitates the entry of these cells into the CNS and the induction of autoimmune processes. Recently, it was reported that MCMV infection of murine fibroblasts altered the expression of 10748 genes (24). Among the most strongly induced genes were those corresponding to Interferon-β, the transcription factor T-bet, and the chemokine CXCL10. The expression levels of all proteins examined correlated with their transcript levels. The role of the chemokine CXCL10 and the transcription factor Tbet, which are markers of Th1 cells, in EAE pathogenesis is well known (25, 26, 27). Immune challenge (immunisation with MOG$_{35-55}$) is assumed to reactivate MCMV infection, leading to the expression of genes whose products affect the polarisation of the adaptive immune response.

We also found that the CNS infiltrates of mice subjected to latent MCMV infection followed by EAE induction contained a significantly higher percentage of Th1, Th17, and Tc17 cells compared with mice subjected to MCMV infection alone. No increase was detected in the percentage of CD8+ cells expressing Tbet and the Th1-related cytokines TNF-α and IFN-γ in the CNS with autoimmune process developed after viral infection compared with mice with new-born MCMV infection. Autoimmune neuroinflammation clearly developed after previous MCMV infection altered the dominant population of CD8+ cells in the CNS. Tc17 cells (IL-17- and RoRγt-expressing CD8+ cells) are required for Th17 accumulation and for the development of EAE (28). Patients with
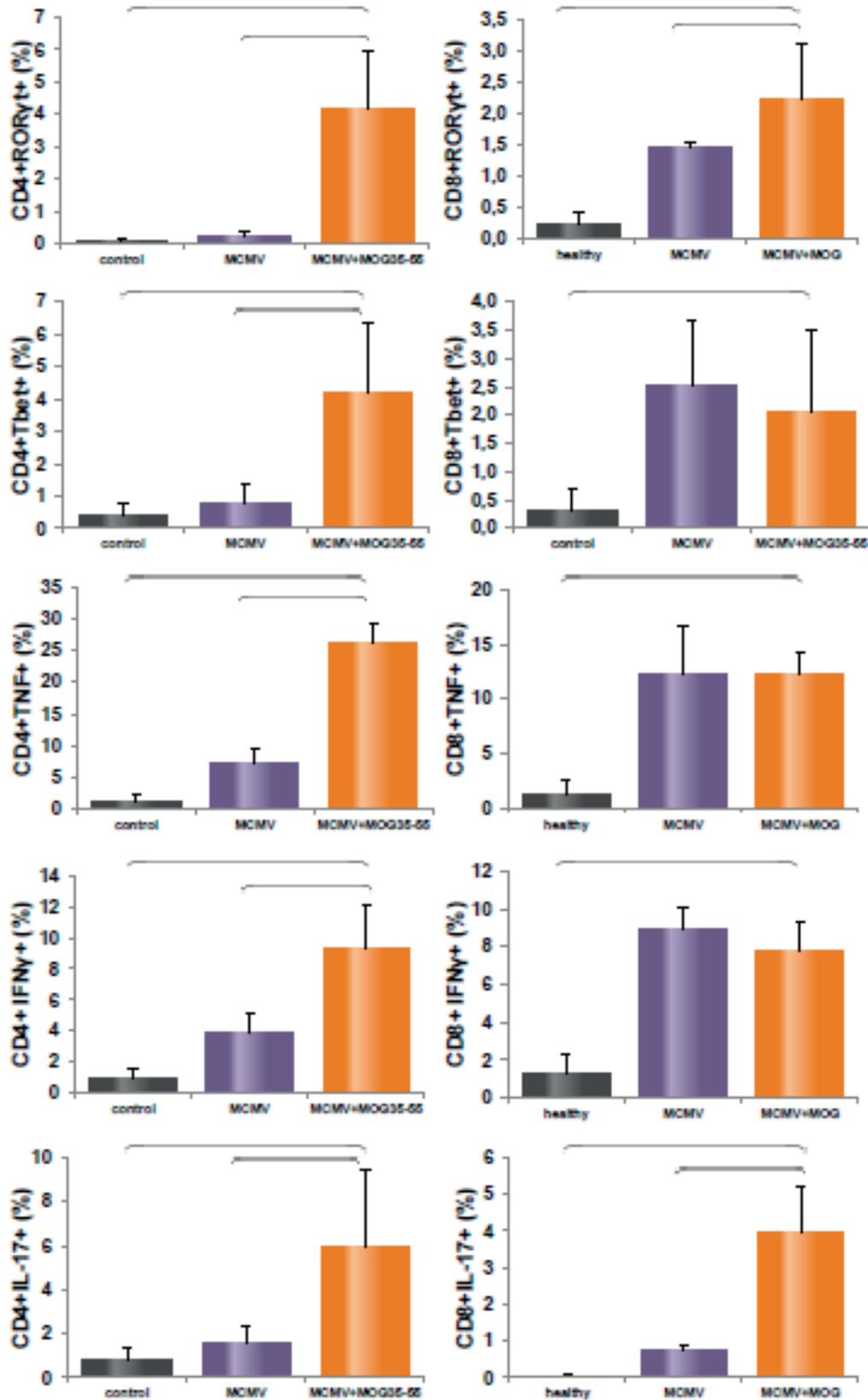
Figure 3. Influx of Tc17 cells following the induction of neuroinflammation in MCMV-infected mice.
Fifteen days after immunisation, mononuclear cells were isolated from the spinal cords and the brains and were used for flow cytometric analysis of the percentages of CD4+Tbet+, CD4+CDRoRγt+ , CD4+TNF-α+, CD4+IFN-γ+, CD4+IL-17+, CD8+Tbet+, CD8+CDRoRγt+, CD8+TNF-α+, CD8+IFN-γ+, and CD8+IL-17+ cells. The data presented were from a representative experiment (means+SD; *P<0. 05; **P<0. 005). Significance was assessed using Student's t-test.

early-stage MS harbour a greater number of Tc17 cells in the cerebrospinal fluid than in the peripheral blood. Tc17 cells contribute to the initiation of CNS autoimmunity by supporting Th17 cell pathogenicity. Our results indicate that latent MCMV infection contributes to the expansion of this subpopulation of CD8+ cells, which is known to play a role in autoimmune neuroinflammation, and the influx of these cells into the CNS.

CONCLUSION

Our findings suggest that latent CMV infection affects EAE development and, possibly, MS pathogenesis, likely via its influence on the initial development of inflammatory lymphocytes that display encephalitogenic potential. Furthermore, this model of EAE may be useful for the examination of the role of CD8+ T cells in CNS autoimmunity.

ACKNOWLEDGMENTS

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