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Role of CD25⁺ CD4⁺ T cells in acute and persistent coronavirus infection of the central nervous system

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ABSTRACT

The influence of CD25⁺ CD4⁺ regulatory T cells (Treg) on acute and chronic viral infection of the central nervous system (CNS) was examined using a glial tropic murine coronavirus. Treg in the CNS were highest during initial T cell mediated virus control, decreased and then remained relatively stable during persistence. Anti-CD25 treatment did not affect CNS recruitment of inflammatory cells. Viral control was initially decreased; however, neither the kinetics of viral control nor viral persistence were affected. By contrast, the absence of Treg during the acute phase resulted in increased demyelination during viral persistence. These data suggest that CNS inflammation, progression of viral control and viral persistence are relatively independent of CD25⁺ CD4⁺ Treg. However, their absence during acute infection alters the ability of the host to limit tissue damage.

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Introduction

Regulatory T cells (Treg) which express the Foxp3 transcription factor, neuropilin 1 (Nrp-1) and the IL-2 receptor (CD25) comprise ~10% of CD4⁺ T cells in the naïve animal and play an essential role in regulating the immune response to infection, progression of clinical disease and tissue damage (Langier et al., 2010; Lourenco and La Cava, 2011; Rouse et al., 2006; Rowe et al., 2012). During viral infection the host is challenged to mount an effective anti-viral immune response while minimizing immune mediated damage. Exuberant T cell effector function and tissue damage are regulated by sustained natural Treg (nTreg), induction of antigen specific Foxp3⁺ Treg (iTreg), secretion of the anti-inflammatory cytokine IL-10 by both Foxp3⁺ and Foxp3⁻ T cells, as well as inhibitory ligand receptor interactions (Belkaid, 2007; Curotto de Lafaille and Lafaille, 2009; Langier et al., 2010; Rowe et al., 2012). Treg influence the immune response during a variety of acute viral infections (Rouse et al., 2006; Rowe et al., 2012; Zelinsky et al., 2009) and are implicated in facilitating persistent infections in both humans and mice (Dittmer et al., 2004; Rowe et al., 2012; Xu et al., 2006). However, their suppressive role and the mechanism(s) of suppression vary depending upon both the pathogen and primary tissue infected. Following mucosal infection by Herpes simplex virus type 2 (HSV-2) Treg facilitate recruitment of virus effectors to the site of infection (Lund et al., 2008). They also enhance the severity of murine hepatitis virus (MHV) induced hepatitis due to their expression of the immunosuppressive cytokine fibrinogen-like protein 2 (Shaley et al., 2009). By contrast, during acute respiratory syncytial virus (RSV) infection of the lung or Herpes simplex virus type 1 (HSV-1) infection of the eye, Treg limit cellular recruitment into the site of infection, diminishing tissue destruction (Lee et al., 2010; Suvas et al., 2004). These findings suggest that Treg play an important role in regulating immunopathology associated with viral infection; however, this anti-inflammatory regulation may also reduce anti-viral activity, leading to delayed clearance and/or viral persistence. The balance between an effective immune response, limited tissue damage, and establishment of viral persistence is especially critical in the central nervous system (CNS), due to its limited regenerative capacity. Theiler's murine encephalomyelitis virus (TMEV) infection produces an acute encephalitis in mouse strains either susceptible or resistant to chronic CNS infection. Depletion and/or functional inactivation of CD25⁺ Treg did not affect CNS inflammation or TMEV replication in mice resistant to chronic infection (Richards et al., 2011). The identical depletion strategy resulted in both enhanced inflammation and control of TMEV replication in the CNS of mice susceptible to chronic infection (Richards et al., 2011). By contrast, Treg depletion...
prior to acute infection with a neurotropic MHV, which also produces chronic demyelination, did not alter CNS inflammation or virus specific T cell responses (Cervantes-Barragan et al., 2012). Following CNS infection with an MHV variant containing a mutation in the immunodominant CD4 T cell epitope which ameliorates disease, anti-CD25 mediated Treg depletion increased both morbidity and mortality (Anghelina et al., 2009). A beneficial role of Treg was also supported by adoptive transfer of nTreg at a time when CNS infection by a sub-lethal, glial tropic JHM strain of MHV (JHMW) was already established. The increased Treg ameliorated clinical disease and immunopathology without altering viral clearance (Trandem et al., 2010). Nevertheless, the role of CD25 Treg in the pathogenesis of acute JHMV encephalomyelitis and progression to persistent CNS infection is unclear. To better define the role of CD25 Treg early during JHMV induced encephalomyelitis and potential consequences on the chronic infection associated with sustained demyelination, the present study examined depletion/function inactivation of CD25 cells in wild type (WT) and syngeneic IL-10 reporter mice. The Treg population, composed of both Nrp1hi and Nrp1low Treg, peaked in the CNS during acute infection, declined as virus was controlled, and was retained in the CNS during viral persistence. The absence of CD25 CD4 T cells did not influence the composition or extent of the CNS inflammatory cells, including virus-specific CD8 T cells. However, CD25 depletion transiently impaired infectious virus control in the absence of detectable differences in ex vivo cytolytic effector function. The transient delay in virus control correlated with increased tissue damage although viral persistence within the CNS was not altered. These data support the concept that the regulation of the immune response within the CNS by CD25 T cells during JHMV infection is confined to a temporally narrow window during the initiation and effector phase of the acute inflammatory response.

Results

Kinetics of CD25 Nrp-1hi nTreg and Nrp-1low iTreg accumulation in the CNS

Prior to initiating anti-CD25 monoclonal antibody (mAb) treatment the kinetics and relative composition of CD25 and Foxp3 CD4 T cells recruited into the CNS were assessed following JHMV infection. CNS accumulation of CD25 and Foxp3 CD4 T cells followed similar patterns throughout infection (Fig. 1A). Total numbers of both CD25 CD4 and Foxp3 T cells peaked at day 7 post infection (p.i.) comprising ~20% of total CD4 T cells (Fig. 1). These populations declined rapidly by day 10 p.i. and stabilized thereafter (Fig. 1B and C). Importantly, >75% of CD25 T cells expressed Foxp3 at day 7 p.i. indicating a minority of ~25% non Treg CD4 effector cells expressed CD25 (Fig. 1A and D). These data show that the vast majority of the Foxp3 population expressed CD25 (Fig. 1A and D) and this proportion remained stable at 75–80% throughout the infection (Fig. 1A and D).

To distinguish a phenotypic transition of Treg populations, possibly accompanied by differential expression of CD25, nTregs were identified based on high Nrp1 expression (Weiss et al., 2012; Yadav et al., 2012). At day 7 p.i., Nrp-1hi nTreg represented the majority (~75%) of Foxp3 Treg within the CNS (Fig. 2A and B). At day 10 p.i., the frequency of Nrp-1hi nTreg declined to ~50% and remained stable at all subsequent time points, resulting in an equal proportion of Nrp-1hi and Nrp-1low Treg (Fig. 2A and B). Similar to the total Foxp3 population (Fig. 1D), CD25 expression remained stable at ~75% on both Nrp-1hi (Fig. 2C) and Nrp-1low Treg during the course of infection (Fig. 2C and D). These data predicted that the majority of CD25 Foxp3 Treg are susceptible to anti-CD25 treatment. Moreover, CD25 treatment at early times during infection was anticipated to primarily target prevailing Nrp-1hi Treg and only a minor population of CD25 effector T cells.

Early CD25 T cell depletion does not alter morbidity or inflammation

The role of CD25 Treg in JHMV induced sub-lethal encephalomyelitis and viral persistence was thus examined by infection of mice treated with anti-CD25 mAb at day -3, 0, and +3 relative to infection. JHMV induces clinical symptoms associated with encephalitis which transitions to predominantly hind limb paralysis. Severity of clinical symptoms reflects both viral load and the antiviral immune response (Bergmann et al., 2006; Kapil et al., 2009; Weiss and Leibowitz, 2011). Anti-CD25 treatment did not alter disease onset, severity or the progression of clinical symptoms
CNS viral replication was increased in anti-CD25 compared to control mAb treated mice (Fig. 5A). A marginal, but not statistically significant, increase in infectious virus was sustained to day 10 p.i. (Fig. 5A). Nevertheless, impaired virus control was transient, as no differences were detected at day 14 p.i. and infectious virus was undetectable in the CNS of either group at day 21 p.i. (Fig. 5A). Consistent with delayed, but eventually effective viral control, no clinical evidence of viral recrudescence was found in CD25 depleted mice.

T cell mediated cytolytic activity and IFN-γ are the primary immune effectors controlling JHMV replication within the CNS (Bergmann et al., 2006) and Treg depletion enhances CD8+ T cell number and activity (Dietze et al., 2011; Haeryfar et al., 2005). Comparison of anti-CD25 treated and control mice indicated no affect on CNS IFN-γ mRNA levels or frequencies of virus-specific T cells secreting IFN-γ (data not shown). The limited control of viral replication at day 7 p.i. in the absence of CD25+ T cells (Fig. 5A) did not coincide with impaired CNS recruitment of total CD8+ T cells (Fig. 5B). Although recruitment of CD8+ T cells into the CNS was increased at days 10 and 14 p.i., these increases did not reach statistical significance. Similarly, the apparent increase in virus specific CD8+ T cells at days 10 and 14 p.i. also did not reach statistical significance (Fig. 5C). During influenza virus infection of the lung, highly cytolytic IL-10 producing CD8+ T cells are dependent upon CD4+ T cells (Sun et al., 2011), suggesting a possible influence of CD4+CD25+ T cells on CD8+ T cell function during JHMV. Furthermore, an IL-10+CD8+ T cell population constituting ~25% of virus-specific CD8+ T cells resides within the JHMV infected CNS at day 7 p.i. (Puntambekar et al., 2011), comprising CD8+ T cells with high cytolytic potential (Trandem et al., 2011a). Anti-CD25 treated and control IL-10 reporter mice were therefore infected to examine the possibility of diminished IL-10+ effector CD8+ T cells. Similar to WT mice, anti-CD25 mAb treatment was effective at eliminating CD25+ CD4+ T cells from the CNS of infected IL-10 reporter mice until at least day 14 p.i. (data not shown). Anti-CD25 treatment reduced the frequency of IL-10+ CD4+ T cells by ~30% at day 7 p.i.; however, the population returned to WT levels by day 10 p.i. (data not shown). In contrast to the CD4+ T cell population, virus specific CD8+IL-10+ were slightly reduced at both days 7 and 10 p.i.; however, these decreases also did not reach statistical significance. CNS derived mononuclear cells from infected anti-CD25 and control mAb treated mice were also tested for direct ex vivo cytolytic activity.
at day 7 p.i. Although virus replication was increased at day 7 p.i. in the anti-CD25 treated group (Fig. 5A), cytolytic activity by CNS derived CD8⁺ T cells was not altered by anti-CD25 treatment (Fig. 5E). Furthermore, cytolytic activity in both anti-CD25 and control mAb treated groups decreased at day 10 p.i. (data not shown), consistent with previous results (Bergmann et al., 1999). Thus the transiently increased viral load in the absence of CD25⁺ CD4⁺ T cells could not be correlated with either reduced IFN-γ, virus-specific CD8⁺ T cell recruitment or cytolytic activity. Transiently decreased anti-viral activity may thus be due to insufficient help provided by CD25⁺ CD4⁺ T cells (Phares et al., 2012) or induction of inhibitory molecules (Phares et al., 2009), neither of which would be directly reflected by in vitro analysis.

**Fig. 3.** Encephalomyelitis is independent of CD25⁺ CD4⁺ T cells. WT mice received 250 μg of anti-CD25 or isotype control mAb i.p. at days −3, 0 and 3 relative to JHMV infection. (A) Clinical symptoms of anti-CD25 treated and control mice. Data are mean ± SEM and represent 3 combined experiments comprising 53 mice per group. (B) Flow cytometric analysis of CD25 expression on CD45⁺CD4⁺ T cells isolated from the infected CNS. Circled populations represent percentages of CD25⁺ T cells within CD4⁺ T cells. Representative data from 2 separate experiments. (C) Bar graph representing the frequency of CD25⁺ T cells within the CNS. Data are mean ± SD and represent a combination of 2–7 experiments with 3 mice per time point in each experiment.

**Early CD25⁺ T cell depletion does not alter persistence but limits demyelination**

A number of chronic viral infections are associated with CD25⁺ CD4⁺ Treg (Belkaid, 2007; Rowe et al., 2012). However, no clinical or virological evidence for viral recrudescence was found in the anti-CD25 treated group (Figs. 3A and 5A), similar to control mice. Therefore, the ability of CD25⁺ T cells to influence...
viral persistence was examined by PCR due to the absence of infectious virus during JHMV persistence (Bergmann et al., 2006; Phares et al., 2010). As expected based on the limited ability of anti-CD25 treated mice to control viral load at day 7 p.i., expression of the mRNA encoding the viral nucleocapsid (N) protein was increased in the brains of anti-CD25 treated mice compared to controls (data not shown). At subsequent times p.i., however, viral mRNA expression was equivalent in the spinal cords of both groups (Fig. 6A), the predominant site of JHMV persistence (Bergmann et al., 2006; Marten et al., 2000). These results indicate that diminished viral control during the acute phase of coronavirus mediated encephalomyelitis in the absence of CD25⁺CD4⁺ T cells did not facilitate viral persistence.

The transient decrease in control of CNS viral replication, decreased demyelination mediated by nTreg adoptive transfer in
a similar model of JHMV infection (Trandem et al., 2010) and the delayed onset of demyelination in anti-CD25 treated TMEV infected mice (Richards et al., 2011) suggested that CD25+CD4+ T cells might influence JHMV induced tissue damage. Therefore, spinal cords from anti-CD25 treated and control mice were compared for inflammation, the extent and distribution of viral antigen, as well as demyelination at day 14 p.i. when viral mRNA levels were equivalent (Fig. 6A). No differences in the extent or distribution of inflammatory cells were detected comparing the two groups (Fig. 6B). Similar inflammation is consistent with the overall recruitment of both inflammatory cells (Fig. 4A) and their composition (Figs. 4 and 5). Although few infected cells were found in either group (Fig. 6B), consistent with low levels of infectious virus (Fig. 5A), the predominant cell type infected exhibited the morphology and anatomical location of oligodendroglia and was similar in both groups (Fig. 6B). These data suggested that anti-CD25 treatment did not alter viral tropism.

To determine if the transiently increased viral load during the acute phase of infection prolonged or worsened tissue damage, demyelination was quantified in both groups at different times p.i. (Lee et al., 2010; Suvas et al., 2004) where they can further dampen T cell effector function to prevent bystander damage. Indeed, recent evidence suggests that suppression is more important at the site of infection (Ding et al., 2012). However, suppressing an anti-viral response may also allow virus dissemination to cells critical for maintaining homeostasis and/or facilitate viral persistence (Belkaid, 2007; Rouse et al., 2006; Rowe et al., 2012). In this regard the CNS presents an especially challenging site to control infection due to the limited capacity for regeneration of specialized cells. JHMV induces an acute encephalomyelitis characterized by the recruitment of a variety of innate and adaptive immune effectors into the CNS (Bergmann et al., 2006), including Treg (Anghelina et al., 2009; Puntambekar et al., 2011). Although reduced tissue damage following adoptive transfer of nTreg at the peak of acute JHMV encephalitis (Trandem et al., 2010) implicated a local protective role, protection was not associated

**Discussion**

During viral infections Treg can suppress T cell activity at multiple levels. They can directly limit T cell priming in the draining lymph nodes and trafficking to the effector site (Lee et al., 2010; Suvas et al., 2004) where they can further dampen T cell effector function to prevent bystander damage. Indeed, recent evidence suggests that suppression is more important at the site of infection (Ding et al., 2012). However, suppressing an anti-viral response may also allow virus dissemination to cells critical for maintaining homeostasis and/or facilitate viral persistence (Belkaid, 2007; Rouse et al., 2006; Rowe et al., 2012). In this regard the CNS presents an especially challenging site to control infection due to the limited capacity for regeneration of specialized cells. JHMV induces an acute encephalomyelitis characterized by the recruitment of a variety of innate and adaptive immune effectors into the CNS (Bergmann et al., 2006), including Treg (Anghelina et al., 2009; Puntambekar et al., 2011). Although reduced tissue damage following adoptive transfer of nTreg at the peak of acute JHMV encephalitis (Trandem et al., 2010) implicated a local protective role, protection was not associated
with either Treg recruitment into the CNS or decreased virus replication (Trandem et al., 2010). These results suggested that after infection is established the primary site of Treg function may be the CLN. Our data demonstrate that the high frequency of Treg recruited into the CNS during the peak of acute inflammation declines as virus replication is controlled. Consistent with previous data (Puntambekar et al., 2011) Treg are nevertheless retained within the CNS during persistence. Moreover, the majority of Treg expressed a Npr-1hi phenotype during peak CNS inflammation, consistent with their previous designation as nTreg based on expression of the helios transcription factor (Zhao et al., 2011).

The Treg population within the CNS declined dramatically by day 10 p.i. as virus replication was controlled. However, the Npr-1low Treg population increased to a stable ~50% of total Treg as virus replication was controlled and similar proportions of Npr-1hi and Npr-1low Treg were retained in the CNS.

To determine if Treg influenced acute JHMV infection and subsequently demyelination, mice were treated with anti-CD25 mAb prior to infection. This well established methodology has been used to minimize the influence of Treg on viral pathogenesis in both the CNS and at other anatomical sites. Inhibition of IL-2/IL-2R interactions may explain the limited inflammation in some viral infections (Fulton et al., 2010; Ruckwardt et al., 2009), but it has no effect on inflammation in others (Betts et al., 2011). In addition, anti-CD25 treatment increases CD8+ T cells responses under some, but not all circumstances (Betts et al., 2011; Dietze et al., 2011; Fulton et al., 2010; Haeryfar et al., 2005; Ruckwardt et al., 2009). Both CD25-CD4+ T cells and CD4-IL-10+ T cells are rapidly recruited into the JHMV infected CNS (Anghelina et al., 2009; Puntambekar et al., 2011). However, both virus control and inflammation are Treg independent following infection with a related MHV (Cervantes-Barragan et al., 2012). Therefore, it was not surprising that anti-CD25 treatment did not alter recruitment of inflammatory cells into the CNS. Limited Treg mediated suppression within the CNS is also consistent with an adverse environment established during CNS inflammation (Korn et al., 2007; Tang et al., 2008). Indeed, induction of IL-12 or IL-6 within the CNS during JHMV infection (Kapil et al., 2009) may contribute to limiting suppressive activity (Longhi et al., 2008; Zhao et al., 2012). Furthermore a variety of inhibitory molecules, i.e. Programmed death-1 ligand (PD-L1), as well as the anti-inflammatory cytokine IL-10 influence JHMV pathogenesis (Phares et al., 2010; Trandem et al., 2011b) potentially exhibiting more potent regulatory effects than Treg. For example, JHMV infection of IL-10 deficient mice demonstrated accelerated viral clearance, yet dramatically enhanced tissue destruction (Trandem et al., 2011a). During JHMV infection IL-10 secretion within the CNS is limited to the T cell compartment with CD25-IL-10+CD4+ T cells, characteristic of nTreg, rapidly recruited into the CNS (Puntambekar et al., 2011). The absence of a suppressive effect suggested by anti-CD25 treatment may reside in the rapid replacement of this population by CD25-IL-10+CD4+ T cells (Puntambekar et al., 2011), or the loss of CD25 expression. Although the precise phenotype of the CD25-IL-10+CD4+ T cell population is unclear, it is possible it comprises virus-specific Foxp3+ Treg (Zhao et al., 2012) or virus-specific IL-10+Foxp3+ cells regulated by IL-27 (Wojno and Hunter, 2012). Interestingly, ~5% of Treg infiltrating the CNS during JHMV infection are virus-specific (Zhao et al., 2011). As CD25 expression was not assessed on this population, our data cannot exclude that enrichment of this population in the CD25 Foxp3+ subset may contribute to JHMV pathogenesis.

Control of CNS virus replication was partially inhibited at the peak of replication, suggesting that CD25+CD4+ T cells enhanced the initial phase of adaptive immunity. Although anti-CD25 treatment did not alter numbers of inflammatory cells recruited into the CNS or the proportions of anti-viral effectors, we cannot rule out a transient CD25 dependent CD4+ helper T cell effect (Phares et al., 2012), independent of the Treg population. This is supported by increased mortality, despite similar JHMV clearance, in transgenic mice lacking both IL-27 and IL-35 (Tirotta et al., 2013) suggesting that neither of these Treg associated cytokines regulates viral clearance. One possibility to account for the limited clearance of infectious virus in the absence of CD25+CD4+ T cells is inhibition of CTL activity in vivo via the inhibitory ligand PD-L1 on CNS targets (Phares et al., 2009) which is not apparent in a direct ex vivo assay. Although PD-L1 mRNA expression was slightly increased in the CNS of the CD25+CD4+ T cell depleted mice relative to controls (data not shown), the difference did not reach statistical significance. Together, these data indicate that Treg exert minimal effects on anti-viral functions during acute JHMV induced encephalomyelitis, similar to their inability to alter TMEV induced encephalitis in resistant mice (Richards et al., 2011). Consistent with functional anti-viral effectors, treatment with anti-CD25 did not enhance or diminish viral persistence. These data contrast with data from a number of both human and viral infections suggesting that CD25+CD4+ Treg facilitate chronic viral infection (Belkaid, 2007; Rouse et al., 2006; Rowe et al., 2012). Although CD25+CD4+ T cells are rapidly recruited to the site of infection, depletion and/or functional inactivation demonstrates the effect on the inflammatory response within the CNS is transient, appearing to decrease the initial set point for control of virus replication without influencing viral persistence.

Transfer of Treg into mice with an established JHMV infection limited demyelination, consistent with the concept that demyelination is mediated by adaptive immunity (Pewe and Perlman, 2002; Savarin et al., 2008). However, infection of mice treated with anti-CD25 did not alter the extent of tissue damage during the acute phase of infection. These data indicate that in contrast to infection of the eye and mucosal sites (Lund et al., 2008; Suvas et al., 2004), Treg do not constitute a primary mechanism of damage control in the CNS during the period of maximal T cell effector function. By contrast, the extent of demyelination increased during viral persistence, even after CD25+ T cells were re-established within the CNS. Thus despite the minimal influence of CD25-CD4+ Treg on JHMV viral load and persistence, these minimal alterations were sufficient to influence the extent of tissue damage. Nevertheless, less extensive tissue damage compared to that found in the total absence of IL-10 (Trandem et al., 2011a), suggests that in addition to Treg, other IL-10 secreting regulatory populations contribute to limiting demyelination following JHMV infection. Overall these results support the notion that the presence of Treg in the CNS during persistence and demyelination is linked to the control of acute viral load and the resulting viral induced tissue damage.

Materials and methods

Mice and virus

C57BL/6 mice (WT) were purchased from the National Cancer Institute (Frederick, MD). IL-10-GFP and Foxp3-GFP reporter mice on the C57BL/6 background (Madan et al., 2009; Korn et al., 2007) were bred locally under pathogen-free conditions. Female and male mice at 6 weeks of age were infected intracranially (i.c.) with 1000 PFU of the sub-lethal glial tropic mAb-selected J2.2v-1 variant of JHMV ( Fleming et al., 1986). Mice were scored daily following infection for clinical signs as follows: 0, healthy; 1, hunched back; 2, partial hind limb paralysis or reduced ability to regain an upright position; 3, complete hind limb paralysis; 4, moribund or dead ( Fleming et al., 1986). All procedures were
conducted in accordance with animal protocols approved by the Institutional Animal Care and Use Committee of the Cleveland Clinic Foundation. To deplete or functionally inactivate CD25+ T cells mice received 3 intraperitoneal (i.p.) injections of 250 μg of anti-CD25 mAb (clone PC61, rat IgG1) obtained from BioXcell (West Lebanon, NH) on days -3, 0 and 3 relative to infection. Controls received 250 μg of an irrelevant rat IgG1 mAb (clone GL113, anti-β-galactosidase), originally obtained from Dr. R. Coffman (DNAX Corporation, Palo Alto, CA) and prepared as described (Phares et al., 2012) on days -3, 0 and 3 relative to infection.

**Tissue processing and virus titration**

Brains were homogenized in Dulbecco’s PBS using ice cold Tenbroeck tissue homogenizers (Weatone Science Products, Milliville, NJ). After centrifugation at 450 g for 7 min at 4 °C supernatants were stored at −80 °C. Cell pellets were resuspended in RPMI medium containing 25 mM HEPES, pH 7.2 and used for the analysis of CNS inflammation as described below. Brain derived supernatants were used to determine virus titers by plaque assay on monolayers of the continuous Delayed Brain Tumor (DBT) astrocytoma cell line as described (Fleming et al., 1986; Kapil et al., 2009; Phares et al., 2012).

**Isolation of CNS cells**

CNS derived cell pellets resuspended in RPMI medium containing 25 mM HEPES (pH 7.2) were adjusted to 30% Percoll (Pharmacia, Piscataway, NJ). Cells were separated from myelin debris by centrifugation at 850 g for 30 min at 4 °C onto a 70% Percoll cushion. Cells from the 30%/70% interphase were collected, washed and analyzed by flow cytometry or for cytolytic activity. Single cell suspensions from CLN were obtained as described (Bergmann et al., 1999; Kapil et al., 2009; Phares et al., 2012)

**Flow cytometric analysis**

Cells were incubated at 4 °C with 1% mouse serum and 1% rat anti-mouse CD16/CD32 mAb to prevent non-specific staining. Surface marker expression was examined using mAb purchased from BD Biosciences (San Diego, CA) unless otherwise noted. Staining used fluorescein isothiocyanate (FITC), phycoerythrin (PE), Peridinin chlorophyll protein (PerCP) and allophycocyanin (APC)—conjugated mAb specific for: CD45 (30-F11), CD4 (L3T4), CD8 (53-6.7), F4/80 (Serotec, Raleigh, NC), MHCI Class II (2G9), CD25 (PC61), CD62L (Mel-14), CD44 (IM7), CD69 (H1-2F3) and Nrp-1 (R&D Systems, Minneapolis, MN). Virus specific CD8+ T cells were identified using D5SS10 MHCI class I tetramers (Bergmann et al., 1999; Beckman Coulter, Fullerton, CA). Cells from IL-10-GFP and Foxp3-GFP reporter mice were analyzed without fixation and GFP expression identified in the FL1/FITC channel as previously described (Puntambekar et al., 2011). Cells were acquired on a FACSCalibur or FACS Aria flow cytometer (BD Biosciences), and the data analyzed by FlowJo 7.6 software (Tree Star Inc., Ashland, OR).

**Cytotoxic T lymphocyte assay**

Specific lysis was determined using EL4 (H-2b) targets pulsed with 1 μM of S510 peptide encoding the H-2Db restricted immunodominant epitope in serum free RPMI medium for 1 h at 37 °C. After washes, S510 coated cells were labeled with high (2.5 μM) and control cells (no peptide) with low (0.125 μM) carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Eugene OR), respectively. High and low CFSE labeled targets (5 × 104) were plated into 96 well plates in a volume of 100 μl. CNS derived cells were added in a volume of 100 μl at various effector: target (E:T) ratios based on the frequency of virus specific CD8+ T cells identified with D5SS510 tetramer (Bergmann et al., 1999). After 6 h incubation at 37 °C, cells were harvested and directly acquired on a FACS Calibur flow cytometer (BD Biosciences). Data were analyzed by FlowJo 7.6 software (Tree Star Inc., Ashland, OR). Specific lysis was calculated based on the following formula: \( [1 - \frac{[\text{ratio of targets only}/\text{ratio of target+T cells}] \times 100}{1} ] \)

**Gene expression**

Spinal cords were homogenized in TRIzol (Invitrogen, Carlsbad, CA) using a TissueLyzer and stainless steel beads (Qiagen, Valencia CA) and RNA isolated as previously described (Ireland et al., 2008). Briefly, following addition of chloroform samples were centrifuged at 12,000 g for 15 min at 4 °C. RNA was precipitated with isopropyl alcohol, washed with 75% ethanol and resuspended in RNase-free water (Gibco/Invitrogen, Grand Island, NY). DNA was eliminated using a DNA-free kit (Ambion, Austin, TX) according to the manufacturer’s instructions. The cDNA was obtained by reverse transcription using 2 μg of RNA, Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA), 10 mM of deoxynucleoside triphosphate mix, and 250 ng of random hexamer primers (Invitrogen, Carlsbad, CA) for 1 h at 37 °C. Real-time quantitative PCR (qRT-PCR) used SYBR® Green in a 7500 Fast Real-time PCR system (Applied Biosystems, Foster City, CA). Primers specific for the JHMV N gene were: (F: forward, R: reverse) 5’-CCCAAGATTTATTCGACAT-3’ (F) and 5’-GAGCTTCTGTCGCTGTGTT-3’ (R). Ct values were normalized to GAPDH mRNA levels using the following formula: \( 2^{-[\text{Ct (GAPDH) - Ct (gene of interest)}]} \times 1000 \) where Ct is the threshold cycle as previously described (Puntambekar et al., 2011).

**Histopathological analysis**

Spinal cords were fixed in 10% Zinc formalin, divided into 6 sections corresponding to cervical, thoracic and lumbar regions and embedded in paraffin as described previously (Hindinger et al., 2012) Sections were stained with hematoxylin and eosin (H&E) to visualize inflammation or Luxol Fast Blue (LFB) to visualize myelin. For viral antigen, sections were incubated with anti-JHMV mAb J.3.3 specific for the viral N protein and visualized using immunoperoxidase-labeled anti-mouse mAb (Vectorstain-ABC kit, Vector Laboratories, Burlingame, CA). Sections were scored in a blind manner for inflammation as described below. Brain derived inflammation was calculated based on the following formula: \( \frac{[\text{average score of all sections in each experimental group}]}{7} \times 100. \)

**Statistical analysis**

Statistical significance was determined using standard two-tailed Student’s t test, assessed by GraphPad Prism 5.0 software (La Jolla, CA). The results are shown as either mean ± SEM or mean ± SD for each group. A value of \( p < 0.05 \) was considered statistically significant.

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