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CD4 and CD8 T Cells Have Redundant But Not Identical Roles in Virus-Induced Demyelination

Gregory F. Wu,* Ajai A. Dandekar, † Lecia Pewe, ‡ and Stanley Perlman* §,8

A chronic demyelinating disease results from murine infection with the neurotropic strain JHM of mouse hepatitis virus (MHV-JHM). Demyelination is largely immune mediated. In this study, the individual roles of CD4 and CD8 T cells in MHV-induced demyelination were investigated using recombinant-activating gene 1−/− (RAG1−/−) mice infected with an attenuated strain of MHV-JHM. These animals develop demyelination only after adoptive transfer of splenocytes from mice previously immunized to MHV. In this study, we show that, following adoptive transfer, virus-specific CD4 and CD8 T cells rapidly infiltrate the CNS of MHV-JHM-infected RAG1−/− mice. Adoptive transfer of CD4 T cell-enriched donors resulted in more severe clinical disease accompanied by less demyelination than was detected in the recipients of undepleted cells. Macrophage infiltration into the gray matter of CD4 T cell-enriched recipients was greater than that observed in mice receiving undepleted splenocytes. In contrast, CD8 T cell-enriched recipients developed delayed disease with extensive demyelination of the spinal cord. MHV-JHM-infected RAG1−/− mice receiving donors depleted of both CD4 and CD8 T cells did not develop demyelination. These results demonstrate that the development of demyelination following MHV infection may be initiated by either CD4 or CD8 T cells. Furthermore, they show that CD4 T cells contribute more prominently than CD8 T cells to the severity of clinical disease, and that this correlates with increased macrophage infiltration into the gray matter.


Demyelination in the CNS is the central pathological feature in humans with multiple sclerosis, rodents with experimental autoimmune encephalomyelitis, and mice infected with a variety of neurotropic viruses. In all of these pathological conditions, demyelination is believed to be in large part immune mediated, with the contributions of myelin- or virus Ag-specific T cells being paramount (1–4). However, the precise mechanisms by which immune cells either initiate or contribute to the progression of disease are not well understood. In particular, little is known about the role of T cells in demyelination after activation but before the onset of frank myelin loss. After activation, T cells enter the infected CNS, and in the case of virus-induced demyelinating diseases, rapidly induce the infiltration of macrophages and microglia into the CNS (1, 2). These cells, in turn, present Ag to T cells, release potentially cytotoxic immune factors, and remove damaged myelin, thereby amplifying the cascade of events that result in widespread demyelination (3). An important unanswered question is to what extent both CD4 and CD8 T cells are able to independently induce macrophage activation and subsequent demyelination once they have been activated.

Mice infected with the JHM strain of mouse hepatitis virus (MHV-JHM) develop acute and chronic demyelinating diseases (4–6). Although initial observations suggested that demyelination in this experimental infection was virus induced (7), recent experiments using irradiated mice or congenitally immunodeficient mice (with SCID) or with genetic disruption of the recombinase-activating gene (RAG1−/−) clearly demonstrated the requirement for host immune function in this process (1, 4, 8). Normal T and B cells do not develop in SCID and RAG1−/− mice, and inoculation with the attenuated J2.2 strain of MHV-JHM (MHV-J2.2-v1) resulted in widespread infection of the white and gray matter without evidence of demyelination. Adoptive transfer of spleen cells from mice immunized with MHV into infected RAG1−/− mice resulted in extensive demyelination at 6–7 days posttransfer (p.t.), accompanied by a large influx of activated macrophages/microglia (1).

The cellular immune response to MHV-JHM infection in C57BL/6 (B6) mice has been extensively characterized. Both CD4 and CD8 T cells are necessary for virus clearance from the CNS of MHV-infected mice (9). Two CD8 T cell epitopes have been identified and are located within the surface (S) glycoprotein of MHV at residues 510–518 (S-510–518) and 598–605 (S-598–605), the former being the immunodominant of the two (10, 11). CD4 T cell epitopes are located in the transmembrane (M) protein of MHV (M-133–147) as well as the S protein (S-333–347, S-358–372) (12).

Although the role of MHV-specific T cells has not been directly addressed, data accumulated from several studies suggest that neither CD4 nor CD8 T lymphocytes are required for demyelination. Infection of mice genetically deficient in either MHC class I (β2-microglobulin−/−) or MHC class II (Aβ−/−) expression with MHV-J2.2-v1 resulted in diminished virus clearance in all mice, with demyelination detected in only some (13). These mice are deficient in CD8 or CD4 T cells, respectively. While these results suggest that neither CD4 nor CD8 T cells are indispensable for demyelination, the variation in the amount of demyelination observed in individual animals may reflect the ability of the immune system to compensate partially for a genetic defect during development. Depletion of either CD4 or CD8 T cells several days after

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1 Abbreviations used in this paper: MHV, mouse hepatitis virus; LFB, luxol fast blue; M, MHV-JHM transmembrane protein; p.t., postinoculation; p.t., posttransfer; RAG, recombinase-activating gene; S, MHV-JHM surface glycoprotein; TMEV, Theiler’s murine encephalomyelitis virus.

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4Abbreviations used in this paper: MHV, mouse hepatitis virus; LFB, luxol fast blue; M, MHV-JHM transmembrane protein; p.t., postinoculation; p.t., posttransfer; RAG, recombinase-activating gene; S, MHV-JHM surface glycoprotein; TMEV, Theiler’s murine encephalomyelitis virus.
infection with MHV did not result in a diminution in the amount of demyelination (14). However, the conclusion that neither CD4 nor CD8 T cells are required for demyelination was recently questioned by Lane et al. (15), who showed that less demyelination developed in mice in which the CD4 gene was genetically disrupted (CD4−/−).

In this study, as an alternative approach to dissecting the requirements for different arms of the immune system in demyelination, splenocytes depleted of either CD4 or CD8 T cells were adoptively transferred into RAG1−/− mice infected with MHV-J2.2-v1. Although depletion experiments have been used widely to assess the role of different lymphocyte subsets in disease, their interpretation is generally tempered by the possibility that a small number of Ag-specific cells might remain in the depleted cell population and contribute to the pathogenic process. Recent advances in the ability to precisely measure the number of Ag-specific CD4 and CD8 T cells have made it possible to address this issue directly. Previously, we have quantified the number of MHV-specific T cells localized in the CNS by direct ex vivo assays using soluble MHC class I/peptide tetramers, IFN-γ enzyme-linked immunospot assays, and intracellular IFN-γ assays. These methods have now been used to quantify the number of MHV-specific CD4 and CD8 T cells in the initial donor splenocyte population and in the CNS of adoptive transfer recipients at the time of harvest. The adoptive transfer model, in conjunction with these methods to track T cells, provides a unique experimental system for determining the extent to which activated, MHV-specific CD4 and CD8 T cells have nonredundant roles in virus-induced demyelination.

Materials and Methods

Virus

The neuroattenuated variant of MHV-JHM, strain J2.2-v1 (16), was generously provided by Dr. J. Fleming (University of Wisconsin, Madison, WI). Virus was grown and titrated as previously described (17). Wild-type MHV-JHM was used to immunize wild-type B6 donor mice, as previously described (1).

Animals

Pathogen-free B6 mice were obtained from the National Cancer Institute (Bethesda, MD). RAG1−/− mice were obtained from The Jackson Laboratory (Bar Harbor, ME). No mature B or T lymphocytes are produced in RAG1−/− mice (18).

Adoptive transfer

RAG1−/− mice were infected with 1 × 10⁷ PFU MHV-J2.2-v1 in 30 µl by intracranial injection (8). Adoptive transfer of splenocytes from B6 mice immunized i.p. with wild-type MHV-JHM to infected RAG1−/− mice was performed as previously described. Demyelination was maximal when cells were transferred to infected RAG1−/− mice 3 days p.i. No infectious virus could be detected by plaque assay in the transferred cells (1). A total of 74 MHV-J2.2-v1-infected RAG1−/− mice was used in these experiments. A total of 23 mice received undepleted donor cells, 19 mice received CD4 T cell-enriched donor cells, 24 mice received CD4 T cell-enriched donor cells, and 5 mice received donor cells depleted of both CD4 T cell subsets. Three RAG1−/− mice infected with MHV and harvested at day 10 p.i. without cell transfer served as controls. Clinical evaluation was based on the following scoring system: 0, asymptomatic; 1, limp tail; 2, wobbly gait with righting difficulty; 3, hind-limb weakness; 4, hind-limb paralysis; 5, moribund/dead.

Antibodies

mAbs F4/80 (macrophage-specific Ab, CLA3-I; Serotec, Oxford, U.K.) and 5B188.2 (Ab to MHV-JHM nucleocapsid protein; kindly provided by Drs. J. Buchmeier, The Scripps Research Institute, La Jolla, CA) were used for immunohistochemical labeling of macrophages/microglia and virus Ag, respectively. Ab to the Fc receptor (2.4G2) and FITC-conjugated Abs to CD4 (GK1.5) and CD8 (Lyt-2) Ags were obtained as previously described (19). For depletion studies, anti-CD4 Ab RL172 and anti-CD8 Ab 3.168 were kindly provided by Dr. John Harty (University of Iowa, Iowa City, IA).

Complement depletion

Donor splenocytes depleted of CD4 or CD8 T lymphocytes were prepared for adoptive transfer by two rounds of complement lysis, as previously described (20). Greater than 99% depletion of CD4 or CD8 T cells was achieved, as assessed by FACS. No adjustment was made in the total number of donor cells delivered, since >90% of the initial cell population was recovered after depletion.

Histology

After perfusion of mice with PBS, brains and spinal cords were bisected in the midsagittal plane. Before embedding in paraffin, half of each sample was fixed in 10% normal buffered Formalin and half in Histochoice fixative (Amresco, Solon, OH). For examination of myelin and cell morphology, 8-µm sections were stained with luxol fast blue (LFB) and counterstained with hematoxylin and eosin.

Immunohistochemistry

Following hydration, 8-µm sections were permeabilized with 0.1% Triton X-100, blocked with CAS Block (Zymed Laboratories, San Francisco, CA), and incubated with primary Ab (F4/80 diluted in 1% normal goat serum at 1/200; 5B188.2 at 1/2000) overnight at 4°C. After washing, sections were incubated with biotinylated goat anti-rat (F4/80) or goat anti-mouse (5B188.2) (Jackson ImmunoResearch, West Grove, PA) diluted at 1/100 for 1 h, at room temperature. Following rinsing, avidin-HRP was applied using the ABC kit (Vector, Burlingame, CA), as directed by the manufacturer. The final substrate utilized for the staining reaction was 3,3’-diaminobenzidine (Sigma, St. Louis, MO).

Imaging

Images of stained spinal cord sections were digitalized using an Optiphot charge-coupled camera attached to a Leitz diaplan light microscope. Quantification of demyelination was done, as previously described (21), using Vtrace software (Image Analysis Facility, University of Iowa). Acquisition of all images was done at the University of Iowa Central Microscopy Research Facility.

FACS analysis

Lymphocytes were prepared from the CNS, as previously described (22), and stimulated with peptide-coated CHB3 or EL-4 cells for Ag presentation to CD4 and CD8 T cells, respectively, in the presence of monensin (Golgistop, PharMingen, San Diego, CA). Peptides corresponding to the CD4 (M-133–147; I-Aα restricted) and CD8 (S-510–518, H-2Db restricted; S-598–605, H-2Kb restricted) T cell epitopes were used at a final concentration of 5 and 1 µM, respectively. After incubation for 4 h at 37°C, cells were washed, blocked with 10% rat serum and anti-FeCyR II Ab 2.4G2 (1/200), and surface stained for CD4 and CD8 Ag (FITC-coupled mAb GK1.5 or Ly-2, respectively). Cells were fixed and permeabilized using the Cytofix/Cytoperm kit according to the manufacturer’s instructions (PharMingen), stained for intracellular IFN-γ using PE-conjugated anti-IFN-γ (PharMingen), and analyzed using a FACSscan flow cytometer (Becton Dickinson, San Jose, CA). The average number of splenocytes isolated from donor spleens following i.p. immunization was 1.4 × 10⁷ cells. Lymphocytes harvested from the brains of two to three mice were pooled together before determining the number of Ag-specific T cells. The range of lymphocytes harvested from the pooled brain populations was 1 × 10⁶ to 2 × 10⁷ (depending on time of harvest). The absolute number of Ag-specific cells was calculated by multiplying the fraction of Ag-specific cells by the fraction of CD4 or CD8 T lymphocytes by the total number of cells per brain or spleen. Negative controls were splenocytes from naive mice incubated with MHV-specific peptide.

Results

Ag-specific T lymphocytes rapidly infiltrate the CNS following adoptive transfer

Previous experiments have shown that the MHV-specific CD8 T cell response is directed at two epitopes (S-510–518 and S-598–605), whereas the CD4 T cell response is dominated by cells recognizing epitope M-133–147 (23). In the initial set of experiments, the relative proportion and temporal appearance of virus-specific CD4 and CD8 T cells in the CNS were quantified using MHV-J2.2-v1-infected RAG1−/− mice following adoptive transfer. Donor splenocytes were isolated from mice immunized i.p. with live wild-type MHV-JHM at 6 days p.i. To identify the number of
Ag-specific CD4 and CD8 T cells within the donor population, intracellular staining for IFN-γ was performed following in vitro stimulation with peptides corresponding to known CD4 and CD8 T cell epitopes, as described in Materials and Methods. Following immunization, a low percentage of T cells responding to these three epitopes was detectable in the initial spleen population (Fig. 1A). No cells responding to other CD4 T cell epitopes (S-333–347, S-358–372) could be detected in this donor cell population (data not shown). From these data, we calculated that there were $\sim1–4 \times 10^5$ cells specific for each epitope per spleen. Since $5 \times 10^6$ splenocytes were delivered to each recipient, $7 \times 10^3$ donor cells responding to M-133–147 and 1–2 $\times 10^4$ CD8 T cells specific for each CD8 T cell epitope were delivered to recipient mice (Tables I and II).

Next, lymphocytes were harvested from the CNS of adoptive transfer recipients at day 4.5 p.t., a time when animals are not yet symptomatic, and day 7 p.t., when they have developed clinical signs of hind-limb paralysis. At 4.5 days p.t., almost one-third of CD8 T cells responded to epitope S-510–518, while a significantly smaller percentage responded to epitope S-598–605 (Fig. 1B, Table II). Furthermore, $\sim20\%$ of CD4 T cells were specific for the immunodominant CD4 T cell epitope, M-133–147 (Fig. 1B, Table I). Approximately the same total number of Ag-specific CD4 or CD8 T cells was observed in the infected CNS at this time p.t. as was detected in the transferred donor population (Tables I and II). Of note, the number of CD4 T cells responding to irrelevant peptide at this time was elevated in both experiments. This response was not seen in the donor populations or in cells harvested at day 7 p.t. from the infected CNS. This result was not expected, since CD8 T cells, and presumably CD4 T cells, rapidly turn off IFN-γ production in the absence of stimulatory peptide (24). By 7 days p.t., a time when mice have developed demyelination, the frequency of epitope M-133–147-specific CD4 T cells increased, while that of epitope S-510–518-specific CD8 T cells decreased (Fig. 1C, Tables I and II). Approximately 20–25% of CD4 T cells were specific for the immunodominant CD4 T cell epitope, while nearly 20% of CD8 T cells were epitope S-510–518 specific. This shift in percentage of Ag-specific CD4 and CD8 T lymphocytes from day 4.5 to day 7 p.t. was not reflected in absolute numbers of cells, however, since on average the absolute number of MHV-specific CD8 T cells in the CNS was greater than that of CD4 T cells. Although the percentage of cells responding to epitope S-598–605 remained low, the number of cells responding to this epitope also increased from day 4.5 to day 7 p.t. These results show that a large increase in number of virus-specific CD4 and CD8 T cells correlated with the development of demyelination and clinical disease after adoptive transfer.

**FIGURE 1.** Analysis of MHV-specific lymphocytes before and after adoptive transfer. Lymphocytes were harvested from spleens 6 days after i.p. inoculation (A) and from the CNS of MHV-infected RAG1−/− mice at 4.5 (B) and 7 (C) days p.t. Cells were stained for CD4 or CD8 Ag and for IFN-γ following stimulation with the indicated MHV-specific peptides. The percentage of M-133–147-specific CD4 T cells remained constant from 4.5 to 7 days p.t., whereas that of S-510–518-specific CD8 T cells decreased during this time. Values for each time represent pooled CNS lymphocytes from three animals.
Ag-specific T cells in CD4 and CD8 T cell-enriched adoptive transfer recipients

Next, the individual roles of MHV-specific CD4 and CD8 T cells in demyelination were determined using the adoptive transfer model. For this purpose, donor cells were depleted of either CD4 or CD8 T cells or treated with complement only before adoptive transfer. Since depletion of either subset resulted in only a small decrease in total number of cells, no compensation was made in the number of cells transferred to infected recipients. Greater than 99% depletion was observed for both CD4 and CD8 T cells following complement lysis (data not shown). To verify that the depleted T cell subset was not present in the CNS of recipient mice, CNS lymphocytes were harvested and assayed for their response to MHV T cell epitopes using IFN-γ intracellular staining. At day 7 p.t., no CD8 T cells (total or MHV specific) were isolated from the CNS of mice receiving CD4 T cell-enriched splenocytes (Fig. 2). However, as expected, a large percentage of CD4 T cells responded to epitope M-133–147 (Table III). Approximately the same absolute number of MHV-specific CD4 T cells was present in the CNS of these mice as was present in the CNS of recipients of undepleted populations (compare Tables I and III).

Complementary results were obtained when lymphocytes were isolated from mice receiving CD8 T cell-enriched populations at day 7 p.t. No CD4 T cells were isolated from the CNS of MHV-infected RAG1−/− mice receiving CD8 T cell-enriched donors (Fig. 2). In comparison with recipients of undepleted splenocytes, the percentage and absolute number of S-510–518-specific CD8 T cells in CD8 T cell-enriched recipients were lower. On day 7 p.t., only 11% of CD8 T cells isolated from the CNS of CD8 T cell-enriched recipients responded to epitope S-510–518 (Table III). Furthermore, at times after day 11 p.t., the percentage of CD8 T cells producing IFN-γ in response to S-510–518 remained low, at ~10%. These results are consistent with previous reports showing that CD4 T cells are important for the trafficking, survival, or effector function of CD8 T cells in the MHV-infected CNS (25).

Clinical disease is different in recipients of CD4 and CD8 T cell-enriched donors

Although neurological disease developed in all mice following adoptive transfer, a distinct difference in clinical signs between CD4 T cell- and CD8 T cell-enriched recipients was apparent. MHV-infected RAG1−/− mice receiving CD8 T cell-enriched donor populations did not exhibit as severe neurological symptoms at day 7 p.t. as did mice receiving an equivalent number of undepleted donor cells (Table IV). On average, the appearance of hindlimb paralysis in CD8 T cell-enriched recipients was delayed 4–5 days. Some animals survived until 15 days p.t., with hindlimb paralysis as the predominant neurological sign. In contrast, CD4 T cell-enriched recipients displayed severe neurological dysfunction at 6 days p.t., often progressing to a moribund state soon after clinical symptoms developed. Although there was a noticeable variability in clinical disease between individual experiments, CD4 T cell-enriched recipients demonstrated severe neurological symptoms within each experiment (data not shown). Analysis of data from all experiments showed that CD8 T cell-enriched and undepleted adoptive transfer groups had significantly less clinical disease when compared with the CD4 T cell-enriched group (Table IV).

Recipients of CD4 and CD8 T cell-enriched populations have different amounts of demyelination

To determine whether levels of demyelination correlated with the clinical differences observed between CD4 and CD8 T cell-enriched recipients, spinal cord sections from each group were examined histologically. LFB staining of spinal cord sections from recipients of undepleted cells revealed extensive demyelination (Fig. 3A). Furthermore, immunohistochemical labeling of F4/80 for macrophages/microglia showed localization of these cells to areas of demyelination (Fig. 3B), as previously described (1). Examination of LFB-stained spinal cord sections from recipients of CD4 T cell-enriched donor cells also demonstrated focal areas of demyelination (Fig. 3C). Quantification of lesion areas throughout the spinal cords of these mice revealed significantly less demyelination than detected in recipients of undepleted populations (Table V). However, in marked contrast, spinal cords from CD4 T cell-enriched recipients exhibited increased cellularity when compared with those receiving undepleted or CD8 T cell-enriched populations. Prominent areas of perivascular cuffing and clusters of mononuclear cells were observed in both gray and white matter (Fig. 3C). Many of these cells were macrophages/microglia.

### Table I. Ag specificity of CD4 T lymphocytes before and after adoptive transfer

<table>
<thead>
<tr>
<th>No. of Expts.</th>
<th>% CD4</th>
<th>% No Peptide</th>
<th>% M-133–147</th>
<th>No. of M-133–147</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donors</td>
<td>3</td>
<td>6.73 ± 1.58b</td>
<td>0.39 ± 0.17</td>
<td>2.18 ± 1.02</td>
</tr>
<tr>
<td>Day 4.5</td>
<td>2</td>
<td>2.58</td>
<td>6.90</td>
<td>22.43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1.48–3.68)d</td>
<td>(5.70–8.10)</td>
<td>(20.36–24.50)</td>
</tr>
<tr>
<td>Day 7</td>
<td>4</td>
<td>16.72 ± 5.56</td>
<td>1.64 ± 0.33</td>
<td>24.50 ± 4.67</td>
</tr>
</tbody>
</table>

* Each group consisted of at least two animals.

b % CD4 cells represents the average percentage (±SE) of CD4 cells in the infiltrates of both the no peptide and M-133–147 groups.

c Absolute number (±SE) of virus-specific cells was calculated as described in Materials and Methods.

d Range for measurements with two experiments.

### Table II. Ag specificity of CD8 T lymphocytes before and after adoptive transfer

<table>
<thead>
<tr>
<th>No. of Expts.</th>
<th>% CD8</th>
<th>% Irrelevant Peptide</th>
<th>% S-510–518</th>
<th>No. of S-510–518</th>
<th>% S-598–605</th>
<th>No. of S-598–605</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donors</td>
<td>4*</td>
<td>10.50 ± 0.94</td>
<td>0.20 ± 0.04</td>
<td>4.21 ± 1.15</td>
<td>2.11 ± 0.47 × 10^4</td>
<td>2.16 ± 0.44</td>
</tr>
<tr>
<td>Day 4.5</td>
<td>2</td>
<td>11.80</td>
<td>1.49</td>
<td>32.34</td>
<td>1.67 × 10^4</td>
<td>3.32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(8.80–15.59)d</td>
<td>(0.30–2.67)</td>
<td>(25.67–39.00)</td>
<td>(1.31–2.03 × 10^4)</td>
<td>(2.10–4.54)</td>
</tr>
<tr>
<td>Day 7</td>
<td>4</td>
<td>41.29 ± 8.80</td>
<td>0.21 ± 0.14</td>
<td>18.67 ± 3.70</td>
<td>9.27 ± 4.67 × 10^4</td>
<td>2.36 ± 0.56</td>
</tr>
</tbody>
</table>

* Each group consisted of at least two animals.

b % CD8 cells represents the average percentage (±SE) of CD8 cells in the infiltrates of irrelevant peptide, S-510–518, and S-598–605 groups.

c Absolute number (±SE) of virus-specific cells calculated as described in Materials and Methods.

d Range for measurements with two experiments.
microglia (Fig. 3D). Most notably, there was a significantly greater infiltration of macrophages/microglia within the gray matter than was observed in mice receiving undepleted populations (compare Fig. 3, B and D). Demyelinating lesions were clearly evident in CD8 T cell-enriched recipients at day 7 p.t. (Fig. 3E), and quantification demonstrated an equivalent percentage of demyelination when compared with mice receiving undepleted cell populations (Table V). Only those animals that exhibited neurological signs at day 7 p.t. were analyzed for demyelination, to observe asymptomatic animals for longer periods of time. Although infiltration of macrophages/microglia into the white matter was detected, strikingly fewer macrophages/microglia were seen in the gray matter than in that of CD4 T cell-enriched recipients (Fig. 3F). In animals that survived more than 11 days p.t., the amount of demyelination increased, with a concomitant increase in numbers of macrophages/microglia (data not shown). These results show that clinical disease and demyelination are not strictly correlated in the recipients of CD4 and CD8 T cell-enriched splenocytes. Furthermore, the substantial increase in demyelination observed between 7 days and

**Table III.** Ag specificity of CD4 and CD8 T cells from CD4 and CD8 T cell-enriched recipients

<table>
<thead>
<tr>
<th>Enrichment</th>
<th>Ag Specificity</th>
<th>% M-133–147</th>
<th>No. of M-133–147</th>
<th>% M-133–147</th>
<th>No. of M-133–147</th>
<th>% Irrelevant Peptide</th>
<th>No. of M-133–147</th>
<th>% S-510–518</th>
<th>No. of S-510–518</th>
<th>% S-598–605</th>
<th>No. of S-598–605</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4 T cell</td>
<td>No Peptide</td>
<td>0.2</td>
<td>0.6</td>
<td>0.2</td>
<td>0.6</td>
<td>0</td>
<td>0</td>
<td>0.18</td>
<td>11.40</td>
<td>3.50</td>
<td>3.50</td>
</tr>
<tr>
<td>(Day 7)</td>
<td>M-133–147</td>
<td>3.0</td>
<td>0.4</td>
<td>3.0</td>
<td>0.7</td>
<td>1.5</td>
<td>0.18</td>
<td>11.44</td>
<td>3.51</td>
<td>3.51</td>
<td>3.51</td>
</tr>
<tr>
<td>CD8 T cell</td>
<td>Irrel. Peptide</td>
<td>0.3</td>
<td>0.9</td>
<td>0.3</td>
<td>1.8</td>
<td>0</td>
<td>0</td>
<td>0.18</td>
<td>11.40</td>
<td>3.50</td>
<td>3.50</td>
</tr>
<tr>
<td>(Day 7)</td>
<td>S-510–518</td>
<td>1.2</td>
<td>0.1</td>
<td>1.2</td>
<td>0.2</td>
<td>0</td>
<td>0</td>
<td>0.2</td>
<td>0.9</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>CD4 T cell</td>
<td>S-510–518</td>
<td>0.2</td>
<td>0.3</td>
<td>0.2</td>
<td>0.5</td>
<td>0</td>
<td>0</td>
<td>0.18</td>
<td>11.40</td>
<td>3.50</td>
<td>3.50</td>
</tr>
<tr>
<td>(Day 11)</td>
<td>S-598–605</td>
<td>1.1</td>
<td>0.1</td>
<td>1.1</td>
<td>0.2</td>
<td>0</td>
<td>0</td>
<td>0.2</td>
<td>0.9</td>
<td>0.9</td>
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</tr>
</tbody>
</table>

* Absolute number (±SE) of virus-specific cells calculated as described in Materials and Methods.
* Each group consisted of at least two animals and values represent the average of three independent experiments.
* SE.
later times p.t. in recipients of CD8 T cell-enriched populations occurred in the absence of any increase in the percentage of MHV-specific CD8 T cells in the CNS (Tables III and V).

CD4 T cell-enriched recipients are not deficient in viral clearance

Since both CD4 and CD8 T cells are necessary for effective clearance of MHV from the CNS of infected mice (9), one possible explanation for enhanced clinical disease in CD4 T cell-enriched recipients is that virus is cleared less efficiently in these mice. No difference in viral titer was observed in CD4 T cell-enriched recipients relative to recipients of undepleted splenocyte populations (Table V). Although CD8 T cell-enriched recipients had greater amounts of virus recovered from the CNS at day 7 p.t., this difference was not present at later times, and was not statistically significant. Furthermore, no difference in localization or quantity of virus Ag was detected in CD4 or CD8 T cell-enriched recipients in comparison with recipients of undepleted spleen cells (data not shown). Therefore, differences in viral load or localization do not explain the differences in clinical disease that were observed.

MHV-induced demyelination is T cell dependent

To show directly that T cells were responsible for the induction of demyelination in MHV-infected RAG1<sup>−/−</sup> mice following adoptive transfer, the donor population was depleted of both CD4 and CD8 T lymphocytes. Again, greater than 99% depletion was achieved (data not shown). In these experiments, control mice received undepleted spleen cells. Mice receiving cells depleted of both CD4 and CD8 T cell lymphocytes died of encephalitis, similar to what was observed in infected mice that did not receive transferred cells. Histological examination showed scattered areas of demyelination in the spinal cord (Fig. 4B). Macrophages/microglia were detected throughout the spinal cord, but most commonly had a spiny morphology resembling microglia, with minimal numbers of rounded macrophages seen within the white matter (Fig. 4C). Virus Ag was abundant in the gray and white matter (Fig. 4D), consistent with the encephalitis that developed in these mice. Although the amount of demyelination detected was much less than observed in recipients of undepleted or singly depleted T cells (Table V), it was greater than the amount detected in MHV-infected RAG1<sup>−/−</sup> mice in the absence of transferred cells. Demyelination is detected in only a minority of infected RAG1<sup>−/−</sup>

### Table IV. Clinical disease after adoptive transfer

<table>
<thead>
<tr>
<th>Enrichment</th>
<th>Clinical Score&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undepleted (Day 7)</td>
<td>3.21 ± 0.22 (19)</td>
</tr>
<tr>
<td>CD8 T cell (Day 7)</td>
<td>2.14 ± 0.36 (14)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD8 T cell (Day 11)</td>
<td>3.04 ± 0.39 (12)</td>
</tr>
<tr>
<td>CD4 T cell (Day 6–7)</td>
<td>4.05 ± 0.26 (19)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Clinical scores were determined as described in Materials and Methods, with number of animals in parentheses.
<sup>b</sup> Significantly less than control group receiving undepleted cells (<i>p</i> < 0.05).
<sup>c</sup> Significantly greater than all other groups (<i>p</i> < 0.05).

---

**FIGURE 3.** Demyelination and macrophage/microglia infiltration in recipients of CD4 or CD8 T cell-enriched populations. MHV-infected RAG1<sup>−/−</sup> mice received undepleted (A and B), CD4 T cell-enriched (C and D), or CD8 T cell-enriched (E and F) splenocytes from immune donors at day 3 p.i. Mice were harvested at 7 days p.i. Representative coronal sections of spinal cord were stained with LFB to detect myelin (A, C, and E). Areas of demyelination are apparent (arrowheads). Extensive cellular infiltrates are evident in CD4 T cell-enriched recipients (arrows) with perivascular cuffing (inset, arrowhead). Longitudinal sections were stained for macrophages/microglia by immunohistochemistry (B, D, and F). Infiltration by macrophages/microglia into the white matter is evident in all groups, but infiltration into the gray matter is prominent only in recipients of CD4 T cell-enriched cells (D, arrow). The number of mice analyzed in these experiments is shown in Table V. Scale bar in A = 100 μm (applies to A–D and F). Scale bar in E = 100 μm.
mice not receiving transferred cells and is always confined to the dorsal root-entry zones (Fig. 4A).

**Discussion**

Central to understanding the mechanisms of immune-mediated demyelination following infection with MHV is determining the individual contributions that CD4 and CD8 T cells make to this process. Our results demonstrate that although CD4 and CD8 T cells are independently capable of producing demyelination, distinct clinical and pathological processes can be attributed to each subset of T cells. The dissociation between demyelination and clinical disease is clearly evident since the recipients of CD4 T cell-enriched donor cells developed more severe clinical disease with significantly less demyelination, while the reverse was true for recipients of CD8 T cell-enriched splenocytes.

These results, showing that neither CD4 nor CD8 T cells are required for demyelination, are in general agreement with a previous study using mice congenitally deficient in CD4 or CD8 T cell function (13). In contrast, Lane et al. (15) recently showed that CD4 T cells are critical for neurological impairment, inflammation, and demyelination in MHV-infected mice. Our results do not necessarily disagree with the data in that report. The adoptive transfer system inherently emphasizes the effector rather than the initiation phase of the MHV-specific immune response. Both CD4 and CD8 T cells were activated before transfer since they were harvested from B6 mice immunized to MHV 6 days previously. In contrast, much of the diminished disease in CD4−/− mice reported by Lane et al. was dependent on the delayed induction of inflammation by CD4 T cells, particularly by the expression of the chemokine, RANTES. In agreement with Lane et al., CD4 T cells very efficiently propagated the pathological process in MHV-infected mice and, in the absence of CD8 T cells, induced the infiltration of large numbers of macrophages into both the gray and white matter (Fig. 3D). The extensive infiltration of macrophages and microglia into the gray matter of these mice may explain the propensity for more severe disease.

Our results show that 15–30% of CD4 and CD8 T cells in the CNS are MHV specific after adoptive transfer. Previous assessment of Ag specificity during MHV infection using soluble MHC class I peptide tetramers revealed a large percentage of virus-specific CD8 T cells during acute and chronic disease, with one-third to one-half of these cells recognizing epitope S-510–518 (22, 26). The kinetics of appearance of MHV-specific CD8 T cells in the CNS are likely to be more rapid than in the natural infection in B6 mice, reflecting both the preexisting viral infection and the fact that these cells are already activated. Between 4.5 and 7 days p.t., during the development of neurological disease and demyelination, this number increases 5-fold, suggesting a rapid recruitment of Ag-specific cells to the infected CNS. MHV-specific CD4 T cells are also rapidly recruited to the CNS. Like MHV-specific CD8 T cells in the CNS of B6 mice with acute encephalitis, MHV-specific CD4 T cells also have an activated phenotype, enhancing their

**FIGURE 4.** Depletion of both CD4 and CD8 T cells before adoptive transfer results in greatly diminished demyelination. Spinal cord sections from MHV-infected RAG1−/− mice were stained for myelin with LFB (A and B), and for macrophages/microglia (A, inset; C) and virus Ag (D). MHV-infected RAG1−/− mice not receiving donor cells do not develop significant amounts of demyelination. Only minor degradation of myelin is detected in the dorsal root entry zone (A), in combination with a delimited infiltrate of rounded, activated macrophages (A, inset). White matter tracks in the ventral spinal cord contain a small portion of demyelination in a mouse receiving splenocytes depleted of both CD4 and CD8 T cells (B). Numerous ramified microglia (C) and MHV-infected cells (D) were detected in both the gray and white matter. The number of mice analyzed in these experiments is shown in Table V. Scale bars = 100 μm.

### Table V. Demyelination and viral titers in adoptive transfer recipients

<table>
<thead>
<tr>
<th>Enrichment Group</th>
<th>% Demyelination*</th>
<th>No. of Samples with Detectable Virus</th>
<th>Titer (log_{10}PFU/g of tissue ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undepleted (Day 7 p.t.)</td>
<td>15.9 ± 1.9 (13)</td>
<td>6/10</td>
<td>3.28 ± 0.63</td>
</tr>
<tr>
<td>CD4 T cell (Day 6–7 p.t.)</td>
<td>10.2 ± 0.6 (9)*</td>
<td>7/8</td>
<td>3.86 ± 0.75</td>
</tr>
<tr>
<td>CD8 T cell (Day 7 p.t.)</td>
<td>15.4 ± 3.2 (7)</td>
<td>5/5</td>
<td>4.42 ± 0.78</td>
</tr>
<tr>
<td>CD8 T cell (&gt;Day 11 p.t.)</td>
<td>25.4 ± 6.1 (4)</td>
<td>6/6</td>
<td>3.41 ± 0.48</td>
</tr>
<tr>
<td>CD4 and CD8 T cell-depleted</td>
<td>1.8 ± 0.3 (5)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Percent demyelination determined as described in Materials and Methods with number of animals in parentheses.

* Significantly lower than undepleted cell recipients (p < 0.05).
trafficking to the CNS (J. Haring, L. Pewe, and S. Perlman, unpublished data).

Following depletion of CD4 T cells from the donor cell population, the percentage of S-510-518-specific CD8 T cells within the infected CNS was decreased relative to recipients of undepleted cells analyzed in parallel. This reduction most likely reflects the requirement for CD4 T cell help for effective trafficking and/or survival of CD8 T cells within the CNS, as described previously (25). This diminished number of MHV-specific CD8 T cells did not affect the demyelinating process since a large increase in demyelination was seen at later time points in these mice. These results suggest that demyelination is induced when the number of MHV-specific CD8 T cells crosses a certain threshold, and that additional numbers of CD8 T cells do not enhance the process.

In another model of CNS demyelination, that induced by Theiler’s murine encephalomyelitis virus (TMEV), depletion of CD4 or CD8 T cells on a resistant background results in decreased virus clearance and increased demyelination. More relevant to the present study are analyses of the role of CD4 and CD8 T cells in mouse strains susceptible to TMEV-induced demyelination (27). In these strains, genetic deletion of CD4 T cells results in increases in demyelination and neurological disease, whereas mice lacking CD8 T cell function develop a significant amount of demyelination with minimal clinical disease after infection with TMEV. Thus, in both MHV- and TMEV-infected mice, CD4 and CD8 T cells are able to initiate demyelination. Additionally, in both cases, the absence of CD8 T cells results in less demyelination than observed in the absence of CD4 T cells. In TMEV-infected mice, a relative paucity of symptoms is observed in the absence of CD8 T cells, even in the presence of significant demyelination. In marked contrast, depletion of CD8 T cells results in an increase in clinical severity in MHV-infected mice (Table IV). The lack of clinical disease in TMEV-infected CD8⁻/⁻ mice has been attributed to maintenance of axonal integrity (28). Axonal dysfunction is detected in MHV-infected mice, but does not appear to explain the more severe disease observed in recipients of CD4 T cell-enriched populations.⁴

CD4 and CD8 T cells may contribute to demyelination by the same or differing mechanisms. However, once the pathogenic process has begun, the pathway to MHV-induced demyelination appears to be redundant, with no single effector molecule being essential for demyelination. TNF-α, IFN-γ, and NO are not required for MHV-induced demyelination (29–32). Furthermore, neither Fas/Fas ligand interactions nor perforin-mediated cytolysis are necessary for this process (33, 34). Consistent with these results, we have observed no decrease in demyelination in infected RAG1⁻/⁻ mice after adoptive transfer of donor cells from perforin⁻/⁻ mice immunized with MHV-JHM (data not shown).

Our results clearly show that T cells are most efficient at propagating the demyelinating process. However, cells that are not thymus derived may substitute in this process since nude rodents infected with MHV also develop demyelination (13). B cells are present in nude rodents, but in the present study, infusion of splenocytes depleted of T cells but not B cells resulted in only a small amount of demyelination. B cells appear to contribute to clinical disease in rats acutely and chronically infected with MHV (35, 36). Although B cells may not be important for initiation of demyelination, they may potentiate the process after initiation by Ag-specific CD4 or CD8 T cells.

A striking difference between recipients of CD4 T cell-enriched splenocytes and those receiving either undepleted or CD8 T cell-enriched populations is the increase in severity of clinical disease. Although the basis for this difference is not known with certainty, the number of macrophages in the gray matter of the CD4 T cell-enriched recipients is markedly increased compared with the other groups. It is clear from our studies and the reports of others that macrophages are key effector cells in the demyelinating process (3, 21, 37, 38). Our results show that either Ag-specific CD4 or CD8 T cells are sufficient for macrophage activation and subsequent demyelination to occur. Future studies directed at determining the precise mechanisms used by MHV-specific T cells to activate macrophages and those involved in destruction of myelin by these macrophages will be key to understanding virus-induced demyelination.

Acknowledgments

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References


