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Characterization of a Severe Parenchymal Phenotype of Experimental Autoimmune Encephalomyelitis in (C57BL6xB10.PL)F1 Mice

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Abstract

We here describe a novel CD4 T cell adoptive transfer model of severe experimental autoimmune encephalomyelitis in (C57BL6xB10.PL)F1 mice. This F1 cross developed severe disease characterized by extensive parenchymal spinal cord and brain periventricular white matter infiltrates. In contrast, B10.PL mice developed mild disease characterized by meningeal predominant infiltrates. As determined by cDNA microarray and quantitative real time PCR expression analysis, histologic and flow cytometry analysis of inflammatory infiltrates, and attenuation of disease in class I-deficient and CD8-depleted F1 mice; this severe disease phenotype appears to be regulated by CNS infiltration of CD8 T lymphocytes early in the disease course.

Keywords

EAE/MS; adoptive transfer; CD8 T cells; cDNA microarray

1. Introduction

Multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE), represent a heterogeneous group of demyelinating, inflammatory diseases of the CNS both clinically and pathologically (Keegan and Noseworthy, 2002; Zamvil and Steinman, 1990). In MS, there are at least four pathologic subtypes of disease (Lucchinetti *et al.*, 2000), whereas in EAE a multitude of potential phenotypes exist dependent on the species, mode of induction, genetic background, and antigen specificity (Gold *et al.*, 2006). How inflammation is initiated in these clinical states remains unclear, but appears to be crucial to determining the disease phenotype.

The most common clinical presentation of MS is relapsing, remitting disease where patients experience exacerbations separated in space and time, usually with near complete resolution of symptoms between attacks. After 10 to 15 years from the time of diagnosis, approximately half of patients will go on to develop secondary progressive disease with incomplete recovery and usually accompanied by insidious progression of disease between exacerbations

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(Weinshenker, 1995). Approximately 10% of patients have a primary progressive course from disease onset (Thompson *et al.*, 1997). However, even among patients within these three categories, substantial heterogeneity exists in the clinical course. For example, a subset of newly diagnosed patients have an early, aggressive clinical course that is only partially responsive to currently available immunotherapies (Weinshenker *et al.*, 1989; Cepok *et al.*, 2001). Even with aggressive immunosuppression, these individuals accumulate profound long term disability and represent the greatest therapeutic challenge within the MS patient population.

One of the goals in EAE disease model development is to replicate these phenotypic differences. Classical EAE induced by active immunization with myelin antigen and adjuvant or by adoptive transfer of myelin-specific CD4 Th1 T lymphocytes results in an ascending myelitis associated with meningeal inflammatory infiltrates (Zamvil and Steinman, 1990; Gold *et al.*, 2006). From a clinical perspective, these lesions are more similar to non-vasculitic autoimmune meningo-encephalitides seen in patients with acute disseminated encephalomyelitis, connective tissue disease or Hashimoto's encephalitis than they are to multiple sclerosis demyelinating plaques (Josephs *et al.*, 2004). Furthermore, they tend to underestimate the role played by CD8 T lymphocytes in the pathogenesis of MS (Friese *et al.*, 2005). More recently developed models demonstrated that CD8 T lymphocytes can also initiate EAE and more closely mimic the axonal pathology seen in patients with MS (Jiang *et al.*, 1992, 2001; Huseby *et al.*, 2001; Koh *et al.*, 1992; Sun *et al.*, 2001).

Other non-classical rodent EAE models have been developed more recently that demonstrate disease localized to the brainstem and cerebellum. In one of these models, on the congenic B10.PL background, spontaneous brainstem-cerebellar EAE develops in immune (RAG^{-/-}) and IFN- γ deficient mice that also express a TCR transgene specific for myelin basic protein (MBP) (Wensky *et al.*, 2005). In contrast, these mice develop spontaneous, classical EAE if they express IFN- γ . These results suggested that disease localization was dependent in part on the homing tendencies of distinct CD4 T cell phenotypes or on regional specific responses to pro-inflammatory cytokines such as IFN- γ .

We reasoned that the earliest responses of the CNS microenvironment to autoreactive T lymphocytes can determine the subsequent pathologic and clinical phenotype in EAE. Previously it was demonstrated that activated T lymphocytes continually cross the blood brain endothelial and blood-cerebrospinal fluid barriers to perform immune surveillance in all mouse strains examined (Hickey *et al.*, 1991; Engelhardt and Ransohoff, 2005; Carrithers *et al.*, 2000, 2002). This surveillance occurs independently of antigen specificity and in the absence of prior inflammation. If a surveillant lymphocyte is specific for CNS antigen, immune activation of the local microenvironment can lead to autoimmune disease. However, the presence of an autoreactive T lymphocyte within the CNS is not sufficient to cause disease. At least three potential outcomes are possible if antigen is encountered: a self-limited local immune activation, clinical disease, or active suppression. In addition, clinical disease can be self-limited and result in complete recovery or lead to a more severe progressive or static deficit.

Our goal here was to develop an adoptive transfer model of EAE in immune competent, genetically related mice and to characterize early inflammatory changes within the CNS that result in a severe disease phenotype. We demonstrate that immune competent (C57BL6xB10.PL)F1 mice develop a severe phenotype of EAE characterized pathologically by inflammatory infiltrates in spinal cord and periventricular white matter. In contrast, control B10.PL mice have a milder disease course associated with meningeal and cauda equina lesions. This difference appears to be due in part to the simultaneous presence of CD8 T lymphocytes in early inflammatory infiltrates along with CD4 cells and expression of MHC class I,

interferon- γ inducible, and CD8-associated genes in the brain parenchyma in (C57BL6xB10.PL)F1 mice.

2. Materials and Methods

Mice

C57BL6/J and C57BL10/J mice were obtained from Jackson Laboratories. K^bD^b deficient mice on the C57BL6 background were from Taconic. Wild type B10.PL mice, B10.PL mice that express a TCR transgene specific for Ac1-11 of MBP (MBP-TCR) (Baron *et al.*, 1993; Lafaille *et al.*, 1994), and (C57BL6xB10.PL)F1 mice (wild type and K^bD^b deficient) were bred in our colony at Yale University School of Medicine. Some of the TCR transgenic mice (B10.PL-MBP-TCR, CJ strain) were a kind gift of T. Brabb and J. Goverman (U. of Washington) to assist our laboratory in restoration of our transgenic colony. All animal studies were reviewed and approved by the Yale School of Medicine institutional review committee (IACUC).

CD4 T Cell Lines

To generate cell lines, spleens were removed from B10.PL MBP-TCR transgenic mice and stimulated with 5 μ g/ml Ac1-11 peptide and 5 U/ml rIL-2. Following 5 days in culture, the cells were re-stimulated and used 3 days later for adoptive transfer. These cells are almost entirely CD4⁺ T lymphocytes that express the clonotypic TCR specific for MBP, express high surface levels of VLA-4 and cause EAE when adoptively transferred.

EAE Induction

To induce EAE, 5×10^6 MBP-specific CD4 cells in 200 μ l PBS containing 20 ng LPS (*s. minnesota*) were adoptively transferred into recipient mice. Animals were observed on a daily basis for signs of clinical EAE beginning the day following transfer. The animals were graded as follows: 1, limp tail; 2, partial hind limb paralysis or ataxia; 3, complete hind limb paralysis or hemiplegia; 4, hind and front limb paralysis; 5, moribund. For CD8-depletion experiments, two days prior to adoptive transfer, (C57BL6xB10.PL)F1 mice were treated with 700 μ g (i.p.) of the CD8-depleting antibody TIB105 (53-6.72) or rat Ig isotype control (Jiang *et al.*, 2001). Animals were cared for in accordance with Yale University guidelines.

Immunocytochemistry

Brain and spinal cord frozen sections (7 μ m) were stained according to standard protocols using bovine serum albumin (BSA) and Triton X-100 as preincubation blockers (Carrithers *et al.*, 2000, 2002). Biotinylated antibodies were diluted in BSA and Triton X-100 solution and added to the sections for 2 hrs. Sections were washed and incubated with avidin-horseradish peroxidase. Sections were then washed, developed and counterstained with hematoxylin. For myelin staining, sections were stained with Luxol Fast Blue and counterstained with cresyl violet.

RT-PCR and real time analysis

Total RNA was isolated from brainstem and spinal cord tissue by guanidine isothiocyanate lysis and solubilization followed by phenol-chloroform extraction. Samples were then cleaned by column purification (Rneasy; Qiagen). Reverse transcription was performed with Superscript II (Gibco). Quantitative PCR was performed using TaqMan primers (FAM-labeled) commercially obtained (Applied Biosystems), and samples were run on a SmartCycler (Cepheid). Samples were normalized to GAPDH Ct values for each experiment. Each experiment was performed at least three times from separate RNA preparations.

cDNA array

Total RNA was isolated as described above using the following conditions: healthy C57BL6 wild type controls, Day 3 post adoptive transfer B10.PL and (C57BL6xB10.PL)F1, and Day 6 (disease onset) B10.PL and (C57BL6xB10.PL)F1. Approximately 100 µg of total RNA for each condition was reverse transcribed in the presence of amino-allyl dUTP and subsequently labeled with Cy-3 (C57BL6 wild type controls, universal control) or Cy-5 (all other conditions). Hybridization was performed on the NIA-15K cDNA microarray with three to four replicates for each condition from separate mRNA preparations. Array slides were scanned on an Axon GenePix 4000A laser confocal scanner, and raw data was extracted using Axon GenePix Pro software.

Expression analysis was performed using GeneSpring GX 7.3.1. For cluster analysis, the similarity measure was a smooth correlation, and the clustering algorithm was average linkage with a separation ratio of 1 and minimum distance of 0.001. This analysis was performed on a subset of 8258 genes that had a $P \leq 0.1$ for at least one of the four conditions analyzed (B10.PL Day 3, (C57BL6xB10.PL)F1 Day 3, B10.PL Day 6, (C57BL6xB10.PL)F1 Day 6).

Flow Cytometry

Cells were stained with the following mAbs: FITC-conjugated anti-CD4 (Gibco-BRL) and PE-conjugated CD8 (Pharmingen). Stained cells were analyzed on a FACS IV and by Cellquest software (Becton Dickinson). For isolation of cells from the CNS, animals were perfused with ice-cold PBS, the spinal cord and brain were removed, the tissue was dissociated and digested with collagenase, and a single cell preparation was isolated following two washes in 37% Percoll (a modification of Brabb *et al.*, 2000).

3. Results

Severe parenchymal EAE in (C57BL6xB10.PL)F1 mice

Our goal in EAE model development was to identify a strain where MS-like parenchymal lesions would occur. We were able to achieve our goal by using an F1 cross between congenic B10.PL (H-2^u) and the common strain, C57BL6 (H-2^b). The advantage of the (C57BL6xB10.PL)F1 cross is that disease can be induced by MBP-specific Th1 CD4 T lymphocytes (H-2^u or H-2^u × H-2^b) in mice that also have additional EAE susceptibility genes from C57BL6.

We transferred 5×10^6 CD4 T lymphocytes specific for Ac1-11 of MBP, generated from TCR transgenic B10.PL mice, to immune competent B10.PL and (C57BL6xB10.PL)F1 mice. As shown in Fig. 1A, B10.PL recipients had a relatively mild disease course limited to tail weakness or mild hindlimb weakness. The F1 mice developed more severe disease characterized by not only hindlimb weakness but forelimb weakness as well as hemiparesis and ataxia in some mice, characteristic of brain and brainstem lesions in addition to more classic spinal cord EAE. Disease incidence was 100% in the F1 group (n=33) with 36% mortality; the incidence of disease was 68% in B10.PL (n=13) with 8% mortality (1/13). Peak disease was reached at Day 12 in B10.PL and was 1.00 ± 0.38 . In the F1, peak disease occurred by Day 13 and was 2.79 ± 0.31 ($P = 0.001$ between the two peak disease scores). Disease was similarly severe in F1 mice if F1 TCR transgenic mice were used as adoptive transfer donors (Fig. 1B).

Histologic analysis of mice at disease onset (days 5–6 post adoptive transfer) demonstrated that the milder disease seen in B10.PL mice was characterized by meningeal predominant infiltrates with minimal local invasion in spinal cord white matter (Fig. 2A). Clinical signs appeared to be due to more extensive infiltration of the cauda equina, a region outside the CNS and classic blood brain barrier (Fig. 2B).

In contrast, the (C57BL6xB10.PL)F1 cross developed severe parenchymal disease characterized by extensive spinal cord and brain periventricular white matter infiltrates at disease onset (days 5 to 6) (Figs. 3A, 4). Immunohistochemical analysis of (C57BL6xB10.PL) F1 mice five to six days following adoptive transfer demonstrated extensive parenchymal spinal cord white matter infiltration with CD4 and CD8 T lymphocytes, B220-positive cells, and Mac-1 positive cells (monocytes/macrophages, activated microglia, and NK cells) (Fig. 3A). In B10.PL mice, although Mac-1 and CD4 positive cells were seen in meningeal infiltrates, there was a minimal amount of local invasion (Fig. 2A). Few, if any, CD8 T lymphocytes were present in B10.PL infiltrates (not shown). Two week following adoptive transfer in (C57BL6xB10.PL)F1 mice, areas of patchy demyelination were seen within the spinal cord parenchymal lesions (Fig. 3B). In addition, at disease onset, inflammatory infiltrates of brain periventricular white matter were seen in (C57BL6xB10.PL)F1 mice, but not B10.PL (Fig. 4).

Enhanced expression of CD8 T lymphocyte, MHC class I, and interferon- γ -associated genes during early EAE in (C57BL6xB10.PL)F1 mice

Since inflammatory initiation by autoreactive Th1 CD4 T lymphocytes causes altered expression patterns of many immune related genes within the CNS microenvironment, we further analyzed the EAE phenotype in B10.PL and (C57BL6xB10.PL)F1 mice to determine which genes are specific to clinically severe disease. Brainstem and spinal cord homogenates at day 3 following adoptive transfer and at disease onset (day 6) were analyzed by the National Institute on Aging (NIA) 15K mouse cDNA array. As compared to untreated mice, many genes demonstrated increased expression in both strains at disease onset; however, a relatively restricted cluster of genes showed increased expression in the F1 cross as compared to wild type controls and B10.PL mice with EAE (Fig. 5; bottom, F1-Day 6). Most notable within this cluster was a 3 to 10 fold increase in the expression of interferon- γ (IFN- γ inducible genes such as MHC class I genes, associated class I genes such as tapasin, and other IFN- γ inducible genes (Table 1).

Given the large data set, there were a relatively small number of genes that demonstrated large changes from baseline at disease onset. As shown in Fig. 6, there were a total of 109 genes that demonstrated a 2-fold or greater increase in expression at disease onset in F1 mice as compared to healthy wild type controls. Fifty of those genes are included in the designated clusters in Fig. 5, and all of these showed the greatest increases as compared to wild type controls (Fig. 6a). In addition, there were a total of 36 genes that demonstrated a 2-fold increase in the F1 at disease onset as compared to B10.PL at disease onset. Half of those genes are included in the clusters shown in Fig. 5, and all of these also showed the largest fold changes in increased expression of F1 genes at disease onset as compared to wild type controls (Fig. 6a). The entire data set is in Appendix 1.

We confirmed the cDNA microarray results by real time RT-PCR for the following genes: β_2 -microglobulin, tapasin, and interferon- γ inducible GTPase (Table 2). All genes demonstrated increased expression at disease onset in both strains as compared to healthy age, sex, and strain matched controls with enhanced expression in F1 mice as compared to B10.PL. In addition, although both strains demonstrated a marked increase in CNS IFN- γ mRNA expression as compared to their healthy controls, there was 2 to 4-fold increased expression of IFN- γ mRNA at disease onset in the F1 ($C_t = 27.2 \pm 0.4$) as compared B10.PL ($C_t = 29.0 \pm 0.7$).

Increased ratio of CD8 to CD4 T lymphocytes in early CNS inflammatory infiltrates in (C57BL6xB10.PL)F1 mice

Based on the results from immunohistochemistry and cDNA array analysis, we hypothesized that CD8 T lymphocytes may have a role in the pathogenesis of severe parenchymal disease

in (C57BL6xB10.PL)F1 mice. We confirmed the observed immunohistochemical differences in CD8 T lymphocyte infiltration by FACS analysis of mononuclear preparations from brain and spinal cord at disease onset (Fig. 7). The total number of CNS T lymphocytes per animal (meningeal and parenchymal combined) was similar between strains. However, approximately 60% of CNS T lymphocytes were CD8 positive in F1 mice, whereas only 30% in B10.PL (Fig. 7A). A higher percentage of the F1 CD8 population were brightly stained, which may reflect differences in activation and also explain the more profound staining differences seen by histology. There were approximately 229 ± 11 CD8 cells in the CNS per animal in F1 mice as compared to 120 ± 11 CD8 cells in B10.PL ($P=0.028$). In contrast, there were 131 ± 16 CD4 cells in F1 mice versus 259 ± 22 in B10.PL ($P=0.022$).

Reduced severity of EAE in K^bD^b-deficient and CD8-depleted (C57BL6xB10.PL)F1 mice, but not (C57BL10xB10.PL)F1 mice

To better define the mechanism of severe EAE in (C57BL6xB10.PL)F1 mice, we performed additional disease experiments to further assess the role of MHC class I and CD8 T lymphocytes in the severe disease course (Fig. 8). We reasoned that if our hypothesis of CD8-mediation of disease phenotype were true, then disease susceptibility should be associated with MHC haplotype and the classical Ia MHC class I locus contributed by C57BL6 in particular (K^bD^b). Transfer of 5×10^6 CD4 T lymphocytes specific for Ac1-11 of MBP, generated from TCR transgenic B10.PL mice, to wild type and K^bD^b-deficient (C57BL6xB10.PL)F1 mice resulted in distinct disease phenotypes in the two groups (Fig. 8). F1 wild type mice developed severe disease with a 32% mortality rate ($n=28$) and a peak disease score of 3.02 ± 0.25 (at day 9). K^bD^b-deficient mice had a 0% mortality rate ($n=27$) with a peak disease score of 1.94 ± 0.13 (at day 10, $P=0.0002$, as compared to F1 wild type). The recovery rate to a score of zero was 56% in the K^bD^b-deficient group and 14% in the F1 group. All K^bD^b-deficient mice improved to a score of at least 1.0.

In contrast, (C57BL10xB10.PL)F1 mice had a similar disease course to (C57BL6xB10.PL)F1 mice (Fig. 8). In the (C57BL10xB10.PL)F1 group, there was 100% disease incidence and 29% mortality ($n=17$). Although peak disease was reached sooner in (C57BL6xB10.PL)F1 wild type mice, the peak disease score was 2.41 ± 0.43 in the (C57BL10xB10.PL)F1 group (day 17), which was not a statistically significant difference as compared to the peak disease score in (C57BL6xB10.PL)F1 wild type ($P=0.24$). Since C57BL10 mice are almost genetically identical to B10.PL with the exception of the MHC locus, these results suggest that the phenotype is primarily driven by MHC-associated genes. However, we cannot rule out a small influence of non-MHC genes that are unique to C57BL6 as compared to C57BL10 that could make peak disease occur earlier. The data from all three groups, K^bD^b-deficient (C57BL6xB10.PL)F1, wild type (C57BL6xB10.PL)F1, and wild type (C57BL10xB10.PL)F1 mice, suggest that MHC class I H2-K^bD^b genes regulate disease severity, possibly through presentation of myelin-related or other CNS-derived antigens within the CNS to CD8 T lymphocytes.

In addition, in (C57BL6xB10.PL)F1 mice, CD8 depletion by antibody treatment prior to post-adoptive transfer resulted in a less severe disease course (Fig. 9). The mortality rate was 13% in the isotype control group ($n=24$) and 0% in the CD8-depleted group ($n=13$). Peak disease score was 1.88 ± 0.24 at day 12 in the isotype control group as compared to 0.31 ± 0.13 in the CD8-depleted group at the same day ($P < 0.0001$). The apparent difference in peak disease score between isotype control-treated mice (Fig. 9) and untreated (C57BL6xB10.PL)F1 mice (Figs. 1A, 8) may be due to non-specific effects of IgG control antibody mediated through Fc receptor interactions. Because the CD8 population begins to recover approximately 10 days following depletion (8 days post-adoptive transfer), this result also suggests that not only do

CD8 T lymphocytes mediate the severity of acute EAE in (C57BL6xB10.PL)F1 mice but that they do so during a specific critical period early in the disease course.

4. Discussion

We here demonstrate that adoptive transfer of MBP-specific CD4 T lymphocytes into immune competent (C57BL6xB10.PL)F1 mice leads to clinically severe EAE associated with parenchymal inflammatory infiltrates in brain and spinal cord. These lesions, similar in some respects to those seen in MS patients with clinically severe disease, are characterized by inflammatory infiltrates that contain a higher proportion of CD8 to CD4 cells and express higher levels of MHC class I, CD8-associated, and IFN- γ -inducible genes. This CD8 predominance is similar to that seen in MS lesions (Booss *et al.*, 1983; McCallum *et al.*, 1987; Babbe *et al.*, 2000; Jacobsen *et al.*, 2002). These lesions also demonstrate a distinct anatomic localization to parenchymal white matter in the brain periventricular regions and in the spinal cord, as compared to the meningeal infiltrates seen in control B10.PL mice.

The timing of early CNS infiltration by CD8 cells suggests that they are activated peripherally and enter the brain simultaneously with secondarily recruited CD4 cells. We hypothesize that, in this study, antigen non-specific stimulation of memory CD8 T lymphocytes by low dose lipopolysaccharide (20 ng) permits rapid homing of these cells to all non-lymphoid tissues including the brain (Tough *et al.*, 1997). Similar activation of memory CD8 cells can also occur following stimulation with viral adjuvants such as the Tlr3 agonist, dsRNA, or by Type I interferons (Tough *et al.*, 1997; Sprent, 2003; Kamath *et al.*, 2005). Here we show that these CD8 cells preferentially home to brain white matter parenchyma rather than the meningeal space.

In other adoptive transfer models of EAE, either CD4 or CD8 T lymphocytes are sufficient to induce CNS inflammatory disease. Although most work has been done with CD4 Th1 lymphocytes, two other studies demonstrate that myelin-specific CD8 T lymphocytes can induce disease in the absence of infection. Huseby *et al.* (2001) isolated MBP-specific CD8 cells from wild type and myelin-deficient C3H mice (H-2^k) immunized with MBP79-87. Transfer of clones activated *in vitro* into irradiated recipients resulted in a predominant brain/brainstem phenotype of EAE, rather than the more classic, myelopathic presentation. Similarly, Sun *et al.* (2001) identified myelin-specific CD8 clones in C57BL6 mice (H-2^b) immunized with MOG35-55. Adoptive transfer of activated cells also caused severe manifestations of EAE, and there was evidence of Wallerian degeneration in some inflammatory, demyelinated lesions.

The use of (C57BL6xB10.PL)F1 mice in this study permits induction of disease by class II H-2^d-restricted, MBP-specific CD4 T lymphocytes and disease amplification by class I H-2^b-restricted CD8 T lymphocytes, which may be specific for the MOG35-55 epitope (Sun *et al.*, 2001). Since myelin antigens such as MBP and MOG can be presented in both the brain parenchyma and the meningeal space, it remains unclear why there is preferential homing of lymphocytes to the parenchymal regions as opposed to the meninges. One possibility is that CNS microenvironment responds more robustly to pro-inflammatory cytokines released by CD8 T lymphocytes such as IFN- γ , another is possible differences in the reactivity of the blood brain endothelium between mouse strains, and a third is altered homing characteristics of CD8 as compared to CD4 T lymphocytes. Our expression data from this study suggests in part a strain-specific role for increased levels of parenchymal IFN- γ and its inducible genes in (C57BL6xB10.PL)F1 mice as compared to B10.PL.

The role of IFN- γ in the pathogenesis of EAE is complex (Wensky *et al.*, 2005; Ferber *et al.*, 1996; Willenborg *et al.*, 1996; Chu *et al.*, 2000; Tran *et al.*, 2000). Absence of IFN- γ can cause

more severe disease despite its pro-inflammatory effects and alter the anatomic disease localization from a classical to a non-classical phenotype (Ferber *et al.*, 1996; Willenborg *et al.*, 1996; Chu *et al.*, 2000). Intrathecal administration of IFN- γ reduces the severity of EAE in C57BL6 mice through enhanced apoptosis of lymphocytes (Furlan *et al.*, 2001). In contrast, intravenous administration of IFN- γ to patients with MS caused a pro-inflammatory effect, and the patients had more frequent clinical exacerbations (Panitch *et al.*, 1987). Our data here suggest that the role of IFN- γ in lesion formation in (C57BL6xB10.PL)F1 mice is more similar to human MS as compared to other rodent models. This difference may be because the source of IFN- γ in CNS lesions in these mice is from both CD8 and CD4 cells rather than either cell type alone and may enhance formation of parenchymal lesions. It is also possible that enhanced release of parenchymal IFN- γ may increase meningeal IFN- γ levels to suppress formation of focal meningeal lesions.

In addition, the role of inflammatory CD8 cells in this EAE model is highly relevant to the pathogenesis of MS, particularly in new lesion formation. Several studies of MS pathology have demonstrated that, as compared to CD4 cells, CD8 T lymphocytes are a dominant component of the demyelinating lesion (Booss *et al.*, 1983; McCallum *et al.*, 1987; Babbe *et al.*, 2000; Jacobsen *et al.*, 2002). In chronic progressive disease subtypes, immunohistochemistry demonstrated a predominance of CD8 cells in parenchymal inflammatory infiltrates (Booss *et al.*, 1983). In a more recent molecular study of active MS lesions, micromanipulation and single cell polymerase chain reaction (PCR) showed that CD8 cells dominate the inflammatory infiltrate and that they are clonally expanded (Babbe *et al.*, 2000). Another study demonstrated oligoclonal expansion of CD8 T lymphocytes within the cerebrospinal fluid from MS patients (Jacobsen *et al.*, 2002). These results suggest that the CD8 cells recognize antigen within the CNS microenvironment.

CD8 T lymphocytes are also highly relevant targets in the treatment of MS. For examples, a more recent treatment, natalizumab, a humanized monoclonal antibody to α -4 integrin, showed exceptional promise in a randomized, placebo controlled trial (Miller *et al.*, 2003). This agent inhibits migration of inflammatory CD4 and CD8 T lymphocytes across the blood brain barrier endothelium. In a phase II trial, natalizumab reduced the relapse rate to approximately two-thirds from baseline and the number of new gadolinium enhancing lesions by approximately 90% (Miller *et al.*, 2003). Because prior trials of CD4-targeted therapy had a less profound impact on clinical outcomes (Van Oosten *et al.*, 1997), natalizumab's efficacy may be due predominantly to its effect on CD8 T lymphocyte trafficking. Unfortunately, in one phase III trial that examined combination treatment of natalizumab with β -interferon, two patients developed progressive multifocal leukoencephalopathy (PML), and one patient died from the complication (Berger and Koranik, 2005; Kleinschmidt-DeMasters and Tyler, 2005; Langer-Gould *et al.*, 2005). In a trial for patients with Crohn's Disease, an additional patient was found retrospectively to have had a fatal case of PML (Van Assche *et al.*, 2005). It has been hypothesized that the underlying pathophysiologic mechanism is reduced immune surveillance by viral-specific CD8 T lymphocytes.

We speculate that, in our EAE model (C57BL6xB10.PL)F1 mice and in some patients with relapsing remitting MS, myelin-specific CD4 T lymphocytes initiate new inflammatory lesions, but that early recruitment of autoreactive CD8 cells to the brain parenchyma during a specific critical period leads to enhanced tissue injury and clinically relevant disease. CD4 cells may be sufficient for localized immune activation, but these localized inflammatory changes may be more self-limited or localized solely to the meningeal compartment unless very large numbers of antigen-specific Th1 CD4 cells enter the brain. Conversely, in the absence of infection, CD8 cells may require the presence of inflammatory CD4 cell help within the CNS microenvironment to activate the local microenvironment and blood brain barrier and permit recruitment of CD8 cells. Amplification of the inflammatory response through locally

enhanced release of IFN- γ and other mediators by CTL's can lead to direct injury of nerve fibers or indirectly through activation of microglia and peripherally recruited macrophages. We believe that this form of EAE more closely mimics the pathogenesis seen during the formation of new lesions in MS. It may also prove to be an excellent model to study acute forms of axonal injury mediated by CD8 T lymphocytes (Kuhlmann *et al.*, 2002).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this paper

MS	multiple sclerosis
EAE	experimental autoimmune encephalomyelitis
MBP	myelin basic protein

References

- Babbe H, Roers A, Waisman A, Lassmann H, Goebels N, Hohlfeld R, Friese M, Schroder R, Deckert M, Schmidt S, Ravid R, Rajewsky K. Clonal expansions of CD8(+) T cells dominate the T cell infiltrate in active multiple sclerosis lesions as shown by micromanipulation and single cell polymerase chain reaction. *J Exp Med* 2000;192:393–404. [PubMed: 10934227]
- Baron JL, Madri JA, Ruddle NH, Hashim G, Janeway CA Jr. Surface expression of $\alpha 4$ integrin by CD4 T cells is required for their entry into brain parenchyma. *J Exp Med* 1993;177:57–68. [PubMed: 7678116]
- Berger JR, Koralnik IJ. Progressive multifocal leukoencephalopathy and natalizumab-unforeseen consequences. *N Engl J Med* 2005;353:414–416. [PubMed: 15947082]
- Booss J, Esiri M, Tourtellotte W, Mason D. Immunohistological analysis of T lymphocyte subsets in the central nervous system in chronic progressive multiple sclerosis. *J Neurol Sci* 1983;62:219–232. [PubMed: 6607973]
- Brabb T, von Dassow P, Ordonez N, Schnabel B, Duke B, Goverman J. In situ tolerance within the central nervous system as a mechanism for preventing autoimmunity. *J Exp Med* 2000;192:871–880. [PubMed: 10993917]
- Carrithers MD, Visintin I, Janeway CA Jr. Dependence of cellular phenotype and genetic background on immune surveillance of the brain by T lymphocytes. *J Neuroimmunol* 2002;129:51–57. [PubMed: 12161020]
- Carrithers MD, Visintin I, Kang SJ, Janeway CA Jr. Differential adhesion molecule requirements for immune surveillance and inflammatory recruitment. *Brain* 2000;123:1092–1101. [PubMed: 10825349]
- Cepok S, Jacobsen M, Schock S, Omer B, Jaekel S, Boddeker I, Oertel WH, Sommer N, Hemmer B. Patterns of cerebrospinal fluid pathology correlate with disease progression in multiple sclerosis. *Brain* 2001;124:2169–2176. [PubMed: 11673319]

- Chu CQ, Wittmer S, Dalton DK. Failure to suppress the expansion of the activated CD4 T cell population in interferon γ -deficient mice leads to exacerbation of experimental autoimmune encephalomyelitis (EAE). *J Exp Med* 2000;192:123–128. [PubMed: 10880533]
- Engelhardt B, Ransohoff RM. The ins and outs of T lymphocyte trafficking to the CNS: anatomical sites and molecular mechanisms. *Trends Immunol* 2005;26:485–495. [PubMed: 16039904]
- Ferber IA, Brocke S, Taylor-Edwards C, Ridgeway W, Dinisco C, Steinman L, Dalton D, Fathman CG. Mice with a disrupted IFN- γ gene are susceptible to the induction of experimental autoimmune encephalomyelitis. *J Immunol* 1996;156:5–7. [PubMed: 8598493]
- Friesen MA, Fugger L. Autoreactive CD8+ T cells in multiple sclerosis: a new target for therapy? *Brain* 2005;128:1747–1763. [PubMed: 15975943]
- Furlan R, Brambilla E, Ruffini F, Poliani PL, Bergami A, Marconi PC, Franciotta DM, Penna G, Comi G, Adorini L, Martino G. Intrathecal delivery of IFN- γ protects C57BL/6 mice from chronic-progressive experimental autoimmune encephalomyelitis by increasing apoptosis of central nervous system-infiltrating lymphocytes. *J Immunol* 2001;167:1821–1829. [PubMed: 11466408]
- Gold R, Linington C, Lassmann H. Understanding pathogenesis and therapy of multiple sclerosis via animal models: 70 years of merits and culprits in experimental autoimmune encephalomyelitis research. *Brain* 2006;129:1953–1971. [PubMed: 16632554]
- Hickey WF, Hsu BL, Kimura H. T-lymphocyte entry into the central nervous system. *J Neurosci Res* 1991;128:254–260. [PubMed: 2033653]
- Huseby ES, Liggitt D, Brabb T, Schnabel B, Ohlen C, Goverman J. A pathogenic role for myelin specific CD8(+) T cells in a model for multiple sclerosis. *J Exp Med* 2001;194:669–676. [PubMed: 11535634]
- Jacobsen M, Cepok S, Quak E, Happel M, Gaber R, Ziegler A, Schock S, Oertel WH, Sommer N, Hemmer B. Oligoclonal expansion of memory CD8(+) T cells in cerebrospinal fluid from multiple sclerosis patients. *Brain* 2002;125:538–550. [PubMed: 11872611]
- Jiang H, Braunstein NS, Yu B, Winchester R, Chess L. CD8(+) T cells control the TH phenotype of MBP-reactive CD4(+) T cells in EAE mice. *Proc Natl Acad Sci USA* 2001;98:6301–6306. [PubMed: 11353822]
- Jiang H, Zhang S, Pernis B. Role of CD8(+) T cells in murine experimental allergic encephalomyelitis. *Science* 1992;256:1213–1215. [PubMed: 1375398]
- Josephs KA, Rubino FA, Dickson DW. Nonvasculitic autoimmune inflammatory meningoencephalitis. *Neuropathology* 2004;24:149–152. [PubMed: 15139593]
- Kamath AT, Sheasby CE, Tough DF. Dendritic cells and NK cells stimulate bystander T cell activation in response to TLR agonists through secretion of IFN $\alpha\beta$ and IFN γ . *J Immunol* 2005;174:767–776. [PubMed: 15634897]
- Keegan M, Noseworthy J. Multiple Sclerosis. *Annu Rev Med* 2002;53:285–302. [PubMed: 11818475]
- Kleinschmidt-DeMasters BK, Tyler KL. Progressive multifocal leukoencephalopathy complicating treatment with natalizumab and interferon beta-1a for multiple sclerosis. *N Engl J Med* 2005;353:369–374. [PubMed: 15947079]
- Koh DR, Fung-Leung WP, Ho A, Gray D, Acha-Orbea H, Mak TW. Less mortality but more relapses in experimental allergic encephalomyelitis in CD8 $-/-$ mice. *Science* 1992;256:1210–1213. [PubMed: 1589800]
- Kuhlmann T, Lingfeld G, Bitsch A, Schuchardt J, Bruck W. Acute axonal damage in multiple sclerosis is most extensive in early disease stages and decreases over time. *Brain* 2002;125:2202–2212. [PubMed: 12244078]
- Lafaille JJ, Nagashima K, Katsuki M, Tonegawa S. High incidence of spontaneous autoimmune encephalomyelitis in immune deficient anti-myelin basic protein T cell receptor transgenic mice. *Cell* 1994;78:399–408. [PubMed: 7520367]
- Langer-Gould A, Atlas SW, Green AJ, Bollen AW, Pelletier D. Progressive multifocal leukoencephalopathy in a patient treated with natalizumab. *N Engl J Med* 2005;353:375–381. [PubMed: 15947078]
- Lucchinetti C, Bruck W, Parisi J, Scheithauer B, Rodriguez M, Lassmann H. Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. *Ann Neurol* 2000;47:707–717. [PubMed: 10852536]

- McCallum K, Esiri M, Tourtellotte W, Booss J. T cell subsets in multiple sclerosis. Gradients at plaque borders and differences in nonplaque regions. *Brain* 1987;110:1297–1308. [PubMed: 2960419]
- Miller DH, Khan OA, Sheremata WA, Blumhardt LD, Rice GP, Libonati M, Hulme Wilmer-AJ, Dalton CM, Mizkiel KA, O'Connor PW. the International Natalizumab Multiple Sclerosis Trial Group. A controlled trial of Natalizumab for relapsing multiple sclerosis. *New Engl J Med* 2003;348:15–23. [PubMed: 12510038]
- Nogai A, Siffrin V, Bonhagen K, Pfueller CF, Honhnstein T, Volkmer-Engert R, Bruck W, Stadelman C, Kamradt T. Lipopolysaccharide injection induces relapses of experimental autoimmune encephalomyelitis in nontransgenic mice via bystander activation of autoreactive CD4+ cells. *J Immunol* 2005;175:959–965. [PubMed: 16002695]
- Panitch HS, Hirsch RL, Haley AS, Johnson KP. Exacerbations of multiple sclerosis patients treated with gamma interferon. *Lancet* 1987;18:893–895. [PubMed: 2882294]
- Sprent J. Turnover of memory-phenotype CD8 T cells. *Microbes and Infection* 2003;5:227–231. [PubMed: 12681412]
- Sun D, Whitaker JN, Huang Z, Liu D, Coleclough C, Wekerle H, Raine CS. Myelin antigen-specific CD8 (+) T cells are encephalitogenic and produce severe disease in C57BL/6 mice. *J Immunol* 2001;166:7579–7587. [PubMed: 11390514]
- Thompson AJ, Polman CH, Miller DH, McDonald WI, Brochet B, Filippi M, Montalban X, De Sa J. Primary progressive multiple sclerosis. *Brain* 1997;120:1085–1096. [PubMed: 9217691]
- Tough DF, Sun S, Sprent J. T cell stimulation in vivo by lipopolysaccharide. *J Exp Med* 1997;185:2089–2094. [PubMed: 9182680]
- Tran EH, Prince EN, Owens T. IFN- γ shapes immune invasion of the central nervous system via regulation of chemokines. *J Immunol* 2000;164:2759–2768. [PubMed: 10679118]
- Van Assche G, Van Ranst M, Sciort R, et al. Progressive multifocal leukoencephalopathy after natalizumab treatment for Crohn's disease. *N Engl J Med* 2005;353:362–368. [PubMed: 15947080]
- Van Oosten BW, Lai M, Hodkinson S, Barkhof F, Miller DH, Moseley IF, Thompson AJ, Rudge P, McDougall A, McLeod JG, Adler HJ, Polman CH. Treatment of multiple sclerosis with the monoclonal anti-CD4 antibody cM-T412: results of a randomized, double-blind, placebo-controlled, MR-monitored phase II trial. *Neurology* 1997;49:351–357. [PubMed: 9270561]
- Weinshenker BG. The natural history of multiple sclerosis. *Neurol Clin* 1995;13:119–146. [PubMed: 7739500]
- Weinshenker BG, Bass B, Rice GP, Noseworthy J, Carriere W, Baskerville J, Ebers GC. The natural history of multiple sclerosis: a geographically based study. 2. Predictive value of the early clinical course. *Brain* 1989;112:1419–1428. [PubMed: 2597989]
- Wensky AK, Furtado GC, Garibaldi-Marconedes MC, Chen S, Manfra D, Lifra SA, Zagzag D, Lafaille JJ. IFN- γ determines distinct clinical outcomes in autoimmune encephalomyelitis. *J Immunol* 2005;174:1416–1423. [PubMed: 15661899]
- Willenborg DO, Fordham S, Bernard CC, Cowden WB, Ramshaw IA. IFN- γ plays a critical down-regulatory role in the induction and effector phase of myelin oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis. *J Immunol* 1996;157:3223–3227. [PubMed: 8871615]
- Zamvil S, Steinman L. The T lymphocyte in experimental allergic encephalomyelitis. *Annu Rev Immunol* 1990;8:579–621. [PubMed: 2188675]

Figure 1. Severe adoptive transfer EAE in (C57BL6/JxB10.PL)F1 mice

5×10^6 cells from myelin basic protein (MBP)-specific CD4 Th1 cell lines were transferred to 10 to 12 wk. old animals. Cell lines were generated from mice that express a TCR transgene specific for Ac1-11 of MBP. A.) Transfer of B10.PL (H-2^u) MBP-specific CD4 Th1 cells to B10.PL and (C57BL6/JxB10.PL)F1 (H-2^u x H-2^b) recipients resulted in moderate to severe disease in F1 animals (n=33) but only mild disease in B10.PL (n=13). Disease incidence was 100% in the F1 group with 36% mortality; there was 62% incidence and 8% mortality in B10.PL. B.) Transfer of (C57BL6/JxB10.PL)F1 MBP-specific CD4 Th1 cells to (C57BL6/JxB10.PL)F1 mice (n=13) also resulted in severe disease with 69% mortality. EAE curves represent aggregate scores from at least three separate adoptive transfer experiments. (EAE

disease scoring: 1, limp tail; 2, hindlimb weakness; 3, paraplegia; 4, quadriparesis; 5, moribund or dead).

Disease Onset in B10.PL

a. Meningeal Spinal Cord Lesions

Hematoxylin

Mac-1

CD4

b. Cauda Equina Infiltrate

Mac-1

CD4

Figure 2. Immunohistochemistry at disease onset demonstrates predominant localization of inflammatory infiltrates in the meninges and cauda equina of B10.PL mice

As in Fig. 1, 10 to 12 wk. old B10.PL mice received 5×10^6 MBP-specific CD4 T lymphocytes. Animals were sacrificed on day 5 or 6 post adoptive transfer (disease onset), and tissue was prepared for frozen sections. Micrographs in (A) are serial sections of the same lesion from a B10.PL mouse (EAE score 0) at day 5 and in (B) are from another B10.PL at day 6 (EAE score 2.0). A.) Histologic analysis of spinal cord from a Day 5 B10.PL mouse reveals meningeal lesions with a mild degree of local invasion. The upper left panel shows a low power view of a hematoxylin stained longitudinal section through the spinal cord showing a meningeal mononuclear inflammatory infiltrate adjacent to white matter tracts. Immunoperoxidase staining of this lesion shows many Mac-1 positive cells (upper right) and a few scattered CD4+ cells (lower left and right). The detail on the lower right shows four CD4+ cells from the region in the box at the lower left. No B220 or CD8+ cells were seen (not shown). B.) Immunohistochemistry of cauda equina in B10.PL mice reveals inflammatory infiltrates at disease onset. Although parenchymal infiltrates of brain periventricular white matter or spinal cord were not seen, some animals had extensive cauda equina lesions associated with hindlimb weakness (EAE score 2). Extensive anti-Mac-1 staining was observed in the mononuclear infiltrate (left panel). Although not as extensive as Mac-1 staining, scattered CD4-positive cells were also seen (right panel) (immunoperoxidase).

a. Spinal Cord Infiltration Day 5 (C57BL6xB10.PL)F1

Hematoxylin

Mac-1

CD4

B220

Figure 3. Immunohistochemistry at disease onset demonstrates invasive parenchymal inflammatory lesions in (C57BL6xB10.PL)F1 mice and subsequent demyelination during the subacute phase

A.) At disease onset (day 5) in a (C57BL6/JxB10.PL)F1 mouse (EAE score 2), hematoxylin staining (upper left) and immunoperoxidase staining for Mac-1 (upper right), CD4 (middle left), B220 (middle right), and CD8 (bottom two panels) demonstrate a large mononuclear inflammatory infiltrate in a longitudinal section from spinal cord white matter. This lesion is primarily parenchymal with some associated meningeal inflammation as well. CD8+ staining was seen throughout the lesion as shown in the bottom panels. The lower right panel is the detail from area designated at the left. B.) Luxol fast blue staining for myelin followed by cresyl violet shows patchy areas of spinal cord white matter demyelination in a (C57BL6xB10.PL)F1 mouse at 14 days post adoptive transfer (EAE disease score 3).

Figure 4. Histologic analysis of brain demonstrates mononuclear inflammatory infiltrates in (C57BL6xB10.PL)F1 mice in periventricular white matter, but not in B10.PL
 Sections are from the same animals as in Figs. 2A and 3A (Day 5 post-adoptive transfer). Immunoperoxidase staining showed extensive periventricular infiltrates containing Mac-1 and CD4+ cells in (C57BL6xB10.PL)F1 mice (middle and left panels). Some of these mice developed ataxia and hemiplegia. B10.PL mice did not demonstrate brain parenchymal infiltrates, although there was some evidence of enhanced Mac-1 staining (right panel). Meningeal infiltrates were also seen as in the spinal cord (not shown).

Figure 5. cDNA microarray expression analysis reveals increased expression of major histocompatibility complex (MHC) class I associated genes and other IFN- γ -inducible genes in (C57BL6xB10.PL)F1 mice at disease onset

Adoptive transfer EAE was induced in B10.PL and (C57BL6xB10.PL)F1 mice as described in Fig. 1. mRNA was prepared from whole brainstem and spinal cord, reverse transcribed, labeled with Cy-5, and hybridized to NIA 15K cDNA mouse arrays using wild type, untreated C57BL/6J brainstem-spinal cord as control cDNA (Cy-3 on all arrays). Day 0 hybridizations for both B10.PL and (C57BL6xB10.PL)F1 mice did not reveal any differences as compared to C57BL/6 control (not shown). The remaining experimental conditions were: B10.PL-Day 3 (far left in panels A and B), B10.PL-Day 6, F1-Day 3, and F1-Day 6 (far right in A and B). Day number is defined as number of days post-adoptive transfer. Multiple arrays (3 to 4) were performed for each experimental condition from different mRNA preparations. Cluster analysis was performed using GeneSpring GX 7.3.1 on a restricted list of genes (8258) that had a $P \leq 0.10$ for at least one experimental condition. Genes were color coded by expression as compared to the C57BL/6 control (red >2 fold increased expression; green <2 fold decreased expression). A.) Within the intensely red-colored cluster at the bottom left (F1-Day 6) are many genes that demonstrate increased expression at disease onset as compared to the other three conditions. B.) A magnified view of the same gene cluster. Within this large cluster are two regions of genes that are strongly upregulated at disease onset in F1; the upper cluster (arrow) contains 43 genes and the lower cluster (arrow) contains 7. The full gene list from these two regions is shown in Table 1. In addition to expressed sequence tags (ESTs), these clusters contain β 2-microglobulin, tapasin, H2 heavy chain k region, H2 heavy chain L region, IFN- γ -inducible GTPase, IFN- γ -induced transmembrane protein 3, dipeptidyl peptidase I, and proteasomal components.

Figure 6. Scatter plot and Venn diagram analysis of cDNA data

A.) In the scatter plot analysis shown, normalized expression data (normalized to expression in untreated control mice) at disease onset was compared between (C57BL6xB10.PL)F1 mice (y-axis) and B10.PL mice (x-axis). Genes are color coded on the basis of the Venn diagram shown in (B). There was a relatively small number of genes that showed both a 2 fold increase in expression from baseline and a 2 fold increase in expression at disease onset in F1 mice as compared to B10.PL (upper left quadrant, white and purple dots). All of those genes which showed a greater than 4-fold increase in gene expression in F1 mice at disease onset as compared to baseline (yellow and white dots in upper quadrants) were included in clusters 1 and 2 shown in Fig. 5. No gene showed a greater than 10-fold increase from baseline. B.) Venn diagram analysis combined with scatter plot analysis demonstrates that the 50 genes in clusters

1 and 2 (Fig. 5) show the greatest degree of increased expression in F1 mice at disease onset as compared to baseline and B10.PL mice at disease onset.

Figure 7. Increased ratio of CD8 to CD4 T lymphocytes in early CNS inflammatory infiltrates in (C57BL6xB10.PL)F1 mice

Single cell suspensions were made from brainstem and spinal cord at disease onset (day 6 post-adoptive transfer), stained with anti-CD4 and CD8 antibodies, and analyzed by flow cytometry. At least 20,000 events were collected in the mononuclear gate as determined by SSC and FSC analysis. A.) The total numbers of CD4 and CD8 T cells were similar between the B10.PL and F1 mice, but the ratio of CD8 to CD4 was slightly greater than 1 in the F1 group (1.17) and was 0.46 in the B10.PL. FACS plots are representative of 6–7 replicates for each condition. B.) Statistical analysis was performed on each replicate. There were approximately 229 ± 11 CD8 cells (graph on left) in the CNS per animal in F1 mice (n=6) as compared to 120 ± 11

CD8 cells in B10.PL (n=7) ($P=0.028$). In contrast, there were 131 ± 16 CD4 cells (graph on right) in F1 mice versus 259 ± 22 in B10.PL ($P=0.022$).

Figure 8. Adoptive transfer EAE in K^bD^b -deficient (C57BL6xB10.PL)F1 and (C57BL10xB10.PL)F1 mice

Disease was induced by adoptive transfer of 5×10^6 MBP-specific CD4 T lymphocytes from B10.PL donors. Wild type (C57BL6xB10.PL)F1 wild type mice developed severe disease with a 32% mortality rate ($n=28$) and a peak disease score of 3.02 ± 0.25 (at day 9). K^bD^b -deficient mice had a 0% mortality rate ($n=27$) with a peak disease score of 1.94 ± 0.13 (at day 10, $P=0.0002$, as compared to F1 wild type). In contrast, although peak disease was reached sooner in (C57BL6xB10.PL)F1 wild type mice, the disease course in (C57BL10xB10.PL)F1 mice was similar to that seen in (C57BL6xB10.PL)F1 mice. There were 17 animals in the (C57BL10xB10.PL)F1 group with 100% disease incidence and 29% mortality. Peak disease score was 2.41 ± 0.43 , which was not a statistically significant difference as compared to the peak disease score in (C57BL6xB10.PL)F1 wild type. EAE curves represent aggregate scores from at least three separate adoptive transfer experiments.

Figure 9. Reduced severity of EAE in (C57BL6xB10.PL)F1 mice depleted of CD8 T lymphocytes
Two days prior to adoptive transfer, (C57BL6xB10.PL)F1 mice were treated with 700 μ g (i.p.) of the CD8-depleting antibody TIB105 (53-6.72) or rat Ig isotype control (Jiang *et al.*, 2001). By FACS analysis of peripheral blood, CD8 cells are depleted within three days (day 1 post-adoptive transfer) and begin to appear again approximately seven days later (day 8 post-adoptive transfer). Although anti-CD8-treated mice developed a mild chronic disease course, peak disease did not exceed a score of 1.0 ($n=5$; 0% mortality). Isotype control-treated mice had a more severe disease course with 13% mortality ($n=24$). Peak disease score was 1.88 ± 0.24 at day 12 in the isotype control group as compared to 0.31 ± 0.13 in the CD8-depleted group at the same day ($P < 0.0001$).

List of genes identified by cluster analysis in Fig. 5 that demonstrate enhanced expression in (C57BL6xB10.PL)F1 mice as compared to B10.PL.

Table 1

Accession Number	Description	B10.PL Day 6	t-test p-value	F1 Day 6	t-test p-value	Ratio of F1/B10.PL
Antigen Processing and Presentation						
BG063994	Beta-2 microglobulin (beta-2m)	4.22	0.074	9.37	0.003	2.21
BG063105	Tapasin	1.74	0.007	4.64	0.041	2.65
BG087558	H2-D1, histocompatibility 2, D region locus 1	2.37	0.053	8.35	0.027	3.51
AU041598	MHC class I heavy chain precursor (H-2K)	1.70	0.147	3.47	0.053	2.04
BG084094	MHC class I heavy chain precursor (H-2D)	2.91	0.084	5.22	0.012	1.79
BG067341	MHC class I, Q region	3.23	0.083	4.69	0.127	1.45
BG067670	EST, MHC class I, Q region	2.02	0.109	3.48	0.042	1.72
BG066678	EST, MHC class I, Q region	2.16	0.020	3.87	0.058	1.79
BG066650	Proteasome (prosome, macropain) 28 subunit, alpha (Psmc1)	1.96	0.077	4.08	0.005	2.08
BG073636	Proteasome (prosome, macropain) 28 subunit, alpha (Psmc1)	1.79	0.033	4.10	0.006	2.29
BG077017	MHC class II region: class II alpha chain (IAalpha) and beta chain (IEbeta) genes	1.79	0.019	4.97	0.004	2.77
Other IFN-gamma Inducible Genes						
BG076245	Interferon gamma induced GTPase (Igp)	3.05	0.074	5.06	0.002	1.66
BG072156	Interferon induced transmembrane protein 3 (Ifitm3)	2.50	0.039	4.49	0.0002	1.79
Accession Number	Description	B10.PL Day 6	t-test p-value	F1 Day 6	t-test p-value	Ratio of F1/B10.PL
BG067655	Stromal cell antigen-2	2.35	0.004	6.28	0.102	2.66
TCR Genes						
BG068648	TCR V-Beta locus	2.50	0.137	4.10	0.029	1.64
BG074388	TCR V-Beta region	3.30	0.124	4.97	0.171	1.50
BG067620	EST, homology to TCR alpha/delta region	1.84	0.029	3.56	0.098	1.93
Other Immune Function Genes						
BG080623	Dipeptidyl peptidase I	2.51	0.082	6.98	0.003	2.78
C77656	Precursor for dipeptidyl peptidase I	4.88	0.003	9.78	0.029	2.00
BG064176	L-34 galactoside- binding lectin (galectin 3)	1.22	0.119	5.26	0.0001	4.30
BG070228	Pre-B-cell colony- enhancing factor (Pbef)	1.72	0.135	2.93	0.027	1.70
BG068980	Lymphocyte cytosolic protein 2 (Lcp2)	1.80	0.086	2.98	0.019	1.64

Accession Number	Description	B10.PL Day 6	t-test p-value	F1 Day 6	t-test p-value	Ratio of F1/B10.PL
BC072793	Lymphocyte antigen 6 complex, locus A	2.08	0.029	3.54	0.008	1.69
BC077355	Beta 1,3-galactosyltransferase, polypeptide 4 (B3gal4)	2.36	0.089	4.88	0.020	2.06
BC070071	Small heat shock protein (HSP25)	2.10	0.056	4.37	0.195	2.08
BC075648	C1 inhibitor	2.02	0.14	4.62	0.109	2.27
BC076653	Peripheral-type benzodiazepine receptor	1.53	0.187	3.90	0.005	2.54
AA08197	Signal transducer and activator of transcription 1 (Stat1)	4.37	0.12	5.89	0.003	1.34
BC068242	Regulatory protein, T lymphocyte 1 (Rpt1)	2.76	0.072	2.94	0.036	1.06
Other Genes						
BC076766	Transthyretin (Ttr)	0.87	0.033	6.76	0.002	7.70
BC087868	Peroxiredoxin 4 (Prdx4)	2.16	0.037	3.85	0.002	1.78
BC067456	Lysozyme M gene,	1.62	0.204	3.95	0.034	2.43
Accession Number	Description	B10.PL Day 6	t-test p-value	F1 Day 6	t-test p-value	Ratio of F1/B10.PL
BC071156	Ninein (Nin)	4.90	0.148	6.23	0.044	1.27
BC063925	Mouse metallothionein II (MT-II)	1.92	0.274	3.32	0.196	1.72
BC077818	Mouse metallothionein-I	2.37	0.08	2.35	0.113	0.99
BC085131	Receptor (calcitonin) activity modifying protein 2 (Ramp2)	1.94	0.137	3.03	0.035	1.55
BC063200	DBY RNA helicase	3.21	0.216	4.61	0.149	1.43
BC074617	Transglutaminase 2, C polypeptide	2.37	0.288	2.83	0.005	1.19
BC070106	Lipocalin (Lcn2)	2.51	0.039	4.59	0.227	1.82
BC080725	Bone morphogenetic protein 15 (Bmp15)	1.70	0.025	3.34	0.058	1.96
BC070855	Glycogenin 1 (Gyg1)	2.89	0.253	5.01	0.129	1.73
BC087418	Mus musculus MS4A11	2.51	0.092	5.38	0.008	2.14
BC072303	Mus musculus deltex 3- like (Drosophila) (Dtx3l)	1.79	0.032	3.11	0.103	1.73
BC064743	EST, homology to Ankyrin 3	1.76	0.075	3.15	0.028	1.78
BC076041	EST	2.49	0.004	4.55	0.142	1.82
AW538759	EST	2.10	0.149	2.16	0.211	1.02
BC068407	EST	2.46	0.095	4.28	0.116	1.74
BC072434	EST	2.10	0.061	3.51	0.0004	1.66
AW545830	MiIP mitochondrion genome	1.90	0.115	3.08	0.046	1.62
C86002	Mitocondrion genome	3.27	0.086	4.44	0.138	1.35

Analysis for data in Table 2 was performed as described in Fig. 9. The numbers represent fold change in gene expression as compared to the universal control, untreated C57BL/6 brainstem and spinal cord. Each data point is the average of 3 to 4 independent experiments from separate RNA preparations.

Table 2

Real time PCR at disease onset.

Strain/Gene	$C_t \pm \text{SEM Disease Onset (Day 6)}$	$C_t \pm \text{SEM Healthy Controls}$	Fold Change
B10.PL/ β_2 -microglobulin	21.4 ± 0.2	24.5 ± 0.1	8
F1/ β_2 -microglobulin	20.6 ± 0.1	24.6 ± 0.1	16
B10.PL/tapasin	25.0 ± 0.2	27.2 ± 0.3	4
F1/tapasin	24.4 ± 0.1	27.5 ± 0.2	8
B10.PL/gamma-induced GTPase	23.9 ± 0.1	30.5 ± 0.1	64
F1/gamma-induced GTPase	23.2 ± 0.1	30.3 ± 0.1	128
B10.PL/IFN- γ	29.0 ± 0.7	41.4 ± 0.6	
F1/IFN- γ	27.2 ± 0.4	40.1 ± 0.3	

Fold change is calculated by the ΔC_t method, where $\Delta C_t = C_t$ in healthy controls – C_t at disease onset. ΔC_t is rounded down to the next whole integer.

Fold change = $2^{\Delta C_t}$. C_t scores were normalized to GAPDH controls ($C_t=20.0$) for each experiment. Each experiment was repeated 4 times for the disease onset condition and 3 times for the control condition.