Kinetics of Virus-Specific CD8⁺-T-Cell Expansion and Trafficking following Central Nervous System Infection

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CD8⁺ T cells control acute infection of the central nervous system (CNS) by neurotropic mouse hepatitis virus but do not suffice to achieve sterile immunity. To determine the lag between T-cell priming and optimal activity within the CNS, the accumulation of virus-specific CD8⁺ T cells in the CNS relative to that in peripheral lymphoid organs was assessed by using gamma interferon-specific ELISPOT assays and class I tetramer staining. Virus-specific CD8⁺ T cells were first detected in the cervical lymph nodes. Expansion in the spleen was delayed and less pronounced but also preceded accumulation in the CNS. The data further suggest peripheral acquisition of cytolytic function, thus enhancing CD8⁺-T-cell effector function upon cognate antigen recognition in the CNS.

The mechanisms of T-cell priming and expansion following infections of the central nervous system (CNS) are poorly understood due to several unique characteristics of the CNS. The blood-brain barrier restricts entry of most circulating resting cells, resulting in very low frequencies of naïve T cells (14, 15). Furthermore, resident cells in the quiescent CNS express very low, if any, basal levels of class I and II molecules (13, 27). Lastly, the CNS lacks conventional regional lymph nodes that drain other tissues and appears to be devoid of resident dendritic cells capable of migrating to lymphoid organs (6, 13). Nevertheless, antigen in the CNS can drain into both the cervical lymph nodes (CLN) and spleen by several routes (6–9, 28). During lymphocytic choriomeningitis virus-induced meningitis and non-neurovirulent influenza virus infection, the deep CLN constitute the primary site of T-cell priming (8, 28); peripherally activated cells then acquire the ability to enter the CNS, where they exert effector function. The relative kinetics of T-cell expansion, migration, and acquisition of effector function following viral infection of the CNS are thus critical for control of replication and potential pathology.

Infection with the neurotropic JHM strain of mouse hepatitis virus (JHMV) was used to study the progression of early T-cell responses preceding virus-induced encephalomyelitis. Mice infected with the reduced-virulence 2.2-V-1 JHMV variant clear infectious virus from the CNS by day 12 postinfection (p.i.) (2, 18, 24) but still develop immune-mediated demyelination (11, 20, 25). Ongoing demyelination is associated with persisting noninfectious viral RNA. Virus replication peaks at day 5 p.i. and is largely controlled by CD8⁺ cytotoxic T lymphocytes (CTL) via both perforin- and gamma interferon (IFN- γ)-mediated mechanisms (18, 20, 24, 30). Cells isolated directly from the brain exhibit virus-specific ex vivo cytotoxicity between days 7 and 9 p.i. (1, 2, 19), a function not observed in cells derived from either spleen or CLN (4, 29). Cytolytic

activity correlates with a high frequency of virus-specific CD8⁺ T cells in the CNS compared with barely detectable levels in the periphery (1, 2, 19). However, it is unknown whether cytolytic activity is acquired in the periphery or at the site of virus replication within the CNS. It is further unclear to what extent virus-specific T cells expand within the CNS or prior to CNS entry.

To trace T-cell expansion and infiltration into the CNS during the course of acute viral infection, BALB/c mice were infected intracerebrally with 1,000 PFU of the JHMV 2.2-V-1 variant (11). Virus-specific CD8⁺-T-cell accumulation within spleen, CLN, and brain was examined at days 3, 4, 5, 6, and 7 p.i. by flow cytometry using *L^d*-N³¹⁸ tetramers (2, 19) comprising the dominant viral nucleocapsid protein epitope (N³¹⁸) (3). Tetramer-positive cells were below the limit of detection prior to day 5 p.i. in all three tissues examined. Nonspecific staining with the *L^d*-N³¹⁸ tetramer is below a frequency of 2/1,000 (0.2%) CD8⁺ T cells; thus, only frequencies above 1/100 (1.0%) CD8⁺ T cells were considered specific. At day 5 p.i., the majority of tetramer-positive CD8⁺ T cells were found in the spleen (2.1×10^5 total cells) and CLN (6.9×10^4 cells) compared with the number in the brain (1.6×10^4 cells) (Fig. 1A). This distribution changed rapidly by day 6 p.i., with tetramer-positive T cells being more abundant within the brain than in the CLN (1.6×10^5 and 5.8×10^4 cells, respectively) but still less than within the spleen (6.2×10^5 cells). In contrast to what was observed in the periphery, tetramer-positive T cells continued to increase in the brain by day 7 p.i. (3.0×10^5 cells). Whereas the total number of cells within the CLN increased considerably early during infection, the number within the spleen declined (Fig. 1B), supporting the CLN as a prominent site of immune activation. N³¹⁸ peptide-induced intracellular IFN- γ production revealed similar kinetics, albeit with lower frequencies (data not shown).

To enhance detection frequencies, IFN- γ ELISPOT assays were conducted at similar time points p.i. (2, 19). Virus-specific T cells first emerged at day 3 p.i. in the CLN (8 spots/10⁶ cells) (Fig. 2A). By day 4 p.i., frequencies within the CLN had increased rapidly (262 spots/10⁶ cells) and were measurable

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FIG. 1. Distribution of virus-specific, tetramer-positive CD8⁺ T cells. Mononuclear cells prepared from brain, CLN, and spleen of JHNV-infected mice on days 5, 6, and 7 p.i. ($n = 3$ per time point) were stained with the L^dN^{318} tetramer and anti-CD8 monoclonal antibody and analyzed by flow cytometry. (A) Total numbers of virus-specific CD8⁺ T cells were calculated by multiplying the frequency of tetramer-positive CD8⁺ T cells within each sample by the number of cells recovered from each organ. Error bars represent the standard error of the average number of total virus-specific CD8⁺ T cells recovered from each organ. Percentages of CD8⁺ T cells and of tetramer-positive CD8⁺ T cells are indicated for each organ. (B) Total numbers of cells recovered per organ. Representative data from one of two experiments are shown.

within the spleen and brain (29 spots/ 10^6 cells and 13 spots/ 10^6 cells, respectively). By day 5 p.i., the frequency of virus-specific T cells in the brain (2,380 spots/ 10^6 cells) exceeded that in either spleen or CLN (433 spots/ 10^6 cells and 534 spots/ 10^6 cells, respectively), which had reached its peak at this time. This dramatic shift in frequencies was even more pronounced on day 7 p.i. These data suggest that recruitment of virus-specific T cells into the CNS is preceded by expansion in the periphery.

Figure 2B presents the total number of virus-specific CD8⁺ T cells per organ so that the relative contribution of trafficking versus local expansion in the alteration of T-cell localization patterns can be more readily distinguished. Coinciding with the frequency data, the total number of virus-specific CD8⁺ T cells was higher in CLN than in spleens and was negligible within the brain at both days 3 and 4 p.i., emphasizing initial expansion within CLN. By day 5 p.i., the number of virus-specific cells within the spleen exceeded that in the CLN, possibly reflecting ongoing expansion in the spleen and increased splenic cellularity. In the same time interval, the number of virus-specific CD8⁺ T cells within the brain increased nearly 200-fold, from 12 to 2.1×10^3 cells. Nevertheless, this constituted fewer cells than were in the spleen and CLN. By day 7 p.i., however, this number increased to 2.5×10^4 cells, which approximated the number found in the spleen a day earlier. The overall 2,000-fold increase in the number of virus-specific T cells within the brain from days 4 to 7 p.i., coincident with a decline in the number of cells within the spleen, strongly implicated migration, as opposed to extensive local cell division,

FIG. 2. Frequencies and relative accumulation of IFN- γ -secreting virus-specific CD8⁺ T cells during CNS infection. Cells isolated from brain, spleen, and CLN of JHNV-infected mice ($n = 3$) at the indicated time points (d, day) were pooled, and serial dilutions were plated in triplicate beginning at 10^6 cells/well. IFN- γ ELISPOT assays were carried out as described previously (2, 17) by using N^{318} peptide-coated syngeneic splenocytes ($1 \mu M$) as stimulators. (A) Frequencies are presented as the number of IFN- γ -producing cells per 10^6 input cells. Error bars represent the standard errors of results for six wells counted per sample. Background spots accounted for less than 12.5% of the number of spots counted in the presence of peptide. (B) Total number of virus-specific CD8⁺ T cells per organ as determined from frequencies depicted in panel A.

within the CNS. This notion is supported by the finding that division times are between 6 to 8 h during peak T-cell expansion following lymphocytic choriomeningitis virus infection (23). A 24- to 30-h time interval would accommodate approximately five division cycles, resulting in a 32-fold increase in the number of virus-specific cells, assuming retention in the respective organ. In the CLN, the increase in frequencies from day 3 to day 4 p.i. was ~ 30 -fold, suggesting that the majority of cells undergo five divisions prior to leaving the CLN. By contrast, the interval between days 4 and 5 p.i. resulted in only a twofold increase, which could be due to either a slower division rate or rapid efflux of expanding cells. The latter possibility is supported by both a lower-than-expected increase in virus-specific CD8⁺ T cells when the total numbers in CLN on days 3 and 4 are compared and their delayed emergence in the spleen. Similarly, despite predominant expansion in the spleen from day 4 to day 5 p.i., the 18-fold increase accounts for only a fraction of the number of theoretically accumulating cells arising from optimal division. A large number of activated cells thus appear to enter the circulation between days 4 and 5 p.i., coincident with the rapid emergence of virus-specific CD8⁺ T cells in the CNS. The 100-fold increase in the frequency of

CNS virus-specific cells during this period vastly exceeds the numbers theoretically achieved by local division only and therefore appears to arise primarily from recruitment. Although this interpretation of the data is oversimplified because novel priming events after day 4 are not taken into account, it is in agreement with the results of recent studies of T-cell turnover during acute peripheral infections (23). These data further support observations from studies with Sindbis virus encephalitis- and JHMV-infected rats, suggesting that the CNS environment does not support T-cell proliferation (9, 16, 17).

Despite the similar overall trend of virus-specific CD8⁺-T-cell accumulation in peripheral tissues prior to CNS entry, the absolute numbers are approximately 10-fold higher by tetramer analysis than by ELISPOT assays. These discrepancies reside in the 8- to 10-fold-lower frequencies detected by ELISPOT compared with tetramer staining, which has been consistently observed following JHMV intracerebral infection (1, 2, 19, 26). As tetramer background staining is below 0.5% within the naive CD8 population, these discrepancies may be attributed to the binding of tetramer to activation markers, IFN- γ secretion at amounts insufficient to form spots, and/or apoptosis of T cells during the 30- to 36-h stimulation period. The fact that 50 to 70% of tetramer-positive cells produce intracellular IFN- γ following peptide stimulation, as measured by flow cytometry (1), suggests that a combination of these factors may be responsible for the lack of a 1:1 tetramer-to-spot correlation. Furthermore, alterations in organ cellularity during infection (Fig. 1B) vary in magnitude between experiments, thus further contributing to variations in the depiction of total cell numbers versus frequencies.

The ELISPOT data indicated that expansion of virus-specific CD8⁺ T cells in peripheral tissues preceded accumulation within the CNS by almost 2 days. The absence of ex vivo cytolytic activity in lymphoid organs may thus reflect low frequencies of virus-specific CTL rather than insufficient differentiation into cytolytic effectors. Cells were therefore isolated from the brain and spleen of JHMV-infected mice on day 7 p.i. and tested for virus-specific cytolytic activity by using N³¹⁸ peptide-coated target cells (3). Brain-derived cells exhibited approximately 12% specific lysis at an effector-to-target cell (E:T) ratio of 15:1 (Fig. 3A). By comparison, no specific lysis was detected from cells isolated from the spleen at an E:T ratio of 100:1 (Fig. 3B). Adjustment of E:T ratios to tetramer-positive CD8⁺ T cells revealed that higher cytolytic activity was due not solely to increased numbers but also to an apparently higher activation state. Whereas CNS CD8⁺ T cells still exhibited lysis at an E:T ratio of 0.4:1, splenic CD8⁺ T cells did not. To enhance the frequency of virus-specific CD8⁺ T cells, splenocytes were depleted of B cells and CD4⁺ T cells by using magnetic beads. The enriched CD8⁺-T-cell fraction, comprising 72% CD8⁺ T cells, 2.1% tetramer-positive CD8⁺ T cells, 12.4% NK cells, and <5% CD4⁺ T cells or B cells, clearly lysed targets in an antigen-specific manner (Fig. 3C). Fifteen percent lysis was achieved at a tetramer-based E:T ratio of 1.6:1, similar to the cytolytic potential observed with CNS-derived cells. However, in contrast to what was seen with the CNS population, lysis rapidly dropped off at lower E:T ratios, supporting a lower differentiation state. No lysis was observed without peptide, suggesting the absence of NK cell-mediated lysis. These data demonstrate that a subset of virus-specific splenic CD8⁺

FIG. 3. Cytolysis of CD8⁺ T cells isolated from the CNS and spleen during acute JHMV infection. Brain-derived cells (A), unfractionated splenocytes (B), and splenocytes depleted of B cells, adherent macrophages, and CD4⁺ T cells (C) were prepared from mice at 7 days p.i. ($n = 6$). Cells were stained with anti-CD8 monoclonal antibody and the L^d-N³¹⁸ tetramer (left panels) and directly assayed ex vivo for lysis of BC10ME target cells in the presence or absence of 100 nM N³¹⁸ peptide (right panels). Percentages of tetramer-positive and tetramer-negative CD8⁺ T cells are indicated in the upper right and lower right quadrants of each plot, respectively. E:T ratios are based on total numbers of effector cells or tetramer-positive CD8⁺ cells as indicated. Background release was less than 10% of the maximal release.

T cells is fully activated into effectors, as defined by the ability to secrete IFN- γ and lyse antigen-presenting cells.

Overall, the kinetics and frequencies of virus-specific CD8⁺ T cells suggest that priming and expansion is initiated in the CLN, followed by delayed recruitment to and/or expansion in the spleen and lastly by rapid accumulation in the CNS. These data thus support the deep cervical nodes as the primary tissue of T-cell expansion via antigen or virus draining into the cerebrospinal fluid (8, 9, 28). Furthermore, the data suggest that virus-specific CD8⁺ T cells undergo at least four local division cycles prior to dissemination. Although differentiation into armed effector cells appears to occur during peripheral expansion, cytolytic potential is optimized at the site of infection within the CNS. Although cells expressing dendritic cell markers are found in the CNS during inflammatory processes (10,

21), there is little evidence that these cells act as stimulator cells for naïve T cells in vivo within the CNS. Indeed, examination of T-cell priming within the CNS by using virus administered by stereotactic microinjection suggests no T-cell activation within the brain parenchyma; only virus within the cerebrospinal fluid initiated immune responses (28). The notion that drainage of JHMV into CLN is the prominent mechanism for peripheral T-cell expansion following intracerebral administration was supported by the detection of viral RNA in CLN (data not shown). The overall kinetics of T-cell expansion, dissemination from CLN to spleen, and acquisition of effector function are reminiscent of T-cell activation following localized peripheral infection, e.g., with herpes simplex virus (5, 12, 22). These data suggest that virus or viral antigen leakage into the cerebrospinal fluid during CNS infection gives rise to T-cell expansion, differentiation, and trafficking similar to events occurring during heterologous, non-CNS-restricted localized infections.

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