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Distinct CD4 T-cell effects on primary versus recall CD8 T-cell responses during viral encephalomyelitis

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Summary

CD4 T-cell help is not a universal requirement for effective primary CD8 T cells but is essential to generate memory CD8 T cells capable of recall responses. This study examined how CD4 T cells affect primary and secondary anti-viral CD8 T-cell responses within the central nervous system (CNS) during encephalomyelitis induced by sublethal gliatropic coronavirus. CD4 T-cell depletion before infection did not impair peripheral expansion, interferon-γ production, CNS recruitment or initial CNS effector capacity of virus-specific CD8 T cells *ex vivo*. Nevertheless, impaired virus control in the absence of CD4 T cells was associated with gradually diminished CNS CD8 T-cell interferon-γ production. Furthermore, within the CD8 T-cell population short-lived effector cells were increased and memory precursor effector cells were significantly decreased, consistent with higher T-cell turnover. Transfer of memory CD8 T cells to reduce viral load in CD4-depleted mice reverted the recipient CNS CD8 T-cell phenotype to that in wild-type control mice. However, memory CD8 T cells primed without CD4 T cells and transferred into infected CD4-sufficient recipients expanded less efficiently and were not sustained in the CNS, contrasting with their helped counterparts. These data suggest that CD4 T cells are dispensable for initial expansion, CNS recruitment and differentiation of primary resident memory CD8 T cells as long as the duration of antigen exposure is limited. By contrast, CD4 T cells are essential to prolong primary CD8 T-cell function in the CNS and imprint memory CD8 T cells for recall responses.

Keywords: CD4 help; CD8 T cells; central nervous system; inflammation

Introduction

A major role of CD4 T cells during infections is to support priming, programming and/or effector function of CD8 T cells. Help can be provided by cytokine production or regulation of co-stimulatory factors, such as CD40/CD40 ligand via antigen-presenting cells.[1,2] Nevertheless, the requirement for CD4 T-cell help in primary CD8 T-cell responses is not universal and depends on the *in vivo* milieu during initial T-cell activation. Primary CD8 T-cell responses against infectious agents are mostly CD4 T-cell independent, whereas responses to non-inflammatory stimulation or non-replicating vaccines are dependent on CD4 T-cell help.[3–6] Irrespective of the requirement for CD4 T-cell help for primary CD8 T-cell responses, it is accepted that CD4 T-cell help is necessary for the generation of memory CD8 T cells capable of efficient recall responses.[5,7,8] CD4 T cells also play a key role in optimal CD8 T-cell expansion in the draining lymph node (LN), subsequent mobilization of activated CD8 T cells into inflamed tissues, as well as their maintenance and survival at effector sites.[1,9–12]

While imprinting of CD4 T cells on CD8 T-cell function and survival has been extensively studied in peripheral

Abbreviations: CNS, central nervous system; JHMV, 2.2v-1 variant of mouse hepatitis virus strain; IFN, interferon; PFU, plaque forming unit; p.i., post infection; i.c., intracranial; SLEC, short-lived effector cells; MPEC, memory precursor effector cells; MFI, mean fluorescence intensity; MHC, major histocompatibility complex

viral infections, how CD4 T cells impact CD8 T cells in the central nervous system (CNS) as a site of effector activity is less well explored. Infection with the neurotropic JHM strain of mouse hepatitis virus (JHMV) produces an acute encephalomyelitis in both C57BL/6 (H-2^b) and BALB/c (H-2^d) mice, which resolves into a persistent infection associated with chronic demyelination.[13] Initial activation of adaptive immunity occurs in the draining cervical LN (CLN).[14] Activated CD4 and CD8 T cells subsequently cross the blood–brain barrier and enter the CNS, where they are re-stimulated to secrete interferon-γ (IFN-γ), express granzyme B, and lyse virus-infected target cells.[9,13] CD8 T cells are the major effectors controlling viral load via both IFN-γ and perforin-mediated mechanisms.[15–17] Nevertheless, sustained viral RNA indicates persistence at low levels.[18] The role of CD4 T cells is complex because they not only promote CD8 T-cell function and survival within the CNS[9,10] and directly contribute to viral control, but also enhance pathology.[19–23]

A recent study to assess whether CD4 T cells influence CD8 T cells at the activation or effector stage during JHMV infection revealed that CD4 T cells not only enhance CD8 T-cell expansion in the CLN during priming, but also exert helper function within the CNS by locally promoting CD8 T-cell effector function and survival.[9] CD8 T cells were incapable of controlling virus in the CNS without CD4 T cells, even when primed in the presence of CD4 T cells.[9] The latter results were obtained in H-2^b mice, in which the dominant CD8 T-cell response is directed to an epitope in a hypervariable region of the viral spike (S) protein restricted to H-2D^b.[24] In the present report, we set out to assess the extent of CD4 T-cell imprinting not only on primary CD8 T-cell responses, but also on memory formation and recall CD8 T-cell responses in the CNS. BALB/c mice were chosen for these studies because they mount a prominent H-2L^d restricted CD8 T-cell response to an epitope in the highly conserved nucleocapsid (N) protein, which is expressed at much higher levels than the S protein,[25,26] potentially leading to distinct T-cell activation requirements. An accelerated CD8 T-cell response to the N relative to S epitope is indicated by earlier detection of N-specific relative to S-specific responses in CLN of infected BALB/c[14] and C57BL/6[9] mice, respectively, as well as an early preponderance of N-specific over S-specific CD8 T cells in the CNS of JHMV-infected (BALB/c $\times$ C57BL/6) F_1 mice.[26] Moreover, adoptive transfers indicate that virus-specific CD8 T cells induced in the context of H-2^d have more potent antiviral activity than virus-specific CD8 T cells induced in the context of H-2^b.[15,27]

Surprisingly, herein we show that peripheral expansion of virus-specific CD8 T cells was not impaired in the absence of CD4 T cells in BALB/c mice, as distinct from C57BL/6 mice. Furthermore, CD4 T-cell help during priming was dispensable for CNS accumulation and initial function of primary virus-specific CD8 effector T cells. However, uncontrolled CNS virus replication in the absence of CD4 T cells ultimately resulted in loss of IFN-γ production, higher CD8 T-cell turnover, and inability to acquire an effector memory phenotype. Nevertheless, the unhelped CD8 T-cell phenotype was rescued when virus replication was controlled by transfer of memory CD8 T cells, indicating that the unhelped CD8 T-cell phenotype during primary responses is regulated by antigen load, rather than lack of CD4 T-cell imprinting. By contrast, unhelped memory CD8 T cells mounted poor recall responses when transferred into CD4 T-cell-sufficient mice and could not be sustained in the CNS, despite efficient virus control.

Materials and methods

Mice, virus and CD4 T-cell depletion

BALB/c (H-2^d) mice were obtained from the National Cancer Institute (Frederick, MD). All mice were used at 6–7 weeks of age and infected intracranially in the right hemisphere with 2000 plaque-forming units of the glia tropic monoclonal antibody (mAb) -derived 2.2v-1 variant of mouse hepatitis virus strain JHM (JHMV).[28] Virus titres were determined as previously described.[28] Briefly, clarified supernatants from individual brain homogenates were used to measure virus by plaque assay on a murine astrocytoma cell line, designated DBT. Plaques were counted after 48 hr incubation at 37°. Clinical disease was scored daily as described elsewhere:[28] 0, healthy; 1, ruffled fur and hunched back; 2, hind limb paralysis/inability to turn to upright position; 3, complete hind limb paralysis and wasting; 4, moribund/dead. CD4 T cells were depleted by intraperitoneal injection with 250 μg purified anti-mouse CD4 mAb (GK1.5; BioXCell, West Lebanon, NH) at days −2 and 0 of infection. CD4 T cells remained below 0·3% in mAb-treated mice for at least 21 days. Controls received the same amount of isotype control anti-β-gal mAb (GL113).

Immunization and isolation of memory CD8 T cells for adoptive transfer

Memory CD8 T cells were generated by intraperitoneal injection of Thy-1.1 BALB/c mice with 2×10^6 plaque-forming units of JHMV. Donor mice were treated with anti-mouse CD4 or control mAb at day −2 and 0 relative to intraperitoneal immunization for comparative analysis of 'unhelped' versus 'helped' CD8 T cells. After 3–4 weeks splenic donor CD8 T cells were isolated by negative selection using FITC-labelled anti-CD4 (clone RM4-5), CD19 (clone 1D3), MHC class II (clone M5/114.15.2), Fcγ III/II receptor (clone 2.4G2) and NK1.1 (clone PK136), in combination with anti-FITC-coated magnetic beads

(Miltenyi Biotec, Inc., Auburn, CA) and transferred into either naive BALB/c mice at day 1 before intracranial infection ($1{\cdot}2 \times 10^6$ to $1{\cdot}5 \times 10^6$/mouse) or at 5 days after intracranial infection ($0{\cdot}25 \times 10^6$/mouse) as indicated. All procedures were carried out under animal protocols approved by the Institutional Animal Care and Use Committees of Cleveland Clinic Foundation.

Isolation of CNS and lymph node cells

For lymphocyte isolation from the CNS, brains and spinal cords were removed from mice perfused with cold PBS (pH 7·2). Tissues were homogenized in RPMI-1640 containing collagenase (1 mg/ml; Roche, Indianapolis, IN) and DNase I (1 mg/ml, Roche) using gentleMACS™ tubes, a gentleMACS Dissociator (Miltenyi Biotec), and cell strainers (BD Falcon, Durham, NC). Homogenates were centrifuged at 450 ***g*** for 10 min at 4°. The cells were resuspended in cold PBS, adjusted to 30% Percoll (GE Healthcare, Uppsala, Sweden), underlayed with 70% Percoll and centrifuged at 850 ***g*** for 30 min at 4° as described previously.[9] Mononuclear cells were collected from the 30%/70% interface, washed with RPMI-1640 and counted before analysis. Peripheral lymphocytes were isolated from CLN.

Quantitative real-time PCR analysis

RNA from brains of naive and JHMV-infected mice was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and cDNA was subsequently generated by Superscript III RTase (Invitrogen) with oligo-dT(12–18) primers (Invitrogen). Taqman primers/probes specific for GAPDH (Mm03302249_g1), IFN-γ (Mm01168134_m1) and CXCL9 (Mm00434946_m1) were purchased from Applied Biosystems (Foster City, CA) and RNA levels were determined using StepOnePlus™ Real-Time PCR Systems (Applied Biosystems) and STEPONE™ Software v2.1 (Applied Biosystems). Gene expression was normalized to GAPDH expression and converted to a linearized value using the formula: $[2e(Ct^{GAPDH} - Ct^{gene})] \times 1000$.

Flow cytometry and ex vivo CTL assay

Single-cell suspensions were blocked with rat anti-mouse CD16/32 mAb (clone 2.4G2: BD Biosciences, San Diego, CA) for 20 min on ice before staining. For four- or five-colour flow cytometry, cells were stained with FITC-, phycoerythrin (PE) -, PE-Cy5-, allophycocyanin- and PE-Cy7-conjugated mAb specific for CD45 (clone 30-F11), CD8 (clone 53-6.7), KLRG1 (clone 2F1), CD127 (clone A7R34) and MHC II (clone M5/114.15.2) (all from eBioscience, San Diego CA) in PBS containing 0·1% BSA. For intracellular staining for Ki67 (clone B56; BD Biosciences), cells were stained with surface molecules before permeabilization with FIX (0·4% PFA)/PERM buffer (PBS containing 0·1% BSA and 0·1% saponin). Virus-specific CD8 T cells were detected with PE-Cy7-conjugated anti-CD8 and PE-conjugated L^d/pN Class I tetramer[26] at 0·1 μg/$0{\cdot}5 \times 10^6$ to $1{\cdot}0 \times 10^6$ cells as directed by the supplier (NIH Tetramer Core Facility, Atlanta, GA). For virus-specific IFN-γ production by CD8 T cells, cells were cultured with or without 1 μM pN peptide for 5–6 hr with 1 μl of Golgi Stop (BD Bioscience)/ml. After stimulation, cells were stained for CD8 surface expression, fixed and permeabilized to detect intracellular IFN-γ (clone 4S.B3; eBiosience). Samples were analysed on a FACS LSRII (BD Biosciences). Forward and side scatter signals obtained in linear mode were used to establish a gate containing live cells, while excluding dead cells and tissue debris. Data were analysed using FLOWJO (9.3.3) software (Tree Star Inc., Ashland, OR).

For cytotoxicity assays target cells were prepared by pulsing ammonium-chloride-potassium lysing buffer (Invitrogen) -treated splenocytes with the immunodominant L^d-restricted pN peptide (5 μM) for 2 hr at 37°. Peptide-pulsed and untreated targets were then labeled in 50 nM CFSE and 2·5 nM CFSE, respectively. Equal numbers of peptide pulsed ($CFSE_{high}$) and control ($CFSE_{low}$) targets were mixed together. Total target cells were plated into a V-bottom 96-well plate at 10^3 per/well, and virus-specific CD8 T cells were added at effector : target ratios of 1 : 1, 2 : 1 or 4 : 1. Target cells incubated alone were used as negative controls. The plate was centrifuged (300 ***g*** for 1 min) to optimize cell contact and incubated for 5 hr at 37°. The proportion of $CFSE_{high}$ and $CFSE_{low}$ cells was determined by flow analysis. Specific lysis was calculated with the formula: [1 − (ratio of targets only/ratio of targets + T cells)] × 100.

Histopathology

Brains and spinal cords from PBS-perfused mice were fixed with 10% neutral zinc-buffered formalin and embedded in paraffin. Sections were stained with either haematoxylin & eosin or luxol fast blue (LFB) to determine inflammation and myelin integrity, respectively, as described previously.[9] Distribution of viral antigen was determined by immunoperoxidase staining (Vectastain-ABC kit; Vector Laboratory, Burlingame, CA) using the anti-JHMV mAb J3.3 specific for the C-terminus of the viral N protein as the primary antibody, and horse anti-mouse as the secondary antibody (Vector Laboratory) as described elsewhere.[9,16,17]

Statistical analysis

Results are expressed as the mean ± SEM for each group of mice. Results are expressed as the mean ± SEM for

each group of mice. Statistics were determined using unpaired two-tailed Student's *t*-test and verified using two-way analysis of variance (ANOVA) with Bonferroni post-test. Any variance between statistical evaluations resulting in a change from significant to non-significant or vice versa are indicated in respective figure legends (Figs 1,6,8). For the data sets indicated in legends, graphs were plotted using a GRAPHPAD PRISM 5.0 software (Graphpad Software, Inc., LA Jolla, CA).

Results

CD4 T cells do not affect CD8 T-cell peripheral expansion

To assess whether CD4 T-cell help for CD8 T cells during JHMV CNS infection is dependent on the nature of the viral antigen and genetic background, BALB/c mice were treated with depleting anti-CD4 or irrelevant anti-β-gal mAb before infection. Distinct from H-2^b mice,[9] the absence of CD4 T cells did not affect expansion of virus-specific CD8 T cells in the CLN (Fig. 1a). Interferon-γ production by virus-specific CD8 T cells was also not affected (Fig. 1b), confirming a redundant role of CD4 T cells in priming effector CD8 T cells in H-2^d mice. These results prompted us to further examine the efficacy of primary anti-viral CD8 T cells within the CNS, when primed in the absence (unhelped) or presence (helped) of CD4 T cells.

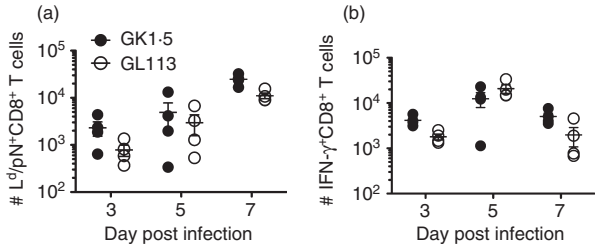

Figure 1. Peripheral virus-specific CD8 T cells expand normally without CD4 T cells. Mice were treated with anti-CD4 (GK1.5) or control (anti-β-gal, GL113) monoclonal antibodies (mAb) at −2 and 0 days before neurotropic JHM strain of mouse hepatitis virus (JHMV) infection. (a) Emergence of virus-specific CD8 T cells in the cervical lymph node (CLN) examined by L^d/pN-tetramer staining at the indicated times post-infection (p.i.). Symbols represent individual animals within one experiment and are representative of two separate experiments with $n = 3$ or $n = 4$ per time-point. (b) Intracellular interferon-γ (IFN-γ) production by CLN-derived CD8 T cells stimulated *ex vivo* in the presence or absence of pN peptide. Events are gated on CD8 T cells; data are the mean ± SEM number of cells producing IFN-γ. While difference was not statistically significant by unpaired Student's *t*-test, evaluation by analysis of variance determined significant differences at day 7 p.i. for L^d/pN-tetramer staining.

Disease severity in CD4-depleted mice is due to the inability to control virus

Disease onset and severity were initially similar in CD4-depleted and control infected BALB/c mice (Fig. 2a). However, whereas controls recovered after day 10 post-infection (p.i.) with a survival rate > 90%, CD4-depleted mice exhibited progressively increased clinical symptoms, with mortality beginning at day 25 p.i. and culminating at 100% before day 40 p.i. (Fig. 2a,b). The sustained symptoms leading to mortality were associated with uncontrolled virus replication (Fig. 2c). Whereas infectious virus in the brain progressively declined to undetectable levels by day 21 p.i. in control mice, CD4-depleted mice failed to control infectious virus after day 7

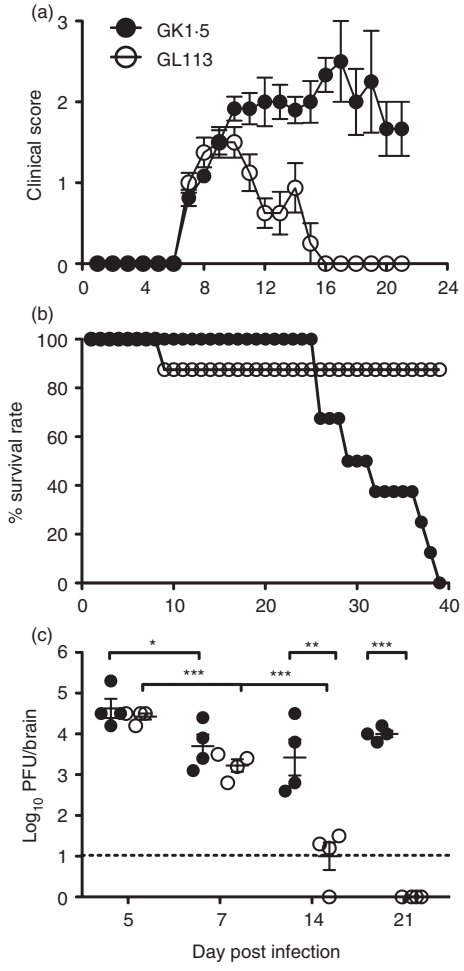

Figure 2. CD4 T-cell depletion during priming impairs virus control. Clinical scores (a) and survival rates (b) of infected CD4-depleted and control mice ($n = 12$ to $n = 20$ per group). (c) Virus titres from individual mice determined by plaque assay. Horizontal bars represent the mean titre and the dashed line marks the limit of detection. Statistical significance determined using an unpaired Student's *t*-test and verified by analysis of variance. $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

p.i. (Fig. 2c). These data demonstrated a critical role of CD4 T cells in controlling CNS virus replication at least to day 14 p.i., when viral clearance in both the H-2^b and H-2^d genetic backgrounds is independent of humoral immunity.[29,30]

Consistent with the inability to clear infectious virus from the brain, immunohistochemistry revealed an increased number of virus-infected cells in CD4-depleted mice at day 21 p.i. (Fig. 3a). Interestingly, in the brain many of the virus-infected cells exhibited the morphology of neurons (Fig. 3a, inset). By contrast, only very rare virus-infected cells were detectable in the brains of control mice at day 21 p.i. Virus-infected cells were also more abundant in spinal cords of CD4-depleted mice compared with controls (Fig. 3b). In CD4-depleted mice virus-infected cells were predominantly glia in appearance (Fig. 3b, left inset) with occasional infected neurons (Fig. 3b, right inset). In control mice the spinal cords showed only rare virus-infected cells with the appearance of macrophages (Fig. 3b, inset). In contrast to the increased number of virus-infected cells and disease severity, overall inflammation in the spinal cord and the extent of demyelination were comparable between the groups (Fig. 3c).

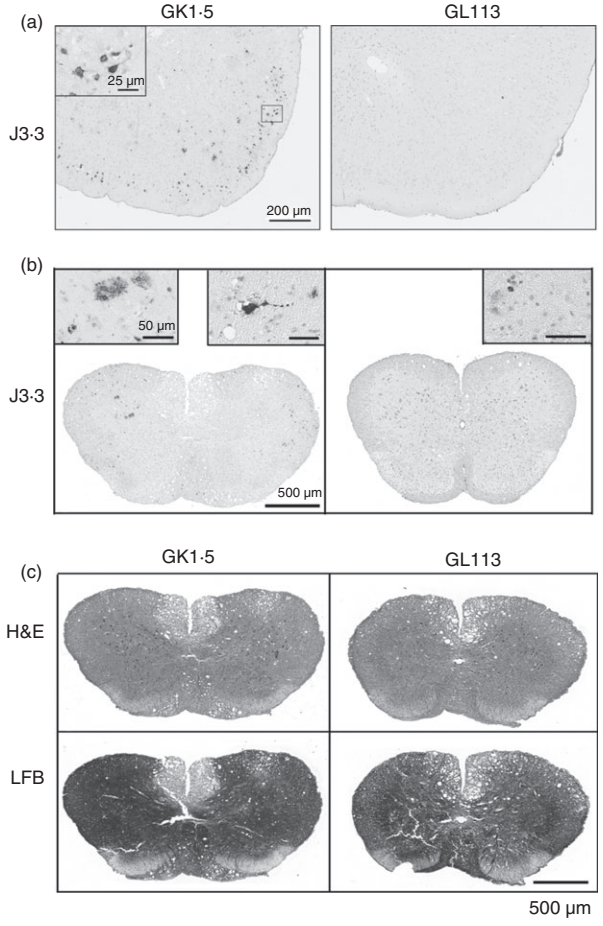

Figure 3. Impaired viral clearance does not enhance inflammation or demyelination. Brains and spinal cords of neurotropic JHM strain of mouse hepatitis virus (JHMV) infected CD4-depleted and control mice analysed for distribution of infected cells, inflammation and demyelination at day 21 post-infection (p.i.). Immunoperoxidase staining with monoclonal antibody (mAb) J.3.3 specific for viral N protein in brain (a) and spinal cord (b). In CD4-depleted mice virus-infected cells in brain exhibit a predominantly neuronal morphology (a, insert), but glia morphology in the spinal cord (b, left insert). (c) Spinal cord sections stained with haematoxylin & eosin for inflammation (top panel) and with Luxol fast blue for demyelination (lower panel).

CD4 T cells do not influence CNS recruitment of virus specific CD8 T cells

As T cells are the primary mediators of JHMV control in the CNS during the first 14 days p.i.[29,30] the inability of CD4-depleted mice to reduce viral load suggested impaired CD8 T-cell recruitment or function. In BALB/c mice infected with a lethal JHMV strain, activated CD8 T cells gained access to the CNS independent of CD4 T cells; however, their numbers in the CNS were reduced by ~50%.[10] Following sublethal JHMV infection of BALB/c mice, CD4 T cells were more prevalent than CD8 T cells in the CNS at day 5 p.i. and accumulated to peak numbers by day 7 p.i. ($\sim 2{\cdot}5 \times 10^5$), similar to CD8 T cells ($\sim 5 \times 10^5$).[29] To distinguish whether CD4 T cells affected CD8 T-cell recruitment and/or effector function following sublethal infection, CNS accumulation of virus-specific CD8 T cells was monitored. CD4 T-cell depletion did not alter the proportion of virus-specific T cells within the CD8 T-cell population infiltrating the brain or spinal cord (Fig. 4a, data not shown). While the frequencies of virus-specific CD8 T cells were equally low at day 5 p.i., peak frequencies were reached by day 7 p.i. in both groups. Moreover, the absolute numbers of virus-specific CD8 T cells were also similar at all times p.i. (Fig. 4b), indicating that CD4 T cells were not required for virus-specific CD8 T-cell recruitment or retention. In the spinal cord virus-specific CD8 T cells were also virtually identical throughout infection, irrespective of the presence or absence of CD4 T cells during priming (Fig. 4b). The failure to clear infectious virus in the absence of CD4 T cells (Fig. 2c) therefore suggested either that the effector functions of CD8 T cells within the target tissue are impaired, or that CD4 T cells contribute directly to virus control.

'Unhelped' CD8 T cells in the CNS are functional

The primary anti-viral mediators reducing infectious JHMV within the CNS are T-cell-derived IFN-γ and perforin.[15–17] The IFN-γ controls virus directly in oligodendrocytes and is also critical to up-regulate MHC class I on oligodendrocytes as well as MHC class II on microglia

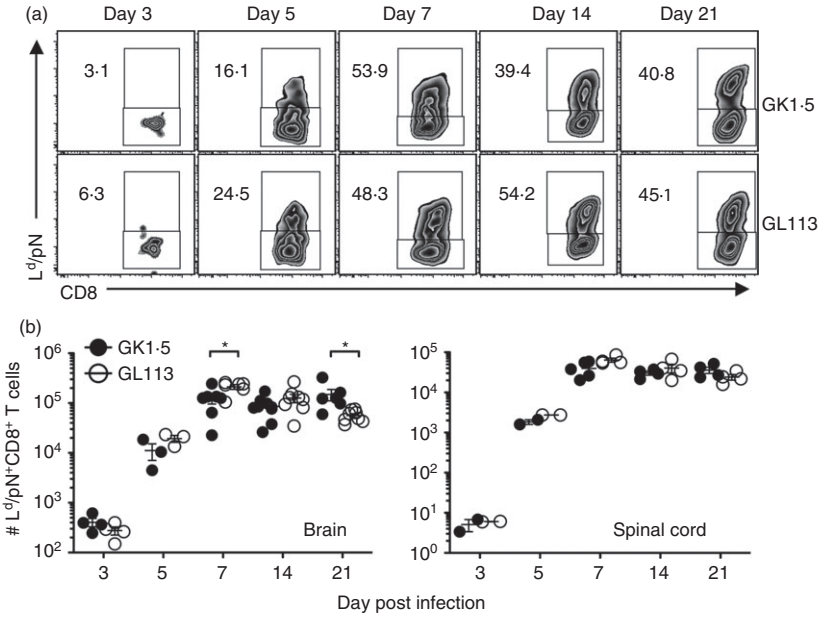

Figure 4. Central nervous system (CNS) accumulation and retention of virus-specific CD8 T cells is CD4 T-cell independent. Cells derived from brains of neurotropic JHM strain of mouse hepatitis virus (JHMV) -infected CD4-depleted and control mice analysed for L^d/pN tetramer$^+$ CD8 T cells by flow cytometry at the indicated days post-infection (p.i.). (a) Representative density plots gated on CD8 T cells; numbers in the upper left corner represent percentages of L^d/pN$^+$ cells within the CD8 T-cell population. (b) Absolute numbers of virus-specific CD8 T cells within brain and spinal cord throughout infection. Symbols represent individual mice. Data are representative of three independent experiments, each with $n = 3$ or $n = 4$ individuals per time-point. Statistical significance determined using an unpaired Student's *t*-test and verified by analysis of variance (ANOVA). $*P < 0{\cdot}05$. While difference was not statistically significant by unpaired Student's *t*-test, evaluation by ANOVA determined significant differences in (b) for spinal cord at day 7 p.i. for L^d/pN-tetramer staining ($*P < 0{\cdot}05$).

and macrophages.[17,31,32] Perforin-mediated cytolysis specifically controls virus in microglia/macrophages, but not oligodendrocytes.[15] We therefore examined potential defects in effector functions of brain-derived unhelped virus-specific CD8 T cells by measurement of *ex vivo* cytolytic activity and IFN-γ expression. Cytolytic capacity of both unhelped and helped CD8 T cells showed no differences between the groups at day 7 p.i. (data not shown). Similarly, CD4 T-cell depletion did not impair the frequency of IFN-γ-producing cells in brain-derived CD8 T cells at day 7 p.i. (Fig. 5a). At day 14 p.i. the frequency of IFN-γ-producing unhelped CD8 T cells remained similar to day 7 p.i., but was reduced relative to helped CD8 T cells. However, by day 21 p.i. the frequency of IFN-γ-producing virus-specific CD8 T cells in the unhelped group declined to < 30%, whereas it was sustained at ~45% in controls. In addition to reduced frequencies, the extent of IFN-γ production as assessed by mean fluorescence intensity (MFI) was also lower in the unhelped groups at days 14 and 21 p.i. (Fig. 5a). In spinal cords the frequencies of IFN-γ-producing CD8 T cells were highest at day 7 p.i. and declined in both groups out to day 21 p.i., with no statistically significant differences between both groups (data not shown). Although a trend towards reduced IFN-γ secretion on a per cell basis was indicated by reduced MFI in the unhelped CD8 T-cell population, differences were not significant (data not shown). These results suggested that CD4 T cells support sustained IFN-γ expression by virus-specific CD8 T cells under conditions of prolonged antigen exposure.

No defects in initial generation and CNS recruitment of IFN-γ-producing virus-specific CD8 T cells suggested that the failure to control infectious virus was attributed to inefficient triggering of T-cell effector function *in vivo*. Interferon-γ mRNA expression in the brain was indeed reduced in the CNS of CD4-depleted mice compared with controls (Fig. 5b). To assess functional IFN-γ protein *in vivo* we further monitored IFN-γ dependent up-regulation of CXCL9 mRNA and MHC class II on microglia. Decreased levels of CXCL9 mRNA supported reduced IFN-γ activity (Fig. 5b). Moreover, MHC class II expression was barely detectable on microglia at day 5 p.i. in CD4-depleted mice, but was already up-regulated on 70% of microglia in controls (Fig. 5c). Although > 98% of microglia in both groups expressed MHC class II by day 7 p.i., overall expression levels assessed by MFI were lower in CD4-depleted mice. CD8 T cells therefore produced sufficient IFN-γ *in vivo* to up-regulate MHC class II on microglia by day 7 p.i. in the absence of CD4 T cells. However, IFN-γ levels were insufficient to achieve optimal MHC class II induction, CXCL9 mRNA up-regulation, or

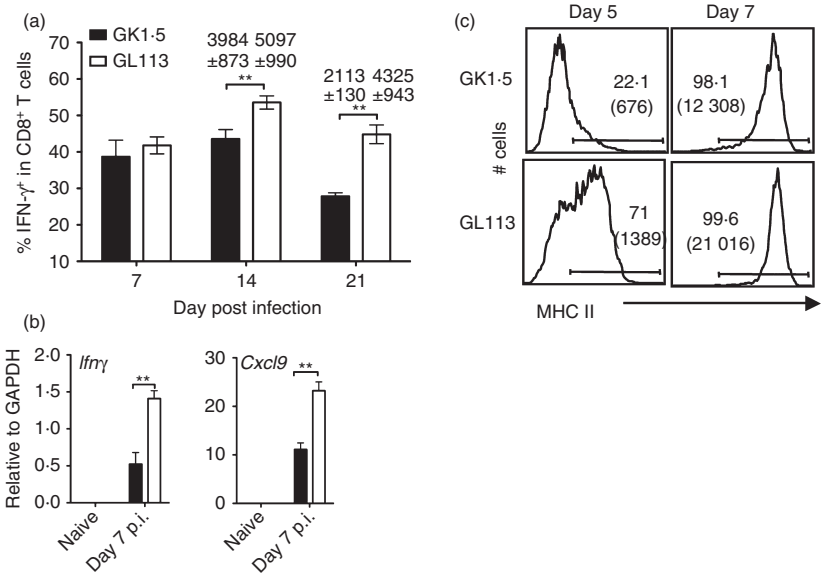

Figure 5. Similar interferon-γ (IFN-γ) production by helped and unhelped CD8 T cells. (a) Central nervous system (CNS) -derived mononuclear cells at days 7, 14 and 21 post-infection (p.i.) were incubated in the absence or presence of pN peptide (1 μM) for 5–6 hr to assess intracellular IFN-γ production by virus-specific CD8 T cells. Number on the top of each bar graph represents mean fluorescence intensity (MFI) of the IFN-γ^+ population. The frequencies of IFN-γ^+ CD8 T cells stimulated in the absence of peptide was < 1·5% during peak viral load. (b) Transcript levels of IFN-γ and CXCL9 relative to GAPDH mRNA in brain of naive or infected mice at day 7 p.i. Data represent the mean ± SEM. Statistical significance was determined using an unpaired Student's *t*-test and verified by analysis of variance for (a). $^{**}P < 0{\cdot}01$. (c) MHC class II up-regulation on microglia from individual mice assessed at days 5 and 7 p.i. Histogram plots are gated on $CD45^{low}$ microglia and are representative of two independent experiments with $n = 4$ per time-point. Numbers in each quadrant are the mean percentage of MHC class II positive microglia and numbers in brackets indicate the MFI of the MHC class II positive population.

control of infectious virus. Overall, these results support a defect in IFN-γ within the CNS, possibly due to the absence of CD4 T cells rather than impaired CD8 T-cell activity.

Unhelped CD8 T cells do not acquire a memory precursor effector phenotype

These data indicated that maintenance of IFN-γ-producing CD8 T cells in the CNS appears optimal when CD4 T cells are present during priming and/or the effector phase. Upon activation, CD8 T cells undergo a complex differentiation programme determined by the nature of inflammatory signals.[33,34] However, two effector cell subsets whose differential expression of the interleukin-7 receptor a chain (CD127) and Killer cell lectin-like receptor G1 (KLRG1) is associated with fate determination and development of memory cells are common to many infections or immunizations. Up-regulation of KLRG1 on effector T cells directly coincides with the magnitude of T-bet expression and serves as a marker of terminally differentiated effector cells.[33,34] By contrast, CD127 is down-regulated upon activation, and activated cells with sustained CD127 expression survive the CD8 contraction phase to form the memory pool.[35] Hence, during differentiation CD8 T cells expressing a $CD127^-$ $KLRG1^+$ phenotype are generally considered to be short-lived effector cells (SLEC), whereas a $CD127^+$ $KLRG1^-$ phenotype is indicative of long-lived memory precursor effector cells (MPEC).[34]

We therefore examined whether the absence of CD4 T cells at priming alters the differentiation phenotype of CNS infiltrated CD8 T cells based on CD127 and KLRG1 expression (Fig. 6a). Although virus-specific CD8 T cells were low at day 5 p.i. (Fig. 4), the majority had down-regulated CD127 (85% versus 70%, in CD4-depleted versus control mice, respectively; data not shown). However, at this early time, only 15% of virus-specific CD8 T cells had a $CD127^-$ $KLRG1^+$ SLEC phenotype in CD4-depleted mice, while ~30% of helped CD8 T cells displayed this terminal effector phenotype (Fig. 6b). Although this profile suggested a more activated virus-specific CD8 T-cell population in CD4-sufficient mice, these early differences resolved by day 7 p.i., when the relative populations of SLEC were similar at ~17%, irrespective of CD4 T cells (Fig. 6b). By days 14 and 21 p.i. the proportion of $KLRG1^+$ cells and SLEC within virus-specific CD8 T cells had dropped significantly in both groups, although SLEC continued to be higher in CD4-depleted mice (Fig 6b). $CD127^+$ $KLRG1^-$ MPEC were similarly low in both groups at day 5 p.i. and gradually increased throughout infection. However, whereas ~70%

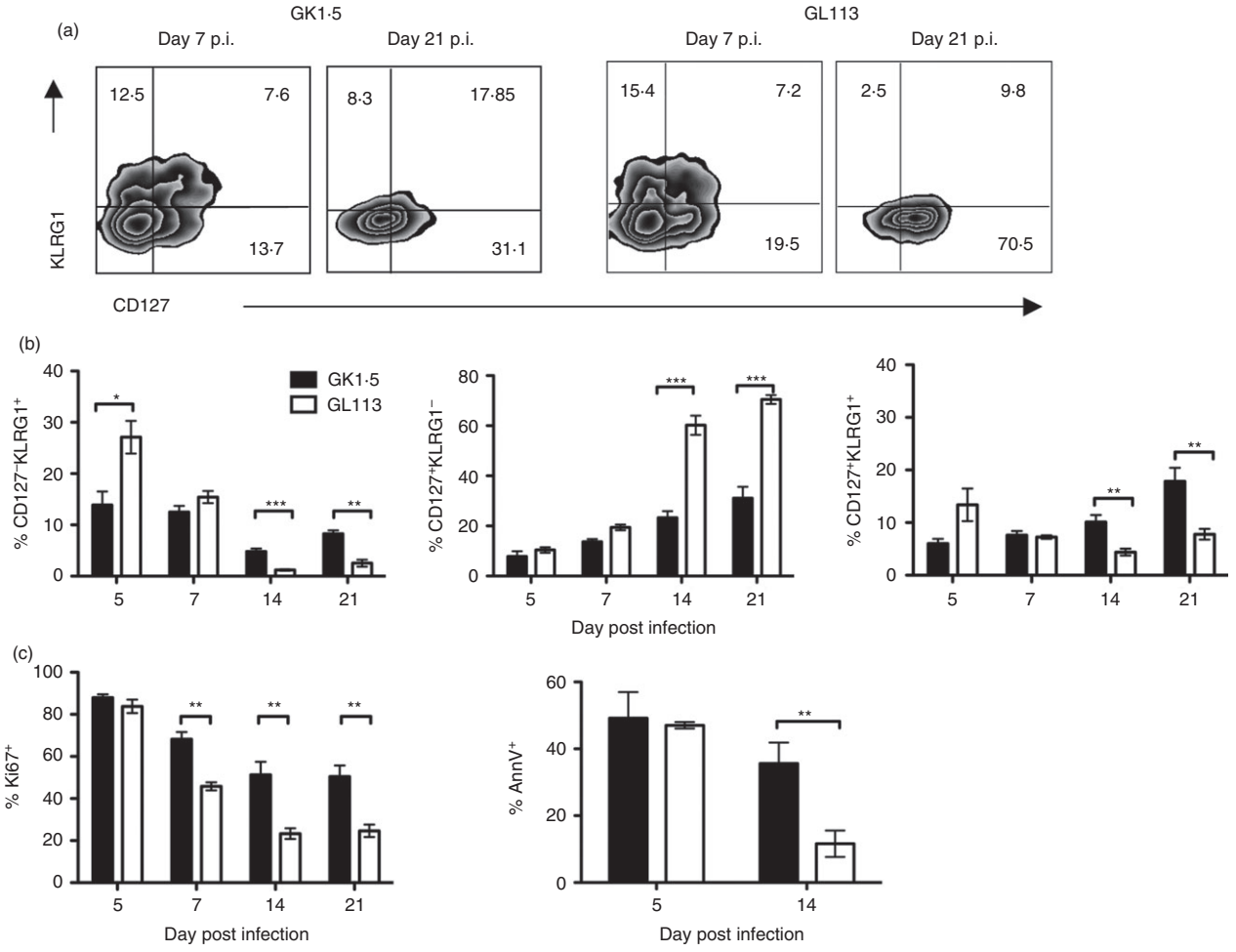

Figure 6. Unhelped CD8 T cells display altered memory differentiation and turnover. Virus-specific CD8 T cells from brain of CD4-depleted and control mice were assessed for acquisition of differentiation phenotypes throughout infection. (a) Representative density plots depicting CD127 and KLRG1 expression on virus-specific CD8 T cells from CD4-depleted and control mice at days 7 and 21 post-infection (p.i.); gating strategy to distinguish CD127$^-$ KLRG1$^+$ short-lived effector cells (SLEC) and CD127$^+$ KLRG1$^-$ memory precursor effector cells (MPEC) is indicated. Numbers represent the proportion of each population in L^d/pN$^+$ CD8$^+$ T cells. (b) Bar graphs show the relative proportions of CD127$^-$ KLRG1$^+$ SLEC, CD127$^+$ KLRG1$^-$ MPEC, or CD127$^+$ KLRG1$^+$ as indicated time-points within helped and unhelped virus-specific CD8 T cells. (c) Proliferation and apoptosis of virus-specific CD8 T cells derived assessed by Ki67 and Annexin V staining at the indicated times p.i. Data are representative of three independent experiments and expressed as mean ± SEM. Statistical significance was determined using an unpaired Student's *t*-test. *$P < 0{\cdot}05$; **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$. Evaluation by analysis of variance showed slightly increased statistical significance (***$P < 0{\cdot}001$) in (b) left graph (SLEC) for days 5 and 21 p.i. and right graph (CD127$^+$ KLRG1$^+$) for day 21 p.i., but no significance in the CD127$^+$ KLRG1$^+$ population at day 14 p.i.

of helped virus-specific CD8 T cells exhibited an MPEC phenotype by day 21 p.i., this population only reached ~30% in CD4-depleted mice (Fig. 6b). A minor fraction ranging from 5% to 20% of virus-specific CD8 T cells expressed both CD127 and KLRG1 throughout infection in both groups. Whereas this population increased with time p.i. in CD4-depleted mice, it decreased in controls that had cleared virus (Fig. 6b). CD127 expression on KLRG1$^+$ cells has been observed under conditions of repetitive antigen re-stimulation and may mark long-lived effector memory T cells.[36,37] Overall, the proportion of KLRG1-expressing JHMV-specific CD8 T cells was relatively low compared with differentiation of SLEC during infection with viruses replicating in non-CNS tissues, e.g. following lymphocytic choriomeningitis virus infection or following malaria parasite immunization.[34,38,39] Weak KLRG1 expression may be attributed to the unique CNS environment and/or the redundant role of interleukin-12, a strong inducer of KLRG1, during JHMV infection.[40] Overall, these results are consistent with the notion that early differences in virus-specific CD8 population arise from the absence of CD4 T cells during priming, whereas the significantly reduced efficacy to acquire an MPEC phenotype, coincident with retention of a

larger proportion of SLEC, is driven by ongoing viral antigen stimulation.

The impaired ability of unhelped virus-specific CD8 T cells to acquire an MPEC phenotype, yet the absence of a preferential decline of unhelped relative to helped CD8 T cells, is consistent with the notion that ongoing exposure to viral antigen drives continual renewal.[34,41] We therefore compared the homeostatic turnover of virus-specific CD8 T cells in the CNS of CD4-depleted and control mice during acute and persistent infection. Consistent with similar numbers of virus-specific CD8 T cells within the CNS, their proliferation was comparable at ~85% at day 5 p.i. (Fig. 6c). The proportions of proliferating virus-specific CD8 T cells within the CNS gradually declined in both groups starting at day 7 p.i. However, ~50% of unhelped CD8 T cells still showed evidence of proliferation at days 14 and 21 p.i., whereas proliferation by helped CD8 T cells had declined to ~25% (Fig. 6c). These data suggest that elevated virus load sustains proliferation, yet also promotes activation-induced cell death. Indeed, while the frequency of apoptotic virus-specific CD8 T cells was similarly high in both groups at day 5 p.i., it remained significantly higher in CD4-depleted mice at day 14 p.i. (Fig. 6c). Therefore, in the absence of CD4 T cells continued proliferation by virus-specific CD8 T cells was balanced by activation-induced cell death, resulting in comparable total numbers of virus-specific CD8 T cells within the CNS.

Viral load, not CD4 T cells, determines the unhelped CD8 T-cell phenotype

Similar phenotypic subsets, as well as activity of helped and unhelped virus-specific CD8 T cells in the CNS at day 7 p.i. suggested that CD4 T cells do not imprint peripheral CD8 T-cell activation or initial CNS effector function. To eliminate the variable effect of viral load in altering CD8 T cells, CD4-depleted infected mice received JHMV-specific memory CD8 T cells to reduce viral load.[15] Memory CD8 T cells were derived from Thy-1.1 mice immunized with JHMV 3–4 weeks post immunization and transferred into JHMV-infected CD4-depleted or control Thy-1.2 recipients at day 5 p.i. This strategy allowed activation of endogenous CD8 T cells and monitoring of both donor and recipient T cells based on the Thy-1 congenic marker. Both CD4-depleted and control recipients developed mild signs of paralysis, and completely recovered from clinical symptoms by day 16 p.i. without mortality (Fig. 7a). Donor memory CD8 T cells prevented mortality consistent with the inability to recover infectious virus at day 16 p.i. Flow cytometric analysis at day 16 p.i. confirmed essentially equivalent CNS recruitment of total as well as virus-specific Thy-1.1 donor CD8 T cells in both CD4-depleted and control recipients (Fig. 7b,c). The vast majority of donor CD8 T cells recruited to the CNS were virus specific (60–70%), despite constituting only 2–3% of the donor population before transfer (data not shown). Virus-specific recipient Thy-1.2 CD8 T cells were slightly higher in the brain of CD4-depleted mice (Fig 7d), whereas those in spinal cord were similar (Fig. 7d, data not shown) confirming that endogenous CD8 T-cell populations were not skewed by the transfer (Fig. 7d). Importantly, > 60% of virus-specific endogenous CD8 T cells in the CNS of CD4-depleted mice expressed a $CD127^+$ $KLRG1^-$ MPEC phenotype at day 16 p.i. (Fig 7e). The SLEC proportion was reduced to < 2%, showing a similar phenotype distribution as in control mice (Fig. 7e). The relative MPEC and SLEC populations were also similar in spinal cords in both recipient groups (data not shown). The capacity of endogenous unhelped CD8 T cells to produce IFN-γ was also restored to that of helped CD8 T cells (Fig. 7f). These findings suggest that the altered phenotype of primary CNS CD8 T cells in CD4-depleted mice is mainly driven by sustained viral load, rather than lack of CD4 T-cell help or imprinting.

CD8 T cells require CD4 T-cell imprinting during priming for recall responses

In peripheral infections, CD4 T-cell imprinting on CD8 T cells is most evident during memory recall responses.[7,8] We therefore determined if CD4 T-cell imprinting is critical for CD8 T-cell recall responses and survival within the CNS. To generate unhelped or helped donor memory CD8 T cells, Thy-1.1 mice were either treated with anti-CD4 or control mAb before JHMV immunization. Unhelped or helped memory Thy-1.1 CD8 T cells were transferred into naive Thy-1.2 recipients 1 day before infection. Mice with no donor T cells served as controls. Recipients of helped memory CD8 T cells displayed enhanced virus control at day 5 p.i., but all groups controlled infectious virus by day 14 p.i. (Fig. 8a). However, in contrast to their helped memory counterparts or primary CD8 T cells (Figs 1 and 4), unhelped virus-specific memory CD8 T cells were significantly impaired in expansion in the draining CLN, as well as early accumulation in the CNS, (Fig. 8b). The relative proportion and total numbers of $Thy\text{-}1.1^+$ CD8 T cells was significantly lower in the unhelped, relative to helped, donor populations in both CLN as well the CNS at days 5 and 7 p.i. (Fig 8b,c). The difference was most apparent at day 5 p.i. in the CNS and was largely resolved by day 14 p.i. Moreover, the number of virus-specific donor CD8 T cells capable of IFN-γ production was reduced in both CLN and CNS of recipients of unhelped versus helped memory CD8 T cells at days 5 and 7 p.i. (Fig. 8d). Despite an apparent equilibration towards similar numbers of unhelped versus helped donor CD8 T cells in the CNS by day 14 p.i., survival of

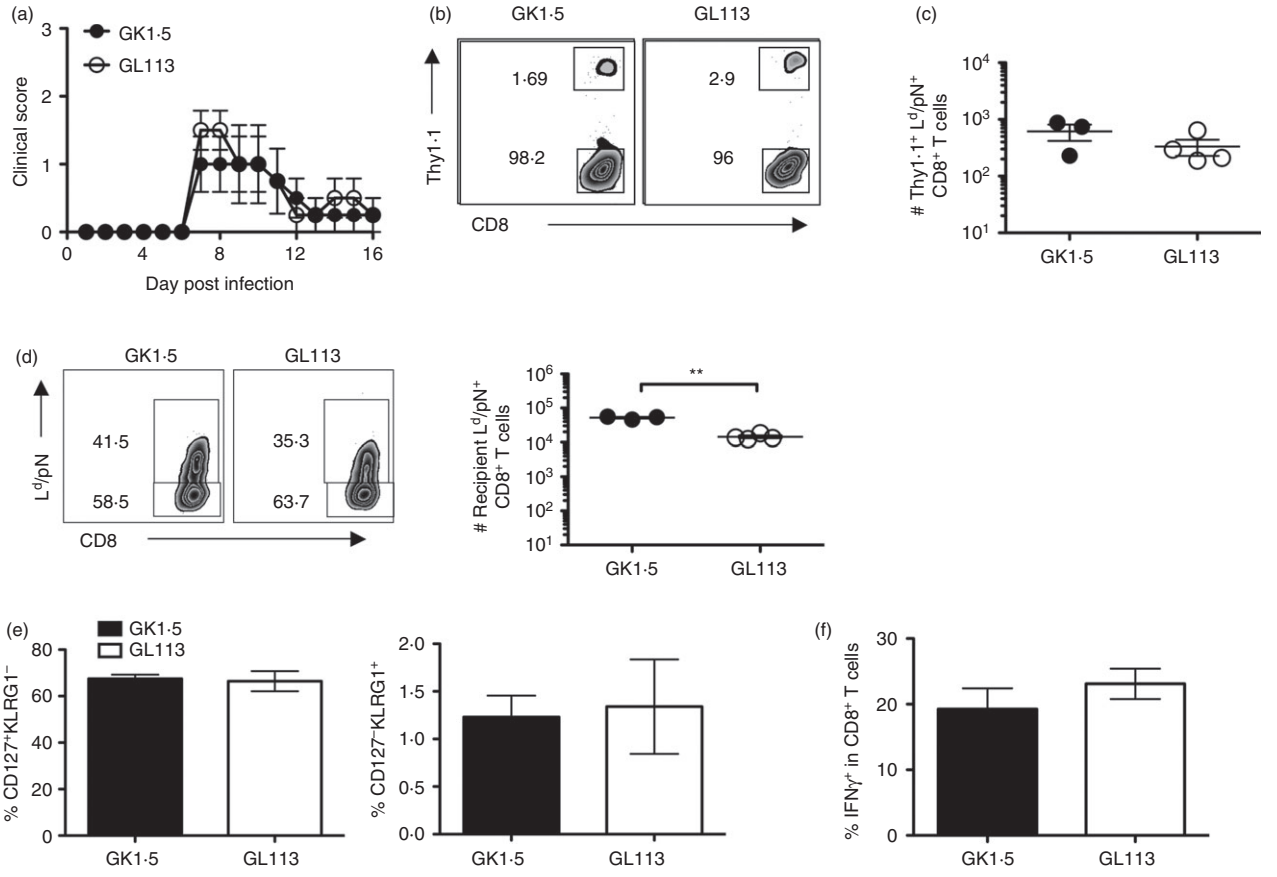

Figure 7. Antigen load imprints the unhelped CD8 T-cell phenotype. Infected CD4-depleted and control mice received memory CD8 T cells ($0{\cdot}25 \times 10^6$) derived from Thy-1.1 mice immunized with neurotropic JHM strain of mouse hepatitis virus (JHMV) intraperitoneally 3–4 weeks previously. (a) Clinical score of infected CD4-depleted or control recipients. (b–d) At 16 days post-infection, brains were analysed for central nervous system (CNS) virus-specific Thy-1.1$^+$ donor accumulation and recipient CD8 T cells as indicated. Representative density plots gated on total CNS-derived CD8 T cells; numbers indicate relative proportion of Thy-1.1$^+$ donor or Thy-1.1$^-$ recipient cells (b) and absolute number of L^d/pN tetramer$^+$ Thy1.1$^+$ CD8 T cells (c). (d) Representative density plots gated on endogenous Thy-1.1$^-$ CD8 T cells; numbers in the upper left corner represent per cent of tetramer$^+$ cells among total CD8 T cells and absolute number of L^d/pN tetramer$^+$ Thy-1.1$^-$ CD8 T cells. (e) Recipient virus-specific CD8 T cells were monitored for CD127 and KLRG1 expression to determine CD127$^+$ KLRG1$^-$ memory precursor effector cells (MPEC) and CD127$^-$ KLRG1$^+$ short-lived effector cells (SLEC) as indicated in Fig. 6. (f) Intracellular interferon-γ (IFN-γ) production by recipient virus specific CD8 T cells were measured by incubation with pN peptide (1 μM) for 5–6 hr. Symbols represent individual mice. Data represent the mean ± SEM. Statistical significance determined using an unpaired Student's *t*-test. $^{**}P < 0{\cdot}01$.

the unhelped memory CD8 T cells was profoundly reduced by day 35 p.i. during the chronic phase. Although helped donor Thy-1.1$^+$ CD8 T cells comprised ~25% of the total CD8 T-cell population, the proportion of unhelped CD8 T cells had declined > 98% (Fig. 8e). This vast reduction was also reflected by the total numbers of unhelped versus helped Thy-1.1$^+$ donor cells within the CNS (Fig. 8e). Unhelped memory cells also failed to survive in the draining CLN (data not shown). Nonetheless, despite their low number, the relative proportion of IFN-γ-producing cells was comparable between the groups (Fig. 8f). These results suggest that CD4 T-cell help at priming plays an essential role in generating long-lasting memory T cells following recall responses within the CNS.

Discussion

Numerous studies on the role of CD4 T cells in regulation of CD8 T-cell immunity reveal that help for functional primary CD8 T-cell responses is dependent on the pathogen and possibly the target tissue. For example, CD4 T-cell help is dispensable for activation and differentiation of naive CD8 T cells during lymphocytic choriomeningitis virus[8] or *Listeria*[7] infection, whereas the induction of primary CD8 T-cell immunity during vaccinia and herpes simplex virus infections[12,42] is CD4 T-cell dependent. Moreover CD4 T-cell help may differentially affect distinct CD8 T-cell effector activities, as shown by diminished IFN-γ production, yet efficient cytolytic activity of CD8 T cells primed without CD4 help

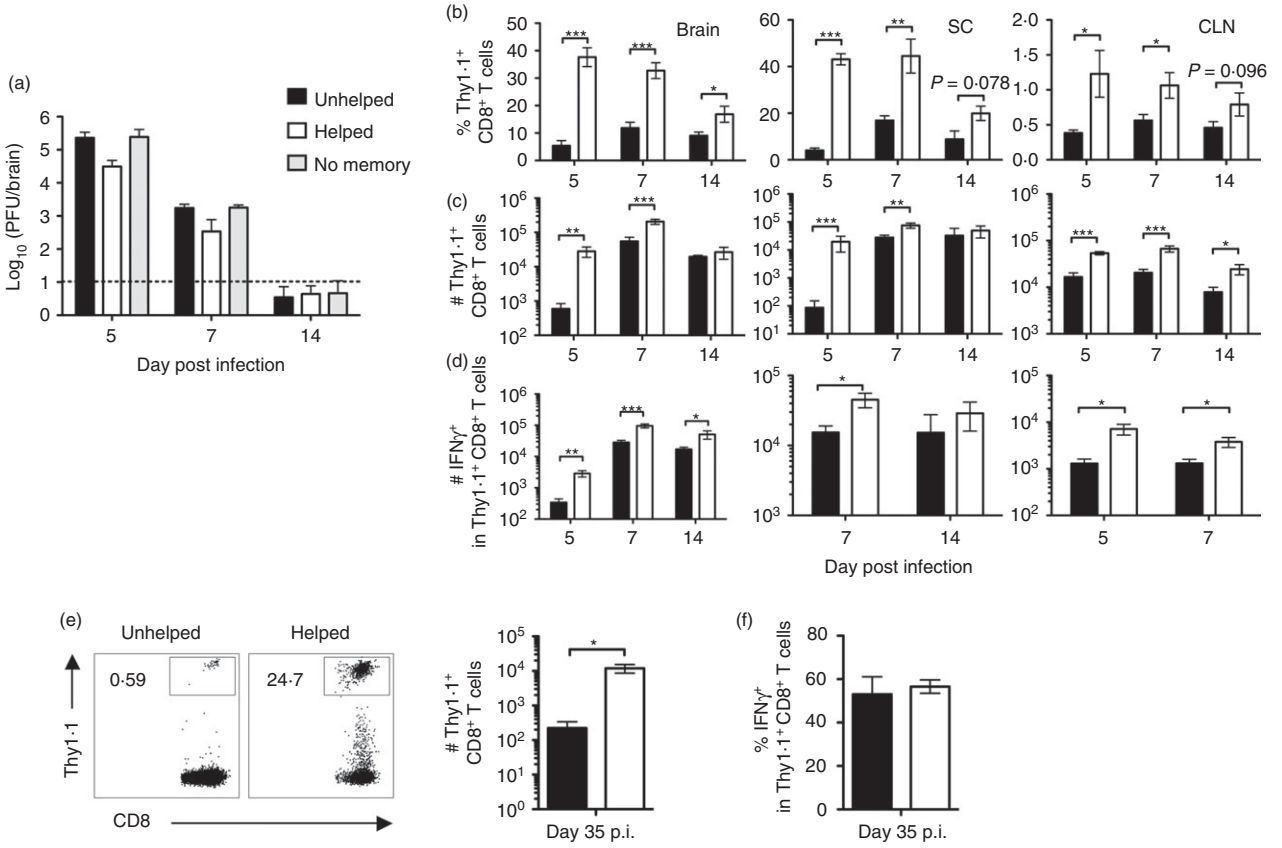

Figure 8. CD8 T-cell recall response requires CD4 help during priming. Helped or unhelped memory Thy-1.1 CD8 T cells ($1{\cdot}2 \times 10^6$ to $1{\cdot}5 \times 10^6$ cells) were transferred into naive wild-type Thy-1.2 recipients 1 day before infection. (a) Virus titres in brains of recipients of helped or unhelped memory CD8 T cells and controls receiving no transfer. (b, c) Accumulation of memory Thy-1.1 donor CD8 T cells in central nervous system (CNS) and cervical lymph node (CLN) of neurotropic JHM strain of mouse hepatitis virus (JHMV) -infected recipients shown by proportion among CD8 T cells (b) and absolute number (c). (d) Cells derived from brain, spinal cord (SC) and CLN at days 5, 7 and 14 post-infection (p.i.) were stimulated with pN peptide (1 μM) for 5–6 hr and analysed for interferon-γ (IFN-γ) production. Data show absolute numbers of IFN-γ producing donor Thy-1.1$^+$ CD8 T cells from CNS and CLN. (e) Brain-derived mononuclear cells assessed for survival of donor Thy-1.1$^+$ cells by flow cytometry at day 35 p.i. Density plot shows representative staining of donor Thy-1.1$^+$ CD8 T cells in the brain. Bar graphs represent absolute numbers of helped or unhelped donor Thy-1.1$^+$ CD8 T cells. (f) CNS-derived cells were incubated with pN peptide to determine IFN-γ-producing Thy-1.1$^+$ CD8 T cells. Data represent the mean ± SEM. Statistical significance was determined using an unpaired Student's *t*-test. *$P < 0{\cdot}05$; **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$. Evaluation by analysis of variance showed slightly increased statistical significance for values in (b) for SC and CLN at day 7 and in (d) for CLN at day 5. By contrast, values in (b) for brain at day 14 and CLN at day 7, in (c) for brain and SC at day 5 and CLN at day 14, as well as (d) for brain at day 5 and 14, and SC and CLN at day 7 did not reach statistically significant differences.

during herpes simplex virus infection.[43] Irrespective of the diverse effects of CD4 T-cell help on primary CD8 T-cell responses, memory CD8 T cells generated in the absence of CD4 T cells are typically defective in recall responses.[5,7,11]

Our studies demonstrate that CD4 T cells differentially influence CD8 T-cell immunity during primary and recall responses mounted to JHMV infection in H-2^d mice and that the necessity of CD4 T-cell help during priming is distinct from infected H-2^b mice.[9] CD8 T cells primed in the absence of CD4 T cells in H-2^d mice had no defects in expansion or IFN-γ expression in the periphery similar to vaccinia virus infection of H-2^b mice.[5] Accumulation and subsequent retention of virus-specific CD8 T cells within the CNS also remained unaffected by the absence of CD4 T cells in H-2^d mice. Moreover, cytolytic activity was similar and IFN-γ-producing virus-specific CD8 effector cells were equally abundant in the CNS regardless of the presence or absence of CD4 T cells during priming and acute infection. CNS CD8 T cells were nevertheless incapable of reducing infectious virus when primed without CD4 T cells, potentially because of delayed and suboptimal MHC up-regulation in the absence of IFN-γ-producing CD4 T cells. A role for CD4 help in maintaining effector function during prolonged antigen exposure was indicated by the decline in both the percentage and

MFI of IFN-γ-producing CD8 T cells later in infection. The failure to clear infectious virus from the CNS in CD4-depleted mice also resulted in higher proliferation and apoptosis of virus-specific CD8 T cells within the CNS, consistent with the inability to acquire an MPEC phenotype. Nevertheless, equilibration of viral load in CD4-depleted mice and controls by transfer of memory CD8 T cells revealed that the unhelped CD8 T-cell phenotype was prominently driven by viral load rather than the absence of CD4 T-cell imprinting.

The mechanisms underlying the differences in CD4 T-cell imprinting during the priming phase in H-2^b versus H-2^d mice are unclear, but highlight critical epitope-specific and genetic influences. The inability to detect JHMV replication in CLN suggests a minimal influence of virus-driven pro-inflammatory responses, especially as type I IFN induction by JHMV is very low.[44,45] Differences may rather reside in the magnitude and kinetics of antigen processing or peptide presentation as L^d restricted N protein-specific responses appear to arise earlier compared with D^b-restricted S protein-specific responses.[26] Moreover, the breadth of CD4 T-cell responses appear to differ, as numerous H-2^b epitopes have been defined, while they appear more limited in H-2^d mice.[46,47] Irrespectively, uncontrolled viral replication despite intact accumulation of virus-specific CD8 T cells was the consequence of CD4 T-cell depletion in both H-2^b and H-2^d mice;[9,10] while an early defect in CNS CD8 T-cell responses in infected H-2^b mice suggested a local beneficial effect of CD4 T cells on CD8 T cells,[9] a similar but more subtle effect in H-2^d mice cannot be excluded. However, we have been unable to distinguish a direct contribution of CD4 T cells to viral control, e.g. via early IFN-γ secretion, from an indirect contribution to CD8 T-cell function involving antigen-presenting cells or soluble mediators. These findings imply that primary CD8 T-cell anti-viral immunity during encephalitis may vary significantly in an outbred population based on provision of CD4 T-cell help at multiple stages and anatomical locations. Nevertheless, irrespective of potentially distinct mechanisms, CD4 T cells appear essential for effective local anti-viral CD8 T-cell function in the CNS.

A number of studies have also revealed that CD4 T cells facilitate the generation of functional memory CD8 T cells during recall responses.[7,8,42] Similar to peripheral infections, the role for CD4 T cells in CD8 T-cell recall responses was essential following JHMV CNS infection. Compared with their helped counterparts, JHMV-specific unhelped memory CD8 cells expanded less efficiently in the CLN, were delayed in CNS accumulation, and exhibited reduced survival. A potential mechanism underlying CD4 help is tumour necrosis factor-related apoptosis-inducing ligand (TRAIL).[48] Since helped CD8 T cells are capable of down-regulating TRAIL expression, they are less vulnerable to TRAIL-mediated apoptosis.[49] However, we previously reported that purified CD8 T cells from the CNS of CD4-depleted H-2^b mice had only slightly increased transcript levels of TRAIL mRNA compared with controls.[9] Whether TRAIL expression influences the limited expansion and survival of unhelped memory CD8 T cells in H-2^d mice remains to be examined.

Distinct from many peripheral infections, both CD4 and CD8 T-cell effector functions are associated with demyelination and clinical disease during JHMV encephalomyelitis. Infection of oligodendrocytes, the primary targets of JHMV infection, is a prerequisite for demyelination but is insufficient to induce myelin stripping without T cells.[13,15] In this context it is of interest to note that uncontrolled virus replication during persistence did not exacerbate demyelination in CD4-depleted mice. This contrasts with increased demyelination associated with similarly uncontrolled viral replication in mice deficient in humoral immunity[29] and supports the notion that CD4 T cells promote pathology.[19,23] Finally, it is interesting to note that uncontrolled viral replication in CD4-depleted H-2^d mice resulted in viral spread to cells with neuronal morphology. While neuronal infection is sparse in immune competent mice, it is clearly evident under conditions of uncontrolled virus replication in infected IFN-α/β receptor-deficient, B-cell deficient, as well as SCID mice,[15,30,45] potentially contributing to the lethal phenotype.

In summary, these data demonstrate that the roles for CD4 T cells in generating fully functional effector/memory CD8 T-cell responses following CNS virus infection could be manifold depending on the stages of activation and differentiation, effector site of CD8 T-cell function, as well as genetic background. Identifying the cellular mechanism by which CD8 T-cell immunity is regulated by CD4 T cells will provide a key insight into understanding CD4–CD8 cooperation for the development of effective primary as well as memory responses within the CNS.

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Disclosure

There are no financial conflicts of interest to declare.

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