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Differential Regulation of Primary and Secondary CD8+ T Cells in the Central Nervous System

Chandran Ramakrishna,* Stephen A. Stohlman,*† Roscoe A. Atkinson,† David R. Hinton,† and Cornelia C. Bergmann2*†

T cell accumulation and effector function following CNS infection is limited by a paucity of Ag presentation and inhibitory factors characteristic of the CNS environment. Differential susceptibilities of primary and recall CD8+ T cells responses to the inhibitory CNS environment were monitored in naive and CD8+ T cell-immune mice challenged with a neurotropic coronavirus. Accelerated virus clearance and limited spread in immunized mice was associated with a rapid and increased CNS influx of virus-specific secondary CD8+ T cells. CNS-derived secondary CD8+ T cells exhibited increased cytolysis activity and IFN-γ expression per cell compared with primary CD8+ T cells. However, both Ag-specific primary and secondary CD8+ T cells demonstrated similar contraction rates. Thus, CNS persistence of increased numbers of secondary CD8+ T cells reflected differences in the initial pool size during peak inflammation rather than enhanced survival. Unlike primary CD8+ T cells, persisting secondary CD8+ T cells retained ex vivo cytolysis activity and expressed high levels of IFN-γ following Ag stimulation. However, both primary and secondary CD8+ T cells exhibited reduced capacity to produce TNF-α, differentiating them from effector memory T cells. Activation of primary and secondary CD8+ T cells in the same host using adoptive transfers confirmed similar survival, but enhanced and prolonged effector function of secondary CD8+ T cells in the CNS. These data suggest that an instructional program intrinsic to T cell differentiation, rather than Ag load or factors in the inflamed CNS, prominently regulate CD8+ T cell function. The Journal of Immunology, 2004, 173: 6265–6273.

M any viral infections of the CNS result in either a lethal outcome or resolve into chronic infections (1–3). The CNS is composed of terminally differentiated resident cells essential to the life of the host. These cells frequently facilitate viral persistence by providing enduring viral reservoirs (2). Although an immune response is mounted, the host appears to favor preservation of function over immune pathology as evidenced by suboptimal or rapid down-regulation of effector function within the CNS (1, 2, 4–6). Contributing to inefficient immunity in the CNS are the necessity to up-regulate MHC molecules, a paucity of professional APC, lack of direct lymphatic drainage, and the blood brain barrier restricting lymphocyte access (2, 7, 8). As a result, primary (1) CD8+ T cells recruited during CNS infection of naive mice frequently exert insufficient effector function to provide sterile immunity (1, 2, 9). By contrast, reactivated Ag-specific memory (2) CD8+ T cells established in primed mice are rapidly recruited, provide protection, and are preferentially retained in the CNS following challenge (10, 11). Thus, despite lethal outcomes associated with primary infections of mice with neurotropic influenza virus, dengue virus, and coronavirus, memory T cells induced by vaccination provide protection from lethal encephalitis following CNS challenge (10–13). Whereas influenza virus-specific memory CD8+ T cells completely cleared the challenge virus (10), coronavirus-specific CD8+ T cells only modestly reduced infectious virus titers (12, 13). Furthermore, both protective influenza and dengue virus-specific memory T cells with effector memory characteristics persisted in the CNS (10, 11). However, despite the common propensity for long-term maintenance of CD8+ T cells within the CNS, 1 and 2 CD8+ T cells appear to differ in the regulation of cytolysis function (10, 14, 15). In contrast to 2 influenza virus-specific CD8+ T cells (10), 1 CD8+ T cells recruited to the CNS of mice infected with a non lethal neurotropic coronavirus, rapidly down-regulate cytolytic effector function as the infectious virus is controlled, independent of virus pathogenicity or ongoing demyelination (14–16). Furthermore, virus recrudescence does not trigger re-expression of cytolysis activity (17), suggesting that Ag alone does not contribute to the regulation of this effector function. The prevalent notion to account for the loss of cytolytic activity is that prolonged cytolytic mechanisms are deleterious to the CNS environment (1, 2) and may predispose to autoimmune responses. The CNS is thus thought to favor noncytolytic mechanisms such as cytokines and Ab to clear virus infections (1, 2, 17, 18). Several candidates involved in the modulation of cytolysis activity by CD8+ T cells include extrinsic factors in the CNS such as lipids, NO, or regulatory cytokines such as IL-10 and TGF-β (2, 6, 19–23). It is also feasible that persisting Ag may account for loss of cytolytic effector function by 1 CD8+ T cells retained in the CNS although studies using the virus completely eliminated from the CNS suggest that this may not be true (15, 16). Alternatively, effector function may be modulated by signals imposed by the prominent cell type infected and/or the activation state of the local CNS environment (6). It is thus unclear whether retention of cytolytic capacity...
is a characteristic of 2'CD8+ T cells residing within the CNS, similar to effector memory T cells in nonlymphoid organs (24).

Direct comparison of 1' and 2' CD8+ T cells is preempted in most CNS viral infections due to a lethal outcome, induction of anti-viral CD4+ T cells, and neutralizing Ab (10, 11, 25). However, CNS infection with the neurotropic JHM strain of mouse hepatitis virus (JHMV) provides a nonlethal model allowing direct comparison of distinct CD8+ T cell differentiation states within the CNS following clearance of the infectious virus. During the acute phase, virus replicates in microglia/macrophages, astrocytes, and oligodendrocytes (26). In naive mice, the virus induces an acute encephalomyelitis, which resolves into a chronic infection characterized by viral RNA and ongoing myelin loss in the absence of infectious virus (1, 15, 16). CD8+ T cells are the major anti-viral effectors and clear infectious virus by 14 days postinfection (p.i.) in a cell type-specific manner via either IFN-γ secretion or perforin-mediated cytolysis (27, 28). Subsequent to virus clearance, the majority of activated T cells disappear from the CNS; however, a population is maintained during persistence (14–16). Ag is generally reduced to undetectable levels by day 30 p.i. (1, 15–17), suggesting minimal Ag-mediated stimulation during persistence. CD8+ T cells in the CNS lose cytolytic activity immediately following virus clearance but retain the capability to secrete IFN-γ ex vivo during persistence (14, 15, 17). Although retention of CD8+ T cells in the CNS correlates with persisting viral mRNA, an association with factors involved in ongoing demyelination has not been excluded (14–16).

JHMV infection was used to compare recruitment kinetics of 1' vs 2' CD8+ T cells, their function, and regulation by the CNS environment. Memory CD8+ T cells were established by immunization with a recombinant vaccinia virus (rVV) encoding an immunodominant CD8+ T cell minigene epitope, thereby precluding JHMV-specific CD4+ T cell and neutralizing Ab responses. Following CNS challenge, 2' CD8+ T cells persist in the CNS rapidly, expressed increased cytolytic activity and IFN-γ secretion, and were more effective in controlling virus replication compared with 1' CD8+ T cells. Although increased numbers of 2' CD8+ T cells persist in the CNS despite rapid elimination of Ag, there was no evidence for enhanced intrinsic survival. However, 2' CD8+ T cells retained ex vivo cytolytic activity and secreted increased IFN-γ compared with 1' CD8+ T cells. These data indicate that modulation of effector function by CD8+ T cells persisting in the CNS is preregulated, rendering the expression of effector function by reactivated memory CD8+ T cells more resistant to CNS suppressive factors.

Materials and Methods

Mice, viruses, and immunizations

BALB/c mice were purchased from the National Cancer Institute (Frederick, MD). Homozygous BALB/c Thy1.1 mice, provided by Dr. J. Harty (University of Iowa City, IA), were bred locally at the University of Southern California (Los Angeles, CA). For immunization, mice of both sexes were used between 6 and 7 wk of age. JHMV-specific memory CD8+ T cells were elicited by i.p. immunization with 1 × 10^6 PFU of a rVV expressing the Ld-restricted immunodominant epitope, designated pN9 (29). This minigene vaccine approach limits JHMV immunity to the CD8+ T cell compartment and results in <2% pN9-specific cells within the splenic CD8+ T cell compartment at the time of challenge. At 4–6 wk postimmunization, immune BALB/c mice were challenged intracerebrally with 500 PFU of J2.2v-1 neutralization mAb-derived variant of neurotropic JHMV (30) as previously described (14). For simplicity, these mice are designated as 2' mice. Age-matched naive mice infected with the identical dose of JHMV were used as primary (1') responders. Thy1.1 mice were injected i.p. with 1–2 × 10^6 PFU of JHMV to provide CD8+ donor cells. Infectious JHMV was determined from clarified homogenates prepared from brains, cervical lymph nodes (CLN), and CNS were preincubated with a mixture (10%) of mouse, rat, and human sera (Atlanta Biologicals, Norcross, GA) and rat anti-mouse CD16/32 (2.442; BD Pharmingen, San Diego, CA) for 20 min on ice to block non-specific binding. PE, FITC, PerCP, and allophycocyanin-labeled mAb specific for CD45 (30-F11), CD4 (RM4-5), CD44 (IN7), CD62L (MEL-14), CD49d (R1-2), Thy1.1 (OX-7), Thy1.2 (30-H12), Ly-6G (1A8), Ly-6C (AL-21), MHC class I (39-10-8), class II (2G9), Bcl2 (3F11), and isotype-control hamster IgG (A9-13) were all obtained from BD Pharmingen. Anti-F4/80 PE was obtained from Serotec (Raleigh, NC). Allophycocyanin-labeled anti-granzyme B (GB12) and isotype-control mouse IgG1 were obtained from Caltag Laboratories (Burlingame, CA). JHMV-specific CD8+ T cells were identified by Ld-pN9 tetramer and pN9 tetramer (29, 31). Function of pN9-specific CD8+ T cells was determined by intracellular cytokine staining for IFN-γ and TNF-α (31). Briefly, 1–2 × 10^6 CNS, spleen, or CLN cells were incubated with 3 × 10^6 J77.4.1 feeder cells with or without 1 μM pN9 peptide for 5 h at 37°C in the presence of Golgistop (BD Pharmingen). Cells were stained for surface markers and permeabilized using Cytofix (BD Pharmingen) for 15 min at 4°C. PE or FITC-labeled IFN-γ (XMG 1.2) and TNF-α (MP6-XT22) mAb were used to assess intracellular cytokine production. Granzyme B and Bcl2 expression were analyzed on cells stained for surface markers before permeabilization with Cytofix. Cells were analyzed with a FACSCalibur flow cytometer using CellQuest Pro software (BD Biosciences, Mountain View, CA).

Histology

Brains, bisected in the mid-coronal plane, and spinal cords were examined for inflammation and distribution of viral Ag. Tissues were fixed for 3 h in 10% buffered formalin (75% ethanol and 25% glacial acetic acid) before embedding. Sections were stained with H&E to determine inflammation. Distribution of viral Ag was determined by immunoperoxidase staining (Vectastain-ABC kit; Vector Laboratories, Burlingame, CA) using the anti-JHMV mAb 3.3.3 specific for the carboxyl terminus of the viral nucleocapsid protein as the primary Ab (32) and horseradish anti-mouse IgG (Vector Laboratories). Sections were scored for inflammation and viral Ag in a blinded fashion. Representative fields were identified based on average scores of all sections in each experimental group.
Results

**Increased CNS virus clearance by 2′ CD8+ T cells**

JHMV-specific memory CD8+ T cells protect immunocompromised mice and reduce CNS virus in the presence or absence of CD4+ T cells (31, 33). To confirm that endogenous CD8+ T cell memory enhances CNS virus clearance in immunocompetent mice, CD8+ T cell memory was established by immunization with an rVV expressing the p99 immunodominant CTL epitope (29). Immune mice were challenged with JHMV at 4 wk postimmunization and virus clearance compared with infected age-matched naive mice. Viral titers were reduced by 40- to 100-fold in the CNS of 2′ compared with 1′ mice at 5 and 7 days p.i. (Fig. 1A). Furthermore, the infectious virus could not be detected in 40% of 2′ mice compared with recovery from 100% of 1′ mice at 10 days p.i. Moreover, the infectious virus was reduced in the remaining 60% of 2′ mice compared with 1′ mice. No infectious virus could be recovered from either group of mice following 14 days p.i., consistent with previous reports (14, 15, 17). Enhanced virus clearance in 2′ mice was associated with earlier onset (days 5–6 p.i.) of clinical disease, characterized by a hunched back and altered gait, compared with similar clinical signs at days 8–9 p.i. in 1′ mice. These observations are consistent with the notion that clinical disease is immune mediated (33, 34). Subsequently, 2′ mice exhibited clinical symptoms similar to 1′ mice through all times points monitored. Histological analysis showed an increased inflammatory response in the CNS of 2′ mice, compared with 1′ mice (Fig. 1, B and C). Consistent with reduced infectious virus in the CNS of 2′ mice (Fig. 1A), fewer Ag-positive glial cells were detected in brains (data not shown) and spinal cords (Fig. 1B) at day 10 p.i. compared with 1′ mice (Fig. 1C). In 2′ mice, relatively few Ag-positive cells were detected in the gray matter. The number of infected white matter cells with the morphological characteristics of oligodendrocytes were also reduced at 10 days p.i. compared with 1′ responders. These data suggested efficient in vivo perforin-mediated cytolysis and IFN-γ secretion (27, 28).

**Memory T cells alter CNS inflammation**

Enhanced virus control, earlier onset of clinical disease, and increased encephalitis in 2′ mice suggested an altered CNS inflammatory response. Increased infiltration of CD45high bone marrow-derived cells was detected in the CNS of 2′ mice (>55% of total CMC), compared with 1′ mice (<20%) as early as 5 days p.i. (Fig. 2). This increase was also reflected by a 5- to 6-fold increase in CD45high cells recovered from the CNS (Fig. 2B). However, despite the rapid onset of inflammation, similar percentages and absolute numbers of CD45high CNS infiltrates were obtained from both groups at day 7 and all subsequent days p.i. (Fig. 2B). The composition of infiltrating cells was analyzed to determine the cell types preferentially recruited into the CNS of 2′ mice. Macrophages constituted the major component in the CNS infiltrates of both groups, with similar frequencies at day 5 p.i. (Fig. 3A). By contrast, the relative frequencies of neutrophils and NK cells were reduced in 2′ vs 1′ CNS infiltrates (Fig. 3A). This predominance of macrophages in the early response contrasts with a lethal neurotropic JHMV infection in which neutrophils are a predominant component of the early CNS infiltrates (35). This discrepancy may be explained by differences in tropism and virulence. The early increase in CNS infiltrates of 2′ responders was thus partially accounted for by increased macrophage recruitment. No differences were evident in the relative CD4+ T cell percentages (Fig. 3B); thus, absolute CD4+ T cell numbers were elevated only 4-fold at day 5 p.i. within the CNS of 2′ mice compared with 1′ mice (Fig. 3B). By contrast, the difference in CD8+ T cell

**FIGURE 1.** JHMV pathogenesis in 1′ and 2′ responder mice. Immunized BALB/c mice harboring JHMV-specific memory CD8+ T cells and age-matched naive mice were infected with JHMV. JHMV replication in the CNS of 1′ and 2′ responder mice was assessed by plaque assay at indicated time points p.i. (A). Data represent mean titers in the CNS of at least three experiments with three to four mice per time point. The dotted line indicates limit of detection. JHMV Ag in the spinal cords of JHMV-infected 2′ (B) and 1′ (C) responder mice (immunoperoxidase stain for JHMV Ag with hematoxylin counterstain). In 2′ responders, viral Ag was limited to a small number of cells localized to the white matter tracks. In 1′ responders, JHMV Ag-positive cells were more numerous and detected within both white and gray matter. Bar, 100 μm.

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recruitment was very distinct (Fig. 3B). CD8\(^+\) T cells comprised a major fraction of the CD45\(^{high}\) population in the CNS of 2\(^{nd}\) mice (\(>30\%\)), compared with 1\(^{st}\) responders (\(<5\%) at day 5 p.i. (Fig. 3B). Thus, absolute CD8\(^+\) T cell numbers in 2\(^{nd}\) responders by far surpassed those in 1\(^{st}\) mice. Even though infiltration equalized by day 7 p.i., the CD8\(^+\) T cell proportion remained elevated at \(>55\%\) of the CD45\(^{high}\) infiltrating cells in 2\(^{nd}\) mice compared with \(<35\%\) in 1\(^{st}\) mice at all subsequent time points (Fig. 3B). Therefore, after day 5 p.i. the relative percentages of total CD8\(^+\) T cells within their respective CD45\(^{high}\) populations remained fairly constant over 90 days p.i. (Fig. 3B).

**Ag-specific 2\(^{nd}\) CD8\(^+\) T cells dominate in quantity and effector function**

The proportion of pN9-specific cells within the total CD8\(^+\) T cells was examined to determine the contribution of virus-specific and bystander CD8\(^+\) T cell recruitment. Tetramer analysis revealed that \(~50\%\) of 2\(^{nd}\) CD8\(^+\) T cells were virus specific at day 5 p.i. and the frequency increased to 65% by day 7 p.i. (Fig. 3C). By contrast, the percentage of tetramer\(^{-}\) cells was lower at day 5 p.i. in the CNS of 1\(^{st}\) responders and only reached 50% of the CD8\(^+\) population at day 7 p.i. The relative proportion of pN9-specific CD8\(^+\) T cells remained consistently elevated in 2\(^{nd}\) mice up to day 90 p.i. (Fig. 3C). Conversion of relative percentages to total numbers of tetramer\(^{+}\) CD8\(^+\) T cells recovered per brain of 2\(^{nd}\) (solid) and 1\(^{st}\) (open) mice are depicted as circles. Data represent an average of two to three independent experiments and SD is \(<10\%\).

**FIGURE 2.** Rapid CNS infiltration of mononuclear cells in 2\(^{nd}\) responders. Single cell suspensions were prepared from the CNS of infected mice undergoing 2\(^{nd}\) and 1\(^{st}\) CD8\(^+\) T cell responses (\(n = 4–5\) per time point). A. Representative density plots of CD45 expression in 2\(^{nd}\) (left plot) or 1\(^{st}\) CNS populations (right plot). Differences in CD45\(^{high}\) infiltrates are highlighted by the R2 and R4 regions. B. Bars depict percentages of infiltrating CD45\(^{high}\) cells in total CNS cells obtained for 2\(^{nd}\) (solid) and 1\(^{st}\) (open) responders. Circles depict total numbers of CD45\(^{high}\)-infiltrating cells recovered per brain from 2\(^{nd}\) (solid) and 1\(^{st}\) (open) responders.

**FIGURE 3.** Altered composition of CNS infiltrates in 2\(^{nd}\) responders. A. Relative percentages of macrophages, neutrophils, and NK cells within the CNS-infiltrating populations of 2\(^{nd}\) and 1\(^{st}\) mice at days 5 and 7 p.i. Data were calculated by setting CD45\(^{high}\)-infiltrating cells to 100%. B. Bars depict relative percentages of CD8\(^+\) (solid) and CD4\(^+\) (open) T cells within the 2\(^{nd}\) and 1\(^{st}\) CD45\(^{high}\) populations (x-axis = day p.i.). C. Bars represent percentages of tetramer\(^{+}\) cells within the CD8\(^+\) T cell population derived from the CNS of 2\(^{nd}\) (solid) or 1\(^{st}\) (open) mice, respectively. Total numbers of infiltrating tetramer\(^{+}\) CD8\(^+\) T cells recovered per brain of 2\(^{nd}\) (solid) and 1\(^{st}\) (open) mice are depicted as circles. Data represent an average of two to three independent experiments and SD is \(<10\%.\)
were detected in the CNS of 2' mice 2 days earlier (day 5 p.i.) and increased a further 2-fold by day 7 p.i. Elevated virus-specific CD8^+ T cells within the CNS of 2' vs 1' mice suggested prominent recruitment and/or expansion of pN9-specific memory T cells. Indeed, increased frequencies of virus-specific CD8^+ T cells were detected both at days 5 and 7 p.i. in the CLN and spleen of 2' responders; expansion was more evident in the CLN compared with spleen (data not shown), consistent with the suggestion that CLN are the prominent site of CNS Ag drainage (7, 36). Despite increased numbers (2- to 3-fold following day 7 p.i.) of tetramer^-specific CD8^+ T cells in 2' mice throughout infection, the overall survival relative to peak CNS accumulation appeared to be similar in both groups (Fig. 3C). Although contraction of the virus-specific CD8^+ T cells was more rapid in 1' responders, resulting in a 70–80% loss between days 7 and 14 p.i., contraction of virus-specific 2' CD8^+ T cells during the same interval was less pronounced with only a ~45% decrease; albeit contraction was prolonged to day 25 p.i. The decline was more gradual and similar comparing 2' and 1' tetramer^+ CD8^+ T cells between 25 and 90 days p.i. (Fig. 3C). Retention of virus-specific CD8^+ T cells following viral clearance thus appeared to correlate with the maximal pool size recruited into the CNS during acute infection, rather than inherently distinct survival capacities.

The increased percentages of tetramer^+ CD8^+ T cells in the 2' CNS population (Figs. 3C and 4A) corresponded with increased CD8^+ T cells producing IFN-γ and TNF-α compared with 1' CD8^+ T cells (Fig. 4, B and C). Although 60–80% of 2' tetramer^+ CD8^+ T cells isolated from the CNS secreted IFN-γ at days 7 and 14 p.i., <60% of 1' tetramer^+ CD8^+ T cells secreted IFN-γ (Fig. 4, B and C). Higher mean fluorescence intensity (MFI) suggested that 2' CD8^+ T cells produced more IFN-γ than 1' CD8^+ T cells (Fig. 4B). Moreover, the majority of 2' tetramer^+ CD8^+ T cells (~70%) retained the ability to produce IFN-γ in contrast to the diminishing numbers (~40–50%) of 1' tetramer^+ CD8^+ T cells at 60 and 90 days p.i. (Fig. 4C). Thus, in addition to an increased percentage of cells capable of IFN-γ secretion, 2' Ag-specific CD8^+ T cells have the capacity to secrete more IFN-γ per cell. A surprising finding was that while >50% of both 1' and 2' tetramer^+ CD8^+ T cells were capable of IFN-γ secretion, only ~20–35% of tetramer^+ CD8^+ T cells produced TNF-α throughout infection (Fig. 4C). Although a higher proportion of cells producing IFN-γ compared with TNF-α is common during many acute infections (37–39), equal frequencies of IFN-γ^+ and TNF-α^+ CD8^+ T cells are observed during memory (37–39). The reduced ability to secrete TNF-α, even following virus clearance, appears to be a unique property of CD8^+ T cells within the CNS, as all splenic memory CD8^+ T cells secreted both IFN-γ and TNF-α at both 2 and 9 mo following peripheral JHMV infection (data not shown).

Virusspecific CD8^+ T cells isolated from both 1' and 2' CNS at days 5–14 p.i. expressed an activated phenotype (CD69^hi, CD44^hi, CD62L^lo, Ly6C^lo, CD49d^hi, and data not shown). Activation in both populations was further supported by high levels of CD43 expression, a marker associated with cytolytic activity (11, 40, 41), and loss of CD127 expression at day 7 p.i. (data not shown). By day 25 p.i., the majority of 1' and 2' CNS CD8^+ T cells expressed CD127, indicative of a memory population (42). By contrast, while the CD43^hi phenotype was retained by ~65% of 2' CD8^+ T cells (MFI = 1024) the majority (~76%) of 1' CD8^+ T cells expressed intermediate CD43 levels (MFI = 520).
Finally, an increased proportion of tetramer$^+$ CD8$^+$ T cells in the 2$^\text{nd}$ immune CNS expressed Bcl2 compared with 1$^\text{st}$ controls at day 7 p.i. (Ref. 43; Fig. 4D). Although increased Bcl2 expression by 2$^\text{nd}$ tetramer$^+$ CD8$^+$ T cells correlated with increased resistance to apoptosis before day 14 p.i., it did not result in increased overall survival in the CNS (Fig. 3C). Furthermore, the majority of 1$^\text{st}$ and 2$^\text{nd}$ CD8$^+$ T cells persisting in the CNS following day 14 p.i. were CD44$^{high}$, CD62L$^{high}$, and retained CD69 expression (data not shown), an early activation marker reported to correlate with retention of memory CD8$^+$ T cells in nonlymphoid organs (10, 14, 44).

2$^\text{nd}$ CD8$^+$ T cells retain cytolytic activity

To determine whether 2$^\text{nd}$ JHMV-specific CD8$^+$ T cells retain cytolytic activity, similar to 1$^\text{st}$ influenza virus-specific CD8$^+$ T cells (10), ex vivo CTL activity was compared in 1$^\text{st}$ and 2$^\text{nd}$-JNS-activated CD8$^+$ T cells. Specific cytolysis was analyzed for ex vivo cytolytic activity at indicated time points p.i. using peptide-coated J774.1 (H-2$^d$) target cells. Specific cytolysis (right plot) was normalized to reflect lysis achieved by one tetramer$^+$ cell per target cell. Data represent one of two to three similar experiments.

Survival and effector function of 1$^\text{st}$ and 2$^\text{nd}$ CD8$^+$ T cells in the same host

To verify that the majority of virus-specific CD8$^+$ T cells recruited and retained in the CNS are derived from the memory population, CD8$^+$ T cells purified from spleens of immunized BALB/c Thy1.1 donors were transferred into naive BALB/c Thy1.2 recipients 24 h before infection. Thy1.1 memory CD8$^+$ T cells comprised $>90\%$ of the CNS-infiltrating CD8$^+$ T cell population at 8 days p.i. (Table I). Furthermore, Thy1.1$^+$ donor CD8$^+$ T cells were retained at high frequencies at 30 days p.i. (Table I), confirming preferential recruitment of memory rather than newly primed cells.

To confirm that increased granzyme B, IFN-$\gamma$, and Bcl2 expression were intrinsic properties of reactivated memory CD8$^+$ T cells, even in a 1$^\text{st}$ CD8$^+$ T cell CNS environment, memory Thy1.1$^+$ CD8$^+$ T cells were transferred at day 2 p.i. into Thy1.2$^+$ recipients. This approach allowed normal priming of endogenous CD8$^+$ T cells and simultaneous recruitment of both 1$^\text{st}$ Thy1.1$^+$ and donor memory Thy1.1$^+$ CD8$^+$ T cells. The CNS CD8$^+$ T cell population was comprised of $66\%$ 1$^\text{st}$ and $34\%$ 2$^\text{nd}$ CD8$^+$ T cells at day 8 p.i. (Table II). The majority of recruited donor CD8$^+$ T cells were virus specific ($>60\%$). By contrast, only $\sim30\%$ of recipient 1$^\text{st}$ CD8$^+$ T cells were virus specific (Table II). Consistent with increased recruitment of memory cells into the infected CNS, a higher percentage of 2$^\text{nd}$ CD8$^+$ T cells expressed granzyme B, IFN-$\gamma$, and the antiapoptotic marker Bcl2, compared with 1$^\text{st}$ recipient CD8$^+$ T cells (Table II). Unaltered ratios of 2$^\text{nd}$ Thy1.1$^+$ to 1$^\text{st}$ Thy1.1$^+$ CD8$^+$ T cells at days 8 and 30 p.i. suggested similar survival capacities by both populations, despite increased Bcl2 expression by 2$^\text{nd}$ CD8$^+$ T cells at day 8 p.i. (Table II). The frequency of tetramer$^+$ cells remained greater in 2$^\text{nd}$ Thy1.1$^+$ CD8$^+$ T cells ($\sim70\%$) compared with recipient Thy1.2$^+$ CD8$^+$ T cells ($\sim30\%$) at day 30 p.i. (Table II and Fig. 6). These data were surprisingly similar to the equivalent survival of 1$^\text{st}$ and 2$^\text{nd}$ CD8$^+$ T cells observed following infection of immune and naive mice. Analysis of the two populations at 30 days p.i. showed an increased percentage of virus-specific 2$^\text{nd}$ CD8$^+$ T cells expressed granzyme B and IFN-$\gamma$ compared with 1$^\text{st}$ CD8$^+$ T cells (Table II and Fig. 6). These results confirm that 2$^\text{nd}$ effectors retain effector function for prolonged periods of time within the CNS compared with 1$^\text{st}$ CD8$^+$ T cells, even when exposed to identical stimuli and suppressive factors. During re-exposure to Ag, these cells overwhelm the primary immune response, clear Ag rapidly, and importantly persist within the CNS in a state of “readiness”.

Discussion

The goal of this study was to investigate quantitative and qualitative differences between 1$^\text{st}$ and 2$^\text{nd}$ CD8$^+$ T cells and their influence on viral CNS pathogenesis. Confirming functional memory, Ag-specific memory CD8$^+$ T cells exhibited a heightened capacity for recall in lymphoid organs following CNS viral challenge, were rapidly recruited to the CNS in increased numbers, and facilitated efficient viral clearance. Comparison of 1$^\text{st}$ and 2$^\text{nd}$ CD8$^+$ populations revealed several novel findings: 1) during acute infection, 2$^\text{nd}$ CD8$^+$ T cells had a greater capacity to secrete IFN-$\gamma$ and express cytolytic activity at the population level and on a single cell basis, despite similar activated phenotypes; 2) survival rates of 1$^\text{st}$ and 2$^\text{nd}$ populations within the CNS were similar following 25 days p.i.; 3) both 1$^\text{st}$ and 2$^\text{nd}$ CD8$^+$ T cells in the CNS had a reduced capacity for TNF-$\alpha$ production, even following viral clearance; and 4) in contrast to 1$^\text{st}$ CD8$^+$ T cells, persisting 2$^\text{nd}$ CD8$^+$ T cells retained cytolytic activity and IFN-$\gamma$ secretion. Therefore, 2$^\text{nd}$ CD8$^+$ T cells residing in the CNS following viral clearance appear to remain on a “higher alert status” compared with 1$^\text{st}$ CD8$^+$ T cells.

Initial activation and proliferation of both 1$^\text{st}$ and 2$^\text{nd}$ CD8$^+$ T cells was detected in the CLN rather than the spleen, supporting CNS Ag drainage primarily to the CLN (7, 36). Vastly increased

### Table I. Memory CD8$^+$ T cells limit the magnitude of primary CNS response

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<th>Days p.i.</th>
<th>Percentage of Donor Thy 1.1$^+$ CD8$^+$</th>
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$^a$ Memory donor CD8$^+$ T cells were transferred i.v. 1 day prior to infection.

$^b$ Percentages within the total CNS CD8$^+$ T cell population.
In contrast to highly efficient IFN-γ class II expression on microglia at both early and late time points, CD8+ T cells were transferred i.v. at 2 days p.i. Data from pooled samples. Representative of two separate experiments. Thy 1.1 memory CD8+ T cells were transferred i.v. at 2 days p.i. Percentages within either donor (Thy 1.1) or recipient (Thy 1.2) CNS CD8+ T cells.

CD8+ T cells were largely derived from the memory pool rather than naive precursors as shown by the dominance of memory donor CD8+ T cells transferred before infection in the recipient CNS-infiltrating T cell population. As the number of virus-specific memory T cells in immune mice is estimated to be at least 20-fold higher than in adoptive transfer recipients, this population is equally likely to out compete 1st responses (50). These data are similar to peripheral models of infection demonstrating that memory CD8+ T cells influence the magnitude of the endogenous 1st response (50). The data suggest that memory cells re-exposed to Ag overwhelm priming and expansion of naive cells in lymphoid organs, possibly by competing for limited APC and are thus prominently recruited into the CNS.

During acute infection, 1st and 2nd CD8+ T cells expressed similar activated phenotypes with the exception of Bcl2 expression. Although 1st tetramer+ CD8+ effectors expressed reduced Bcl2 levels compared with naive and memory cells (43), a significant population of 2nd virus-specific CD8+ T cells within the CNS retained Bcl2 expression. One possible reason could relate to the distinct histories of Ag exposure and proliferation during re-challenge, resulting in CD8+ T cell subsets with distinct survival capacities. Alternatively, this population may be enriched for Ag-specific memory T cells recruited directly from the periphery, which are more resistant to apoptosis compared with effector cells (24). Nevertheless, Bcl2 expression during acute infection correlated with prolonged contraction in 2nd CD8+ T cells, but not with enhanced overall survival at later time points, similar to the prolonged contraction of 2nd CD8+ T cells following either Listeria monocytogenes or lymphocytic choriomeningitis virus infection (50).

Enhanced effector function inherent to 2nd CD8+ T cells was evident by both increased frequencies of IFN-γ-secreting cells and also by increased expression on a per cell basis. This difference in IFN-γ secretion was even more evident following virus clearance. These results were confirmed by increased IFN-γ expression by CNS-derived 2nd donor CD8+ T cells compared with 1st recipient CD8+ responses. Increased IFN-γ biological activity in the CNS of 2nd responders was also reflected by enhanced IFN-γ-dependent class II expression on microglia at both early and late time points p.i. (C. Ramakrishna and C. Bergman, unpublished observations).

In contrast to highly efficient IFN-γ production, there was a clear paucity of TNF-α production by both 1st and 2nd virus-specific CD8+ T cells throughout the course of CNS infection. This effect does not appear to be a virus-mediated decoy mechanism (51), as equal numbers of memory CD8+ T cells secreting IFN-γ and TNF-α were present in the spleens of JHMV-immunized mice. TNF-α down-regulation is Ag independent and occurs early following Ag recognition (37, 38). Although TNF-α producers are fewer than IFN-γ producers during many acute responses, restimulation of memory T cells isolated from both lymphoid and non-lymphoid organs is associated with equivalent responsiveness (37–39). Down-regulation of TNF-α during acute responses may thus be explained by Ag encounter in both the periphery and CNS. However, the inability to recover TNF-α production once viral Ag is cleared from the CNS remains unsolved. Inefficient priming has been shown to affect the capacity of T cells to efficiently secrete TNF-α and/or establish efficient memory (52). Although Ag drainage into the CLN and the absence of professional APC in the CNS may indeed lead to suboptimal priming following CNS infection, IFN-γ/TNF-α competent memory T cells should not be regulated by this mechanism during a recall response. It has also been suggested that partially exhausted memory CD8+ T cells lose the ability to secrete TNF-α under conditions of subsaturating signal strength and long-term exposure to the virus (53). Although this may be true for 1st CD8+ T cells, 2nd CD8+ T cells in the CNS are exposed to even lower levels of virus, implying exhaustion is unlikely to play a role. One possible explanation is regulation via adhesion molecules. Both CD69 and CD43 are highly expressed on 1st and 2nd CD8+ T cells persisting in the CNS and both molecules have been implicated in down regulating lymphocyte responses (41, 54). Alternatively, signaling by CNS resident cells or factors present in the environment may provide negative signals for T cell TNF-α production. Irrespective of the mechanism, the reduced capacity of 2nd CD8+ T cells in the CNS to produce TNF-α upon Ag stimulation is inconsistent with conversion to a resting effector memory CD8+ T cell following viral clearance.

A crucial finding is retention of ex vivo cytolytic activity by 2nd CD8+ T cells, correlating with increased CD43 and granzyme B expression. Distinct regulation of cytolytic activity may either be intrinsic to differentiation of 1st vs 2nd CD8+ T cells, or regulated by reduced exposure to Ag in the CNS during the 2nd response (53). Down-regulation of cytolytic activity by 1st CD8+ T cells following infection with both pathogenic and nonpathogenic, nonpersisting JHMV variants (15, 16) suggest that prolonged Ag exposure does not correlate with modulation of this effector function. Conversely, the inability of 1st CD8+ T cells to re-express cytolytic activity following virus recrudescence in the CNS of B cell-deficient mice does not exclude Ag-mediated suppression (17).

The present data imply that CD8+ T cell intrinsic factors regulate cytolytic activity because both 1st and 2nd CD8+ T cells are exposed to the same inflamed CNS environment. This notion is supported by increased granzyme B+ CD8+ T cell frequencies in 2nd tetramer+ CD8+ T cells compared with 1st recipient CD8+ T cells in the CNS. Thus, these results favor a distinct susceptibility to the
down-regulation of effector function, rather than the action of omnipotent suppressive factors, cytokines, or T regulatory cells in the CNS. Furthermore, they suggest that vaccination strategies designed to elicit vigorous T cell responses to CNS pathogens is a dual edged sword. Although a peripheral pool of memory CD8\(^+\) T cells is clearly beneficial for rapid viral clearance, early recruitment coincides with significantly enhanced macrophage infiltration, potentially enhancing immune-mediated pathology. Although retention of highly armed effector cells in the CNS long after viral clearance may provide an early line of defense upon re-infection, potential cross-reactivity with self-Ag may also facilitate development of autoimmune disease.

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