JCI The Journal of Clinical Investigation

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J Clin Invest. 1999;104(5):599-610. https://doi.org/10.1172/JCI7292.

Article

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Endogenous presentation of self myelin epitopes by CNS-resident APCs in Theiler's virus–infected mice

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Received for publication May 10, 1999, and accepted in revised form July 19, 1999.

The mechanisms underlying the initiation of virus-induced autoimmune disease are not well understood. Theiler's murine encephalomyelitis virus–induced demyelinating disease (TMEV-IDD), a mouse model of multiple sclerosis, is initiated by TMEV-specific $CD4^+$ T cells targeting virally infected central nervous system–resident (CNS-resident) antigen-presenting cells (APCs), leading to chronic activation of myelin epitope–specific $CD4^+$ T cells via epitope spreading. Here we show that $F4/80^+$, $I\text{-}A^{s+}$, $CD45^+$ macrophages/microglia isolated from the CNS of TMEV-infected SJL mice have the ability to endogenously process and present virus epitopes at both acute and chronic stages of the disease. Relevant to the initiation of virus-induced autoimmune disease, only CNS APCs isolated from TMEV-infected mice with preexisting myelin damage, not those isolated from naive mice or mice with acute disease, were able to endogenously present a variety of proteolipid protein epitopes to specific Th1 lines. These results offer a mechanism by which localized virus-induced, T cell–mediated inflammatory myelin destruction leads to the recruitment/activation of CNS-resident APCs that can process and present endogenous self epitopes to autoantigen-specific T cells, and thus provide a mechanistic basis by which epitope spreading occurs.

J. Clin. Invest. **104**:599–610 (1999).

Introduction

The mechanisms underlying the initiation of autoimmunity are not well understood, but studies in a number of human autoimmune diseases strongly suggest that infections often precipitate clinical episodes in genetically predisposed individuals. This has perhaps been best documented in multiple sclerosis (MS), a human autoimmune disease characterized by $CD4^+$ T cell–mediated demyelination of the central nervous system (CNS) and by autoimmune responses to myelin proteins such as myelin basic protein (MBP), proteolipid protein (PLP), and myelin oligodendrocyte glycoprotein (MOG) (1–4). In MS, epidemiological evidence strongly supports the hypothesis that the disease may be initiated by a virus infection (5), although no single virus has been consistently found in MS lesions. It is therefore of great interest to explore mechanisms by which infectious agents trigger autoimmune disease. Potential mechanisms include direct activation of autoreactive T cells that are cross-reactive with a viral epitope(s) (molecular mimicry; ref. 6); nonspecific activation of autoreactive T cells by virus-encoded superantigens (7); and de novo activation of autoreactive T cells by autoepitopes released secondary to virus-specific T cell–mediated bystander damage to self tissues (epitope spreading). We have recently shown that in SJL mice, epitope spreading plays a major role in the progression of 2 clinically distinct T cell–mediated demyelinating diseases that resemble the 2 distinct clinical presentations of MS: relapsing experimental autoimmune encephalomyelitis (R-EAE) and chronic-progressive Theiler's murine encephalomyelitis virus–induced demyelinating disease (TMEV-IDD) (8–11).

R-EAE in the SJL mouse is a $CD4^+$ T cell–mediated autoimmune demyelinating disease that can be induced with a variety of myelin proteins/peptides. The disease presents with a relapsing-remitting paralytic course, characterized clinically by flaccid hindlimb paralysis and histologically by mononuclear cell infiltration and demyelination (12). In PLP139-151–induced R-EAE, the initial clinical relapse is mediated primarily by T cells specific for the secondary, non–cross-reactive PLP178-191 epitope that are activated after acute myelin destruction mediated by $CD4^+$ T cells specific for the initiating PLP139-151 determinant (13).

More relevant to initiation of virus-induced autoimmune diseases, we have also recently shown that the chronic stages of TMEV-IDD involve the activation of autoreactive $CD4^+$ T cells specific for a variety of myelin epitopes (14). TMEV-IDD is the most relevant of the available virus-induced animal models of immune-mediated demyelination (8), and serves as an excellent system in which to assess the potential contribution of

antimyelin autoimmune responses to initiation and progression of clinical disease. TMEV, a picornavirus and natural mouse pathogen, induces a lifelong persistent infection of central nervous system–resident (CNS-resident) antigen-presenting cells (APCs) (15–17), and results in a chronic immune-mediated CNS demyelinating disease when inoculated intracerebrally into susceptible strains of mice. Infected SJL mice develop progressive symptoms of gait disturbance, spastic hindlimb paralysis, and urinary incontinence (18), histologically related to perivascular and parenchymal mononuclear cell infiltration and demyelination of white matter tracts within the spinal cord (19–21). In the highly susceptible SJL mouse strain, current evidence indicates that the myelin damage is initiated by TMEV-specific $CD4^+$ T cells targeting persistent virus antigen (22–26), whereas the chronic stage of the disease also involves the activity of $CD4^+$ myelin epitope–specific T cells primed by epitope spreading (14).

These results illustrate that autoimmune responses are not static, as T-cell responses to endogenous self epitopes emerge during the chronic course of these 2 T cell–mediated CNS inflammatory diseases. As importantly, they indicate that bystander myelin damage initiated by virus-specific immune responses can lead to the activation of naive autoreactive T cells, demonstrating that epitope spreading is an important alternate mechanism to molecular mimicry for explaining the etiology of certain virus-induced, organ-specific autoimmune diseases. Here we show that $F4/80^+$, $I\text{-}A^{s+}$ macrophages/microglia isolated from the CNS of TMEV-infected SJL mice have the ability to endogenously process and present virus epitopes at both acute and chronic stages of the disease. Relevant to the initiation of virus-induced autoimmune disease, CNS APCs isolated from the spinal cords of TMEV-infected mice with preexisting myelin damage, but not CNS APCs isolated from mice at disease initiation or microglia from naive brain, were able to endogenously present a variety of PLP epitopes to specific Th1 lines. To our knowledge, this is the first demonstration of presentation of endogenous autoepitopes in the target organ of a virus-induced disease. These results offer a mechanism by which a localized virus-induced inflammatory myelin destruction results in processing and presentation of endogenous self epitopes by CNS-resident APCs that can activate autoantigen-specific T cells.

Methods

Mice. Female SJL/J mice, 6–7 weeks old, were purchased from Harlan Laboratories (Indianapolis, Indiana, USA). All mice were housed in the Northwestern animal care facility and maintained on standard laboratory chow and water ad libitum. Severely paralyzed mice were afforded easier access to food and water.

Proteins and peptides. Peptides used in this study were synthesized using a Synergy Peptide Synthesizer (ABI, Columbia, Maryland, USA). The sequences were as follows: VP2 70-86 (WTTSQEAFSHIRIPLPH); PLP56-70 (DYEYLINVIHAFQYV); PLP104-117 (KTTICGKGLSATVT); PLP139-151 (HSLGKALGHPDKF); PLP178-191 (NTWTTCQSIAFAPSK); and MBP84-104 (VHFFKNIVTPRT*P*SQGKGR). The amino acid composition and purity (>97%) of these peptides was confirmed by mass spectroscopy at the University of North Carolina–Chapel Hill Biotechnology Center. MP-4, a recombinant fusion protein comprising the 21.5-kDa isoform of human MBP and a recombinant variant of human PLP, was produced and purified as described previously (27, 28).

Antibodies. Anti–B7-1 (clone 16.10.A1), anti–B7-2 (clone 1G10), and the murine CTLA4-Ig fusion protein were supplied by J. Bluestone (University of Chicago, Chicago, Illinois, USA) or purchased from PharMingen (San Diego, California, USA). An mAb directed against the macrophage marker F4/80 was purchased from Caltag Laboratories Inc. (South San Francisco, California, USA). Anti-CD45 (clone 30F11) was purchased from PharMingen. Anti–$I\text{-}A^s$ (clone MKS4) was purified from culture supernatants by using a protein G Sepharose column (Pierce Chemical Co., Rockford, Illinois, USA). Hamster control Ig was purchased from Cappel Research Products (Durham, North Carolina, USA). Avidin-R-phycoerythrin (A-PE) was obtained from Molecular Probes Inc. (Eugene, Oregon, USA). Isotype controls of irrelevant specificity, conjugated to FITC, PE, or biotin as appropriate, were purchased from PharMingen. For FACS staining, all antibodies were titrated using SJL/J spleen cell suspensions. Culture supernates containing anti–$I\text{-}A^s$ (clone MKS4) and anti–$I\text{-}A^d$ (clone 34-4-20) were used for inhibition of MHC class II molecules.

Virus. The BeAn 8386 strain of TMEV is a tissue culture–adapted strain of TMEV that has been plaque purified and passaged in BHK-21 cells grown in DMEM (29). Working stocks of virus were purified by polyethylene glycol precipitation of total BHK-21 cell lysates, sonication in the presence of SDS, and centrifugation over successive sucrose and $CsSO_4$ gradients.

Induction and clinical evaluation of TMEV-IDD. Mice were anesthetized with methoxyflurane (Mallinckrodt Veterinary, Mundelein, Illinois, USA) and inoculated in the right cerebral hemisphere with 9×10^7 plaque-forming units (PFUs) of TMEV, strain BeAn 8386, in 30 μL DMEM. Mice were examined 2–3 times per week for the development of chronic gait abnormalities and spastic paralysis indicative of demyelination (30), and were assigned a clinical score of 0–6 as follows: 0 = asymptomatic; 1 = mild waddling gait; 2 = severe waddling gait, intact righting reflex; 3 = severe waddling gait, spastic hindlimb paralysis, impaired righting reflex; 4 = severe waddling gait, spastic hindlimb paralysis, impaired righting reflex, mild dehydration, and/or malnutrition; 5 = total hindlimb paralysis, severe dehydration, and/or malnutrition; and 6 = death. The data are plotted as the mean clinical score for each group of animals. Each group of animals displayed clinical signs representative of the entire population.

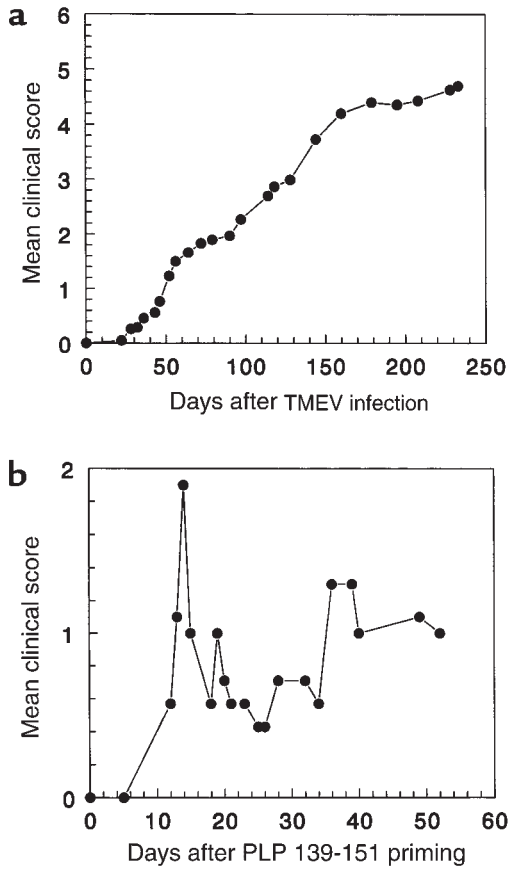

Figure 1
TMEV-IDD and PLP139-151–induced R-EAE have distinct clinical disease courses in SJL mice. TMEV-IDD (**a**) and R-EAE (**b**) were induced in 2 groups of 20 SJL/J mice, and all animals were graded for clinical signs of demyelination as described in the Methods. Results are expressed as mean clinical score of affected animals versus days after immunization/infection. Disease incidence in both groups was 100%.

Induction and scoring of R-EAE. R-EAE was induced by the subcutaneous administration of 0.1 mL of an emulsion containing 50 μg PLP139-151 and 200 μg *Mycobacterium tuberculosis hominis* H37Ra (Difco Laboratories, Detroit, Michigan, USA) divided between 3 sites on the shaved flank (12). Clinical severity was assessed daily and assigned a numerical grade of 0–5 as follows: 0 = asymptomatic; 1= loss of tail tone; 2 = ataxic gait; 3 = hindlimb weakness; 4 = total paralysis of both hindlimbs; and 5 = death.

Isolation of CNS-resident mononuclear cells. For spinal cord APCs, mice were anesthetized with methoxyflurane and perfused through the left ventricle with cold PBS until the effluent ran clear. Spinal cords were extruded by flushing the vertebral canal with cold PBS; they were then rinsed in PBS. The spinal cords were forced through 100-mesh stainless steel screens to give a single-cell suspension, in a BSS containing 300 U/mL per cord of type 4 clostridial collagenase (Worthington Biochemical Corp., Freehold, New Jersey, USA), and were then incubated for 75 minutes (37°C). The spinal cord homogenate was resuspended in 30% Percoll (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA), divided into tubes (equivalent to 5 spinal cords per 5 mL per tube), and underlaid with 70% Percoll (5 mL per tube). The gradients were centrifuged at 500 *g* at 24°C for 20 minutes. CNS mononuclear cells were collected from the 30%/70% interface and were washed and resuspended in RPMI-1640 + 10% FBS. Enrichment of the macrophage/microglia population was accomplished by allowing the cells to adhere to 10-cm plastic tissue culture dishes (75 minutes at 37°C in a humidified CO_2 incubator). Thereafter, the nonadherent cells were removed, and the dish was washed gently with RPMI-1640 + 10% FBS. Cold RPMI was then added to the dish, which was placed on ice for 10 minutes, after which the adherent cells were removed by scraping. The cells were centrifuged and cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 μg/mL streptomycin, and 100 U/mL penicillin (DMEM-10; all from Sigma Chemical Co., St. Louis, Missouri, USA). For naive brain microglia, brains from perfused naive SJL mice were harvested and put in cold 10% FBS in HBSS. The brains were forced through 100-mesh stainless steel screens to give a single-cell suspension, in 10% FBS in HBSS. The brain cells were washed once and incubated for 1 hour at 37°C in digestion media (10 mL per brain) composed of HBSS containing 0.2 mg/mL type 4 clostridial collagenase, and 28 U/mL DNase I (Sigma Chemical Co.). The brain homogenates were washed twice and resuspended in 256 mg/mL Percoll (5–10 brains per 20 mL per tube), and underlaid with 1.088 g/mL Percoll (7 mL per tube). The gradients were centrifuged at 1,000 *g* at 24°C for 20 minutes. Brain mononuclear cells were collected from the interface and washed and resuspended in compete tissue culture media, as already described here.

Isolation of splenocytes. Spleens were removed from naive mice, placed in BSS, and forced through 100-mesh stainless steel screens to yield a single-cell suspension. Erythrocytes in the spleen cell preparations were lysed by hypotonic shock in Tris-NH_4Cl for 5 minutes at 37°C; thereafter, isotonic buffered saline was added, and the cells were washed and resuspended in DMEM-10.

Flow cytometry. The cells to be stained were resuspended in isotonic buffered saline containing 0.1% NaN_3 (IBS; Baxter Diagnostics Inc., McGaw Park, Illinois, USA) and 1.0% normal goat serum (NGS; Pel-Freez Biologicals, Rogers, Arkansas, USA). The cells to be stained were incubated first with anti-FcRγII/III (2.4G2) hybridoma supernatant. The cells (0.5×10^6 to 1×10^6) were stained with F4/80-FITC and a predetermined concentration of anti–I-A^s-biotin, anti–CD45-PE, anti–B7-1-PE, or anti–B7-2-PE and propidium iodide for 30 minutes at 4°C and washed in IBS/NGS; the anti–I-A^s group was incubated with A-PE for 30 minutes at 4°C in the dark, washed again, and resuspended in IBS. Data collection and analysis were performed on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, California, USA) with CellQuest software (Becton

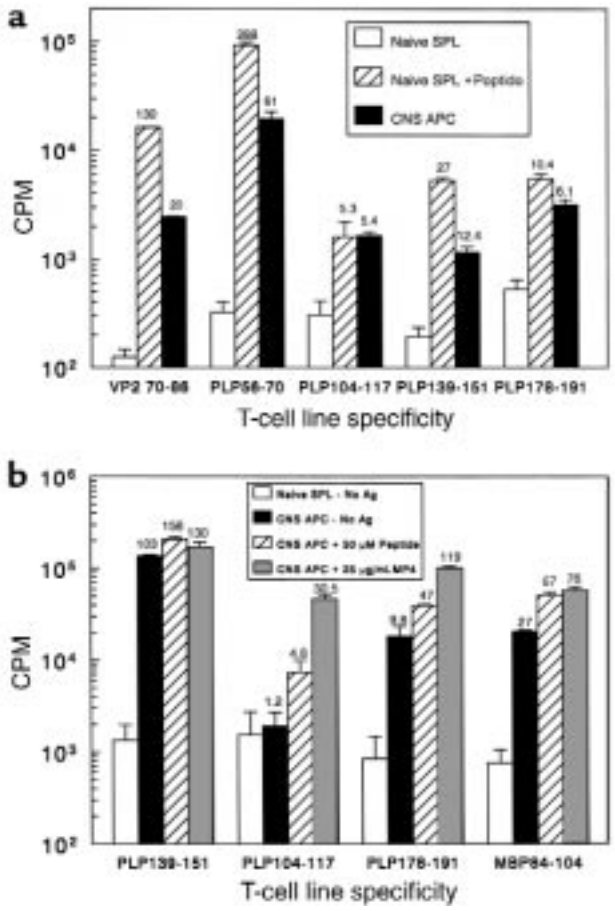

Figure 2
Endogenous presentation of myelin epitopes by CNS-resident APCs from the spinal cords of mice with ongoing TMEV-IDD and PLP139-151–induced R-EAE. (**a**) Plastic-adherent spinal cord mononuclear cells were prepared from SJL mice 88–95 days after intracerebral infection with TMEV. A total of 3×10^4 to 4×10^4 irradiated (30 Gy) CNS APCs were cultured with 4×10^4 of the indicated T-cell lines for 60 hours, and pulsed with 1 μCi of [^{3}H]TdR for the final 16 hours of culture. T-cell lines cultured with 4×10^4 irradiated splenocytes + 50 μM of the appropriate peptide served as the positive control. (**b**) Plastic-adherent spinal cord mononuclear cells were prepared from SJL mice during the recovery period after the acute phase of PLP139-151–induced R-EAE (18 days after immunization). A total of 4×10^4 of the indicated T-cell lines were cultured with either 4×10^4 irradiated naive splenocytes or 2×10^4 irradiated CNS APCs, without the addition of antigen, with 25 μg/mL of the MP-4 MBP/PLP fusion protein, or with 50 μM of the appropriate peptide. Cultures were pulsed with 1 μCi of [^{3}H]TdR at 48 hours and harvested 24 hours thereafter. Values for both groups represent the mean cpm ± SEM of duplicate or triplicate cultures. Stimulation indices are indicated above each bar, and were calculated using the cpm of the T-cell line cultured with naive splenic APCs in the absence of peptide.

Dickinson Immunocytometry Systems). Nonspecific staining was determined by incubating cells with directly labeled, isotype-matched control antibodies.

Maintenance of T-cell lines. Long-term Th1 lines specific for TMEV VP2 70-86 and for 5 encephalitogenic myelin peptides (PLP56-70, PLP104-117, PLP139-151, PLP178-191, and MBP84-104) were established from the lymph nodes of SJL/J mice primed 10 days earlier with 100 μg of the respective peptide emulsified in incomplete Freund's adjuvant supplemented with 200 μg of *M. tuberculosis* H37Ra. Every 3–4 weeks, live T cells were isolated on Ficoll-Histopaque (Amersham Pharmacia Biotech) by centrifugation at 500 *g* at 24°C for 15 minutes and propagated by in vitro stimulation of 10^6 T cells with 5×10^6 irradiated syngeneic splenic cells with 25 μM of the respective peptide for 72 hours. All stimulation assays were performed in DMEM (Sigma Chemical Co.) supplemented with 10% FBS (Sigma Chemical Co.), 2×10^{-3} M L-glutamine (GIBCO BRL, Grand Island, New York, USA), 100 U/mL penicillin (GIBCO BRL), 100 μg/mL streptomycin (GIBCO BRL), 5×10^{-5} M 2-mercaptoethanol, and 0.1 mM nonessential amino acids (Sigma Chemical Co.). Between expansions, T-cell lines were maintained in DMEM supplemented with 10% FBS, 2×10^{-3} M L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 5×10^{-5} M 2-mercaptoethanol, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate (GIBCO BRL), MEM essential vitamins (GIBCO BRL), 0.1 mM asparagine (GIBCO BRL), 0.1 mg/mL folic acid (GIBCO BRL), 0.8% T-STIM (Collaborative Biomedical Research, Bedford, Massachusetts, USA), and recombinant IL-2 (0.2 U/mL) (Boehringer Mannheim Biochemicals, Indianapolis, Indiana, USA). All antigen-presentation assays using T-cell lines were conducted 14–20 days after stimulation.

Antigen-presentation assays. The plastic-adherent fraction of isolated CNS-infiltrating mononuclear cells was assayed for the ability to stimulate the virus and myelin epitope–specific T-cell lines in comparison with naive SJL splenocytes. Irradiated (30 Gy) CNS adherent cells (1.5×10^4 to 4×10^4 per well) isolated from spinal cords of mice with TMEV-IDD or R-EAE, or irradiated SJL splenocytes (3×10^4 to 2×10^5 per well), were cultured with 3×10^4 to 4×10^4 T cells from the different lines in the presence or absence of 5 μg ultraviolet-inactivated TMEV virions, 25 μg/mL MP-4, or varying concentrations (0.5–50 μM) of the appropriate peptide. In all experiments, triplicate cultures at each condition were carried out in flat-bottom 96-well microtiter plates in DMEM-10 supplemented with aminoguanidine (1 mM) to suppress nitric oxide synthetase activity. Proliferative responses were determined by [^{3}H]TdR (0.1 μCi/well) incorporation during the final 16–24 hours of the 48- to 72-hour culture period. Cultures were harvested on 96-well filter plates (Uni-plates; Packard Instrument Co., Meriden, Connecticut, USA) for liquid scintillation counting, and the results were expressed as cpm ± SEM and as stimulation indices (SI = (cpm + Ag)/(cpm – Ag).

Statistical analyses. Differences in T-cell proliferation were analyzed using a 1-tailed Student's *t* test assuming equal variances. Values of $P < 0.05$ were considered statistically significant.

Results

Distinct clinical disease patterns in SJL mice with TMEV-IDD and PLP139-151–induced R-EAE. Groups of 15–20 SJL mice were monitored for development of clinical signs

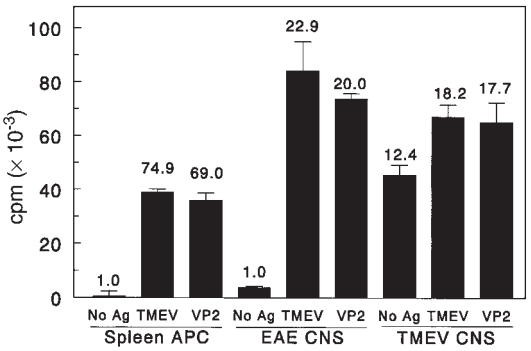

Figure 3
Specificity of endogenous antigen presentation by CNS-resident APCs from the spinal cords of mice with ongoing TMEV-IDD and PLP139-151–induced R-EAE. Plastic-adherent spinal cord mononuclear cells were prepared from groups of SJL mice both 90 days after intracerebral infection with TMEV and 17 days after PLP139-151 immunization. A total of 10^4 T cells from the sTV1 T-cell line, specific for TMEV VP2 70-86 epitope, were cultured with either 5×10^4 irradiated (30 Gy) naive SJL splenocytes or 5×10^4 irradiated CNS APCs, without the addition of antigen, with 5 μg of purified ultraviolet-inactivated TMEV virions, or with 25 μM of VP2 70-86 peptide. Cultures were pulsed with 1 μCi of [^{3}H]TdR at 48 hours, and were harvested 24 hours thereafter. Values represent the mean cpm ± SEM of triplicate cultures. Stimulation indices are indicated above each bar. The background for both CNS-derived APC groups was the value obtained from the culture of the sTV1 T-cell line with CNS APCs from R-EAE mice in the absence of added antigen.

of demyelination after infection with TMEV and active immunization with PLP139-151/CFA. The TMEV-IDD and R-EAE models demonstrate 2 distinct clinical disease courses that resemble the chronic-progressive and relapsing-remitting forms of human MS. Figure 1a shows a typical disease course of SJL/J mice, inoculated intracerebrally with the BeAn strain of TMEV. Initial clinical symptoms of a mild waddling gait first appear approximately 30 days after infection, and follow a chronic-progressive disease course, with most animals progressing to total hindlimb paralysis by 150–200 days after infection. The relapsing-remitting nature of R-EAE is demonstrated in Figure 1b, which shows that the acute clinical disease signs appear around 12–13 days after disease induction and peak on day 14. This is followed by a remission and a number of subsequent relapses peaking on days 20, 29, and 37.

Endogenous presentation of myelin epitopes by APCs isolated from the spinal cords of SJL mice with ongoing TMEV-IDD and PLP139-151–induced R-EAE. Because the progression of both TMEV-IDD and R-EAE are characterized by the activation of CD4$^+$ T cell–mediated immune responses to endogenous myelin epitopes (13, 14), we asked whether CNS APCs isolated from the spinal cords of mice undergoing either or both of the demyelinating diseases could endogenously present self myelin epitopes. We recently reported that endogenously acquired TMEV epitopes could be presented by CNS-resident microglia/macrophages isolated from the spinal cords of TMEV-infected mice around the time of onset of clinical disease (40 days after infection) (31). To test the possibility of endogenous antigen presentation in the CNS of affected mice, plastic-adherent CNS-resident mononuclear cells were purified from the spinal cords of mice undergoing both TMEV-IDD and R-EAE, irradiated, and used as APCs to stimulate a panel of T-cell lines and hybridomas specific for various viral and myelin epitopes.

Plastic adherent CNS-resident mononuclear cells isolated from mice 88–95 days after infection with TMEV (about 60 days after initiation of clinical symptoms) were irradiated and cultured with a panel of SJL-derived Th1 lines specific for the immunodominant TMEV epitope (VP2 70-86) and for a number of encephalitogenic epitopes on PLP (PLP56-70, PLP104-117, PLP139-151, and PLP178-191). As seen in Figure 2a, 3×10^4 to 4×10^4 irradiated APCs from the CNS of the TMEV-infected mice were able to activate significant proliferative responses of 3×10^4 to 4×10^4 Th1 line cells specific for both the virus and each of the PLP epitopes in the absence of addition of exogenous antigen (Figure 2, filled bars). In general, the level of activation was not as great as that achieved by irradiated splenic APCs pulsed with the relevant peptides (Figure 2, hatched bars), but it was nevertheless highly significant in comparison with naive splenocytes in the absence of antigen (open bars) and was reproducible in multiple experiments. Endogenous activation of the Th1 lines depended on the number of CNS APCs added to the culture, and these APCs induced the secretion of IL-2 from the Th1 lines (data not shown). We have recently reported (31) that CNS APCs from TMEV-infected mice do not trigger an I-A^s–restricted horse myoglobin–specific Th1 line, indicating that activation is specific to TMEV and endogenous myelin epitopes. The current results indicate that there is a significant amount of processed, MHC class II–associated VP2 70-86 peptide endogenously present on mononuclear APCs from the spinal cords of mice infected with TMEV almost 3 months previously. This supports the documented ability of TMEV to persist long-term in macrophages/microglia in the CNS target organ (15). Most significantly, APCs resident in the inflammatory CNS environment of TMEV-infected mice were able to process and present a number of endogenous PLP epitopes that have been associated with chronic disease (14).

Plastic-adherent CNS-resident mononuclear cells harvested from mice recovering from acute R-EAE were also able to activate myelin-specific Th1 lines in the absence of added antigen. As seen in Figure 2b, CNS APCs from mice with PLP139-151–induced R-EAE triggered proliferation of a Th1 line specific for the initiating PLP139-151 epitope, as we have shown previously (32). More significantly, these APCs activated Th1 lines specific for relapse-associated, non–cross-reactive epitopes on both PLP (PLP178-191) and MBP (MBP84-104) (filled bars). However, at this point in disease, the CNS APCs did not endogenously activate proliferation of a Th1 line specific for

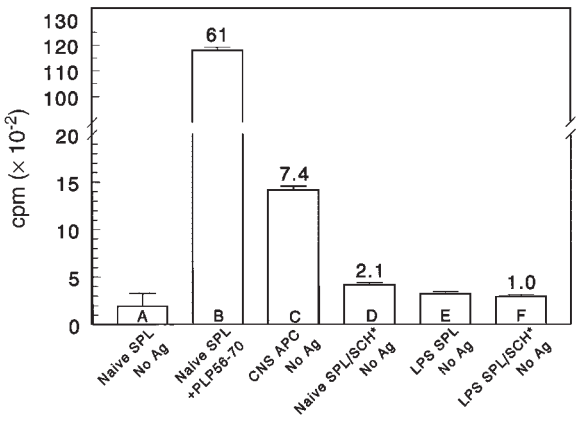

Figure 4
Myelin epitopes are not generated during the isolation of CNS-resident APCs. Spinal cords from naive SJL mice and a group of SJL mice infected with TMEV 85 days previously were dissociated and treated with collagenase. The spinal cord suspension from the naive mice was split and mixed with either naive splenocytes or LPS-preactivated splenocytes at the ratio of 1 spleen equivalent per spinal cord. All 3 suspensions were separated on discontinuous Percoll gradients. The cells were incubated in plastic dishes for 1.5 hours, and the plastic-adherent cells were irradiated (30 Gy). A total of 2.8×10^4 cells from a PLP56-70–specific T-cell line were cultured with the following: 3×10^4 irradiated naive SJL splenocytes in the absence of added peptide (group A); 3×10^4 irradiated naive SJL splenocytes + 50 μM PLP56-70 (group B–positive control); 3×10^4 irradiated, plastic-adherent CNS APCs from TMEV-infected mice (group C); 3×10^4 irradiated naive splenocytes recovered from Percoll gradients after admixture with the naive spinal cord suspension (group D); 3×10^4 irradiated, LPS-preactivated splenocytes (group E); and 3×10^4 irradiated, LPS-preactivated splenocytes recovered from Percoll gradients after admixture with the naive spinal cord suspension (group F). Cultures were pulsed with 1 μCi of [^{3}H]TdR at 48 hours, and were harvested 16 hours thereafter. Values represent the mean cpm ± SEM of triplicate cultures. Stimulation indices are indicated above each bar, and were calculated using the cpm of the PLP56-70–specific T-cell line cultured with irradiated naive splenic APCs in the absence of peptide. *Naive and LPS-preactivated splenocytes in these groups were processed similarly to the CNS APCs.

the less dominant PLP104-117 epitope. It is significant that these CNS APCs could both present exogenous peptide (hatched bars) and process and present the MP-4 fusion protein (gray bars), containing the 21.5-kDa isoform of MBP and a recombinant variant of human PLP (27, 28), to all of the different T cells. Thus, a variety of immunodominant epitopes within the major myelin proteins PLP and MBP are endogenously displayed by CNS APCs isolated from mice during the acute phase of PLP139-151–induced R-EAE, and these cells are highly efficient at processing encephalitogenic myelin determinants.

Analysis of specificity of presentation of endogenous myelin epitopes by CNS-resident APCs. To determine the specificity of endogenous antigen presentation, we compared the ability of CNS APCs from mice with TMEV-IDD (90 days after infection) to APCs from mice with PLP139-151–induced R-EAE to activate a Th1 line (sTV1) specific for the immunodominant TMEV VP2 70-86 epitope. As shown in Figure 3, irradiated splenocytes from naive mice were able to process and present ultraviolet-inactivated TMEV virions and present the VP2 70-86 peptide to the sTV1 line. CNS APCs from mice with both TMEV-IDD and R-EAE were also able to process efficiently intact virions and present peptide to the VP2 70-86–specific T-cell line. However, only CNS APCs from TMEV-infected mice were able to endogenously activate the virus-specific Th1 line. As expected, CNS APCs from mice with R-EAE, which had never been exposed to TMEV, did not induce proliferation of the virus-specific cells, indicating the specificity of the endogenous presentation.

To ensure that the endogenous activation of virus- and myelin-specific T cells was due to preexisting peptide/I-A^s complexes on the cell surface of CNS APCs and not due to the uptake of myelin and/or viral fragments during cell isolation, we performed several control experiments. First, we dissociated spinal cords from naive mice in parallel with spinal cords from TMEV-infected donors. Before Percoll gradient separation, either naive splenocytes or splenocytes preactivated with LPS for 3 days in vitro were added to the naive spinal cord suspension. Figure 4 demonstrates that both peptide-pulsed splenocytes and CNS APCs isolated from TMEV-infected donors (85 days after infection) could activate a Th1 line specific for the PLP56-70 epitope. However, neither naive nor LPS-preactivated splenocytes mixed with dissociated spinal cords from normal mice and later reisolated on Percoll gradients were capable of activating the myelin peptide–specific T-cell line. Similar results were obtained with T cells specific for the immunodominant PLP139-151 epitope (data not shown). Thus, uptake of myelin proteins during APC isolation appears to be minimal. We also showed the endogenous presentation of myelin epitopes by CNS APCs from TMEV-infected mice was not diminished by the addition of the antigen-processing inhibitor leupeptin during the entire isolation procedure (data not shown).

Efficient endogenous presentation of myelin epitopes by CNS APCs from TMEV-infected mice requires preexistent myelin damage. It is logical to assume that loading of myelin peptides on APCs in the CNS of TMEV-infected mice would require prior myelin damage, whereas endogenous presentation of virus epitopes should be independent of the level of myelin destruction. We thus isolated CNS APCs from TMEV-infected mice that displayed minimal clinical and histological signs of disease (days 40–42 after infection). Unlike APCs derived from mice with severe clinical disease at 88–95 days after infection (Figures 2a, 3, and 4), APCs isolated from mice at onset of TMEV-induced disease (Figure 5a) stimulated vigorous proliferation (SI = 41) and IL-2 secretion (data not shown) of the sTV1 VP2 70-86–specific T-cell line but were incapable of activating a T-cell line specific for the immunodominant PLP139-151 epitope to either proliferate (SI = 1.5) or secrete IL-2. CNS APCs purified from TMEV-infected mice at 45–50 days after

infection also failed to activate a PLP56-70–specific T cells (data not shown). This result indicates that a threshold level of myelin destruction must occur before CNS APCs can efficiently activate myelin-specific T cells. In addition, this result again indicates that there is little or no exogenous uptake of myelin proteins during isolation of the CNS APCs. These conclusions are supported by the fact that microglia isolated from the brains of naive mice (it was not possible to isolate sufficient cells from naive spinal cords) failed to endogenously activate either a PLP139-151–specific T-cell line (Figure 5b) or a VP2 70-86–specific T-cell hybridoma (Figure 5c). In contrast, naive microglia could present myelin and virus peptides in a dose-dependent manner, and were capable of processing the MP-4 fusion protein for presentation of PLP139-151.

Endogenous presentation of myelin epitopes by CNS APCs from TMEV-infected mice is B7 dependent and MHC class II restricted. The CD28/B7 costimulatory pathway is critical for T-cell activation, particularly activation of naive T cells (33). To characterize further the in vivo potential of APCs resident in the CNS of mice with ongoing TMEV-IDD to activate naive T cells specific for endogenous myelin epitopes, we examined the B7 dependency and MHC restriction of the CNS APCs. As seen in Figure 6a, endogenous presentation of myelin epitopes by CNS APCs from TMEV-infected mice is MHC class II restricted, as activation of a PLP139-151–specific T-cell line was specifically inhibited by a monoclonal anti–I-A^s antibody. The B7 dependence of the presentation is illustrated by data showing that T-cell activation is significantly inhibited both by the combination of anti–B7-1 and anti–B7-2 mAb's (Figure 6b) and by murine CTLA4-Ig (Figure 6a), which blocks B7-1 and B7-2. A predominance of B7-2–mediated costimulation on the CNS APCs is suggested by the enhanced ability of anti–B7-2 (72% inhibition), compared with anti–B7-1 (44% inhibition), to block activation of the PLP139-151–specific Th1 line (Figure 6b).

Phenotypic characterization of CNS-resident mononuclear APCs. Flow cytometric analysis of the CNS-resident mononuclear cells isolated from the spinal cords of TMEV-infected SJL mice 90 days after infection on discontinuous Percoll gradients was carried out. In general, these preparations yielded between 5×10^4 and 5×10^5 total mononuclear cells per spinal cord. Approximately 50% of these cells bore the F4/80 marker (Figure 7a) and Mac1 (data not shown), which are specific macrophage/microglia lineage markers (34). The percentage of F4/80$^+$/Mac1$^+$ cells is enriched to 70–80% of the total lymphoid cells following the plastic-adherence step, with a usual yield of 1.25×10^4 to 2.5×10^4 cells per spinal cord (data not shown). These cells are large and vacuolated under the light microscope. The majority of CNS-resident F4/80$^+$ cells (70–85%) express I-A^s (Figure 7b), B7-1 (Figure 7c), and B7-2 (Figure 7d). Based on mean fluorescence intensity, relatively more B7-2 than B7-1 molecules are expressed on the cell surface of the F4/80$^+$ population. This correlates with the

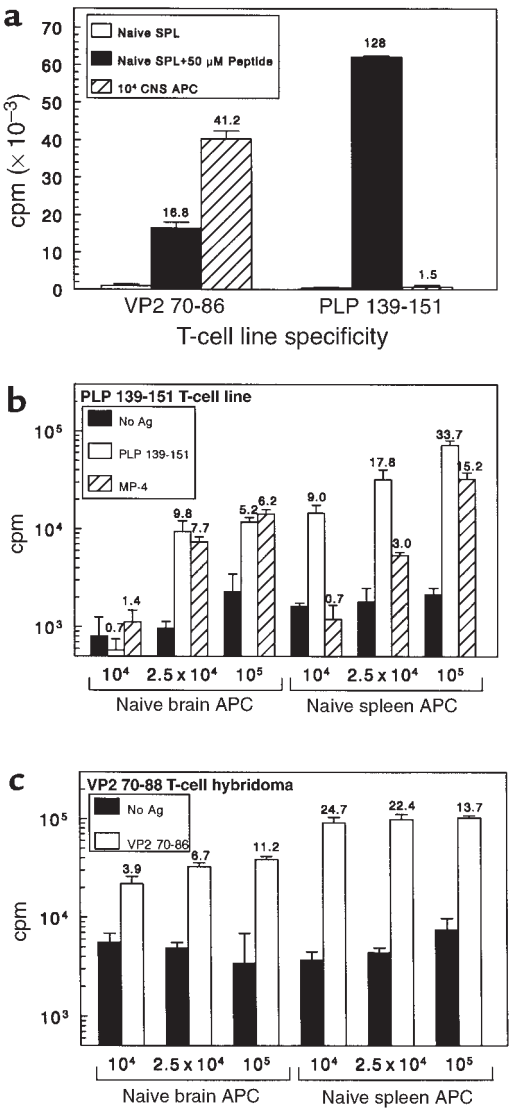

Figure 5
Viral, but not myelin, epitopes are endogenously presented by CNS-resident APCs isolated from SJL mice at the onset of TMEV-IDD. (**a**) A total of 1.8×10^4 plastic-adherent spinal cord mononuclear cells were prepared from SJL mice 42 days after intracerebral infection with TMEV. The CNS APCs were irradiated and cultured with either 3×10^4 sTV1 T cells (specific for VP2 70-86) or the same number of T cells from a long-term PLP139-151–specific line. T-cell lines cultured with 1.8×10^4 irradiated splenocytes + 50 µM of the appropriate peptide served as the positive control. (**b**) A total of 10^4 to 10^5 irradiated microglia isolated from the brains of naive SJL mice or irradiated naive splenocytes were cultured with 3×10^4 T cells from a long-term PLP139-151–specific line in the presence or absence of 50 µM of PLP139-151 or 25 µg/mL of the MP-4 MBP/PLP fusion protein. (**c**) A total of 10^4 to 10^5 irradiated microglia isolated from the brains of naive SJL mice or irradiated naive splenocytes were cultured with 3×10^4 cloned T-cell hybridoma cells specific for TMEV VP2 70-86 in the presence or absence of the peptide. For **a** and **b**, cultures were pulsed with 1 µCi of [^{3}H]TdR at 48 hours, and were harvested 16–24 hours thereafter. For **c**, culture supernates were harvested after 48 hours and tested for their ability to support the proliferation of the IL-2–dependent CTLL-2 cell line. Values represent the mean cpm ± SEM of triplicate cultures. Stimulation indices are indicated above each bar.

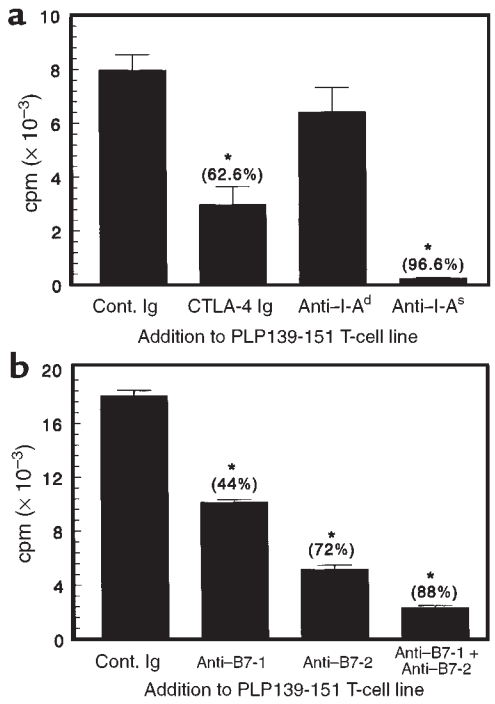

Figure 6
Endogenous presentation of myelin epitopes by CNS-resident APCs isolated from SJL mice with ongoing TMEV-IDD is B7 dependent and MHC class II restricted. (**a**) A total of 5×10^4 irradiated, plastic-adherent CNS APCs isolated from mice infected 115 days previously with TMEV were cultured with 3×10^4 PLP139-151–specific T cells for 72 hours and pulsed with 1 μCi of [^{3}H]TdR for the final 24 hours of culture. CNS APCs were incubated with 10 μg/mL of mouse control Ig, mCTLA-4 Ig, anti–I-A^d, or anti–I-A^s for 30 minutes before the addition of the T-cell line. (**b**) A total of 3×10^4 irradiated, plastic-adherent CNS APCs isolated from mice infected 125 days previously with TMEV were cultured with 3×10^4 PLP139-151–specific T cells for 72 hours and pulsed with 1 μCi of H]TdR for the final 24 hours of culture. CNS APCs were incubated with 10 μg/mL of hamster control Ig, anti–B7-1, anti–B7-2, or a combination of anti–B7-1 and anti–B7-2 for 30 minutes before the addition of the T-cell line. Values for both experiments represent the mean cpm ± SEM of triplicate cultures. Figures in parentheses above each bar indicate the percentage inhibition in comparison to the addition of control Ig. *Inhibition is statistically significant ($P < 0.01$).

enhanced ability of anti–B7-2, compared with anti–B7-1, to inhibit endogenous activation of the PLP56-70–specific Th1 line by CNS APCs from TMEV-infected mice (Figure 6b). Our previous analyses (31) have shown that only 30% of F4/80$^+$ cells from the spleen of TMEV-infected SJL mice express MHC class II, and that F4/80$^+$ cells account for only 4% of the I-A^{s+} cells.

The CNS F4/80$^+$ population could be separated into 2 subpopulations based on surface expression of the leukocyte common antigen CD45. Approximately 60% of these cells bear intermediate levels of CD45, whereas around 30% express high levels of CD45 (Figure 7e). Gating on the CD45$^+$ subpopulations revealed that expression of both B7-1 and B7-2 was higher on the CD45^{hi} population than on the CD45^{lo} population. This is significant in that Ford et al. (35) have previously shown that the CD45^{hi} population represents perivascular macrophages in brain tissue that are functionally capable of presenting MBP to a specific T-cell line, whereas the CD45^{lo} population represents microglia that are relatively poor APCs. It is therefore of interest to compare the relative ability of sorted CD45^{hi} and CD45^{lo} populations to present endogenous myelin epitopes.

Discussion

The initiation and progression of a number of autoimmune diseases, including MS (5, 36), are strongly suspected to arise secondary to virus infections. Therefore, determining the mechanisms by which infections lead to autoimmune sequelae is an area of great interest, and may provide important clues about the pathogenesis and regulation of autoimmune diseases. Molecular mimicry has been postulated to be a prime mechanism whereby infections can lead to the initiation of T-cell autoreactivity (6, 37–39), but in general there has been a paucity of direct evidence supporting this hypothesis. Alternatively, epitope spreading — the process whereby epitopes distinct from, and non–cross-reactive with, an inducing epitope become major targets of an ongoing immune response — has been implicated in the pathogenesis of both human autoimmune diseases and their experimental models. In animal models of autoimmunity, there is mounting evidence that chronic inflammatory-mediated tissue damage can lead to de novo activation of autoreactivity via epitope spreading. The primary examples of epitope spreading have been in T cell–mediated autoimmune models, such as R-EAE (13, 40, 41) and diabetes in nonobese diabetic mice (42–44). Epitope spreading has also been demonstrated after infection with the picornaviruses, TMEV (14), and Coxsackie virus (45). Recent evidence has suggested that local expression of proinflammatory cytokines such as TNF-α promotes autoimmunity by enhancing presentation of autoantigens (46). Although epitope spreading has generally been described as a Th1 phenomenon, recent evidence shows that Th2 epitope spreading may serve as an intrinsic negative feedback mechanism to regulate autoimmune pathology (47). Epitope spreading has also been described at the B-cell level in systemic lupus erythematosis, which is mediated by pathogenic autoantibodies (48, 49). Although there is yet no conclusive proof that epitope spreading is a general feature in human autoimmune diseases, recent evidence suggests that it may play a role in autoimmune skin diseases (50) and in the chronic-progressive course of MS (51).

Our laboratory has used the R-EAE and TMEV-IDD models of CD4$^+$ T cell–mediated CNS demyelination to study the functional significance of T-cell responses specific for endogenous myelin epitopes that arise during the chronic course of CNS damage in these 2 diseases. R-EAE in the SJL mouse is a Th1-mediated autoimmune demyelinating disease, useful in dissecting the immune response to chronic tissue damage because disease can be induced in a peptide-specific

manner, and the identity and relative dominance of encephalitogenic epitopes on a variety of myelin proteins, including MBP, PLP, and MOG, have been well defined. Both intra- and intermolecular epitope spreading play important pathologic roles in the progression of ongoing autoimmune disease in R-EAE, because blockade of T-cell reactivity to the relapse-associated myelin epitopes, either by costimulatory antagonists (52, 53) or by antigen-specific tolerance (13, 41, 54) (C.L. Vanderlugt et al., unpublished study), results in inhibition of clinical relapses. Myelin damage in TMEV-infected SJL mice is initiated by TMEV-specific $CD4^+$ T cells targeting virus persisting in CNS-resident APCs, leading to upregulation of proinflammatory cytokines in the CNS (22–26, 55). Similar to R-EAE, the chronic stage of TMEV-IDD is associated with the activation of $CD4^+$ myelin epitope–specific T cells primed by epitope spreading, as there are no apparent virus epitopes that are shared with the encephalitogenic myelin epitopes on PLP, MBP, or MOG (14).

There are a variety of cells within the normal CNS with antigen presentation potential, including astrocytes, microglia, and macrophages. IFN-γ–treated primary astrocytes (56, 57) and microglia (58, 59) cultured from neonatal mouse brain upregulate MHC class II and can present antigens to T cells in vitro. It is important to realize that antigen presentation by neonatal cells in long-term culture may not faithfully reproduce the in vivo state in adult animals. Microglia directly isolated from adult rats can more efficiently present MBP to T-cell lines in vitro than can neonatally derived microglia (35), but they are inefficient in endogenously activating myelin-specific T cells (Figure 5b). Studies using allogeneic bone marrow chimeras have supported the idea that cells of hematopoietic origin (i.e., microglia and macrophages) are the principal APCs active in the CNS during the initiation of EAE (60–62). Although they are much more abundant than microglia, astrocytes are significantly less potent when inducing EAE in chimeras (62).

How and where do T cells specific for endogenous myelin epitopes become activated during the initiation and progression of R-EAE and TMEV-IDD? It is possible that T cells specific for relapse-associated epitopes are activated in the peripheral immune organs. After inflammatory disruption of the blood-brain barrier, myelin debris and/or macrophages/microglia that have ingested myelin proteins within the CNS may gain access to the cervical lymph nodes, which drain the cerebrospinal fluid (63), or to the spleen, which concentrates blood-borne material. In support of the idea that APCs can traffic from the CNS to peripheral lymphoid organs, it has been reported that donor cells from alloantigen-disparate solid CNS grafts placed intracerebrally can be later identified in the host spleen and lymph nodes (64). It is also possible that T cells specific for endogenous myelin epitopes are activated in the local inflammatory environment within the CNS. In both R-EAE and TMEV-IDD, the lymphocytic infiltrate

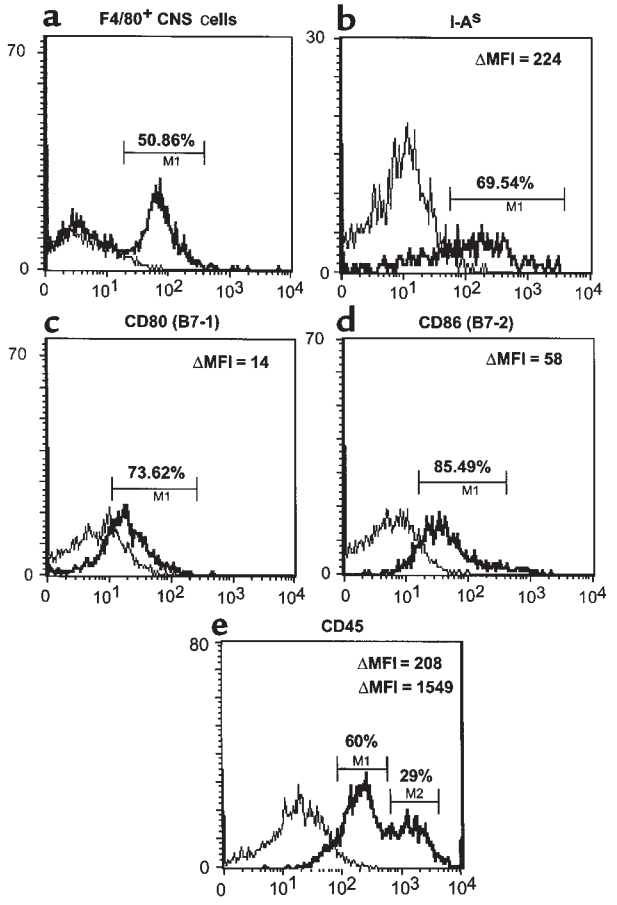

Figure 7
Phenotypic characterization of CNS APCs isolated from SJL mice with ongoing TMEV-IDD. Spinal cord CNS mononuclear cells were isolated on discontinuous Percoll gradients from 25 SJL mice infected 102 days previously with TMEV. The cells were stained with anti–F4/80-FITC and anti–I-A^s-biotin, anti–B7-1-PE, anti–B7-2-PE, or anti–CD45-PE followed by A-PE (anti–I-A^s group only). Approximately 50% of the CNS mononuclear cells expressed F4/80 (**a**), and the remainder were $CD3^+$ (data not shown). By electronic gating on the F4/80$^+$ cells, it can be seen that 70–85% of the CNS-resident macrophage/microglial cells expressed I-A^s (**b**), B7-1 (**c**), and B7-2 (**d**). Approximately 90% of the F4/80$^+$ cells were $CD45^+$, with 60% bearing intermediate levels and 30% bearing high levels of this marker (**e**).

is composed of numerous I-A^{s+}, $B7^+$-activated microglia, and macrophages, derived from both the CNS-resident pool and macrophages or monocytes infiltrating from the peripheral blood (31, 32, 52). Macrophages/microglia within the demyelinated areas also contain phagocytized myelin debris (65). Considered together with the fact that the majority of T cells infiltrating the CNS during immune-mediated demyelinating diseases are thought to be naive "bystander" cells that are not specific for the inducing antigen, the CNS theoretically provides an ideal environment for the activation of naive autoreactive $CD4^+$ T cells.

In the present report, we examined the potential presentation of viral and myelin epitopes within the CNS of mice with ongoing T cell–mediated demyelinating disease. After a recent report in which we

demonstrated endogenous presentation of an immunodominant viral epitope by CNS APCs from TMEV-infected mice (31), we asked whether myelin destruction initiated by TMEV-specific $CD4^+$ T cells would also lead to the uptake, processing, and presentation of myelin epitopes. The current results clearly indicate that a variety of self myelin epitopes are endogenously processed and displayed in the context of MHC class II molecules on the surface of plastic-adherent, $F4/80^+$, $I\text{-}A^{s+}$ mononuclear APCs isolated from the CNS of SJL mice with preexisting myelin damage (Figure 2a), but not from naive mice (Figure 5, b and c) or mice in the initial stages of TMEV-IDD (Figure 5a). In contrast, TMEV epitopes were associated with CNS-resident mononuclear APCs both before the onset of clinical disease and in mice with extensive myelin damage (85–120 days after TMEV infection) (Figure 2a), supporting previous findings that TMEV persist long term in macrophages/microglia in the CNS (15). The temporal difference in the availability of TMEV versus myelin epitopes indicates a lack of cross-reactivity between viral and self myelin epitopes. This lack of cross-reactivity is supported by the failure of APCs from mice with R-EAE to activate a TMEV VP2-specific T-cell line (Figure 3), and by our previous report showing that T-cell clones and lymph node T cells specific for immunodominant TMEV and myelin epitopes do not cross-react (14).

The phenotypic characteristics of the cells presenting endogenous myelin epitopes (i.e., $F4/80^+$, MHC class II^+, $B7\text{-}1^+$, $B7\text{-}2^+$; Figure 7) are characteristic of activated macrophages and microglia. This conclusion is also supported by the bimodal distribution of the leukocyte common antigen CD45, expressed on the APCs (Figure 7). Previous studies have indicated that naive CNS-resident microglia express low levels of CD45, whereas activated microglia express intermediate levels of this marker. Both naive and activated parenchymal microglia are relatively poor APCs, as determined by induction of T-cell proliferation, but they are more efficient in stimulating Th1 differentiation, as assessed by IFN-γ production. In contrast, $CD45^{hi}$ perivascular microglia and infiltrating macrophages have been shown to be highly efficient APCs (35, 66, 67). Our finding that the CNS APCs capable of efficient presentation of endogenous myelin epitopes were of the macrophage/microglia lineage is consistent with previous studies. Using immunohistochemistry, we showed that MHC class II was predominantly expressed on macrophages/microglia in spinal cord demyelinating lesions in TMEV-infected mice, and that the majority of cells in these lesions were sialoadhesin-bearing infiltrating macrophages (31). Macrophages/microglia are the cells that predominantly harbor persistent TMEV infection (15–17), and the number of $F4/80^+$ cells increases to 2- to 3-fold the number of $CD4^+$ T cells in spinal cords 50–60 days or more after infection (55), around the time when endogenous myelin epitopes are functionally expressed (Figure 1a). In addition, these MHC class II–bearing cells expressed B7-1 and B7-2 at levels exceeding those in the spleens of infected mice (31). B7-1 was expressed predominantly on infiltrating macrophages, whereas B7-2 was expressed on both $F4/80^+$ macrophages/microglia and a subpopulation of $CD4^+$ T cells. Thus, CNS-resident mononuclear APCs present virus and myelin epitopes, and express the requisite costimulatory molecules required for activation of naive myelin epitope–specific $CD4^+$ T cells.

Comparison of MHC class II and B7 expression of CNS-resident APCs between TMEV-IDD and R-EAE is important in relation to our data on the manipulation of B7 molecules in vivo during R-EAE in the SJL mouse (52). The current functional (Figure 6) and FACS (Figure 7) analyses indicate that both B7-1– and B7-2–mediated costimulatory processes are important on CNS-resident $F4/80^+$ cells isolated from SJL mice with ongoing TMEV-IDD. In contrast, our recent studies have shown that that B7-1 becomes the dominant costimulatory molecule in SJL mice with ongoing R-EAE, as its surface expression level and functional role in T-cell activation are significantly increased relative to B7-2 both in the CNS and in the peripheral lymphoid tissues (32, 52). Moreover, blockade of B7-1 by treatment of SJL mice with anti–B7-1 F(ab) fragments during R-EAE remission inhibited subsequent disease relapses by preventing activation of T cells specific for endogenous myelin epitopes (13, 52). Thus, the costimulatory dependence of epitope spreading differs in these 2 models of T cell–mediated demyelination.

For multiple reasons, the finding that CNS mononuclear APCs from TMEV-infected mice contain and present viral and myelin antigens to T cells ex vivo is significant to understanding the pathogenesis of MS. First, MHC class II–bearing macrophages, astrocytes, and endothelial cells have been observed in lesions from patients with MS (68–70). Expression of B7 costimulatory molecules has also been demonstrated in MS lesions (71–73). Therefore, multiple cells in MS lesions are equipped to activate fully both naive and memory T cells within the CNS. Second, IL-2R–bearing cells have been observed in the lesions (68), as have products of activated T cells, including IFN-γ (74) and IL-2 (68), further suggesting local antigen presentation. Third, the epidemiology of MS strongly suggests a role for an infectious agent, perhaps a virus, that is widespread, chronic, and usually subclinical (5). Presentation within the CNS of viral antigens (leading to bystander demyelination), of neuroantigens cross-reactive with viral antigens (molecular mimicry), or of neuroantigens liberated by virus-induced CNS damage (epitope spreading) are all possible mechanisms by which pathogenic immune reactions could be initiated by viruses within the CNS. Our results showing the temporal availability of first, virus epitopes, and later, both virus and myelin epitopes, on CNS APCs support a chronic CNS infection, such as that observed in SJL mice infected with TMEV, as a plausible initiating event in the autoimmune pathogenesis of MS.

Acknowledgments

This work was supported by the United States Public Health Service/National Institutes of Health (grants NS23349, NS26543, and NS34819) and a by National Multiple Sclerosis Society Postdoctoral Fellowship (to Y. Katz-Levy).

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