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A Pathogenic Role for CD8$^+$ T Cells in a Spontaneous Model of Demyelinating Disease$^1$

Marcel Brisebois,* Simone P. Zehntner, †José Estrada,* Trevor Owens, † and Sylvie Fournier$^2*$

Transgenic (Tg) mice that overexpress the costimulatory ligand B7.2/CD86 on microglia spontaneously develop a T cell-mediated demyelinating disease. Characterization of the inflammatory infiltrates in the nervous tissue revealed a predominance of CD8$^+$ T cells, suggesting a prominent role of this T cell subset in the pathology. In this study, we show that the same neurological disease occurred in Tg mice deficient in the generation of CD4$^+$ T cells, with an earlier time of onset. Analysis of the CD8$^+$ T cell repertoire at early stage of disease revealed the presence of selected clonal expansions in the CNS but not in peripheral lymphoid organs. We further show that Tg animals deficient in IFN-γ receptor expression were completely resistant to disease development. Microglia activation that is an early event in disease development is IFN-γ-dependent and thus appears as a key element in disease pathogenesis. Collectively, our data indicate that the spontaneous demyelinating disease in this animal model occurs as a consequence of an inflammatory response initiated through the activation of CNS-specific CD8$^+$ T cells by Tg expression of B7.2 within the target organ. Thus, autoreactive CD8$^+$ T cells can contribute directly to the pathogenesis of neuroinflammatory diseases such as multiple sclerosis. The Journal of Immunology, 2006, 177: 2403–2411.

M ultiple sclerosis (MS)$^3$ is an inflammatory disease of the CNS that leads to demyelination and axonal damage, resulting in permanent neurological deficits. The etiology of MS is unknown but autoreactive T lymphocytes are considered to play a pivotal role in disease pathogenesis (1). Consistent with this hypothesis, experimental autoimmune encephalomyelitis (EAE), an animal model that exhibits several clinical and pathological features found in MS patients, can be induced in rodents upon transfer of CD4$^+$ T cell lines specific for various myelin Ags (2–4). Most studies in MS and EAE have consequently focused on the contribution of CD4$^+$ T cells to immune-mediated nervous tissue injury. However, several indirect observations suggest that CD8$^+$ T cells could also be involved in the pathogenesis of MS. Myelin-specific MHC class I-restricted CD8$^+$ T cells can be derived from MS patients and healthy individuals (5–8). Cytokine-producing CD8$^+$ T cells were shown to directly correlate with the development of magnetic resonance imaging features of demyelination and axonal loss (9). Oligoclonal expansions of CD8$^+$ T cells have been repeatedly detected in the blood and/or cerebrospinal fluid of MS patients (10–12). In active MS brain lesions, CD8$^+$ T cells were shown to predominate over CD4$^+$ T cells and to have undergone clonal expansion presumably in situ (13). However, the function and antigenic specificity of the clonally expanded CD8$^+$ T cells in MS patients are still unknown. A pathogenic role for CD8$^+$ T cells has been suggested in other human autoimmune diseases of the nervous tissue such as paraneoplastic neurological syndromes, Rasmussen’s encephalomyelitis and Guillain-Barré syndrome (14–18).

The functional role of CD8$^+$ T cells in brain inflammation has been investigated using the EAE model. It has been shown that CD8$^+$ T cells may have beneficial effects and down-regulate inflammation in the CNS (19–23). In contrast, studies in CD4$^+$ or CD8$^+$ gene-deficient mice indicated that CD8$^+$ T cells might govern the extent of CNS demyelination during the effector phase of the disease (24). Furthermore, adoptive transfer of freshly activated myelin-specific CD8$^+$ T cell lines has been shown to induce CNS pathology in mice (25–27), suggesting that CD8$^+$ T cells may have a direct role in nervous tissue destruction. However, little is known about the potential of CD8$^+$ T cells to induce demyelinating disease in situations that do not require in vitro activation of cells. Moreover, the choice of the neuroantigen in these animal models is deliberate, and may not reflect the natural CD8$^+$ T cell repertoire involved in a spontaneous autoimmune disease such as MS.

We recently reported that transgenic (Tg) mice constitutively expressing the T cell costimulatory ligand B7.2/CD86 on microglia (herein referred to as B7.2 Tg mice), spontaneously develop neurological symptoms and demyelinating lesions in the spinal cord (28). The CNS pathology in this animal model is driven by T lymphocytes as B7.2 Tg mice deficient in the generation of TCRβ$^+$ T cells were resistant to disease development. Tg expression of B7.2 by resident APCs of the nervous tissue is a key determinant of disease induction. Tg T cells induced disease when adoptively transferred into T cell-deficient B7.2 Tg recipients but not into non-Tg recipients (28), showing that T cell activation within the CNS by B7.2-expressing microglia is a prerequisite for disease development. Interestingly, characterization of CNS-infiltrating T lymphocytes revealed a dramatic preponderance of CD8$^+$ T cells that correlated with the symptomatic state, suggesting an active role for this T cell subset in disease development.

In this study, we provide evidence that the demyelinating disease in B7.2 Tg mice is mediated by the activation of Ag-specific CD8$^+$ T cells within the CNS and that IFN-γ receptor signaling is a key element in disease pathogenesis. This novel animal model for

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$^5$Abbreviations used in this paper: MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; Tg, transgenic.
spontaneous incidence of CD8+ T cell-mediated demyelinating disease provides a unique tool to define the specificities of CD8+ T cells participating in CNS autoimmune responses and to dissect their role in the pathogenesis of autoimmune demyelinating diseases.

Materials and Methods

**Mice**

B7.2 Tg lines 31 and 27 have been described previously (28, 29) and were maintained by successive backcross to C57BL/6 mice. Transgene-negative littermates were used as controls in all experiments. I-Ab−/−, CD4−/−, IFN-γ−/−, and OT-I TCR Tg mice were purchased from The Jackson Laboratory. All procedures were in accordance with guidelines of the Canadian Council on Animal Care, as approved by the Animal Care Committee of McGill University. Initial clinical symptoms are defined as hind limb clamping while full-blown neurological symptoms also include uncoordinated hind-limb spaying and poor proprioception when walking on cage bars, as described previously (28).

**Immunohistochemistry**

Spinal cord sections were stained as previously described (28). Following blocking, sections were incubated with the SMI-32 mAb (Sternberger Monoclonals) or the RIP Ab (Chemicon International), washed, and incubated with goat anti-rat IgG conjugated to Cy3 (Jackson Immunoresearch Laboratories). Sections stained with the RIP Ab were counterstained with Hoechst (Molecular Probes).

**Flow cytometry**

Single-cell suspensions of the CNS and lymphoid tissue were prepared as described previously (28, 29). Flow cytometry was performed on a FACScan (BD Immunocytometry Systems). Data were collected with live gating on 10,000 cellular events and analyzed using CellQuest software. The following mAbs were used: biotin-conjugated anti-CD4 (IM7), anti-CD62L (MEI-14), anti-CD4 (GK1.5), anti-H-2Kk (AF6-88.5), anti-I-Ak (KH74), and anti-CD40 (3/23); PE-conjugated anti-CD68 (53-6.7), anti-B7.1 (RMMP-1), anti-B7.2 (RMMP-2); FITC-conjugated anti-CD4 (GK1.5) and anti-CD11b (M1/70.15); PE-Cy5-conjugated anti-CD45 (30-F11). All mAbs were purchased from BD Biosciences except anti-B7.1 and anti-B7.2 mAbs (Cedarlane Laboratories).

**RT-PCR and primary PCR amplification**

Total RNA was isolated using TRIZol reagent (Invitrogen Life Technologies) and 5 µg of RNA was converted to cDNA using Superscript II Reverse Transcriptase (Invitrogen Life Technologies) according to the manufacturer's instructions. Saturating PCR amplification of cDNA was performed as follows: 5 µl of cDNA was incubated with 2.5 µl of 10× PCR buffer, 1 µl of 50 mM MgCl2, 1 µl of dNTPs (6.25 mM), 1 µl of sense sequence primer of the individual Vβ (25 µM): Vβ1, -2, -3, -14, -5, -1, -2, -6, -7, -8, -9, -10, -11, -12, -13, -14, -15, -16, -17, -18, -20 (30), 1 µl of a common Cβ antisense primer (25 µM) (30). A 0.2 µl (0.2 U) of Taq polymerase and water to a final volume of 25 µl. Amplification program: 94°C for 5 min, 60 cycles of 45 s at 94°C, 45 s at 60°C, 1 min at 72°C, and a final extension at 72°C for 10 min.

**Run-off reaction and CDR3 spectratyping**

Using the same amplification program, 5 µl of each primary PCR product were subjected to three cycles of a run-off reaction with a 32P end-labeled common Cβ antisense primer. Radioactive PCR products were mixed with equal volumes of formamide denaturing buffer and heated at 94°C for 5 min. Eight microliters of the samples were loaded onto a prewarmed 6% acrylamide sequencing gel and were analyzed with the NIH Image software 1.62 after exposure to Imaging Screen K (Bio-Rad).

**Cloning and sequencing of PCR products**

The PCR products amplified with the Vβ-Cβ pairs of interest were directly ligated in TOPO vector and transformed in E. coli using the TOPO TA Cloning kit (Invitrogen Life Technologies). The plasmid DNA was isolated using QIAprep Spin Miniprep columns (Qiagen) followed by insert sequencing using M13 reverse primers.

Results

**Deficiency in CD4+ T cells does not prevent disease development in B7.2 Tg mice**

To examine the contribution of the CD4+ T cell subset to disease development, we produced B7.2 Tg mice deficient in the generation of CD4+ T cells by crossing them with mice carrying a targeted deletion of the gene encoding the β-chain of the MHC class II molecule I-A (I-Ab−/− mice) (31). B7.2 Tg mice are on the C57BL/6 background, in which expression of the MHC class II molecule I-E is defective. Consequently, I-Ab−/−B7.2 Tg mice have a profound defect in differentiation to the CD4+ T cell lineage in the thymus and only 3–4% of CD4+ T cells are found in the peripheral lymph nodes of these mice (data not shown).

Interestingly, I-Ab−/− B7.2 Tg mice exhibited identical neurological symptoms to those of I-Ab−/− B7.2 Tg. Mice showed clamping of the hind limbs when lifted by the tail, hind-limb weakness, difficulty to right when overturned, weak tail movement, uncoordinated hind-limb spaying, and poor proprioception when walking over cage bars. In both strains of B7.2 Tg mice, disease was progressive and resulted in death unless mice were euthanized. However, disease onset was significantly accelerated in I-Ab−/− B7.2 Tg mice compared with B7.2 Tg mice having a full complement of CD4+ T cells (Fig. 1). In I-Ab−/− B7.2 Tg mice (n = 23), neurological symptoms appeared at the age of 59 ± 10 days whereas I-Ab−/− B7.2 Tg animals (n = 31) displayed the first clinical signs at 133 ± 26 days (p < 0.001). In addition, deficiency in CD4+ T cells shortened the time span by which 100% of animals exhibited neurological symptoms (Fig. 1). All I-Ab−/− B7.2 Tg mice developed clinical symptoms within a 10-wk period while clinical manifestations of disease followed a much broader distribution in animals having a full complement of CD4+ T cells (Fig. 1). That the effect observed on disease development in I-Ab−/− B7.2 Tg mice was due to a deficiency in the generation of CD4+ T cells was confirmed by the fact that CD4 gene-deleted B7.2 Tg mice showed the same accelerated disease onset (66 ± 20 days, n = 20) compared with control B7.2 Tg animals (Fig. 1).

Flow cytometry analysis of spinal cord cell suspensions revealed that the CNS infiltrate in both I-Ab−/− B7.2 Tg mice exhibited predominantly of lymphocytes (CD45high) with a paucity of CD45−CD11bhigh macrophages (Fig. 2A). As previously reported and shown in Fig. 2B, the lymphocytes found in the CNS of I-Ab−/− B7.2 Tg mice were predominantly CD8+ T cells. The number of CD8+ T cells was significantly higher in I-Ab−/− B7.2 Tg mice compared with age-matched Tg animals having a full complement of CD4+ T cells (Fig. 2B). Furthermore, the increased expansion/accumulation of CD8+ T cells in the CNS occurred rapidly in I-Ab−/− B7.2 Tg mice, between 4 and 6 wk of age, while it was more progressive in control B7.2 Tg mice (Fig. 2B).
We previously showed that the neurological disease in B7.2 Tg mice was associated with loss of myelin figures in spinal cord parenchyma (28). Therefore, it was of interest to determine whether demyelination could also be observed in symptomatic B7.2 Tg mice which were deficient in CD4⁺ T cells. Immunostaining of frozen spinal cord sections with the RIP mAb, which labels the cell body and processes of mature oligodendrocytes (32), revealed extensive loss of RIP-positive cells in the spinal cord of B7.2 Tg mice (Fig. 2A). Staining of frozen spinal cord sections with the RIP mAb, anti-CD4, and anti-CD8 (B) mAbs and analyzed by flow cytometry. As shown in Fig. 2B, the frequency of CD8⁺ T cells in the CNS of age-matched OT-I B7.2 Tg was very low and comparable to that found in OT-I TCR Tg wild-type littersmates. Because CNS-specific T cells do not exist in OT-I mice, these data suggest that a CNS-specific CD8⁺ T cell response is involved in the expansion/accumulation of CD8⁺ T cells in the CNS of B7.2 Tg mice and the spontaneous development of neurological symptoms.

Oligoclonal expansions of CD8⁺ T cells in the CNS of I-Aβ⁻/⁻ B7.2 Tg mice at early stage of disease

To have a better understanding of the nature of the CD8⁺ T cell expansion/accumulation in the CNS of I-Aβ⁻/⁻ B7.2 Tg mice, we analyzed the CDR3 length distribution of the Vβ chain TCR transcripts from the spinal cord and brain tissues directly ex vivo. If specific Ags were involved in the expansion of CD8⁺ T cells in the CNS, we reasoned that this would be reflected as a skewed distribution of CDR3 lengths. Conversely, a Gaussian distribution of CDR3 lengths, reflecting a polyclonal TCR repertoire, would indicate that the accumulation of CD8⁺ T cells in the CNS of these mice occurs independently of specific Ag recognition. We performed CDR3 length distribution analysis for 21 Vβ TCR transcripts from the spinal cord cDNA of preclinical mice, mice at the disease development

To examine whether the development of the CD8⁺ T cell-mediated CNS pathology in B7.2 Tg mice required antigenic stimulation provided by specific environmental and/or self-Ags, we restricted the CD8⁺ T cell repertoire of B7.2 Tg mice by crossing them with a TCR Tg line. We choose the OT-I TCR Tg line because nearly all (~95%) of the CD8⁺ T cells in this line are clonotype positive (Vo₂⁺Vβ⁺) (35). The frequency of peripheral CD8⁺ T cell coexpressing the Tg Vo₂⁺Vβ⁺ TCR was similar in B7.2 Tg and control animals (Fig. 3A). In contrast to B7.2 Tg animals, which all developed neurological symptoms at 4–8 mo of age, none of the OT-I B7.2 Tg mice monitored for up to 12 mo (n = 12) exhibited any neurological dysfunction (Fig. 1). We harvested brain and spinal cord tissues of 5-mo-old perfused animals, an age at which B7.2 Tg mice have extensive infiltration of CD8⁺ T cells in the CNS, and analyzed the frequency of T cells by flow cytometry. As shown in Fig. 3B, the frequency of CD8⁺ T cells in the CNS of age-matched OT-I B7.2 Tg was very low and comparable to that found in OT-I TCR Tg wild-type littersmates. Because CNS-specific T cells do not exist in OT-I mice, these data suggest that a CNS-specific CD8⁺ T cell response is involved in the expansion/accumulation of CD8⁺ T cells in the CNS of B7.2 Tg mice and the spontaneous development of neurological symptoms.
early stage of disease (7 wk of age) and of animals displaying full-blown neurological symptoms (10 wk of age). Pooled lymph node cell suspensions were prepared from the same animals to compare the CNS infiltrating and peripheral CD8+ T cell repertoire. Spectratypes from individual representative animals are shown in Fig. 4 and the results are summarized in Table I.

CDR3 spectratype analysis of CNS-infiltrating CD8+ T cells from preclinical and symptomatic 7-wk-old I-\(\text{A}\)^\(-/-\) B7.2 Tg mice indicated that several V\(\beta\) families showed CDR3 spectratypes with either single peaks or clearly skewed distributions (Fig. 4B and Table I). The percentage of V\(\beta\) families with altered CDR3 length distribution was similar in preclinical animals and in mice at early stage of disease. Some of the V\(\beta\) families with altered CDR3 length distribution, such as V\(\beta\)11 and V\(\beta\)10, were detected in several individuals (data not shown). However, no consistent altered V\(\beta\) patterns were observed across all animals. In contrast, peripheral CD8+ T cells from the same animals displayed a Gaussian distribution for all V\(\beta\) families, which was identical with that observed in control animals and therefore typical of a diverse and unbiased TCR repertoire (Fig. 4A and data not shown). These data indicate Ag-driven expansion of selected CD8+ T cell clones in the CNS of I-\(\text{A}\)^\(-/-\) B7.2 Tg mice during the early stage of disease.

At 10 wk of age, the percentage of V\(\beta\) families in the CNS exhibiting a Gaussian CDR3 length distribution was significantly higher (\(p<0.01\)) compared with 7-wk-old animals (Table I and Fig. 4C). More than 70% of the V\(\beta\) families showed a Gaussian distribution of CDR3 lengths (Table I). These data indicate that a more diversified and polyclonal T cell repertoire is present in the CNS of I-\(\text{A}\)^\(-/-\) B7.2 Tg mice with overt disease and/or that the focus of T cell activation has narrowed at this stage of the disease.

To determine whether the V\(\beta\) families that exhibited spectratypes with biased CDR3 length distribution in 7-wk-old animals corresponded to oligoclonal populations, we performed sequence analysis of cloned cDNA derived from selected V\(\beta\) families with biased CDR3 length distribution or Gaussian distribution. The data obtained for five animals are shown in Table II. The predominance of a particular clonotype was observed for each of the V\(\beta\) families exhibiting a biased CDR3 length distribution. As a striking example, in one mouse (M1) a single clonotype accounted for 81% (26 of 32) of all V\(\beta\)6 cDNA clones (Table II). Other mice showed overrepresented clonotypes, ranging from 16 to 53% of the PCR-amplified cDNA clones sequenced. In contrast, all the cDNA clones sequenced from TCR V\(\beta\) families that exhibited a Gaussian distribution in the CNS showed unique CDR3 sequences with the same size. From these data, we conclude that the skewed CDR3 length distribution of particular TCR V\(\beta\) families observed at early stage of disease reflects expansion of selected T cell clones within the CNS.

**Microglia activation is an early event in disease development**

Given the key role that activated microglia play in CD4+ T cell-mediated EAE (36), we investigated whether microglial activation was also observed in our model of CD8+ T cell-mediated demyelinating disease. Activated microglia can be identified through up-regulation of MHC expression and other cell surface molecules (37). We found that expression of MHC, CD40, and B7.1/CD80 was significantly up-regulated on CD45lowCD11bhigh microglia in I-\(\text{A}\)^\(-/-\) (data not shown) and I-\(\text{A}\)^\(+/-\) B7.2 Tg mice (Fig. 5A) with overt disease. To determine whether microglia activation was merely a consequence of the massive influx of CD8+ T cells that occurs in the CNS of animals with overt disease, we assessed expression of these cell surface markers on microglia isolated from young (3-wk-old) B7.2 Tg animals that had no clinical signs and fewer infiltrating CNS T cells (Fig. 5B). Interestingly, levels of MHC class I expression on CD45\(^{\text{low}}\)CD11b\(^{\text{high}}\) microglial cells in these young preclinical animals were as high as those observed in symptomatic mice (Fig. 5B). Expression of B7.1 and CD40 was also up-regulated on microglia isolated from young preclinical animals compared with control animals but to a lesser extent than in symptomatic animals (Fig. 5B).

Because in vitro studies have reported that activated T cells can promote microglia activation in absence of Ags (38), it was possible that the activation status of microglia was due to a non-specific increased frequency of memory T cells in the CNS of these young preclinical animals. To test this possibility, we analyzed MHC class I expression on microglia in an independent B7.2 Tg mouse line (line 27). We have shown previously that, in contrast to
the B7.2 Tg mouse line studied in this report (line 31), line 27 mice do not constitutively express B7.2 on microglia and do not develop disease over time (28). However, line 27 mice have an increased frequency of activated/memory T cells in peripheral lymphoid organs and harbor an increased proportion of memory T cells in the nervous tissue compared with wild-type mice (28, 29). As shown in Fig. 5C, MHC expression on microglia was not up-regulated in young preclinical B7.2 Tg mice. Thus, the increased expression of MHC class I or class II in spleen and lymph nodes of preclinical or symptomatic B7.2 Tg mice was not up-regulated (data not shown). Together, these data indicate that microglia activation is an early event in disease pathogenesis and occurs as a consequence of a CNS-specific CD8\(^{+}\) T cell response driven by Tg expression of B7.2 within the target organ.

Expression of MHC class I or class II in spleen and lymph nodes cells of preclinical or symptomatic B7.2 Tg mice was not up-regulated (data not shown). Together, these data indicate that microglia activation is an early event in disease pathogenesis and occurs as a consequence of a CNS-specific CD8\(^{+}\) T cell response driven by Tg expression of B7.2 within the target organ.

**IFN-\(\gamma\) receptor signaling is required for disease development in B7.2 Tg mice**

We previously reported that a much higher percentage of CD8\(^{+}\) T cells produced IFN-\(\gamma\) in the CNS of preclinical and symptomatic B7.2 Tg mice in comparison to peripheral organs (28). To determine whether responsiveness to this cytokine was a critical element in the pathogenesis of the CD8\(^{+}\)-mediated demyelinating disease, we assessed disease development in B7.2 Tg mice deficient in the expression of the IFN-\(\gamma\) receptor (IFN-\(\gamma\)R\(^{-/-}\) B7.2 Tg). Remarkably, none of the IFN-\(\gamma\)R\(^{-/-}\) B7.2 Tg mice (\(n = 10\)) exhibited clinical manifestations of the neurological disease over a period of 9 mo. Flow cytometric analysis of CNS mononuclear cells revealed that the frequency of CNS-infiltrating T cells was significantly reduced in IFN-\(\gamma\)R\(^{-/-}\) B7.2 Tg mice when compared with IFN-\(\gamma\)R\(^{-/-}\) B7.2 Tg littermates (\(n = 4\)) (Fig. 6A). The few T cells residing in the CNS of IFN-\(\gamma\)R\(^{-/-}\) B7.2 Tg mice were dominated by the CD8\(^{+}\)CD44\(^{high}\) T cell subset (Fig. 6B). It has been shown that activated/memory T cells have an increased

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**Table II. CDR3 amino acid sequences of TCR\(\beta\) chain transcripts derived from CNS-infiltrating T cells of I-A\(\beta\)-/ B7.2 Tg mice**

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<th>I(\beta)</th>
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*Number of times sequence found per total number of clones sequenced.

*Unique sequences.
An increased frequency of memory phenotype CD8⁺/H11001 and CD4⁺/H11001 T cells in lymphoid tissues of B7.2 Tg mice has been shown to result from transgene-derived expression of B7.2 on T cells that provides a costimulatory signal (29, 40). To determine whether the reduced frequency of CD8⁺/H11001 T cells in the CNS of IFN-γ/H9253⁻/H11002/⁻/H11002 B7.2 Tg mice could be due to a decreased frequency of activated/memory CD8⁺/H11001 T cells in the periphery, we assessed expression of various activation markers on CD8⁺/H11001 T cells isolated from spleen and

FIGURE 5. Microglia activation occurs early in disease development. H2-Kb, I-Ab, CD40, or B7.1 expression on CD11b⁺/H11001 CD45lo-gated microglia from the spinal cord of symptomatic B7.2 Tg mice (A), 3-wk-old B7.2 Tg animals (B), 3-mo-old line 27 mice (C), Rag1⁻/⁻ (D), and OT-I TCR (E) B7.2 Tg animals. Shaded histograms in A–C represent background stainings of CD11b⁺/H11001 CD45lo-gated microglia using isotype-matched control Abs. Shaded histograms in D and E represent stainings of non-Tg littermates. Dot plots in B and C represent CD4 and CD8 expression on gated lymphocytes from the CNS of 3-wk-old B7.2 Tg animals and non-Tg littermates (B, upper panels) and 3-mo-old line 27 mice (C, left panels). One representative experiment of three to six is shown.

FIGURE 6. B7.2 Tg mice deficient in IFN-γ signaling are resistant to disease development. Frequency of T cells in cellular isolates from the spinal cord of 5-mo-old IFN-γR⁻/⁻ mice (n = 5), IFN-γR⁻/⁻ × B7.2 Tg mice (n = 5), and IFN-γR⁻/⁻ × B7.2 Tg littermates (n = 4) (A). Expression of CD44 by CD8⁺ T cells from the CNS (B, upper panels) and spleen (B, lower panels). H2-Kb and I-Aβ expression on CD11b⁺/CD45lo-gated microglia from the spinal cord of B7.2 Tg animals (C). Shaded histograms represent staining of CD11b⁺/CD45lo-gated microglia from non-Tg littermates.
of regulatory CD4+ T cells in mice. As expected and shown in Fig. 6B, the frequency of CD8+ T cells expressing high levels of CD44 was as markedly increased in peripheral lymphoid organs of IFN-γR−/−/B7.2 Tg mice as in the IFN-γR−/− mice described previously. Importantly, in the absence of IFN-γ signaling, the frequency of CD8+ CD44high T cells remained elevated in spleen and lymph node equivalent to that in IFN-γR−/−/B7.2 Tg mice (Fig. 6B). We also did not observe any difference between peripheral CD8+ T cells from IFN-γR−/− and IFN-γR−/−/B7.2 Tg mice in the expression of other activation/memory markers (CD69, CD62-L, CD43, and LFA-1) (data not shown). Thus, the difference in the extent of expansion/accumulation of CD8+ T cells in the CNS of IFN-γR−/− and IFN-γR−/−/B7.2 Tg mice cannot be attributed to a disparity in the peripheral population.

To establish whether IFN-γ could play a role in the activation of microglia, we assessed MHC class I expression on CD45+Mac-1+ microglial cells in IFN-γR−/− and IFN-γR−/−/B7.2 Tg mice. As shown in Fig. 6C, absence of IFN-γ signaling completely abolished up-regulation of MHC expression by this cell type. These data establish that IFN-γ signaling is required for microglia activation and is a crucial element in the pathogenesis of this CD8+ T cell-mediated demyelinating disease model.

**Discussion**

We previously demonstrated that constitutive expression of the costimulatory ligand B7.2/CD86 on microglia drives the spontaneous development of a T cell-mediated demyelinating disease (28). In this report, we have shown that CD8+ T cells represent the primary effector T cell subset in the pathogenesis of the neurological disease in this experimental model. Clonal dominance of CD8+ T cells in the CNS, which was independent of the peripheral repertoire, was observed at the early stage of disease. The CD8+ T cell-mediated neurological disease was associated with early activation of microglia and showed a complete dependence on IFN-γ receptor signaling.

That B7.2 Tg mice genetically deficient in the generation of CD4+ T cells developed the same pathological features as those observed in animals having a full complement of CD4+ T cells clearly indicates that CD8+ T cells are the active mediator in this experimental system. Our finding that the CD8+ T cell subset is sufficient to promote demyelination and axonal damage is in line with recent studies showing that brain inflammation and demyelination occur in mice upon adoptive transfer of in vitro-activated myelin-specific CD8+ T cell lines (25–27). Accumulating evidence from multiple studies also suggest that CD8+ T cells may participate in the pathogenesis of CNS disease in humans (41, 42). Our demonstration of a pathogenic role for CD8+ T cells in a spontaneous CNS disease therefore provides strong support for the development of therapeutic approaches that target this T cell subset in MS and other human autoimmune diseases of the nervous tissue (43).

Another significant finding of our study was the earlier onset and increased penetrance of neurological symptoms in B7.2 Tg mice lacking a full complement of CD4+ T cells. Accelerated disease development in I-Aβ−/−/B7.2 Tg mice was associated with hastened enrichment of CD8+ T cells in the CNS, suggesting that a regulatory process that normally controls the T cell autoimmune response is lacking in these mice. Thus, the most likely explanation for disease exacerbation in these mice is the absence of regulatory CD4+ T cells, which have been shown to regulate T cell responses in various models of autoimmune diseases (44). It is not clear whether regulatory CD4+ T cells control the activation of autoreactive T cells in peripheral lymphoid organs and/or the CNS.

We observed a markedly increased frequency of CD8+ T cells in the CNS of preclinical animals deficient in CD4+ T cells in comparison with age-matched control B7.2 Tg, suggesting that the extent of local CD8+ T cell expansion in the target tissue may be controlled by regulatory CD4+ T cells. Alternatively, the rapid disease development in B7.2 Tg mice deficient in CD4+ T cells may simply reflect an increased activation of peripheral T cells allowing them to migrate more effectively within the CNS. Of note, adoptive transfer of in vitro activated myelin-Ag-specific CD8+ T cells into scid or RAG-deficient recipients did not result in accelerated disease onset (25, 26), indicating that regulatory CD4+ T cells may primarily influence the activation of autoreactive CD8+ T cells in the periphery rather than at the site of inflammation. In a CD8+ T cell-mediated model of type 1 diabetes, Green et al. (45) showed that CD4+CD25+ T regulatory cells control autoaggressive CD8+ T cell responses through a TGF-β-dependent pathway. Experiments are underway to determine whether this regulatory CD4+ T cell subset and the TGF-β/TGF-βR pathway are similarly involved in the control of spontaneous CD8+ T cell-mediated CNS inflammation in our experimental system.

The CDR3 spectratyping study of I-Aβ−/−/B7.2 Tg mice provided important insights into the nature of the CD8+ T cells involved in the pathogenesis of the demyelinating disease. Our data indicated either selective expansion of certain T cell clones or discriminatory entry of certain Vβ families into the CNS. Sequencing of TCR Vβ families with skewed CDR3 length distribution revealed the predominance of identical CDR3 sequences, which is consistent with the former possibility. These results are consistent with the concept that Ag recognition by T cells within the CNS promotes their retention in the organ (46) and thus suggest that, in this experimental system, B7.2-expressing microglia drive the activation and proliferation of Ag-specific CD8+ T cell clones in the CNS.

The skewed TCR repertoire in the CNS involved distinct Vβ families in different animals that were genetically identical. This was not due to problems of reproducibility with the technique as repeated amplification of the same CNS RNA samples generated identical results (data not shown). Most likely, these findings reflect that the T cell repertoire specific for a given autoantigen is highly heterogeneous. For instance, encephalitogenic myelin oligodendrocyte glycoprotein 33–55-specific T cell lines isolated from immunized mice used a diverse set of Vβ genes (47). Similarly, diabetogenic CD8+ T cells isolated from early insulitic lesions of NOD mice showed a diverse TCR Vβ chain repertoire while the α-chain usage was more restricted (48). In addition, it has been shown that the CD8+ T cell naïve repertoire specific for individual viral epitopes may be largely nonoverlapping even among genetically identical animals raised in similar environments (49, 50). It is of note that high variability in myelin basic protein-specific T cell clones has been observed in monozygotic twins (51).

As disease progresses, the CNS CD8+ T cell population demonstrated a more diverse repertoire as shown by the increased frequency of Vβ families exhibiting a Gaussian CDR3 length distribution. Presumably, the activation of Ag-specific CD8+ T cells by B7.2-expressing microglia generates an inflammatory environment into which bystander T cells are nonspecifically recruited. Several studies have shown that bystander T cells can indeed be nonspecifically recruited to the CNS but that their accumulation within the CNS is dependent on local Ag-specific interactions (52–55). Despite the increased heterogeneity of the overall CNS CD8+ T cell repertoire in mice with overt disease, some Vβ families nevertheless exhibited skewed CDR3 size distribution. This may indicate that the Ag-driven response at this stage of disease is more
focused. We do not know whether this reflects persistence of selected T cell clones within the CNS, or expansion of new clones through the process of epitope spreading. This would require longitudinal sampling of the CNS in individual animals, which is technically impossible.

The delineation of the Ag specificity of the CNS CD8+ T cell clones that initiate the pathological process will be addressed in future studies. Whether the MHC class I epitopes recognized by these clones are derived from myelin Ags remains to be determined. The cellular and molecular processes by which microglia can present myelin peptides in association with MHC I include cross-presentation after clearance of apoptotic debris resulting from normal turnover (43, 56, 57).

In addition to microglia, astrocytes express MHC class I molecules and therefore have the potential to activate CD8+ T cells providing that they express costimulatory ligands. Immunohistochemistry studies indicated that B7.2 expression was not detected on astrocytes in 4-wk-old preclinical B7.2 Tg mice (data not shown), indicating that they are unlikely to play a role in the initial activation of CD8+ T cell clones in the CNS. However, it is not excluded that the activation of Ag-specific CD8+ T cells by B7.2-expressing microglia generates an inflammatory environment that leads to the induction of costimulatory molecules on astrocytes and that they contribute to the activation of CD8+ T cell clones later in the pathological process.

We have established that IFN-γ responsiveness is an essential factor for disease pathogenesis. We showed previously that B7.2 Tg mice deficient in the generation of αβ TCR+ T cells are resistant to disease development (28). Because mice deficient in αβ TCR+ T cells have normal numbers of NK cells and γδ TCR+ T cells and that the disease in B7.2 Tg mice is mediated by the CD8+ T cell subset, the most likely source of IFN-γ are CD8+ αβ TCR+ T cells. We found that IFN-γ-dependent microglia activation is an early event in pathogenesis of the demyelinating disease since it was detected in young preclinical animals in which the frequency of CNS CD8+ T cells was not as high as in symptomatic animals but was nevertheless higher than in wild-type animals (data not shown). Microglia activation does not occur in B7.2 Tg animals deficient in lymphocytes (Rag-/- B7.2 Tg mice) or that have a highly restricted T cell repertoire (OT-1 TCR B7.2 Tg mice) or in B7.2 Tg animals that do not constitutively express B7.2 on microglia (line 27 mice). Thus, these data clearly show that microglia activation requires the presence of CNS-specific T cells and the constitutive expression of B7.2 by these CNS-resident APCs. We have also provided evidence of oligoclonal expansions of CNS CD8+ T cells at the early stage of disease. In addition, IFN-γ-dependent up-regulation of MHC molecules was apparent in the CNS but not in peripheral lymphoid organs of preclinical animals. Thus, collectively, our data indicate that the initial event that drives the cascade of the later pathogenesis is an Ag-specific interaction between CD8+ T cells that access the CNS and the B7.2-expressing microglial cells.

The requirement for IFN-γ in the pathogenesis of this CD8+ T cell-mediated demyelinating animal model is in line with recent reports showing that blocking IFN-γ signaling in the CNS greatly diminished CNS inflammation induced by adoptive transfer of myelin-specific CD8+ T cell lines (25) and that IFN-γ is essential for CD8+ T cell-mediated demyelination in mice infected with a neurotropic coronavirus (58). This is in contrast to CD4+ T cell-mediated EAE, in which IFN-γ primarily plays a negative regulatory role (59). Interestingly, the IFN-γ dependency of our and other model of CD8+ T cell-mediated brain inflammation parallels findings in MS where administration of IFN-γ resulted in disease exacerbation (60) and anti-IFN-γ Ab therapy reduced disability in secondary progressive MS (61).

The exact mechanism by which Ag-specific CD8+ T cell responses in the CNS lead to demyelination and axonal loss in our model system remains to be elucidated. IFN-γ produced by the Ag-specific CD8+ T cell response in the CNS may promote demyelination and axonal damage through microglia-induced activation and subsequent release of neurotoxic mediators. Such a mechanism has been shown to play a key role in the effector processes of CD4+ T cell-mediated EAE (36) and is proposed for MS (62). Consistent with this possibility, we have observed in preliminary studies significant NO production by CNS mononuclear cells isolated from disease-affected animals. An alternative, not mutually exclusive, possibility is that IFN-γ promotes MHC class I up-regulation on oligodendrocytes and neurons allowing a direct MHC/TCR contact-dependent cytotoxic effect. In vitro studies have shown that IFN-γ-stimulated oligodendrocytes and neurons can be damaged by CD8+ CTLs, in an MHC class I-restricted fashion (63, 64). Furthermore, in actively demyelinating MS lesions, CD8+ T cells have been frequently observed in close contact with injured axons with their cytotoxic granules oriented toward the CTL-neuron contact zone (41).

In summary, we describe a novel mouse model for CD8+ T cell-mediated CNS inflammation in which the pathological process appears to be initiated by the activation of Ag-specific CD8+ T cells within the CNS. This experimental system shows highly relevant pathological features to MS, including spontaneous development of demyelination and axonal damage, and dependency on IFN-γ. The study of this model may shed new light on the Ag-specific CD8+ T repertoire recognized in MS and the mechanisms by which they cause immunopathology.

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Disclosures
The authors have no financial conflict of interest.

References


