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Regulatory T Cells Selectively Preserve Immune Privilege of Self-Antigens during Viral Central Nervous System Infection

Luisa Cervantes-Barragán,*† Sonja Firner,*‡ Ingo Bechmann, † Ari Waisman, ‡ Katharina Lahl, § Tim Sparwasser, ‡ Volker Thiel,*∥ and Burkhard Ludewig*∗

Regulatory T cells (Tregs) are important for the attenuation of immune reactions. During viral CNS infections, however, an indiscriminate maintenance of CNS immune privilege through Treg-mediated negative regulation could prevent autoimmune sequelae but impair the control of viral replication. We analyzed in this study the impact of Tregs on the development of acute viral encephalomyelitis, T cell-mediated antiviral protection, and prevention of CNS autoimmunity following intranasal infection with the gliatropic mouse hepatitis virus strain A59. To assess the contribution of Tregs in vivo, we specifically depleted CD4*Foxp3* T cells in a diphtheria toxin-dependent manner. We found that depletion of Tregs had no impact on viral distribution and clearance and did not significantly alter virus-specific CD4+ and CD8+ T cell responses. However, Treg depletion led to a more severe CNS inflammation associated with neuronal damage. Dissection of the underlying immunopathological mechanisms revealed the elaborate Treg-dependent regulation of self-reactive CD4+ T cell proliferation within the CNS-draining lymph node and downtuning of CXCR3 expression on T cells. Taken together, these results suggest that Tregs preserve CNS immune privilege through selective control of CNS-specific Th cells while keeping protective antiviral immunity fully operative. The Journal of Immunology, 2012, 188: 3678–3685.

Viral infections have been implicated in a variety of autoimmune diseases, including immune-mediated demyelinating disorders such as multiple sclerosis (1, 2). A number of viruses, including EBV (3), human herpes virus-6 (4), human coronavirus OC-43 (5), and human coronavirus 229E (6), have been found in the CNS of multiple sclerosis patients. Furthermore, durable immune responses against these viruses have been detected in affected individuals (4, 7, 8). Infection with neurotropic viruses may not only induce specific antiviral immune responses in the CNS, but also most likely predisposes to a stereotyped immunopathological tissue response that precipitates de novo immune reactions against genuine self-antigens (2, 9). Thus, damage control mechanisms in organs with poor regenerative capacity, such as the CNS, have to guarantee efficient control of the pathogen and, at the same time, should attenuate immunopathological bystander and autoimmune reactions. In other words, dedicated regulatory circuits should establish a relative immune privilege in the CNS (10) that shields self-determinants from overshooting immune responses. The delicate balance between T cell-mediated immunopathology/autoimmunity and pathogen control is maintained by different layers of negative regulatory processes including T cell-intrinsic inhibitory signaling pathways (11) or, more global, T cell-extrinsic processes such as distinct regulatory cell populations (12, 13). Regulatory T cells (Tregs) are a subset of CD4+ T cells that can attenuate T cell proliferation and/or function (14). Tregs are characterized by the expression of particular surface molecules including the IL-2Rα (CD25), CTLA-4, and glucocorticoid-induced TNFR and the transcription factor Foxp3 (15). The multiple mechanisms employed by Tregs to downregulate CNS function include secretion of suppressive cytokines or expression of ligands for coinhibitory molecules that either directly act on T cells or inhibit the function of APCs (14, 16). The general immunosuppressive function and hence protective role of Tregs has been demonstrated in various settings of experimental autoimmune diseases such as experimental autoimmune encephalomyelitis (17) or transplant rejection (18). In viral infections, Tregs can attenuate virus-mediated tissue damage through improved innate (19) or CD8+ T cell-mediated (20) virus control. However, in other viral infections, the tradeoff for Treg-reduced immunopathology and attenuated disease severity can be a delay in viral clearance and global attenuation of antiviral T cell responses (21, 22), indicating that the function of Tregs in viral infections is context dependent. It is likely that differential Treg-mediated control of T cell reactivity during viral infection is particularly important in organs in which infection-associated immunopathology and initiation of autoimmune reactions would be most detrimental.

To assess the importance of Tregs during viral CNS infection and clarify the role of Tregs in the initiation of CNS-specific T
cell responses, we used the mouse hepatitis virus (MHV) as a model infection. MHV belongs to the family of coronaviridae and permits studies of natural host–pathogen interactions. CNS infection with MHV causes acute encephalomyelitis and can lead to a chronic disease with demyelination and ascending paralysis (23). To specifically deplete Tregs in vivo, we employed DEREG mice that express the human diphertheria toxin (DT) receptor and the enhanced GFP (eGFP) under the control of the Foxp3 promoter (24). We found that depletion of Tregs did neither alter viral distribution and clearance kinetics, nor did it significantly promote the virus-specific CD4+ and CD8+ T cell response. However, Treg-depleted mice showed a more severe CNS pathology that was due to increased recruitment of T cells that did not respond to the known MHV epitopes. Mechanistic dissection of the Treg-controlled inflammation during coronaviral CNS infection revealed that Tregs control the inflammatory processes mainly in the CNS-draining lymph node by suppressing the proliferation of self-reactive Th cells and by global downtuning of the expression of CXCR3 on both CD8+ and CD4+ T cells in secondary lymphoid organs. Taken together, this study shows that during viral CNS infection, Tregs can selectively control infection-associated autoimmune immune responses while efficient antiviral immunity is maintained.

Materials and Methods

Ethics statement

Experiments were performed in accordance with federal and cantonal guidelines under permission numbers SG09/83 and SG09/87 following review and approval by the Cantonal Veterinary Office (St. Gallen, Switzerland).

Mice and Treg depletion

C57BL/6 (B6) mice were purchased from Charles River Laboratories (Sulzfeld, Germany). Myeloid oligodendocyte glycoprotein (MOG(35–55)-specific CD4 TCR-transgenic mice expressing the allelic marker Thy1.1, (2D2 × Thy1.1) were described previously (25). Depletion of Treg mice (DEREG) was described previously (24). 2D2 and DEREG mice were crossed to obtain double-transgenic 2D2 × DEREG mice. CXCR3−/− mice were obtained from the Institut für Laboratoriumsmedizin (University of Zürich). All mice were maintained in individually ventilated cages and used between 6 and 9 wk of age. To deplete Treg cells, DEREG mice were injected with DT (List Biological Laboratories). Mice were injected i.p. with 1 μg DT every other day over a period of 9 d starting with the first injection 1 d prior MHV AS99 infection. For adoptive transfer experiments, recipients were treated with DT on days 5, 7, and 9 postinfection (p.i.). Control mice (B6) received the same DT treatment as DEREG mice. 2D2 × DEREG donor mice were injected with 1 μg DT 1 d prior to cell isolation.

Virus infections and determination of virus titers

Mice were infected intranasally (i.n.) with 5 × 10^3 PFU or i.p. with 50 PFU MHV AS99 as previously described (26). Mice were sacrificed at the indicated time points, and organs were stored at −70°C until further analysis. MHV titers were determined by standard plaque assay using L929 cells (27). L929 were purchased from the European Collection of Cell Cultures.

Histology

For histological analysis, mice were sacrificed, immediately perfused with PBS (PBS, pH 7.5), and organs were fixed in 4% formaldehyde for at least 12 h and embedded in paraffin. Five-micrometer-thick sections were stained with H&E. Images were acquired using a Leica DMRA microscope (Leica Microsystems) and processed using Adobe Photoshop (Adobe Systems). For immunohistochemical analysis, mice were sacrificed and immediately perfused with PBS, followed by PBS/4% paraformaldehyde (pH 7.5). Organs were further fixed in PBS/4% paraformaldehyde for at least 12 h and embedded in 4% low-melting agarose (USB). Twenty-micrometer sections were cut using a vibratome (Leica VT 1200S; Leica Microsystems). Free-floating sections were permeabilized in PBS/1% Triton X-100 (Sigma-Aldrich) and blocked with PBS/0.1% Triton X-100 and 1% normal goat serum (Brunschwig). Sections were further incubated with anti-human CD3 (1:50; AbD Serotec), followed by a Dyelight 647-labeled anti-rat secondary Ab (Jackson Immunoresearch Laboratories). Nuclei were stained with DAPI (Sigma-Aldrich), and sections were mounted with fluorescence mounting solution (DakoCytomation). Images were acquired using Zeiss LSM210 microscope (Zeiss) and processed using ZEN software (Zeiss) and Adobe PhotoShop (Adobe Systems). Vessel widths were determined by measuring the perpendicular diameter of cross-sectioned infiltrated vessels including the infiltrates using the IM1000 software (Leica Microsystems).

Cell isolation and flow cytometry

Mice were sacrificed and immediately perfused with PBS. Single-cell suspensions from spleen and cervical lymph nodes (CLNs) were prepared by mechanical disruption of the organs. Brains were mechanically disrupted, and the leukocytes were further enriched using a 70–30% Percoll gradient (GE Healthcare) and centrifugation for 25 min at 800 × g. Analysis of MHV-specific CD8+ T cell responses was performed using PE-conjugated MHV S998/H-2K^b tetramers (Sanquin, Amsterdam, The Netherlands). For surface staining, the following mAbs were used: PE-labeled anti-CD8, anti-CD4 (BioLegend), allyllophycocyanin-labeled anti-Thy1.1 (eBioscience), anti-CD8, anti-CD4, anti-CXCR3 and, and anti-Ly5.1 (BioLegend). 7-Aminoacinoctymycin D (Calbiochem) was used to discriminate dead cells in flow cytometric analysis. For peptide-specific cytokine production, 10^5 splenocytes were restimulated with S598 or M133 peptides in the presence of brefeldin A (5 μg/ml) for 5 h at 37°C. Cells were stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml; both purchased from Sigma-Aldrich) as positive control or left untreated as a negative control. For intracellular staining, restimulated cells were surface stained and fixed with Cytofix-Cytoperm (BD Biosciences) for 20 min. Fixed cells were incubated at 4°C for 40 min with permeabilization buffer (2% FCS/0.5% saponin/PBS) containing anti-IFN-γ, anti-IL-4, anti-IL-17, or anti-IL-10 mAb (BD Biosciences). Foxp3 staining was performed with the mouse regulatory T cell staining kit (eBioscience) using the Foxp3 PE (FJK-16) Ab. Samples were analyzed by flow cytometry using a FACScalibur (BD Biosciences); data were analyzed using Cell Quest (BD Biosciences) and FlowJo software (Tree Star). Both M133 (TVYRPYIEDYHTLT) and S598 (RCQIFANI) peptides were purchased from Neosystem (Strasbourg, France). For adoptive transfer experiments, single-cell suspensions from spleens of Thy1.1, 2D2 × Thy1.1, or 2D2 × DEREG × Thy1.1 mice were stained with CFSE (Molecular Probes, Leiden, The Netherlands), and 1.5 × 10^7 CFSE-labeled splenocytes were transferred i.v. to the recipient mice.

Statistical analysis

All statistical analyses were performed with Prism 4.0 (Graphpad). Data were analyzed with the nonpaired Student t test. A p value of <0.05 was considered significant.

Results

MHV-induced CNS inflammation is aggravated in the absence of Tregs

Access of lymphocytes to the CNS is tightly controlled under homeostatic conditions (28). During infection with the neurotropic MHV, activated CD4+ and CD8+ T cells gain access to the CNS and contribute to viral control (23). In this study, infection with MHV strain AS99 via the i.n. route was used to grant the virus access to the CNS. The ensuing acute encephalomyelitis was associated with a significant accumulation of Foxp3+ Tregs that constituted ~7% of the CNS-infiltrating CD4+ T cells at day 8 p.i. (Fig. 1A). Because DEREG mice express the eGFP/DT receptor transgene only in the CD4+ T cell compartment (Supplemental Fig. 1), administration of DT to MHV-infected mice permitted efficient and specific ablation of Tregs in the CNS and secondary lymphoid organs such as CLNs and spleen, as shown by the almost complete loss of the eGFP signal (Fig. 1B). To assess whether Tregs modulate the magnitude of acute MHV-induced CNS inflammation, B6 and DEREG mice were i.n. infected with MHV, treated with DT, and acute CNS inflammation was assessed on day 8 p.i. In situ analysis revealed significantly increased perivascular (Fig. 2A, 2B) and intraparenchymal inflammation (Fig. 2C) in MHV-infected DEREG mice whereby the majority of the CNS-infiltrating cells expressed the T cell marker...
CD3 (Fig. 2C). However, DT treatment of DEREG mice did not enhance infiltration with other immune cells such as macrophages, dendritic cells, or neutrophils (data not shown). Furthermore, Treg-depleted mice showed accumulation of extravascular erythrocytes (Fig. 2D, arrows in top panel) and increased numbers of apoptotic neurons (Fig. 2D, arrows in bottom panel, 2E) in areas of high MHV replication such as the hypothalamus. These data indicate that the absence of Tregs during MHV CNS infection exacerbated the T cell-dominated inflammatory response in the CNS.

**Impact of Tregs on antiviral immunity**

Because the lack of Tregs improved access of T cells to the CNS during MHV infection, we tested whether viral replication and distribution would be affected by the altered immune cell distribution. Interestingly, viral peak titers on day 6 p.i. and the kinetics of viral clearance were not different in DT-treated B6 and DEREG mice (Fig. 3A). To assess whether the presence of Tregs could result in more efficient viral clearance from particular regions of the CNS, we determined viral titers on days 6 and 8 in the olfactory bulb, hippocampal region, cortex, cerebellum, and spinal cord. As shown in Fig. 3B and 3C, Treg deletion did not result in an altered distribution of the virus, suggesting that the enhanced T cell infiltration in the absence of Tregs did not significantly affect the host’s ability to cope with the viral infection.

To further delineate to which extent Tregs impact on the antiviral T cell response against MHV, we assessed the CD8+ T cell response against the S598 epitope (29) and the CD4+ T cell response against the M133 epitope (30) in the presence and absence of Tregs. Although the overall T cell density in the absence of Tregs was increased (Fig. 2C), the relative abundance of virus-specific T cells in the CNS was reduced in Treg-depleted mice (Fig. 4A–C). Interestingly, the expected phenotype, namely increased antiviral T cell responses, was only observed in the spleen, where M133-specific CD4+ T cells were increased in the absence of Tregs, whereas similar numbers of M133-specific CD4+ T cells were found in the CNS of B6 and DEREG mice (Fig. 4C, left panel). As described previously (24), a residual population of 1 to 2% of Foxp3+CD4+ T cells could not be depleted in DEREG mice (Fig. 4C, right panel). Nevertheless, enumeration of the total CD4+ T cells revealed no significant change in the absence of Tregs (data not shown).

![FIGURE 1.](http://www.jimmunol.org/)

**FIGURE 1.** Treg distribution and depletion in MHV-infected DEREG mice. (A) B6 mice were infected i.n. with $5 \times 10^4$ PFU MHV, and Foxp3+ CD4+ T cells in CNS and spleen were enumerated at days 0 and 8 p.i. Results represent mean percentage of Foxp3+CD4+ T cells ± SEM ($n = 8$ mice, pooled data from two independent experiments). (B) Following i.n. MHV infection, DEREG mice were treated with DT on days −1, 1, 3, 5, and 7 or were left untreated. The efficiency of Treg depletion in the indicated organs was analyzed at day 8 p.i. Values in the upper right quadrant represent mean percentage ± SEM of eGFP+ cells (Tregs) within the CD4+ T cell compartment ($n = 8$ mice, pooled data from two independent experiments).

**FIGURE 2.** Aggravated CNS inflammation in the absence of Tregs during MHV infection. B6 and DEREG mice were i.n. infected with MHV or received PBS i.n. as control and were treated on days −1, 1, 3, 5, and 7 with DT. In situ analysis was performed on day 8 p.i. (A) H&E-stained sections from different CNS regions. Scale bar, 100 μm. (B) Determination of vessel width. Data represent mean width ± SEM of all cross-sectioned vessels on one whole brain section per mouse ($n = 6$ mice). (C) Representative sections from immunofluorescence analysis of perivascular T cell infiltration using anti-CD3 (red) and DAPI (blue) in parenchymal areas within the tegmentum. Scale bar, 50 μm. (D) High-resolution analysis of H&E sections from the hypothalamus revealing increased extravascularization of RBCs (arrows in the top panel) and higher prevalence of apoptotic neurons (arrows in the bottom panels) in the absence of Tregs. Original magnification ×40 (top panels), ×60 (bottom panels). (E) Quantification of neuronal cell death. Data represents the mean ± SEM of apoptotic neurons per high-power field of the hypothalamic region. Twenty high-power fields from six mice were analyzed. Statistical analysis was performed using the Student t test ($^{*}p < 0.05$, $^{***}p < 0.001$). ST, Striatum; TE, tegmentum; TH, thalamus.
numbers of CNS-infiltrating cells revealed that recruitment of virus-specific CD8+ and CD4+ T cells to the CNS was not affected by the Treg depletion (Fig. 4D, 4E), whereas the numbers of CD8+ (Fig. 4D) and CD4+ T cells (Fig. 4E) that were not specific for the known viral epitopes were significantly increased. To assess whether T cells that do not recognize the known MHV epitopes would be under Treg control, we assessed T cell proliferation using BrdU incorporation and determined the expression of the activation markers CD69 and CD44 on the different T cell populations. We found that the absence of Tregs permitted increased T cell proliferation in CLNs (Supplemental Fig. 2A, 2B). However, both antiviral CD8+ (Supplemental Fig. 2C) and CD4+ T cells (Supplemental Fig. 2D) were exempt from the increased BrdU incorporation, indicating that mainly cells with other specificities were controlled by Tregs. Furthermore, tetramer-negative CD8+ T cells in CLNs showed significantly increased CD44 (Supplemental Fig. 2E) and CD69 expression (Supplemental Fig. 2F), indicating that Tregs controlled both proliferation and activation of T cells that did not respond to the known MHV epitopes.

To exclude that the observed effects were a consequence of Foxp3 promoter activity-dependent depletion in non-Tregs, we generated mixed bone marrow chimeric mice using mice with the scurfy mutation (31) as donors. Again, DT-treated DEREG + scurfy→B6 chimeras did not show an increased recruitment of virus-specific CD8+ T cells into the CNS compared with B6 + scurfy→B6 chimeras, but a significantly elevated fraction of cells that did not bind the MHV-specific tetramer (Supplemental Fig. 3). Taken together, these data suggest that the aggravated CNS inflammation in Treg-depleted mice during MHV infection was not due to alterations in the antiviral T cell response, but rather a consequence of activation or differential recruitment of virus-unrelated T cells.

**Tregs efficiently attenuate CNS infiltration by self-reactive CD4+ T cells**

To assess whether MHV infection is able to induce the activation and infiltration of truly self-reactive T cells into the CNS, B6 mice were infected with MHV, and, at the peak of infection at day 6 p.i., CFSE-labeled MOG peptide (MOG35–55)-specific CD4+ T cells from 2D2-transgenic mice (25) were adoptively transferred into infected recipients. As shown in Fig. 5A (top panel), proliferating MOG-specific CD4+ T cells could be detected in CLNs and CNS of MHV-infected mice on day 10 p.i., whereas nontransgenic CD4+ T cells were not activated (Fig. 5A, middle panel), hence excluding inflammation-driven bystander activation. Importantly, i.p. MHV infection, which restricts viral replication to spleen, liver, and lung (32), did not drive 2D2 cells into the CNS (Fig. 5A, bottom panel) indicating that MHV infection drives autoreactive T cells into the CNS only when the cytopathic virus replicates in this compartment. Moreover, although MHV infection in the CNS was cleared around day 12 p.i. (Fig. 3), MOG-specific CD4+ T cells prevailed in the CNS for a prolonged period of time (Fig. 5B). Thus, MHV infection precipitated not only the activation of MOG-specific CD4+ T cells and facilitated their access to the infected tissue, but also fostered the persistence of autoreactive T cells in this vulnerable organ.

To assess whether and to which extent Tregs control the proliferation and infiltration of autoreactive T cells into the CNS, MHV-infected B6 and DEREG mice were treated with DT at days 5, 7, 9, and 11 p.i., and at day 6 p.i., CFSE-labeled splenocytes from 2D2 × DEREG mice (treated with DT to eliminate Tregs from the donor population) were adoptively transferred into the infected mice. At day 12 p.i., activation and migration of MOG-specific CD4+ T cells in CNS, CLNs, and spleen was assessed. Treg depletion had a significant effect on both 2D2 T cell activation and their migration into the CNS, with the most pronounced effect on CD4+ T cell activation in the CLNs (Fig. 6A, 6B). However, the absence of Tregs did not significantly alter the differentiation pattern of CNS-infiltrating MOG-specific CD4+ T cells because IFN-γ was the dominant cytokine produced both in the absence and in the presence of Tregs (Fig. 6C). Taken together, these data indicate that Tregs can efficiently restrict the proliferation of CNS-specific CD4+ T cells and that this activity is exerted mainly within the CNS-draining lymph node.

To assess whether Tregs impact on the migration of self-reactive CD4+ T cells from the CNS-draining lymph nodes to the CNS, we first analyzed the expression of chemokine receptors on CNS-infiltrating T cells during MHV infection. Although CCR6 (33) has been implicated in the migration of CD4+ T cells to the CNS, this receptor was not expressed on CD4+ or CD8+ T cells in the CNS during MHV infection (data not shown). Because previous reports have shown that CXCL10 (i.e., one of the ligands of CXCR3) is upregulated in the CNS of MHV-infected mice and critically regulates MHV-induced pathology (34, 35), we assessed whether Tregs have an impact on the expression of CXCR3 on CD8+ and CD4+ T cells during MHV infection. Indeed, ablation of Tregs resulted in a pronounced upregulation of CXCR3 on both CD4+ (Fig. 7A, 7B) and CD8+ T cells (Fig. 7C, 7D) in spleen and CNS-draining lymph nodes. It is noteworthy that CXCR3 expression in CNS-infiltrating T cells was not significantly affected
by the depletion of Tregs (Fig. 7A–D), suggesting that Tregs selectively downregulate CXCR3 expression on activated T cells within secondary lymphoid organs. The importance of CXCR3-mediated T cell migration during MHV infection is illustrated by the significantly reduced accumulation of T cells within the CNS in the absence of this chemokine receptor (Fig. 7E–H), indicating that, under these conditions, Tregs selectively impact on homing of effector T cells to control virus-mediated CNS immunopathology.

Discussion

Because the CNS is a vital organ with poor regenerative capacity, immune privilege preservation for self-antigens is essential to limit inflammation-associated damage. This study reveals the elaborate regulation of CNS immune privilege by Tregs via two distinct mechanisms: first, by attenuating the proliferation of self-reactive CD4+ T cells within the CNS-draining lymph node, and second, by downregulating the migration efficacy of T cells to the CNS by pre-empting exaggerated CXCR3 expression on T cells within secondary lymphoid organs.

CNS immune privilege can be regarded as a selective advantage in the protection from immunopathological/autoimmune disease (10). The efficient self-protection from overshooting inflammatory reactions has been attributed to the segregation of the CNS parenchyma from peripheral circuits through the blood–brain barrier. A recent conceptual revision suggests that the blood–brain barrier should be referred to as a capillary barrier for soluble substances, whereas control over cellular neuroinflammatory reactions is maintained by dedicated cell populations at different locations (28). For example, perivascular APCs can regulate access of activated T cells from the vasculature to the CNS parenchyma (36). The present study reveals that Tregs contribute to preservation of CNS immune privilege during viral infection by acting on the activation of self-reactive T cells within the CNS-draining lymph node.

The importance of Tregs for the maintenance of immune privilege has been demonstrated for other tissues and inflammatory settings including transplant rejection (reviewed in Ref. 18) and chronic parasite infection (reviewed in Ref. 37). It is particularly interesting that Tregs can even facilitate immune privilege of Leishmania Ags during skin infection by attenuating inflammatory reactions. Such limited antimicrobial protection permits establishment of the chronic low-level persistence of the pathogen and facilitates long-term protection through the maintenance of T and B cell memory (38). Hence, in this particular setting of chronic pathogen infection, Treg-mediated functional immune privilege (37) can secure coexistence of host and pathogen.

In viral infections, the impact of Tregs on the host–pathogen relationship is strongly context dependent and most likely geared
toward the optimal net effect for host survival. For example, Treg depletion during respiratory syncytial virus infection can lead to a lag in antiviral T cell recruitment to infected lungs and hence results in transiently elevated viral titers in this organ. However, the overall effect of Treg depletion in this system is, despite a delayed clearance of the virus, a reduced inflammatory lung disease (20). Likewise, during cytopathic HSV-2 infection, Tregs are essential to instruct innate immune cells to migrate to the inflamed mucosal surface and thereby to control the pathogen more efficiently (19). A more classical role of Tregs in terms of global immune suppression has been demonstrated for acute and chronic retroviral infection in which Treg depletion results in

![Figure 5](http://www.jimmunol.org/)

**Figure 5.** Virus-induced recruitment of self-reactive CD4+ T cells to the CNS. B6 mice were infected with MHV either i.n. with 5 \times 10^4 PFU or i.p. with 50 PFU. Six days p.i., CFSE-labeled splenocytes obtained from 2D2 mice or from transgene-negative littermates expressing the congenic marker Thy1.1 were adoptively transferred into infected mice. (A) Flow cytometric analysis of CD4+ T cell proliferation in the indicated organs at day 10 p.i. (B) Flow cytometric analysis of 2D2 CD4+ T cell proliferation in the CNS of i.n. infected B6 mice at the indicated days p.i. Representative FACS plots from one out of three independent experiments with three mice per group.

![Figure 6](http://www.jimmunol.org/)

**Figure 6.** Tregs restrict proliferation and CNS infiltration of autoreactive CD4+ T cells. B6 and DEREG mice were infected i.n. with 5 \times 10^4 PFU MHV A59 and treated with DT on days 5, 7, 9, and 11 p.i. At day 6 p.i., CFSE-labeled splenocytes from DT-treated 2D2 × DEREG mice were adoptively transferred into infected mice. Flow cytometric analysis from CNS, CLNs, and spleens was performed at day 12 p.i. Proliferation of MOG-specific Thy1.1+ CD4+ T cells was determined by CFSE dilution. (A) Representative dot plots (top and middle panels) and histograms (bottom panel, DEREG [gray] and B6 [black line]) showing the CFSE dilution profiles. Values in the histograms represent the mean fluorescence intensity of the total cell population. (B) Frequencies of proliferated MOG-specific Thy1.1+ CD4+ T cells in CNS, CLNs, and spleens. Data represent mean values ± SEM from three pooled experiments (n = 9 mice/group). (C) Phenotype of CNS-infiltrating self-reactive T cells. Data represent the mean percentage ± SEM of IFN-γ+, IL-17+, IL-4+, and IL-10+ in the Thy1.1+CD4+ T cell population from two experiments with pooled cell preparations from seven mice. Statistical analysis was performed using the Student t test (**p < 0.01, ***p < 0.001). n.d., Not detectable.
higher T cell responses and improved control of the virus (21, 39). We present in this paper a third scenario for the impact of Tregs on virus–host interaction: the absence of a significant modulation of antiviral immunity. We observed almost indistinguishable viral distribution patterns, clearance kinetics, and virus-specific CD8+ and CD4+ T cell responses in MHV-infected Treg-depleted DEREG mice when compared with B6 mice. Similar findings were obtained in a recent study by Trandem et al. (40) using adoptive transfer of Tregs into RAG-deficient hosts together with intracranial infection with an attenuated MHV JHM mutant. Although CD4+Foxp3+ T cell-reconstituted RAG-deficient mice showed no altered viral replication pattern compared with mice reconstituted with CD4+Foxp3+ T cell-reconstituted recipients, a pronounced impact of Tregs on CNS inflammation and demyelination was observed. Likewise, adoptive transfer of Tregs attenuated lethal intracranial infection of B6 mice with MHV JHM without altering viral clearance (41). The results provided in our study provide a mechanistic explanation for these previous findings; namely, that Tregs mainly act on self-reactive T cells during acute viral encephalomyelitis but spare the protective antiviral immune response from negative regulation.

During MHV infection, virus-specific CD4+ T cells can acquire a Treg phenotype (42). Thus, the interpretation that Tregs can selectively contribute to immune privilege of CNS self-antigens during viral infection whereas the initiation of protective T cell responses remains unaltered raises the question of how such selectivity is achieved. We can offer two possible explanations: first, Ag dose has been shown to be an important determinant for Treg differentiation in which high Ag doses favor the induction of effector T cells, whereas low Ag doses lead to increased Treg differentiation (43). Second, it has been shown that the inflammatory environment, in particular the levels of the proinflammatory cytokine IL-6, critically influence the balance of Treg versus T effector differentiation (44, 45). Interestingly, DC–T cell interaction in the presence of high Ag doses favors the production of IL-6 and hence the suppression of Treg induction (43). Conversely, the activation (or action) of Tregs under high Ag dose/high inflammation conditions within secondary lymphoid organs
during acute MHV infection is kept in check by IL-6 or other proinflammatory mediators. In summary, this study reveals a novel immune privilege paradigm for Treg function; namely, the ability of these cells to select-attenuate CNS-specific Th cells while keeping antiviral immunity fully operational.

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Disclosures

The authors have no financial conflicts of interest.

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Supplementary Figure 1: Expression of eGFP in DEREG mice during MHV infection. DEREG and B6 mice were infected with $5 \times 10^4$ pfu MHV A59 i.n. At day 8 post infection, flow cytometric analysis of eGFP expression in CD4$^+$, CD8$^+$, F4/80$^+$ and CD11c$^+$ cells from the CNS was performed. Values in the upper right quadrant represent mean ±SEM of eGFP$^+$ cells within the respective compartment (n=3).
Supplementary Figure 2: T cell proliferation and activation in the absence of Tregs. B6 and DEREG mice were infected i.n. with $5 \times 10^4$ pfu MHV on day 0 and treated on days -1, 1, 3, 5, and 7 with DT. An i.p. BrdU pulse (1 mg diluted in 100 μl PBS) was injected i.p. into was provided on day 7 and flow cytometric analysis was performed at day 8 post infection. Proliferation of CD8$^+$ T cells (A), CD4$^+$ T cells (B) as determined by BrdU incorporation. Anti-BrdU stains were performed according to the manufacturer’s instruction (APC BrdU Flow kit; BD Pharmingen). Proliferation of virus epitope-specific T cells was assessed by determining S598 (C), and M133 (D) peptide-reactive IFN-γ producing cells that had incorporated BrdU. The activation status of virus-specific CD8$^+$ T cells was determined by combining S598 tetramer stain with anti-CD44 (1M7, Biolegend) (E) and anti-CD69 (H1.2F3, eBioscience) (F) staining. Data represent the mean ± SEM from 1 experiment with 5-6 mice per group. Statistical analysis was performed using the Student’s t test (*, p<0.05; **, p<0.01; n.s., not significant).
Supplementary Figure 3: MHV induced-CNS inflammation in Treg-depleted mice. B6 mice were irradiated and reconstituted with either a mixture of B6 and Scurfy bone marrow or with a mixture of DEREG and Scurfy bone marrow. For the generation of bone marrow-chimeric mice, recipients were lethally irradiated with 900 rad and injected i.v. 1 day later with $2 \times 10^7$ of the indicated donor bone marrow cells. Mice were used for experiments 8–10 wk after bone marrow reconstitution. Bone marrow chimeras were infected with $5 \times 10^4$ pfu MHV i.n. on day 0 and treated with DT on day -1, 1, 3, 5 and 7 p.i. Lymphocytes of the brain and spleen were isolated and analyzed on day 8. (A) Frequencies of S598 tetramer-positive CD8$^+$ T cells and (B) enumeration of CNS-infiltrating S598-specific CD8$^+$ T cells. Data represent the mean ± SEM of 5 B6+Scurfy→B6 BM chimeras and 4 DEREG+Scurfy→B6 BM chimeras. Statistical analysis was performed using the Student’s t test (**, p<0.01; n.s., not significant).