Disproportionate Recruitment of CD8⁺ T Cells into the Central Nervous System by Professional Antigen-Presenting Cells

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Inappropriate immune responses, thought to exacerbate or even to initiate several types of central nervous system (CNS) neuropathology, could arise from failures by either the CNS or the immune system. The extent that the inappropriate appearance of antigen-presenting cell (APC) function contributes to CNS inflammation and pathology is still under debate. Therefore, we characterized the response initiated when professional APCs (dendritic cells) presenting non-CNS antigens were injected into the CNS. These dendritic cells expressed numerous T-cell chemokines, but only in the presence of antigen did leukocytes accumulate in the ventricles, meninges, subarachnoid spaces, and injection site. Within the CNS parenchyma, the injected dendritic cells migrated preferentially into the white matter tracts, yet only a small percentage of the recruited leukocytes entered the CNS parenchyma, and then only in the white matter tracts. Although T-cell recruitment was antigen specific and thus mediated by CD4⁺ T cells in the models used here, CD8⁺ T cells accumulated in numbers equal to or greater than that of CD4⁺ T cells. Few of the recruited T cells expressed activation markers (CD25 and VLA-4), and those that did were primarily in the meninges, injection site, ventricles, and perivascular spaces but not in the parenchyma. These results indicate that 1) the CNS modulates the cellular composition and activation states of responding T-cell populations and that 2) myelin-restricted inflammation need not be initiated by a myelin-specific antigen. (*Am J Pathol* 1999, 154:481–494)

Because it is essential for an organism's survival, the central nervous system (CNS) must be defended from pathogens and other insults. However, inappropriate immune responses within the CNS are thought to initiate or exacerbate neurotoxic and neurodegenerative symptoms observed in a wide variety of neuropathologies. Until recently, the active participation of the CNS in these

immune processes was largely ignored. The CNS was assumed to be isolated passively behind the blood-brain barrier (BBB) and to be uninvolved in regulation of immune responses. It has now been realized that leukocyte migration into the CNS is not blocked by the BBB, and the CNS itself suppresses or fails to support the initiation of immune responses. For example, allografts transplanted into the CNS, unlike those transplanted into peripheral sites, are not rejected.^{1,2} Similarly, rapid T cell responses are mounted against bacillus Calmette-Guerin (BCG) injected into peripheral sites but not sites in the CNS.³ The CNS is not completely impervious to T-cell responses; peripheral initiation of allograft- or BCG-directed immune responses does lead to T-cell recruitment in the CNS.^{1–3} Yet, as in experimental autoimmune encephalomyelitis (EAE), demyelinating perivenous encephalomyelitis, and multiple sclerosis, T-cell recruitment is anatomically restricted.^{4–10} In the early stages of disease, lymphocyte recruitment is enriched in the perivascular, meningeal, and ventricular spaces as compared with the parenchyma. Thus, although some T cells infiltrate the CNS parenchyma, most do not^{4–10}.

Several possible mechanisms are thought to contribute collectively to the high threshold for the activation and infiltration of T cells into the CNS parenchyma. First, the presence of a BBB coupled with the absence of draining lymphatics reduces the number of immune cells and the rate at which immune cells interact with the CNS as compared with other tissues.^{1,2,4} Second, major histocompatibility (MHC) class I and MHC class II proteins are expressed at very low levels in the CNS parenchyma as compared with other tissues^{2,4,5,11–13}. As a consequence, under normal, nonpathological circumstances, the CNS lacks a resident cell population that is equipped to present antigen to T cells.^{11,13,14} By contrast, macrophages in the perivascular spaces, meninges, and choroid plexus do express MHC class I and II proteins and thus may be able to act as antigen-presenting cells (APCs).^{2,11–13} Hence, allografts can be rejected and T-cell responses can be initiated in these latter re-

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gions.^{2,3,11,13} Third, the CNS environment may inhibit T-cell activation directly. Ceramides, transforming growth factor (TGF)- β 1 production, and antigen-specific interactions between T cells and either astrocytes or microglia have all been suggested to suppress T-cell activation or even to induce T-cell apoptosis in the CNS.^{15–19} Thus, although antigen-specific responses may be generated in the perivascular spaces, meninges, ventricles, or even within allografts that contain their own resident APCs, T-cell responses may have difficulty spreading into the CNS parenchyma.

In vivo, the relative contributions of these factors have been difficult to ascertain because it is rarely possible to manipulate the parameters individually without global activation of the peripheral immune system or gross changes in CNS physiology. It is unclear whether the development of spontaneous CNS pathologies such as multiple sclerosis are due more to the failures of the CNS to limit immune responses or to the overactivation of leukocytes outside the CNS. Studies of early cases of multiple sclerosis favor the former possibility because the initial demyelinating lesions are composed primarily of activated microglia/macrophages and not lymphocytes.^{4,5,9} Lymphocyte accumulation appears fairly restricted to perivascular cuffs in the early stages of disease.

Similarly, it is unclear whether the higher failure rate of xenografts as compared with allografts is primarily T cell mediated. Suggestively, allografts in the CNS fail with an increasing rate correlating with increasing antigenic differences between donor and host.^{1,2} However, in most types of CNS graft failure, very few lymphocytes are found; rather, activated microglia/macrophages appear to mediate most of the graft damage. It is unclear whether the few lymphocytes recruited are sufficient to direct the microglia/macrophage-inflicted damage or whether their contribution to graft failure is of low significance.^{2,8,20}

Here, we have tested whether CNS immune privilege results primarily from the absence of an effective resident APC population by injecting bone-marrow-derived dendritic cells directly into the CNS. These dendritic cells are 100- to 1000-fold more potent APCs than macrophages.¹² In contrast to the CNS, most tissues have similar resident populations of tissue dendritic cells that can efficiently process and present antigen and that frequently recirculate to the lymph nodes.^{12,13} Many of the myelin and neural antigens usually used as T-cell targets in EAE are also expressed at very high levels in the thymus and spleen where their presence has been suggested to regulate ongoing T-cell responses.^{21–26} Consequently, to study CNS-specific modulation of immune responses independent of CNS epitopes, we used two types of stimuli that have been well characterized in both *in vitro* assays and peripheral tissue immune responses: 1) allogeneic stimuli in which fewer than 5% to 10% of the T-cell population are potential responders and 2) the moth cytochrome C peptide, in a transgenic mouse model in which greater than 90% of the T cells are potential responders. We found that, whereas potent APCs expressed numerous T-cell chemokines, T cells accumulated in the CNS only in an antigen-specific manner. The antigenic stimuli used were not myelin related and should

have triggered primarily CD4⁺ T cell responses. Surprisingly, although leukocyte recruitment occurred predominantly in the ventricles, meninges, subarachnoid spaces, and the injection site, parenchymal leukocyte infiltration occurred almost exclusively in white matter tracts and consisted of equal numbers of CD4⁺ and CD8⁺ cells.

Materials and Methods

Mice

Dendritic-cell cultures were prepared from C57Bl/6, B10.(A)5R, B10.D2, and 107KO mouse strains and injected into C57Bl/6, 107KO, or ANDB6 mice. The AND transgenic mice express a transgenic TCR specific for moth cytochrome c peptide 88–102 presented on class II I-E^b.²⁷ The mice are maintained on a C57Bl/6 background; therefore, the TCR-transgenic T cells are positively selected on I-A^b even in the absence of the restricting element I-E^b. Thus, these mice contain clonotype-positive CD4⁺ T cells (>90% of all T cells) but do not contain APCs that can stimulate antigen-specific responses. Dendritic cells prepared from either the B10.(A)5R and 107KO mouse strains express I-E^b and can act as APCs to transgenic AND T cells.²⁸ The 107KO mouse line is maintained on a C57Bl/6 background and does not express any MHC class II other than I-E^b.^{14,28}

Dendritic Cell Isolation

Dendritic cells were isolated from bone marrow cultures essentially as described.¹² Briefly, marrow from femur bones was eluted in RPMI 1640. Cells were recovered by centrifugation and cultured at 1 mouse equivalent per 150-mm plate in RPMI 1640 plus 10% fetal bovine serum (FBS), 25 mmol/L Hepes, 1 mmol/L glutamine, 50 μ mol/L 2-mercaptoethanol, 50 U/ml granulocyte/macrophage colony-stimulating factor (GM-CSF), and 100 U/ml interleukin (IL)-4. After 2 days, non-adherent cells were transferred into a new 150-mm plate. Five days after the initiation of the bone marrow cultures, the cells were placed in AIM V media (Gibco/BRL, Gaithersburg, MD), and 48 hours later, dendritic cells were isolated from the non-adherent population in both 150-mm plates by flow cytometric sorting. Dendritic cells were identified by size, side scatter, and high B7.2 expression using fluorescein isothiocyanate (FITC)-conjugated antibodies against B7.2 (Pharmingen, San Diego, CA) and using a FACS Vantage or FACStar Plus with CellQuest acquisition software (Becton Dickinson, Mountain View, CA). FACS analysis demonstrated that these sorted dendritic cell preparations consisted of greater than 95% N418/CD11c⁺ and NLDC145/Dec 205⁺ cells.

Intrathecal Injections

After isolation, dendritic cells were allowed to recover for 20 minutes at 37°C in RPMI plus 2% FBS, 25 mmol/L Hepes, 50 μ mol/L 2-mercaptoethanol in the presence or absence of 2 μ g/ml moth cytochrome c peptide 88–102. Cells were

washed and resuspended at approximately 2000 to 5000 cells per μl in the same media plus or minus peptide. Approximately 10 μl were injected intrathecally using a 26-gauge needle into metophane-anesthetized mice.

Fluorescent Cell Labeling

Dendritic cells were incubated in RPMI plus 10% FBS and 25 $\mu\text{mol/L}$ Cell Tracker Green (Molecular Probes, Eugene, OR) at 37°C for 20 minutes. As the dye must be cleaved intracellularly to become fluorescent, only viable cells are labeled. By this measure, greater than 90% of the sorted dendritic cells were viable.

Microglia Isolation from Mixed Glial Cultures

Mixed glial cultures were prepared as previously described.²⁹ Briefly, CNS from newborn mice were stripped of meninges, mechanically dissociated, seeded into T-75 flasks, and maintained in OM5 media with 10% FBS. After 2 to 4 weeks, cultures were trypsinized and incubated in RPMI (10% FBS, without phenol red) in suspension for 60 minutes at 37°C to allow for the re-expression of trypsinized surface markers. Microglia were then purified by flow cytometry using phycoerythrin (PE)-conjugated antibodies against FcR/CD16/CD32 (Pharmingen) as previously described.¹⁴ An extensive characterization of the antigenic phenotype and APC potential of microglia prepared by this method has been previously described.¹⁴ The cells were suspended in RPMI plus 2% FBS, 25 mmol/L HEPES, 50 $\mu\text{mol/L}$ 2-mercaptoethanol at approximately 2000 to 5000 cells per μl before intrathecal injection.

Preparation of Brain Cell Suspensions

The brains were removed from halothane-euthanized mice, the meninges were rapidly removed, and the brain tissue was mechanically dissociated into a single-cell suspension in PBS plus 10% serum. The cell suspension was washed twice to remove small debris and was suspended in RPMI plus 2% FBS, 25 mmol/L HEPES, 50 $\mu\text{mol/L}$ 2-mercaptoethanol at approximately 2000 to 5000 cells per μl before intrathecal injection.

Immunohistochemistry

Mice were sacrificed by halothane inhalation. The brains were rapidly removed and snap-frozen in OCT (Miles Laboratories, Elkhart, IN). Cryostat sections (25 μm) were post-fixed in ice-cold 4.5% paraformaldehyde for 1 minute and then blocked in serial incubations with avidin, biotin, 5% goat serum, and 0.01% Triton X-100. Sections were incubated with primary antibodies overnight at 4°C, followed by incubations with secondary biotin-conjugated antibodies and tertiary streptavidin-horseradish peroxidase-conjugated antibodies. Immunoreactivity was visualized by using 3-amino-9-ethyl carbazole as the chromogen. For GFAP immunoreactivity, CNS tissue was

prepared from mice that were perfused through the heart with saline followed by 4.5% paraformaldehyde. Brains were removed and post-fixed overnight in 4.5% paraformaldehyde at 4°C before cryosectioning. Sections were processed as described above.

Reverse Transcription Polymerase Chain Reaction

RNA was isolated from cells as previously described,³⁰ and cDNA was prepared using the Pharmacia first-strand kit according to the manufacturer's directions. Polymerase chain reactions (PCRs) were performed in a Perkin Elmer 9600, with a denaturing temperature of 94°C (15 seconds), annealing temperature of 58°C (15 seconds), and extension temperature of 72°C (30 seconds) repeated for 35 cycles. All reactions were hot started by adding primers during an initial denaturation step (4 minutes at 94°C) that preceded the cycle program. Primers for each of the chemokines were as follows: lymphotactin (CATGGGTTGTGGAAGGTG and GCTGTGCTGGTG-GACCTC), RANTES (GCTGCCCTCACCATCATCCTC and ACTTCTTCTCTGGGTTGGCAC), MIP-1 α (AGGTC-TCCACCACTGCCCTTG and TCAGGCATTGAGTTCC-AGGTCAGT), MIP-1 β (CTCTGCGTGTCTGCCCTCTC-TCTC and CTGTCTGCCTCTTTTGGTCAGGAAT), and C10 (GCAACAGAGACAAAAGAAGT and GGAAGACCAAGAAAGTAGC). As a control for nonspecific amplification and to ensure that only in the presence of both primers would a PCR product of the correct size be generated, each of the primers was used separately in PCR reactions. To test for genomic DNA contamination, the RNA sample used to generate the cDNA was also used as a PCR template.

Results

Bone-Marrow-Derived Dendritic Cells Display a Mature APC Phenotype

To test whether the mere presence of an APC was detrimental to CNS function and sufficient to initiate a T-cell-mediated immune response in the CNS, we prepared dendritic cells from murine bone marrow cultures. Dendritic cells are efficient and potent APCs.¹² Although activated B cells and macrophages can act as APCs, dendritic cells are approximately 100-fold more capable of stimulating naive T cells in an antigen-specific manner.^{12,31,32} Dendritic cells prepared from bone marrow cultures were non-adherent with multiple processes characteristic of a mature dendritic cell (Figure 1B). MHC class II immunoreactivity was observed on the cell surface and throughout the dendritic cell processes (Figure 1B) and was not localized only to the cytoplasm of the cell body as seen in immature dendritic cells. Flow cytometric analysis of the isolated dendritic cells demonstrated that more than 95% of the isolated cells expressed high levels of the molecular components required to present antigen, namely, MHC class II and co-stimulatory molecules B7.1,

A

B

C

Figure 1. Bone-marrow-derived dendritic cells express the molecules required to act as antigen-presenting cells. **A:** Filled histograms represent flow cytometric analysis of all viable cells (as judged by forward and side scatter parameters) in the dendritic cell preparations that were reacted with fluorescently labeled antibodies directed against MHC class II, CD40, B7.1, and B7.2. Unfilled histograms in the FL2-H (PE) channel depict the fluorescence observed when dendritic cells were reacted with the a control antibody directed against B220, a molecule not expressed by dendritic cells. The unfilled histogram in the FL1-H (FITC) channel shown in the B7.2 panel depicts the autofluorescence of the unlabeled cells. **B:** Cytopins of dendritic cells isolated by flow cytometry and reacted with antibodies against MHC class II (M5114). **C:** RT-PCR analysis of dendritic cell chemokine mRNA expression. Products of appropriate sizes were observed when primer pairs specific for RANTES, MIP-1 β , MIP-1 α , C10, or lymphotactin were used. No PCR products were observed in samples in which either primer was omitted or in samples in which RNA rather than cDNA was used as the amplification template. We have confirmed these results using RNase protection assay analysis.

B7.2, and CD40 (Figure 1A). Reverse transcription PCR analysis of RNA prepared from these dendritic cells demonstrated that they expressed mRNAs for numerous chemokines capable of recruiting T lymphocytes, including lymphotactin, RANTES, MIP-1 α , MIP-1 β , and C10 (Figure

1C), and thus were capable of inducing T-cell extravasation. Although dendritic cells prepared by this method expressed F4/80 (Figure 1A), more than 95% of the isolated cells were positive for NLDC145/Dec 205 and N418/CD11c (Figure 1A).

Table 1. Injected Dendritic Cells Can Home to the Cervical Lymph Node and Preferentially Migrate in the Myelin-Rich Regions of the CNS

	% of cells in T-dependent regions of lymph node	% of cells in non-T-dependent regions of lymph node	% of cells at the injection site	% of cells in the nonparenchymal CNS sites	% of cells in white matter tracts
Dendritic cells injected into the CNS	100%	0%	36.6%	35%	28.4%
Dendritic cells injected into the ear	100%	0%	None	None	None
107KO LN T cells injected into the CNS	NA	NA	100%	0%	0%

Dendritic cells were labeled with the fluorescent intracellular dye Cell Tracker Green before their injection. Intracellular cleavage of the dye is required to generate fluorescence; thus, only viable cells are labeled. Sections of lymph node and brain were examined, and fluorescent cells were counted. In the lymph node, these cells were mapped to either the T-dependent or non-T-dependent regions. In the CNS, these cells were mapped to the injection site, nonparenchymal regions (meninges, subarachnoid spaces, and ventricles), or white matter tracts. 107KO lymph node (LN) T cells were identified by CD3⁺ immunoreactivity. Sections for all experiments were prepared from mice sacrificed 48 hours after injection. NA, not applicable. None indicates that there were no fluorescent cells detected in the entire cryosection.

Within the CNS Parenchyma, Functionally Mature Dendritic Cells Preferentially Infiltrate Myelin-Rich Regions

One of the hallmark characteristics of mature dendritic cells is their ability to migrate from the site of antigen to the T-dependent regions of lymphoid organs. Therefore, we tested whether dendritic cells injected either subcutaneously in the ear or into the CNS would home to the cervical lymph nodes. For definitive identification, dendritic cells were labeled with an intracellular, fluorescent dye, Cell Tracker Green, just before injection. In both types of injections (either into the ear or the CNS), labeled dendritic cells could be found in cryosections of cervical lymph nodes from mice sacrificed 48 hours after injection (Table 1). In each case, labeled cells were found only in the T-dependent and not the B-dependent regions of the cervical lymph nodes. At 48 hours after injection, fluorescently labeled cells could be detected in cryosections of the injected tissue only in those mice injected in the CNS and not in the ear. Labeled dendritic cells were found throughout the CNS, but in a regionally restricted pattern. Fluorescently labeled cells were found in the meninges and subarachnoid spaces and along the length of the white matter tracts, including the optic chiasm, striatum, corpus colosum, and fimbria. Labeled cells were rarely found in the gray matter regions of the brain and then only in regions that appeared damaged by the injection process.

Dendritic Cells Recruit a Diverse Leukocyte Population, but Only in an Antigen-Specific Manner

Two different paradigms of antigenic stimulation, alloreactivity and peptide-specific interactions with a transgenic TCR, were used to characterize the ability of the CNS environment to modulate two qualitatively different T-cell responses. Alloreactivity involves high-ligand-density, low-receptor-affinity interactions of various T-cell receptors specific for the foreign MHC molecules on the APC.^{33,34} Alloreactive responses can involve both CD4⁺ and CD8⁺ T-cell populations if the APC and T cells differ

in both MHC class I and II alleles, or only the CD4⁺ T-cell population if the differences are limited to MHC class II.³⁴ In contrast, the peptide-specific interactions with a MHC-class-II-restricted transgenic TCR involves high-receptor-affinity interactions with the CD4⁺ T cell population.²⁷ The transgenic TCR used here is specific for moth cytochrome c presented by I-E^b; however, the TCR transgene is maintained on a C57BL/6 backcross that is I-E^b negative.^{27,28} Consequently, none of the cells in the transgenic mice, including microglia, macrophages, and astrocytes, can act as APCs. The sizes of the potential responder populations also differ between the two types of stimuli; in alloreactive models, fewer than 5% to 10% of the T-cell population are potential responders, as opposed to greater than 90% of the T-cell population in the transgenic TCR model.

Injection of C57Bl/6 dendritic cells into the CNS of syngeneic C57Bl/6 mice induced astrogliosis and microgliosis as judged by respective increases in GFAP and F4/80 immunoreactivity in the regions surrounding the injection site relative to more distal regions (Figure 2, B and D). Minimal neutrophil infiltration (GR-1⁺ cells) was observed near the injection site and meninges (Figure 2A). Although mechanical destruction of both neuronal and glial cells was caused by the injection process, the syngeneic dendritic cells failed to initiate a CNS-directed T-cell response. Only an occasional T cell (CD3⁺ cell) could be found in the injection site or the meninges (Figure 2C). B cells (B220⁺ cells) were not detected (data not shown).

In striking contrast, injection of C57Bl/6 dendritic cells into the CNS of either B10.D2 (alloreactive for both MHC class I and class II) or 107KO mice (alloreactive for only MHC class II) induced the accumulation of a large population of cells immunoreactive for several leukocyte markers, but the accumulation was in an anatomically restricted pattern. The accumulated cells were immunoreactive for CD3 and CD4 (Figure 3, A and C), GR-1 (Figure 3B), and B220 (Figure 3D), indicative of T cells, neutrophils, and B cells, respectively. These cells were found primarily in the meninges, subarachnoid spaces, ventricles, and injection site. Only a small percentage (~5%) of the recruited T and B cells were found within the

Figure 2. Injection of C57Bl/6 dendritic cells into syngeneic C57Bl/6 CNS induces microgliosis and astrogliosis without significant T cell accumulation. Serial brain sections are from a mouse sacrificed 2 days after injection, reacted with antibodies that label neutrophils (GR-1 monoclonal; **A**), macrophages and microglia (F4/80 monoclonal; **B**), and T cells (anti-CD3; **C**). The upper left portion of each panel (marked with an asterisk) represents the area near the injection site. Arrows indicate examples of immunopositive cells. **D** represents a region near an injection site similar to that depicted in **A** to **C** that was reacted with antibodies that label astrocytes (anti-GFAP). CNS tissue was flash-frozen in sections shown in **A** to **C** whereas CNS tissue was perfused with paraformaldehyde in the section shown in **D** before cryosectioning. Different preparations of CNS tissue were required for optimal antibody labeling. Histology was representative of at least three experiments with at least two animals per experiment.

CNS parenchyma and then only in the white matter tracts and not the gray matter regions.

This pattern of leukocyte recruitment was mirrored in the TCR transgenic mice injected with moth cytochrome c peptide and I-E^p-expressing dendritic cells from 107KO mice (Figure 3, E–H). The number but not the percentage (~5%) of T cells migrating into white matter tracts was higher in the TCR transgenic mice. This is most likely a consequence of the 10- to 20-fold larger responding T-cell population in the transgenic model (Figures 3E and 6, A, C, and E) as compared with the allogeneic model (Figure 3, A and C). Injection of dendritic cells alone, the moth cytochrome c peptide alone, or the moth cytochrome c peptide in combination with dendritic cells expressing a MHC class II inappropriate for the transgenic TCR all failed to induce an accumulation of T and B cells within the CNS. Thus, leukocyte recruitment in both the allogeneic and TCR transgenic models was dependent on specific antigen.

In both models, large-scale leukocyte recruitment was evident within 24 hours of injection and peaked between 1 and 3 days after injection. After 1 week, few of the injected dendritic cells or recruited leukocytes were detected in the CNS.

Priming Mice with Allogeneic Cells Decreases the Requirement for a Potent APC

Although dendritic cells are highly potent APCs, many other cell types can act as partial or inefficient APCs. For example, microglia from mixed glial cultures express the molecular machinery to present antigen but are relatively weak APCs as tested in T-cell proliferation assays.¹⁴ Although these microglia are weak stimulators of T-cell proliferation, they display an activated phenotype that resembles microglia isolated from mice with severe demyelination induced by the overexpression of IL-3 in the CNS.³⁵ Therefore, we injected into the CNS of allogeneic 107KO mice three types of weak APCs prepared from C57Bl/6 mice (microglia from mixed glial cultures, 3T3 fibroblasts, or whole-brain cell suspensions) in the same concentrations and sites as had been done with the dendritic cells. The site near the injection was examined by immunohistochemistry for evidence of gliosis and leukocyte accumulation. As with the injection of syngeneic dendritic cells, only an occasional T or B cell could be detected by immunohistochemistry. Increases in GFAP, F4/80, and GR-1 immunoreactivity on both amoeboid and

Figure 3. Leukocyte accumulation is antigen specific. Brain sections were reacted with antibodies that label T cells (anti-CD3; **A**), neutrophils (GR-1 monoclonal; **B**), CD4⁺ T cells (anti-CD4; **C** and **E**), B cells (B220 monoclonal; **D**), CD8⁺ T cells (anti-CD8; **F**), microglia and macrophages (F4/80 monoclonal; **G**), MHC class II (M5114 monoclonal; **H**). **A** to **D**: Sections from the CNS of 107KO mice injected with allogeneic C57Bl/6 dendritic cells and sacrificed 2 days after injection. **E** to **H**: Serial sections from the CNS of the TCR-transgenic mouse injected with 107KO dendritic cells plus the moth cytochrome C peptide antigen sacrificed 2 days after injection. **A** and **B**: Serial coronal sections of the lateral ventricle (LV). **C** and **D**: Serial coronal section of the ventral surface of the forebrain (V). **E** to **H**: Serial coronal sections of the corpus callosum (CC). Histology is representative of at least six experiments with at least two animals per experiment.

Figure 4. C57Bl/6 CNS cells that lack the ability to present antigen can induce the accumulation of leukocytes when injected into the CNS of allogeneic 107KO mice previously primed with allogeneic stimulus. **A** and **B**: Serial sections reacted with antibodies that label CD4⁺ T cells (anti-CD4) and B cells (B220 monoclonal), respectively. **C** and **D**: Serial sections reacted with antibodies that label CD4⁺ T cells (anti-CD4) and CD8⁺ T cells (anti-CD8).

stellate-shaped cells surrounding the injection site were indicative of astrogliosis, microgliosis, and neutrophil recruitment, respectively (data not shown).

As weak APCs could not recruit naive T cells into the CNS, we asked whether they could recruit T cells from mice that had been primed with antigen at peripheral sites. For this experiment, 107KO mice were primed by subcutaneous injections of 2×10^7 C57Bl/6 spleen cells 2 weeks before intrathecal injections. As in previous experiments, CNS cryosections prepared from mice sacrificed 48 hours after intrathecal injections were examined for cells immunoreactive for the B cell marker B220 and the T cell markers CD4 and CD8. After priming, all three types of weak APCs, including C57Bl/6 brain cell suspensions, triggered the accumulation of cells immunoreactive for CD4, CD8 (Figure 4, A, C, and D) and B220 (Figure 4B) in the CNS. B and T cell accumulation was not limited to nonparenchymal CNS sites but extended into the white matter tracts. In sections from primed 107KO mice injected with potent APCs (C57Bl/6 dendritic cells), greater numbers of cells immunoreactive for CD4 (Figure 5, A and C) and CD8 (Figure 5, B and D) were found in the CNS white matter than had been found in similarly treated unprimed mice. T and B cell accumulation in white matter did not appear diminished over 200 μ m distant from the injection site.

Conversely, we tested whether the T-cell accumulation in the CNS was dependent on the presence of a potent

APC by injecting 107KO lymph node T cells intrathecally into allogeneic C57Bl/6 mice. In cryosections prepared from mice sacrificed 24 hours after injection, very few cells immunoreactive for the T-cell marker CD3 remained in the CNS of allogeneic C57Bl/6 mice. The few CD3⁺ cells present in the section remained at the injection site; none were found in the meninges, ventricles, or white matter (Table 1).

CD8⁺ T Cells Were Recruited Disproportionately

Antigen-specific effects should be mediated by CD4⁺ T cells in both the transgenic TCR model and the allogeneic model in which the dendritic cells differ from the CNS only in MHC class II haplotype. Surprisingly, immunohistological characterization of the recruited and infiltrating T cells revealed that the T-cell population consisted of roughly equivalent numbers of CD4⁺ and CD8⁺ cells (Figures 3, E and F; 4, C and D; and 5, A–D). Leaky expression of the MHC-class-II-restricted TCR transgene in CD8⁺ cells is insufficient to account for their large-scale antigen-specific recruitment into the TCR-transgenic CNS. Flow cytometric analysis of the TCR-transgenic mice indicated that CD4⁺ cells outnumbered CD8⁺ cells by at least 58:1 in peripheral blood, spleen,

Figure 5. T cell accumulation was localized to white matter regions without causing white matter pallor. All panels depict sections from the CNS of 107KO mice previously primed with allogeneic stimulus and injected with allogeneic C57Bl/6 dendritic cells. Blue regions represent Luxol Fast Blue labeled myelin. **A and C:** Labeled with antibodies against CD4. **B and D:** Labeled with antibodies against CD8. **E and F:** Labeled with an antibody against MHC class II (M5114). **Arrows** indicate CD4⁺ (**C**) and CD8⁺ (**D**) cells localized to white matter regions.

and lymph node (Table 2). Flow cytometric analysis also indicated that up to 66% of the CD8⁺ cells expressed the transgenic TCR. Hence, the predicted ratio of CD8:CD4 cells appearing in the CNS infiltrates would be lower than 1:80 if recruited by direct antigen-TCR interactions and lower than 1:50 if recruited secondarily by antigen-specific CD4 interactions. It is unlikely that the disproportionate detection of CD8⁺ T cells in immunohistochemical as compared with flow cytometric analysis because similar CD4:CD8 ratios were detected in lymph node and spleen taken from unmanipulated ANDB6 mice whether analyzed by im-

munohistochemistry or by flow cytometry. Thus, the observed approximate 1:1 ratio of CD4⁺:CD8⁺ cells in both the TCR-transgenic model and the allogeneic model involving only MHC class II indicates that CD8⁺ T cells are preferentially and nonspecifically recruited.

T Cell Recruitment without T Cell Activation

Activated T and B cells can be distinguished histologically from naive cells by their intense labeling with antibodies against the IL-2 receptor (CD25) and the late

Table 2. CD4⁺ T Cells Constitute the Majority of T Cells in the ANDB6 (TCR Transgenic) Mouse

	Ratio of CD4 ⁺ :CD8 ⁺ cells	% of CD4 ⁺ cells expressing transgenic TCR	% of CD8 ⁺ cells expressing transgenic TCR	Ratio of transgenic CD4 ⁺ : transgenic CD8 ⁺ cells
Peripheral blood	58:1	93.3%	48.2%	112:1
Lymph Node	60:1	92.7%	66.1%	84:1
Spleen	122:1	92.1%	30.2%	372:1

Suspensions of cells prepared from peripheral blood, lymph node, and spleen of 9.5-week-old ANDB6 mice were labeled with antibodies against CD4, CD8, and V α 11 (RR8-1). Ratios of positive cells to total cells were then determined by flow cytometric analysis. The percentage of CD4⁺ or CD8⁺ cells that express the transgenic TCR was determined using the RR8-1 antibody, which specifically recognizes the transgenic TCR.

activation marker VLA-4.^{4,7} We examined the T and B cells found within the white matter, meninges, ventricles, or injection site with antibodies against CD25, VLA-4, LFA-1, and CD62L (Mel 14, the leukocyte homing receptor). Although CD62L immunoreactivity was readily detected in lymph node sections, we failed to detect CD62L immunoreactivity in CNS sections from either the TCR-transgenic or alloreactive models (data not shown). However, we could detect LFA-1 (a T-cell integrin; data not shown). The absence of CD62L immunoreactivity coupled with LFA-1 expression was consistent with the extravasation of T and B cells from the bloodstream. Fewer than 10% of these T and B cells were labeled by antibodies directed against CD25 or VLA-4 (Figure 6). Positive cells were not scattered stochastically throughout the infiltrating population. Rather, cells positive for VLA-4 and CD25 were found primarily in the ventricles, injection sites, and perivascular regions whereas T cells found within the CNS parenchyma white matter rarely expressed VLA-4 or CD25 (Figure 6 and data not shown). These data suggest that healthy CNS parenchyma may be able to down-regulate T-cell activation markers.

T- and B-cell infiltration was associated with macrophage infiltration and microglial activation as judged by increased F4/80, MHC class II, and CD45 immunoreactivity in the white matter tracts and the areas surrounding the injection sites in the TCR-transgenic model (Figure 7) and the alloreactive model (Figure 5E). Ameboid cells that labeled intensely for CD45 were presumed to be of peripheral origin, whereas stellate cells that labeled weakly for CD45 were presumed to be resident microglia, based on previous measurements on F4/80⁺ cells isolated from noninjected mice.^{13,14,18} Under normal conditions, CD45 expression by microglia is too low to be detected by immunohistochemistry. On activation, microglial CD45 levels increase but remain much lower than the levels found on peripheral immune cells. Microglia in the surrounding gray matter regions appeared normal, although occasional clusters of MHC-class-II-positive cells with stellate morphology were found in the cerebral cortex (Figure 5F).

Although T cell accumulation (both CD4⁺ and CD8⁺ cells) occurred preferentially within the white matter tracts of the CNS (Figure 5, A–D), there were no gross changes in myelination as judged by luxol fast blue staining, even in the mice previously primed with allogeneic stimulus. Similarly, the presence of MHC-class-II-positive cells was not associated with large-scale changes in luxol fast blue staining in either myelin-rich areas such as

the striatum (Figure 5E) or in relatively myelin-poor areas such as the cerebral cortex (Figure 5F).

Discussion

Prolonged allograft survival and an inability to initiate T-cell responses are the defining features of CNS immunological privilege and have been thought to result largely from the absence of a resident population of antigen-presenting cells (APCs).^{1–3,11,13} Conversely, inappropriate acquisition of APC function or the inappropriate infiltration of APCs into the CNS have been surmised to contribute to chronic CNS pathology and xenograft rejection.^{2,4,5,8,13} As a direct test of these hypotheses, we placed the most efficient and potent APC found in the body, the bone-marrow-derived dendritic cell, directly into the CNS.

Unexpectedly, we found that efficient APCs, capable of recruiting and activating naive T cells, were insufficient to initiate a T-cell-mediated response in the CNS of syngeneic mice. The injection of dendritic cells into the CNS caused tissue damage, yet the dendritic cells apparently could not present CNS antigens from the damaged tissue. Although this could be due to peculiarities of CNS antigens or the frequency of CNS-specific T cells, more probably it was a consequence of the maturation state of the dendritic cells used in our studies. As dendritic cells mature and become more potent APCs, they lose the ability to process antigen, relying on phagocytosing macrophage for that function.¹² Consistent with this interpretation, the dendritic cells used in our studies displayed a mature phenotype and were capable of correctly homing to the T-dependent regions of the cervical lymph nodes whether they had been injected into the CNS or the ear. However, we did find that dendritic cells could recruit large numbers of leukocytes into the CNS when presenting allogeneic or peptide stimuli.

We do not address here how the injected dendritic cells can induce the antigen-specific accumulation of T cells into the CNS. It is possible that they merely trap the T cells that routinely enter the CNS under nonpathological conditions. It is also possible that dendritic cell chemokine expression serves to induce T-cell extravasation and subsequent CNS infiltration. Alternatively, the migration of injected dendritic cells to the T-dependent area of the cervical lymph nodes may serve to inform and activate lymph node T cells to migrate in search of the presented antigen (either allogeneic or peptide).

Figure 6. Few of the T cells that accumulate in the CNS display an activated phenotype. All panels depict sections from the CNS of a TCR-transgenic mouse injected with 107KO dendritic cells plus moth cytochrome C peptide. **A and B:** Serial sections of the lateral ventricle. **C and D:** Serial sections of the fimbria. **E and F:** Serial sections near the injection site. **A, C, and E:** Labeled with anti-CD4 antibodies. **B and D:** Labeled with anti-VLA-4 antibodies. **F:** Labeled with anti-CD25 antibodies. **Arrows** indicate examples of immunopositive cells.

These studies revealed three other unexpected findings. First, dendritic-cell migration and leukocyte recruitment into the CNS was anatomically restricted to the meninges, subarachnoid spaces, ventricles, and white matter of the CNS. Second, CD8⁺ T cells were recruited into the CNS in numbers vastly disproportionate to their numbers in peripheral blood, spleen, or lymph node. In the TCR-transgenic model, some of these CD8⁺ T cells could have been recruited via direct peptide-TCR inter-

action on the CD8⁺ T cells; however, their recruitment in the allogeneic model in which the allogeneic differences were solely in MHC class II, recruitment should only be secondary to the antigen-specific interactions with CD4⁺ T cells.^{27,34} Third, although numerous T and B cells were recruited to the CNS in an antigen-specific manner by a potent APC, very few of the cells expressed the activation markers CD25 (IL-2 receptor) or VLA4, even in mice previously primed with antigen. Consistent with this un-

Figure 7. Microglia are activated in regions with leukocyte recruitment. All panels depict sections from the CNS of TCR-transgenic mice injected with 107KO dendritic cells plus moth cytochrome C peptide. **A to C:** Serial sections of the optic chiasm. **D and E:** Serial sections of the corpus collosum. **A and E:** Sections reacted with antibodies that label all nucleated cells of hemopoietic origin (anti-CD45). **Arrows** indicate examples of intensely immunopositive cells, and **arrowheads** indicate examples of weakly immunopositive cells. **B and D:** Sections reacted with antibodies that label microglia and macrophages (F4/80). **C** Section reacted with an antibody against MHC class II (M5114).

activated T-cell phenotype, luxol fast blue staining of myelin, animal appearance, and behavior appeared to be unaffected by the robust T-cell recruitment. By contrast, mice injected in the CNS with lipopolysaccharide/interferon- γ or activated T cells rapidly show signs of illness, developing a hunched posture, failing to groom properly, and becoming less motile.³⁶

The mechanism by which CD8⁺ T cells preferentially accumulated within the CNS is unclear, but the CNS itself must play a role shaping this response because initiation of CD4⁺ T-cell responses does not lead to a disproportionate accumulation of CD8⁺ T cells in other tissues.^{37,38} For example, in our studies, a preferential accumulation of CD8⁺ T cells was not observed when allogeneic dendritic cells were injected in the ear. Similarly, CD8⁺ T cells accumulate only in relatively small numbers as compared with CD4⁺ T cells during the initiation and progression of diabetes in a MHC-class-II-restricted TCR-transgenic model.³⁸ At present, only IL-15 has been identified as a chemokine that may preferentially attract and activate CD8⁺ T cells as compared with CD4⁺ T cells.³⁹ A preliminary comparison between CNS injected with dendritic cells plus antigen or CNS injected with dendritic cells in the absence of antigen failed to show any differences in IL-15 expression (unpublished observations).

Cumulatively, these data suggest that the spontaneous development of myelin-directed diseases such as multiple sclerosis need not be triggered by myelin-specific antigens. Rather, an initial myelin-restricted pattern of inflammation could result simply from the preferential leukocyte (both APC and responder cell) migration through white matter regions as opposed to gray matter regions. For example, if infiltrating APCs happen to initiate antigen-specific immune responses that are not directed against myelin or CNS antigens while in these areas, myelin-specific responses could result from deter-

minant spreading.⁴⁰ Such a phenomenon is observed in Theiler's virus infection of CNS oligodendrocytes.⁴¹ Although the initial immune response is directed solely against viral antigens, myelin-directed T-cell responses begin to emerge during the later stages of disease progression. In addition, our data indicate that significant CD8⁺ T-cell recruitment is not necessarily indicative of MHC-class-I-restricted stimuli such as is associated with viral antigens or molecular mimicry.^{4,7,34} Thus, it is possible that CD8 recruitment in diseases such as multiple sclerosis can be nonspecific and secondary to a MHC-class-II-restricted stimulus.

Although the functional significance of this disproportionate CD8⁺ T cell accumulation is unknown, several studies have suggested that CD8⁺ T cells can play a protective role in CNS pathology. For example, the frequency and severity of relapses in EAE is much higher in CD8 knockout mice as compared with wild-type mice.⁴² Conversely, studies by Weiner and colleagues have suggested that CD8⁺ T cells can acquire the ability to suppress CD4⁺ T cell responses in both rodent and human CNS demyelinating disease.^{43,44} In addition, these authors also found that CD8⁺ T cells from patients with chronic progressive multiple sclerosis were deficient in this immunosuppressive activity as compared with CD8⁺ T cells from patients with relapsing-remitting multiple sclerosis or from normal individuals.⁴⁴

Finally, these data suggest that the absence of a resident population of APCs does play a protective role in the CNS but that additional mechanisms exist that shape the immune response and that set a high threshold for lymphocyte activation. It is not only inappropriate activation of the immune system in the periphery that can lead to neuropathology but also malfunctions of these protective CNS mechanisms.

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