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CD4 T Cells Promote CD8 T Cell Immunity at the Priming and Effector Site during Viral Encephalitis

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CD4 T cell activation during peripheral infections not only is essential in inducing protective CD8 T cell memory but also promotes CD8 T cell function and survival. However, the contributions of CD4 T cell help to antiviral CD8 T cell immunity during central nervous system (CNS) infection are not well established. Encephalitis induced by the sublethal coronavirus JHMV was used to identify when CD4 T cells regulate CD8 T cell responses following CNS infection. Peripheral expansion of virus-specific CD8 T cells was impaired when CD4 T cells were ablated prior to infection but not at 4 days postinfection. Delayed CD4 T cell depletion abrogated CD4 T cell recruitment to the CNS but only slightly diminished CD8 T cell recruitment. Nevertheless, the absence of CNS CD4 T cells was associated with reduced gamma interferon (IFN-γ) and granzyme B expression by infiltrating CD8 T cells, increased CD8 T cell apoptosis, and impaired control of infectious virus. CD4 T cell depletion subsequent to CD4 T cell CNS migration restored CD8 T cell activity and virus control. Analysis of γc-dependent cytokine expression indicated interleukin-21 (IL-21) as a primary candidate optimizing CD8 T cell activity within the CNS. These results demonstrate that CD4 T cells play critical roles in both enhancing peripheral activation of CD8 T cells and prolonging their antiviral function within the CNS. The data highlight the necessity for temporally and spatially distinct CD4 T cell helper functions in sustaining CD8 T cell activity during CNS infection.

CD4 T cells play critical roles in controlling viral infections by promoting CD8 T cell responses as well as humoral immunity. While primary antiviral CD8 T cell immunity is elicited prior to production of neutralizing antibodies (Ab), humoral responses provide a first line of defense against secondary infection. Nevertheless, reactivation of CD8 T cell memory is vital to control viruses escaping neutralizing Ab due to genetic variation or inadequate humoral memory. CD4 T cells provide help to establish functional CD8 T cell memory during a primary response. While mice mount a robust primary CD8 T cell response to *Listeria monocytogenes*, vaccinia virus, murine gammaherpesvirus, and lymphocytic choriomeningitis virus infection in the absence of CD4 help, they fail to develop protective memory to a secondary challenge (19, 34, 35, 46, 52, 53). In contrast, the dependence of primary CD8 T cell responses on CD4 T cells is variable and depends on the virus, duration of antigen (Ag) exposure, tissue environment at the priming/expansion, as well as effector sites. Activation of naïve CD8 T cells is relatively T helper independent in situations in which virus replication induces potent activation of antigen-presenting cells. Other viruses or vaccine vectors require CD4 T cell licensing of dendritic cells (DC) for effective CD8 T cell priming and effector function (40, 48). Irrespective of their role in CD8 T cell activation, CD4 T cells also promote activity and survival of effector CD8 T cells during primary responses, a function especially critical in maintaining effective CD8 T cells during chronic infections. Last, CD4 T cells can be critical in catalyzing recruitment and/or entry of CD8 T cells primed in draining lymphoid tissue to localized infections, such as that of vaginal tissue (33). CD4 T cells thus have numerous roles in promoting CD8 T cell immunity, especially during infection in nonlymphoid organs.

The central nervous system (CNS) constitutes an exceptionally challenging environment for T cells to efficiently exert antiviral functions, based on several physiological and immunological features. These include limited T cell access due to the blood brain barrier, limited numbers and location of antigen-presenting cells, low expression of major histocompatibility complex (MHC) molecules, and little, if any, production of γc-dependent cytokines supporting T cell activity and survival. Both recruitment and survival of activated CD8 T cells may thus be exquisitely dependent on CD4 help during viral encephalomyelitis. Recent data from the peripheral nervous system indeed demonstrate that early CD4 T cell help prevents partial CD8 T cell exhaustion and promotes maintenance of herpes simplex virus 1 (HSV-1) latency in sensory ganglia (11). Furthermore, CD4 T cell depletion abrogates control of neurotropic coronavirus infection (22). However, the mechanisms and temporal constraints imposed upon CD4 T cell regulation of CD8 T cell functionality in the CNS are not established.

The present study uses a nonlethal, glia-tropic mouse hepatitis virus (JHMV) to identify the stages at which CD4 T cells influence CD8 T cell responses following CNS infection. In this model, virus control in the CNS requires a collaborative effort between CD4 and CD8 T cells (44, 50, 54, 61). Control of virus replication in astrocytes and microglia/macrophages is mediated via perforin-mediated CD8 T cell cytolysis, while gamma interferon (IFN-γ) secretion by T cells controls viral replication in oligodendroglia, the cellular targets of viral persistence (4, 13, 25, 36). Although adoptive transfer into immunodeficient hosts revealed direct antiviral mechanisms for both memory CD8 and CD4 T cells (4, 50), the contribution of direct CD4 T cell antiviral activity in the pres-

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ence of CD8 T cells is unknown. It is important to note that neither oligodendrocytes nor astrocytes express MHC class II molecules during viral encephalitis (14, 29), supporting a more prominent helper role of CD4 T cells. Earlier studies in which CD4 T cell-depleted mice were infected with a lethal strain of JHMV suggested that CD4 help contributes to but is not absolutely required for CD8 T cell effector function and survival in the CNS (49). However, the results did not exclude impaired peripheral CD8 T cell activation in imprinting a less effective functional phenotype within the CNS. Thus, to define the temporal stages at which CD4 T cells contribute to CD8 T cell immunity during sublethal viral encephalitis, CD4 T cells were depleted prior to infection or at 4 days or 7 days postinfection (p.i.). The results demonstrate that CD4 T cells exert critical helper functions early during CD8 T cell priming and expansion in cervical lymph nodes (CLN) and later promote CD8 T cell function and survival within the CNS. The critical role for CD4 T cells in promoting antiviral CD8 T cell responses in the priming as well as target tissues during viral encephalitis thus highlights the necessity to incorporate CD4 T cell helper function for immune-based approaches to combat CNS infections and minimize persistence.

MATERIALS AND METHODS

Mice, virus infection, and CD4 depletion. Wild-type C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD). Mice were housed under pathogen-free conditions at an accredited facility at the Cleveland Clinic Lerner Research Institute. Mice were infected at 6 to 7 weeks of age by intracranial injection with 250 PFU of the gliatropic JHM variant V2.2-1 of mouse hepatitis virus (JHMV). Recipients were depleted of CD4 T cells by intraperitoneal injection with 250 μg of anti-CD4 (α-CD4) monoclonal Ab (MAb) GK1.5 either at -2, 0, and 2 days p.i. during initial T cell activation, at 4 and 6 days p.i. during T cell expansion and trafficking, or at 7 and 9 days p.i. coinciding with peak T cell accumulation in the CNS. Control animals received the same amount of α-β-galactosidase (α-βgal) control MAb GL113. Animals were scored daily for clinical signs of disease as follows: 0, healthy; 1, ruffled fur and hunched back; 2, hind limb paralysis or inability to turn to upright position; 3, complete hind limb paralysis and wasting; 4, moribund or dead. All procedures were conducted in accordance with animal protocols approved by the Cleveland Clinic Lerner Research Center Institutional Animal Care and Use Committee.

Virus titers, cytokine determination, and serum Ab. Virus titers within the brain were determined from clarified supernatants by plaque assay using the murine delayed brain tumor (DBT) astrocytoma as detailed previously (9). Plaques were counted after 48 h of incubation at 37°C. Clarified supernatants were also used to measure IFN-γ by enzyme-linked immunosorbent assay (ELISA) as described previously (38). Briefly, 96-well plates were coated overnight at 4°C with 100 μl of a 1 μg/ml concentration of α-IFN-γ (R4-6A2; BD Bioscience, San Jose, CA). Nonspecific binding was blocked with 10% fetal calf serum (FCS) in phosphate-buffered saline (PBS) for 1.5 h before the addition of IFN-γ recombinant cytokine standard (BD Bioscience) and samples. After 2 h of incubation at room temperature, bound IFN-γ was detected using biotinylated α-IFN-γ Ab (XMG1.2; BD Bioscience) and avidin peroxidase followed by 3,3′,5,5′ tetramethylbenzidine (TMB reagent set; BD Bioscience) 1 h later. Optical densities were read at 450 nm with a Bio-Rad model 680 microplate reader and analyzed using Microplate Manager 5.2 software (Bio-Rad Laboratories, Hercules, CA).

Neutralizing Ab was measured as described previously (58). Briefly, following heat inactivation, triplicate serial 2-fold dilutions of serum from individual mice ($n = 3$ to 5) were incubated with 50 PFU of JHMV in 96-well plates for 90 min at 37°C. DBT cells (8×10^4 cells/well) were then added, and plates were incubated at 37°C for 48 h. Neutralization titers represent the log of the highest average serum dilution that inhibited cytopathic effect.

Isolation of mononuclear cells. CNS-derived cells were isolated as described previously (3). Briefly, brains from PBS-perfused mice ($n = 3$ to 6) were homogenized in ice-cold Tenbroeck grinders in Dulbecco's PBS. Homogenates were clarified by centrifugation at 400 $\times$ *g* for 7 min, and supernatants were collected and stored at -80°C for further analysis. Cell pellets were resuspended in RPMI supplemented with 25 mM HEPES, adjusted to 30% Percoll (Pharmacia, Piscataway, NJ), and underlaid with 1 ml of 70% Percoll. After centrifugation at 800 $\times$ *g* for 30 min at 4°C, cells were recovered from the 30%-70% interface, washed once, and resuspended in fluorescence-activated cell sorter (FACS) buffer (PBS with 0.5% bovine serum albumin [BSA]). CNS-derived cell populations for PCR analysis were isolated from infected mice as described above. For flow cytometric analysis of Annexin-V and Ki-67 expression by CD8 T cells, brains or spinal cords were homogenized in RPMI containing collagenase (1 mg/ml; Roche, Indianapolis, IN) and DNase I (1 mg/ml; Roche) using a gentleMACS dissociator (Miltenyi Biotec, Inc., Auburn, CA). Homogenates were centrifuged at 450 $\times$ *g* for 10 min at 4°C. Pelleted cells were resuspended in ice-cold PBS, and mononuclear cells were separated by a Percoll gradient as described above. Cell suspensions from CLN were prepared from identical animals as previously described (3).

Flow cytometric analysis and FACS. Cells were incubated with mouse serum and rat α-mouse FcγIII/II MAb for 20 min on ice prior to staining. Expression of cell surface markers was determined by incubation of cells with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, peridinin chlorophyll protein (PerCP)-, or allophycocyanin-conjugated MAb specific for CD45 (30-F11), CD4 (L3T4), CD8 (53-6.7), CD62L (MEL-14), I-A/I-E (2G9) (BD Bioscience), PD-1 (RMP1-30) (eBioscience, San Diego, CA) and F4/80 (CI:A3-1; Serotec, Raleigh, NC) for 30 min on ice. Virus-specific CD8 T cells were identified using D^b/S510 MHC class I tetramers (Beckman Coulter, Inc., Fullerton, CA) as described previously (3). Stained cells were washed twice with FACS buffer and fixed in 2% paraformaldehyde. For intracellular detection of granzyme B or IFN-γ, cells were stained for cell surface markers prior to permeabilization with Cytofix/Cytoperm reagent (BD Bioscience) and staining with allophycocyanin-labeled α-granzyme B Ab (GB12, isotype-control mouse IgG1; Caltag Laboratories, Burlingame, CA) or α-IFN-γ Ab (BD Bioscience). For detection of Annexin-V, cells were stained with α-CD8 Ab and α-CD45 Ab, washed, and then resuspended in 1$\times$ Annexin-V binding buffer containing Annexin-V (BD Bioscience) and incubated for 15 min. For detection of Ki-67, cells were stained with α-CD8 Ab and α-CD45 Ab. After fixation with 0.4% paraformaldehyde, cells were permeabilized with 0.5% BSA and 0.5% saponin buffer and stained with α-Ki67 Ab for 45 min. A minimum of 2×10^5 viable cells were stained and analyzed with a FACS Calibur flow cytometer (BD, Mountain View, CA). Data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR). CNS monocyte-derived $CD45^{high}$ ($CD45^{hi}$) $F4/80^+$ macrophages, $CD45^{low}$ ($CD45^{lo}$) microglia, as well as CD4 and CD8 T cells were purified from pooled brains ($n = 6$) using a BD FACS Aria (BD). A minimum of 5×10^4 cells were collected per pooled sample and frozen in 400 μl TRIzol (Invitrogen, Carlsbad, CA) at -80°C for subsequent RNA extraction and PCR analysis as described previously (17).

Virus specific IFN-γ production by CLN-derived CD8 T cells was evaluated after peptide stimulation. Briefly, 2×10^6 CLN cells were cultured in the absence or presence of 1 μM S510 peptide encompassing the H-$2D^b$ restricted CD8 T cell epitope in a total volume of 200 μl RPMI supplemented with 10% fetal calf serum for 5 h at 37°C with 1 μl Golgi Stop (BD Bioscience)/ml. After stimulation, cells were stained for surface expression of CD8, CD44, and CD62L, fixed, and then permeabilized to detect intracellular IFN-γ as recommended by the supplier (BD Bioscience).

Histopathology. Spinal cords from PBS-perfused mice were fixed in 10% formalin and embedded in paraffin for distribution of viral Ag. Viral nucleocapsid protein was detected by immunoperoxidase staining using

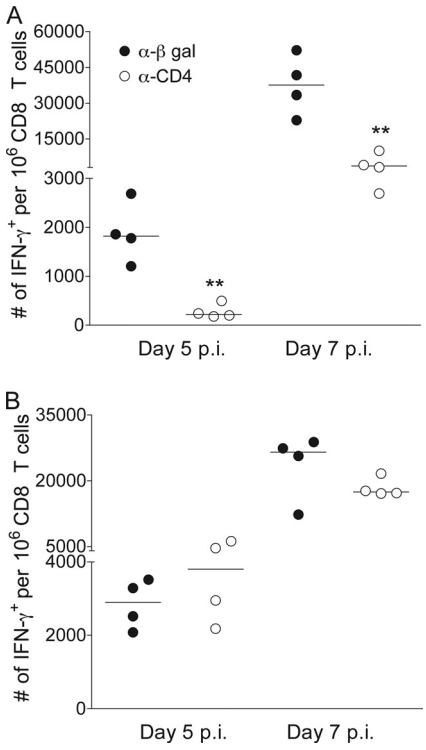

FIG 1 Early but not delayed CD4 T cell depletion affects priming and expansion of peripheral naïve CD8 T cells. CD4 T cell were depleted either at −2, 0, and +2 days p.i. (A) or at 4 and 6 days p.i. (B). Frequencies of CLN-derived IFN-γ-secreting CD8 T cells at days 5 and 7 p.i. were determined by flow cytometry after stimulation with S510 peptide. Data are representative of results of two independent experiments and expressed as the numbers of IFN-γ^+ per 10^6 $CD8^+$ T cells. Statistically significant differences between the control and CD4 T cell-depleted samples are indicated as follows: **, $P < 0.005$.

the α-JHMV MAb J.3.3 as the primary Ab, horse α-mouse as the secondary Ab, and 3,3′-diaminobenzidine substrate (Vectastain-ABC kit; Vector Laboratories, Burlingame, CA). Sections were scored in a blinded fashion, and representative fields were identified based on the average score of all sections in each experimental group. For analysis of T cell distribution, mice were perfused with ice-cold PBS. Brains were snap-frozen in Tissue-Tek OCT compound (Sakura Finetex, Torrance, CA) and sectioned at 10 μm using a Thermo Shandon cryostat. Sections were fixed with 4% paraformaldehyde for 20 min, treated with 1% Triton X-100 in PBS at room temperature, blocked for 30 min, and then stained with rabbit α-mouse laminin Ab (Cedarlane Laboratories, Ontario, Canada) and rat α-mouse CD3 MAb (eBioscience) overnight at 4°C. Alexa Fluor 594 goat α-rabbit (Invitrogen) and Alexa Flour 488 goat α-rat (Invitrogen) Ab were added, and the samples were incubated for 1 h at room temperature. Sections were mounted with ProLong Gold antifade reagent with 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen) and analyzed using a Leica DM2500 microscope.

PCR. Snap-frozen brains or spinal cords from individual PBS-perfused mice ($n = 3$ to 4) were placed in 1 ml TRIzol (Invitrogen) and homogenized in Tenbroeck glass grinders, and RNA was isolated as described previously (16). DNA contamination was removed by treatment with DNase I for 30 min at 37°C (DNA-free kit; Ambion, Austin, TX), and cDNA was synthesized from RNA using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen), oligo(dT) primers (Promega Madison, WI), and random primers (Promega). Quantitative real-time PCR was performed using 4 μl of cDNA and SYBR green Master Mix (Applied Biosystems, Foster City, CA) in duplicate on a 7500 Fast real-time PCR system (Applied Biosystems). PCR conditions were 10 min at 95°C followed by 40 cycles at 95°C for 15 s, 60°C for 30 s and 72°C for 30 s. Previously described primers were used for transcripts encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH), JHMV nucleocapsid, interleukin-10 (IL-10), CCL2, CCL5, CXCL9, CXCL10, tumor necrosis factor (TNF), and IL-21 (37, 39, 45). Other real-time primer sequences were as follows: CCL3 sense, 5′-CCAAGTCTTCTCAGCGCCAT-3′, and antisense, 5′-GAATCTTCCGGCTGTAGGAGAAG-3′; CCL4 sense, 5′-CCCGGCAGCTTCACAGAA-3′, and antisense, 5′-ACAGCTGGCTTGGAGCAAA-3′; CCL7 sense, 5′-GGGAAGCTGTTATCTTCAAG

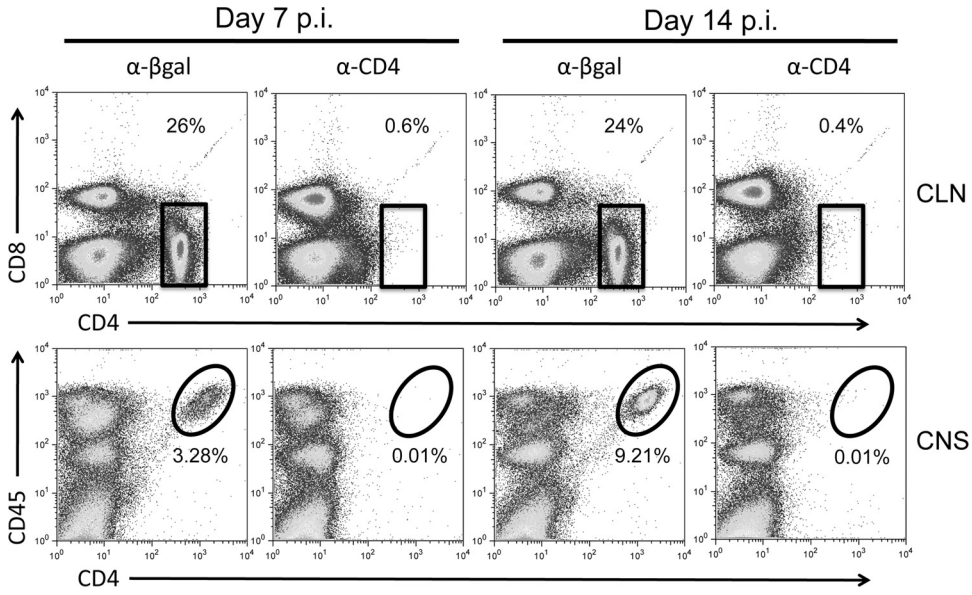

FIG 2 α-CD4 MAb treatment prevents CD4 T cell accumulation in the CNS. JHMV-infected mice were treated with α-CD4 or control MAb at 4 and 6 days p.i. Pooled CLN (upper row) or brain cells (bottom row) ($n \geq 3$/group) isolated at days 7 and 14 p.i. were stained for CD4, CD8, or CD45. Percentages of $CD4^+$ T cells within the CLN (boxed cells) are shown in the upper right corner. Percentages of $CD4^+$ T cells within the CNS (circled cells) are shown below the ellipse. Data are representative of results of three independent experiments.

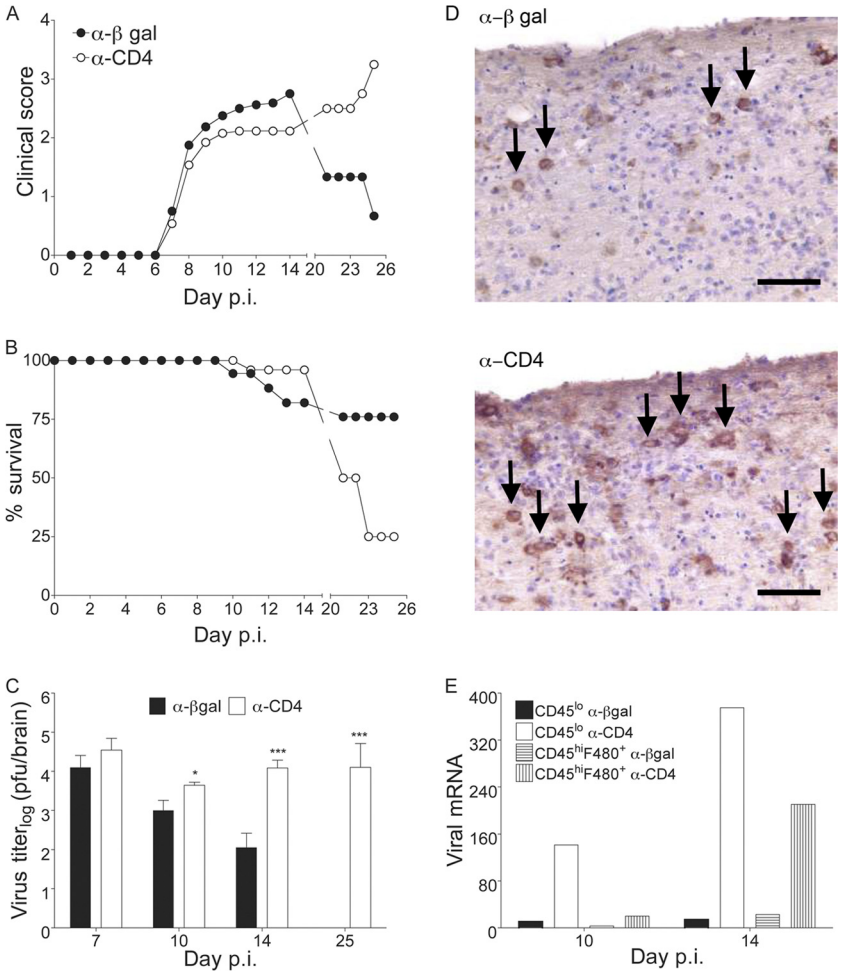

FIG 3 Delayed depletion of CD4 T cells impairs viral control in the CNS. JHMV-infected mice were treated with α-CD4 or control MAb at 4 and 6 days p.i. and monitored for clinical symptoms (A), survival (B), and virus titers in brains by plaque assay (C). Titers are expressed as the means ± SEM. (D) Virus-infected cells in spinal cord white matter tracks of infected mice at 10 days p.i. Immunoperoxidase stain using α-nucleocapsid MAb (brown) with hematoxylin counterstain. Arrows indicate viral nucleocapsid Ag-positive cells with morphology consistent with oligodendroglia. Scale bar, 100 μm. (E) Relative transcript levels of viral nucleoprotein in purified CD45^{lo} microglia and CD45^{hi} F4/80^{+} monocyte-derived macrophages from pooled brains ($n = 6$) 10 and 14 days p.i. were assessed by real-time PCR. Transcript levels are relative to GAPDH. Statistically significant differences in overall clinical score up to day 14 p.i. between control and CD4 T cell-depleted mice were determined by Wilcoxon matched pairs test. **, $P < 0.005$. Statistically significant differences in viral titers between control and CD4 T cell-depleted mice were determined by unpaired *t* test. *, $P < 0.5$; ***, $P < 0.001$.

ACAAA-3′, and antisense, 5′-CTCCTCGACCCACTTCTGATG-3′; IL-7 sense, 5′-AGTGCCACATTAAAGACAAAGAAGGT-3′, and antisense, 5′-ATTCGGGCAATTACTATCAGTTCCT-3′; IL-15 sense, 5′-TCTCGT GCTACTTGTGTTTCCTTCT-3′, and antisense, 5′-CAGCCCAAAATG AAGACATGAA-3′; and TNF-related apoptosis-inducing ligand (TRAIL) sense, 5′-CCTCTCGGAAAGGGCATTC-3′, and antisense, 5′ TCCTGCTCGATGACCAGCT-3′. GAPDH, IL-2, IFN-γ, IgG, kappa-light chain, and perforin mRNA levels were determined using Applied Biosystems gene expression arrays with Universal TaqMan Fast Master Mix (Applied Biosystems). PCR conditions were 20 s at 95°C followed by 40 cycles at 95°C for 3 s and 60°C for 30 s. Transcript levels were calculated relative to the GAPDH housekeeping gene using the following formula: 2[CT(GAPDH) − CT(target gene)] × 1,000, where CT represents the threshold cycle at which the fluorescent signal becomes significantly higher than that of the background.

Statistical analysis. Results are expressed as the means ± standard errors of the means (SEM) for each group of mice. In all cases, a *P* value of <0.05 was considered significant. Graphs were plotted and statistics assessed using GraphPad Prism 3.0 software.

RESULTS

CD4 T cells regulate initial priming of CD8 T cells. During infection with a lethal JHMV strain, CD4 T cells enhance, but are not absolutely required for, peripheral CD8 T cell activation (49). To determine the role of CD4 help in promoting CD8 T cell function at several stages during sublethal JHMV infection, CD4 T cells were initially deleted at −2, 0, and +2 days relative to infection. The magnitude of virus-specific CD8 T cell expansion in CLN, the primary site of T and B cell priming during JHMV infection (30, 59), was monitored. The absence of CD4 T cells reduced virus-specific CD8 T cells in the CLN ~7- to 8-fold at days 5 and 7 p.i. (Fig. 1A). This requirement of CD4 T cells to efficiently prime naïve CD8 T cells preempted a direct analysis of their role in CD8 T cell entry and effector function in the CNS. Thus, to allow CD4 T cell imprinting during the priming phase, CD4 depletion was delayed until day 4 p.i. When this approach was used, expansion of virus-specific CD8 T cells in CLN was comparable to that of con-

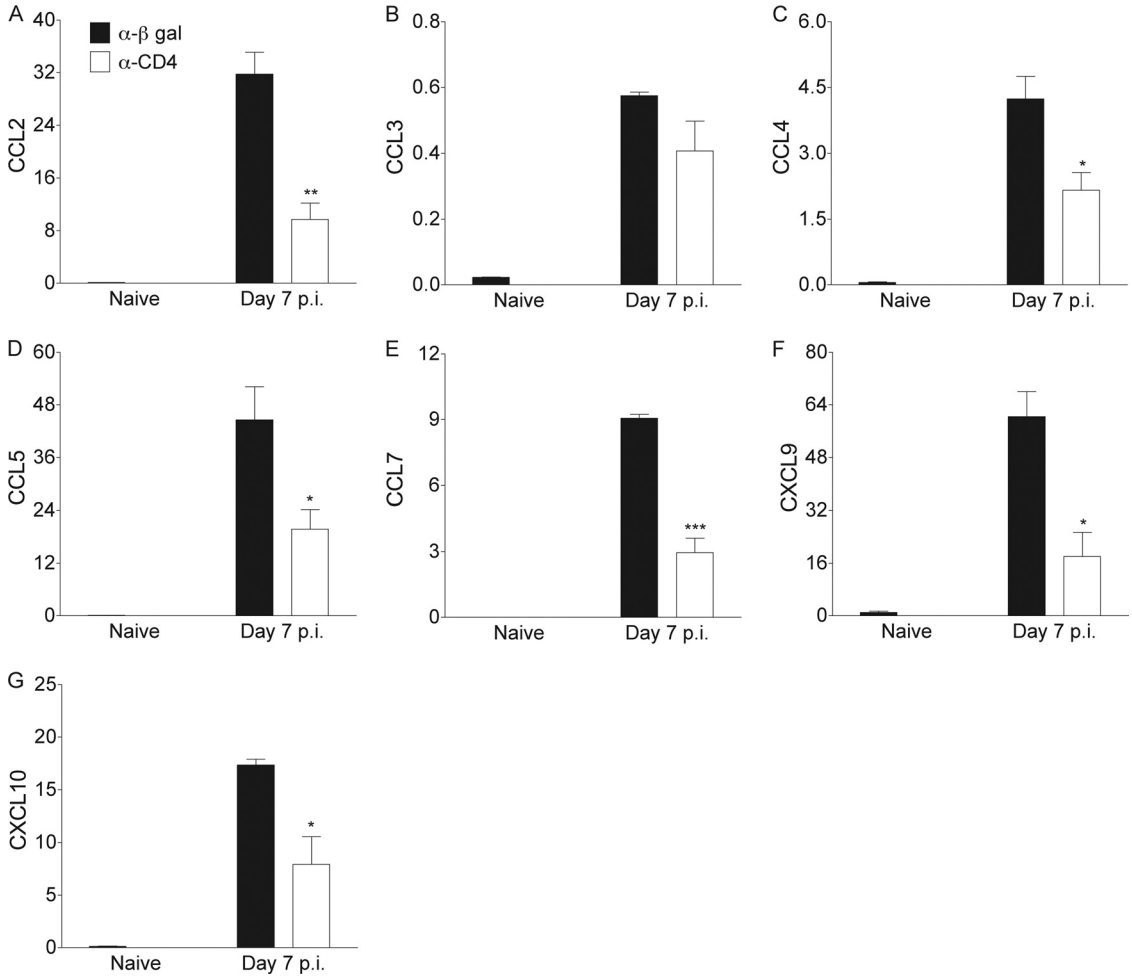

FIG 4 CD4 depletion reduces chemokine production in the CNS. JHMV-infected mice were treated with α-CD4 or control MAb at 4 and 6 days p.i. Relative transcript levels of CCL2 (A), CCL3 (B), CCL4 (C), CCL5 (D), CCL7 (E), CXCL9 (F), and CXCL10 (G) in spinal cords of naïve and infected mice were assessed by real-time PCR. Data are expressed as the mean transcript levels ± SEM relative to GAPDH mRNA from three to four individual mice and are representative of results of two independent experiments. Statistically significant differences between control and CD4 T cell-depleted mice were determined by unpaired *t* test. *, $P < 0.5$; **, $P < 0.005$; ***, $P < 0.001$.

trols at days 5 and 7 p.i. (Fig. 1B). Similar numbers of virus-specific D^b-S510 tetramer$^+$ CD8 T cells were also identified by flow cytometry between groups (data not shown). These results suggested that CD4 T cells exerted CD8 T cell helper function mainly within the first 3 days of infection during the initial activation phase in CLN. Although this is consistent with prominent recruitment of T cells to the priming site within days of infection (7, 10), the possibility that additional imprinting on CD8 T cell function may occur at day 4 p.i. or later cannot be excluded.

Sustained CD4 T cell help is required to control virus. CD4 T cells are detected in the CNS as early as day 5 p.i. and peak at approximately day 10 p.i. (45). In contrast, CD8 T cells reach maximal levels at day 7 p.i. (45). Depletion of CD4 T cells initiated at day 4 p.i. thus affects the initial migration of CD4 T cells into the CNS, and flow cytometry confirmed the absence of CD4 T cells in both the CLN and the CNS (Fig. 2). This regimen resulted in a >99% depletion of CD4 T cells (Fig. 2). To determine whether the delayed depletion of CD4 T cells alters infection, disease severity and viral control were compared to those of control mice. Disease onset and progression were initially comparable between CD4 T cell-depleted and control mice. Although CD4 T cell-depleted mice displayed an overall reduction in acute clinical symptoms (Fig. 3A), the majority ultimately succumbed to infection, with mortality increasing between days 21 and 24 p.i. (Fig. 3B). To assess if mortality was associated with impaired viral control in the CNS, similar to results for CD4 T cell-depleted mice infected with lethal JHMV (49), viral loads in the brain and spinal cord were monitored. Infectious virus levels in the brain samples of the two groups were similar at day 7 p.i. and were reduced ~10-fold by day 10 p.i. for both groups, irrespective of the presence or absence of CD4 T cells, supporting potent CD8 T cell effector function within this time span (Fig. 3C). However, CD4 T cell-depleted mice harbored slightly more virus at day 10 p.i. and subsequently lost viral control, as demonstrated by viral titers in the CNS at day 14 p.i. that were ~2 $\log_{10}$ higher than those of the controls (Fig. 3C). Consistent with elevated CNS viral titers, the number of infected cells in the spinal cord was also increased in the absence of CD4 T cells (Fig. 3D). Notably, in both groups, the majority of virus-infected cells exhibited morphology consistent with oligodendrocytes, the predominant cell type infected in immunocompetent

mice (13, 36). To further assess viral control in myeloid targets susceptible to CD8 T cell-mediated cytolysis, viral mRNA was measured for CD45^{lo} microglia and CNS-infiltrating CD45^{hi} F4/80^{+} monocyte-derived macrophages purified from the infected CNS. Viral mRNA was 14- and 27-fold higher in microglia derived from CD4 T cell-depleted mice than in control mice at days 10 and 14 p.i., respectively (Fig. 3E). Although not as robust as in microglia, viral mRNA was also increased 6- and 9-fold in infiltrating macrophages derived from the CNS of CD4 T cell-depleted mice. These results clearly demonstrate that CD4 T cells are indispensable contributors to viral control within the CNS.

Notably, T cell function is critical to reduce viral titers to undetectable levels by day 14 p.i., independent of humoral responses (44, 50, 54, 61). However, subsequently, local Ab production by invading B cells is required to maintain persisting virus at levels detectable only by PCR (24, 41, 42). As CD4 T cell help is essential for high-affinity virus-specific Ab production and generation of long-lived plasma cells, we determined how depletion of CD4 T cells at days 4 and 6 p.i. alters humoral immunity. Significantly lower titers of neutralizing Ab were detected in infected CD4 T cell-depleted mice at day 14 p.i. (2.2 ± 0.2 for the control versus 1.4 ± 0.3 for the CD4 T cell-depleted mice; $P < 0.005$). Moreover, transcript levels of IgG and kappa-light chain were also severely diminished at day 21 p.i. in the CNS of day 4 and 6 p.i. CD4 T cell-depleted mice compared to those for control mice (data not shown). These data suggest that impaired Ab production and accumulation of Ab-secreting cells within the CNS contribute to uncontrolled viral replication and mortality after 14 days p.i. but that reduced CD8 T cell activity is responsible for impaired viral control prior to day 14 p.i.

Virus-specific CD8 T cell recruitment into the CNS is independent of CD4 T cell help. In addition to affecting CD8 T cell priming and expansion, CD4 T cells may promote mobilization of effector CD8 T cells to the site of infection. Following HSV-1 infection, IFN-γ secretion by early migratory CD4 T cells enhanced local chemokine production and CD8 T cell migration (33). Similarly, enhanced CD4 T cell accumulation relative to that of CD8 T cells in the CNS during early JHMV infection (45) may promote inflammatory responses via IFN-γ production. CD4 T cells also enhance monocyte recruitment during JHMV infection (22), suggesting that CD4 T cells can affect CNS inflammation at multiple levels. To assess how CD4 T cell depletion initiated at day 4 p.i. affects proinflammatory chemokine expression, CCL2, CCL3, CCL4, CCL5, CCL7, and IFN-γ-inducible CXCL9 and CXCL10 transcripts in the CNS were compared. All proinflammatory chemokines, with the exception of CCL3, were reduced by at least 50% (Fig. 4), with CCL2, CCL7, and CXCL9 most prominently affected. As CCL2-recruited monocytes enhance CD8 T cell access to the CNS parenchyma (45), and CXCR3 ligands can affect T cell recruitment (26–28), these data suggested that ineffective viral clearance may be a consequence of impaired CD8 T cell recruitment, resulting in decreased antiviral effector function.

Analysis of CNS inflammation revealed that total numbers of CD45^{hi} CNS-infiltrating cells were slightly lower in CD4 T cell-depleted mice than in control mice at day 7 p.i. (Fig. 5A), consistent with decreased expression of chemokine transcripts (Fig. 4). Most notably, the number of infiltrating CD45^{hi} F4/80^{+} macrophages was diminished by ~45% in CD4 T cell-depleted mice (Fig. 5D), consistent with decreased CCL2 expression (Fig. 4). Numbers of total and virus-specific CD8 T cells were comparable

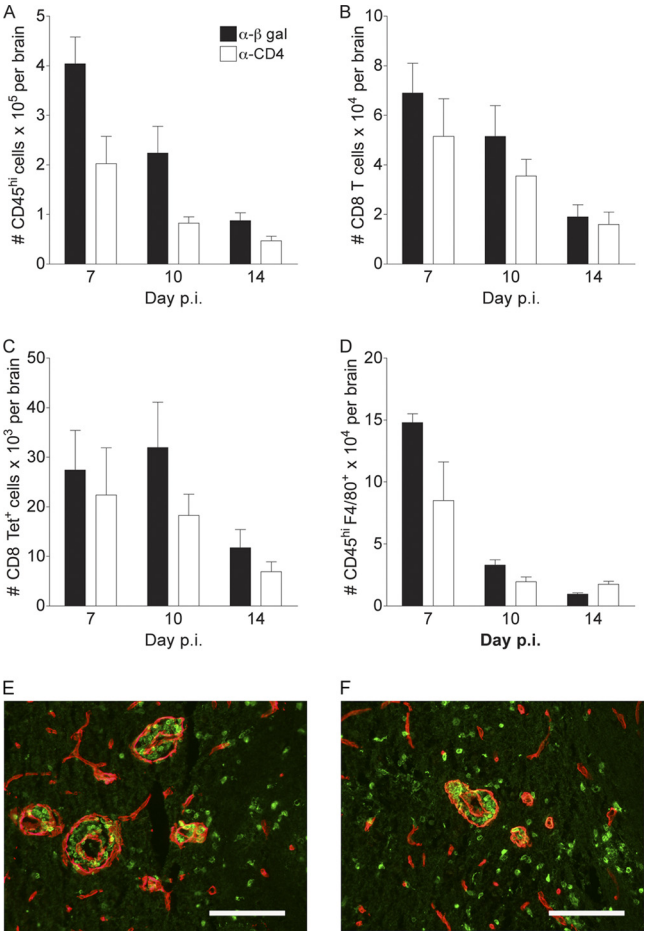

FIG 5 CD4 T cells within the CNS are not required for CNS CD8 T cell recruitment. JHMV-infected mice were treated with α-CD4 or control MAb at 4 and 6 days p.i. CNS inflammation was analyzed by flow cytometry from pooled tissues of $\geq$3 mice and immunohistochemistry at the indicated time points. Numbers of total CD45^{hi} CNS-infiltrating cells (A), CD8 T cells (B), Db/S510 tetramer^{+} virus-specific CD8 T cells (C), and CNS-infiltrating CD45^{hi} F4/80^{+} macrophages (D) are shown. The data are expressed as the means $\pm$ SEM from two independent experiments with at least 3 mice per time point per experiment. CD3 localization in the brain of control (E) or CD4 T cell-depleted (F) mice at day 7 p.i. was analyzed using α-CD3 (green) and α-laminin (red) Ab. Scale bar, 50 μm.

between CD4 T cell-depleted and control mice (Fig. 5B and C). Furthermore, CD4 T cell depletion did not impair CD8 T cell access to the CNS parenchyma, as evident by similar anatomic distributions of T cells in the CNS of the two groups at days 7 (Fig. 5E and F) and 10 (data not shown) p.i. Taken together, these data indicate that failure to control virus replication, despite early CD4 T cell help, could not be attributed to impaired peripheral expansion or altered CNS accumulation of virus-specific CD8 T cells.

The absence of CNS CD4 T cells compromises CD8 T cell effector function and survival. The absence of functional CD4 T cells compromises CD8 T cell effector function during many chronic infections associated with elevated viral Ag load (1, 31, 63). During sublethal JHMV infection, T cells control infectious virus by day 14 p.i. CD8 T cells exert a prominent antiviral function based on MHC class I, but not MHC class II, upregulation on oligodendrocytes and astrocytes (14, 29). Nevertheless, T cell

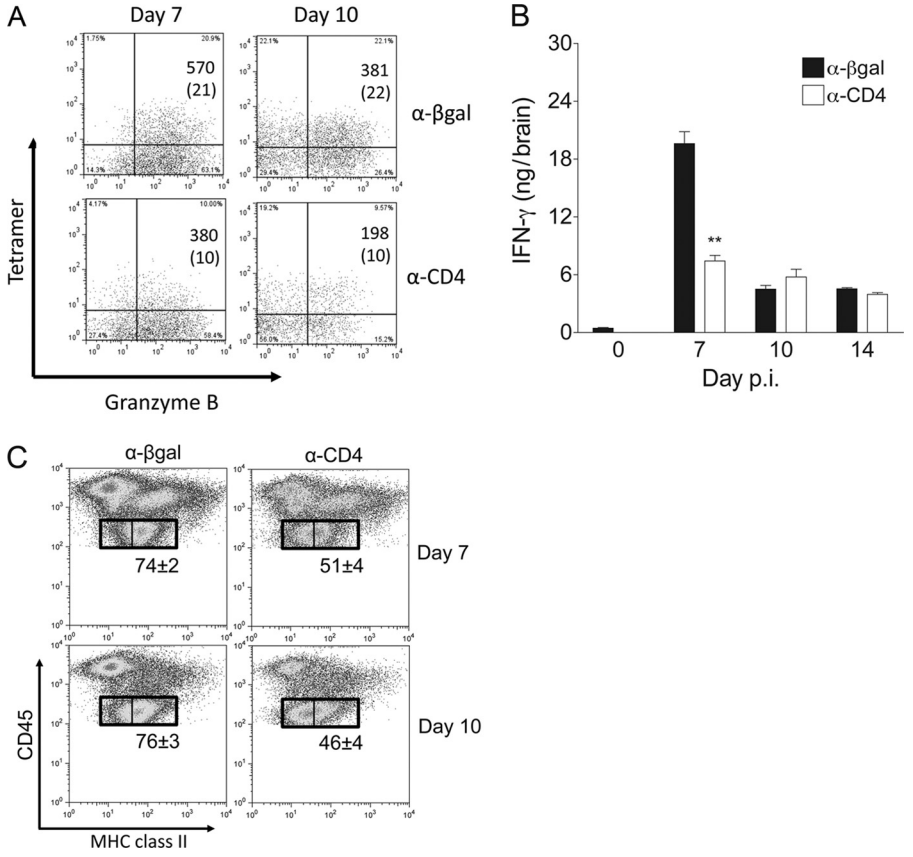

FIG 6 Delayed CD4 ablation impairs antiviral effector function in the CNS. JHMV-infected mice were treated with α-CD4 or control MAb at 4 and 6 days p.i. (A) Pooled brain cells ($n \geq 4$/group) isolated at days 7 and 10 p.i. were stained for CD8, D^b/S510-specific T cell receptor (TCR) and intracellular granzyme B. Representative density plots depict staining with α-granzyme B Ab and Db/S510 tetramer. Plots are gated on CD8 T cells. Mean fluorescence intensity of granzyme B staining in D^b/S510 tetramer$^+$ virus-specific CD8 T cells is shown in the upper-right quadrant. Percentage of granzyme B$^+$ D^b/S510 tetramer$^+$ virus-specific CD8 T cells is shown in parentheses. Data are representative of results of three independent experiments. (B) Brain IFN-γ levels were assessed by ELISA at the indicated days p.i. Data are means ± SEM ($n \geq 6$/group) from two separate experiments. Statistically significant differences were determined by unpaired *t* test. **, $P < 0.005$. (C) Pooled brain cells ($n \geq 4$/group) isolated at days 7 and 10 p.i. were stained for CD45 and MHC class II. Density plots are representative of two independent experiments and depict staining with α-CD45 and MHC class II Ab, with CD45^{lo} microglia boxed. Microglia positive for MHC class II are depicted by the population falling into the right side of the box based on the separation of the MHC class II$^+$ cells in the CD45^{hi} population. Numbers are mean percentages ± SEM of MHC class II$^+$ cells within the CD45^{lo} microglia of both experiments.

function declines rapidly, and low levels of persisting virus are controlled by humoral immunity (24, 41, 42) despite the presence of virus-specific CD8 and CD4 T cells in the CNS (30). Impaired viral control at day 10 p.i. despite effective CD8 T cell recruitment thus indicated an expedited decline in CD8 T cell function in the absence of CNS CD4 T cells. Granzyme B, an effector molecule of cytolysis, was assayed directly *ex vivo* without peptide stimulation. Granzyme B was expressed by a smaller percentage of CNS-derived CD8 T cells, and levels at day 7 p.i. were lower in CD4 T cell-depleted mice than in controls (Fig. 6A). This deficiency was evident at day 7 p.i. and exacerbated by day 10 p.i. (Fig. 6A).

IFN-γ, secreted by both CD8 and CD4 T cells within the CNS of JHMV-infected mice (20, 39), is critical for upregulation of MHC molecules on CNS-resident cells (14, 29) and viral control in oligodendrocytes (13). The level of IFN-γ was significantly lower in the CNS of CD4 T cell-depleted mice at day 7 p.i., but similar to that of controls by days 10 and 14 p.i. (Fig. 6B). Consistent with IFN-γ-dependent upregulation of MHC class II expression on microglia (4), the percentage of MHC class II$^+$ microglia was decreased in the absence of CNS CD4 T cells (Fig. 6C).

During JHMV infection, all resident glia cells, including oligodendrocytes, upregulate MHC class I and have the capacity to directly trigger CD8 T cell effector activity. However, CD4 T cells are likely engaged only by MHC class II$^+$ microglia/macrophages or DC, due to sparse, if any, MHC class II expression on other CNS-resident cells (14, 29). To assess whether reduced IFN-γ was primarily attributed to the absence of CD4 T cells or reduced IFN-γ production by CD8 T cells *in vivo*, CD8 T cells were purified from the infected CNS at days 7 and 10 p.i. Levels of IFN-γ transcripts were indeed ~2- to 3-fold lower in CD8 T cells from CD4 T cell-depleted mice than those of their control counterparts (Fig. 7A). Similarly, transcript levels of perforin were also reduced ~2.3-fold at day 10 p.i. (Fig. 7B), correlating with diminished expression of granzyme B (Fig. 6A). The recent finding that CNS-derived IL-10$^+$ CD8 T cells are highly cytotoxic during JHMV infection (55) suggested that the impaired viral control in CD4 T cell-depleted mice is associated with lower expression of IL-10. Indeed IL-10 transcripts were decreased ~3-fold in CD8 T cells from CD4 T cell-depleted mice (Fig. 7C), supporting a role of CD4 T cells in influencing IL-10 production by CD8 T cells (51). Nev-

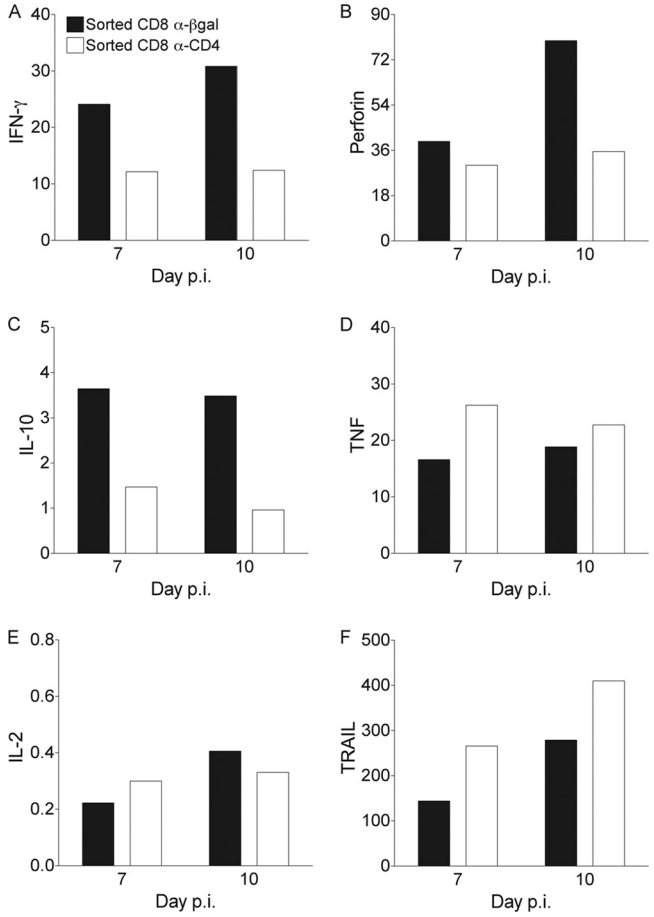

FIG 7 Absence of CD4 T cells in the CNS impairs CD8 T cell activity. JHMV-infected mice were treated with α-CD4 or control MAb at 4 and 6 days p.i. (A to F) Relative transcript levels of IFN-γ (A), perforin (B), IL-10 (C), TNF (D), IL-2 (E), and TRAIL (F) in FACS-purified CD8 T cells from pooled brains of 6 to 8 mice collected at 7 and 10 days p.i. were assessed by real-time PCR. Data are representative of results of two independent experiments, and trends for each transcript were similar between experiments. Transcript levels are relative to GAPDH.

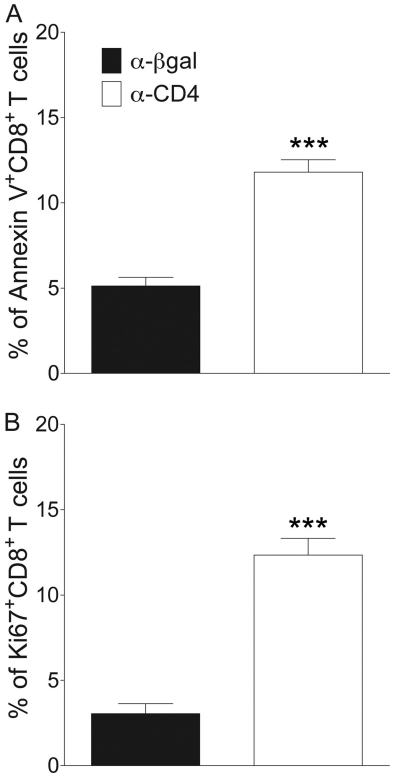

FIG 8 Absence of CNS CD4 T cells compromises CD8 T cell survival. JHMV-infected mice were treated with α-CD4 or control MAb at 4 and 6 days p.i. Brain cells from individual mice ($n = 3$ to 4) isolated at day 10 p.i. were stained for CD8, Annexin-V, and Ki-67. Percentages of Annexin-V$^+$ (A) or Ki-67$^+$ (B) CD8 T cells are shown. Data are representative of results of two independent experiments. Statistically significant differences were determined by unpaired *t* test. ***, $P < 0.005$.

ertheless, analysis of TNF and IL-2 mRNA as additional indicators of T cell activity demonstrated that decreased effector function of CD8 T cells in the absence of CD4 T cells is not global (Fig. 7D and E). These data thus support a critical role for CD4 T cells in optimizing CD8 T cell antiviral effector function at the effector site, in addition to early activation.

CD4 T cell help also supports CD8 T cell survival. Depletion of CD4 T cells prior to infection with lethal JHMV increased the frequency of apoptotic cells in the CNS, although the cell type was not characterized (49). CD8 T cells purified from day 4 p.i. CD4 T cell-depleted mice have slightly higher transcript levels of TRAIL than that of their control counterparts (Fig. 7F), suggesting an increase in the number of apoptotic CD8 T cells in the absence of CD4 T cells. To further determine whether CD4 T cells withdrawn after the initial expansion influence CD8 T cell survival, CNS-derived CD8 T cells were tested for Annexin-V expression. CD8 T cells from infected control mice contained ~5% Annexin-V-positive (Annexin-V$^+$) cells at day 10 p.i. (Fig. 8A). In contrast, the percentage of Annexin-V$^+$ CD8 T cells increased ~2.5-fold in the absence of CD4 T cells (Fig. 8A). The percentage of proliferating CD8 T cells was also greater in CD4 T cell-depleted mice, with ~12% of the CD8 T cells expressing Ki-67 compared to ~3% in controls (Fig. 8B). Increased apoptosis, yet increased CD8 T cell proliferation within the CNS in the absence of CD4 T cells, explained the numbers similar to those for control mice. How the continued presence of viral Ag contributes to proliferation yet reduced effector function remains unclear.

The mechanism(s) by which CD4 T cells contribute to CD8 T cell effector function and survival in the CNS may involve γc-dependent cytokines, including IL-2, IL-7, IL-21, and IL-15, although IL-15 and IL-7 are not produced by T cells (43). The absence of CNS CD4 T cells did not alter IL-2 mRNA levels, but led to a significant reduction in IL-7 mRNA in the CNS at day 7 p.i. (Fig. 9A&B). Nevertheless, by days 10 and 14 p.i., levels were comparable to those for controls. In contrast, IL-15 mRNA expression was significantly higher at day 7 p.i. in the CNS of CD4 T cell-depleted mice (Fig. 9C). In stark contrast to results for the other γc-dependent cytokines, IL-21 mRNA was markedly diminished in the CNS of CD4 T cell-depleted mice (Fig. 9D), confirming CD4 T cells as the primary source of IL-21 in the CNS (37). Furthermore, in contrast to the decline of IL-2 and IL-7 transcripts after day 7 p.i. in the CNS of infected control mice, IL-21 mRNA was sustained at elevated levels throughout day 14 p.i. (Fig. 9D). Elevated IL-2 expression, even in the absence of CD4 T cells, suggests that CD8 T cell-derived autonomous IL-2 and increased

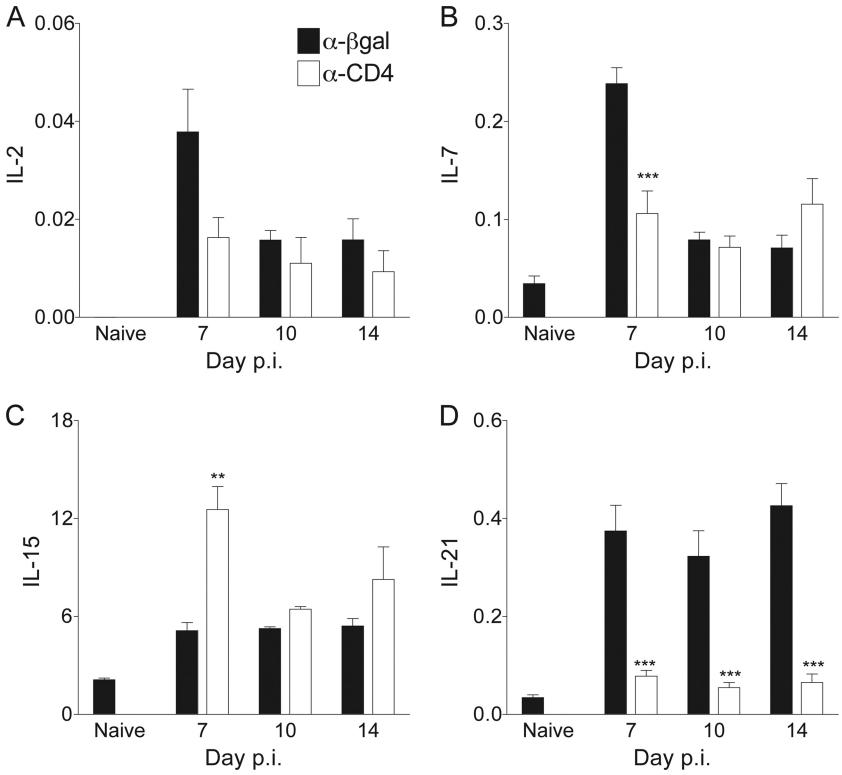

FIG 9 Absence of CNS CD4 T cells abrogates IL-21 expression in the CNS. JHMV-infected mice were treated with α-CD4 or control MAb at 4 and 6 days p.i. Relative transcript levels of IL-2 (A), IL-7 (B), IL-15 (C), and IL-21 (D) in spinal cords of naïve and infected mice were assessed by real-time PCR. Data are expressed as the mean transcript level relative to GAPDH mRNA from 3 to 4 individual mice ± SEM and are representative of results of two independent experiments. Statistically significant differences between control and CD4 T cell-depleted mice were determined by unpaired *t* test. **, $P < 0.005$; ***, $P < 0.001$.

IL-15 may support ongoing CD8 T cell proliferation. However, neither IL-2 (66) nor IL-15 (68) are required for CD8 T cell activity in the CNS, implicating IL-21 in promoting CD8 T cell effector function.

Depletion of CD4 T cells following CNS T cell infiltration rescues viral control and CD8 T cell effector function. Efficient CD8 T cell recruitment but impaired function within the CNS imply that CD4 T cell help acts at the site of infection. We therefore tested if peripheral CD4 T cell depletion subsequent to CNS infiltration rescued CD8 T cell-mediated viral control. CD4 T cells emerge in the CNS as early as day 5 p.i. but do not peak until day 10 p.i. (45). To allow initial accumulation of CD4 T cells concomitant with CD8 T cells within the CNS, CD4 T cells were not depleted until day 7 p.i. Total numbers of $CD45^{hi}$ CNS-infiltrating cells were slightly decreased at day 10 p.i. following CD4 depletion at days 7 and 9 p.i. ($1.3 \pm 0.2 \times 10^5$ per control mouse brain versus $0.9 \pm 0.1 \times 10^5$ per CD4 T cell-depleted mouse brain). Importantly, although >99% of CD4 T cells were depleted in lymphoid tissue, CD4 T cells in the CNS were reduced by only ~50% at day 10 p.i. ($5.6 \pm 1.2 \times 10^4$ per control mouse brain versus $2.6 \pm 0.3 \times 10^4$ per depleted mouse brain). Partial reduction of CD4 T cells in the CNS suggested that the remaining CD4 T cells were derived from cells infiltrating prior to day 7 p.i. and were not affected by MAb treatment due to diminished ability of the MAb to cross the blood brain barrier. Alternatively, MAb depleted only a subset of CD4 T cells, most likely those within the perivascular space (45). Irrespectively, the retention of limited numbers of CD4 T cells within the CNS was sufficient to mediate viral control and enhance CD8 T cell function (Fig. 10). Unlike CD4 depletion following initial CD8 T cell expansion at day 4 p.i., infectious virus in the CNS of day 7 p.i. CD4 T cell-depleted mice was controlled as effectively as in control mice at days 10 and 14 p.i. (Fig. 10A). Furthermore, the magnitudes of granzyme B expression (Fig. 10B) as well as the percentages of granzyme B^+ CD8 T cells (data not shown) at day 10 p.i. were comparable for the two groups. CD8 T cells purified from the infected CNS at day 10 p.i. further demonstrated IFN-γ, perforin, and IL-10 mRNA levels similar to those for CD8 T cells from undepleted infected mice (Fig. 10C to E), in contrast to results for CD4 T cell-depleted mice at day 4 p.i. prior to T cell infiltration (Fig. 7).

DISCUSSION

CD4 T cells regulate CD8 T cells at numerous levels, including priming, expansion, migration, effector function, survival, and establishment of memory. However, the parameters dictating the various stages of CD4 T cell imprinting are still unclear and vary with distinct infection models. Following sublethal JHMV infection, T cells are primed in CLN, although infectious virus is undetectable in the periphery (30). The requirement of CD4 T cells for optimal priming and expansion of virus-specific CD8 T cells, demonstrated by CD4 T cell depletion prior to infection, is consistent with poor DC licensing, analogous to HSV-1 infection (48). Priming of CD8 T cells specific for CNS-derived Ag in CLN is also CD4 dependent (60), suggesting that DC licensing by helper T cells provides a mechanism to overcome weak induction of innate immune responses. Numerous members of the coronavirus

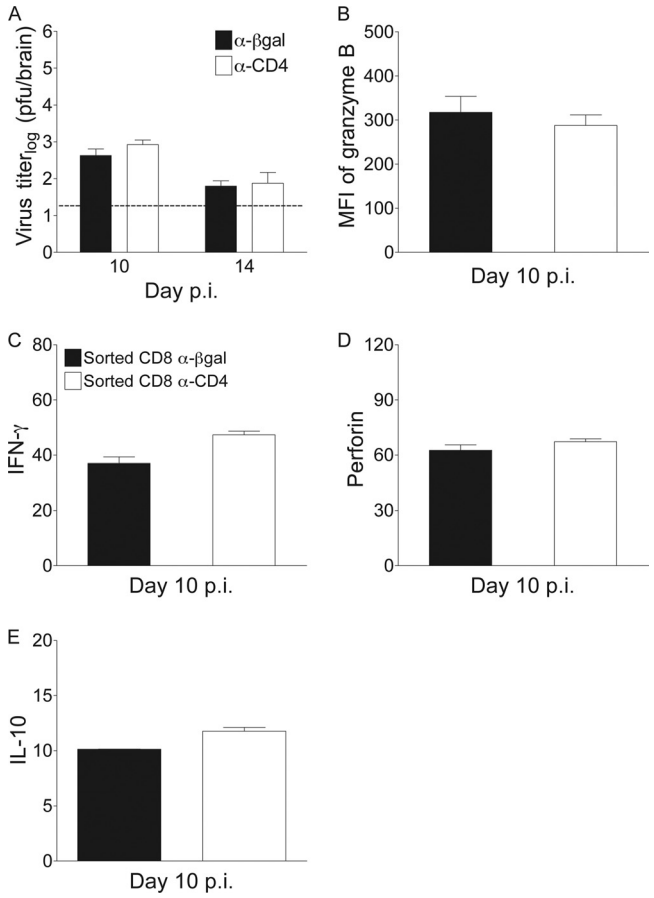

FIG 10 Depletion of CD4 T cells subsequent to CNS T cell infiltration restores viral control and CD8 T cell effector function. JHMV-infected mice were treated with α-CD4 or control MAb at 7 and 9 days p.i. (A) Virus titers in the brains were determined by plaque assay. Titers are expressed as means ± SEM. (B) Pooled brain cells ($n \geq 4$/group) isolated at day 10 p.i. were stained for CD8 and intracellular granzyme B. Mean fluorescence intensity of granzyme B staining in CD8 T cells is shown. Relative transcript levels of IFN-γ (C), perforin (D), and IL-10 (E) in purified CD8 T cells from pooled brains on day 10 p.i. were assessed by real-time PCR. Transcript levels are relative to GAPDH, and data are representative of results of two independent experiments. Trends for each transcript were similar between experiments.

family, including JHMV, are poor inducers of type I IFN (67, 68), supporting a role for CD4 T cells in efficient DC licensing during JHMV infection. This is distinct from lymphocytic choriomeningitis virus infection, for which virus-induced type I IFNs are sufficient for cross-priming of CD8 T cells in the absence of CD4 T cells (23). Chemokine production triggered by Ag-specific DC-CD4 T cell interactions may further contribute by attracting naïve CD8 T cells to competent DC (5). Additionally, recent data show that CD4 T cells support CD8 T priming by controlling lymph node input of naïve lymphocytes (21). Although our data cannot distinguish the relative CD4 T cell contribution to DC licensing from the migratory influence on naïve CD8 T cells, effective rescue of primary virus-specific CD8 T cell expansion by delayed CD4 T cell depletion at day 4 p.i. supports the notion that early signals arising from DC-helper T cell interactions are critical for efficient activation of virus-specific CD8 T cells during CNS infection.

During genital HSV-1 infection, early migratory CD4 T cells enhance local chemokine production and mobilization of effector CD8 T cells to the site of infection (33). Similarly, CNS expression of CCL2, CCL3, CXCL9, and CXCL10 during neurotropic coronavirus infection promotes T cell trafficking to the CNS (6, 15, 28, 56). Reduced chemokine expression in the CNS devoid of CD4 T cells supported local CD4 T cell-mediated augmentation of proinflammatory responses. Consistent with decreased CCL2 expression, recruitment of blood-derived CD45^{hi} F4/80^{+} infiltrating macrophages was reduced (45). Although not addressed in this study, recruitment of peripheral DC to the infected CNS (57) may be diminished in the absence of CD4 T cells and thereby affect CD8 T cell function. Whether CNS-infiltrating DC influence local T cell function during JHMV remains to be elucidated. Nevertheless, chemokine expression was sufficient to recruit CD8 T cells into the CNS parenchyma, excluding impaired trafficking as the cause of diminished virus control.

In contrast to the apparently redundant role of CD4 T cell help in promoting CD8 T cell accumulation, CD4 T cells were crucial to enhance local antiviral CD8 T cell responses. CD8 T cells control JHMV replication in MHC class I-expressing glia via both perforin- and IFN-γ-mediated activities (4, 13). Decreased granzyme B protein as well as IFN-γ and perforin mRNA levels in CNS-derived CD8 T cells from CD4 T cell-depleted mice demonstrated that both effector functions were significantly reduced *in vivo*. Impaired CNS viral control between days 10 and 14 p.i. is thus likely a direct consequence of reduced IFN-γ and cytolytic activity at the CD8 T cell level, although a direct contribution of CD4 T cell antiviral activity cannot be excluded. Interestingly IL-10 transcript levels were also reduced, in agreement with markedly impaired production of IL-10 by CD8 T cells at the effector site of influenza virus-infected mice depleted of CD4 T cells (51). As CD4 T cell depletion also includes removal of T regulatory CD4 T cells, our data further suggest that CD8 T cell effector function-promoting activities override potentially dampening effects exerted by T regulatory CD4 T cells (64, 65).

CD4 T cells also play a role in promoting CD8 T cell survival (18). This role has been implicated in the CNS during West Nile virus (WNV) infection (47), as well as during acute, lethal JHMV infection (49). The increased frequency of Annexin-V^{+} CD8 T cells in the absence of CD4 T cells supports a direct local role of CD4 T cells in promoting CD8 T cell survival. Moreover, increased apoptosis correlated with the absence of IL-21 in the CNS of CD4 T cell-depleted mice. IL-21 is known to support CD8 T cell survival and functionality during viral infection (2, 8, 12, 34, 62). One suggested mechanism is the regulation of the expression of TRAIL (2, 32). Purified CD8 T cells from the CNS of CD4 T cell-depleted mice had slightly higher transcript levels of TRAIL than those of controls; however, the differences were not significant. Nevertheless, TRAIL mRNA levels were equivalent in purified CD8 T cells when CD4 T cell depletion was initiated at day 7 p.i., subsequent to CNS T cell infiltration (data not shown). A direct correlation between increased apoptotic CD8 T cells and TRAIL during viral encephalitis thus remains tentative. Moreover, the precise contribution of IL-21 to CD8 T cell function during JHMV infection warrants further investigation.

In summary, temporally distinct CD4 T cell depletions reveal that CD4 T cells provide crucial helper functions in optimizing activation of CD8 T cells in CLN, as well as maintaining antiviral CD8 T cell function within the CNS, throughout acute viral encephalitis. Moreover, while early help is likely to involve direct DC licensing, based on IL-2, IL-12, or IL-15 independent peripheral

activation and CNS accumulation of virus specific CD8 T cells (20, 66, 68), IL-21 may enhance effector function within the CNS. Prolonged CD8 T cell effector function is vital, as protective humoral immunity within the CNS does not emerge until after 14 days p.i. (24, 41, 42). Targeted enhancement of CD4 T cell help to promote CD8 T cell responses at various levels should thus be considered to minimize the establishment of persistent infections in nonlymphoid organs.

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