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Inverted Immunodominance and Impaired Cytolytic Function of CD8+ T Cells During Viral Persistence in the Central Nervous System

Cornelia C. Bergmann,*† John D. Altman,§ David Hinton,*§ and Stephen A. Stohlman*†

Mice infected with the neurotropic JHM strain of mouse hepatitis virus (JHMV) clear infectious virus; nevertheless, virus persists in the CNS as noninfectious RNA, resulting in ongoing primary demyelination. Phenotypic and functional analysis of CNS infiltrating cells during acute infection revealed a potent regional CD8+ T cell response comprising up to 50% virus-specific T cells. The high prevalence of virus-specific T cells correlated with ex vivo cytolytic activity and efficient reduction in viral titers. Progressive viral clearance coincided with the loss of cytolytic activity, but retention of IFN-γ secretion and increased expression of the early activation marker CD69, indicating differential regulation of effector function. Although the total number of infiltrating T cells declined following clearance of infectious virus, CD8+ T cells, both specific for the dominant viral epitopes and of unknown specificity, were retained within the CNS, suggesting an ongoing T cell response during persistent CNS infection involving a virus-independent component. Reversed immunodominance within the virus-specific CD8+ T cell population further indicated epitope-specific regulation, supporting ongoing T cell activation. Even in the absence of infectious virus, the CNS thus provides an environment that maintains both unspecific and Ag-specific CD8+ T cells with restricted effector function. Chronic T cell stimulation may thus play a role in preventing viral recrudescence, while increasing the risk of pathological conditions, such as demyelination. *The Journal of Immunology, 1999, 163: 3379–3387.

Effective control of viral infection is dependent on tropism, the rate of replication, and the host’s ability to mount an effective immune response. Many acute infections are controlled by specific CD8+ T cells, which are primed in the draining lymph nodes and home to the site of infection, where they execute effector functions (1–3). Concurrent with viral clearance, significant numbers of highly activated CD8+ T cells undergo apoptosis (1, 4). Following localized infections, such as in the lung, there is little evidence for retention of Ag-specific T cells at the site of infection after virus is cleared (3). Surviving virus-specific T cells are redistributed to secondary lymphoid organs and blood where they comprise a stable memory pool, which is readily reactivated following Ag challenge (1, 3). By contrast, during systemic infections associated with high Ag load, chronic TCR stimulation may lead to anergy or clonal exhaustion and failure to control infection (5, 6). The relationships between stimulation, function, and survival of CD8+ T cells during chronic regional infections in the liver or CNS, or during latent infections of lymphoid cells, appear more complex, as both the tissue and cell type infected contribute to immune regulation (7–13). The stringent regulation of T cell invasion, effector functions, and subsequent apoptosis minimizes tissue damage, but also provides the opportunity for virus to sequester into tissue or cell types more resistant to CTL-mediated clearance.

The CNS is a preferred anatomical site for persistent viral infections (14, 15). The absence of classical lymphatic drainage, the presence of the blood brain barrier, low levels of MHC expression, and relative resistance of resident CNS cells to apoptosis are all factors contributing to inefficient immunity, thus providing a suitable environment for persistence (14, 16, 17). The mechanisms regulating the interactions between the immune response and the CNS as a target are poorly understood. Specifically, how the balance between viral replication, lymphocyte recruitment, and expression of effector function during acute infection contributes to the control of infection, viral persistence, and ultimately the severity of chronic disease has not been extensively explored. The recently discovered long-term presence of activated CD8+ T cells in the apparent absence of viral Ag or RNA (11) confirmed previous evidence that CD8+ T cells within the CNS are regulated differently compared with those in the periphery (10).

Intracerebral infection of rodents with the neurotropic JHM strain (JHMV)3 of mouse hepatitis virus (MHV) provides a paradigm for an acute CNS infection in its natural host resulting in persistence (18, 19). The acute infection, characterized by encephalitis associated with demyelination, is primarily controlled by CD8+ T cells infiltrating the parenchyma (20, 21). Infectious virus is typically cleared within 2–3 wk after infection, and mice eventually recover from hind limb paralysis (22). However, viral RNA (vRNA) can persist in the CNS up to 2 yr after clearance of infectious virus (23). Persistence is further characterized by ongoing

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3 Abbreviations used in this paper: JHMV, JHM strain of mouse hepatitis virus; MHV, mouse hepatitis virus; CLN, cervical lymph nodes; N, nucleocapsid protein; S, spike protein; p.i., postinfection; DBT, delayed brain tumor; SC, spinal cord; vRNA, viral RNA; ELISPOT, enzyme-linked immunospot; APC, allophycocyanin; HPRT, hypoxanthine phosphoribosyltransferase; LCMV, lymphocytic choriomeningitis virus.
primary demyelination (24). Both virus segregation to cells resistant to CTL-mediated lysis (20, 25) and vRNA sequence variability (23, 26–28) may contribute to the inability to provide sterile immunity. JHMV replicates preferentially in microglia, astrocytes, and oligodendrocytes, but only rarely in neurons (22, 29). During acute infection, the host immune response, specifically the CD8 component, clears virus from microglia and partially from astrocytes, and persisting RNA or Ag is predominantly detected in oligodendrocytes and astrocytes (25, 29, 30). Even following adoptive transfer of activated CD8+ T cells, oligodendrocytes remain relatively resistant to viral clearance; nevertheless, decreased demyelination suggests that potent early CD8+ T cell functions provide a more favorable clinical outcome (20). Although the peak incidence of CD8+ T cells coincides with a reduction in viral titers (31), CD8+ T cells are still found within the CNS of clinically recovered mice at 35 days postinfection (p.i.) (32). The role of virus-specific CD8+ T cells in the CNS during persistence is less clear, as very little is known about their functional state or specificity.

To begin to understand the relationship between viral replication and CD8+ T cell regulation within the CNS, this study characterizes CD8+ T cells throughout the acute and persistent infection. Class I tetramer staining combined with functional analysis revealed that up to 50% of CD8+ T cells in the CNS of acutely infected mice were virus-specific. Concomitant ex vivo cytolytic activity suggested that persistence is not attributed to inadequate T cell recruitment or expression of effector function. The CD8+ population in the CNS declined during the course of infection; however, the percentage of tetramer+ cells remained high, even after infectious virus was completely eliminated. IFN-γ secretion was maintained throughout. In contrast, Ag responsiveness was impaired at the cytolytic level, indicating differential regulation of distinct effector functions. Ongoing chronic activation was further evident by a switch in immunodominance from nucleocapsid (N) protein-specific CD8+ T cells during the acute infection to spike (S) protein-specific CD8+ T cells during persistence. Mechanisms underlying the regulation of CD8+ T cells at the initial site of infection may reside in increased CD69 expression and delayed clearance of Ag from oligodendrocytes, compared with astrocytes and macrophages. Although persisting CD8+ T cells in the CNS may play a role in preventing viral recrudescence, chronic immune stimulation and concomitant cytokine secretion may also constitute a higher risk for immune-mediated pathogenesis than previously appreciated.

**Materials and Methods**

**Mice, viruses, and virus titration**

Male CB6 F1 (H-2d/b) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at 6 wk of age and certified naive to prior MHV exposure. Mice were housed in microisolation cages in an accredited animal facility at the University of Southern California (USC; Los Angeles, CA) and infected within 1 wk of arrival. Sublethal CNS infections were induced by intracranial injection of 32 μl containing 200 PFU of the 2.2v-1 mAb-derived variant of JHMV, as previously described (22). This variant produced paralysis associated with demyelination and replicates predominantly in oligodendrocytes. Viruses were propagated in the presence of neutralizing mAb J2.2 and quantified by plaque assay using the murine delayed brain tumor (DBT) astrocytoma cell line, as described (29). For determination of tissue virus titers, one-half of the brain was homogenized in 2.0 ml of Dulbecco’s PBS (pH 7.4) using Tenbrock tissue homogenizers. Following centrifugation at 1500 × g for 7 min at 4°C, supernatants were assayed immediately or frozen at −70°C. Data presented are the average titers of individuals from groups of three or more mice.

**Tissue sampling and isolation of lymphocytes**

CNS mononuclear cells were obtained from pooled brains and spinal cords (SC) of 6–10 mice per group at various time points p.i., as described (32), briefly, tissues were minced and homogenized in Tenbrock homogenizers. Cells were adjusted to 30% Percoll (Pharmacia, Uppsala, Sweden) and pelleted onto a 70% Percoll cushion, washed, and resuspended in RPMI medium. Typical yields were 0.8–2.0 × 106 cells per mouse, depending upon the disease state. Single cell suspensions were prepared from the spleens and cervical lymph nodes (CLN) from identical groups of mice, as previously described (33).

**CD8+ T cells during CNS persistence**

**CTL assays and synthetic peptides**

CTL assays were performed as described (34). Briefly, J774.1 (H-2d) or EL-4 (H-2b) target cells were labeled with 100 μCi Na1CrO4 (New England Nuclear, Boston, MA) and peptides added to washed target cells at a final concentration of 1 μM before addition of CTL at the indicated E:T ratios. After 4 h of incubation, 100 μl supernatant was removed and specific 1Cr release determined. Specific lysis was defined as: 100 × [(experimental release) – (spontaneous release)]/[determinant release + (spontaneous release)]. Maximum spontaneous release values were <15% of the total values in all experiments.

**Enzyme-linked immunospot (ELISPOT) assays**

**Flow cytometry**

Single cell suspensions were blocked with purified anti-mouse CD16/CD32 (2.4G2; PharMingen, San Diego, CA). For two- or three-color flow cytometric analysis, cells were stained with PE- or APC-conjugated tetramers (MultiScreen HA; Millipore, Bedford, MA) precoated with R4-624 mAb (PharMingen, San Diego, CA) and stained in the presence of irradiated (25 Gy) splenocytes from naive mice (5 × 106/well), either pulsed with 1 μM pS510 or pN318 peptide or left untreated. EL-4 supernatant was added as a source of IL-2 to a final 2.5%, and cultures were incubated for 36 h at 37°C. Bound IFN-γ was detected by overnight incubation at 4°C with biotinylated anti-IFN-γ mAb (0.5 μg/ml XMGL2; PharMingen), followed by consecutive incubations with streptavidin-peroxidase (Sigma, St. Louis, MO) and 3,3′-diaminobenzidine as substrate (Sigma).

**L. N318 and D. S510 tetramers**

MHC class I heavy chains Lα and Dα associated with viral 9-mer peptides pN318 and pS510, respectively, were generated as previously described (31). Monomers were converted to tetramers using either allophycocyanin (APC)-labeled streptavidin or R-PE-labeled Neutra-Lite avidin (Molecular Probes, Eugene, OR) at a molar ratio of 4:1.

**RNA extraction and RT-PCR analysis**

One-half of the brain or SC per mouse was processed individually for RNA isolation as previously described (26). Synthesis of cDNA was conducted by RT using 2 μg total RNA and 1 μg random primer, as previously described (26), and amplification of primers spanning N gene nucleotides 854–875 (forward, 5′-CTG GCT TAC GCC CAG CCT AAG C-3′; reverse, 5′-CTG TTC TTC CCT CAC CTT CAC-3′), G gene nucleotides 586–535 (forward, 5′-CAG TTC CCG CTC GAG GTG CCG GAG C-3′; reverse, 5′-CGG GAC TCT GGA TCC CAG G-3′), and S gene nucleotides 1390–1895 (forward, 5′-GTT GGT GCC TAC GCC CAG C-3′; reverse, 5′-GAT ACC CAT AGA GTG CAT TGC GCG-3′). The amplified products were analyzed by agarose gel electrophoresis (2.5% gel) and stained with ethidium bromide.
Pathogenesis and viral replication in CB6 F1 mice

Infection of mice with the JHMV variant 2.2v-1 produces an acute, nonfatal encephalomyelitis with extensive demyelination (22, 39). CD8+ T cells comprise the major antiviral effectors by reducing virus replication in astrocytes and microglia via a perforin-dependent mechanism and in oligodendroglia via cytokine secretion (25, 29). C57BL/6 (H-2b) mice mount a dominant CTL response to a Db-restricted epitope (S510) contained within the viral S protein (33, 34), while BALB/c (H-2d) mice respond predominantly to an Ld-restricted epitope in the viral N protein (N318) (33, 34). CB6 F1 (H-2d<sup>−</sup>;H-2b<sup>+</sup>) mice were chosen to examine the expansion and fate of CD8<sup>+</sup> T cell populations specific for the abundant and conserved N protein (40), compared with the less abundant, hypervariable S protein (28, 41). Following infection of CB6 F1 mice, virus titers in the brain declined to undetectable levels by day 16 p.i. (Fig. 1), similar to parental C57BL/6 and BALB/c mice (38). All mice developed clinical symptoms with varying severity by day 12 and partially recovered from hind limb paralysis by day 21. Few mice developed clinical symptoms with varying severity by day 12 and similar to parental C57BL/6 and BALB/c mice (38). All mice died by day 21. Viral titers in brain tissue were determined by plaque assay on DBT cell monolayers. Each time represents the mean of three samples including SEs. The dashed line indicates the limit of detection.

Results

Infection of mice with the JHMV variant 2.2v-1 produces an acute, nonfatal encephalomyelitis with extensive demyelination (22, 39). CD8<sup>+</sup> T cells comprise the major antiviral effectors by reducing virus replication in astrocytes and microglia via a perforin-dependent mechanism and in oligodendroglia via cytokine secretion (25, 29). C57BL/6 (H-2b) mice mount a dominant CTL response to a Db-restricted epitope (S510) contained within the viral S protein (33, 34), while BALB/c (H-2d) mice respond predominantly to an Ld-restricted epitope in the viral N protein (N318) (33, 34). CB6 F1 (H-2d<sup>−</sup>;H-2b<sup>+</sup>) mice were chosen to examine the expansion and fate of CD8<sup>+</sup> T cell populations specific for the abundant and conserved N protein (40), compared with the less abundant, hypervariable S protein (28, 41). Following infection of CB6 F1 mice, virus titers in the brain declined to undetectable levels by day 16 p.i. (Fig. 1), similar to parental C57BL/6 and BALB/c mice (38). All mice developed clinical symptoms with varying severity by day 12 and partially recovered from hind limb paralysis by day 21. Few mice showed minor residual signs of paralysis by day 45 p.i., the last time point analyzed.

Effect function and quantitation of CD8<sup>+</sup> T cells in the CNS during acute infection

Mononuclear cells isolated from the CNS of acutely JHMV-infected BALB/c or C57BL/6 mice exhibit ex vivo cytolytic activity. By contrast, lymphocytes from the spleen or CLN require in vitro stimulation to detect cytolytic function (33, 42). Priming of CD8<sup>+</sup> T cells specific for both epitopes and recruitment into the CNS was confirmed in acutely infected CB6 F1 mice (Fig. 2A). Mononuclear cells from the CNS demonstrated similar cytolytic activities specific for both the S510 and N318 epitopes, whereas no ex vivo cytolytic activity was recovered from either the spleen or CLN. These data support previous suggestions that JHMV-specific CTL rapidly accumulate to high numbers within the CNS and/or acquire Ag-driven effector function at the site of viral replication (33, 42). To examine whether ex vivo cytolyis reflects a higher proportion of Ag-specific CD8<sup>+</sup> T cells in the CNS vs periphery, CD8<sup>+</sup> T cells localizing to the CNS during acute infection were compared with those present in the peripheral lymphoid organs. Mononuclear cells were stained for both CD8 and either D<sup>b</sup>-S510- or L<sup>d</sup>-N318-specific TCRs using class I tetramers (4, 43). At day 8 p.i., ~40% of CNS-derived CD8<sup>+</sup> T cells were virus-specific, with 26% specific for the N epitope and 13% specific for the S epitope (Fig. 2B). By contrast, neither CD8<sup>+</sup> T cells present in the spleen nor CLN exhibited tetramer staining above 1%. Ex vivo cytolytic effector function of CNS-derived T cells therefore directly coincided with a high frequency of Ag-specific T cells within the CNS compared with the periphery.

Persisting CD8<sup>+</sup> T cells exhibit impaired cytolytic function

Clearance of infectious virus is generally associated with a severe decline in Ag-experienced T cell concomitant with entry into a quiescent memory phase (1). However, data obtained from immune mice challenged with a neurotropic influenza virus suggest that virus-specific CD8<sup>+</sup> T cells may be maintained in the CNS in a chronically activated, yet nondividing state for extended periods of time in the absence of detectable vRNA (11). JHMV RNA...
CNS-derived mononuclear cells contained a significant proportion of tetramer staining revealed a slight increase in virus-specific CD8\(^+\) population decreased from 26% to 18%, S-specific CD8\(^+\) cells increased from 13% to 30%. Similar to the acute phase, peripherally virus-specific CD8\(^+\) T cells that expressed CD69 were consistently elevated at ~90% in both the total CD8\(^+\) T cells and the tetramer\(^+\) populations (Fig. 4). Thus, the majority (55–70%), but not all, of tetramer\(^+\) CD8\(^+\) T cells expressed CD69 at day 8 p.i., coincident with clearance of infectious virus from the CNS. Thereafter, the numbers of CD69\(^+\) CD8\(^+\) T cells declined, but remained constantly elevated at ~90% in both the total CD8\(^+\) T cells population and the tetramer\(^+\) subsets. Although the CD69\(^+\) CD8\(^+\) T cell population likely comprises cells recently stimulated within the CNS, only a comparatively low percentage of CD8\(^+\) T cells expressed the IL-2R \(\beta\)-chain, even at day 8 p.i. (<1.0%), consistent with phenotypic analysis of CD8\(^+\) T cells from the CNS of JHMV-infected rats (45).

**Inverted immunodominance during persistence**

An inverse frequency of epitope-specific CD8\(^+\) T cell populations during the transition from acute infection to persistence suggested a distinct Ag-driven turnover process of N- vs S-specific CD8\(^+\) T cells (see Fig. 3 above). Examination of the frequency of N- and S-specific CD8\(^+\) T cells within the CNS during the course of infection revealed that the transition coincided with the clearance of infectious virus (Fig. 5). Initially N-specific CD8\(^+\) T cells dominated up to day 12 p.i. Between days 14 and 21, this population declined, concomitant with an increase in S-specific CD8\(^+\) T cells. The transition exhibited fluctuating percentages within different groups of mice (Fig. 5); however, the final phase after day 43 p.i. was characterized by a consistent prevalence of S-specific CD8\(^+\) T cells.

The switch in epitope immunodominance suggested that CD8\(^+\) T cells retained within the CNS during persistence exhibit some Ag-driven functional activity. Abrogation of cytolytic activity during persistence (Fig. 3B) and similar potency during acute infection (Fig. 2A) showed that altered epitope hierarchy was not evident at the cytolytic level. To relate the altered pattern of persisting vRNA, leading to Ag expression and chronic T cell stimulation, or of other local suppressive factors in inducing this anergic state, is unclear (10, 44).

Phenotypic comparisons of CD8\(^+\) T cells from the CNS during acute and persistent infection revealed similar expression patterns of activation/memory markers. The majority of CNS-infiltrating CD8\(^+\) T cells during acute infection were CD44\(^{high}\) (93.5%) and CD62\(^{low}\) (82.8%), CD11a \(^{high}\) (87.9%) and CD49d\(^{+}\) (79.4%), characteristic of an activated/memory phenotype (1). Furthermore, as only 40–50% of the CD8\(^+\) T cells were tetramer\(^+\), these markers did not discriminate virus-specific from nonspecific CD8\(^+\) T cells. However, reduction of infectious virus was associated with an increased frequency of CD69\(^+\) CD8\(^+\) T cells, revealing a distinct phenotypic difference between CD8\(^+\) T cells isolated early and late during infection (Fig. 4). In contrast to the other activation markers examined, the early activation Ag CD69 was consistently expressed on <55% of CD8\(^+\) T cells recovered on day 8 p.i. Although this implicated CD69 as a potential marker to distinguish the virus-specific population, examination of both N- and S-specific TCR subsets using three-color flow cytometry revealed CD69 expression on both the tetramer\(^+\) and tetramer\(^-\) populations (Fig. 4).

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immunodominance to an independent immune function, the frequency of IFN-γ-responsive cells specific for each epitope was determined by ELISPOT (Fig. 6). A high frequency of T cells responding to both CTL epitopes was observed at day 8 p.i. At this time, N-responsive cells prevailed over S-responsive cells, consistent with the phenotypic immunodominance demonstrated by tetramer staining. Although the frequencies of both subsets declined markedly by days 12 and 16 p.i., the N-responsive population remained dominant at day 12 and declined to the frequency of S-responsive cells at day 16. By contrast, an increased number of IFN-γ secreting S-specific T cells was found at day 21 p.i., at which time phenotypic staining was unable to distinguish an altered frequency of Ag-specific CD8+ T cell subsets (Fig. 5). The overall higher frequency of IFN-γ-secreting cells at day 21 compared with day 16 p.i., suggested the possibility of a second wave of infiltrating virus-specific T cells. The dominance of S-specific CD8+ T cells, compared with the acute phase of infection, was maintained to day 45 p.i. Overall, the inverse ratios of S- vs N-specific IFN-γ-secreting cells confirmed the phenotypic difference at the functional level. Importantly, the total frequency of JHMV-specific IFN-γ-secreting cells was only slightly lower during persistence at day 45 p.i. compared with the acute phase at day 8 p.i., despite a ~2-fold drop in CD8+ T cells. Furthermore, cells isolated from the CNS at day 21 p.i. showed a 2-fold increase in the frequency of S- over N-specific cells, despite evidence of codominant populations by phenotypic analysis (see Fig. 5). These data indicated that the transition in immunodominance was evident earlier at a functional level compared with phenotypic analysis.

**FIGURE 4.** Increased CD69 expression on CNS-infiltrating CD8+ T cells during acute and persistent infection. Mononuclear cells, harvested from the CNS of infected mice (n = 8–10) at days 8, 12, 16, 21, and 45 p.i., were stained for expression of CD8 (PE-labeled anti CD8), CD69 (FITC-labeled anti CD69), and N-epitope-specific TCR (APC-labeled Ld-N318 tetramer). A. The top row depicts forward scatter and side scatter characteristics of representative mononuclear cell preparations. Gate A represents the population analyzed for marker expression. The middle row represents flow cytometry density plots showing expression of CD69 (x-axis) vs CD8 (y-axis) in the gated population. Numbers represent percentages of positive cells in each quadrant. Cells stained with CD8 only are shown for day 8 in the left panel to set quadrants. The bottom row represents flow cytometry density plots showing expression of CD69 (y-axis) vs Ld-N318-specific TCR (y-axis) gated on CD8+ T cells. The data show that CD69 expression peaks at day 16 in both the tetramer+ and tetramer− CD8+ population coincident with viral clearance. B. Percentage of CD69-expressing cells in total CD8+ or the individual Ld-N318 and Db-S510 tetramer+ populations depicted schematically.
To correlate altered patterns of epitope-specific responsiveness to differences in Ag load and/or ongoing viral gene expression, brain and SC tissues were analyzed for vRNA by RT-PCR. Comparison of PCR products specific for the viral N and S genes and the housekeeping gene HPRT showed that vRNA is prominent at day 6 p.i. in both brain and SC (Fig. 7A). However, at day 8 p.i., vRNAs decreased in the brain and to a lesser extent in the SC. Only one mouse had prominent levels of N gene sequences in the brain, although the identical sequence was readily detected in the SC of all four animals analyzed. By contrast, S gene sequences were only found in the SC of a single mouse at day 8 p.i. At days 12 and 14 p.i., RNAs encoding both the N and S genes were found predominantly within the SC. Whereas RNA containing N gene sequences was still detected in the SC at day 21 p.i., S gene sequences dropped to below detection. At day 45 p.i., both gene sequences had dropped below detection levels, although increased amplification confirmed their persistence (data not shown). As mutations in the S epitope (27), but not N epitope (26), play a role in JHMV pathogenesis, dominance of S-specific CD8\(^+\) T cells within the CNS due to epitope mutations, thereby preempting activation-induced cell death, was examined. However, sequence analysis of S gene-specific PCR products derived from the CNS of four individual mice on day 45 p.i. revealed no evidence for mutations within the S epitope (data not shown). CTL escape mutations therefore do not account for preferential survival of S-specific CD8\(^+\) T cells during persistence. Relative to HPRT encoding RNA, vRNA within the SC peaked at day 8 and 12 p.i.; RNA encoding the N protein was prominent at both days, compared with delayed detection of S protein encoding RNA (Fig. 7B), likely reflecting the predominance of N gene sequences in the nested set of subgenomic RNAs expressed during the replication cycle (40). These data suggest that the rapid accumulation and subsequent loss of N-specific CD8\(^+\) T cells within the CNS coincides with clearance of infectious virus. Mononuclear cells from the CNS of infected mice were stained for expression of CD8 (FITC-labeled anti CD8), and either N- or S-epitope-specific TCRs (PE-labeled L\(^d\)-N318 and D\(^b\)-SS10 tetramer, respectively) at the indicated time points. The percentage of CD8\(^+\) T cells positive for either tetramer stain are indicated by filled (L\(^d\)-N318) and open (D\(^b\)-SS10) symbols at each time point analyzed. Different symbols characterize data obtained from separate groups of infected mice (n = 6–10).
were found abundantly in glial and occasionally ependymal cells and were distributed equally in the brain and SC at day 6 p.i. Whereas a transient decrease in Ag-positive cells was observed at day 8 p.i. in both the brain and SC, Ag localized increasingly to the SC between days 12 and 21 p.i. In the SC, the number of Ag-positive cells increased up to day 12 and 14 and declined by day 21 p.i. Only very rare positive cells expressing either protein were detected at day 45 p.i. Most importantly, no differences were detected in the regional distribution or cell types expressing either protein; however, highly focused tropism for oligodendrocytes was clearly evident by day 14 p.i. Localization of vRNA to the SC concurrent with the delayed peak in S gene-specific RNA may contribute to the increase in S-specific T cells during persistence.

Discussion
The CNS is a favored anatomical site for persistent infections due to its unique cellular environment and interactions with cells of the immune system (14, 15). This study examined the regulation CD8+ T cells during acute and persistent CNS infection with the natural rodent pathogen JHMV. During the peak of inflammation, up to 50% of CNS-infiltrating CD8+ T cells are virus-specific by class I tetramer staining. A vigorous immune response at the site of virus replication is also functionally evident by ex vivo cytolytic activity of CNS-derived lymphocytes. Potent cytolytic activity of CNS-derived, but not peripheral T cells, has been demonstrated for other viral CNS infections (45, 47–49). However, the tetramer-staining technique allowed a direct correlation between cytolsis and the quantity of virus-specific CD8+ T cells. Thus, virus-specific CTL from the CNS are cytotytic at E:T ratios of 0.5:1, and their apparent low frequency in lymphoid tissues is consistent with the absence of cytolytic activity.

Potent local effector function associated with a high frequency of tetramer+ T cells has also been demonstrated in other nonlymphoid tissues such as the lung (3). A further characteristic of tissue-restricted infections is the paucity of Ag-specific CD8+ T cells in peripheral lymphoid organs. During primary influenza virus infection, CD8+ T cells within the lung contain ~20% virus-specific T cells, compared with only 1–2% in spleen and regional nodes (3). By contrast, during primary lymphocytic choriomeningitis virus (LCMV) infection, >50% of splenic CD8+ T cells are virus-specific (4). However, unlike influenza virus and JHMV, productive LCMV replication in secondary lymphoid organs may contribute to the potent splenic response. Localized infections in nonlymphoid organs thus recruit virus-specific T cells into the infected tissue, giving rise to effector function concentrated at the site of Ag. The high frequency of tetramer+ CD8+ T cells within the JHMV-infected CNS further suggests highly efficient Ag presentation, despite relatively low levels of class I expression (16, 50).

Following clearance of infectious virus, a significant population of CD8+ T cells is retained within the CNS. Furthermore, the percentage of tetramer+ cells did not decline. This contrasts with nonpersistent infections in which the vast majority of Ag-experienced CD8+ T cells undergo apoptosis following virus clearance, leaving a small population of quiescent memory cells and little evidence for retention of virus-specific cells at the previous site of infection (1, 3, 4). Similar to our results, tetramer+ cells are maintained at relatively high percentages following recovery of acute EBV infection, evoking viral persistence as a driving force (8). The degree to which viral persistence or the unique anatomic properties of the CNS drive T cell maintenance during persistent JHMV infection is unclear. RNA, but not infectious JHMV, can be detected 2 yr p.i. (23). However, nonproliferating CD8+ T cells may reside in the CNS even in the absence of viral persistence (11). This contrasts with the regulation of CD4+ T cells, which efflux from the CNS in the absence of cognate Ag recognition (51–53). Persisting CD8+ T cells within the CNS may result from trapping or ongoing recruitment from the periphery. The latter would involve only memory cells, as there is no evidence for influenza virus or JHMV replication at sites outside the CNS in these models. A potential efflux of viral Ag during persistence may further be too low to trigger naive T cell activation. In addition, constitutive recruitment of memory cells would lead to an enrichment of virus-specific CD8+ T cells and eventually to CTL exhaustion (2, 5). Neither possibility is supported by experimental data, as splenocytes from JHMV persistently infected mice contain virus-specific CD8+ T cells (32) and persisting influenza virus-specific CTL retain effector function up to 160 days p.i. (11).

The biological significance of persisting CD8+ T cells at the site of previous infection appears complex. The loss of ex vivo cytolytic activity by CNS-derived T cells following clearance of infectious JHMV clearly demonstrates diminished effector function, in contrast to retention of cytolytic capacity by influenza virus-specific CD8+ T cells (11). Based on the differences in tropism and vRNA load during influenza virus and JHMV infection, the functional CD8+ T cell discrepancies may reflect Ag-induced anergy within the JHMV-specific CD8+ T cell population rather than reduced frequency. Persisting JHMV-specific CD8+ T cells may nevertheless play a role in preventing viral recrudescence via their ability to secrete IFN-γ. The total frequency of IFN-γ-secreting cells was only marginally decreased at day 45 compared with day 8 p.i., despite a decline in the CD8+ population. This apparent selective regulation may be beneficial to the host. The early loss of cytolytic function may minimize tissue damage following perforin-mediated clearance of infectious virus from microglia and astrocytes (20, 29). However, retention of IFN-γ secretion during persistence may be crucial in restraining virus replication in oligodendrocytes. These cells are refractory to perforin-mediated lysis, but sensitive to IFN-γ-mediated viral clearance (25). IFN-γ secretion in vivo is supported by the continued detection of IFN-γ-encoding mRNA in the persistently infected CNS (38).

The high frequency of tetramer+ CD8+ T cells within the CNS may reflect regional expansion or efficient recruitment of T cells activated in the CLN. However, the presence of a tetramer- CD8+ T cells during acute JHMV infection suggests nonselective recruitment. Interestingly, the “nonspecific” CD8+ T cell component is retained within the CNS throughout the course of infection. Nonselective lymphocyte recruitment has been demonstrated during the initial phase of CNS inflammation involving antiviral or autoimmune responses (51–55). Subsequent expansion of distinct T cell subsets within the CNS may result from Ag-driven proliferation (55) or from Ag-mediated retention concomitant with efflux of nonspecific T cells, as shown for CD4+ T cells (51, 52). Similar ratios of tetramer+ vs tetramer- CD8+ T cells within the CNS throughout the course of JHMV infection argue against selective enrichment of virus-specific CD8+ T cells. These data indicate that CD8+ T cells in the CNS may be regulated differently than CD4+ T cells (53), a notion supported by the presence of CTL with no apparent viral specificity during Thielers’ virus infection (56). The identity of the tetramer- CD8+ T cells remains unresolved. Although this population appears to be a nonviral component, responses to epitopes other than the dominant N318 and S510 peptides (33, 34, 36) cannot be ruled out in CB6 F1, mice. One candidate is the K b-restricted S598 epitope within the viral S protein, identified as subdominant by analysis of CNS-infiltrating cells from mice infected with a neurovirulent JHMV variant (36). However, neither ex vivo CTL or ELISPOT assays confirmed a S598-specific response in CB6 F1, mice (data not shown). In addition to

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the potential existence of as yet unidentified epitopes, an underestimation of tetramer + cells due to down-regulation of reactive TCRs may contribute to the apparently nonspecific CD8+ T cell population. The tetramer - CD8+ population may further contain autoantigen-specific T cells, although mice do not show symptoms of autoimmune disease.

The majority of CNS-derived CD8+ T cells, including the tetramer - subset, were characterized by CD62Llow, CD44high, CD11ahigh, and very late Ag-4 expression, characteristic of activated/memory T cells accessing the CNS (1, 16, 53, 57). These markers were unable to distinguish T cells isolated from the acutely or persistently infected CNS. The most striking phenotypic change during viral clearance was increased expression of the early activation marker CD69 in both the tetramer - and tetramer + subsets. CD69 is rapidly up-regulated after stimulation in vitro and is thereby implicated as an early marker of activation (58, 59). However, CD69 did not correlate with expression of the high-affinity IL-2Rα as a marker for recent activation and proliferation, consistent with minimal IL-2Rα expression on CD8+ T cells during other CNS infections (45, 60). CD69 - CD8+ T lymphocytes have been associated with chronic activation (11). However, CD69 expression may also identify cells in a state of "anergy post function" (58), and its expression has been implicated in several immunopathological diseases (59, 61). CD69 expression on CNS-infiltrating CD8+ T cells may therefore characterize cells following prolonged Ag contact, resulting in diminished effector function. This is supported by a high proportion of functionally unresponsive CD69+ CD8+ T cells in CD4-deficient mice compared with normal control mice persistently infected with LCMV (6). This is also consistent with the finding that splenic CD8+ T cells do not express CD69 during acute peripheral JHMV infection (data not shown), presumably due to rapid and complete viral clearance. However, CD69 expression on tetramer - CD8+ T cells in the present study, in addition to cytolytic activity of persisting influenza virus-specific CD69 - CD8+ T cells, indicates that CD69 regulation is only indirectly Ag-driven or associated with functional down-regulation. Other components in the local CNS environment, e.g., TNF-α, may also play a regulatory role (62).

An intriguing element of persisting CD8+ T cells is the relationship between biological function, specificity, and clonotypic makeup. Recent evidence from both human and murine models of persistent infections suggests that distinct subsets of primary CTL prevail during memory (6, 12, 13, 32, 63). The inverted ratio of CD8+ T cells responding to two, individually dominant CTL epitopes during the acute and persistent infection demonstrates that immunodominance within the CNS is dynamically regulated during an ongoing response, and thus not strictly dictated by precursor frequencies. The mechanisms responsible for the altered composition of T cell subsets are speculative. Early dominance of N-specific T cells may reflect the large pool of N protein in JHMV-infected cells, giving rise to a high density of L3-N peptide complexes. The decline of this population may be a consequence of activation-induced cell death or the preferential location of vRNA in the SC at day 12 p.i. However, these factors do not provide a satisfactory explanation for the increased frequency of S-specific T cells, as immunocytochemistry revealed no differences in either the cell type or quantity of N vs S protein distribution. Although both proteins are still present in all cell types at day 12 p.i., expression is limited to oligodendrocytes by day 14 p.i. It is therefore conceivable that oligodendrocytes may favor presentation of the S epitope, as class I Ag processing and presentation may be cell type-specific (13, 64). Changes in cell tropism and thereby class I presentation may provide a mechanism for the retention of responsive "memory" T cell subsets during persistent CNS infection. Furthermore, the existence of cross-reactive CNS epitopes cannot be excluded as a mechanism for preferential maintenance of S-specific T cells (65).

Despite a strong quantitative and qualitative local CD8+ T cell response, JHMV persists in the CNS in a noninfectious form. These data demonstrate the retention and/or ongoing recruitment of activated CD8+ T cells at the site of infection, which may be attributed to viral persistence, as evidenced by the switch in immunodominant T cell populations. However, contributions of the unique environment of the CNS as a target organ cannot be ruled out. Loss of cytolytic function, but maintenance of Ag-specific IFN-γ secretion, further suggests that the CNS has the propensity to rapidly adjust ongoing immune responses to minimize tissue destruction. Distinct tissue-specific regulation of CD8+ T cells is supported by comparative analysis of T cells derived from the CNS and peripheral tissues during autoimmune, tumor, viral, or bacterial-induced inflammation (10, 52, 66). As chronic activation of both T cells and macrophages/microglia have been implicated in pathological conditions such as autoimmune disease and ongoing primary demyelination, the extent to which persisting, chronically activated T cells exert effector function may be larger than previously anticipated and critical for disease outcome.

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References


