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CNS immune privilege: hiding in plain sight

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Summary

Central nervous system (CNS) immune privilege is an experimentally defined phenomenon. Tissues that are rapidly rejected by the immune system when grafted in sites, such as the skin, show prolonged survival when grafted into the CNS. Initially, CNS immune privilege was construed as CNS isolation from the immune system by the blood–brain barrier (BBB), the lack of draining lymphatics, and the apparent immunoincompetence of microglia, the resident CNS macrophage. CNS autoimmunity and neurodegeneration were presumed automatic consequences of immune cell encounter with CNS antigens. Recent data have dramatically altered this viewpoint by revealing that the CNS is neither isolated nor passive in its interactions with the immune system. Peripheral immune cells can cross the intact BBB, CNS neurons and glia actively regulate macrophage and lymphocyte responses, and microglia are immunocompetent but differ from other macrophage/dendritic cells in their ability to direct neuroprotective lymphocyte responses. This newer view of CNS immune privilege is opening the door for therapies designed to harness autoreactive lymphocyte responses and also implies (i) that CNS autoimmune diseases (i.e. multiple sclerosis) may result as much from neuronal and/or glial dysfunction as from immune system dysfunctions and (ii) that the severe neuronal and glial dysfunction associated with neurodegenerative disorders (i.e. Alzheimer's disease) likely alters CNS-specific regulation of lymphocyte responses affecting the utility of immune-based therapies (i.e. vaccines).

Keywords

immune privilege; central nervous system; experimental autoimmune encephalitis; immunotherapy; microglia

Introduction

Throughout the body, inflammation has two general purposes: tissue homeostasis and tissue defense against pathogens (1-4). Inflammation contributes to tissue homeostasis by (i)

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Supplementary material

The following supplementary material is available for this article online: [**Video Clip S1**]. Heterogeneity of Iba-1 positive cells within a single microscopy field.

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removing dead tissue, (ii) promoting the rapid death of irretrievably damaged cells, and (iii) promoting wound healing. Inflammation contributes to tissue defense by the production of (i) cytotoxic substances, (ii) chemokines for the recruitment of specific immune cell populations, and (iii) cytokines for coordinating the immune responses of local stromal cells, vasculature, and the immune system itself.

The central nervous system (CNS) is an organ essential for survival and clearly must be defended from pathogens (5). Yet, the role of the immune system in maintaining homeostatic CNS function versus promoting neurodegeneration has been a subject of debate for several reasons (5). Activated immune cells (both lymphocytes and macrophages) produce inflammatory molecules with demonstrated neurotoxicity (6). The brain is encased in a non-elastic skull and thus cannot tolerate excessive cellular accumulation and swelling associated with many peripheral inflammatory reactions. Finally, inflammation can promote both cell death and cell regeneration (4). CNS function is dependent on experience-driven (activity-dependent) development of precise neural circuitry (7). Removal of key neurons clearly has the potential to inactivate specific neural processing pathways, leading to cognitive and/or motor deficits. However, inappropriate induction of CNS ‘healing’ responses associated with new neuronal synaptic formation is not necessarily adaptive. For example, many neurological disorders including autism are associated with inappropriate formation of synapses and dendritic arborization (8-10).

For the better part of the last century, the CNS was believed to be both immunologically inert and immunologically separated from the peripheral immune system (11,12). It was viewed primarily as a tissue of postmitotic cells that were highly vulnerable to the onslaught of activated immune cells, if and when these immune cells infiltrated the CNS. In support of this view, T cells are found in perivascular cuffs and within the demyelinated lesions in patients with multiple sclerosis (MS), an autoimmune demyelinating disorder (6,13). Furthermore, transfer of activated T cells specific for components of CNS myelin into genetically susceptible rodent strains is sufficient to trigger CNS inflammation and clinical onset of motor disabilities (hindlimb paralysis), referred to as experimentally induced autoimmune encephalomyelitis (EAE)(6,14). In this view, the only elements protecting the CNS from immune-mediated harm was the presence of an intact blood–brain barrier (BBB) and the absence of an immune-competent population of tissue macrophages/tissue dendritic cells.

Over the last 20 years, there has been a dramatic reevaluation of the type of cellular immune responses that can and do occur within the CNS (15-17). Current data indicate that the CNS is both immune competent and actively interactive with the peripheral immune system (16). Inflammation is also recognized to be a prominent feature of many classic neurodegenerative disorders even in the absence of substantial infiltration of peripheral immune cells (2,18-22). In stark contrast to the older view, neuroinflammation is now realized to have both neuroprotective and neurotoxic aspects (23-28). Indeed, recent data suggest that maintenance of normal CNS function and induction of regeneration may be absolutely dependent on the initiation of specific types of immune response (26,29,30). The documentation of neuroinflammation in diverse CNS disorders such as Alzheimer's disease, stroke, Parkinson's disease, and autism has led some to conclude that the CNS cannot be considered an immune-privileged site (16,17).

In this review, we discuss the unique tissue-specific interactions that occur between the CNS and peripheral immune systems and that define the phenomenon of CNS immune privilege. We illustrate that immune privilege is not immune isolation. Rather, immune privilege is a collection of CNS-driven mechanisms that actively regulate T-cell responses within the CNS. These CNS-driven mechanisms even have demonstrated systemic effects on the effector functions of autoreactive T cells in non-CNS sites. In large part, these mechanisms are driven

by neuronal health and activity. Finally, we discuss the physiological consequences of immune privilege for CNS regeneration, pathogen defense, and immune-based therapies for CNS neurodegeneration.

The CNS: is it or isn't it an immune-privileged site?

Numerous experiments performed by many different investigators over the last 100 years have clearly demonstrated that it is much more difficult to initiate destructive T-cell responses from inside the CNS parenchyma than from many other nonCNS sites (11,12,31). Foreign tissue grafts survive for more prolonged periods when placed within the parenchyma of the CNS as compared with being placed under the skin. Similarly, injection of heat-killed bacille Calmette-Guérin (BCG) bacteria under the skin leads to rapid recruitment of neutrophils and macrophages within the first few hours and later leads to the initiation of a proinflammatory T-cell response directed against the BCG bacteria (31,32). Injection of the same BCG preparation into the CNS parenchyma does initiate macrophage recruitment to the CNS, but it fails to initiate a proinflammatory T-cell response.

A comparable difference is seen with destructive autoimmune responses. T-cell receptor (TCR) transgenic mice in which >95% of the T cells are specific for CNS antigens, such as myelin basic protein (MBP), do not spontaneously develop T-cell infiltrates in MBP-expressing brain regions, demyelinating lesions, or clinical signs of EAE when maintained under specific-pathogen-free (SPF) conditions (33,34). This response is not limited to just myelin antigens. Transgenic mice expressing TCRs specific for influenza hemagglutinin (HA) spontaneously develop autoimmune diabetes when HA is expressed in the pancreatic islets, but they fail to develop any signs of autoimmunity when HA is expressed by CNS astrocytes or when HA-expressing islets are grafted into the CNS of TCR transgenic mice (35-37, Ploix et al., unpublished observations).

Taken together, these data clearly indicate a differential ability to initiate T-cell responses against antigens located in the CNS as compared with outside the CNS. Considering only these experiments, one might conclude (incorrectly) that T cells cannot detect antigenic targets located within the healthy CNS.

Peripheral infections and molecular mimicry

T cells activated outside the CNS can readily detect their antigenic targets even when located within the CNS parenchyma (38). Foreign tissue grafts within the CNS parenchyma are rapidly rejected by the immune system (even after prolonged periods of survival), immediately after the same type of tissue is grafted under the skin (11,12). The similar rejection kinetics of both the primary CNS and secondary skin tissue grafts indicate that activated T cells can detect antigens within the CNS as readily as antigens in non-immune-privileged sites. The ability of peripherally activated T cells to enter and attack tissue within the CNS has led to the molecular mimicry hypothesis of CNS autoimmunity (39-42).

The molecular mimicry hypothesis suggests that immune responses raised against pathogenic epitopes with structural similarity to endogenous CNS molecules will not only clear the pathogen from the body but also successfully attack the CNS (39-42). In support of this hypothesis, epidemiological studies have suggested a potential viral contribution for at least one CNS autoimmune disorder, MS (43-45). Experimental mouse models have illustrated both the plausibility of this hypothesis and also its limits. Most notably, Miller and colleagues (43) have infected SJL mice with non-pathogenic variants of Theiler's murine encephalomyelitis virus (TMEV) that were genetically engineered to express the immunodominant myelin epitope proteolipid protein $PLP_{139-151}$. Mice infected with the PLP-

expressing TMEV variant developed early-onset demyelinating disease associated with the activation of $PLP_{139-151}$-specific $CD4^+$ T cells.

However, generation of peripheral immune responses sufficient to clear a pathogen from the body is not always sufficient for destructive responses against the same epitopes when expressed in the CNS. For example, transgenic mice expressing the nucleoprotein of lymphocytic choriomeningitis virus (LCMV) in CNS oligodendrocytes generate immune responses able to clear a peripheral LCMV infection without developing overt CNS autoimmunity or demyelination (46). Even after priming the T-cell response with multiple peripheral LCMV infections, only modest transient demyelinating lesions could be detected in the CNS of mice expressing LCMV nucleoprotein.

In humans, a similar discrepancy is seen between the efficacy of autoimmune responses against antigens expressed in and outside the CNS. Type 1 diabetes, also referred to as juvenile diabetes, is an autoimmune disorder targeting the insulin-producing cells of the pancreas: the β cells of the islets (47,48). Most individuals with type 1 diabetes develop strong immune responses against several antigens expressed in common between the islets and the CNS, most notably glutamic acid decarboxylase 65 (GAD65), tyrosine phosphatase IA-2, and glial fibrillary acidic protein (GFAP), but fail to develop CNS autoimmune disease (47-49). Fewer than 0.001% of individuals with autoimmune diabetes and strong anti-GAD immune responses develop stiff person syndrome (SPS), an autoimmune CNS disorder associated with strong detectable GAD65 responses (47,50). Surprisingly, approximately 30% of individuals with SPS do develop diabetes in adulthood. SPS individuals also have anti-GAD antibodies with a higher titer and increased epitope recognition as compared with those detected in individuals with autoimmune diabetes. These data suggest that even in the same individual, the threshold for destructive autoimmune responses is higher in the CNS than in the periphery.

From these types of studies, it is important to stress that the phenomenon of CNS immune privilege refers only to the specific inhibition of initiating adaptive (antigen-driven lymphocyte) proinflammatory immune responses. Innate immune responses, most notably the recruitment and activation of peripheral and resident macrophages, are readily initiated within the CNS by bacterial components, tissue damage, cytokine overexpression, or the deposition of amyloid plaques (5,16,17). However, the kinetics of innate immune responses can be slightly delayed as compared with other tissues. It is unclear to what extent this is a cause of or a response to decreased propensity for destructive lymphocyte responses. In addition, granulocyte responses tend to be substantially reduced as compared with other tissues. Although, it is important to note that neutrophils are prominently recruited to the CNS in response to many insults (intracerebral injection of lipopolysaccharide, spinal cord injury, ischemia, bacterial brain abscess) (51-53). To emphasize this often overlooked distinction that many types of immune responses do occur in the CNS, there has been a trend to refer to the CNS as immune specialized rather than as immune privileged (16). In this review, the two terminologies are viewed as equivalent because both terms define the CNS as having a unique tissue-specific relationship with the immune system.

CNS immune privilege: location, location, location

The phenomenon of immune privilege that was just described does not apply to all regions of the CNS. Robust proinflamma-tory T-cell responses to grafted tissue and pathogens are readily triggered within the non-parenchymal sites of the CNS: the ventricles, the meninges, and the subarachnoid spaces (31). Consistent with the difference is the apparent difference in the propensity of the CNS versus peripheral tissues to organize inflammatory infiltrates into structures reminiscent of lymphoid tissue. Within the CNS, such features have been detected within the meninges but not in the CNS parenchyma of patients with secondary progressive

MS (54). It is uncertain whether the lower incidence of lymphoid neogenesis in the inflamed CNS is a cause or a symptom of CNS immune privilege. However, transgenic overexpression of tumor necrosis factor (TNF) was sufficient to promote neurodegeneration and the formation of lymphoid-like structures within the spinal cord (55). Interestingly, the vasculature within inflammatory infiltrates did at least partially display the high endothelial venule phenotype characteristic of lymphoid tissue. However, only a few of the T and B cells displayed an activated phenotype. The majority displayed an unactivated memory phenotype. Somewhat surprisingly, genetic deletion of the T-cell population demonstrated that in this TNF-induced model of neurodegeneration, T cells played a non-inflammatory, neuroprotective role (55).

How is CNS immune privilege maintained?

Regardless of the terminology being used, the question remains: how does the CNS inhibit the initiation of proinflammatory T-cell responses directed against targets found within the CNS? To begin to answer this question, one must first define on the most basic terms what a T cell requires to respond to antigens and then define if the CNS lacks any of these essential elements (1,56). First, the T cell must have access to its antigenic targets. With regard to the CNS, CNS antigens must drain to the cervical lymph nodes and/or T cells must be able to enter the CNS environment. Second, T cells require antigen-presenting cells (APCs), because the TCR cannot bind free antigen in solution. TCRs only recognize antigens located within the binding cleft of major histocompatibility complex (MHC) expressed on the surface of an APC. Specifically, $CD4^+$ T cells require macrophages or immature dendritic cells to capture molecules from the environment (i.e. from cell debris) to intracellularly process the captured material into smaller fragments able to fit within the MHC class II binding cleft. For most efficient $CD4^+$ T-cell activation, after antigen capture, APCs then travel from the starting tissue (for example, the skin) to the draining lymph node (where naive unactivated T cells reside). For $CD8^+$ T cells, any pathogen-infected cell induced to express MHC class I can serve as an APC. The ultimate ability of APCs to effectively drive proinflammatory T-cell responses is also tightly coupled to levels and types of costimulatory molecules expressed by the APC [i.e. CD40, B7.1, B7.2, intercellular adhesion molecule-1 (ICAM-1)](1,56).

Are CNS antigens sequestered or hidden from the immune system?

Medawar (12) was one of the first to suggest that the inability of foreign tissue grafts to elicit immune responses from within the CNS was a consequence of CNS architecture and of CNS isolation from the immune system. As subsequently refined by others, the high threshold for initiating lymphocyte responses from within the CNS has often been hypothesized to be dependent on (i) the presence of the BBB that would impede T-cell entry into the CNS, thus preventing T-cell encounter with CNS antigens; (ii) the absence of draining lymphatics that would prevent cervical lymph node T cells from becoming exposed to antigens located within the CNS parenchyma; and (iii) the inability of microglia, the resident tissue macrophage of the CNS, to present antigen to T cells.

In this framework, robust activation of the immune system from outside the CNS (i.e. in the skin) would circumvent all three barriers. Activated T cells express high levels of molecules such as very late antigen-4 (VLA-4) and leukocyte-function-associated antigen-1 (LFA-1) that facilitate their transmigration across the BBB (1,25,56). In addition, activation of the peripheral immune system can lead to BBB leakiness caused by systemic production of inflammatory mediators [TNF, nitric oxide (NO)] and can lead to transmigration of activated macrophages and dendritic cells (competent APCs) across the BBB (25,57,58). This general three-part scheme of immune privilege was the predominant viewpoint for most of the last century. However, careful analysis of recent data does suggest that each of these elements plays a role

in CNS-specific regulation of T-cell responses but that the roles involve much more than serving as passive barriers separating the CNS from the immune system.

The BBB is a barrier for molecules: is it really an impermeable barrier to lymphocytes?

The existence of BBB was first intimated by Paul Ehrlich in 1885 by his observation that systemically injected dye failed to penetrate the brain as readily as it penetrated the other tissues. Although the term *Bluthirnschranke* (blood–brain barrier) was first used as early as 1900 by Lewandowski, the existence and cellular structure of the BBB was debated well into the 1960s (59). Research by numerous groups has now revealed that the BBB is a complex structure consisting of specialized cerebrovascular endothelial cells in contact with pericytes and astrocytes (60-63). In contrast to the vasculature in other organs, CNS endothelial cells lack transendothelial fenestrations, have low pinocytotic activity, have high numbers of mitochondria, and are tightly joined to adjacent endothelial cells by complex tight occluding junctions with very high electrical resistance. The parenchymal side (abluminal) of these endothelial cells is surrounded by basement membranes and further encased by the endfeet of astrocyte processes. Pericytes are bone-marrow-derived cells found in the perivascular space between the basement membranes separating the endothelial cells from the astrocytic endfeet (64,65). In humans, this space is referred to as the Virchow-Robin space. The Virchow-Robin spaces are most prominent along the vasculature that enters the brain from the cortical surface and are continuous with the subarachnoid space [the space between the CNS and meningeal membranes filled with cerebrospinal fluid (CSF)]. *In vitro* studies have revealed that the specialized phenotype of the CNS cerebrovascular endothelial cells is dependent on the interactions between these different cell types and can be altered by astrocytic activation (59, 63).

The presence of endothelial tight junctions in the intact BBB has long been presumed to prevent leukocyte movement from the blood into the CNS (63,66). In support of this hypothesis, several *in vivo* and *In vitro* studies have demonstrated increased leakiness of the BBB to dyes and other small molecules at sites associated with leukocyte transmigration. Disruptions in the BBB detected by magnetic resonance imaging are often among the earliest signs of MS and tightly correlate with macrophage infiltration and lesion development in rodent EAE models (67). Several studies have attempted to link the onset of CNS autoimmunity with the changes in systemic levels of molecules able to disrupt endothelial tight junction structure. A small clinical study has monitored the serum levels of matrix metalloproteinase-9 (MMP-9), a molecule shown *In vitro* to facilitate T-cell migration across brain microvascular endothelial cells, and of tissue inhibitor of matrix metalloproteinase 1 (TIMP-1), its natural inhibitor, in patients with severe relapsing-remitting forms of MS (68). Suggestively, MS patients had higher levels of MMP-9 and higher ratios of MMP-9/TIMP-1 than healthy controls. Strikingly, treatment of these same patients with interferon-β (IFN-β) resulted in a decrease in their serum levels of MMP-9 and their MMP-9/TIMP-1 ratios. In separate studies, Minagar *et al.* (69) have shown that serum from MS patients, but not from patients in remission or from normal healthy controls, had decreased expression of two tight junction components, occludin and vascular endothelial (VE)-cadherin, in cultured cerebral microvessels and endothelial cells.

It must also be cautioned that the cause/effect relationship between BBB disruption and subsequent CNS infiltration of leukocytes in these situations is uncertain. Merely breaking the BBB does not automatically result in autoimmunity. Indeed, grafting foreign tissue into the CNS causes damage to the CNS vasculature (by direct mechanical disruption and subsequent trauma-induced TNF production). This transient rupture in the BBB was clearly insufficient to facilitate destructive proinflammatory T-cell responses (11). Induction of ischemia or treatment with hyperosmolar agents in several different animal models leads to more global

and prolonged damage to the cerebrovasculature (25). Yet, obligate induction of proinflammatory T-cell responses is still not observed. Finally, regions of the healthy CNS with normally incomplete or less developed 'leaky' BBBs are not associated with increased rates of lymphocyte accumulation or autoimmunity.

From an immunological perspective, the function of the BBB has been defined primarily in terms of limiting leukocyte movement into the CNS. Evolutionary evidence suggests that at best this role is a secondary one because the BBB arose prior to the adaptive immune system [reviewed by Lowenstein and Castro (70)]. The BBB performs several essential non-immune cell-related functions necessary for CNS function including limiting uncontrolled entry of blood-borne metabolites and toxins into the brain (60,71). The luminal (blood facing) side of the BBB has specialized transporters to regulate the movement of specific classes of nutrients (for glucose, amino acids, vitamins, and nucleosides) into the CNS. Conversely, the abluminal side of the BBB has specific transporters to move potentially toxic molecules (excitatory transmitters, metabolic waste products, and even Aβ, a major component of amyloid plaques in Alzheimer's disease) out of the CNS (72). Thus, the loss of BBB integrity is not a simple case of opening the CNS borders to non-CNS citizens such as the leukocytes. Rather, the loss of BBB integrity more closely approximates the sudden influx of toxic pollutants into a neighborhood from an unregulated industrial factory discharges. Acute exposure to blood-borne products and/or a decrease in the export of toxic metabolites directly induces neuronal stress and activation of CNS innate immune response (i.e. astrogliosis and microgliosis). Thus, by itself BBB disruption may be injurious and even predisposing for autoimmunity but alone is not sufficient to initiate autoimmune responses to CNS antigens (73).

BBB integrity is also insufficient to prevent leukocyte trafficking into the CNS. Using various genetic and fluorescent markers, several groups have demonstrated that pericytes and perivascular macrophages are continuously being replaced within the extensive vasculature of the healthy CNS (62,74,75). As it is a normal ongoing physiological process, by definition, this migration across the cerebrovascular endothelium occurs without detectable changes in normal BBB function.

Rare T cells can also be detected by histological analysis of CNS tissue from healthy individuals and healthy rodents kept under SPF conditions. Although most of these cells have apparently extravasated from the bloodstream, they tend not to enter the healthy CNS and remained located in perivascular, subarachnoid, or meningeal spaces (53,76,77). Flow cytometric analysis indicates that most of these cells are unactivated memory $CD4^+$ T cells. Much more prominent lymphocyte accumulation is observed when fully activated $CD4^+$ T cells are transferred into an otherwise unmanipulated recipient. Interestingly, several such studies using T cells of defined antigen specificity have revealed that activated CNS-specific $CD4^+$ T cells can apparently chaperone naive non-CNS-specific T cells across the BBB into the CNS (78,79).

We have also found that naive antigen-inexperienced T cells can enter the healthy CNS under lymphopenic conditions. Under normal conditions, T cells do not undergo proliferation in the absence of antigenic stimulus. In part, this condition is due to T-cell competition for survival factors such as CCL21, interleukin-7 (IL-7), and IL-15 (36). Naive $CD4^+$ T cells express CCR7, the receptor for CCL21. With normal numbers of naive $CD4^+$ T cells in circulation, there is insufficient CCL21 to promote antigen-independent homeostatic proliferation of $CD4^+$ T cells (36). However, when T-cell numbers drop below normal, as in genetically T-cell-deficient recombination-activating gene knockout mice or in response to sublethal irradiation, the increased availability of CCL21 triggers proliferation. Mice lacking CCL21 are unable to support homeostatic proliferation of $CD4^+$ T cells, while overexpression of CCL21 is sufficient to support homeostatic proliferation in the absence of lymphopenia (36). One consequence of homeostatic proliferation is the induction of VLA-4 and LFA-1 expression, adhesion molecules

required for lymphocyte migration across the BBB. Strikingly, CCL21 is expressed in the preclinical stages of EAE and many other spontaneous autoimmune diseases (80,81).

Wolburg *et al.* (82) have provided somewhat surprising data indicating how leukocyte migration across the BBB can occur without disruption of the complex tight junctions joining adjacent cerebrovascular endothelial cells. They performed histological analysis of serial tissue sections from inflamed CNS tissue taken from mice with EAE. In their studies, they provide clear evidence for transcellular migration of leukocytes through pore-like structures formed within the endothelial cells rather than between endothelial cells. A similar phenomenon has been observed *In vitro*. In culture models of cerebrovascular endothelial cells, transcellular migration has been found to be regulated by endothelial expression of several adhesion molecules: E-selectin, platelet-endothelial cell adhesion molecule, vascular cellular adhesion molecule-1 (VCAM-1), and ICAM-1 (83,84). Using *In vitro* models, Carman and Springer have further demonstrated that upon contact with leukocytes, the endothelial cells create membrane microdomains enriched with VACM-1 and ICAM-1 that surround the leukocytes and form a transient pore-like structure similar to that seen in the later studies by Wolburg (83). As yet, it is unclear to what extent this mechanism contributes to the routine and continual repopulation of pericytes and perivascular macrophages in the absence of inflammatory signals or the occasional appearance of memory T cells in healthy CNS tissue. Although transcellular migration in human tissue has also not yet been demonstrated, the studies of Wolburg *et al.* (82) provide further indications that leukocyte entry into the CNS is not absolutely dependent on prior breakdown of BBB integrity.

Considered together, these data indicate that BBB disruption is neither an automatic cause nor a requirement for autoimmunity. However, these data also do not exclude a role for the BBB in modulating the rate or propensity that leukocytes and particularly lymphocytes may enter the CNS. Furthermore, regulation may be in part under the control of CNS-resident cells such as astrocytes and microglia (85). For example, only a very small number of activated myelin-specific T cells enter the CNS immediately after adoptive transfer into a susceptible recipient animal. Russell and colleagues (79) have recently demonstrated that for the majority of transferred T cells, entry into the CNS was dependent on TNF-mediated induction of VCAM-1 not only on the endothelial cells but also on the astrocyte endfeet.

Ransohoff *et al.* (16) have recently reviewed the evidence for an alternative route of T-cell entry into the CNS that bypasses the BBB entirely: from the blood to the CSF via the choroid plexuses within the ventricles of the CNS. Based primarily on tracing studies using fluorescently labeled T cells, several investigators have shown that leukocytes can also extravasate across the fenestrated endothelium of the choroid plexuses through the epithelium directly into the CSF.

Does the absence of draining lymphatics keep the immune system ignorant of antigens located in the CNS?

The initial site of antigen encounter for most unactivated, naive, antigen-inexperienced T cells is in the secondary lymphoid tissues. However, it is an undisputed anatomical fact that the CNS lacks a traditional lymphatic system (16). For decades, the prevailing view was that the absence of a defined lymphatic system absolutely prevented CNS antigens from draining into the cervical lymph nodes. This position has been dramatically reversed by data accumulated during the last 20 years indicating that soluble CNS antigens can drain via the CSF along the perivascular and subarchnoid spaces through the cribriform plate into the lymphatics of the nasal submucosa (86,87). Unlike the BBB, the ependymal lining of the ventricles lack tight junctions. Therefore, soluble CNS antigens within the interstitial fluid of the brain can either drain into the perivascular spaces or into the CSF of the ventricles (16).

Most of these data rely on tracing the migration of injection of dyes and tagged molecules from the CNS parenchyma to the cervical lymph nodes. However, we and others have also detected CNS antigens in the cervical lymph nodes (88). In a marmoset model of EAE, de Vos *et al.* (88) detected myelin antigens associated with cervical lymph node dendritic cells. In our studies, we measured the *in vitro* proliferation of HA-specific T cells stimulated by APCs isolated from the lymph nodes of unmanipulated healthy mice expressing a HA transgene in either the pancreas (by insulin-producing β cells: Ins:HA mice) or the CNS (by astrocytes: GFAP:HA mice). We found that APCs from cervical lymph node draining the CNS of GFAP:HA mice could stimulate HA-specific T cells to a similar extent as the APCs from pancreatic lymph nodes of Ins:HA mice. Cervical and pancreatic lymph node APCs from non-HA-expressing mice failed to stimulate HA-specific T-cell proliferation. These data indicated that the cervical lymph node APCs were preloaded with the HA antigen expressed in the CNS. Thus, even in a healthy animal in the absence of inflammation-induced disruptions in BBB structure, CNS-expressed antigens appeared in the cervical lymph nodes. As yet, it is unclear whether these antigens passively drained into the lymph nodes or whether pericytes, perivascular macrophages, or dendritic cell populations within non-parenchymal CNS sites captured and actively transported the astocyte-derived HA antigen to the cervical lymph node.

Are CNS APCs really that incompetent?

The discussion to this point has illustrated that the BBB and absence of traditional lymphatics prevent neither the T cells from entering the CNS nor CNS antigens from reaching the cervical lymph nodes. Therefore, if CNS antigens do drain into the cervical lymph nodes, why do foreign tissue grafts fail to elicit a primary proinflammatory T-cell response? The answer to this question may lie in part with the nature of the APC populations found within the healthy CNS. Efficient initiation of T-cell responses or even the retention within a tissue of previously activated T cells relies on the presence of tissue APCs able to capture and process antigen from the environment. In contrast to soluble antigens, cell-associated antigens are actively transported by an activated APC to the draining lymph node (1). The ability to drive T-cell proliferation, differentiation, and cytokine production (in the lymph node or in the tissue) is highly dependent on the activation state of the APC and the array of costimulatory molecules expressed by the APC. The obvious question is: does the CNS have such a population of APCs able to process antigens from phagocytosed cell debris, to migrate to the cervical lymph nodes, and to express sufficient MHC and costimulatory molecules to drive robust proinflammatory T-cell responses?

Like all other tissues, the CNS is populated by cells of stellate morphology that can be labeled with most of the common macrophage markers, including Iba-1, cd11b, or F4/80 [reviewed by Melchior *et al.* (89)]. However, very little expression of MHC or costimulatory molecules can be detected in the CNS parenchyma of most strains of healthy rodents maintained under SPF conditions. By definition, the absence of MHC expression indicates that these CNS macrophage populations cannot act as APCs under these SPF conditions. However, this oft quoted characteristic of the healthy CNS is insufficient to explain the failure to initiate T-cell responses against the foreign tissue graft discussed at the beginning of this article. MHC class II expression is rapidly induced in response to nearly any acute insult to the CNS, including surgically induced trauma associated with placing a graft within the CNS parenchyma, or even alterations in neuronal activity (90). Furthermore, susceptibility to CNS autoimmunity does not correlate with basal or inducible CNS MHC expression (91). For example, EAE-resistant brown Norway rats express much higher basal levels of MHC in the CNS than EAE-susceptible Lewis rats (92).

Not all CNS APCs are created equal

Older literature refers to all macrophage populations located within the CNS with stellate morphologies as microglia (89). Considered as a single population, they comprise approximately 15% of all CNS cells and are found in all regions of the brain and spinal cord (89). There has been substantial debate on the origin of these CNS macrophages. Four major questions have dominated this discussion: are these CNS cells of neuroectodermal origin? Are they derived from macrophage precursors? Are they constantly being renewed from blood-derived cells, or are they a self-renewing population? Are the cells found in the healthy CNS functionally distinct from macrophages that are recruited to the CNS? The answers to these questions indicate that the microglia are a heterogeneous population of CNS-specific macrophages that play an important part in maintaining CNS immune privilege.

It was long recognized that these CNS macrophages could be induced to express nearly all markers of the macrophage lineage including CD45, also known as leukocyte common antigen, a tyrosine phosphatase expressed by all nucleated cells of hematopoietic lineage (93). These data implied that they were of the same mesenchymal origin as other non-CNS macrophage populations. The inability to generate microglia from neuroectodermal stem cells (*in vitro* or when implanted in the CNS) strongly supports a non-neuroectodermal origin for these cells (94). Today, the consensus is that all CNS macrophage populations are derived from hematopoietic precursors.

By and large, most studies use morphological criteria to distinguish CNS macrophage populations as CNS-resident microglia versus acutely infiltrating macrophage populations. In the confocal images presented in Fig. 1, we illustrate the inherent inaccuracies caused by the reliance on morphology as the primary identifying or parsing parameter. Three Iba-1-labeled myeloid cells are indicated in Fig. 1A. At first glance, all three cells are separated in distance by only a few cell bodies and thus they appear to be in essentially the identical CNS microenvironment. Cell 2 is much more highly ramified than cells 1 and 3 and displays the stereotypical morphology of a classic parenchymal microglia. Cells 1 and 3 appear somewhat elongated but still display stellate projections.

A cursory histological analysis might categorize all three cells as parenchymal microglia. However, in Fig. 1B,weshow the same section labeled with tomato lectin, which labels microglia, macrophages, and blood vessels. Fig. 1D shows the confocally merged images and dramatically reveals the difference between cell 1 and cells 2 and 3 (Supplemental Movie 1 available online is the three-dimensional rotation movie of these confocal images that clearly illustrates the relative location of the cells to the vasculature). The cell body and processes of cell 1 are tightly wrapped around a blood vessel. By contrast, cell 2 has endfeet extending to the blood vessel, while cell 3 is merely in close proximity to a blood vessel but does not come in contact with it (Supplemental Movie 1). This single image therefore depicts three cells that have very different basal contacts with the vasculature and therefore with the immune system.

Irradiation bone marrow chimeric rodents have been used to determine the functional similarity of cells in these three different locations (Fig. 2). In brief, bone marrow chimeric rodents are generated by first administering a dose of whole-body irradiation that is sufficient to kill the bone marrow. This dose must be sufficient to eliminate the primary source of immune cell stem cells in the adult animal. However, the dose cannot be too high such that it causes permanent radiation-induced damage to the intestine, tissue scarring, and non-specific innate inflammatory responses within internal organs. Animals receive replacement bone marrow from a genetically distinct donor immediately following irradiation. The turnover rate of macrophages within each tissue is then determined by monitoring the time required to replace macrophages displaying the recipient genotype with macrophages displaying the donor

genotype. These types of studies using chimeric rats and mice have demonstrated that CNS macrophages can be divided into at least two major populations (74,75,95): (i) a population that in the adult is rarely replenished from bone-marrow-derived cells, is primarily located within the parenchyma, and displays a $CD45^{lo}$ to $CD45^{intermediate}$ phenotype when assayed by flow cytometry (Fig. 1, cells 2 and 3) and (ii) a population that is rapidly replaced by bone-marrow-derived cells and displays a $CD45^{hi}$ phenotype. This second population is located in perivascular regions, meninges, choroid plexuses, and the subarachnoid spaces (Fig. 1, cell 1) and comprises pericytes, macrophages, and dendritic cells.

Does the APC source have consequences for APC function?

Vallieres and Sawchenko (95) have further refined this general picture. Their careful analysis of long-term irradiation bone marrow chimeric mice studies has provided suggested links between areas prone to experimentally induced autoimmunity and areas receiving substantial donor-derived contributions to parenchymal microglial populations. They noted that donor-derived cells never contributed to the parenchymal microglial population in some brain regions such as the cerebral cortex, caudoputamen, and hippocampal formation. By contrast, they found that bone-marrow-derived cells did contribute to the parenchymal microglial population in one brain region with demonstrated susceptibility to T-cell-mediated responses in EAE: the cerebellum. Within the cerebellum, donor-derived microglia were always located in the molecular layer and most abundantly the paraflocculus.

Can any of the CNS APC populations initiate T-cell responses against CNS antigens?

Nearly 20 years ago, Hickey and Kimura (74) used irradiation chimeric rats to differentially manipulate the ability of either resident parenchymal microglial or the bone-marrow-derived cells to express the appropriate MHC class II to act as APCs in MBP-induced EAE (Fig. 2). In their seminal studies, they found that limiting MHC class II expression to the CNS-resident cells was not permissive for the induction of EAE. By contrast, limiting MHC class II expression to just the peripheral immune system was sufficient for the initiation of EAE. These studies were the first to demonstrate clearly that peripheral and not CNS endogenous APCs were necessary and sufficient for the generation of destructive, proinflammatory, T-cell-mediated anti-CNS immune responses. However, these original studies did not fully discern whether different subsets of bone-marrow-derived APCs (pericytes, perivascular macrophages, and/or infiltrating dendritic cells) were equivalent in their abilities to initiate versus sustain the proinflammatory response. These studies also did not determine whether initiation of destructive proinflammatory responses had to occur in secondary lymph-oid organs or within the CNS itself.

Approximately 30 years later, studies by McMahon *et al.* (96) and Greter *et al.* (97) have partially addressed these two issues. The studies by Greter *et al.* (97) essentially repeat the same experiments of Hickey and Kimura (74) using a transgenic mouse line in which MHC class II expression is restricted to $CD11c^{+}$ cells, characterized as consisting primarily of dendritic cells. Using the same bone marrow chimeric approach as Hickey and Kimura (74), Greter *et al.* (97) demonstrate that of all the possible MHC class-II-expressing peripheral immune cell populations, CNS-infiltrating $CD11c^{+}$ dendritic cells are by themselves sufficient to support EAE caused by transfer of preactivated myelin oligodendrocyte/glycoprotein (MOG)-specific T cells into pertussis-toxin-treated recipient mice. In addition, Greter *et al.* (97) also generated mice that lacked secondary lymphoid tissue by splenectomizing *aly* mice that lack lymph nodes and Peyer's patches. Using these mice, they demonstrated that sustaining the activity of preactivated MOG-specific T cells does not require T-cell trafficking to APCs located in secondary lymphoid tissues.

Perhaps one of the most surprising results from this study is the ability to induce clinical MOG-EAE in the absence of any B-cell responses. The absence of MHC class II expression on B cells in their models prevents B cells from acting as APCs to MOG-specific T cells and requesting $CD4^+$ T-cell help for antibody production. The data of Greter *et al.* (97) are unexpected because several studies have strongly correlated MOG antibody production with disease severity and even the generation of a spontaneous transgenic model of EAE (98,99). It would be informative in future studies to add back APC function in the B cell and pericyte populations to test how these cells may modify the initiation of anti-CNS immune responses. This examination may be especially important in the dissection of pathogenesis in spontaneous, immunization-induced, and adoptive transfer models of autoimmune disease.

Caveats for the use of irradiation bone marrow chimeras

A few key caveats for these types of studies do need to be mentioned. First, irradiation leads to widespread if transient activation of glia and endothelial cells throughout the CNS. Most notably, transient disruptions in the BBB have been reported. For this reason, some investigators choose to shield the head during the irradiation procedure. At this time, the extent that shielding the head limits disruptions in the BBB that might be caused by irradiation-induced increases in systemic cytokines is uncertain. Second, sufficient time (at least 8 weeks) must be allowed for full reconstitution of the peripheral immune cell by the donor bone marrow. Finally, investigators need to verify the effectiveness of their irradiation, by testing for chimerism in the peripheral immune cell compartment. Chimerism in the peripheral immune cell compartment can lead to the mis-identification of cells recently derived from the bone marrow as being of the recipient (non-bone marrow)-derived source.

Antigen presentation can occur in the CNS even in the presence of intact secondary lymphoid organs

McMahon *et al.* (96) performed a set of experiments complementary to those of Greter *et al.* (97). McMahon *et al.* (96) used two different models of anti-CNS T-cell responses: a $PLP_{178-191}$-induced relapsing EAE in SJL mice and TMEV-induced demyelinating diseases. Unlike the adoptive transfer model of MOG-EAE, epitope spreading beyond the initial immunogen is a prominent pathogenic feature of both models. McMahon *et al.* (96) induced relapsing EAE or TMEV infection prior to transferring dye-labeled naive T cells of known $PLP_{139-151}$ specificity. The studies by Greter *et al.* (97) demonstrated that adoptive transfer EAE could be supported in the absence of secondary lymphoid tissue. The studies by McMahon *et al.* (96) conclusively demonstrate that even in the presence of secondary lymphoid tissue, proliferation of (and thus presumably antigen presentation to) the dye-labeled naive $PLP_{139-151}$ occurs within the CNS in the perivascular regions in close proximity to $CD11c^+$ cells, presumably the CNS-infiltrating dendritic cells identified by Greter *et al.* (97). *Ex vivo* tests of the ability of $CD11c^-$ microglia, $CD11c^-$ macrophages, and $CD11c^+$ dendritic cells revealed that all three types of cells could drive proliferation of PLP-specific cells, but the dendritic cells were most effective.

Microglia can act as APCs in vivo but differ from other APCs

The *in vivo* measures of CNS APC function discussed so far examined the differential ability of resident versus peripheral cells to act as effective APCs for CNS antigens within mice treated with adjuvants (pertussis toxin, virus, or complete Freund's adjuvant). We reexamined the ability of intracerebrally injected $CD45^{hi}$ dendritic cells versus $CD45^{lo}$ microglia to act as APCs in the healthy CNS (53). To strictly limit APC function to just the injected cells, we used 107KO mice as a source of APCs and ANDB6 mice as the recipient host. The ANDB6 transgenic mouse line expressed a transgenic TCR specific for moth cytochrome *c* peptide 88–

102 presented on the MHC class II haplotype I-E^b. The AND transgene was maintained on a C57Bl/6 genetic background that does not express I-E^b. However, ANDB6 T cells are still positively selected in the thymus of C57Bl/6 mice on the MHC class II haplotype I-A^b. Thus, 90% of the $CD4^+$ T cells in ANDB6 mice are specific for moth cytochrome *c* peptide, but 0% of the MHC class-II-expressing APCs in these mice can present this target antigen to the ANDB6 T cells. 107KO mice are maintained on a C57Bl/6 genetic background and do not express any other MHC class II except I-E^b (the MHC allele required to present the moth cytochrome *c* peptide to ANDB6 T cells).

Using this model, we intracerebrally injected both peptide-loaded 107KO dendritic cells and activated MHC class-II-expressing microglia into the ANDB6 CNS (53). Injected dye-labeled cells migrated throughout the CNS along the white matter tracts, and these cells could also be observed in the subarachnoid spaces and perivascular regions. Despite expressing high levels of T-cell chemokines, injected dendritic cells failed to recruit T cells into the CNS in the absence of antigen. Only antigen-primed dendritic cells were able to recruit naive ANDB6 cells into the CNS. Intracerebrally injected microglia could only recruit T cells previously primed in the periphery by injected splenic APCs.

The previous studies suggested that in adjuvant-primed and/or inflamed CNS, all presentation of CNS antigens occurred only in the CNS. The current model used recipient mice that did not have an ongoing inflammatory response and that were not treated with adjuvants. Thus in the ANDB6 model, the differential ability of dendritic cells and microglia to transport antigen to the draining lymph node may underlie their differential ability to initiate unprimed T-cell responses. In our assays, microglial migration to the cervical lymph nodes was never detected (53). Therefore, we concluded that either microglia never leave the CNS or they do leave at a rate that was at least two orders of magnitude lower than that of injected dendritic cells. Regardless of the type of APC injected, the healthy CNS parenchyma apparently modulated the activation state of the recruited cells. Few of the recruited ANDB6 T cells expressed activation markers, and those that did were preferentially located in non-parenchymal sites (i.e. meninges, ventricles, subarachnoid spaces, perivascular cuffs). Therefore, this study indicates that even in contact with mature antigen-bearing dendritic cells, proinflammatory T-cell responses are less likely to be activated from within the CNS parenchyma.

Are microglia irrelevant as APCs in CNS-specific T-cell responses?

Considered together, do all these studies indicate that CNS-resident microglia are irrelevant as CNS APCs? Or to restate, is there any potential evolutionary selection pressure driving the well-characterized propensity of microglia to express MHC class II in response to nearly any insult to the CNS? Even upon full activation, the levels of MHC and costimulatory molecules are much lower than that seen of ‘professional’ dendritic cell APCs. Irradiation bone marrow chimeric mice have illustrated that microglial expression of costimulatory molecules, such as CD40, and/or cytokines that drive T-cell effector function, IL-12 and/or IL-23, are essential for the development of EAE of equivalent clinical severity as seen in wildtype controls (100, 101). In the presence of microglia unable to express these molecules, peripheral immune cells were still able to infiltrate into the CNS. These data support the suggestion that resident microglia are not playing the primary role in antigen-specific recruitment of lymphocytes into the CNS. Rather, they are more likely to play significant roles in either modifying or directing T-cell effector function.

Ex vivo APC assays of microglia function provide data consistent with these observations. For *Ex vivo* assays, CNS-resident microglia are isolated from the adult rodent CNS and separated from the CNS-infiltrating macrophages/dendritic cells by flow cytometry based on their relative levels of CD45 expression (93,102-104). Using cells isolated from adult murine CNS,

two different studies demonstrated that CNS inflammation and demyelination induced by either overexpression of IL-3 or induction of EAE do increase the APC function of CNS-resident microglia (103,105). However, the ability of activated $CD45^{lo/intermediate}$ microglia to drive T-cell proliferation was much lower than that of $CD45^{hi}$ CNS-infiltrating macrophages and dendritic cells. By contrast, the ability of activated microglia to drive T-cell effector function toward polarized Th1 cytokine production following primary and secondary stimulation was much greater than that of the $CD45^{hi}$ cells. In large part, their relatively poor stimulation of T-cell proliferation was due to their production of prostaglandins and NO (103,105). These classic proinflammatory molecules not only directly inhibit T-cell proliferation but also decrease APC expression of MHC and costimulatory molecules. Thus, microglia appear specialized to promote short self-limiting T-cell responses.

Microglia may also play significant roles in terminating ongoing CNS T-cell responses and in promoting clinical remission during EAE and/or MS. For example, APCs can inhibit proinflammatory T-cell responses by expressing inhibitory costimulatory molecules such as B7-H1 (also called PDL1) or anti-inflammatory molecules such as indolemine (106,107). Magnus *et al.* (106) found that exposing microglia to IFN-γ or to the supernatants from activated proinflammatory T-helper 1 cells induced microglial expression of B7-H1 (106). In the presence of B7-H1 blocking antibodies, microglia were much more effective in inducing T-cell activation.

Antigen presentation is a two-way street

As a consequence of APC-T-cell interactions, activated T cells are also likely to play prominent roles in regulating microglial immune functions. T-cell expression of CD40 ligand (also known as CD154) drives microglial production of NO and TNF (108). Again, using bone marrow chimeric mice, Ponomarev *et al.* (109) demonstrated that in the absence of microglial CD40 expression, activated encephalogenic T cells cannot increase the microglial expression of CD45, MHC class II, and B7.2 *in vivo*. Unexpectedly, CD40-deficient microglia proliferated within the inflamed CNS at a much higher rate than wildtype microglia. A careful quantification of peripheral cell infiltration revealed that as a result of microglial CD40 deficiency, fewer T cells and peripheral macrophages accumulated in the CNS. For T cells, this deficiency was associated with decreased proliferation. The demonstration of a progression in microglial activation associated with pathology-specific interactions is also consistent with the previous reports of Miller and colleagues (110), indicating that microglial APC function in TMEV was dependent on disease stage (110).

A novel role for microglia in promoting neuroprotection and clonal diversion of T-cell effector function

To this point, we have concentrated the discussion on the initiation of proinflammatory, anti-CNS T-cell responses. However, antigen presentation can also have at least two additional outcomes: initiation of anti-inflammatory, immunosuppressive regulatory T-cell (Treg) function and promotion of non-inflammatory, neuroprotective T-cell responses. For immune responses occurring outside of the CNS, an active and as yet unresolved area of research is the phenotype(s) of Treg-promoting APCs (111). However, APCs that express low levels of MHC and costimulatory molecules can direct T-cell function toward a T-helper 2, anergic, or Treg phenotype, depending on the context and history of the T cell.

Within the CNS, Streilein and colleagues (112) were among the first to provide evidence that CNS antigens were not hidden from the immune system. They demonstrated that in the absence of preexisting infection/inflammation or other experimentally added amplifying factors (i.e. complete Freund's adjuvant and pertussis toxin), presentation of CNS antigens promoted

immunosuppression. In an elegant series of studies spanning several decades, Streilein and colleagues (113) characterized this phenomena, termed anterior-chamber-induced immune deviation within the eye and brain-induced immune deviation within the brain. In brief, they demonstrated that a T-cell response was initiated against antigens placed into the healthy CNS. However, instead of stimulating a pro-inflammatory response, injection of antigen into either the anterior chamber of the eye or the brain of rodents primed T cells to produce high levels of IL-10 and transforming growth factor-β if they subsequently encounter that same peptide outside the CNS (for example, if injected within the footpad). They demonstrated that the effect on the antigen-specific lymphocytes was sufficient to dramatically inhibit delayed-type hypersensitivity responses to antigen reencountered outside the CNS.

Brabb *et al.* (34) subsequently used MBP-TCR transgenic mice to illustrate a similar phenomenon. In these mice, approximately 95% of the $CD4^+$ T cells are specific for the MBP. She and her colleagues not only discovered that naive MBP-specific T cells could enter the CNS but also that in young mice, the naive T cells isolated from the CNS could not be stimulated to proliferate in response to MBP, even in the presence of IL-2. As the mice aged, most of the MBP-specific T cells that were isolated from the CNS increasingly displayed a memory phenotype. From their studies, they concluded that antigen presentation within the healthy CNS and in the absence of amplifying adjuvant factors led to the generation of anergized T cells. Although untested, these T cells may have had the same Treg functions as those described in the studies by Streilein and colleagues.

More recently, several reports have suggested that presentation of CNS antigens in the absence of adjuvant may do more than promote immunosuppression [reviewed by Carson (5)]. It may even lead to the generation of neuroprotective T-cell responses. Interestingly, following facial axotomy, the generation of neuroprotective $CD4^+$ T-cell responses was found to depend on antigen presentation by both peripheral APCs and CNS-resident microglia (26). Motoneuron cell bodies are located within the CNS, while their axons project outside of the CNS. Facial axotomy leads to microglial activation and CNS infiltration of macrophages and T cells (114). In a wildtype mouse, only 15% of motoneurons die 4 weeks after axotomy (115). By contrast, approximately 50% of motoneurons die in mice that lack $CD4^+$ T cells or are unable to present antigen to $CD4^+$ T cells (MHC class II knockout mice) (115). Using irradiation bone marrow chimeric mice, we generated mice in which either only microglia or only peripheral immune cells could express MHC class II and act as APCs (26). Mice that do not express MHC class II in the thymus failed to generate $CD4^+$ T cells. Therefore, T cells were isolated from healthy unmanipulated wildtype mice and transferred into axotomized mice expressing MHC only in the CNS or only in the peripheral immune system.

We found that $CD4^+$ T cells were unable to inhibit motoneuron cell death in axotomized chimeric mice in which MHC class II expression was limited to either the CNS or the peripheral immune system. These data indicated that neither the CNS-resident APCs nor the peripheral APCs could by themselves drive neuroprotective T-cell responses. Speculating that microglia were unable to traffic to the lymph nodes and initiate the T-cell responses, we transferred T cells isolated from axotomized wildtype mice into our axotomized chimeric mice. In this paradigm, T cells could be activated by peripheral APCs in wildtype axotomized mice. Subsequent transfer into axotomized mice tested whether CNS-infiltrating APCs or CNS-resident microglia would be sufficient to inhibit motoneuron cell death. Neuroprotection was only seen in mice expressing MHC class II in the CNS-resident microglia. In sum, macrophages were required to initiate the lymphocyte response. However, despite their infiltration into the CNS of axotomized chimeric mice, peripheral macrophages were insufficienttosupport$CD4^+$ T-cell-mediated neuroprotection of motoneurons; however, once T-cell activation was initiated by peripheral macrophages, protection of motoneurons following facial axotomy was absolutely dependent on antigen presentation by microglia activated by local

neurodegenerative signals. As yet, the mechanism of protection is undefined. Tantalizingly, at least one report suggests that antigen presentation by microglia may promote T-cell production of neurotrophic factors.

State of the CNS defines CNS-specific regulation of immune responses

As the concept of CNS immune privilege evolves beyond immune isolation, research has begun to elucidate the extent that CNS neurons and macroglia (astrocytes and oligodendrocytes) actively regulate immune responses. Intercerebral injections and *in vivo* tracking studies have demonstrated that the CNS can be cytotoxic for lymphocytes. Depending on the activating stimulus and the genetic background, CNS inflammation can be associated with an increase in the global proapoptotic environment of the CNS. In part, this inflammation is due to increased astrocytic expression of CD95L (17,116).

In vitro studies have also revealed robust regulation of microglial MHC class II expression by neuronal activity (90,117). For example, neuronal and macroglial production of neurotrophins inhibits the IFN-γ-induced microglial expression of MHC class II *In vitro*. Conversely, in microglial–neuronal cocultures, blocking neuronal electrical activity induces microglial expression of MHC class II. Restoration of neuronal activity is sufficient to reduce microglial expression of MHC. Neuropeptides such as alpha-melanocyte stimulating hormone (α-MSH) and vasoactive intestinal peptide (VIP) inhibit proinflammatory cytokine and NO production by lipopolysaccharide-activated cultured microglia, while GABA has immunosuppressive effects on T and B-cell function (117). Not all neuropeptides are immunosuppressive. Norepinephrine and dopamine facilitate lymphocyte activation, while substance P augments microglial production of proinflammatory factors.

Although the ligand has not yet been identified, Daws *et al.* (118) have reported that a binding activity for the triggering receptor expressed on myeloid cells-2 (TREM-2) is expressed on the surface of activated astrocytes. TREM-2 is an orphan receptor that forms a signaling complex with DAP12 (119,120). Both TREM-2 and DAP12 are expressed by microglia and other myeloid cells (89,121). Antibody cross-linking studies demonstrated that the TREM-2/DAP12 pathway induces MHC class II expression and APC function of dendritic cells, macrophages, and microglia. We previously reported that TREM-2 expression is somewhat heterogeneous throughout the CNS (Table 1), being lowest in CNS regions with incomplete BBB. Somewhat unexpectedly, microglial expression of TREM-2 was highest in areas with greatest susceptibility to Alzheimer's disease pathology (89,121). Humans lacking a functional TREM-2/DAP12 pathway develop an early onset form of cognitive dementia (122). Recently, we also found that level of expression of TREM is high on $CD45^{lo/intermediate}$ microglia than on CNS-infiltrating $CD45^{hi}$ macrophages. While activated astrocytes express many proinflammatory cytokines, these data suggest that astrocytes have the potential to regulate APC function of myeloid cells in a spatially restricted cell-contact-dependent mechanism.

A very recent publication provides indirect evidence that neurons may also regulate TREM-2-mediated activation of microglia (123). In this study, Takegahara *et al.* (123) demonstrate that TREM-2 forms a heterocomplex with the semaphorin receptor, plexin-A1, and thus links plexin-A1 to DAP12-mediated intracellular signaling. Within the CNS, neurons express a ligand for plexin-A1: semaphorin 3A (124). In Alzheimer's disease, semaphorin expression increases in the same regions where TREM-2 is highly expressed (124). It is tempting to speculate that in Alzheimer's disease, CNS neurons are attempting to promote their survival by triggering increased neuroprotective APC function in microglia via the TREM-2 pathway.

Three additional molecules have been implicated in neuronal regulation of microglial and CNS-infiltrating immune cell function: CD22, CD200, and fractalkine. As mentioned earlier in this article, all nucleated differentiated cells of hematopoietic origin express CD45, an inhibitory

receptor for CD22 (125). CD22 has classically been viewed as a B-cell antigen, but Mott *et al.* (125) have recently shown that CNS neurons also express high levels of CD22. CD45 function has been studied in the greatest depth using peripheral macrophages. In these cells, CD45 functions as both an activating signal and a Janus kinase tyrosine phosphatase that negatively regulates cytokine receptor signaling involved in the differentiation, proliferation, and antiviral immunity of hematopoietic cells (126). Taken together, these observations suggest that CD22-expressing neurons would be more efficient at inhibiting macrophage activation than microglia activation for the simple reason that macrophages express an order of magnitude higher level of CD45 than microglia. This in turn supports the concept that activated resident microglia may be performing different functions that are perhaps less destructive or more beneficial than those performed by CNS-infiltrating macrophages.

Similarly, CNS neurons express both CD200 and the membrane-bound chemokine fractalkine, while microglia express CD200R and CX3CR1, the respective receptors for these molecules (127-129). In mice lacking either of these molecules, microglia display an increased activation profile in unmanipulated mice, while induction of EAE and neurodegeneration was much more rapid and severe (127,129). As yet, it is incompletely explored how aging coupled with the onset of neurodegenerative disorders (i.e. Alzheimer's and Parkinson's diseases) alters the coordinated neuronal expression of these immune regulatory neuropeptide and immune ligands. The recent series of clinical trials aimed at promoting anti-amyloid responses to breakup and remove plaques in aged Alzheimer's disease patients underscores the obvious need to clarify the likely synergistic actions of these multiple neuronal mechanisms modulating immune cell function (130).

Immune privilege is actively maintained: implications for CNS immunotherapies

The last 20 years have seen a dramatic reorientation in the field of neuroimmunology. The CNS is no longer viewed as being cloistered away from inappropriate encounters with the peripheral immune system. Rather, new evidence implies that CNS–immune system interactions may actually be a normal ongoing mechanism for the maintenance of CNS integrity. As yet, it is largely unconsidered whether therapies for CNS autoimmune disease, such as Tysabri, that inhibit T-cell entry into the CNS will have long-term effects on the maintenance of CNS function by impeding neuroprotective T-cell responses.

In this review, we have detailed the multiple mechanisms by which the CNS actively regulates immune cell entry into the CNS, the detection of antigens located within the CNS, and the type of immune response elicited by antigen detection. By and large, for most of us, for most of our lives, CNS immune privilege contributes to the healthy function of brain and spinal cord. Unfortunately, the active maintenance of immune privilege by the coordinated actions of CNS neurons and glia also indicates that the CNS inflammatory and autoimmune disorders may result as much from CNS dysfunction as from immune cell function. When the normal immune regulatory mechanisms of the CNS are rendered dysfunctional by age, pathogen exposure, or neurodegeneration, these events by themselves may not be sufficient to trigger autoimmunity or chronic neuroinflammation. However, they are likely to alter the threshold for CNS inflammation and the ability of the CNS to direct lymphocyte effector functions. The outcome may ultimately be a decrease in the ability to both promote neuroprotection and support controlled proinflammatory responses against pathogens and other insults. Data from multiple inbred animal studies identify the strong influence of genetic background as a further complication in determining the highly variable effectiveness of lymphocyte responses in clearing pathogens from the CNS and in promoting neurosurvival versus neurotoxcity (91).

In sum, the new realization that CNS immune privilege is not equivalent to immune isolation is leading to the development of radically new therapeutic approaches for Alzheimer's disease/spinal cord injury and MS that involve stimulating or blocking anti-CNS immune responses, respectively. While both approaches are likely to ultimately lead to the development of successful therapies, extreme caution will need to be taken to develop biomarkers that will provide readily interpretable signs of distress caused by these new immune-based therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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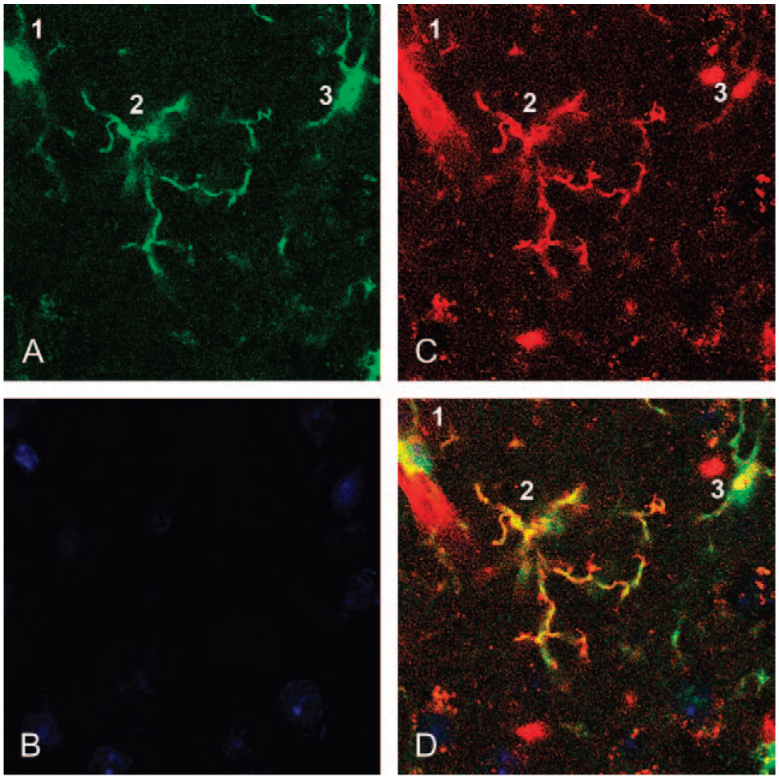

Fig. 1.

Microglial morphology is an inaccurate predictor of juxtaposition with cerebrovasculature. (A) Three apparently stellate Iba-1^{+}cells in the CNS of a healthy adult mouse are identified in green. (B) All nuclei in the same tissue section are identified in blue with DAPI. (C) All myeloid cells and blood vessels are identified in red with tomato lectin. (D) Merged images of all three panels illustrates close juxtaposition of cell 1 but not that of cells 2 and 3 with blood vessels. Confocal images were taken using a Zeiss. A rotating three-dimensional image is provided online as Supplemental Movie 1.

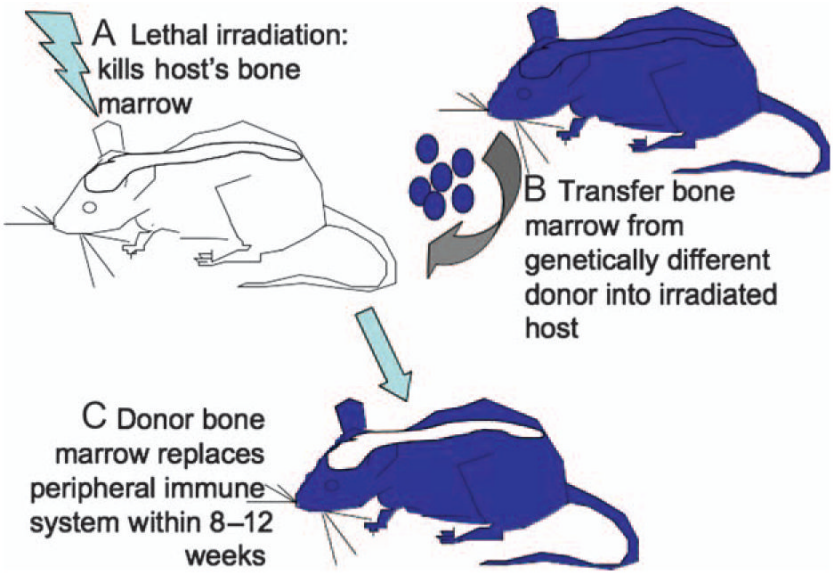

Fig. 2. The genotype of resident microglia and peripheral immune cells can be differentially manipulated using irradiation bone marrow chimeric methodology.

(A) Rodents receive whole-body irradiation sufficient to kill the adult bone marrow stem cell population. (B) To survive, irradiated rodents must receive bone marrow from a donor. The genotype of the donor and recipient can differ. (C) The donor bone marrow contributes to the repopulation of the peripheral immune system but not to that of most the CNS myeloid population (see text for discussion).

Table 1

Microglial population density and TREM-2 expression is region dependent

Brain regions	Relative microglial density (no. of cells/mm^2)	Relative percentage of TREM-2+ microglia	Relative expression level per microglia
Cingulated cortex	40	45	+++
Nucleus accumbens	60	13	+++
Medial hippocampal formation	30	<1	++
Laterodorsal thalamic nucleus	45	<1	−
White matter tracts			
Fimbria	25	20	+++
Corpus collosum	25	28	++
Cerebellum molecular layer	20	13	++
Areas with incomplete BBB			
Vascular organ	Not determined	<1	−
Subfornical organ	Not determined	<1	−
Medial accessory optic tract	70	<1	−
Median eminence	Not determined	<1	−
Anterior hypothalamic nucleus	45	10	++