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# The art of survival during viral persistence

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**Central nervous system infection by the neurotropic JHM strain of mouse hepatitis virus (JHMV) results in chronic demyelination characterized by viral persistence in the absence of infectious virus. CD8$^+$ T cells inhibit acute viral replication via cell type–specific effector mechanisms. Perforin-mediated cytolysis controls virus in microglia/macrophages and astrocytes, whereas interferon (IFN)-$\gamma$ regulates viral replication in oligodendroglia. JHMV infection of antibody-deficient mice confirmed a primary role of cellular immunity and a redundant role for humoral immunity during acute infection. However, infectious virus reactivates in antibody-deficient mice following viral clearance. This observation suggests that virus-specific T cells in the central nervous system are unable to control viral persistence. Reactivation in antibody-deficient mice is not associated with increased T-cell infiltration, but is prevented via transfer of neutralizing antibody. A vital role for humoral immunity during persistence is supported by the accumulation and retention of virus-specific antibody secreting cells following clearance of infectious virus. Thus, cell-mediated immune responses control acute infection, whereas humoral immunity maintains viral persistence. Therefore, although the central nervous system provides an environment for prolonged retention of both T cells and plasma cells, plasma cells are critical in maintaining persistent virus at undetectable levels. The low turnover of virus, T cells, and B cells constitute a unifying feature of persistent infection, illustrating the dichotomy between distinct immune effectors in regulating acute and persistent central nervous system infection.**
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## Introduction

Infection of mice with the neurotropic JHM strain of mouse hepatitis virus (JHMV) results in a biphasic disease. Initially, virus replication in the central nervous system (CNS) results in an acute encephalomyelitis associated with myelin loss. The immune response controls virus replication but is unable to completely eliminate virus, resulting in chronic CNS infection. Both the acute and the persistent phase are associated with CNS demyelination and are widely used as a model of the human demyelinating disease multiple sclerosis (Stohlman *et al*, 1999).

JHMV-induced encephalomyelitis results in the accumulation of immune cells, including neutrophils, natural killer (NK) cells, B cells, macrophages, and CD4$^+$ and CD8$^+$ T cells within the CNS. Investigating the contribution of these individual cell types in suppressing virus replication is critical in understanding the mechanisms underlying incomplete virus clearance. The critical role of the adaptive immune response is demonstrated by the inability of immunodeficient mice to control JHMV replication (Wang *et al*, 1990; Houtman and Fleming, 1996; Wu *et al*, 2000). Uncontrolled replication in immunodeficient mice, which have intact NK cells and macrophages, suggests that these two effectors play little or no role in inhibiting virus replication. However, a minor role of the innate immune response was indicated by

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the partial inhibition of virus replication in T cell–deficient mice (Williamson and Stohlman, 1990). By contrast, mice deficient in either $CD4^+$ or $CD8^+$ T-cell subsets do not control virus replication, demonstrating a vital anti-viral role for both $CD4^+$ and $CD8^+$ T cells–mediated effector functions (Williamson and Stohlman, 1990; Fleming *et al*, 1993; Stohlman *et al*, 1999; Lane *et al*, 2000). Virus titers decrease as $CD4^+$ T cells, $CD8^+$ T cells, and virus-specific $CD8^+$ T cells begin to accumulate within the CNS. However, peak virus titers precede peak T-cell accumulation by 3 to 4 days, suggesting that T-cell accumulation continues as virus replication is suppressed (Bergmann *et al*, 1999; Marten *et al*, 2000a). Although virus-specific T cells within the CNS decline following clearance of infectious virus, they are retained during JHMV persistence as long as viral antigen (Ag) or RNA is present (Marten *et al*, 2000b).

A primary role for $CD8^+$ T cells in controlling acute virus replication is supported by *ex vivo* cytolytic activity at the peak of viral clearance and by effective clearance of infectious virus by adoptively transferred $CD8^+$ T cells (Stohlman *et al*, 1995a; Bergmann *et al*, 1999; Ramakrishna *et al*, 2002). $CD8^+$ T cells use a variety of mechanisms to control viral replication. Analysis of JHMV infection in Fas-deficient mice revealed no role for Fas/FasL interactions (Parra *et al*, 2000). Tumor necrosis factor (TNF)-$\alpha$, suggested to play a prominent role in autoimmune T cell–mediated demyelination (Ruddle *et al*, 1990), does not influence viral replication, recruitment of CNS inflammatory cells, or JHMV-induced demyelination (Stohlman *et al*, 1995b). By contrast, analysis of mice deficient in perforin-mediated cytolysis not only demonstrated that cytolysis was critical for viral clearance, but also that the ability to lyse infected cells preferentially inhibited virus replication in astrocytes and microglia/macrophages but not oligodendroglia (Lin *et al*, 1997). Furthermore, analysis of mice deficient in interferon (IFN)-$\gamma$ clearly demonstrated that this effector molecule, and not cytolysis, was important for controlling virus replication in oligodendroglia (Parra *et al*, 1999). These data indicate that $CD8^+$ T cells are not only critical for the control of infectious virus, but that different CNS cell types are susceptible to distinct effector mechanisms.

$CD4^+$ T cells appear to have multiple accessory roles in enhancing $CD8^+$ T cell–mediated immunity, rather than having a major direct antiviral function. Although $CD8^+$ T cells are induced in the absence of $CD4^+$ T cells, the magnitude of the response is diminished (Stohlman *et al*, 1998; Bergmann *et al*, 2001; Neumann *et al*, 2002). In addition, analysis of the infected CNS indicated that the majority of $CD4^+$ T cells remain in perivascular and subarachnoid areas, similar to the distribution observed in multiple sclerosis (Lin *et al*, 1997; Stohlman *et al*, 1998). By contrast, $CD8^+$ T cells are widely distributed within the parenchyma (Lin *et al*, 1997; Stohlman *et al*, 1998). Furthermore, the number of apoptotic $CD8^+$ T cells was vastly increased in the absence of $CD4^+$ T cells (Stohlman *et al*, 1998). These data indicate that $CD4^+$ T cells provide two essential roles during acute infection. First, they enhance the frequency of virus-specific $CD8^+$ T cells, and secondly, they provide a tropic factor required for $CD8^+$ T cell viability once they enter the parenchyma.

Passive transfer of antiviral antibody (Ab) prior to infection increases survival, and in some cases, limits virus replication in a complement-independent manner (Buchmeier *et al*, 1984; Nakanaga *et al*, 1986; Fleming *et al*, 1989). However, the inability to detect antiviral Ab prior to the decrease in infectious virus within the CNS (Parra *et al*, 1999; Tschen *et al*, 2002) suggested that Ab played either a limited or no role in regulating acute virus replication. JHMV pathogenesis in mice deficient in B cells or transgenic mice with B cells unable to secrete Ab confirmed the role of cellular immunity in regulating virus within the CNS (Lin *et al*, 1999; Ramakrishna *et al*, 2002). All mice unable to secrete antiviral Ab cleared infectious virus from the CNS with kinetics identical to wild-type (wt) mice. However, in contrast to the inability to recover infectious virus from the CNS of wt mice, the absence of humoral immune responses allows virus reactivation in the CNS (Lin *et al*, 1999; Ramakrishna *et al*, 2002). Furthermore, passive transfer of antiviral Ab following initial viral clearance prevented reactivation (Lin *et al*, 1999) suggesting that Ab is the critical effector maintaining chronic CNS infection.

The data in this manuscript demonstrate that JHMV reactivation within the CNS of B cell–deficient mice does not change either the retention or the recruitment of $CD4^+$ or $CD8^+$ T cells following acute infection. T cells within the CNS of B cell–deficient mice decline with kinetics similar to the gradual reduction found in the CNS of persistently infected wt mice. These data suggest that once the cellular immune response has been down-regulated concomitant with virus clearance (Bergmann *et al*, 1999), it is incapable of responding to increased antigen load. Data are also presented indicating that Ab-secreting cells (ASCs) specific for JHMV are only recruited into the CNS after the majority of virus has been cleared. They continue to accumulate until well after the CNS T-cell population has begun to decline and are retained as plasma cells at relatively high levels within the CNS during viral persistence. Finally, passive transfer of JHMV-specific monoclonal antibodies (mAbs) with different specificities and biological activities demonstrates that the ability of Ab to neutralize infectivity *in vitro* correlates directly with the ability to prevent virus reactivation *in vivo*. These data are consistent with a role of neutralizing Ab in preventing reactivation and/or maintaining a chronic infection in the absence of infectious virus.

## Results

*Increased viral antigen does not alter CNS T cells*

Reduction of infectious virus from the CNS of B cell–deficient mice suggests that the cellular immune compartment functions effectively in the absence of B cells during acute infection (Figure 1). In addition, accumulation of $CD4^+$ T cells, total $CD8^+$ T cells, and virus-specific $CD8^+$ T cells within the CNS during initial viral clearance was similar to wt mice (Ramakrishna *et al*, 2002). Nevertheless, JHMV reactivates within the CNS of B cell–deficient mice following initial viral clearance, approaching levels found during acute infection (Lin *et al*, 1999; Ramakrishna *et al*, 2002). Despite increased virus replication in these mice, there was little evidence of alterations in CNS inflammation (Lin *et al*, 1999). Flow cytometric analysis was used to quantitatively assess potential alterations in the T-cell subsets during virus reactivation (Figure 1). The frequency of total T cells within the CNS of $\mu$MT mice undergoing virus reactivation was compared to the CNS of wt mice, which had cleared infectious virus at approximately 2 weeks post infection (p.i.). Consistent with previous data (Bergmann *et al*, 1999; Marten *et al*, 2000a), the frequency of T cells within the CNS of wt mice declined following acute infection (Figure 1). No increase in T cells was associated with JHMV reactivation in the CNS of B cell–deficient mice relative to the acute phase (Figure 1). Analysis of the T-cell subsets showed no alteration in $CD4^+$ T cells, $CD8^+$ T cells, or virus-specific $CD8^+$ T cells (data not shown). These data demonstrate that the increase of viral antigen in the CNS during virus reactivation does not lead to enhanced accumulation of T-cell effector populations capable of controlling viral replication.

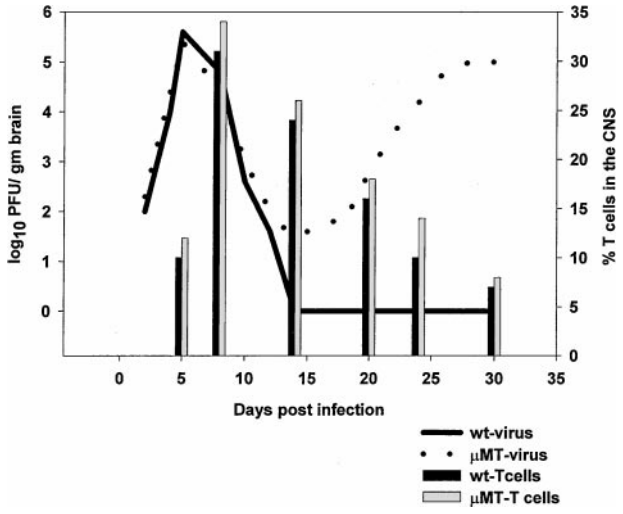


**Figure 1** JHMV pathogenesis in wild-type (wt) and B cell–deficient ($\mu$MT) mice. Virus replication in the CNS of wt (solid line) and $\mu$MT mice (dotted line) as well as total T-cell accumulation in the CNS of wt (solid bar) and $\mu$MT mice (gray bar) are depicted. Data represent one of more than three separate experiments, with three to four mice per time point.

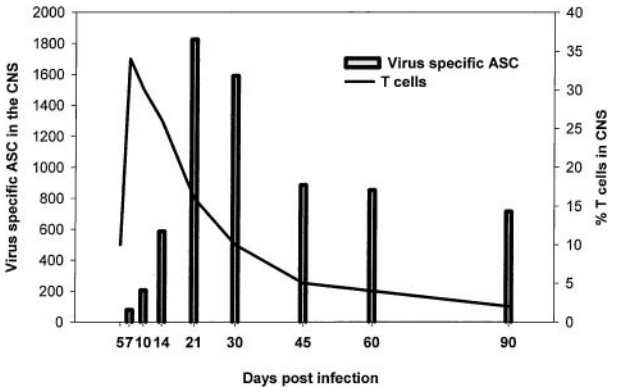


**Figure 2** JHMV-specific ASC and T cells within the CNS. Frequency of virus-specific ASC in the CNS of wild-type C57BL/6 mice determined by ELISPOT is depicted as bars (*left axis*). The frequency of T cells in the CNS following JHMV infection is shown as a solid line. Data represent three to five separate experiments.

*ASCs are retained in the CNS*

ASCs are recruited into the CNS with kinetics that differ from the recruitment of T cells, i.e., accumulation is delayed relative to both T-cell accumulation and virus clearance (Tschen *et al*, 2002). These data are consistent with a primary role of cellular immunity in regulating virus replication. As T cells are retained for at least 3 months p.i. in persistently infected wt mice, infected mice were examined to determined if ASCs are also retained in the CNS after their delayed infiltration. Consistent with previous data (Tschen *et al*, 2002), plasma cells secreting Ab-specific for JHMV were recruited into the CNS prior to day 30 p.i. (Figure 2). Furthermore, they were retained within the CNS for at least 90 days p.i. (Figure 2). Retention of T cells is dependent upon the presence of viral antigen (Marten *et al*, 2000b). Although plasma cell recruitment is not dependent upon antigen, it is interesting that the frequency of ASCs retained in the CNS ultimately declines, albeit with delayed kinetics compared to the decline of T cells. Whether retention is driven by the presence of viral antigen or influenced by the T-cell compartment is not clear; however, these data are consistent with the notion that local secretion of Ab within the CNS maintains viral persistence.

*Neutralizing Ab prevents JHMV reactivation*

Polyclonal anti-JHMV Ab inhibits virus reactivation in B cell–deficient mice (Lin *et al*, 1999). Therefore, the contribution of antibodies specific for individual virus structural proteins as well as the biological activity required to prevent virus reactivation was examined via the passive transfer of mAb into JHMV infected B cell–deficient mice at 9, 12, and 17 days p.i. Virus reactivation in the CNS of mAb recipients was compared at day 21 p.i. to control groups that received injections of either the polyclonal anti-JHMV Ab or phosphate-buffered saline (PBS). Consistent with previous data (Lin *et al*, 1999), passive

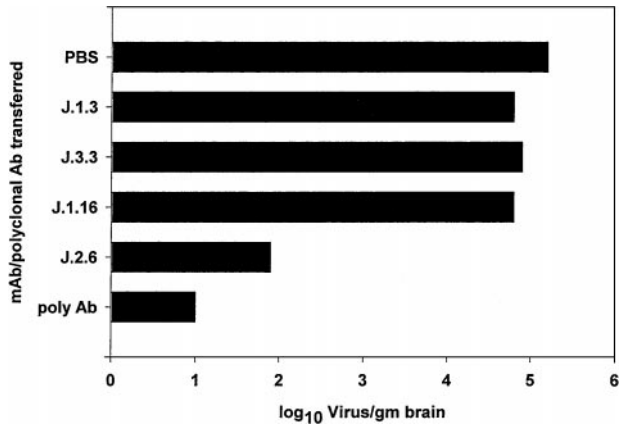


**Figure 3** Virus reactivation in B cell–deficient $\mu$MT mice following Ab transfer. Virus replication in the CNS of infected B cell–deficient mice at day 21 p.i. that received mAb at days 9,12, and 17 p.i. are shown as bars. Control mice received only PBS. Data represent average of three to five experiments.

transfer of polyclonal Ab suppressed virus reactivation in the CNS of infected $\mu$MT mice (Figure 3). In contrast to their ability to alter acute infection (Fleming *et al*, 1989), mAb specific for either the matrix protein (mAb J.1.3) or the nucleocapsid protein (mAb J.3.3) were unable to suppress virus reactivation. Similar to the matrix and nucleocapsid protein specific mAb, mAb specific for the viral spike protein (mAb J.1.16) lacking neutralizing activity was unable to prevent viral recrudescence (Figure 3). Only mAb specific for the viral spike protein, which exhibits high *in vitro* neutralizing activity (mAb J.2.6), was nearly as efficient as polyclonal Ab at suppressing JHMV reactivation. These data suggest that recruitment and retention of JHMV-specific plasma cells secreting Ab with neutralizing activity regulate persistent CNS infection.

## Discussion

Analysis of JHMV infection of the CNS has demonstrated the complexity of the immune responses involved in controlling this infection temporally as well at the level of cellular tropism. Viral replication during acute infection is controlled by the cellular components of the immune response. $CD8^+$ T cells inhibit virus replication via two effector mechanisms applied to distinct CNS cell types. Virus clearance from astrocytes and microglia/macrophages is susceptible to perforin-mediated cytolysis, whereas clearance from oligodendroglia is only susceptible to IFN-$\gamma$–mediated mechanisms. Although the $CD8^+$ T cells are the primary effectors of virus clearance, there is a clear dependence on $CD4^+$ T cells. $CD4^+$ T cells not only enhance $CD8^+$ T cell expansion (Stohlman *et al*, 1995a; Bergmann *et al*, 2001), but also appear to regulate $CD8^+$ T cell viability within the CNS (Stohlman *et al*, 1998). The mechanism of interdependence between the $CD4^+$ and $CD8^+$ T cell responses are unclear. The requirement for sustaining viability within the CNS parenchyma poses an enigma, because the $CD4^+$ T cells appear to preferentially localize to the perivascular and subarachnoid spaces, whereas the $CD8^+$ T cells migrate in a diffuse pattern throughout the CNS parenchyma (Stohlman *et al*, 1998).

JHMV persists in most cell types in the CNS (Stohlman *et al*, 1999); however, it is not clear whether the chronic demyelination is associated with persistence of virus in a noninfectious form in a specific cell type. The recovery of wt infectious virus from the CNS of B cell–deficient mice during reactivation suggests that persistence is not associated with the selection of mutants (Lin *et al*, 1999; unpublished data). The basis for persistence in astrocytes and microglia/macrophages appears to be due to the rapid loss of $CD8^+$ T cell cytolytic activity as virus is cleared from the CNS (Bergmann *et al*, 1999; Marten *et al*, 2000b; Ramakrishna *et al*, 2002). Although IFN-$\gamma$ mRNA appears to increase during viral persistence, the inability of cell mediated immunity to eliminate virus from oligodendroglia remains an unresolved issue (Lin *et al*, 1999; Parra *et al*, 2000). The data presented here demonstrate that the increased viral burden within the CNS does not alter the gradual loss of T cells from the CNS found in the absence of virus reactivation. Increased viral burden thus cannot overcome the mechanism employed to down-regulate $CD8^+$ T cell effector activity within the CNS during acute infection. These results suggest that the down-regulation of $CD8^+$ T cell effector function is independent of viral burden, but rather results from the establishment of a T-cell inhibitory state once a threshold of antiviral activity has been achieved.

Reactivation of infectious virus in the CNS of B cell–deficient mice following clearance (Lin *et al*, 1999; Ramakrishna *et al*, 2002), compared to the persistence of noninfectious virus in wt mice, suggests that the loss of expression of the major antiviral effector mechanisms are compensated for by the humoral immune response. Comparison of the kinetics of ASC accumulation in the CNS of wt mice suggests that ASCs are recruited into the CNS following viral clearance and during reestablishment of blood-brain barrier integrity (Tschen *et al*, 2002). The present data demonstrate that Ab specific for the major virus structural proteins are unable to maintain chronic JHMV infection of the CNS. Only Ab specific for the viral spike protein that express neutralizing activity are as efficient as polyclonal antiviral Ab at preventing virus reactivation. ASCs secreting antispike protein Ab are recruited into the CNS following resolution of the acute encephalomyelitis (Tschen *et al*, 2002). The data presented here demonstrate that although the frequency of JHMV-specific ASCs decline

during viral persistence, they remain at detectable levels at least until 90 days p.i. Although it is not clear whether antispike protein–specific ASCs persist with the same kinetics as the total population, serum-neutralizing Ab is maintained at high levels during persistent infection (Stohlman *et al*, 1999; unpublished data). Whether the maintenance of JHMV in a noninfectious state during chronic infection is due to the retention of ASCs specific for the viral spike protein within the CNS, the accumulation of serum neutralizing Ab within the CNS, or a combination of both, is not clear. However, these data suggest that JHMV persistence within the CNS is initially established via the inability of the cellular immune response to effect complete viral clearance is then maintained via antiviral Ab expressing neutralizing activity.

## Materials and methods

*Mice*
Homozygous B cell–deficient C57BL/6-Igh-6$^{tm1Cgn}$ ($\mu$MT) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred under pathogen-free conditions at the University of Southern California, Keck School of Medicine (Los Angeles, CA). Syngeneic C57BL/6 mice were obtained from the National Cancer Institute (Frederick, MD). Mice of both sexes were used between 7 to 8 weeks of age.

*Virus*
Mice were infected intracerebrally (IC) with 250 plaque forming units (PFU) of the J.2.2v-1 neutralizing mAb-derived variant of JHMV (Fleming *et al*, 1987) in 30 $\mu$l of Dulbecco's PBS, pH 7.4, in the left hemisphere. Virus was propagated and plaque assayed as previously described (Fleming *et al*, 1986). CNS virus titers were determined by plaque assay using monolayers of the murine DBT astrocytoma (DBT) cells as previously described (Fleming *et al*, 1986). Briefly, one half of the brains were homogenized in RPMI medium containing 25 mM HEPES, pH 7.2, using Tenbrock tissue homogenizers. Following clarification by centrifugation at 500 $\times$ *g* for 7 min, homogenates were either assayed directly or stored at $-70^{\circ}$C. Mice received polyclonal Ab or mAb intraperitoneally (IP) on days 9, 12, and 17 p.i.

*CNS mononuclear cells*
Infected mice were perfused with PBS at various times p.i. CNS mononuclear cells (CMCs) were isolated from one half of the brain and spinal cord as described (Bergmann *et al*, 1999). Briefly, tissues were dissociated in Tenbroeck homogenizers and adjusted to 30% Percoll (Amersham Pharmacia Biotech, Sweden). A 1.0-ml 70% Percoll underlay was added before centrifugation at 800 $\times$ *g* for 25 min at 4$^{\circ}$C. Mononuclear cells were recovered from the 30%/70% interface.

*Ab secreting cells*
JHMV-specific ASCs were detected by ELISPOT as previously described (Tschen *et al*, 2002). Briefly, 96-well plates (Millipore Multiscreen, Bedford, MA) were coated with clarified serum-free supernatant collected from JHMV-infected DBT cells (approximately $5 \times 10^5$ PFU/well) and control wells were coated with virus-free medium. Wells were blocked with modified Eagle's medium (MEM) containing 2% bovine serum albumin (BSA) (Sigma, St. Louis, MO). CMCs suspended in RPMI medium containing 10% fetal calf serum (FCS) were incubated at 37$^{\circ}$C for 4 h followed by the addition of biotinylated goat anti-mouse immunoglobulin (Ig)M and IgG (Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. After washing, spots were developed with avidin peroxidase (Sigma) diluted in PBS-TWEEN containing 10% FCS and 3,3′-diaminobenzidine (Sigma) as substrate and were counted using a dissecting microscope.

*Flow cytometry*
CMCs were preincubated with a mixture (10%) of polyclonal mouse and human sera (Atlanta Biologicals, Norcross, GA) and rat anti-mouse Fc$\gamma$-III/IIR mAb (2.4G2) (BD PharMingen, San Diego, CA) for 20 minutes on ice to block nonspecific binding. Phycoerythrin-, fluorescein isothiocyanate-, or cychrome *c*–labeled mAb specific for CD4 (GK1.5), CD8 (53.67), CD44 (IM7), CD62L (MEL-14), CD43 (S7), CD19 (1D3), B220 (RA3-6B2), and IgM (R6-60.2) were all obtained from BD PharMingen. JHMV-specific CD8$^+$ T cells were identified by labeling with the D$^b$-S510 class I tetramer as previously described (Bergmann *et al*, 1999). Cells were analyzed with a FACSCalibur flow cytometer (Becton Dickenson, San Jose, CA) using CellquestPro software.

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