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Mouse hepatitis virus pathogenesis in the central nervous system is independent of IL-15 and natural killer cells

Jun Zuo [a], Stephen A. Stohlman [a,b], Jason B. Hoskin [a], David R. Hinton [a], Roscoe Atkinson [a], Cornelia C. Bergmann [a,b,*]

[a] Department of Pathology, University of Southern California Keck School of Medicine, Los Angeles, CA 90033, USA
[b] Department of Neurology, University of Southern California Keck School of Medicine, Los Angeles, CA 90033, USA

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Abstract

Infection by the neurotropic JHM strain of mouse hepatitis virus (JHMV) results in an acute encephalomyelitis associated with demyelination. T cells are critical in controlling viral replication, but also contribute to central nervous system (CNS) pathogenesis. To reveal a role for innate effectors in anti-viral immunity and neurological disease, JHMV pathogenesis was studied in mice deficient in interleukin-15 (IL-15$^{-/-}$) and natural killer (NK) cells. Clinical disease, CNS inflammation and demyelination in infected IL-15$^{-/-}$ mice were similar to wild-type mice. Despite the absence of NK cells and suboptimal CD8^{+} T cell responses, IL-15$^{-/-}$ mice controlled JHMV replication as efficiently as wild-type mice. Similar kinetics of class I and class II upregulation on microglia further suggested no role of NK cells in regulating major histocompatibility complex (MHC) molecule expression on resident CNS cells. IL-15 and NK cells thus appear dispensable for anti-viral immunity and CNS pathogenesis during acute JHMV infection.

Keywords: Interleukin-15; Natural killer cells; CD8^{+} T cells; Coronavirus; Encephalitis

Introduction

The neurotropic JHM strain of mouse hepatitis virus (JHMV) infects multiple cell types in the central nervous system (CNS) and causes acute encephalomyelitis and demyelination in rodents (Marten et al., 2001; Wang et al., 1990). Acute inflammatory responses in the CNS involve production of matrix metalloproteinases, pro-inflammatory chemokines and cytokines, and recruitment of both innate and adaptive immune cells (Bergmann et al., 2003; Glass et al., 2002; Li et al., 2004; Marten et al., 2001; Zhou et al., 2005b). JHMV replication is controlled by T cells, prominently CD8^{+} T cells, about 2 weeks after infection (Bergmann et al., 2003; Marten et al., 2001). Nevertheless, viral RNA and inflammatory cells persist in the CNS long after clearance of infectious virus (Marten et al., 2001). Neutralizing antibodies are crucial for the final stage of clearance of infectious virus and prevent recrudescence of persisting virus (Lin et al., 1999; Ramakrishna et al., 2003). A pathological hallmark of JHMV infection is ongoing immune-mediated demyelination, similar to the human demyelinating disease, multiple sclerosis. Although there is little evidence for demyelination in immune-deficient severe combined immunodeficiency (SCID) or recombination activating gene 1$^{-/-}$ (RAG1$^{-/-}$) mice, numerous depletion and adoptive transfer studies implicate both innate and adaptive cell types in contributing to demyelination (Fleming et al., 1993; Houtman et al., 1995; Marten et al., 2001; Stohlman and Hinton, 2001). Although NK cells and γδ T cells display anti-viral activity during CNS infection in the absence of αβT cells (Dandekar and Perlman, 2002; Paya et al., 1989; Trifilo et al., 2004), a protective role for these innate cells has yet to be confirmed in a non-lymphopenic host. Similarly, although γδ T cells can influence demyelination during JHMV infection of immunocompromised hosts (Dandekar et al., 2005; Dandekar and Perlman, 2002), their pathogenic contribution in the

* Corresponding author. Department of Neurosciences, Cleveland Clinic Foundation, 9500 Euclid Avenue NC30, Cleveland, OH 44195, USA. Fax: +1 216 444 7927.
E-mail address: bergmac@ccf.org (C.C. Bergmann).

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presence of αβ T cells, which constitute a vastly larger inflammatory population, remains to be confirmed.

NK cells and γδ T cells both require IL-15 for development and homeostatic maintenance (Kennedy et al., 2000). IL-15 can be produced by many cell types, including microglia and activated astrocytes (Hanisch et al., 1997; Kurowska et al., 2002; Lee et al., 1996; Li et al., 2004; Maslinska et al., 2002; Nagai et al., 2001). This pleiotropic factor further supports NK cell expansion during infection (Lodolce et al., 1998; Nguyen et al., 2002) and is important for homeostatic maintenance of memory $CD8^+$ T cells (Becker et al., 2002; Tan et al., 2002). Induction of IL-15 following mouse hepatitis virus (MHV) infection of CNS-derived cells (Li et al., 2004) and during experimental autoimmune encephalitis (Tran et al., 2000) implies a role of this cytokine in enhancing both innate and adaptive CNS immune responses. This notion is consistent with reports suggesting a correlation between increased IL-15 expression and progression of multiple sclerosis (Blanco-Jerez et al., 2002; Kivisakk et al., 1998; Losy et al., 2002; Pashenkov et al., 1999), which may reflect pro-inflammatory properties of IL-15 (Ma et al., 2000).

Based on the role of IL-15 in both innate and adaptive peripheral immune responses, as well as its potential regulation of CNS inflammatory disease, the contribution of IL-15 to JHMV pathogenesis was examined in IL-$15^{-/-}$ mice. IL-15 may affect CNS inflammation, viral clearance and demyelination directly or via activities of NK cells, γδ T cells and $CD8^+$ T cells. Naive IL-$15^{-/-}$ mice have no NK cells and diminished peripheral $CD8^+$ T cells, especially within the $CD122^+$ (IL-2/15 Rβ chain), $CD44^+$ memory subset (Kennedy et al., 2000). However, following peripheral infections with lymphocytic choriomeningitis virus (LCMV), recombinant vaccinia virus and recombinant Sindbis virus, no defects in $CD8^+$ T cell expansion were detected (Becker et al., 2002; Zuo et al., 2005). Furthermore, initial expansion of $CD8^+$ T cells was only marginally affected following vesicular stomatitis virus infection (Schluns et al., 2002). Confirming effective $CD8^+$ T cell function, peripheral infections were all controlled in IL-$15^{-/-}$ mice with kinetics similar to wild-type (wt) mice (Becker et al., 2002; Schluns et al., 2002; Zuo et al., 2005). These data predicted that virus-specific $CD8^+$ T cell expansion should not be significantly affected following JHMV infection, providing a model to monitor the influence of NK and γδ T cells during CNS infection of a relatively immunocompetent host.

The present study demonstrates that IL-15 plays a redundant role in JHMV pathogenesis and control of virus replication in the CNS. Although the frequency of virus-specific effector $CD8^+$ T cells recruited to the CNS was reduced compared to wt mice, clearance of infectious virus was only slightly delayed. Despite the absence of NK cells, there was no evidence for delayed IFNγ-induced MHC expression on CNS resident cells, suggesting that these effectors do not contribute significantly to viral clearance or enhanced adaptive immune effector functions within the CNS. Furthermore, the absence of innate lymphocytes and reduced $CD8^+$ T cell numbers did not ameliorate clinical disease or demyelination. Thus, despite the pleiotropic role of IL-15 in regulating innate and adaptive immune responses (Ma et al., 2000), little evidence was obtained supporting a role of either NK cells or γδ T cells in JHMV-induced encephalomyelitis or demyelination in otherwise immunocompetent hosts.

Results

Inflammation and pathogenesis in the CNS

IL-$15^{-/-}$ mice were infected with JHMV to examine effects of IL-15 deficiency, associated with absence of NK and γδ T cells, on CNS inflammation and pathogenesis. The mean day of onset, extent of clinical disease, morbidity and recovery from acute JHMV infection of IL-$15^{-/-}$ mice were similar to those of wt mice (data not shown). To determine how the composition of bone marrow-derived $CD45^{hi}$ inflammatory cells was altered in the absence of IL-15, CNS inflammatory cells were characterized by flow cytometry. The absence of NK cells and reduced peripheral $CD8^+$ T lymphocyte subsets in IL-$15^{-/-}$ mice only slightly diminished the overall numbers of inflammatory cells recruited into the CNS during acute JHMV infection (Table 1). NK cells were rapidly recruited into the CNS in wt mice, peaked at day 5 p.i. and then declined to barely detectable levels by day 14 p.i., in contrast to their absence from the CNS of infected IL-$15^{-/-}$ mice. Macrophages constituted the vast majority of CNS infiltrating cells in both groups early following infection, suggesting IL-15-independent recruitment. Although the percentage of macrophages declined as inflammation increased, they were retained at similar levels in both groups (Table 1). The absence of IL-15 resulted in diminished $CD8^+$ T cell recruitment, possibly reflecting the overall reduction in peripheral $CD8^+$ T cells (Kennedy et al., 2000; Tan et al., 2002; Zuo et al., 2005). No significant differences in either percentages or expression levels of activation markers, i.e., CD43, CD44 and CD62L, on infiltrating $CD8^+$ T cells were detected comparing the two groups (data not shown). Although there was no apparent increase in $CD4^+$ T cells early in infection, substantially higher percentages of $CD4^+$ T cells were evident in the CNS of IL-$15^{-/-}$ mice during peak inflammation (Table 1). Surprisingly, although IL-15 affects the γδ T cell

Table 1
CNS infiltrating cells during acute JHMV infection of IL-$15^{-/-}$ mice[a]

Days p.i.[b]	Mice	$CD45^{hi}$	% within $CD45^{hic}$			
		Cells ($\times 10^4$)[d]	NK1.1	F4/80	CD4	CD8
5	wt	50.0 ± 15.2	8.0	65.0	8.6	2.8
	IL-$15^{-/-}$	32.7 ± 5.7	ND[e]	68.8	9.8	1.2
7	wt	92.3 ± 6.3	4.9	49.2	16.0	21.1
	IL-$15^{-/-}$	92.3 ± 6.3	ND[e]	55.5	19.6	10.3
9	wt	119.0 ± 26.9	2.1	34.3	25.5	29.1
	IL-$15^{-/-}$	92.3 ± 6.3	ND[e]	36.6	44.9	16.5
14	wt	45.1 ± 9.8	1.4	16.6	43.8	28.1
	IL-$15^{-/-}$	43.1 ± 13.7	ND[e]	15.3	57.8	17.0

[a] Infected with 250.
[b] Days post-infection.
[c] Only means are shown.
[d] Averages of 6 experiments.
[e] Not detected.

population associated with the gastrointestinal track (Kennedy et al., 2000), γδ T cells constituted between 1.4 and 2.1% of the inflammatory population at day 5 p.i. in the CNS of wt and IL-15$^{-/-}$ mice and remained between 0.8 and 2.1% out to day 17 p.i. (data not shown). There was no evidence for decreased numbers in the CNS of IL-15$^{-/-}$ compared to wt mice at any single time point, contrasting the NK cell population.

Induction of JHMV-specific CD8$^+$ T cells in lymphoid organs

A considerable proportion of bystander CD8$^+$ T cells, characterized by a non-activated memory phenotype, are recruited during CNS JHMV infection (Chen et al., 2005). The reduced CD8$^+$ T cell percentage in the CNS of infected IL-15$^{-/-}$ mice thus likely reflects the reduced memory cells in lymphoid organs (Kennedy et al., 2000; Tan et al., 2002). Furthermore, expansion of virus-specific CD8$^+$ T cells in IL-15$^{-/-}$ mice following peripheral infection is not compromised (Becker et al., 2002; Schluns et al., 2002; Zuo et al., 2005), consistent with the concept that IL-15 acts primarily to sustain memory cells (Prlic et al., 2002). To test how IL-15 deficiency impacts virus-specific CD8$^+$ T cell expansion following an infection restricted to the CNS, JHMV-specific CD8$^+$ T cells were compared in the CNS, spleen and CLN, the primary site of T cell priming following CNS infection (Dorries, 2001; Marten et al., 2003). The proportions of JHMV spike protein-specific CD8$^+$ T cells were comparable in spleen and CLN in IL-15$^{-/-}$ and wt mice at day 7 p.i. (Fig. 1A), supporting IL-15-independent expansion (Becker et al., 2002; Zuo et al., 2005). Analysis of the CNS at day 7 p.i. revealed slightly reduced percentages of tetramer$^+$ cells within the CD8$^+$ T cell population derived from the infected IL-15$^{-/-}$ mice. Surprisingly, although the majority of tetramer$^+$ cells in secondary lymphoid organs expressed CD122 (IL-2/15Rβ), virus-specific CD8$^+$ T cells recruited into the CNS were CD122$^{-/dim}$ in both groups (Fig. 1). These data imply either preferential recruitment of the CD122$^{-/dim}$ population or downregulation of CD122 expression upon CNS entry. Overall, the relative number of virus-specific CD8$^+$ T cells within the CNS was reduced 2–3-fold in the infected IL-15$^{-/-}$ mice compared to wt mice during peak inflammation at day 7 p.i. (Fig. 1B). Although the CD8$^+$ T cells in the CNS of wt mice declined as infectious virus and viral antigen was reduced, the total number of virus-specific CD8$^+$ T cells in the CNS of the IL-15$^{-/-}$ mice remained constant (Fig. 1B). These data suggest that induction, expansion and recruitment of virus-specific CD8$^+$ T cells in response to an infection localized to the CNS are independent of IL-15.

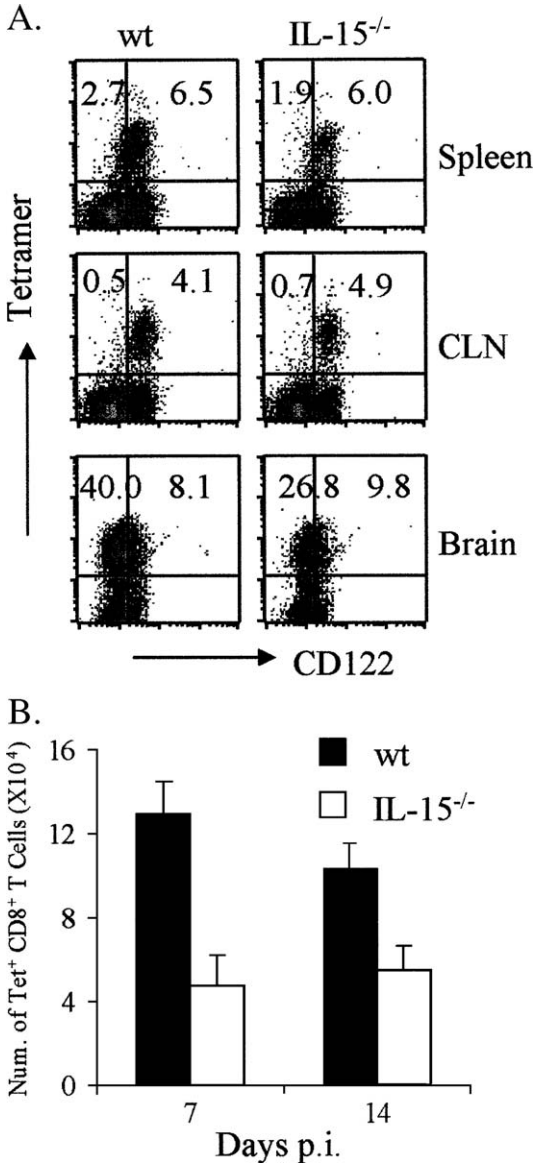

Fig. 1. Distinct phenotypes and frequencies of JHMV-specific CD8$^+$ T cells in lymphoid organs and the CNS. (A) JHMV-specific CD8$^+$ T cells in the spleen, cervical lymph nodes and brain were assessed by Dd/S510 tetramer staining and flow cytometry at day 7 p.i. The plots are gated on CD8$^+$ T cells. A representative of 3 independent experiments using mononuclear cells from pooled animals ($n \geq 3$) is shown. The numbers in each quadrant represent percentages within CD8$^+$ T cells. (B) Absolute numbers of tetramer$^+$ CD8$^+$ T cells in the brain were obtained based on yields of cell preparations and flow cytometric analysis at days 7 and 14 p.i. Data are presented as mean ± SEM of 3 independent experiments in which more than 3 mice were used at each time point per group.

Efficient control of JHMV infection of the CNS

NK cells played a major role in control of a recombinant MHV expressing CXCL10 in the CNS of RAG1$^{-/-}$ mice (Trifilo et al., 2004). The absence of NK cells, coupled with reduced virus-specific CD8$^+$ T cells within the CNS of infected IL-15$^{-/-}$ mice suggested that control of virus replication may be defective. Nevertheless, JHMV replication in the CNS of IL-15$^{-/-}$ mice was not significantly affected at days 3 or 5 p.i. and only slightly increased at day 7 p.i. compared to wt mice (Fig. 2A). Overall, IL-15$^{-/-}$ mice controlled infectious virus within the CNS with kinetics very similar to wt mice (Fig. 2). Viral antigen in the CNS of IL-15$^{-/-}$ and wt mice was compared at day 7 p.i. (Fig. 2B) to determine if the slight increase in infectious virus represented altered viral tropism. However, no difference in either the amount or distribution of viral antigen, predominantly in astrocytes and oligodendroglia, was detected when comparing both groups (Fig. 2B). This suggests an

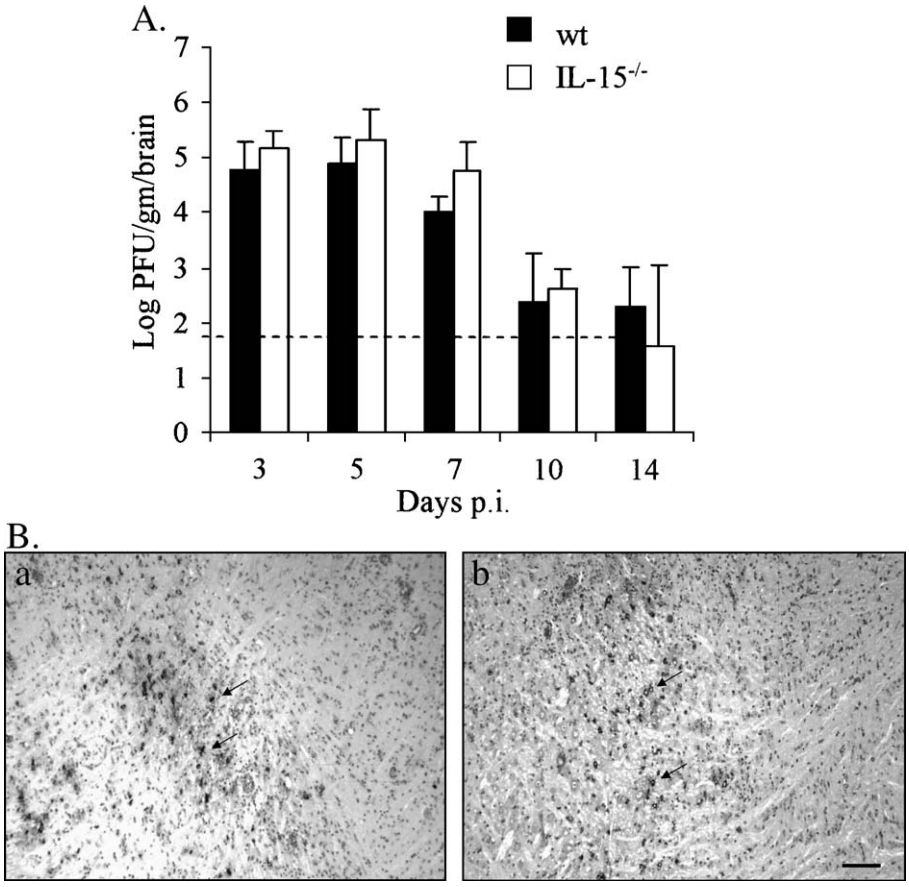

Fig. 2. Control of JHMV replication in the CNS of IL-15$^{-/-}$ mice. (A) Infectious virus within the CNS of JHMV-infected IL-15$^{-/-}$ and wt mice was determined by plaque assay as described in Materials and methods. The dashed line marks the detection level. Data represent mean ± SD ($n \geq 3$/group) and are representative of 3 separate experiments. (B) Distribution of viral antigen in brains of JHMV infected wt (a) and IL-15$^{-/-}$ mice (b) determined at day 7 p.i. by immunoperoxidase staining using anti-JHMV mAb J.3.3. Antigen positive cells are demonstrated by the presence of chromogen aminoethylcarbzole (arrows). Sections counterstained with hematoxylin. The pattern and extent of antigen staining were similar in wt and IL-15$^{-/-}$ mice. Scale bar = 100 μm.

apparent redundancy of NK cells, and a sufficient virus-specific CD8$^+$ T cell response to mediate virus control in the CNS in the absence of IL-15.

IL-15-independent MHC expression on microglia

In the quiescent CNS, resident glial cells express few if any MHC molecules. However, pro-inflammatory cytokines, including interferons and TNF, induce increased expression of MHC molecules in the CNS during inflammation (Bergmann et al., 2003; Liu and Lane, 2001; Zhou et al., 2005b). JHMV infection induces MHC class I molecules on microglia, independent of IFNγ; however, expression of MHC class II molecules depends exclusively on IFNγ (Bergmann et al., 2003). Although NK cells appeared to play no direct role in viral clearance, they may nevertheless enhance early MHC molecule expression on glial cells via IFNγ secretion and thus optimize αβ T cell effector function. To evaluate a potential role of NK cells in enhancing antigen presentation capacity, the kinetics of MHC class I and II upregulation were assessed on microglia (Fig. 3). Class I molecule expression emerged as early as day 3 p.i. in both IL-15$^{-/-}$ and wt mice (Fig. 3A). Although microglia derived from infected IL-15$^{-/-}$ expressed less class I molecules at day 5 p.i., expression was maximal on microglia in both groups of mice by day 7 p.i. In contrast to class I expression, class II molecules remained below detection on microglia from either group at day 3 p.i. and only increased slightly by day 5 p.i. This delay in class II expression, relative to class I, was overcome at day 7 p.i. when ~90% of microglia derived from both groups of JHMV-infected mice expressed MHC class II molecules (Fig. 3B). The absence of an evident difference in MHC molecule upregulation suggests little, if any, contribution of innate lymphocytes to IFNγ secretion early during infection. Maximal MHC molecule expression coincident with peak CNS T cell numbers underscores the critical role of αβ T cells as a source of IFNγ in regulating both class I and class II molecule expression on CNS resident cells.

JHMV-specific CD8$^+$ T cell responses in the CNS

CNS infiltrating CD8$^+$ T cells in both IL-15$^{-/-}$ and wt mice were CD44^{hi}, CD62L$^{lo/-}$, expressed similar levels of CD43, and were CD127$^-$ (data not shown), a pattern associated with effector function (Kaech et al., 2003; Onami et al., 2002). Furthermore, the majority of CNS CD8$^+$ T cells in both groups apparently downregulated CD122 expression (Fig. 1A),

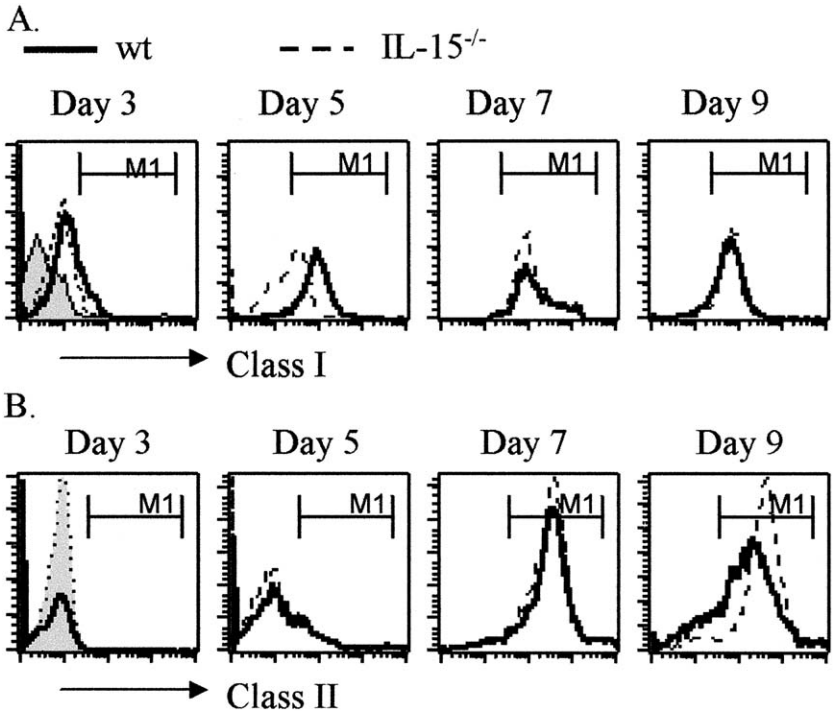

Fig. 3. MHC expression on microglia. Expression of MHC class I (A) and MHC class II (B) on CD45^{lo} microglia derived from infected IL-15$^{-/-}$ and wt mice at indicated time points. Shaded areas represent histograms of control isotype antibodies. Solid lines and dashed lines represent histograms of wt mice and IL-15$^{-/-}$ mice, respectively. Representative of 5 separate experiments.

suggesting low responsiveness to ligands involved in CD122 binding. CNS CD8$^+$ T cell functions were evaluated to support phenotypic similarities observed in both groups. Virus-specific IFNγ production was detected in ~20% of infiltrating CD8$^+$ T cells in both IL-15$^{-/-}$ and wt mice at day 7 p.i. (Fig. 4A). Consistent with the slight reduction in CD8$^+$ T cells infiltrating the CNS of the infected IL-15$^{-/-}$ mice, CNS mononuclear cells from IL-15$^{-/-}$ mice expressed reduced ex vivo cytolytic activity compared to wt mice at the population level (Fig. 4B). However, adjustment to cytolysis per tetramer$^+$ CD8$^+$ T cell revealed no differences in lytic capacity between cells from the CNS of IL-15$^{-/-}$ and wt mice (Fig. 4B). Therefore, despite decreased CD8$^+$ T cell numbers within the CNS in the absence of IL-15, differentiation to anti-viral effector T cells, based on both the ability to secrete IFNγ and express cytolytic activity, was not affected. Similar kinetics of class I expression (Fig. 3)

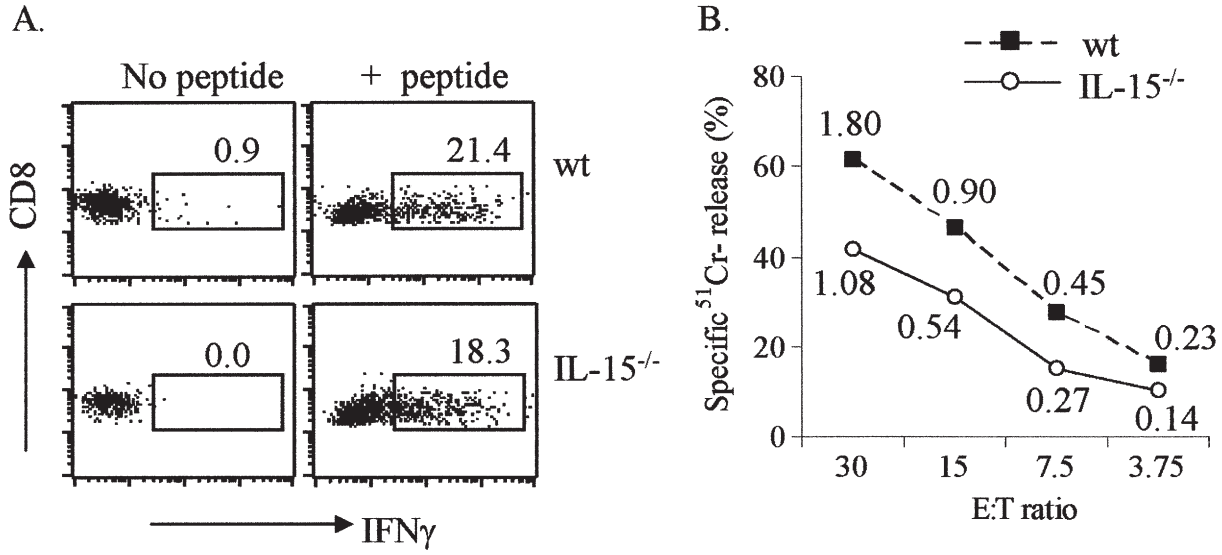

Fig. 4. Effector function expression by CNS-derived virus-specific CD8$^+$ T cells. (A) Frequency of virus-specific IFNγ-secreting CD8$^+$ T cells within the CNS of infected wt and IL-15$^{-/-}$ mice at day 7 p.i. Percentages of IFNγ$^+$ CD8$^+$ T cells (inside rectangles) within infiltrating CD8+ T cells are shown. (B) Ex vivo virus-specific cytolytic activity mediated by CNS-derived CD8$^+$ T cells from wt and IL-15$^{-/-}$ mice at day 7 p.i. Cytolysis was measured as described in Materials and methods. Frequencies of virus-specific CD8$^+$ T cells in CNS-derived mononuclear cells were determined by tetramer-staining and flow cytometry. E:T ratios based on tetramer$^+$ CD8$^+$ T cells are shown. Representative of 3 independent experiments.

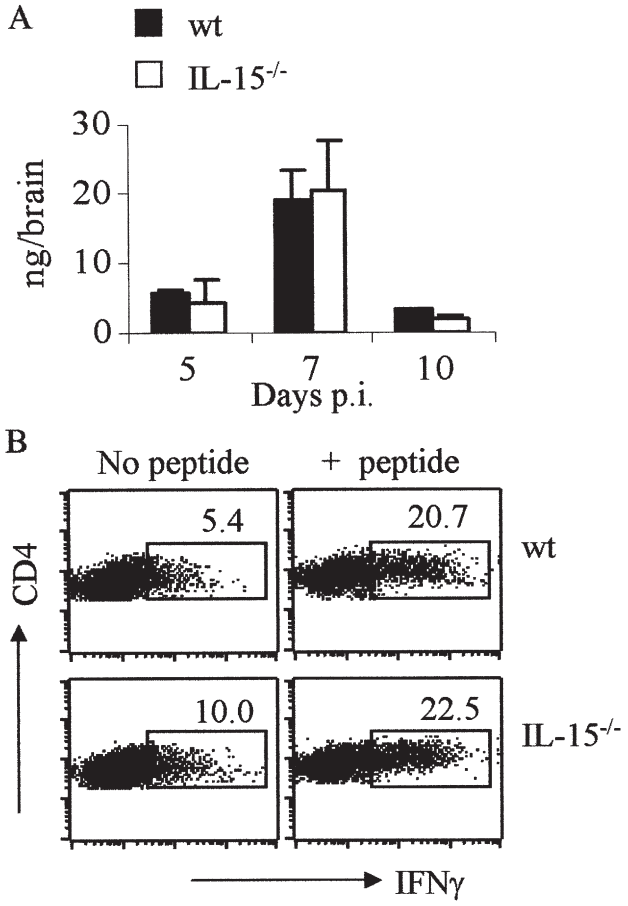

Fig. 5. IFNγ within the CNS of IL-15$^{-/-}$ and wt mice. (A) CNS IFNγ was assessed by ELISA at various times p.i. in infected wt and IL-15$^{-/-}$ mice. Data represent mean ± SEM of individual mice ($n \geq 3$). (B) Frequencies of virus-specific IFNγ secreting CD4$^+$ T cells (inside rectangles) within the CNS of JHMV-infected wt and IL-15$^{-/-}$ mice at day 7 p.i. CNS-derived mononuclear cells from pooled animals ($n \geq 3$) were stimulated with or without peptide for 6 h and then stained for intracellular IFNγ. Plots are gated on infiltrating CD4$^+$ T cells and percentages of IFNγ$^+$ CD4$^+$ T cells within CD4$^+$ T cells are shown. Representative of 3 independent experiments.

supports the notion that CD8$^+$ T cells are a prominent source of IFNγ secretion at day 5 p.i. as only few class II expressing cells are available to trigger IFNγ secretion by CD4$^+$ T cells.

Potent IFNγ production within the CNS in the absence of IL-15

IFNγ is crucial for the control of JHMV in the CNS, as it enhances both CD4$^+$ and CD8$^+$ T cell function by increasing MHC molecule expression on target cells (Bergmann et al., 2003, 2004). It is furthermore vital for viral clearance from oligodendrocytes (Bergmann et al., 2004; Parra et al., 1999). Similar patterns of MHC expression on microglia suggested comparable IFNγ production in both groups, despite the absence of NK cells and reduced virus-specific CD8$^+$ T cells within the inflamed CNS (Table 1; Fig. 1). Therefore, CNS IFNγ levels were measured to test whether MHC upregulation was saturated, thus under representing in vivo IFNγ levels. IFNγ was already present in the CNS of both groups at day 5 p.i., increased sharply at day 7 p.i., coincident with the peak of T cell responses (Table 1), and rapidly declined at day 10 p.i. (Fig. 5A). These data suggest a downregulation of IFNγ secretion within the CNS as both infectious virus and viral antigen are cleared, even though both CD4$^+$ and CD8$^+$ T cells were still abundant (Table 1). The slight over-representation of CD4$^+$ T cells within the CNS inflammatory population in IL-15$^{-/-}$ mice suggested the possibility that this population may compensate for decreased CD8$^+$ T cells. This interpretation is supported by the ability of nearly equivalent frequencies (~20%) of CNS-derived virus-specific CD4$^+$ T cells to secrete IFNγ (Fig. 5B).

These data suggest that neither the absence of IL-15 nor absence of NK cells affects virus clearance from the CNS. Although NK cells have not directly been implicated in the process of myelin loss following JHMV infection, recent data suggested that γδ T cells influence JHMV-induced demyelination (Dandekar et al., 2005; Dandekar and Perlman, 2002). To examine the possibility that IL-15 deficiency influenced the loss of myelin in a relatively immunocompetent host, demyelination was compared in infected IL-15$^{-/-}$ and wt mice. Focal areas of demyelination were present in the brains and spinal cords of both groups at day 10 p.i. (data not shown). Consistent with previous data (Wang et al., 1990), the extent of spinal cord demyelination increased with time p.i. in both groups. Although focal areas of myelin loss and inflammation were evident in the spinal cords of both groups (Fig. 6), no differences in the extent or distribution of inflammatory cells or demyelinated lesions

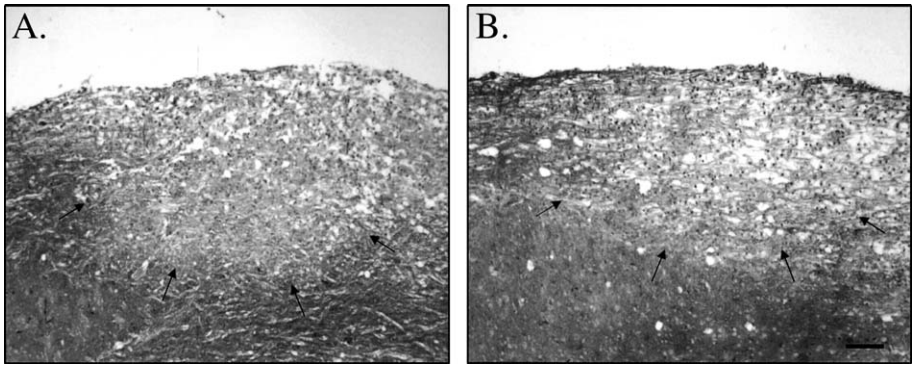

Fig. 6. Inflammation and demyelination in the CNS of JHMV-infected wt and IL-15$^{-/-}$ mice. Extent of demyelination and inflammation in spinal cords of infected wt (A) and IL-15$^{-/-}$ (B) mice at day 14 p.i. by Luxol fast blue staining. Size and distribution of plaques of demyelination (outlined by arrows) and extent of mononuclear cell infiltration are similar in both groups. Scale bar = 100 μm.

were detected at day 14 p.i. In addition, no differences in the extent or distribution of F4/80$^+$ macrophages, CD4$^+$ or CD8$^+$ T cells were detected comparing the CNS of infected IL-15$^{-/-}$ and wt mice (data not shown). These data suggest that neither the absence of IL-15 nor absence of NK cells influences myelin loss following JHMV infection of otherwise immunocompetent hosts.

Discussion

IL-15 is an important pleiotropic cytokine crucial for NK cell development and homeostatic maintenance of memory CD8$^+$ T cells (Kennedy et al., 2000; Ma et al., 2000; Prlic et al., 2002). Despite the profound defect in these cell populations, IL-15$^{-/-}$ mice mount effective virus-specific CD8$^+$ T cell responses and control numerous experimental peripheral viral infections (Becker et al., 2002; Obar et al., 2004; Schluns et al., 2002; Zuo et al., 2005). An exception is the increased mortality following neurovirulent vaccinia virus infection (Kennedy et al., 2000). This report is the first to demonstrate that IL-15 is not required to control a coronavirus infection, confined to the CNS and dependent on CD8$^+$ T cell responses for anti-viral control (Bergmann et al., 2003; Marten et al., 2001).

JHMV infection is restricted to the brain and spinal cord and results in the recruitment of multiple innate and adaptive effector cells, including both NK and CD8$^+$ T cells (Marten et al., 2001). Several observations suggested that NK cell activity may be important in early control of JHMV, possibly prior to induction of adaptive immunity. Among these are the slight reduction in virus replication in SCID mice (Houtman et al., 1995) and the apparent partial control of infectious virus in the CNS of mice deficient in either CD4$^+$ or CD8$^+$ T cells (Williamson and Stohlman, 1990). Early control of a less neurovirulent MHV-A59 strain engineered to express CXCL10 in RAG1$^{-/-}$ mice also supports NK cell-mediated anti-viral function (Trifilo et al., 2004). By contrast, analysis of NK deficient IL-15$^{-/-}$ mice failed to detect any evidence for delayed or reduced IFNγ production or other anti-viral activity following JHMV infection. Both direct analysis of IFNγ and indirect analysis via MHC class II upregulation on microglia revealed no differences during the time of maximal NK cell recruitment. The notion that NK cells do not exert detectable effector function within the inflamed CNS in immunocompetent hosts was further supported by the inability to detect NK cytolytic activity in splenocytes of infected wt mice (data not shown). The discrepancy between the apparently redundant role of NK cells in immunocompetent mice and the data obtained in RAG1$^{-/-}$ mice (Trifilo et al., 2004) may reside in the more rapid and increased NK cell inflammation achieved by over-expression of CXCL10.

The absence of IL-15 did not affect virus-specific CD8$^+$ T cell expansion, CNS recruitment or differentiation into antigen-specific effector cells. Rapid activation of CD8$^+$ T cells was also evident following peripheral JHMV infection (unpublished data). Induction and expansion of T cells in response to CNS infection occur in secondary lymphoid organs (Dorries, 2001; Marten et al., 2003). Consistent with signaling through the IL-2/15Rβ chain CD122 during the expansion phase, possible via IL-2, the majority of JHMV-specific CD8$^+$ T cells in the spleen and CLN expressed CD122 in both IL-15$^{-/-}$ and wt mice. Interestingly, the vast majority of CD8$^+$ T cells recruited into the CNS in the brain were CD122$^-$. CD122 expression is thus either downregulated after CD8$^+$ T cells enter the CNS, or alternatively, the CD122$^{-/lo}$ population preferentially traffics into the CNS. The first explanation is favored by the low frequency of CD122$^{-/lo}$ virus-specific CD8$^+$ T cells in lymphoid organs as well as the downregulation of CD122 on splenic CD8$^+$ T cells after 6 h in vitro stimulation with peptide (data not shown). Whether CD122 is downregulated as a result of abundant Ag stimulation or withdrawal of CD122 ligands, e.g., IL-2, is presently unclear. However, the dispensability of CD122 of CNS localized T cells is consistent with the notion that T cell expansion is inhibited following entry into infected non-lymphoid parenchymal tissues. In the CNS, T cell expansion appears to be inhibited by prostaglandins and other down-regulatory factors (Irani and Griffin, 2001). However, recent evidence from the Theiler's murine encephalitis virus model disputes this notion by demonstrating de novo priming and proliferation of auto-reactive TCR transgenic T cells within the CNS (McMahon et al., 2005). Irrespectively, the apparent survival of CD122$^{-/lo}$ polyclonal CD8$^+$ T cells in the CNS is consistent with the absence of an adverse effect of IL-2 inhibition during JHMV infection of the CNS (Zhou et al., 2005a).

Reduced absolute numbers of virus-specific CD8$^+$ T cells recruited into the CNS of IL-15$^{-/-}$ mice compared to wt mice likely reflect the overall reduced peripheral CD8$^+$ T cell population in naive IL-15$^{-/-}$ mice. The small, but reproducible, increase in infectious virus in the CNS of the IL-15$^{-/-}$ mice at day 7 p.i. may thus reflect the overall reduction in the CNS infiltrating CD8$^+$ T cells. Nevertheless, IL-15$^{-/-}$ mice controlled JHMV replication with kinetics similar to wt mice, disputing an overall defect in anti-viral activity. JHMV clearance from the CNS in wt mice is dependent upon both perforin-mediated cytolysis and IFNγ secretion (Bergmann et al., 2004; Lin et al., 1997; Parra et al., 1999). Direct measurement of IFNγ showed comparable amounts of IFNγ suggesting that virus-specific CD4$^+$ T cells may compensate for reduced CD8$^+$ T cells in IL-15$^{-/-}$ mice. Although a considerable fraction of IL-15$^{-/-}$ CD4$^+$ T cells produced IFNγ following Ag stimulation, no evidence for altered virus tropism or enhanced MHC class II expressing macrophages/microglia was detected. Overall, this finding underscores flexibility of T cell responses and a synergy between CD4$^+$ and CD8$^+$ T cells in controlling CNS viral infections.

IL-15 and γδ T cells have been implicated in regulating the process of myelin loss (Blanco-Jerez et al., 2002; Dandekar et al., 2005; Dandekar and Perlman, 2002; Kivisakk et al., 1998; Losy et al., 2002; Pashenkov et al., 1999). However, no differences in the extent or distribution of demyelinated lesions were found in either brains or spinal cords of infected IL-15$^{-/-}$ mice compared to wt mice. Although the gastrointestinal tract of IL-15$^{-/-}$ mice is deficient in γδ T cells (Kennedy et al., 2000), it was surprising to detect similar, albeit small numbers of γδ T cells in the CNS of both wt and IL-15$^{-/-}$-infected mice

throughout acute infection. However, no significant differences between the groups could be detected in these small populations at any given time point. Therefore, the analysis of JHMV pathogenesis in IL-15$^{-/-}$ was unable to shed light on the potential role of this T cell subset, implicated in JHMV induced demyelination in nude mice (Dandekar et al., 2005; Dandekar and Perlman, 2002). In conclusion, our data demonstrate that IL-15 is not essential for effective anti-viral immunity against a neurotropic coronavirus infection. Although NK cells are recruited into the infected CNS, no evidence could be found to support either direct anti-viral effector functions or indirect function mediated via enhanced adaptive immunity. Both IL-15 and NK cells are thus dispensable for inflammation, demyelination and pathogenesis in the CNS. These data underscore the importance of adaptive immune responses during JHMV infection of the CNS.

Materials and methods

Mice and infection

Homozygous C57BL/6 IL-15$^{-/-}$ (IL-15$^{-/-}$) mice (Kennedy et al., 2000), originally provided by Amgen Corporation (Seattle, WA), were bred locally. Syngeneic C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD) at 5–6 weeks of age. Mice were housed in an accredited animal facility at the University of Southern California and were used between 6 and 8 weeks of age. All procedures were performed in compliance with protocols approved by the Keck School of Medicine Institutional Animal Care and Use Committee. For each experiment, mice were both sex- and age-matched, and groups of at least 3 individuals were analyzed per time point. No gender-dependent differences were observed. Infections were initiated by intracerebral injection of 250 PFU of the J.2.2v-1 monoclonal antibody (mAb) neutralization-derived JHMV variant as previously described (Fleming et al., 1986). Infected mice were scored for clinical symptoms daily as previously described (Lin et al., 1997).

Virus titers and IFNγ ELISA

Infectious JHMV within the CNS was determined in clarified supernatants derived from homogenates of one-half brain by plaque assay on monolayers of DBT cells as previously described (Lin et al., 1997). Plaques were counted after 48 h incubation at 37 °C. Supernatants were also tested for IFNγ by ELISA. Briefly, 96 well plates (Dynex Technologies, Chantilly, VA) were coated for 18 h at 4 °C with 100 μl containing 1.5 μg/ml of anti-IFNγ (R4-6A2). Non-specific binding was blocked with PBS containing 10% FCS. Samples and recombinant cytokines (BD PharMingen) were adsorbed overnight 4 °C. Color was developed by sequential addition of biotinylated detection anti-IFNγ (XMG1.2) followed by avidin peroxidase and hydrogen peroxide (TMB Substrate Reagent A, BD PharMingen) and 3,3′,5,5′ Tetramethylbenzidine (TMB Substrate Reagent B) 30 min later. Color was developed for 15 min and results read at 405 nm using a Bio-Rad Model 680 microplate reader and analyzed using Microplate Manager 5.2 software (Bio-Rad Laboratories, Hercules, CA).

Mononuclear cell preparations

CNS mononuclear cells were isolated at various time points post-infection (p.i.) from brains or spinal cords (SC) of 3–6 mice per group, as previously described (Bergmann et al., 2003; Marten et al., 2000). Briefly, tissues were homogenized in ice-cold Tenbrock homogenizers, adjusted to 30% Percoll (Pharmacia, Uppsala, Sweden) and suspensions layered onto a 1 ml 70% Percoll cushion. Following centrifugation at 800×g for 25 min at 4 °C, cells were recovered from the 30%/70% Percoll interface and resuspended in RPMI medium. Single cell suspensions were prepared from RBC-depleted spleens or cervical lymph nodes (CLN) as previously described (Marten et al., 2000).

Flow cytometric analysis

Expression of cell surface markers was determined as described previously (Zuo et al., 2005). Briefly, non-specific antibody binding was inhibited by incubation with anti-mouse CD16/CD32 (2.4G2, BD PharMingen) for 15 min at 4 °C prior to staining. For flow cytometric analysis, cells were stained with FITC-, PE- or CyChrome-conjugated mAb (for 3 color analysis), or FITC-, PE-, Percp- or APC-conjugated mAb (for 4 color analysis) for 30 min at 4 °C in PBS containing 0.1% BSA using mAb specific for CD8 (53–6.7), CD4 (GK1.5), NK1.1 (PK136), γδ TCR (GL3), CD43 (1B11), CD44 (IM7), CD45(30–F11), CD62L(MEL-14), CD122 (TM-β1) (all from BD PharMingen), CD127(A7R34, eBioscience, San Diego, CA) and F4/80 (A3-1, Serotech, Raleigh, NC). Virus-specific CD8$^+$ T cells were detected with PE labeled Db-S510 class I tetramer as previously described (Zuo et al., 2005). Stained cells were fixed with 2% paraformaldehyde and analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA). Forward and side scatter signals obtained in linear mode were used to establish a gate containing live lymphocytes. A minimum of 2.5 × 10^5 viable cells were stained and 0.5–2 × 10^5 events per sample analyzed using CellQuestPro software (Becton Dickinson). Intracellular staining for IFNγ was performed as described previously (Zuo et al., 2005). Briefly, 1 × 10^6 CNS mononuclear cells were stimulated in 200 μl of RPMI 1640 supplemented with 10% FCS, containing 1 μM S510 peptide (CSLWNGPHL) (Zuo et al., 2005) for CD8$^+$ T cells or the I-A^b-restricted M133 peptide (TVYVRPIIE-DYHTLT) (Haring et al., 2001) presented by CHB3 cells (I-A^b) (Bishop et al., 1993) for CD4$^+$ T cells and 1 μl/ml Golgistop (BD PharMingen) for 6 h. Peptides were synthesized by the University of Southern California Norris Cancer Center Microchemistry Laboratory and purity assessed by HPLC and mass spectrometry. CHB3 cells (I-A^b) were derived from a B-cell tumor (Bishop et al., 1993) and used at a ratio of 5 CHB3 cells to 1 lymphocyte (Haring et al., 2001). Cells were surface-stained with anti-CD8 or CD4 mAbs, fixed and permeabilized with cytofix/cytoperm reagents, and finally stained with anti-

IFNγ mAb (XMG1.2) according to the suppliers' instructions (BD PharMingen).

Ex vivo cytolysis

CNS mononuclear cells were evaluated for CD8+ T cell-mediated ex vivo cytolytic activity at the indicated time points p.i. as previously described (Zuo et al., 2005). Briefly, $Na^{51}CrO_4$-labeled EL-4 (H-2^b) target cells were coated with S510 peptide at 1 μM prior to addition of the CNS mononuclear cells at various Effector:Target (E:T) ratios. ^{51}Cr release was determined in 100 μl of supernatant following 4 h incubation at 37 °C. Specific lysis is defined as 100 × (experimental release-spontaneous release)/(maximal release-spontaneous release). Spontaneous release values were *b*10% of detergent release.

Histopathology

Brains, bisected in the mid-coronal plane, and spinal cords were examined for inflammation, demyelination and viral Ag as previously described (Lin et al., 1997). Briefly, tissues were fixed for 3 h in Clark's solution (75% ethanol and 25% glacial acetic acid) before paraffin embedding. Deparaffinized sections were stained with hemotoxylin and eosin to determine inflammation or luxol fast blue for demyelination. Distribution of viral Ag was determined by immunoperoxidase staining (Vectastain-ABC kit; Vector Laboratories, Burlingame, CA) using the anti-JHMV mAb J.3.3 specific for the carboxyl terminus of the viral nucleocapsid protein as primary Ab and horse anti-mouse secondary Ab (Vector Laboratories). Sections were scored for inflammation, demyelination and viral Ag in a blinded fashion. Representative fields were identified based on average scores for all sections in each experimental group.

Statistical analysis

Means from 2 groups of mice were compared by two-tailed Student's *t* test and a difference was statistically significant when *P* value is *b*0.05.

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