Memory CD4⁺ T-Cell-Mediated Protection from Lethal Coronavirus Encephalomyelitis[▽]

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The antiviral role of CD4⁺ T cells in virus-induced pathologies of the central nervous system (CNS) has not been explored extensively. Control of neurotropic mouse hepatitis virus (JHMV) requires the collaboration of CD4⁺ and CD8⁺ T cells, with CD8⁺ T cells providing direct perforin and gamma interferon (IFN- γ)-mediated antiviral activity. To distinguish bystander from direct antiviral contributions of CD4⁺ T cells in virus clearance and pathology, memory CD4⁺ T cells purified from wild type (wt), perforin-deficient (PKO), and IFN- γ -deficient (GKO) immune donors were transferred to immunodeficient SCID mice prior to CNS challenge. All three donor CD4⁺ T-cell populations controlled CNS virus replication at 8 days postinfection, indicating IFN- γ - and perforin-independent antiviral function. Recipients of GKO CD4⁺ T cells succumbed more rapidly to fatal disease than untreated control infected mice. In contrast, wt and PKO donor CD4⁺ T cells cleared infectious virus to undetectable levels and protected from fatal disease. Recipients of all CD4⁺ T-cell populations exhibited demyelination. However, it was more severe in wt CD4⁺ T-cell recipients. These data support a role of CD4⁺ T cells in virus clearance and demyelination. Despite substantial IFN- γ -independent antiviral activity, IFN- γ was crucial in providing protection from death. IFN- γ reduced neutrophil accumulation and directed macrophages to white matter but did not ameliorate myelin loss.

T-cell-mediated immunity plays a pivotal role during viral infections. For many viruses, CD8⁺ T cells are the major effectors of the antiviral immune response mediating cytolysis of infected cells and secreting antiviral cytokines such as tumor necrosis factor (TNF) or gamma interferon (IFN- γ) (17, 23, 36, 37). However, CD4⁺ T cells, in addition to their “helper” functions, can also display antiviral activity (9, 22). CD4⁺ T-cell-mediated antiviral responses involve varied effector mechanisms, including cytokine secretion (i.e., IFN- γ [10, 12]), and cytolysis, preferentially mediated by Fas-FasL (39). In addition, perforin-mediated cytolysis has been implicated in CD4⁺ T-cell-mediated virus clearance during Sendai virus, lymphocytic choriomeningitis virus infections, and mouse hepatitis virus (MHV)-induced liver disease (19, 29, 45). However, along with their antiviral functions, T cells play a central role in tissue damage (28, 30). A contribution of T cells to tissue alteration has been demonstrated during many chronic virus infections: i.e., hepatitis C virus infection (31), Theiler’s murine encephalomyelitis virus infection (13), and human T-cell lymphotropic virus type I-associated myelopathy (21). Thus, it is essential to distinguish T-cell immune mechanisms implicated in virus clearance from those associated with pathogenesis, especially during infection of the central nervous system (CNS), which can compromise cognitive, motor, and sensory functions.

The neurotropic JHM strain of MHV (JHMV) produces an acute encephalomyelitis accompanied by both acute and

chronic demyelination (3). Acute infection is characterized by an extensive CNS mononuclear cell infiltration. Cells initially recruited into the CNS comprise components of the innate response, i.e., neutrophils, NK cells, and macrophages, followed by adaptive components: i.e., B cells and CD4⁺ and CD8⁺ T cells (3). Analysis of CD8-deficient mice (24), transfer of CD4⁺ T-cell-depleted activated spleen cells into Rag^{-/-} recipients (49), and transfer of memory CD8⁺ T cells into SCID recipients (5) all indicate that CD8⁺ T cells are the primary effector mechanism controlling infectious virus within the CNS. Both perforin- and IFN- γ -dependent mechanisms are essential: JHMV replication in astrocytes and microglia is controlled via a perforin-dependent mechanism, whereas IFN- γ secretion controls viral replication in oligodendrocytes (3, 5). Analysis of CD8-deficient mice, as well as adoptive transfers into immunodeficient mice, also provided evidence that CD8⁺ T cells contribute to the demyelinating process (5, 24, 35).

In contrast to CD8⁺ T cells, the role of CD4⁺ T cells in JHMV pathogenesis is less well understood. Analysis of CD4-deficient mice (24, 47) suggested a role for CD4⁺ T cells in JHMV clearance from the CNS. However, the ability of CD4⁺ T cells to promote migration and survival of CD8⁺ T cells within the CNS during JHMV infection (41) implicated a supportive role, rather than antiviral function. However, CD4⁺ T cells transferred into recipients deficient in both IFN- γ (GKO) and perforin (PKO) exhibited antiviral activity (42). IFN- γ secretion by CD4⁺ T cells was not required for initial control of viral replication but was essential in maintaining antiviral activity (42). These data confirmed the antiviral function of wild-type (wt) CD4⁺ T cells. In contrast, transfer of CD4⁺ T-cell-enriched splenocytes obtained during acute infection

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into immunodeficient recipients provided only limited antiviral activity (49). Surprisingly, transfer of CD8⁺ T-cell-depleted CD4⁺ T-cell-enriched populations from IFN- γ -deficient donors into Rag^{-/-} recipients resulted in a rapidly fatal disease. Mortality did not coincide with uncontrolled virus replication but with increased demyelination, suggesting a protective role of IFN- γ both in preventing myelin loss and in antiviral activity (34). These data suggest that the function of CD4⁺ T cells within the CNS may be regulated by their activation state and/or the local environment (e.g., the presence of other inflammatory cells, as well as local immunomodulatory responses).

To examine the basis of CD4⁺ T-cell function within the CNS during JHMV infection, the ability of purified CD4⁺ T cells to control virus replication and affect pathogenesis was analyzed in SCID mice devoid of adaptive immunity. Memory CD4⁺ T cells from syngeneic wt, PKO, and GKO donors were transferred into immunodeficient SCID mice prior to JHMV infection. The results demonstrate that CD4⁺ T cells control JHMV replication in glial cells in an IFN- γ - and perforin-independent manner, but contribute to demyelination. Whether control of virus replication was maintained in the absence of IFN- γ could not be determined due to rapid mortality in recipients of GKO CD4⁺ T cells. However, the data support the concept that CD4⁺ T cells contribute to the control of CNS virus replication independent of perforin and IFN- γ . Although this antiviral pathway limits the extent of tissue destruction, IFN- γ is clearly required to protect from inflammation-induced mortality.

MATERIALS AND METHODS

Mice. SCID mice were obtained from the National Cancer Institute (Frederick, MD). Homozygous BALB/c Thy1.1, GKO, and PKO mice were previously described (5). Recipients and donors were maintained under sterile conditions. Recipients were used at 6 weeks of age. Donors were immunized at 6 to 8 weeks of age. Procedures were performed in compliance with the University of South California Keck School of Medicine and Cleveland Clinic Institutional Animal Care and Use Committee-approved protocols.

Virus. SCID mice were injected in the left hemisphere with a 30- μ l volume containing 500 PFU of the neutralizing monoclonal antibody (MAb)-derived JHMV variant designated 2.2v-1 in endotoxin-free Dulbecco's modified phosphate-buffered saline (PBS). JHMV was propagated in the presence of MAb J.2.2 and plaque assayed on monolayers of DBT cells, a continuous murine astrocytoma cell line (4, 42). Clinical disease was graded as previously described (4, 11): 0, healthy; 1, hunched back; 2, partial hind limb paralysis or inability to maintain the upright position; 3, complete hind limb paralysis; 4, moribund or dead. For determination of CNS virus, one-half of the brains were homogenized on ice in 4 ml of Dulbecco's PBS using Ten Broeck tissue homogenizers. Following clarification by centrifugation at 400 $\times$ g for 7 min at 4°C, supernatants were stored at -70°C. Pellets containing CNS-derived cells were used as a source of infiltrating cells for flow cytometry analysis (see below). Virus titers were determined by plaque assays on monolayers of DBT cells as previously described (4, 11, 42).

T-cell purification and adoptive transfer. BALB/c Thy1.1, GKO, and PKO donors were immunized by intraperitoneal (i.p.) injection with 2 $\times$ 10⁶ PFU of JHMV as previously described (42). Virus administration by this route produces a self-limiting peripheral infection and induces excellent memory T-cell responses (4, 5, 42). At 3 weeks postimmunization, no infectious virus can be recovered from spleen or liver, nor can mRNA encoding the viral nucleocapsid protein be detected by PCR (42). Virus-specific CD4⁺ T cells in the donor populations were enumerated by enzyme-linked immunospot assay at 2 months postimmunization to ensure similar frequencies. Briefly, 96-well plates were coated overnight at 4°C with anti-IFN- γ MAb at 10 μ g/ml or anti-TNF MAb (both from BD PharMingen, San Diego, CA) at 5 μ g/ml. Dilutions of donor CD4⁺ T cells were mixed with irradiated BALB/c splenocytes (5 $\times$ 10⁵ per well) that had been preincubated in the presence or absence of JHMV (~2 $\times$ 10⁴ PFU

equivalents per 10⁶ splenocytes) for 60 min at 4°C. After 36 h at 37°C, spots were developed via incubation overnight at 4°C with biotinylated anti-IFN- γ or anti-TNF MAb (BD PharMingen). Following addition of streptavidin peroxidase and 3,3'-diaminobenzidine (Sigma Aldrich, St. Louis, MO), spots were counted using an ImmunoSpot analyzer (Cellular Technology). Equivalent frequencies of CD4⁺ T cells secreting IFN- γ (~600/10⁶) and TNF (~900/10⁶) were present in the wt and PKO donor populations. TNF enzyme-linked immunospot assays indicated that the frequency of virus-specific CD4⁺ T cells in the GKO population was increased by ~2-fold (~2,000/10⁶) compared to wt and PKO donors. Donor splenocytes were prepared at 4 to 16 weeks postimmunization. CD4⁺ T cells were purified by positive selection using anti-CD4-coated magnetic beads (Miltenyi Biotec, Inc., Auburn, CA) according to the manufacturer's instructions. Purity was assessed by flow cytometry using fluorescein isothiocyanate (FITC)-labeled anti-CD4 (clone GK1.5), phycoerythrin-labeled anti-CD8 (clone 53-6.7), and peridinin chlorophyll protein (PerCP)-labeled anti-CD19 (clone 1D3) MAb (BD PharMingen, San Diego, CA). CD4⁺ T cells were enriched to >96%. Each recipient received 5 $\times$ 10⁶ donor CD4⁺ T cells by intravenous injection and was challenged with virus 2 to 3 h after adoptive transfer. In addition, initial experiments demonstrated that although >96% of CD4⁺ T cells were transferred for each experiment, virus-specific CD8⁺ T cells, defined by staining with major histocompatibility complex (MHC) class I tetramers (2), were detected within the CNS of each recipient group (data not shown). Therefore, to prevent CD8⁺ T-cell expansion, recipients were injected i.p. with 250 μ g of anti-CD8 MAb 2.43 (obtained from ATCC, Rockville, MD) immediately after CD4⁺ T-cell transfer. Anti-CD8 MAb treatment of infected SCID mice in the absence of CD4⁺ T-cell transfers had no effect on JHMV pathogenesis (data not shown). Data were all obtained from recipients in which neither CD8⁺ T cells nor virus-specific CD8⁺ T cells were detected within the CNS.

Isolation of CNS mononuclear cells. Cells were isolated from the CNS by homogenization on ice with Ten Broeck tissue homogenizers as described above. Briefly, following centrifugation to obtain brain supernatants for virus titer determination, cell pellets were resuspended in RPMI medium containing 25 mM HEPES (pH 7.2) and adjusted to 30% Percoll (Pharmacia, Uppsala, Sweden). A 1-ml underlay of 70% Percoll was added prior to centrifugation at 800 $\times$ g for 30 min at 4°C. Cells were recovered from the 30% to 70% interface and washed in RPMI medium prior to analysis.

Flow cytometry. CNS-derived cell suspensions were blocked with anti-mouse CD16/CD32 (clone 2.4G2; BD PharMingen) MAb on ice for 15 min prior to staining. For four-color flow cytometry, cells were stained with FITC-, phycoerythrin-, PerCP-, or allophycocyanin (APC)-conjugated MAb 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 PharMingen except when indicated): anti-CD45 (clone Ly-5), anti-CD4 (clone GK1.5), anti-CD8 (clone 53-6.7), anti-CD11b (clone M1/70), anti-F4/80 (Serotec, Raleigh, NC), anti-Ly6G (clone 1A8), and anti-I-A/I-E (clone 2G9). Samples were analyzed on a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA). Forward and side scatter signals obtained in linear mode were used to establish a region (R1) containing live cells, while excluding dead cells and tissue debris. A minimum of 2.5 $\times$ 10⁵ viable cells were stained, and 5 $\times$ 10⁴ to 1 $\times$ 10⁵ events were analyzed per sample.

Gene expression analysis. RNA was prepared from individual brains of three or more mice per group at day 8 postinfection (p.i.). RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. First-strand cDNA synthesis used SuperScript II reverse transcriptase (Invitrogen) with oligo(dT)₁₂₋₁₈ primers (Invitrogen). Semiquantitative RNA expression was assessed using a LightCycler and Sybr green kit (Roche, Basel, Switzerland). Samples from each mouse were analyzed in duplicate, and the data are presented as the average of duplicates from three or more mice per group. Linearity of each primer pair was confirmed to have a correlation coefficient of >0.98 by measuring fivefold dilutions of cDNA samples. Threshold cycle (C_T) values are defined as the cycle number at which fluorescence exceeded a threshold value of 0.5. Levels of mRNA expression were normalized to ubiquitin mRNA and converted to a linearized value using the following formula: [1.8e ($C_{Tubiquitin} - C_{Tgene_x}$)] $\times$ 10⁵ as previously described (50).

Histopathological analysis. Brains and spinal cords were fixed with Clark's solution (75% ethanol and 25% glacial acetic acid) or 10% neutral buffered formalin and embedded in paraffin. Sections were stained with either hematoxylin and eosin or Luxol fast blue as described previously (2, 25, 32) to study inflammation or demyelination, respectively. 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 carboxyl terminus of the viral nucleocapsid protein (40) as the primary antibody and horse anti-mouse as the secondary antibody (Vector Laboratory). Sections were scored

FIG. 1. IFN- γ -sufficient CD4⁺ T cells decrease virus-mediated mortality. Shown is the mortality of control SCID mice ($n = 10$) and recipients of wt ($n = 45$), PKO ($n = 32$), and GKO ($n = 25$) CD4⁺ T cells following JHMV infection.

for inflammation, viral antigen, and demyelination in a blinded fashion. Representative fields were identified based on a average scores of all sections in each experimental group.

CD4 and F4/80 staining was performed on snap-frozen tissue. Sections were cut at 10 μ m on a cryostat and fixed with ice-cold acetone for 10 min. Rat anti-mouse antibodies specific for CD4 (L3T4, H129.19; BD Biosciences) or F4/80 (Serotec) were incubated at a 1:100 dilution for 3 h. Immunoreactivity was detected by immunoperoxidase staining (Vectastain-ABC kit; Vector Laboratory). Cleaved caspase-3 immunoreactivity was evaluated on formalin-fixed paraffin-embedded tissue sections. Prior to immunostaining, sections were processed for antigen retrieval. Dewaxed paraffin sections were rehydrated and immersed in Trilogy citrate buffer (Cell Marque, Hot Springs, AZ) and incubated for 10 min at 100°C. Sections were cooled in fresh Trilogy buffer and then rinsed with PBS. Rabbit antibody specific for cleaved caspase-3 (Cell Signaling Technology, Danvers, MA) was incubated for 2 h, and immunoreactivity was detected by immunoperoxidase staining (Vectastain-ABC kit; Vector Laboratories).

RESULTS

IFN- γ prevents fatal CD4⁺ T-cell-mediated disease. CD8⁺ T cells control JHMV replication even in the CNS of SCID mice which lack adaptive immunity (5). In contrast, the role of CD4⁺ T cells in expression of clinical disease, control of virus replication, and myelin loss is unclear (34, 42, 47, 49). To determine how CD4⁺ T cells influence clinical disease, viral replication, and pathological changes within the CNS in the absence of other adaptive immune components, SCID recipients were reconstituted with CD4⁺ T cells from immunized donors prior to JHMV infection. In the absence of adaptive immunity, infected SCID mice exhibit little or no signs of clinical disease until day 10 p.i., at which time signs of encephalitis increase rapidly. Mortality appears at day 10 p.i. and rapidly increases, as less than 40% of infected SCID mice survive to day 14 p.i. (Fig. 1). To compare infected SCID mice to CD4⁺ T-cell recipients, day 14 p.i. was chosen as the few remaining infected SCID mice succumbed by day 18 p.i. In contrast to infected controls, clinical disease was apparent by 3 days p.i. in recipients of CD4⁺ T cells from wt donors. However, in these recipients, clinical signs of encephalitis diminished after day 10 p.i. (data not shown). Furthermore, few fatalities were observed in the wt CD4⁺ T-cell recipient group (Fig. 1). Recipients of CD4⁺ T cells deficient in perforin (PKO CD4⁺ T cells) exhibited progressive clinical disease and minimal mortality similar to the recipients of wt-derived CD4⁺ T cells (Fig. 1). These data suggest that perforin-mediated cytotoxicity does not contribute to clinical disease following JHMV infection. In stark contrast, infection of recipients of CD4⁺ T cells unable to secrete IFN- γ (GKO CD4⁺ T cells), resulted in a rapid onset of clinical symptoms, initially apparent by day

FIG. 2. CD4⁺ T cells infiltrate the CNS. CD4⁺ T-cell recruitment into the CNS of infected SCID mice (Control) or recipients of wt, PKO, or GKO CD4⁺ T cells at day 8 p.i. (A) CNS mononuclear cells obtained at day 8 p.i. analyzed by flow cytometry. CD4⁺ T cells in the CD45^{hi} infiltrating population. Numbers represent percentage of cells within each quadrant. (B) Spinal cords stained immunohistochemically for CD4⁺ cells. The border between gray and white matter is shown by a horizontal double-headed arrow in each image. Perivascular and infiltrating CD4⁺ cells (arrow head) are present in wt and PKO recipients, while GKO hosts show predominant localization of CD4⁺ cells in gray matter. Bar, 200 μ m.

2 p.i. Clinical disease increased dramatically with time and was associated with a uniformly fatal outcome by day 9 p.i. (Fig. 1). These data are similar to the fatal outcome observed in infected Rag^{-/-} recipients of activated splenocytes from GKO donors depleted of CD8⁺ T cells (34). Together, these data suggest that IFN- γ provides protection from a CD4⁺ T-cell-mediated fatal outcome.

CD4⁺ T-cell recruitment and antiviral activity. The fatal outcome in infected SCID recipients of GKO CD4⁺ T cells compared to control mice and recipients of wt and PKO CD4⁺ T cells suggested potential alterations in CD4⁺ T-cell recruitment into the CNS. To test this hypothesis, infiltration of CD4⁺ T cells into the CNS of wt, PKO, and GKO SCID recipients was analyzed. No CD4⁺ or CD8⁺ T cells were detected in the CNS-infiltrating leukocyte population (CD45^{hi}) of control infected SCID mice (Fig. 2). CD4⁺ T cells com-

FIG. 3. CD4⁺ T cells inhibit CNS virus replication. Virus replication in brains of untreated SCID mice and recipients of wt, PKO, and GKO CD4⁺ T cells analyzed at days 8, 10, and 14 p.i. Data represent the average of three to five mice per group ($\pm$ standard deviation) and are representative of four separate experiments. n.a., not available. ***, $P < 0.001$.

prised an equal percentage of CNS-derived cells in all recipients (Fig. 2A). The increased mortality in GKO CD4⁺ T-cell recipients was thus not due to altered CD4⁺ T-cell recruitment into or expansion in the infected CNS. The CD4⁺ T-cell distribution in the CNS of wt, PKO, and GKO recipients was examined by immunohistochemistry to determine a possible correlation with increased mortality in the infected GKO recipients. CD4⁺ T cells were present in the perivascular cuffs and both gray and white matter in all recipient groups (Fig. 2B). In both protected groups (i.e., wt and PKO recipients), CD4⁺ T cells were distributed equally in white matter and gray matter (Fig. 2B). This distribution contrasts with the predominant localization of CD4⁺ T cells in gray matter of the GKO CD4⁺ T-cell recipients (Fig. 2B), suggesting that mortality may be due to preferential retention within gray matter.

Virus load in the CNS was analyzed to determine if the inability of CD4⁺ T cells to secrete IFN- γ altered virus replication. Uncontrolled virus replication in the CNS of infected SCID mice (Fig. 3) confirms the requirement for adaptive immunity to control CNS virus replication (5). CD4⁺ T cells derived from wt donors efficiently controlled virus replication at day 8 p.i. and continued to exert antiviral activity at day 10 p.i. By day 14 p.i., infectious virus was below the limit of detection (Fig. 3). In contrast, recipients of CD4⁺ T cells purified from naïve donors exhibited clinical disease similar to untreated infected SCID mice and no antiviral activity at days 8 and 10 p.i. (data not shown).

Secretion of IFN- γ by CD4⁺ T cells (23, 36) and the protection afforded by CD4⁺ T cells in the clearance of hepatotropic MHV from liver via a perforin-dependent mechanism (45) suggested these effector mechanisms may contribute to the CD4⁺ T-cell antiviral response within the CNS. Virus clearance in PKO CD4⁺ T-cell recipients (Fig. 3) indicates that control of virus replication within the CNS is independent of perforin-mediated cytolysis. Although PKO CD4⁺ T cells were slightly more effective at day 8 p.i. than wt cells, the difference did not reach statistical significance. GKO CD4⁺ T cells were also as efficient as wt CD4⁺ T cells at reducing CNS virus replication at day 8 p.i. (Fig. 3). The antiviral activities of T cells exerted in recipients of wt, PKO, and GKO CD4⁺ T cells suggest that the initial mechanism controlling JHMV replica-

FIG. 4. CNS MHC class II expression requires IFN- γ . (A) Relative MHC I-A^d class II mRNA expression in the CNS of naïve SCID mice ($n = 4$), untreated JHMV-infected SCID mice ($n = 3$), and wt ($n = 3$), PKO ($n = 3$), and GKO ($n = 8$) recipients at day 8 p.i. determined by quantitative real-time PCR. (B) Flow cytometric analysis of class II expression on CNS cells from control infected SCID mice and recipients of CD4⁺ T cells from wt, PKO, and PKO immune donors at day 8 p.i. Quadrants separate class II expression on infiltrating leukocytes (CD45^{hi} [upper right quadrant]) and microglia (CD45^{lo} [lower right quadrant]). The numbers represent percentages of positive cells within each quadrant.

tion in the absence of CD8⁺ T cells is independent of perforin-mediated cytolysis and IFN- γ . Unfortunately, sustained antiviral control by GKO CD4⁺ T cells could not be analyzed due to the mortality within this group (Fig. 1). Nevertheless, these data suggest that increased mortality does not correlate with defective virus clearance (Fig. 1).

Expression of MHC class II mRNA was examined to determine the availability of recognition structures for T-cell-mediated antiviral activity. A minor but reproducible increase in class II mRNA (~ 6 -fold) was detected following infection of control SCID mice (Fig. 4A), presumably due to infiltration of class II-expressing dendritic cells (DCs) or monocytes. In contrast, class II mRNA was upregulated >170 -fold in the CNS of SCID recipients of both wt- and PKO-derived CD4⁺ T cells (Fig. 4A). Only an ~ 13 -fold increase in class II mRNA was detected in the CNS of GKO CD4⁺ T-cell recipients (Fig. 4A), consistent with the dependence of MHC class II expression on IFN- γ (4). Class II was not detected on microglia (CD45^{lo}) derived from the CNS of control infected SCID mice or infected GKO recipients by flow cytometry (Fig. 4B), consistent with the minimal increase in class II mRNA (Fig. 4A). In contrast, class II protein was upregulated on microglia derived from the CNS of infected wt and PKO recipients (Fig. 4B), coincident with the increase in class II mRNA expression (Fig. 4A). The minimal increase of MHC class II mRNA and protein expression in the CNS of GKO recipients relative to wt and PKO recipients supports the concept of an MHC class II-independent CD4-mediated effector mechanism.

The relative levels of expression of TNF, inducible nitric oxide synthase (iNOS), Fas, and FasL mRNAs were examined

FIG. 5. CNS expression of mRNA in JHMV-infected SCID mice. Relative mRNA expression levels of Fas, Fas-L, TNF, and iNOS were determined by quantitative real-time PCR. Total RNA was extracted from brains of naïve SCID mice ($n = 4$), untreated JHMV-infected SCID mice ($n = 3$), and SCID recipients of wt ($n = 3$), PKO ($n = 3$), or GKO ($n = 8$)-derived CD4⁺ T cells at day 8 p.i. Data are represented as levels relative to ubiquitin.

to gauge potential IFN- γ - and perforin-independent CD4⁺ T-cell-mediated effector mechanisms. TNF mRNA expression increased markedly following infection but was independent of CNS infiltration by CD4⁺ T cells (Fig. 5). Importantly, these data suggest that increased TNF is not associated with either antiviral activity or increased morbidity and mortality in the infected GKO recipient group. In contrast to TNF mRNA, iNOS mRNA increased only ~2 fold following the infection of control SCID mice, but increased dramatically in the CNS of wt (>30-fold) and PKO (>20-fold) recipients (Fig. 5). The level of expression in the CNS of GKO CD4⁺ T-cell recipients approximated the level detected in the CNS of the infected control group (Fig. 5), consistent with the concept that IFN- γ is necessary for induction of iNOS (8). Similarly, no increases in mRNA encoding interleukin-6 (IL-6) or IL-1 β nor differential type I IFN secretion were associated with clearance of JHMV from the CNS following the transfer of wt, PKO, or GKO donor CD4⁺ T cells (data not shown). Fas and FasL mRNAs increased slightly following infection of control SCID mice and increased further in the CNS of all CD4⁺ T-cell recipient groups (Fig. 5). The greatest increase was detected in the CNS of GKO CD4⁺ T-cell recipients (Fig. 5). Although Fas-FasL interactions play no role in JHMV pathogenesis in otherwise immunologically intact hosts (33), a contribution of these interactions to viral control or pathogenesis in the CNS of reconstituted SCID mice cannot be excluded.

CD4⁺ T-cell-mediated demyelination. Pathological changes in the CNS of JHMV-infected SCID mice were examined at day 8 p.i. to determine if the increased mortality in the absence of IFN- γ was associated with altered viral tropism and if the

reduction in infectious virus influenced myelin loss. Infected control SCID mice were compared to recipients of all three types of CD4⁺ T cells. Minimal inflammation was present in the CNS of control infected SCID mice (Fig. 6). In contrast, the presence of lymphocytes adjacent to blood vessels and within the CNS parenchyma, especially in areas of tissue destruction, in all recipient groups was consistent with a vigorous inflammatory response (Fig. 6). Virus-infected cells, indicative of extensive virus replication, were localized throughout the CNS of control SCID mice (Fig. 6). Focal areas of virus infection were most prominent in the white matter, with few infected cells present in the gray matter of all CD4⁺ T-cell recipient groups (Fig. 6). Reduced numbers of foci of infected cells as well as reduced numbers of infected cells per focus compared to those in control SCID mice reflect reduced recovery of infectious virus (Fig. 3). Importantly, the distributions of virus-infected cells were similar in all groups, suggesting that the mortality of GKO CD4⁺ recipients is not due to altered viral tropism. Few apoptotic cells were detected in the CNS of control SCID mice; however, no difference was found comparing the recipient groups (data not shown), suggesting that mortality in the GKO recipients did not correlate with increased cell death. Extremely rare areas of focal myelin loss were found in the CNS of infected SCID mice in the absence of apparent inflammatory cells (Fig. 6). This was consistent with very few macrophages, implicated in myelin loss (14), localizing within the CNS of control infected SCID mice (data not shown). In contrast, focal areas of demyelination were clearly evident in the CNS of all recipient groups (Fig. 6). The extent of myelin loss and macrophage infiltration was increased in the CNS of wt recipients relative to PKO and GKO CD4⁺ T-cell recipients (Fig. 6). Although myelin loss was prominent in the CNS of GKO recipients (Fig. 6), macrophage infiltration was reduced compared to that of the other two recipient groups, with an increased frequency in gray matter (data not shown).

IFN- γ regulation of CNS inflammation. IFN- γ shapes the composition of the inflammatory response within the CNS during autoimmune encephalitis (44). To determine if IFN- γ influenced the composition of the inflammatory response during virus-induced encephalitis, the extent of inflammation and the composition of bone marrow-derived (CD45^{hi}) inflammatory cells recruited into the CNS were examined. A limited number of bone marrow-derived cells were recruited into the CNS of infected control SCID mice (Fig. 7A). Inflammatory cells increased ~2-fold between days 8 and 10 p.i., which correlated with increased neutrophils (Fig. 7B). Equivalent frequencies of CD4⁺ T cells were observed in all three recipient groups at day 8 p.i., suggesting that the inability to secrete IFN- γ did not alter T-cell recruitment into the CNS. CD4⁺ T cells increased in both the wt and PKO recipient groups with time p.i. (Fig. 7B), consistent with their continued ability to control virus replication (Fig. 3). Recruitment of macrophages into the CNS of GKO recipients was slightly reduced compared to that in the control and other recipient groups at day 8 p.i. (Fig. 7B). It is interesting to note that the frequency of macrophages present in the CNS of control mice remained relatively constant, while the percentages declined in the wt and PKO recipient groups, consistent with the suppression of infectious virus (Fig. 3). Neutrophils represented a substantial

FIG. 6. CD4⁺ T cells reduce virus infected cells and induce demyelination. Shown are spinal cords of infected control SCID mice or recipients of wt, PKO or GKO CD4⁺ T cells at day 8 p.i. Sections were stained with hematoxylin and eosin (HE) for inflammation, by immunoperoxidase staining with MAb J.3.3 for viral antigen, and by Luxol fast blue (LFB) for demyelination. Perivascular inflammation was prominent in wt, PKO, and GKO recipients compared to controls (arrowheads). Images of representative foci of infected cells show viral antigen was predominantly expressed in oligodendroglia and was reduced in wt, PKO, and GKO CD4⁺ T-cell recipients compared to control infected SCID mice. LFB stains show no demyelination in control infected SCID mice; however, very rare foci of early demyelination are seen on hematoxylin and eosin (arrowheads). Prominent demyelination is seen in wt, PKO, and GKO CD4⁺ T-cell recipients on both HE and LFB (arrows). Bar, 200 μ m.

component of the inflammatory cells in untreated mice and GKO recipients (Fig. 7B). In contrast, in the CNS of wt and PKO CD4⁺ T-cell recipients, neutrophils decreased as infectious virus was reduced (Fig. 7B). Together, these data suggest the possibility that macrophage recruitment into gray matter and increased recruitment of neutrophils may contribute to JHMV-associated mortality in SCID mice reconstituted with GKO CD4⁺ T cells.

CD4⁺ T-cell-mediated virus control. Histological analysis at day 14 p.i. showed residual inflammation, predominantly in white matter of the wt and PKO recipient groups, compared to minimal inflammation in control SCID mice (Fig. 8). Little or no demyelination was detected in the CNS of control SCID mice at day 14 p.i., similar to day 8 p.i. (Fig. 4). This contrasts with the demyelination present in the CNS of wt and PKO recipients (Fig. 8). Consistent with the uncontrolled virus replication in control SCID mice, virus-infected cells were prominent throughout the white and gray matter and appeared to involve primarily glial cells, with only few infected cells showing neuronal morphology (Fig. 8). Virus-infected cells were reduced in the wt and PKO recipient groups and localized primarily within the white matter tracks (Fig. 8). These diminished numbers of virus infected cells in the CNS of both wt and PKO recipients (Fig. 8) are consistent with the dramatically reduced infectious virus recovered at day 14 p.i. (Fig. 3).

DISCUSSION

To better define CD4⁺ T-cell effector mechanisms in both virus clearance and demyelination during JHMV infection, SCID mice were reconstituted with purified virus-specific memory CD4⁺ T cells derived from wt, PKO, and GKO donors. All CD4⁺ T cells exerted antiviral activity in the absence of CD8⁺ T cells, whereas naïve CD4⁺ T cells had no effect. This demonstrates that protection is dependent upon viral antigen-experienced CD4⁺ T cells. Surprisingly, initial antiviral activity was independent of the ability of the CD4⁺ T-cell population to secrete IFN- γ or perforin. Although a contribution of endogenous NK cells to antiviral activity in SCID mice cannot be excluded, there were no differences in NK cell infiltration in the CNS, irrespective of the presence of CD4⁺ T cells (data not shown). A negligible role, if any, for NK cells is consistent with the analysis of SCID recipients of memory CD8⁺ T cells, and NK cell-deficient mice, which both revealed no evidence for direct antiviral activity or a role for NK cell-secreted IFN- γ -mediated MHC class II upregulation on microglia (5, 51).

The mechanism(s) of virus clearance mediated by CD4⁺ T cells remains unresolved, although several effector molecules are excluded. Antiviral activity by the three CD4⁺ T-cell populations could not be linked to differences in TNF or iNOS expression. That there were no differences in IFN- β in brain

FIG. 7. Recruitment of donor CD4⁺ T cells into the CNS of infected SCID mice. CNS inflammation of untreated SCID mice and wt, PKO, and GKO recipients was analyzed by flow cytometry at days 8, 10, and 14 p.i. (A) Total leukocyte (CD45^{hi}) infiltration. (B) Percentage of CD4⁺ T cells (CD4⁺), macrophages (F4/80⁺), and neutrophils (Ly6G⁺) in the infiltrating population. Data represent means ($\pm$ standard deviations) of six mice per group for two separate experiments at each time point. n.d., not detected; n.a., not available.

homogenates of CD4⁺ T-cell recipients (data not shown) further suggested that IFN- α/β , critical in controlling JHNV infection of the CNS (20), does not contribute to differential disease outcome. Although IFN- γ is a critical determinant of sustained viral clearance by CD8⁺ T cells, the contribution of CD4⁺ T cells as a source of IFN- γ was precluded in the present study by the early mortality of GKO CD4⁺ T-cell recipients. IFN- γ was clearly not essential in initial viral reduction in infected SCID hosts; however, hosts deficient in IFN- γ and perforin demonstrated an essential role for IFN- γ in sustained antiviral activity by CD4⁺ T cells (42). The absence of class II on microglia in GKO CD4⁺ T-cell recipients, coupled with the lack of class II expression by astrocytes and oligodendroglia during JHNV-induced inflammation (16, 26), is consistent with an IFN- γ -independent CD4⁺ T-cell-mediated antiviral component. The similar distributions of virus-infected and apoptotic cells in all recipient groups and the presence of little or no mRNA or surface class II expression in the CNS of GKO recipients support the apparent class II-independent viral control. A potential contribution of MHC-independent

Fas-FasL-induced cytotoxicity in reducing JHNV burden is indeed supported by increased Fas and FasL mRNA expression in the CNS of all CD4⁺ T-cell recipient groups. Although Fas-FasL interactions do not contribute to JHNV clearance in immunocompetent hosts, studies with bone marrow chimeric mice suggested that Fas-dependent cytotoxicity can compensate for the absence of perforin-mediated cytotoxicity (33). The unavailability of Fas- or FasL-deficient mice on the BALB/c background precluded further analysis.

CD4⁺ T-cell antiviral function raises the issue of CD4⁺ T-cell receptor engagement within the CNS. Sustained effector function by both CD4⁺ and CD8⁺ T cells requires T-cell receptor engagement (7, 38). The necessity of T-cell receptor/class II engagement for antiviral activity within the CNS is supported by recruitment, but not retention of activated CD4⁺ T cells in the CNS in the absence of cognate antigen (18). Purified memory CD4⁺ T cells did not express activation markers prior to transfer (data not shown), suggesting antigen-driven activation in the draining cervical lymph nodes (27). However, the identity of the class II-expressing cells interacting with CD4⁺ T cells invading the CNS is unclear. During JHNV infection of immunocompetent hosts, a fraction of microglia and macrophages differentiate into a cell type with characteristics of an immature DC, expressing CD11c, class II, and costimulatory molecules (43). Although microglia do not express class II in the absence of IFN- γ and only a small fraction of CNS-infiltrating monocytes expressed class II (4), class II is constitutively expressed by a small population of infiltrating mature DCs. There is no evidence for DC infection *in vivo*, but cross-presentation of virus antigen by DCs trafficking to the CNS may provide a crucial stimulus for GKO CD4⁺ T cells in the CNS (1). In the CNS of wt and PKO SCID recipients, microglia and infiltrating cells provide additional recognition structures resulting in local IFN- γ secretion, thus amplifying class II expression.

In addition to demonstrating an antiviral role for CD4⁺ T cells, these results confirm their contribution to tissue pathology. Myelin loss during JHNV infection is a complex process (3). While demyelination requires viral infection of oligodendrocytes, infiltrating macrophages, and T cells (5, 14), CD8⁺ T-cell-mediated demyelination is not necessarily coupled to antiviral activity (15). This may also be true for CD4⁺ T cells. In stark contrast to SCID mice, foci of demyelination were present in all groups of SCID recipients, but most prevalent in wt CD4⁺ T-cell recipients. These data contrast with other studies using adoptive transfer approaches. In IFN- γ /perforin-deficient recipients, protection against myelin loss was afforded by donor CD4⁺ T cells, irrespective of their ability to secrete IFN- γ (42), suggesting that endogenous immune components may temper donor CD4⁺ T-cell activity. Furthermore, whereas the absence of IFN- γ partially protects against myelin loss in SCID recipients of memory CD4⁺ T cells, transfer of activated GKO CD4⁺ donor T cells into Rag^{-/-} mice increased demyelination and mortality. In contrast, Rag^{-/-} recipients of activated wt CD4⁺ T cells exhibited reduced demyelination (34). These distinct results may be due to the different genetic backgrounds between Rag^{-/-} and SCID mice, the distinct activation states and virus specificities of the transferred populations, and/or an effect by additional cell populations present in the CD4⁺ T-cell-enriched splenocytes on T-cell-mediated myelin

FIG. 8. CD4⁺ T cells clear virus from the CNS. Shown are spinal cords of infected control SCID mice and recipients of wt- and PKO-derived CD4⁺ T cells at day 14 p.i. Sections were stained with hematoxylin and eosin (HE) for inflammation, by immunoperoxidase staining with MAb J.3.3 for viral antigen, and by Luxol fast blue (LFB) for demyelination. Perivascular inflammatory infiltrates (arrowheads) and infiltrating cells (arrows) appeared more prominent in wt and PKO recipients compared to control infected SCID mice. Viral antigen was present in both gray matter and white matter in control infected SCID mice. In comparison, viral antigen, predominantly in white matter, was reduced in wt and PKO CD4⁺ T-cell recipients (arrows). Luxol fast blue stains show no demyelination in control infected SCID mice compared to prominent areas of myelin loss in wt and PKO CD4⁺ T-cell recipients. Bar, 200 μ m.

loss. However, SCID recipients of GKO CD4⁺ T cells presented more severe clinical disease, but slightly reduced demyelination, compared to the other recipient groups. Similar results were found in Rag^{-/-} recipients of activated wt CD4⁺ T cells (49). Both Rag^{-/-} recipients of wt CD4⁺ T cells and SCID recipients of GKO CD4⁺ T cells were characterized by a preferential localization of T cells and macrophages in gray matter rather than white matter. The predominant localization of macrophages and CD4⁺ T cells to white matter tracks in the wt and PKO groups suggests that the absence of IFN- γ secretion by virus-specific CD4⁺ T cells prevents focused recruitment of both CD4⁺ T cells and macrophages into the white matter.

Increased Fas-FasL expression in the CNS of SCID mice with GKO CD4⁺ T cells suggested that excessive apoptosis could be linked to the fatal disease outcome. However, no apoptotic cell increase was noted in either the gray or white matter of GKO compared to wt CD4⁺ T-cell recipients (data not shown). The other prominent factor that distinguishes recipients of GKO CD4⁺ T cells was the increase in CNS neutrophil infiltration. A crucial contribution of neutrophils to CNS pathology is supported by the association of neutrophilia with rapid mortality during experimental autoimmune encephalitis in GKO mice (44, 46). The large number of neutrophils in the CNS of GKO CD4⁺ T-cell recipients compared to other groups may thus participate in tissue damage by secreting proteases, free radicals, or proinflammatory cytokines (6, 48). Moreover, these data are consistent with the role of IFN- γ in limiting neutrophil recruitment/retention in the CNS by regulating chemokine production (44).

In summary, these data demonstrate that CD4⁺ T cells me-

diates virus clearance. The apparent IFN- γ - and perforin-independent mechanism suggests a contribution of Fas-FasL interactions. CD4⁺ T cells also contribute to myelin loss. A role of IFN- γ in macrophage localization within the CNS was revealed by decreased demyelination in GKO CD4⁺ T-cell recipients, coincident with preferential localization of CD4⁺ T cells and macrophages in the gray matter. Nevertheless, the lack of IFN- γ aggravated CNS inflammation, particularly by neutrophils, and resulted in increased mortality in SCID hosts. These data suggest that IFN- γ , by regulating chemokine production, controls the inflammatory response within the CNS during viral encephalomyelitis and also limits disease severity.

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