Published in final edited form as:
Immunol Rev. 2011 May ; 241(1): 228–240. doi:10.1111/j.1600-065X.2011.01016.x.

Immune Tolerance in Multiple Sclerosis

Joan M. Goverman
Dept. Immunology, University of Washington, Seattle, WA USA

Summary

Multiple sclerosis is believed to be mediated by T cells specific for myelin antigens that circulate harmlessly in the periphery of healthy individuals until they are erroneously by an environmental stimulus. Upon activation, the T cells enter the central nervous system and orchestrate an immune response against myelin. To understand the initial steps in the pathogenesis of multiple sclerosis, it is important to identify the mechanisms that maintain T-cell tolerance to myelin antigens and to understand how some myelin-specific T cells escape tolerance and what conditions lead to their activation. Central tolerance strongly shapes the peripheral repertoire of myelin-specific T cells, as most myelin-specific T cells are eliminated by clonal deletion in the thymus. Self-reactive T cells that escape central tolerance are generally capable only of low-avidity interactions with antigen-presenting cells. Despite the low avidity of these interactions, peripheral tolerance mechanisms are required to prevent spontaneous autoimmunity. Multiple peripheral tolerance mechanisms for myelin-specific T cells have been indentified, the most important of which appears to be regulatory T cells. While most studies have focused on $CD4^+$ myelin-specific T cells, interesting differences in tolerance mechanisms and the conditions that abrogate these mechanisms have recently been described for $CD8^+$ myelin-specific T cells.

Keywords

tolerance; multiple sclerosis; myelin; EAE

Introduction

Multiple sclerosis (MS) is an inflammatory, demyelinating disease of the central nervous system (CNS) that is believed to be initiated by self-reactive T cells that recognize myelin antigens infiltrating the CNS. Entry of naive T cells into the CNS is limited by the tight junctions between endothelial cells that comprise the blood brain barrier. In contrast, activated T cells express an array of cell-surface proteins that facilitate both their adhesion to vascular walls and extravasation across this barrier. Therefore, the first event thought to occur in the pathogenesis of MS is a breakdown in tolerance that allows activation of naive myelin-specific T cells circulating in the periphery of healthy individuals. Epidemiological and genetic studies indicate that MS is triggered in genetically susceptible individuals following exposure to environmental factors, which are likely responsible for loss of tolerance and activation of the myelin-specific T cells. Once the activated T cells have infiltrated the CNS, they encounter local antigen-presenting cells (APCs) that are processing and presenting epitopes of myelin proteins synthesized within the CNS. The re-activation of the myelin-specific T cells within the CNS triggers effector functions that initiate the

Corresponding author: Joan Goverman, Box 357650, University of Washington, Seattle WA 98195, Tel.: +1 206 685 7604, Fax: +1 206 543 1013, goverman@u.washington.edu.

The author declares no conflicts of interest.

recruitment of a host of inflammatory leukocytes and ultimately lead to the destruction of myelin (1).

Much of our understanding of the pathogenesis of MS is based on studies using the animal model experimental autoimmune encephalomyelitis (EAE). EAE is induced by stimulating an immune response directed against CNS antigens (2). EAE can be actively induced by immunization with myelin-derived antigens in adjuvant. The immunization protocol causes the myelin antigens to be presented primarily by MHC class II molecules, therefore, the primary effector T cells in this model are $CD4^+$ myelin-specific T cells. EAE can also be passively induced by adoptive transfer of activated myelin-specific T cells obtained from an immunized animal into a naive recipient animal. Either method of induction results in a disease in which the inflammatory infiltrates and demyelination in the CNS exhibit many similarities to the pathology of multiple sclerosis (3).

The ability to induce EAE by adoptive transfer of myelin-specific T cells reinforced the idea that MS is a T cell-mediated autoimmune disease. The observation that transfer of $CD4^+$ T cells alone was sufficient to trigger EAE focused most studies investigating pathogenic mechanisms to concentrate on the pathogenic role of $CD4^+$ T cells. However, analyses of CNS tissues from MS patients have shown that $CD8^+$ T cells are more abundant in MS lesions than $CD4^+$ T cells. Clonal expansion is also detected more frequently among $CD8^+$ T cells isolated from MS lesions compared to $CD4^+$ T cells. In both the CSF and blood of MS patients, preferential enrichment of memory $CD8^+$ T cells exhibiting evidence of oligoclonal expansion compared with $CD4^+$ memory T cells is observed (4,5). The frequency of $CD8^+$ but not $CD4^+$ T cells specific for CNS antigen-derived peptides was also shown *in vitro* to be higher in patients with MS compared with healthy individuals (6). Collectively, these observations suggest that $CD8^+$ T cells may play an important role in the pathogenesis of MS. Consistent with this hypothesis; our laboratory has recently developed an animal model that demonstrates a pathogenic role for $CD8^+$ T cells in CNS autoimmunity. Accordingly, tolerance mechanisms that restrain activity of both $CD4^+$ and $CD8^+$ myelin-specific T cells are reviewed here.

Mechanisms of central and peripheral immune tolerance

The first opportunity to eliminate self-reactive T cells occurs in the thymus, where thymocytes undergoing high-avidity interactions with APCs presenting a self-antigen are eliminated by clonal deletion. Thymocytes experiencing interactions with APCs that while not strong enough to trigger cell death are sufficiently strong to indicate an unacceptable level of self-reactivity may undergo induction of anergy, TCR revision, or be diverted into alternative lineages, such as $FoxP3^+$ regulatory T cells or CD8αα T cells instead of undergoing deletion. Most of the proteins present in CNS myelin are not found in peripheral myelin, therefore, they can be considered tissue-specific antigens (TSAs). Ectopic expression of many TSAs occurs in a specialized subset of thymic epithelial cells, allowing these cells to induce central tolerance and purge the TCR repertoire of many T cells specific for TSAs. The importance of this mechanism in maintaining tolerance to TSAs was confirmed when mutations in AIRE, a protein that regulates the ectopic expression of many TSAs in the thymus, were shown to cause autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy in humans (7).

Despite the numerous mechanisms of central tolerance, it is clear that self-reactive T cells still escape to the periphery. This may occur stochastically for some thymocytes simply because they did not encounter the limited number of APCs expressing their cognate ligand as they matured in the thymus. In other cases, the avidity between the self-reactive thymocyte and the APC presenting the self-antigen may not be quite high enough to trigger

the normal mechanisms of central tolerance. Low avidity interactions may occur because the TCR has relatively low affinity for the peptide/MHC complex. Alternatively, the peptide may have low affinity for the MHC molecule, and this unstable interaction may result in a low abundance of peptide/MHC complexes available on the APC cell surface. Normally, these low avidity interactions would not pose a threat when the T cells enter the periphery, as the threshold for T cell activation in the periphery is believed to be higher than the threshold for induction of central tolerance. However, T cells with low avidity for their self-antigen/MHC complex could contribute to autoimmunity if the abundance of self-antigen/MHC complexes increases in the periphery relative to the amount found in the thymus, or a post-translational modification of the self-antigen occurs in the periphery that increases the affinity of the TCR for the self-antigen/MHC complex. Furthermore, antigens that are developmentally expressed may not be present in the thymus when some thymocytes are subjected to negative selection, resulting in a failure to induce central tolerance. Cells expressing these antigen/MHC complexes later in life could become the targets of self-reactive T cells that matured in the thymus and entered peripheral circulation prior to the expression of the self-antigen. Finally, it may not be possible to exert central tolerance with sufficient stringency to eliminate all self-reactive T cells and still generate a peripheral T-cell repertoire capable of exhibiting broad specificity for pathogens.

Because central tolerance is not 100% efficient, mechanisms of peripheral T-cell tolerance are required to prevent autoimmunity. Peripheral tolerance mechanisms that occur when a T cell engages a self-peptide/MHC complex on the surface of a ‘quiescent’ APC that has not sensed the presence of a pathogen include clonal deletion, induction of anergy, downregulation of the TCR and/or downregulationCD4/CD8 coreceptors. Expression of Aire by cells in peripheral lymphoid organs may also contribute to tolerance to TSAs, as observed in the thymus (8). Active peripheral tolerance is also maintained by numerous types of regulatory T cells, the best known of which are FoxP3$^+$ Tregs that develop naturally in the thymus or can be induced in the periphery. As discussed, below, all of these mechanisms contribute to maintaining tolerance to myelin antigens.

Mechanisms for tolerizing myelin-specific CD4$^+$ T cells

Historically, the CNS was considered an ‘immunologically privileged’ site in which immune responses were difficult to mount because of the ability of the blood brain barrier to limit T-cell trafficking of the CNS, and because of the almost complete lack of MHC expression on cells in the CNS. These features also suggested that immune tolerance to myelin antigens would be difficult to induce within the CNS itself. Furthermore, the lack of formal lymphatic drainage of the CNS suggested that transport of myelin protein epitopes to lymph nodes might be inefficient, which would impair the function of peripheral tolerance mechanisms that normally operate in lymphoid tissues when naive T cells encounter self-antigen. Lack of immune tolerance to myelin proteins would be dangerous because myelin-specific T cells that are fortuitously activated in the periphery have acquired the capability to transverse the blood brain barrier much more readily than naïve T cells and would be poised to initiate an autoimmune attack. It is now clear that antigens can be transported from the CNS to deep cervical lymph nodes via drainage of cerebral spinal fluid through the cribiform plate (9); however, it was not known if this potential mechanism was sufficient to induce tolerance to myelin proteins. The extent and mechanisms underlying immune tolerance have been investigated in animal models for three proteins of the CNS myelin sheath that function as target auto-antigens in CD4$^+$ T-cell-mediated EAE: proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG), and myelin basic protein (MBP).

$CD4^+$ T-cell tolerance to PLP

PLP is the major integral membrane protein in CNS myelin and comprises about 50% of the CNS myelin protein mass (10). Two transcripts are generated from the PLP locus; a longer transcript encoding full-length PLP and a shorter transcript formed by alternative splicing encoding an isoform referred to as DM20 (11,12). The DM20 isoform is missing 35 amino acids (residues116–150) encoded by exon 3B of the PLP locus (12). PLP and DM20 are predominantly expressed in the CNS; however, both isoforms are incorporated at very low levels into the peripheral myelin sheath (less than 1% of peripheral myelin protein) (13,14). DM20 appears to be the dominant PLP isoform expressed in non-CNS tissues, particularly in the thymus. DM20 transcripts were readily detected in mouse thymus while PLP transcripts were barely detectable (15,16). The PLP transcripts appear to be synthesized by both thymic epithelial cells and bone marrow-derived APCs.

Thymic expression of PLP isoforms plays an important role in mediating immune tolerance. In B6 mice that are not susceptible to PLP-induced EAE, PLP-specific T cells were readily generated in $PLP^{-/-}$ but not wildtype mice, and all of the responses in $PLP^{-/-}$ mice were directed toward epitopes within DM20 (16). In contrast, wildtype SJL mice that are susceptible to PLP-induced EAE readily generated T-cell responses to PLP139–151, a region that is contained only in full length PLP, but no responses to DM20 (15,16). These data suggest that the low level of expression of full-length PLP detected in the thymus is not sufficient to tolerize PLP139–151-specific T cells, but the higher expression level of DM20 in the thymus induced tolerance to epitopes within this isoforms. SJL mice expressing a transgenic T-cell receptor (TCR) specific for PLP139–151 do not exhibit central tolerance, supporting the notion that tolerance to PLP correlated with the expression level of PLP isoforms in the thymus (17). The TCR transgenic mice exhibit spontaneous EAE, demonstrating a need for peripheral tolerance to restrain self-reactive T cells that escape tolerance (discussed below). The initial site of PLP-specific T-cell activation in the periphery is the cervical lymph nodes, which are considered the draining lymph nodes for the CNS and thus are likely to have increased presentation of CNS myelin epitopes (18).

$CD4^+$ T-cell tolerance to MOG

MOG is a very minor component (0.01–0.05% wt of membrane protein) that is expressed on the outer surface of CNS myelin (19). While MOG transcripts have been detected in the thymus (20), the T cell responses in $MOG^{-/-}$ mice do not appear to differ from those observed in wildtype mice (21). A MOG-specific TCR transgenic mouse was first developed on the B6 background and these mice did not exhibit central tolerance (22). The frequency of spontaneous EAE is very low in these mice although almost half of the transgenic mice exhibit histological evidence of optic neuritis. MOG-specific TCR transgenic mouse have also been generated in the SJL background that develop much higher rates of spontaneous EAE. Different founder lines of the transgenic mice were studied and each line exhibited a different frequency of transgenic T cells in the periphery; however, none of the mice demonstrated strong central tolerance despite the expression of MOG transcripts in the thymus (23).

$CD4^+$ T-cell tolerance to MBP

MBP is the second most abundant protein (30–40% wt of membrane protein) in CNS myelin and is a more significant component of peripheral myelin (5–15%) relative to other CNS myelin antigens (24). In addition, the expression pattern of MBP proteins is complex. Within the MBP locus, there are eleven exons that encode two families of proteins, referred to as classic-MBP and golli-MBP. Each family is transcribed using different promoters embedded within the locus, and both families are composed of multiple isoforms generated via alternative splicing (25,26). There is extensive exon-sharing between classic and golli-

MBP transcripts such that the isoforms in both families contain long stretches of identical amino acid sequence (26). However, the expression patterns and function of the golli- and classic-MBP proteins are very different. The name golli-MBP derives from 'gene in the oligodendrocyte lineage' because of the initial detection of these transcripts in oligodendrocytes (27). Golli-MBP transcripts are the only transcripts initiated from the most upstream promoter in the MBP locus; therefore, sequences encoded by the first three exons are expressed only in golli-MBP isoforms. Golli-MBP isoforms are expressed in the nervous system, thymus, and peripheral lymphoid tissues where they modulate calcium influx in different cell types (28,29). Classic MBP isoforms are initiated from distinct promoters that are located down-stream of the golli-MBP promoter. Because classic MBP isoforms are incorporated into the myelin sheath, their promoters are active primarily in myelin-forming cells in both the central and peripheral nervous systems and their developmental regulation controls the rate of myelination in the animal. Classic MBP is first detected in the mouse brain between postnatal day 6–10, coinciding with the start of active myelin formation. Accumulation of classic MBP peaks at day 30 and reaches steady state levels at day 60 (30,31).

The extensive exon-sharing between classic and golli-MBP suggested that central tolerance to epitopes derived from classic MBP could be mediated by the expression of golli-MBP in the thymus, similar to the central tolerance induced to most epitopes of full-length PLP by thymic DM20 expression. The potential for golli-MBP to induce tolerance in classic MBP-specific T cells was explored in both mice and humans by analyzing the ability of T-cell clones specific for classic MBP epitopes to respond to golli-MBP isoforms (32–34). In some cases, the classic MBP-specific T cells were able to respond to epitopes derived from golli-MBP (although the magnitude of the response to golli-MBP was always lower), but in other cases, they were not. Interestingly, T cells specific for a classic MBP epitope have been described that can respond to one golli-MBP isoform containing the same sequence but not a different golli-MBP isoform containing the same sequence (32). As the golli-MBP isoforms differ in sequences distal to the T-cell epitope, this observation suggested that sequences flanking an epitope can influence either its processing or the efficiency of presentation. Thus, expression of golli-MBP may not necessarily mediate tolerance in all T cells specific for a shared epitope present in classic MBP. In fact, a determinant present in golli-MBP whose sequence overlaps a determinant present in classic MBP has been suggested to outcompete the classic MBP epitope for MHC binding, indicating that classic MBP epitopes may be prevented from inducing tolerance when they are contained in golli-MBP proteins (35). To define the mechanisms of central and peripheral tolerance to classic MBP, we and others have generated a series of TCR transgenic mice specific for different MBP epitopes that allowed us to follow the fate of MBP-specific T cells as they mature in the thymus and circulate in the periphery. These models have provided a detailed understanding of how multiple tolerance mechanisms restrain immune responses directed toward a myelin antigen.

Differential central tolerance in MBP-specific $CD4^+$ T cells

The first insight into the fate of myelin-specific T cells generated in the thymus was provided by a TCR transgenic mouse model that expressed a MHC class II-restricted transgenic TCR that recognized MBPAc1–11 associated with I-A^u (36). This epitope is the primary target of T cells that mediate EAE induced by immunization with MBP in B10.PL and PL/J mice. Thymocytes expressing the transgenic TCR mature into the $CD4^+CD8^-$ lineage, as expected for a MHC class II-restricted TCR, and the percentage of $CD4^+$ single positive thymocytes is over-represented in the thymi of the TCR transgenic mice relative to non-transgenic mice. This result demonstrated that MBPAc1–11-specific thymocytes are positively selected and not subjected to clonal deletion. Two other TCR transgenic models specific for the same AcMBP-11/I-A^u epitope were subsequently developed by other

laboratories; in each case clonal deletion of the self-reactive thymocytes was not observed (37,38). A lack of central tolerance may seem surprising because the MBPAc1–11 sequence of classic MBP is contained in all isoforms of golli-MBP that are expressed in the thymus. In addition to the presence of epitopes within golli-MBP that overlap with this classic-MBP epitope (35) and may compete for binding to I-A^u, a likely explanation for the inability of golli-MBP to contribute to central tolerance induction to T cells specific for this classic MBP epitope is that classic MBP isoforms are acetylated on the amino terminus and this acetyl group is required for T-cell recognition of this peptide/MHC complex. Therefore, even if the MBPAc1–11 peptide was generated by processing golli-MBP, the peptide would not be acetylated and therefore would not be recognized by MBPAc1–11-specific thymocytes.

Although tolerance to this epitope requires presentation of the epitope derived from classic MBP isoforms, the lack of central tolerance in these TCR transgenic mice does not necessarily mean that classic MBP isoforms are not expressed in the thymus. Central tolerance depends on enough peptide/MHC complexes being available on the APC surface to engage developing thymocytes and trigger a strong enough signal to induce negative selection. Peptides with low affinity for the MHC molecule may not generate enough peptide/MHC complexes for this to occur. This appears to be the case for MBPAc1–11 as this peptide forms very unstable complexes with I-A^u due to the unfavorable positioning of a charged lysine residue in the peptide into the hydrophobic p6 pocket of I-A^u (39–42). The weak binding of MBPAc1–11 to I-A^u would result in few MBPAc1–11/I-A^u complexes on the surface of APCs, which in turn would result in thymocyte/APC interactions that are too weak to induce clonal deletion.

The observations made in MBPAc1–11-specific TCR transgenic mice support the paradigm that the majority of self-reactive T cells found in the peripheral repertoire escaped central tolerance because they exhibit low avidity interactions with APCs presenting the self-antigen. However, other mechanisms could account for escape of self-reactive T cells from central tolerance. MBP84–102 is a dominant epitope in HLA-$DR2^+$ individuals, and T cells specific for this region are easily detected in both MS patients and healthy controls (43,44). However, peptides within MBP84–102 bind to DR2 with high affinity (45,46), raising the question of why T cells specific for these peptides do not undergo central tolerance. The detection in thymic APCs of an asparagine endopeptidase that cleaves MBP at asparagine 94 thereby destroying the epitope suggested an explanation for how these thymocytes escape central tolerance (47). The fact that MBP85–99/DR2 complexes are detected in the CNS of MS patients (48) suggested that this enzyme may be differentially expressed in thymic APCs relative to the central nervous system.

The availability of the $MBP^{-/-}$ (*shiverer*) mouse, a mouse in which a naturally occurring mutation deleted exons 7–11 in the MBP locus (49–51), provided a valuable tool to determine the extent to which central tolerance shapes the peripheral MBP-specific T-cell repertoire. We compared the immune responses elicited by immunization with whole murine MBP in $MBP^{-/-}$ versus wildtype mice and found that $MBP^{-/-}$ B10.PL mice generate a dominant response to two overlapping peptides, MBP121–140 and MBP131–150, while the response directed to MBPAc1–11 is very minor in comparison (52). In wildtype mice, responses to either peptide within the MBP121–150 region are barely detectable, while MBPAc1–11-specific responses are equivalent between wildtype and $MBP^{-/-}$ mice. This result indicates that MBP121–150-specific T cells are efficiently tolerized in mice that synthesize endogenous MBP. T-cell hybridomas were generated from T cells that responded to MBP121–140 and 131–150 *in vitro*, and these hybridomas were used to identify MBP125–135 and MBP136–146 as core peptides sufficient to stimulate T-cell responses. Both of these peptides exhibit high affinity for I-A^u (53). Thus, the differential tolerance

induction that allows T cells specific for MBPAc1–11 to escape tolerance and T cells specific for epitopes within MBP121–150 to be tolerized correlates with different peptide binding affinities for I-A^u. Interestingly, despite the fact that there is no overlap between MBP125–135 and 136–146, about one third of the hybridomas responded to both peptides. TCR contact residues were identified in both of these epitopes and the results indicated that there was no chemical similarity between the contact residues (53). The basis for this high degree of degenerate recognition of non-overlapping MBP peptides in this region is not known. Other groups compared T-cell responses in $MBP^{-/-}$ and wildtype Balb/c and C3H mice immunized with MBP and found that strong responses were detected in the $MBP^{-/-}$ but not wildtype mice (54,55). Accordingly, both of these strains are resistant to EAE induced by MBP immunization.

To provide direct support for the model that the varying affinities of peptide binding for the MHC molecule results in differential abundance of peptide/MHC complexes on the surface of APCs, we used mass spectrometry to analyze peptides eluted from I-A^u-containing complexes purified from MBP-pulsed APCs. Our prediction was that peptides within the MBP121–150 region would be more abundant than peptides containing MBPAc1–11. Surprisingly, MBPAc1–17 and MBPAc1–18 were the most abundant peptides detected, while peptides containing MBP125–135 were detected at 20-fold lower levels (56). Subsequent analyses suggested that MBPAc1–17 and MBPAc1–18 bound to I-A^u in an alternate register from the MBPAc1–11 register in which MBP5–16 occupies the binding cleft in I-A^u. This alternate register places the tyrosine at position 12 in the MBP sequence into the hydrophobic p6 pocket of I-A^u instead of the lysine that occupies this pocket when the peptide is bound in the MBPAc1–11 binding register. Accordingly, peptides bound in the MBP5–16 register bind to I-A^u with high affinity, increasing their abundance on the APC surface and increasing their ability to induce tolerance in T cells specific for MBPAc1–17 and MBPAc1–18 bound in this register. Dissociation kinetics of MBPAc1–18 from I-A^u indicated that most, but not all, of MBPAc1–18 is bound to I-A^u in the MBP5–16 register. Thus, T cells specific for MBPAc1–11 escape tolerance because they do not recognize the MBPAc1–17 and MBPAc1–18 peptides when they are bound in the MBP5–16 register, and the complexes containing MBPAc1–17 and MBPAc1–18 bound in the MBPAc1–11 register are very unstable.

Tolerance to classic MBP is developmentally regulated

To determine if T cells specific for peptides within MBP121–150 undergo central tolerance, we generated TCR transgenic mice using a TCR that recognizes both MBP125–135 and MBP136–146. In contrast to MBPAc1–11-specific transgenic T cells, we found that T cells in MBP121–150-specific TCR transgenic mice are efficiently deleted in the thymus (57). Tolerance could not be mediated by golli-MBP isoforms in this case because MBP121–150 epitopes are contained only within classic-MBP. To determine the cell type within the thymus that mediated negative selection and the source of the MBP121–150 epitope, a series of bone marrow chimeras were generated using $MBP^{+/+}$ and $MBP^{-/-}$ bone marrow and recipients, as well as bone marrow cells that expressed the correct MHC class II molecules (I-A^u) or irrelevant MHC class II (I-A^k). These experiments showed that the thymic epithelial cells mediated positive but not negative selection of MHC class II-restricted MBP121–150-specific T cells, and that central tolerance was induced only by bone marrow-derived cells (57). These results appear to differ from studies showing that DM20 transcripts are synthesized by both thymic epithelial cells and bone marrow-derived APC; however, our experiments identified APCs that induce functional tolerance, which may be a more restricted phenomenon than expression of myelin transcripts. Interestingly, bone marrow-derived APCs from $MBP^{+/+}$ and $MBP^{-/-}$ were equally efficient in mediating negative selection, suggesting that bone marrow-derived APCs process and present MBP acquired

from the host. In fact, negative selection of MBP121–150-specific T cells did not occur when bone marrow-derived cells were the only source of MBP, indicating that MBP derived from myelin is the most likely source of the MBP that induces central tolerance. Because myelination begins shortly after birth in the mouse and levels of MBP in myelin do not peak until approximately 30 days after birth, our findings suggested that central tolerance of MBP121–150-specific T cells might be developmentally regulated. Indeed, we observed that deletion of MBP121–150-specific thymocytes is barely detectable at two weeks of age, moderate at four weeks of age, and extensive at 10 weeks of age, correlating with the levels of expression of MBP in myelin. Consistent with this observation, the MBP121–150-specific TCR transgenic mice were more susceptible to induction of EAE at four weeks of age than at 10 weeks of age (57).

Peripheral $CD4^+$ MBP-specific T-cell tolerance

TCR transgenic models specific for many myelin proteins exhibit what has been referred to as ‘split tolerance’ in that the T cells do not engage APCs presenting their cognate antigen *in vivo* with sufficient strength to induce central tolerance or deletion or anergy in the periphery; however, they respond vigorously to stimulation with myelin antigen in vitro. This ‘ignorance’ *in vivo* was believed to be due primarily to the low avidity of interactions between T cells (such as MBPAc1–11-specific T cells) and APCs presenting endogenous ligand *in vivo*. However, the higher concentrations of antigen used to stimulate the T cells *in vitro* appears to be sufficient to trigger T-cell activation. Importantly, this state of ignorance can be broken in vivo because MBP-, PLP-, and MOG-specific TCR transgenic mice all exhibit spontaneous EAE, although the frequency varies among the different models. Because the incidence of spontaneous EAE was not 100% in any of the models, these observations indicate that peripheral tolerance mechanisms must exist to prevent autoimmunity in the transgenic mice that remain healthy.

Studies comparing $Rag^{-/-}$ to $Rag^{+/+}$ MBPAc1–11-specific TCR transgenic mice provided the first insight into peripheral tolerance mechanisms that operate on myelin specific T cells. The incidence of spontaneous EAE rose to 100% on the $Rag^{-/-}$ background due to a loss of regulatory T cells (38,58,59). Regulatory T-cell activity capable of suppressing spontaneous EAE in this model is contained within both the $CD4^+CD25^+$ and $CD4^+CD25^-$ subsets (60,61). Both natural Tregs (nTegs) that develop in the thymus and peripherally induced Tregs (iTregs) associated with IL-10 production have been shown to suppress spontaneous EAE in a PLP-specific TCR transgenic model (18), and this is likely to be the case for Treg-mediated suppression of MBP-specific spontaneous EAE as well. Recent studies using a MBP-specific TCR transgenic model showed that regulatory T cells are critical to prevent activation of the MBP-specific T cells in the cervical lymph nodes because T cells expressing activation markers are detected in these CNS-draining lymph nodes before being detected in either the CNS or other peripheral lymphoid tissues (62). Removal of cervical lymph nodes delayed the onset of spontaneous EAE and was also associated with reduced severity of disease. These studies showed that activated MBP-specific T cells entered the CNS in a ‘wave’ 24 h after their appearance in the cervical lymph nodes (62).

In addition to the peripheral tolerance mediated by regulatory T cells that is required to prevent spontaneous EAE, our laboratory showed that MBPAc1–11-specific T cells undergo tolerance *in situ* within the CNS (63). T cells isolated from the lymph nodes of MBPAc1–11-specific TCR transgenic mice proliferate well in response to MBP peptide *in vitro* but T cells isolated from the CNS of the same mice are anergic to peptide stimulation *in vitro* even in the presence of exogenously added IL-2. Interestingly, proliferation of MBP-specific transgenic T cells isolated from lymph nodes was suppressed when they were co-cultured with mononuclear cells isolated from the CNS of MBPAc1–11 TCR transgenic mice, and

this suppression was abrogated when the CNS cells were depleted of T cells. This result implicates an active tolerance mechanism that is enriched within the CNS itself.

The data described above indicate that a significant degree of $CD4^+$ T-cell tolerance to CNS myelin proteins occurs in the thymus, such that the majority of T cells that escape to the periphery are low avidity. However, our studies using the MBP121–150-specific TCR transgenic model demonstrated that tolerance to MBP is developmentally regulated, allowing high avidity MBP-specific T cells to escape central tolerance early in life before endogenous MBP levels reach steady state (57). Because MBP is a component of peripheral myelin and almost all tissues are innervated, we hypothesized that endogenous MBP would be presented constitutively by APCs throughout the periphery. Indeed, we found that naive MBP121–150-specific T cells isolated from $MBP^{-/-}$ mice proliferated strongly in the spleen and lymph nodes when transferred directly into $MBP^{+/+}$ wildtype mice but not in $MBP^{-/-}$ control mice (57). This observation suggested that MBP-specific T cells that escape central tolerance at a young age should respond to endogenous MBP in the periphery of older mice when the level of MBP has increased. Peripheral tolerance mechanisms must exist to regulate this response because autoimmunity does not occur in wildtype mice expressing MBP without exogenous stimulation. To investigate how high avidity MBP-specific T cells that encounter MBP for the first time in the periphery (as would be the case for early thymic emigrants that escaped central tolerance in young mice) are regulated, we transferred transgenic MBP121–150-specific T cells isolated from $MBP^{-/-}$ mice into both wildtype and $Rag^{-/-}$ mice. While wildtype recipients remained healthy, $Rag^{-/-}$ mice succumbed to severe, multi-organ autoimmune disease (64). Regulatory T cells could protect the $Rag^{-/-}$ recipients from disease even if the Tregs had matured in a $MBP^{-/-}$ animal, suggesting that MBP expression is not required in the thymus to generate Tregs capable of suppressing MBP-specific autoimmunity. Interestingly, the tolerance mediated by regulatory T cells in wildtype mice was abrogated if the endogenous APCs encountered by the transferred MBP-specific T cells were activated by administration of LPS and anti-CD40 five days after transfer. However, no disease occurred if the APCs that are constitutively presenting endogenous MBP were activated with the same stimuli in recipient mice 30 days post transfer. This observation indicated that the MBP121–150-specific T cells responded very differently to activated APCs presenting endogenous MBP if it was their first encounter with cognate antigen compared to their second encounter. Further studies showed that regulatory T cells induced the MBP-specific T cells encountering MBP presented by non-activated APCs to differentiate into a tolerized phenotype such that a subsequent encounter with activated APCs presenting MBP elicited the suppressive cytokines IL-10 and TGF-β rather than T_H1 cytokines (64). Both the initial suppression and the maintenance of this unique tolerant state of MBP-specific T cells require the presence of regulatory T cells, underscoring their importance in maintaining self-tolerance, even under inflammatory conditions.

Identification of APCs presenting endogenous MBP

Because the MBP121–150-specific transgenic T cells have such high avidity for APCs presenting endogenous MBP, they provided a useful tool to identify which APCs presented MHC class II-restricted epitopes derived from endogenous MBP *in vivo*. We found that lymph node cells stimulated much stronger proliferation that splenocytes when used directly ex vivo as APCs (65). $CD8\alpha^+$ and $CD8\alpha^-$ dendritic cells (DCs) were the most potent APCs in the lymph node for triggering MBP-specific T-cell proliferation. Surprisingly, resting B cells purified from lymph nodes also stimulated proliferation of both naive and previously activated/memory MBP-specific T cells *ex vivo*. Expression of an MBP-specific B-cell receptor did not appear to be necessary for B cells to acquire and present endogenous MBP. Interestingly, neither naive nor previously activated/memory MBP-specific T cells produced

cytokines in response to stimulation with B cells. Activated/memory T cells that had been stimulated with B cells presenting steady-state levels of endogenous MBP became refractory to subsequent stimulation with bulk lymph node cells, indicating that in the absence of ongoing demyelination, encounter with B cells presenting endogenous MBP may exert a tolerogenic influence on MBP-specific T cells (65). However, adding exogenous MBP peptide to resting B cells in culture caused the activated/memory MBP-specific T cells to produce effector cytokines rather than become refractory, suggesting that when the amount of MBP available for presentation by B cells increases during ongoing CNS autoimmunity, antigen presentation by B cells could become pathogenic rather than tolerogenic (65).

It is interesting to compare our observations regarding presentation of MBP by B cells with results from a study in which TCR transgenic mice specific for MOG35–55 were bred to mice expressing a transgenic heavy chain obtained from a MOG-specific B-cell receptor. In the double transgenic mice, the frequency of spontaneous EAE was significantly enhanced compared to single TCR transgenic or IgH transgenic mice (66,67). Although the mice expressing the transgenic IgH chain contained a high precursor frequency of MOG-specific B cells and high titers of anti-MOG antibodies, they did not exhibit spontaneous EAE until they were bred to the TCR-transgenic mice. Interestingly, the MOG-specific antibodies were predominantly IgM in the single IgH transgenic mice, however, the MOG-specific antibodies became predominantly IgG1 in the double transgenic mice (66). This observation suggested that extensive MOG-specific interactions between T and B cells occurred in the mice that developed spontaneous EAE (66). These findings suggest that increasing the precursor frequency of MOG-specific B cells directly facilitated the development of spontaneous EAE in MOG-specific TCR transgenic mice because the B cells functioned as APCs presenting endogenous MOG. Thus, interactions between myelin-specific T cells and B cells presenting myelin epitopes can be tolerogenic or pathogenic, depending on the amount of available myelin and the precursor frequency of myelin-specific B cells.

Immune tolerance of CD8+ T cells specific for myelin proteins

Effector T cells that mediate disease in most models of EAE are primarily $CD4^+$ T cells because the disease is induced by immunization with myelin antigen, a route that results in presentation of the antigen in the MHC class II pathway. Thus, the attention of most researchers using an animal model to study MS has focused on the role of $CD4^+$ T cells. However, observations made over the last several decades from MS patients strongly suggest a role for $CD8^+$ T cells in MS. $CD8^+$ T cells outnumber $CD4^+$ T cells in MS lesions, and the $CD8^+$ T cells within these lesions exhibit more clonal expansion compared to $CD4^+$ T cells (4,68,69). Memory $CD8^+$ T cells are also preferentially enriched compared to memory $CD4^+$ T cells in the CSF and blood of MS patients, and memory $CD8^+$ T cells show evidence of oligoclonal expansion, consistent with activation by cognate antigen (4,68). Interestingly, depletion of $CD4^+$ T cells in patients with MS did not improve disease, however, treatment with alemtuzumab, which depletes several leukocyte populations (including both $CD4^+$ and $CD8^+$ T cells), appears beneficial (70). This observation suggested that $CD8^+$ T cells may exert a pathogenic function in MS. However, initial studies in mouse models suggested an immunoregulatory role for $CD8^+$ T cells. Mice that were deficient in $CD8^+$ T cells either via genetic manipulation or depletion exhibited less mortality but more relapses in EAE (71,72). $CD8^+$ T cells of all specificities were depleted in these studies; therefore, it is possible that the pathogenic versus regulatory function of $CD8^+$ T cells in MS may depend on their antigen specificity.

Studies that focused on $CD8^+$ T cells specific for myelin antigens demonstrated a pathogenic rather than regulatory role for these T cells. Our laboratory isolated $CD8^+$ T cells specific for MBP and found that adoptive transfer of the $CD8^+$ T cells induced a demyelinating

disease in mice that exhibited pathological features distinct from $CD4^+$ T cell-mediated EAE that resembled some aspects of pathology seen in MS patients (73). Pathogenic MOG-specific $CD8^+$ T cells in B6 mice (74,75), and human $CD8^+$ PLP-specific T cells have also been described (76). Novel animal models based on both the murine $CD8^+$ MBP-specific and the human PLP-specific T cells have been generated that yielded surprising insights into how $CD8^+$ myelin-specific T cells are tolerized and how this tolerance can be compromised to generate CNS autoimmunity.

Tolerance to PLP-specific $CD8^+$ T cells

Friese and colleagues (76) generated a humanized TCR transgenic mouse model in which T cells expressing a human TCR specific for both PLP45–53 associated with HLA-A3 and an unknown antigen associated with HLA-A2 were expressed. In mice expressing only the HLA-A3 allele, the T cells were positively selected in the thymus and circulated in the periphery in an apparent state of ignorance, similar to many $CD4^+$ TCR transgenic models specific for myelin proteins. Interestingly, when the PLP45–53-specific TCR was expressed in mice that also expressed the HLA-A2 allele, the T cells were negatively selected in the thymus. The elimination of the PLP-specific T cells from the repertoire was caused by degenerate recognition of a non-myelin peptide presented by HLA-A2, which may explain some of the recently reported protective effect of this allele in MS (77). Spontaneous EAE was rare in this model and immunization of the TCR transgenic mice with the PLP45–53 peptide in complete Freund's adjuvant induced only mild disease initially. However, a more severe $CD4^+$ T-cell-dependent disease developed later, suggesting that $CD4^+$ T cells may be required for $CD8^+$ T cells to participate in chronic EAE (76). A similar finding has been reported for MOG-specific $CD8^+$ T-cell-mediated EAE (78).

Central tolerance of MBP-specific $CD8^+$ T cells

Our laboratory isolated MBP-specific T cells using recombinant vaccinia and adeno viruses expressing a cDNA encoding MBP to prime $CD8^+$ T cells in both wildtype and $MBP^{-/-}$(shiverer) mice on the C3HeB/Fej background. $CD8^+$ MBP-specific T cells were readily isolated from $MBP^{-/-}$ but the precursor frequency of these T cells was much lower in wildtype mice, reflecting the same strong tolerance observed for high avidity $CD4^+$ MBP-specific T cells. The $CD8^+$ T cells recognized a single epitope in MBP, MBP79–87, restricted by K^k (79). To define the tolerance mechanisms that eliminate these T cells *in vivo*, we developed TCR transgenic models using two different TCRs specific for MBP79–87/K^k (80). One TCR is comprised of $V_\alpha 8$ and $V_\beta 6$ TCR chains (designated 8.6 mice), and the other TCR is comprised of $V_\alpha 8$ and $V_\beta 8$ (designated 8.8 mice). Surprisingly, these TCR transgenic models exhibited very different phenotypes. $CD8^+$ MBP-specific T cells in 8.6 mice were subjected to strong central tolerance, with deletion occurring at the transition from the $CD4^+CD8^+$ stage to the mature CD4 $CD8^+$ stage. Any transgenic T cells that escaped thymic deletion and exited to the periphery appeared to downregulate expression of the transgenic $V_\alpha 8$ chain, indicating that peripheral tolerance mechanisms also functioned to restrain the activity of the $CD8^+$ MBP-specific T cells.

The 8.6 mice provided an excellent tool to investigate the mechanisms responsible for central tolerance of $CD8^+$ MBP-specific T cells. Our previous studies of central tolerance of $CD4^+$ MHC class II-restricted T cells focused on T cells specific for MBP121–150, an epitope present in classic but not golli-MBP. Central tolerance of MBP121–150-specific T cells was mediated only by bone marrow-derived cells that obtained the MBP from an exogenous source, most likely degraded myelin, rather than synthesizing the MBP isoform themselves (57). MBP79–87 differs from MBP121–150 in that it is expressed by both classic and golli-MBP isoforms. Furthermore, because MBP79–87 is a MHC class I-

restricted epitope, it may be more efficiently presented by APCs that synthesize classic or golli-MBP isoforms compared to MHC class II-restricted epitopes.

To investigate how tolerance to this MHC class I-restricted epitope of MBP is induced, we employed the 8.6 TCR transgenic mice bred onto a wildtype, $\beta 2m^{-/-}$, $MBP^{-/-}$ (lacks both classic- and golli-MBP isoforms containing MBP79–87) and $golli^{-/-}$ (unaltered classic MBP expression but no golli isoforms) (81) background. These studies revealed two novel mechanisms for inducing tolerance to a naturally expressed TSA. Using a series of bone marrow chimeric mice generated with mice of these different genotypes combined with transplantation of thymic grafts of different genotypes, we generated experimental models in which the expression of classic- and/or golli-MBP was restricted to bone marrow-derived cells, non-bone marrow-derived cells in the both the thymus and periphery, non-bone marrow-derived cells in the periphery only or non-bone marrow-derived cells in the thymus only (82). We found that the expression of either classic- or golli-MBP alone is sufficient to mediate efficient clonal deletion of $CD8^+$ MBP79–87-specific thymocytes; however, the mechanisms differed depending on whether classic- or golli-MBP was the source of the tolerogenic antigen. Surprisingly, our data showed that bone marrow-derived cells induced deletion of 8.6 thymocytes by synthesizing classic MBP containing MBP79–87 and presenting it directly in the MHC class pathway. This finding indicates that tolerance to TSAs can be induced in the thymus by bone marrow-derived cells as well as by the previously described thymic epithelial cells, at least in the MHC class I pathway. This result differs from our observation that bone marrow-derived cells do not synthesize classic MBP that can be used to induce tolerance in MHC class II-restricted MBP121–140-specific thymocytes. This difference could reflect inefficiency in presentation of endogenously synthesized antigen in the MHC class II pathway in these APCs. Alternatively, more isoforms of classic MBP contain MBP79–87 compared to MBP121–150, and the differences in abundance of these epitopes may influence the extent to which they are directly presented to thymocytes. In contrast to bone marrow-derived cells, non-boned marrow-derived cells in the thymus (presumably thymic epithelial cells) do not mediate deletion via synthesis and direct presentation of classic MBP, although they do induce tolerance by synthesizing and directly presenting golli-MBP to developing thymocytes. Nevertheless, we were surprised to find that TECs can induce central tolerance using only classic MBP as a source of antigen. Using thymic grafts and bone marrow chimeric recipients, our data demonstrated that the TECs acquire exogenous classic MBP from myelin *in situ* rather than from the periphery and cross-present the MBP79–87 epitope to the MHC class I-restricted thymocytes. This is the first description of tolerance induced *in vivo* to a TSA via cross-presentation by TECs, and the mechanism of MBP uptake by TECs is not yet known. The fact that TECs do not induce tolerance to the MHC class II-restricted classic MBP121–150 epitopes suggests a nonphagosomal mechanism of antigen acquisition.

The difference between $CD4^+$ and $CD8^+$ T-cell tolerance mechanisms results in distinct patterns of tolerance to classic MBP observed during development. Because tolerance to the MHC class II-restricted MBP121–140 epitope is mediated only by bone marrow-derived cells that must acquire exogenous MBP from myelin, the extent of tolerance to this epitope parallels the gradual rate of myelination. In contrast, multiple types of APCs can synthesize and induce tolerance to classic MBP presented directly in the MHC class I pathway; accordingly, strong negative selection of MHC class I-restricted classic MBP-specific T cells is already established in 3-day-old mice (82).

$CD8^+$ MBP-specific T cells can avoid negative selection

Although the 8.6 and 8.8 T-cell clones used to generate our TCR transgenic mouse models exhibited similar responses to MBP79–87 peptide *in vitro*, 8.8 T cells differed from 8.6 T cells in that they were not negatively selected in the thymus or in the periphery (80). 8.8 T

cells exhibited a mostly naïve phenotype *in vivo* yet proliferated strongly to MBP79–87 peptide *in vitro*, similar to the ignorant phenotype seen in most $CD4^+$ T cells that escape tolerance because of their low avidity for APCs presenting their myelin epitope. However, in contrast to the $CD4^+$ MBP-specific T-cell models, we found that 8.8 T cells exhibited a higher affinity compared to 8.6 T cells for MBP79–87/K^k in experiments that measured dissociation rates of a MBP79–87/K^k tetramer (80). Further studies using mixed bone marrow chimeras in which 8.8 and 8.6 thymocytes matured in the same thymus revealed that 8.8 thymocytes had the surprising ability to prevent clonal deletion of 8.6 thymocytes. The number of 8.6 T cells in the periphery of $MBP^{+/+}$ mixed bone marrow chimeras was also significantly increased compared to the number in $MBP^{+/+}$ 8.6 mice, and the 8.6 T cells in the periphery of the bone marrow chimeras displayed a naïve phenotype. Thus, the presence of 8.8 T cells in the mixed bone marrow chimeric mice prevented both central and peripheral tolerance of 8.6 T cells. Interestingly, 8.8 T cells could inhibit 8.6 T cells from responding to MBP in the periphery when both 8.8 and 8.6 T cells were isolated from $MBP^{-/-}$ mice and adoptively transferred into wildtype mice. Therefore, the ability to rescue 8.6 T cells from peripheral tolerance did not require that 8.8 and 8.6 T cells mature together in the thymus. The tolerizing ability of 8.8 T cells applied only to 8.6 T cells; 8.8 T cells did not affect the responses of T cells specific for other epitopes associated with K^k or T cells specific for MHC class II-restricted MBP epitopes (80).

We hypothesized that the 8.8 TCR might exhibit sufficiently high avidity that it engages the MBP peptide/MHC complex and removes it from the surface of APCs. Removal of the ligand by 8.8 T cells would prevent 8.6 T cells from interacting with the same peptide/MHC complex on the APC surface, thereby facilitating the escape of 8.6 T cells from tolerance. We established an *in vitro* system to study the mechanism by which 8.8 T cells exert their tolerogenic effect on 8.6 T cells using DCs isolated from wildtype mice to stimulate the T cells without addition of exogenous MBP peptide. In this system, the 8.6 T cells proliferated in response to the DCs but the 8.8 T cells did not proliferate. Importantly, 8.8 T cells were also able to inhibit the proliferation of the 8.6 T cells in this *in vitro* system. Our hypothesis predicted that stimulation of 8.6 T cells with DCs from 8.8 mice would result in reduced proliferation compared to stimulation with wildtype DCs because the DCs in 8.8 mice would be relatively "stripped" of the MBP79–87/K^k complex. This prediction was confirmed; however, the 8.8 DCs were still able to present endogenous MBP121–150 in the MHC class II pathway. This observation indicated that the MBP79–87/K^k epitope had been selectively removed by the 8.8 T cells and that the suppression of 8.6 T cell activity induced by 8.8 T cells was not mediated by production of soluble factors. The mechanism by which 8.8 T cells can engage the MBP79–87/K^k complex and remove it from the APC surface without initiating 8.8 T-ell activation is not yet understood. Addition of IL-2 to cultures containing 8.8 T cells and DCs from wildtype mice restored the ability of 8.8 T cells to proliferate, suggesting that there is a defect in IL-2 production following 8.8 T-cell stimulation with DCs presenting endogenous MBP such that proliferation of the 8.8 T cells is not sustained (80).

The novel form of tolerance described above for the 8.8 T cells allowed these T cells to leave the thymus and circulate in the periphery. In contrast to $CD4^+$ TCR transgenic T cells specific for myelin antigens, spontaneous EAE was not observed in 8.8 mice, even on a $Rag^{-/-}$ background. Thus, regulatory T cells do not seem to be critical in restraining the activity of 8.8 T cells *in vivo*. Furthermore, neither immunization with MBP79–87 in CFA with pertussis toxin nor injections of pertussis toxin alone induced disease, although these protocols are efficient in triggering EAE in $CD4^+$ TCR transgenic models (36). Infection of 8.8 mice with a recombinant vaccinia virus expressing MBP did efficiently induce CNS autoimmunity. Interestingly, infection with wildtype vaccinia virus that did not express MBP was equally efficient in inducing autoimmune disease (83). The mechanism by which

wildtype vaccinia virus induced disease did not involve molecular mimicry or bystander activation. Instead we found that co-expression of endogenous TCR chains with the transgenic MBP-specific TCR on a $Rag^{+/+}$ background was required for viral infection to induce disease. The expression of endogenous TCR chains allows a subset of the transgenic T cells to co-express both a MBP-specific and a virus-specific TCR. While engagement of the MBP-specific TCR alone does not trigger T cell activation, engagement of the virus-specific TCR does cause T cell activation and also releases the block in signaling by the MBP-specific TCR such that the activated T cells can respond to both viral and MBP epitopes. These activated T cells with dual specificity not only participate in clearing the virus, but also induce an autoimmune attack against MBP (83). This model illustrates one mechanism by which a common virus could trigger autoimmune disease in only a subset of genetically-predisposed people because the co-expression of TCR chains that confer both myelin-specific self-reactivity and reactivity to a particular pathogen should occur at a low, stochastic frequency.

Concluding remarks

To understand the initial steps in the pathogenesis of MS, it is important to define the mechanisms that allow myelin-specific, self-reactive T cells to escape tolerance and circulate in the periphery. Central tolerance is an important first step that purges many myelin-specific T cells from the repertoire. Although myelin proteins are considered TSAs whose expression is largely restricted to the central nervous system, their expression within the thymus has been shown to induce negative selection. Among the myelin-specific T cells shown to escape central tolerance, most have been characterized as low avidity T cells that do not engage their antigen during maturation with sufficient strength to induce deletion. Despite this state of “ignorance”, active tolerance mediated by regulatory T cells is still needed to prevent autoimmunity. MBP is more abundant in peripheral myelin compared to other proteins that comprise CNS myelin, and MBP derived from myelin has been implicated as a source of tolerogenic antigen in both the thymus and the periphery. While so far only bone marrow-derived cells have been implicated as APCs involved in tolerance induction of CD4+ classic-MBP-specific thymocytes, both bone marrow-derived and non-bone marrow-derived cells synthesize and directly present MBP epitopes to $CD8^+$ MBP-specific thymocytes, revealing a wide range of mechanisms to induce central tolerance to TSAs. Recently, high-avidity T cells specific for a MHC class I-restricted epitope of MBP have been shown to escape central tolerance by engaging their cognate antigen with sufficient avidity to remove it from the surface of APCs without triggering T cell activation. This unusual form of tolerance leaves the T cells vulnerable to activation by viral infection, which has so far not been demonstrated as a direct trigger for activating myelin-specific $CD4^+$ T cells. Together these data indicate that multiple layers of tolerance are needed to prevent CNS autoimmune disease, and that the events that lead to a loss of tolerance may differ depending on the particular mechanism that is breached.

Acknowledgments

I am grateful to all of the current and former students and post-doctoral fellows in my laboratory whose work has contributed greatly to this review. Our work discussed here has been supported by the National Multiple Sclerosis Society (RG 3851-A-5) and the National Institutes of Health (AI072737).

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