CD4 T Cells Contribute to Virus Control and Pathology following Central Nervous System Infection with Neurotropic Mouse Hepatitis Virus†

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Replication of the neurotropic mouse hepatitis virus strain JHM (JHMV) is controlled primarily by CD8+ T-cell effectors utilizing gamma interferon (IFN-γ) and perforin-mediated cytotoxicity. CD4+ T cells provide an auxiliary function(s) for CD8+ T-cell survival; however, their direct contribution to control of virus replication and pathology is unclear. To examine a direct role of CD4+ T cells in viral clearance and pathology, pathogenesis was compared in mice deficient in both perforin and IFN-γ that were selectively reconstituted for these functions via transfer of virus-specific memory CD4+ T cells. CD4+ T cells from immunized wild-type, perforin-deficient, and IFN-γ-deficient donors all initially reduced virus replication. However, prolonged viral control by IFN-γ-competent donors suggested that IFN-γ is important for sustained virus control. Local release of IFN-γ was evident by up-regulation of class II molecules on microglia in recipients of IFN-γ producing CD4+ T cells. CD4+ T-cell-mediated antiviral activity correlated with diminished clinical symptoms, pathology, and demyelination. Both wild-type donor CD90.1 and recipient CD90.2 CD4+ T cells trafficked into the central nervous system (CNS) parenchyma and localized to infected white matter, correlating with decreased numbers of virus-infected oligodendrocytes in the CNS. These data support a direct, if limited, antiviral role for CD4+ T cells early during acute JHMV encephalomyelitis. Although the antiviral effector mechanism is initially independent of IFN-γ secretion, sustained control of CNS virus replication by CD4+ T cells requires IFN-γ.

Control of infectious virus by T cells is a critical component of the pathogenesis elicited by neurotropic mouse hepatitis virus (MHV) infection. The neurotropic JHM strain of MHV (JHMV) replicates primarily in astrocytes, microglia/macrophages, and oligodendroglia. It induces an acute encephalomyelitis accompanied by both acute and chronic demyelination (4). Acute central nervous system (CNS) infection is characterized by a mononuclear cell infiltrate which includes neutrophils, NK cells, macrophages, B cells, and CD4+ and CD8+ T cells (1, 4). Analysis of infected gene-deficient mice, as well as adoptive transfer of CD8+ T cells into immunodeficient hosts, supports the concept that virus replication in astrocytes and microglia is controlled via CD8+ T-cell perforin-dependent cytolyis (2, 4, 21). By contrast, gamma interferon (IFN-γ) secretion controls replication in oligodendrocytes, the cell type which synthesizes and maintains myelin (2, 3, 13, 27). Despite elimination of detectable infectious virus by CD8+ T-cell effector function, sterile immunity within the CNS is not achieved, resulting in a persistent viral infection associated with chronic ongoing demyelination (4). Distinct from control of acute infection by cellular immune responses, viral persistence is maintained by local secretion of neutralizing antibody (4, 31).

CD4+ T cells not only are critical for control of CNS viral replication but also are key mediators of the pathological changes associated with encephalitis and demyelination (7, 12, 25). The contributions of CD4+ T cells to JHMV pathogenesis are controversial. Infection of mice deficient in CD4+ T cells results in uncontrolled CNS virus replication (19, 43). JHMV-specific CD4+ T-cell clones protect with (18, 45) or without (36) reducing viral replication. Similar to CD8+ T cells, JHMV-specific CD4+ T cells are present in the CNS at a high frequency during acute encephalitis and are retained within the CNS during viral persistence (1, 15, 22, 32). However, in contrast to CD8+ T cells, CD4+ T cells transiently accumulate in the perivascular and subarachnoid spaces following JHMV infection, which is associated with expression of the tissue inhibitor of matrix metalloproteinase-1 early (39, 46). As infection progresses they eventually enter the CNS parenchyma, localizing to both the gray and white matter (19, 39). In the absence of CD4+ T cells, CD8+ T-cell apoptosis is increased, coupled with a decrease in the ability of CD8+ T cells to traffic into the CNS parenchyma (39). These data suggest that CD4+ T cells are integral to virus clearance during JHMV-induced encephalitis but that they may play a supportive rather than a primary effector role.

CD4+ T cells can exert multiple antiviral functions, including cytokine secretion (i.e., IFN-γ secretion) (5, 9, 11, 16) and cytotoxicity, primarily via Fas-FasL interactions with infected...
targets (35). JHMV is cleared from the CNS of Fas−/− mice as efficiently as from that of wild-type (wt) mice (28), suggesting that these cell-cell interactions do not contribute to viral clearance. Class II-restricted CD4+ T cells expressing perforin-mediated cytolytic activity have been detected during Sendai virus and lymphocytic choriomeningitis virus infections (16, 24) and have been implicated in the clearance of a hepatotropic MHV from the liver (42). Delayed but eventual clearance of JHMV from the CNS of perforin−/− (PKO) mice was attributed to a defect in CD8+ T-cell-mediated clearance (21). However, the potential role of class II-restricted cytosis mediated during JHMV-induced encephalomyelitis has not been explored. JHMV infection of immunodeficient hosts results in uncontrolled CNS virus replication with minimal clinically apparent disease (2, 29, 30, 44). Adoptive transfer of activated spleen cells into immunodeficient hosts enriched for CD4+ T cells by CD8+ T-cell depletion suggested that CD4+ T cells were as effective as CD8+ T cells in controlling initial virus replication, although the effector mechanism was not identified (29, 44). Activated CD4+ T-cell enriched spleen cells from IFN-γ−/− (GKO) donors induced a rapidly fatal disease, which was not associated with increased virus replication (29). Interestingly, this rapid fatal infection was associated with increased myelin loss (29), suggesting that IFN-γ secretion plays a protective role during JHMV encephalomyelitis. Furthermore, the latter data suggest that CD4+ T cells influence the extent of tissue destruction in immunodeficient hosts independent of antiviral activity.

The present experiments tested the ability of memory CD4+ T cells to influence JHMV CNS replication and pathogenesis in hosts that were unable to exert antiviral IFN-γ- or perforin-mediated effector function but were otherwise immune competent. These mice, designed PKO/GKO, contain lymphoid compartments with normal cellularity but succumb to a JHMV infection that otherwise is nonlethal in wt mice (3). CD4+ T cells were thus purified from JHMV immune wt, GKO, and PKO donors and transferred to PKO/GKO recipients prior to infection to provide evidence for a direct protective role. CD4+ T cells from wt donors controlled virus replication within the CNS, albeit less efficiently than CD8+ T cells. Although GKO and PKO CD4+ T cells also limited CNS virus replication, only IFN-γ-competent CD4+ T cells had sustained protective capacity. Coincident with partially effective clearance of infectious virus, PKO/GKO recipients of all memory CD4+ T cells showed reduced clinical symptoms. However, only IFN-γ-secreting CD4+ T cells correlated with decreased demyelination. The incomplete yet protective capacity of GKO memory CD4+ T cells contrasted with the increased clinical symptoms and rapidly fatal disease mediated by activated CD4+ T-cell-enriched splenocytes from GKO donors in infected Rag−/− recipients (29). This suggested that an IFN-γ-independent inflammatory component not present in Rag−/− recipients down-regulates the pathogenic potential of GKO CD4+ T cells in PKO/GKO recipients. Overall, comparison of the pathological changes induced by JHMV infection in untreated or CD4+ T-cell-treated recipients demonstrated a correlation with the ability to control virus replication and reduce bone marrow-derived neutrophil/macrophage infiltration.

Materials and Methods

Mice. BALB/c (H-2b) wt mice congenic for CD90.1 and IFN-γ-deficient (GKO), perforin-deficient (PKO), and perforin/IFN-γ-deficient (PKO/GKO) mice, all on the BALB/c genetic background, were previously described (3). GKO, PKO, and PKO/GKO mice were maintained under sterile conditions. Recipients and control PKO/GKO mice were infected at 6 weeks of age. All procedures were performed in compliance with Keck School of Medicine Institutional Animal Care and Use Committee-approved protocols.

Virus. The neutralizing monoclonal antibody (MAb)-derived JHMV variant designated 2.2v-1, which produces a sublethal infection followed by viral persistence (10), was used for intracranial infection. Virus was propagated in the presence of MAb J.2.2 and plaque assayed on monolayers of DBT, a continuous murine astrocytoma cell line, as previously described (1, 2, 22, 27). Mice were injected in the left hemisphere with 30 μl containing 500 PFU of JHMV in endotoxin-free Dulbecco’s modified phosphate-buffered saline (PBS). Clinical disease was graded as previously described (1, 2, 10): 0, healthy; 1, hunched back; 2, partial hind limb paralysis or inability to attain the upright position; 3, complete hind limb paralysis; 4, moribund or dead. For determination of CNS virus titers, one half of the brain was homogenized on ice in 4 ml of Dulbecco’s PBS using Tenbroeck tissue homogenizers. Following clarification by centrifugation at 400 × g for 7 min, supernatants were stored at −70°C and virus titers determined by plaque assay on monolayers of DBT cells as previously described (2, 3, 10). Cell pellets were used as a source of CNS-infiltrating cells for flow cytometric analysis (see below). BALB/c, GKO, and PKO mice were immunized by intraperitoneal infection with 2 × 106 PFU of the neurotropic JHMV. This produces a self-limiting peripheral infection and induces excellent memory T-cell responses to JHMV infection (16, 23). No evidence for infectious virus is detected in the peripheral lymphoid organs of mice infected intraperitoneally by 14 days postinfection (p.i.). Donors immunized by peripheral infection were used at 4 to 16 weeks p.i.

T-cell purification and adoptive transfer. Donor spleenocytes were depleted of B cells by adsorption onto 150-mm plates coated with goat anti-mouse immuno-globulin (ICN Pharmaceuticals, Costa Mesa, CA). Following B-cell depletion, CD4+ T cells were purified by positive selection using anti-CD4 (MAb GK1.5)-coated magnetic beads (Miltenyi Biotec Inc., Auburn, CA). Purity was assessed by flow cytometry using anti-CD4 (clone 45-576, anti-CD4 (clone GK1.5), and anti-CD19 (clone 1D3) MAbs (all from BD Pharamingen, San Diego, CA). CD4+ T cells were enriched to >98%. Donor CD4+ T cells (5 × 106/recipient) were adoptively transferred into GKO/PKO recipients by intravenous injection at 3 to 6 h prior to viral challenge.

Isolation of CNS-derived cells. CNS-derived cells were isolated from mice perfused with PBS by homogenization on ice with Tenbroeck tissue homogenizers and subsequently purified by centrifugation. CNS-infiltrating cells were cultured with fluorescein isothiocyanate-, phycoerythrin-, or cytochrome-conjugated MAbs at 4°C for 30 min in PBS containing 0.1% bovine serum albumin. Expression of surface molecules was characterized using the following MAbs (all obtained from BD Pharamingen except where indicated): anti-CD8 (clone 53-6.7), anti-CD4 (clone GK1.5), anti-CD19 (clone 1D3), and anti-Perforin (clone DX5). Neutrophils were identified using anti-Ly6G/6C (clone RB6-8C5). Virus-specific CD8+ T cells were detected by staining with fluorescein isothiocyanate-labeled anti-CD8 and phycoerythrin-labeled L929/18 tetracycline as described previously (3). Phycoerythrin- or cytochrome-labeled anti-CD45 (Ly-5) and anti-F4/80 (Serotec, Raleigh, NC), distinguished microglia (CD45+ F4/80+) from infiltrating/perivascular macrophages (CD45+ F4/80−, CD45+). Major histocompatibility complex (MHC) expression on microglia was determined using anti-H-2 Ld-specific MAb (clone 28-14-8; eBiosciences, San Diego, CA), the restriction element for the immunodominant CD8 T-cell nucleocapsid protein-derived epitope, anti-H-2 Dd (clone 24-2-12), and anti-I-A/I-E (clone M5-114; eBiosciences). Samples were analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) using CellQuest Pro software. Forward- and side-scatter signals were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA) using CellQuest Pro software. Forward- and side-scatter signals were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA) using CellQuest Pro software.

Flow cytometry. Brain-derived cells were blocked with 10% mouse serum and anti-mouse CD16/CD32 (clone 2.4G2; BD Pharamingen) on ice for 15 min prior to staining. For three-color flow cytometric analysis, cells were stained with fluorescein isothiocyanate-, phycoerythrin-, or cytochrome-conjugated MAbs at 4°C for 30 min in PBS containing 0.1% bovine serum albumin. Expression of surface molecules was characterized using the following MAbs (all obtained from BD Pharamingen except where indicated): anti-CD8 (clone 53-6.7), anti-CD4 (clone GK1.5), anti-CD19 (clone 1D3), and anti-Perforin (clone DX5). Neutrophils were identified using anti-Ly6G/6C (clone RB6-8C5). Virus-specific CD8+ T cells were detected by staining with fluorescein isothiocyanate-labeled anti-CD8 and phycoerythrin-labeled L929/18 tetracycline as described previously (3). Phycoerythrin- or cytochrome-labeled anti-CD45 (Ly-5) and anti-F4/80 (Serotec, Raleigh, NC), distinguished microglia (CD45+, F4/80+) from infiltrating/perivascular macrophages (CD45− F4/80+, CD45+). Major histocompatibility complex (MHC) expression on microglia was determined using anti-H-2 Ld-specific MAb (clone 28-14-8; eBiosciences, San Diego, CA), the restriction element for the immunodominant CD8 T-cell nucleocapsid protein-derived epitope, anti-H-2 Dd (clone 24-2-12), and anti-I-A/I-E (clone M5-114; eBiosciences). Samples were analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) using CellQuest Pro software. Forward- and side-scatter signals obtained in linear mode were used to establish a region (R1) containing live lymphocytes, macrophages, and neutrophils, while excluding dead cells and tissue debris. A minimum of 2.5 × 106 viable cells were stained and 5 × 104 to 1 × 105 events analyzed per sample.
Histopathological analysis. Brains and spinal cords were fixed with Clark’s solution (75% ethanol and 25% glacial acetic acid) and embedded in paraffin. Sections were stained with either hematoxylin and eosin or luxol fast blue as described previously (1, 21, 27). The distribution and extent of viral antigen were determined by immunoperoxidase staining (Vectastain-ABC kit; Vector Laboratories, Burlingame, CA) using the anti-JHMV MAb J3.3, specific for the capsid protein (37), as the primary Ab and horse anti-mouse as the secondary Ab (Vector Laboratories). Apoptotic cells were detected by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling using the NeuroTACS II in situ apoptosis detection kit (R&D Systems). To prepare frozen sections, either brains and spinal cords were snap frozen or mice were perfused with 4% paraformaldehyde in PBS (pH 7.4). Following perfusion, brains and spinal cords were removed, postfixed with 4% paraformaldehyde at 4°C for 24 h, and then equilibrated with 30% sucrose in PBS (pH 7.4) overnight at 4°C. Oligodendroglia were identified with allophycocyanin MAb (Ab-7; Oncogene Research Products, Cambridge, MA) and visualized with the peroxidase Vector NovaRED substrate kit (Vector Laboratories). Viral antigen was detected with J.3.3 MAb, which is specific for the viral nucleocapsid protein, and visualized with the peroxidase Vector SG substrate kit (Vector Laboratories) with 3,3′-diaminobenzidine (Sigma Chemicals, St. Louis, MO). CD4+ T-cell infiltration was examined by immunoperoxidase staining of acetone-fixed frozen sections using anti-CD90.1 (HIS51) (BD PharMingen). Primary MAbs were detected by immunoperoxidase staining with biotinylated rabbit anti-Ab (Vector Laboratories) and theVectastain-ABC kit. Aminoethyl carbazol was used as the chromogen substrate (Vector Laboratories). Sections were scored for inflammation, viral antigen, and demyelination in a blinded fashion by a board-certified neuropathologist. Representative fields were identified based on average scores for all sections in each experimental group. The types of infected CNS cells initially identified during a blinded screen were confirmed independently by a second board-certified neuropathologist. The identities of infected cells identified viral antigens, and apoptotic cells were quantified manually in serial spinal cord sections and are reported as positive cells/mm2 as previously described (13).

Statistical analysis. Statistical differences between the groups were evaluated using the Student t test with equal variance. Statistical significance was accepted for a P value of ≤0.05.

RESULTS

CD4+ T cells control JHMV replication within the CNS. PKO/GKO mice, deficient in both IFN-γ secretion and perforin-mediated cytotoxicity, are unable to control JHMV replication within the CNS, resulting in a lethal encephalomyelitis by 16 days p.i. (2). Virus replication peaks in the CNS at 5 days p.i. and remains essentially uncontrolled until day 10 p.i. (2). Prior to the fatal outcome, virus declines by ∼2 log10 at day 14 p.i. (2). Memory CD8+ T cells derived from wt donors are sufficient to control CNS virus replication in the CNS of PKO/GKO recipients (2), confirming the importance of both IFN-γ and perforin-mediated cytotoxicity in the control of JHMV-induced encephalomyelitis (4). To assess direct antiviral roles of CD4+ T cells in JHMV pathogenesis, purified CD4+ T cells from immunized donors were adoptively transferred into PKO/GKO recipients prior to infection. In contrast to untreated JHMV-infected PKO/GKO mice, recipients of wt CD4+ T cells from immune donors exhibited little or no clinical signs of acute disease at days 8 and 10 p.i. (Fig. 1A). By contrast, recipients of CD4+ T cells from naive donors displayed signs of acute encephalitis that were similar to those in infected untreated PKO/GKO mice (reference 2 and data not shown). These data contrast with the previous suggestion that CD4+ T cells were primary inducers of JHMV-induced clinical symptons in Rag−/− mice (29). The viral burden within the CNS was tested to determine whether reduced clinical disease correlated with reduced virus replication. Virus replication was diminished at 8 days p.i. and declined further at 10 days p.i. in recipients of wt memory CD4+ T cells (Fig. 1B). By contrast, CD4+ T cells derived from naive donors exerted no antiviral activity at day 8 p.i. but reduced virus by <1 log10 at day 10 p.i. (data not shown), indicating a minimal induction of antiviral activity.

IFN-γ secretion by CD8+ T cells is critical for the control of JHMV replication within the CNS and enhances perforin-mediated antiviral activity (2, 3). Although virus-specific CD4+ T cells recruited into the CNS during JHMV infection are capable of IFN-γ secretion (15, 29), their contribution to pathogenesis is not clear. Furthermore, CD4+ T-cell-mediated cytolysis controls MHV replication in liver (42), although no evidence for CD4+ T-cell cytotoxicity has been detected following JHMV infection of the CNS (unpublished observation). To determine the contribution(s) of IFN-γ secretion and perforin-mediated cytotoxicity by CD4+ T cells, memory cells from immunized GKO or PKO donors were transferred into PKO/GKO recipients prior to infection. Clinical signs in GKO CD4+ T-cell recipients were comparable to those in untreated JHMV-infected PKO/GKO mice (Fig. 1A). GKO CD4+ T cells reduced CNS virus at day 8 p.i. but were less efficient than wt CD4+ T cells (Fig. 1B), consistent with an antiviral role for
IFN-γ (3). However, in contrast to enhanced viral control provided by wt CD4+ T cells by day 10 p.i., control by GKO-derived CD4+ T cells remained limited (Fig. 1B), but it was statistically significant compared to that in untreated mice.

IFN-γ-independent CD4+ T-cell-mediated control of virus replication within the CNS at day 8 p.i. suggested a possible role for perforin-dependent cytolyis. Recipients of PKO memory CD4+ T cells exhibited little or no clinical signs of acute disease at day 8 or 10 p.i. (Fig. 1A), similar to wt CD4+ T-cell recipients. CD4+ T cells from PKO donors were slightly less efficient than wt CD4+ donor cells in controlling virus replication at day 8 p.i., although this did not reach statistical significance (Fig. 1B). However, by day 10 p.i. CNS virus replication was reduced to similar levels as in recipients of wt CD4+ T cells (Fig. 1B). The data combined from all groups suggest a complex pattern of limited antiviral CD4+ T-cell activity, which is initially independent of either IFN-γ or perforin. Nevertheless, IFN-γ secretion is critical to sustain antiviral activity as indicated by enhanced viral clearance and reduced clinical disease in the presence of IFN-γ. IFN-γ thus plays a protective role in diminishing acute disease.

To ensure the presence of biologically active IFN-γ secreted by CD4+ T-cell donor cells, IFN-γ inducible MHC class II expression was examined on microglia, the resident CNS macrophages (Fig. 2A and B). Consistent with the absence of IFN-γ, microglia in infected PKO/GKO mice did not express class II. By contrast, recipients of IFN-γ-competent CD4+ T cells up-regulated class II expression on the vast majority of microglia at day 8 p.i. and maintained class II expression by day 10 p.i. (Fig. 2A and B), when virus was already considerably reduced (Fig. 1). These data are consistent with CD4+ T-cell-mediated IFN-γ secretion within the CNS and suggest that enhanced and prolonged antiviral function may result from T-cell receptor target cell recognition. As initial class I up-regulation on microglia of JHMV-infected PKO/GKO mice is transient (2), class I Ld expression was monitored in untreated and CD4+ T-cell recipient mice (Fig. 2C and D). Although class I expression was detected on ~50% of microglia in the absence of IFN-γ, it was further enhanced in the presence of IFN-γ at day 8 p.i. However, class I expression was considerably reduced in all groups by day 10 p.i., with the reduction being most severe in mice lacking IFN-γ. Analysis of class I Dd expression in a separate experiment also revealed reduced expression on microglia derived from GKO CD4+ T-cell recipients compared to wt recipients at day 10 p.i. (data not shown). Sustained expression of class II, but not class I, on CNS resident microglia supports direct class II-mediated T-cell receptor interactions between CD4+ T cells and viral antigen-presenting CNS resident cells.

CD4+ T cells alter inflammatory components within the CNS. JHMV infection of PKO/GKO mice results in a prominently increased Gr-1hi neutrophil/monocyte population and increased CD8+-to-CD4+ T-cell ratios in the CNS compared to those in wt mice (2). Neutrophils may contribute to CNS pathology via release of proteases and breakdown of the extracellular matrix (46). Inflammation in untreated mice was thus compared to that in CD4+ T-cell recipients at days 8 and 10 p.i. to assess whether donor CD4+ T cells reduced the Gr-1hi population and skewed the T-cell population in favor of CD4+ T cells. The recovery of CNS cells was reduced in all recipient groups compared to untreated infected PKO/GKO mice, consistent with a ~50% reduction in the percentage of CD45hi infiltrating cells (data not shown). Total yields in recipient groups varied two- to threefold (3.3 × 10^6 to 9.0 × 10^6) between individual experiments; however, yields between groups within each experiment did not deviate by more than 30%. Comparison of the compositions of infiltrating CD45hi cells revealed that Gr-1hi populations were significantly reduced in all recipient groups (Fig. 3), similar to the case for recipients of memory CD8+ T cells (2). Thus, Gr-1hi cells, which represented ~15% and 70% of bone marrow-derived CNS inflammatory cells in untreated PKO/GKO mice at days 8 and 10 p.i., respectively, decreased in all recipient groups. This reduction was more prominent at day 10 p.i. than at day 8 p.i., suggesting that uncontrolled virus replication maintains an environment that supports ongoing neutrophil/monocyte infiltration or survival. These data suggest that neutrophil recruitment, previously linked to IFN-γ secretion (39), is regulated independently of IFN-γ in this model.

The frequencies of CD4+ T-cell populations in CD45hi CNS cells were elevated in all CD4+ T-cell recipients at both days 8 and 10 p.i. compared to untreated controls, coinciding with the drop in Gr-1hi cells (Fig. 3). CD8+ T cells remained largely unaffected, and the frequencies of Ld(p) tetramer+ cells within CD8+ T cells were only slightly elevated in control PKO/GKO mice (54 to 63%) compared to CD4+ T-cell recipient mice (44 to 58%). Ratios of CD8 to CD4 T cells thus dropped from ~5.0 in control mice to ~1.7 in recipients of IFN-γ-competent CD4+ T cells at day 8 p.i. Transfer of IFN-γ-deficient CD4+ T cells diminished the ratio to <1.0. These ratios prevailed at day 10 p.i., at which time untreated infected mice had increased their CD8-to-CD4 ratio to 9.4. These data suggest that donor memory CD4+ T cells, even in the absence of IFN-γ, have an enhanced capacity for expansion, recruitment, and/or survival compared to endogenous CD4+ T cells in GKO/PKO recipients. Reduced viral replication and clinical disease thus correlated with reduced neutrophil inflammation and CD8+-to-CD4+ T-cell ratios.

Expression of the CD90.1 marker in wt CD4+ T-cell transfers allowed assessment of the relative proportions of transferred versus endogenous CD4+ T cells accumulating in the CNS of recipient mice. The slightly higher proportion of CD90.1+ endogenous cells (62%) versus CD90.1+ donor cells (38%) indicated that memory CD4+ T cells did not preferentially expand compared to naïve T cells. Furthermore, similar proportions of host- and donor-derived cells were present at day 10 p.i., suggesting similar survival within the infected CNS. Although these relative proportions could not be measured in the recipients of PKO and GKO CD4+ T cells, it is expected that PKO CD4+ T cells behave similarly to wt CD4+ T cells. By contrast, GKO CD4+ T cells have been described as exhibiting enhanced proliferative capacity in other models (6).

To confirm that reduced virus replication in recipients of memory CD4+ T cells was correlated with their trafficking into the CNS parenchyma, memory CD4+ T cells from immunized CD90.1 donors were transferred into CD90.2 PKO/GKO recipients. Analysis of the distribution of CD4+ CD90.1+ T cells within the CNS of infected PKO/GKO mice showed that CD90.1 CD4+ T cells were preferentially localized within the white matter, often in close proximity to oligodendroglia
Furthermore, the simultaneous presence of CD4<sup>+</sup>CD90.1<sup>-</sup> cells within the white matter demonstrates that both host (CD90.1<sup>-</sup>) and donor (CD90.1<sup>-</sup>) T cells localized to the white matter during JHMV-induced inflammation (Fig. 4). The proportions of CD4<sup>+</sup>CD90.1<sup>-</sup> and CD4<sup>+</sup>CD90.1<sup>-</sup> cells within white matter determined by immunohistochemistry approximated the relative percentages determined by flow cytometry, indicating similar trafficking capacities. These data suggest that although CD4<sup>+</sup> T cells localize to perivascular areas and subarachnoid spaces early in infection, they subsequently traffic into the CNS parenchyma, where they are able to inhibit virus replication.

**CD4<sup>+</sup> T cells inhibit virus-induced demyelination.** Uncontrolled JHMV replication in the CNS of PKO/GKO mice is accompanied by recruitment of inflammatory cells, widespread viral antigen in both brain and spinal cord, and demyelination...
As infection progresses, viral antigen increases in the spinal cord concomitant with a dramatic increase in the frequency of infected oligodendroglia (Fig. 5) (3). CNS inflammation was not dramatically altered in infected PKO/GKO mice versus recipients of CD4+ T cells derived from wt mice. However, the prominent perivascular and subarachnoid space accumulation of inflammatory cells observed in untreated PKO/GKO mice (3) was decreased in all CD4+ T-cell recipients (Fig. 5). Although the mechanism limiting perivascular retention is not clear, both CD4+ (Fig. 4) and CD8+ (3) T cells from immune donors facilitate the trafficking of inflammatory cells into the CNS parenchyma.

CD4+ T cells derived from wt mice protected from clinical disease and reduced virus replication within the CNS (Fig. 1). Sections of brain and spinal cord were examined to determine the localization of inflammatory cells within the CNS, the frequency and distribution of infected cells, and the extent of myelin destruction. A reduction in virus-infected cells in the CNS of wt CD4+ T-cell recipients was apparent by day 8 p.i. and was more dramatic by day 10 p.i. (Fig. 5). Consistent with the glial tropism of this JHMV variant, a variety of CNS resident cells were infected at day 8 p.i.; however, the majority were within the white matter with a morphology consistent with oligodendroglia (Fig. 5). By day 10 p.i. the number of infected cells was dramatically reduced in wt CD4+ T-cell recipients compared to untreated infected PKO/GKO mice.

These cells were exclusively within the white matter and exhibited both anatomical and morphological characteristics of oligodendroglia. Consistent with the reduction in infected oligodendroglia, the extent of demyelination in recipients of wt-derived CD4+ T cells was decreased at both days 8 and 10 p.i. (Fig. 5). These data contrast with the effect of virus-specific CD8+ T cells, which were more efficient at controlling virus replication but were associated with increased myelin loss (3). Myelin loss is dependent upon infection of oligodendroglia (4, 10) but is independent of the absolute frequency of infected oligodendroglia (13). The relative frequencies of infected oligodendroglia in untreated, infected PKO/GKO mice were thus compared with those in wt CD4+ T-cell recipients as previously described (13). The data suggest that CD4+ T cells derived from wt donors reduced the number of infected oligodendroglia by ~95% at day 10 p.i. (256 ± 177 versus 14 ± 18 infected oligodendroglia/mm²), although there was considerable variation in individual spinal cord sections. CD4+ T cells from PKO donors were similar to wt CD4+ T cells in their ability to control infectious virus within the CNS (Fig. 1). The inflammatory response, frequency of infection, cell types infected, and extent of myelin loss within the CNS of PKO CD4+ T-cell recipients were identical to those in recipients of wt CD4+ T cells (data not shown).

In GKO CD4+ T-cell recipients, inflammation, distribution,
extent of viral antigen, and demyelination were similar to those in wt and PKO recipients at day 8 p.i., consistent with control of infectious virus (Fig. 1). However, at day 10 p.i. the pathology in the CNS of GKO recipients diverged dramatically from the pathology found in the other recipient groups. Although some antiviral activity was evident by reduced numbers of virus-infected cells compared to those in untreated PKO/GKO mice, GKO cells were inferior to wt CD4+/H11001 T cells at reducing the numbers of infected cells (Fig. 5). The majority of infected cells also localized to the white matter. However, in addition to infected oligodendrocytes, small numbers of infected cells with morphology consistent with astrocytes and microglia/macrophages were noted in GKO recipients. The frequencies of apoptotic cells within the CNS in untreated, wt, and GKO recipients at day 10 p.i. were compared to determine if the relative increase in GKO CD4+ T cells (Fig. 2) was due to increased apoptosis in wt recipients. Apoptosis of CD4+ T cells within the CNS during experimental autoimmune encephalitis is associated with IFN-γ (6). Frequency analysis with a limited number of mice (n = 3) indicated that the frequency of apoptotic cells was reduced in both the wt and GKO recipients compared to infected PKO/GKO mice. However, a <2-fold increase in apoptotic cells was found in the CNS of GKO compared to wt CD4+ T-cell recipients (Fig. 5), suggesting the absence of an influence of IFN-γ on activation-induced cell death during virus-induced encephalomyelitis. Although the number of virus-infected cells was increased in spinal cords relative to that in the other treated groups, the extent of myelin loss was similar (Fig. 5). These data suggest that virus-specific CD4+ T cells contribute to the inhibition of JHMV replication within the CNS, although they are not as efficient as CD8+ T cells (2, 5). Furthermore, CD4+ T cells appear to regulate the infection of all cell types, with IFN-γ contributing to enhanced virus clearance from astrocytes and microglia/macrophages.

**DISCUSSION**

Virus-specific CD8+ and CD4+ T cells both participate in the pathogenesis and resolution of acute disease induced by JHMV infection (4). Deletion of either CD4+ or CD8+ T cells abrogates effective virus clearance (20, 43), and either population can mediate demyelination (2, 44). Numerous efforts have thus been made to dissect the contribution of each T-cell subset and their effector functions in viral clearance and pathogenesis by using adoptive transfer and gene knockout approaches. These studies revealed that CD8+ T cells protect from lethal acute disease by direct antiviral effector mechanisms. Perforin-mediated cytolysis controls infectious virus in astrocytes, microglia, and macrophages (2, 3, 21), while IFN-γ is critical for control of infectious virus in oligodendroglia (2, 3, 13, 27). CD4+ T cells were suggested to play a supporting role in viral clearance by providing CD8+ T-cell help, increasing CD8+ T-cell access into the CNS parenchyma, and limiting CD8+ T-cell activation-induced apoptosis (40). However, the role of CD4+ T cells as direct antiviral effectors remains obscure. Potential antiviral CD4+ T-cell effector functions in-
clude cytokine secretion as well as perforin- and Fas/Fas ligand-induced cytotoxicity (5, 8, 9, 11, 16, 35). However, abrogation of tumor necrosis factor alpha, Fas/FasL interactions, and IFN-γ-induced inducible nitric oxide synthase does not affect JHMV clearance or virus-induced pathology (20, 28, 38), ruling out a role for these effector mechanisms. Nevertheless, transfer of clonal CD4⁺ T cells provided protection and correlated with control of infectious JHMV, even in the absence of CD8⁺ T cells (18, 36, 45). Similarly, the reduced viral titers following adoptive transfer of CD4⁺ T-cell enriched splenocytes into Rag⁻⁻ mice supported direct antiviral activity (29).

The present study took advantage of PKO/GKO mice to examine the role of virus-specific memory CD4⁺ T cells in the control of infectious JHMV in mice with normal lymphoid architecture and endogenous CD4⁺ and CD8⁺ T-cell numbers (3). In contrast to infected immunodeficient Rag⁻⁻ or SCID mice (3, 29, 30, 44), which show little or no clinical evidence of acute disease, PKO/GKO mice develop clinical symptoms similar to those of infected wt mice, but they cannot control virus (2). CD4⁺ T cells from wt immune donors inhibited CNS virus replication almost as effectively as donor CD8⁺ T cells in these hosts (2). Control of MHV infection in the liver by cytolytic CD4⁺ T cells (42) suggested that perforin-dependent class II-mediated cytosis might contribute to antiviral activity within the CNS. However, PKO CD4⁺ T cells were nearly as effective as wt CD4⁺ T cells in controlling virus replication. In addition, the types of CNS cells infected remained similar, suggesting that class II-mediated cytotoxicity is not a critical feature of CD4⁺ T-cell-mediated antiviral activity within the CNS. This is consistent with the lack of class II expression by astrocytes and oligodendrocytes during CNS infection (14, 33). Similar to wt and PKO CD4⁺ T-cell recipients, recipients of GKO CD4⁺ T cells limited viral replication initially but were less efficient in sustaining antiviral activity. A modest IFN-γ-independent antiviral CD4⁺ T-cell effect was also evident in Rag⁻⁻ mice (29). Our data indicated that an IFN-γ-independent CD4⁺ T-cell component contributed to viral control but was less effective than IFN-γ. In the CNS, IFN-γ influences recruitment of innate and adaptive components (40), in addition to enhancing MHC expression (2) and mediating activity against many viruses, including JHMV (5, 9, 11, 27). In infected wt mice, IFN-γ mRNA and protein expression within the CNS peak coincident with maximum T-cell infiltration (14, 26). Furthermore, a similar MHC expression pattern and viral clearance in NK-deficient interleukin-15⁻⁻ mice negates any contributions by NK cells (47). More rapid accumulation of reactivated donor memory T cells, relative to primary virus specific T-cells in the CNS (32), is consistent with CD4⁺ T-cell-mediated partial clearance. Indeed donor CD4⁺ T cells predominantly infiltrated white matter areas, not only the CNS parenchyma. These data suggest that local IFN-γ secretion by CD4⁺ T cells contributes to control of infectious virus in oligodendroglia. Although the mode of IFN-γ action in JHMV-infected PKO/GKO hosts remains unclear, increased detection of viral antigen in cells with astrocyte and microglia morphology in the absence of IFN-γ suggests a role in early virus clearance from these cell types, thus reducing subsequent infection of oligodendrocytes. Alternatively, CD4⁺ T-cell stimulation by class II-expressing cells may directly trigger sufficient IFN-γ to control virus in proximal oligodendrocytes. Although donor memory CD4⁺ T cells could potentially affect endogenous CD8⁺ T cells, the potential antiviral effector molecules involved are elusive.

A critical aspect of JHMV infection of the CNS is the loss of myelin (4). The mechanisms which damage oligodendroglia and facilitate myelin removal appear to have at least two major components. One is viral infection of oligodendroglia (10). The second is a requirement for an adaptive immune response within the infected CNS (2, 4, 44). Demyelination in infected PKO/GKO mice (2) is consistent with infection of oligodendroglia, T-cell accumulation, and participation of activated macrophages in myelin loss (17), even in the absence of antiviral effector function. Nevertheless, the mechanisms by which CD4⁺ and CD8⁺ T-cell subsets regulate macrophage/microglia-mediated demyelination remain unclear. Following JHMV infection of Rag⁻⁻ mice, demyelination induced by donor CD8⁺ T cells is proposed to be IFN-γ dependent, but that induced by donor CD4⁺ T cells is proposed to be IFN-γ independent (29, 30, 44). However, as virus titers remained high in this system, the relative contributions of viral load versus T-cell accumulation could not be assessed. By contrast, the present data demonstrate that myelin loss is drastically diminished in all three recipient groups relative to infected PKO/GKO mice. These results suggest that reduced demyelination is directly linked to CD4⁺ T-cell-mediated virus control. By contrast, transfer of CD8⁺ T cells into PKO/GKO recipients demonstrated that both wt- and GKO-derived CD8⁺ T cells enhanced demyelination, while PKO-derived CD8⁺ T cells did not alter myelin loss (2). Although the current data suggest a correlation between CD4⁺ T-cell antiviral activity and protection from myelin loss, demyelination did not correlate with CD8⁺ T-cell antiviral activity (2). Furthermore, PKO/GKO recipients of IFN-γ-sufficient memory CD4⁺ T cells exhibited decreased clinical symptoms and survived at least until the termination of the experiment (10 days p.i.). Recipients of GKO CD4⁺ T cells also showed decreased clinical symptoms. However, the degree of viral control cannot be the sole determinant for demyelinating pathology, as transgenic mice deficient in IFN-γ signaling in oligodendroglia did not exhibit increased myelin loss or apoptosis of oligodendroglia despite vast numbers of infected oligodendroglia compared to wt mice (13). Another critical consequence of CD4⁺ T-cell transfer in GKO/PKO mice was the prominent reduction of Gr-1⁺ neutrophils/macrophages. Ameliorated pathogenesis may thus also be correlated with reduced protease activity in the local CNS environment. Overall, these results contrast sharply with the increased demyelination and rapidly fatal outcome associated with JHMV-infected Rag⁻⁻ recipients of activated donor GKO relative to wt CD4⁺ T cells (29). The apparent discrepancies observed in the Rag⁻⁻ and GKO/PKO models may be reconciled by the vastly different endogenous lymphoid tissue environments and activation states of donor populations. Whereas adoptive transfer into lymphopenic environments leads to rapid viral antigen-independent expansion of T cells, cells transferred into an established homeostatic environment need to compete for viral antigen and survival factors (23). Furthermore, endogenous T cells and donor T cells presumably cross regulate each other, as evidenced by reduced CD8/CD4 ratios in CD4⁺ T cells in GKO/PKO recipients compared to untreated groups.
Lastly, reactivated memory CD4+ T cells may have enhanced antiviral function on a per-cell basis compared to primary effector CD4+ T cells, similar to CD8+ T cells in the CNS (32). Distinct activation profiles may thus account for differential affects on viral clearance and pathogenesis.

In summary, the present data indicate a direct antiviral role for memory CD4+ T cells during acute encephalomyelitis induced by JHMV in hosts deficient in perforin and IFN-γ secretion. Neither IFN-γ or perforin alone appeared to be essential for initial reduction of virus replication. However, IFN-γ was essential for sustained control of CNS virus replication and concomitant protection from myelin loss. This report thus confirms the protective antiviral role of CD4+ T cells observed in other CNS infections, including measles virus, West Nile virus, and Theiler’s murine encephalomyelitis virus infections (8, 12, 34). Although a supportive role of CD4+ T cells for CD8+ T-cell function appears to be common to other CNS viral infections, direct antiviral function may be limited to infections involving class II target cells, such as macrophages/microglia in the CNS; e.g., CD4+ T cells did not exert a direct antiviral function during neuronal West Nile virus infection (41). Furthermore, although CD4+ T cells have been targeted as main players in propagating demyelination and disease (29, 44), the data presented here clearly demonstrate their potential to act as protective mediators.

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