The health of an organism is dependent on the ability of all of the components of the immune system to function together to protect from and control pathogenic organisms as well as cancerous tissue. At the same time there must be mechanisms to protect the organism from developing inappropriate immune responses that are harmful to ones own body (allergy, autoimmunity) and those that help to silence the inflammatory responses and allow their resolution. Tolerance to self-antigens is a result of central tolerance (negative selection) and various mechanisms of peripheral tolerance that include anatomical sequestration of self-antigens, deletion of peripheral autoreactive lymphocytes, the development of lymphocyte functional unresponsiveness and action of T regulatory (Treg) cells. This article summarizes current knowledge about mechanisms of immunological tolerance that protect from development of immune responses to self-antigens present in the central nervous system (CNS). Finally, it discusses the subject of skin-induced tolerance as demonstrated in an animal model of autoimmune disease of the CNS, experimental autoimmune encephalomyelitis (EAE). The potential clinical use of this approach to regulate disease will be discussed.

**Key words:** multiple sclerosis, self-tolerance, skin-induced tolerance, T regulatory (Treg) cells

**INTRODUCTION**

The health of an organism is dependent on the ability of all of the components of the immune system to function together to protect from and control pathogenic organisms as well as cancerous tissue. At the same time there must be mechanisms to protect the organism from developing inappropriate immune responses that are harmful to ones own body (allergy, autoimmunity) and those that help to silence the inflammatory responses and allow their resolution. Tolerance to self-antigens is a result of central tolerance (negative selection) and various mechanisms of peripheral tolerance that include anatomical sequestration of self-antigens, deletion of peripheral autoreactive lymphocytes, the development of lymphocyte functional unresponsiveness and action of T regulatory (Treg) cells. This article summarizes current knowledge about mechanisms of immunological tolerance that protect from development of immune responses to self-antigens present in the central nervous system (CNS). Finally, it discusses the subject of skin-induced tolerance as demonstrated in an animal model of autoimmune disease of the CNS, experimental autoimmune encephalomyelitis (EAE). The potential clinical use of this approach to regulate disease will be discussed.

**MECHANISMS OF SELF-TOLERANCE**

T lymphocytes develop in the thymus, whereas B cells develop in the liver or adult bone marrow in mammals. During development immature lymphocyte precursors begin to differentiate and rearrange antigen receptor loci. T cell antigen receptors (TCR) and B cell antigen receptors (BCR) are generated by joining V, D and J segments of TCR and BCR loci (1). These random mechanisms of TCR and BCR creation result in the generation of many unique antigen receptors that can recognize non-self antigens but also, potentially, self antigens. Thus, there must be some mechanisms that can control these potentially dangerous self-reactive (autoreactive) lymphocytes. The mechanisms that keep autoreactive lymphocytes under control are known as "self tolerance", and are best studied for T lymphocytes. Tolerance to self-antigens is a result of central tolerance (negative selection) and various mechanisms of peripheral tolerance that include anatomical sequestration of self-antigens, deletion of peripheral autoreactive lymphocytes, the development of lymphocyte functional unresponsiveness and action of T regulatory (Treg) cells. In this article, tolerance will be discussed with a primary emphasis on tolerance to the antigens of the central nervous system (CNS).
“autoimmune regulator” (Aire) (4). Aire mutations result in incomplete silencing of autoreactive T cells in the thymus, and in development of multiorgan autoimmunity in both mouse and human (5).

A variety of CNS self-antigens that are relevant for multiple sclerosis (MS) pathogenesis are also expressed in the thymus (6). Both myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG) are expressed in the thymus especially by mTEC (7, 8). Although expression of MBP and MOG in the thymus seems to induce negative selection of self-reactive T cells with high affinity TCRs, autoreactive T cells with low affinity are spared and leave the thymus once they finish maturation. Indeed, potentially harmful MBP-specific T cells are present in the circulation of healthy mice and humans (9, 10). Thus there must be other mechanisms of control of these CNS-specific T cells in the periphery.

PERIPHERAL TOLERANCE

Regulatory T cells

In 1970 Gershon, was the first to propose that T cells, in addition to their helper activity, may also play a role of regulatory cells capable of suppressing immune responses (11). Since then, many scientists focused their attention on T suppressor cells (Ts), trying to characterize both their phenotype and mechanism of action. After a time of huge interest in suppression, the world of cellular immunology started to doubt if Ts really existed and many findings were considered to be over-interpretation of inconclusive data. Since the late 1990s, Ts cells came back on stage with a new name – T regulatory (Treg) cells (12). T regulatory cells are a population of CD4+ CD25+ T cells that express FoxP3, a transcription factor essential for their development and function (13). FoxP3+ T cells are generated in the thymus (natural Treg (nTreg)) but also in the periphery (induced Treg (iTreg)). There are many reports showing that Treg cells keep self-reactive T lymphocytes under control and thereby protect from autoimmunity (14).

Natural Treg develop in the thymus and their TCRs show high affinity to self-antigens. The development of nTreg is guided by nTEC expressing self-antigens and is also supported by various co-stimulatory and adhesion molecules and cytokines (15). It has been shown that FoxP3+ Treg cells exert suppression via cell contact-dependent mechanisms e.g. killing of either antigen presenting cells (APC) or T effector cells via release of granzyme and perforin, as well as mechanisms mediated by soluble factors such as IL-10, IL-35, TGF-β and galectin-1, or deprivation of IL-2 (15, 16). Additional suppressive functions of FoxP3+ Treg include modulation of APC via CD39/CD73 and LAG-3 (17, 18). However, it is believed that CTLA-4-dependent down-regulation is a core mechanism of Treg mediated suppression. It was found that CTLA-4 mediated suppression is caused by down-regulation of CD80 and CD86 by DC, induction in DC indoleamine 2,3-dioxigenase-dependent catabolism of tryptophan and production of immunosuppressive kynureine and inhibition of proinflammatory cytokines synthesis (18). It is worth noting that FoxP3+ Treg lymphocytes can differentiate to acquire the ability to specifically control Th1, Th2 and Th17 cells by modifying their expression of Th lineage- specific transcription factors such as T-bet, IRF-4 and RORγt. These transcription factors may influence chemokine receptor expression patterns that facilitate Treg cell migration to a particular inflammatory site (15, 19-21). Indeed the presence of Treg cells in the CNS and their role in controlling autoimmunity have been well documented in experimental models. Treg cells have been shown to accumulate during the recovery from experimental autoimmune encephalomyelitis (EAE) (22).

Immune privilege

Numerous sites in the body possess varying degrees of immune privilege including the brain, the anterior chamber of the eye, pregnant uterus, hair follicles and hamster cheek pouch. The advantage of immune privilege to the tissue is that damage generated during a normal immune response is attenuated and non-renewable tissues e.g. brain are protected (23).

It is believed that several mechanisms are involved in immune privilege of CNS. First, the tight junction between vascular endothelial cells in the brain creates a blood-brain barrier that retards extravasation of leukocytes into the brain. Second, the absence of lymphatic vessels prevents antigens from leaving the brain and reaching regional lymph nodes (24). Third, the immune responses cannot develop in the CNS because only few resident cells constitutively express major histocompatibility complex (MHC) molecules in the steady state. Fourth, local tolerogenic mechanisms exist within the CNS (6). It was shown that multiple cells in the CNS such as astrocytes, oligodendrocytes, microglia and the vascular endothelium express FasL (25). It is believed that endothelial cells in the CNS reduce the risk for inflammation by expressing FasL, which limits extravasation of inflammatory cells (26). Additionally, CNS expression of PGE2, TGF-β and galectin-9 is associated with functional silencing of incoming T lymphocytes (27-29).

At present there are several lines of evidence that indicate that the immune privilege of the CNS is not absolute. First, access of T lymphocytes to the CNS is limited and involves active transendothelial migratory process but is not completely forbidden (6). It was already shown that the endothelial cells of blood-brain barrier having only limited expression of endothelial P-selectin, E-selectin and VCAM-1 are not resistant to the development of immunopathology once inflammation within the organ itself has begun (30). Additionally, there is strong evidence that both naive CD4+ and CD8+ T lymphocytes are able to patrol non-lymphoid tissues, including the CNS (31). However, while naïve T lymphocytes can circulate in the CNS without triggering a deleterious response, activation of myelin-specific T cells is not always sufficient to allow self-reactive T lymphocytes to enter the CNS and additional signals are required (6). Second, there is substantial, lymphatic drainage connecting the meninges and ventricular system, if not the brain parenchyma, directly through the cribriform plate to the deep cervical lymph nodes (23). It was also shown that antigens escape the CNS and accumulate in cervical lymph nodes where they induce a form of immune deviation (32). It is believed that brain-associated immune deviation contributes to the immune privilege of the brain reduces the risk for immune-mediated inflammation in the CNS. Third, antigen presentation may occur in the CNS. It was shown that oligodendrocytes and neurons exposed to proinflammatory environment express MHC I, whereas astrocytes and microglial cells express MHC II.

In summary, while the CNS is not favorable for development of immune responses, current research suggests that, under inflammatory conditions, T cell mediated responses can develop within this tissue.

OTHER MECHANISMS OF PERIPHERAL TOLERANCE

Anergy and deletion

Ligation of TCR by antigen-MHC in the presence of costimulation (CD80/CD86 molecules and proinflammatory
cytokines) results in T cell activation. However, recognition of peptide-MHC by T lymphocytes in the absence of costimulatory signals leads to functional anergy or T cell depletion. Thus, in the absence of inflammation, lymph node and spleen immature DCs induce tolerance in naive T cells that express TCR with high affinity for self-antigen-MHC complexes. Indeed, it has been shown that autoreactive T cells recognizing self-antigen-MHC complexes die by apoptosis as a result of Fas engagement by FasL and Bim-dependent triggering of a Bel-2 Bel-xL-regulated mitochondrial death pathway (33).

Induction of tolerogenic dendritic cells

It is believed that certain dead or dying cells can reinforce a tolerogenic DC phenotype (34). Apoptotic cells, unlike necrotic cells, are insufficient to trigger DC maturation. Moreover, the uptake of apoptotic cellular material suppresses TLR signaling and results in induction of tolerogenic DCs. Recognition of self-antigen-MHC complexes on tolerogenic mature DCs by autoreactive T cells leads to a functional inactivation and/or peripheral depletion in secondary lymphoid organs.

Expression of Aire and TSA outside the thymus

In the periphery, the role of Aire remains not fully understood but increasing evidence suggests that secondary lymphoid organs are sites of both ectopic TSA and Aire expression. The presence of Aire transcripts and nuclear AIRE protein was demonstrated in a unique population of tolerogenic stromal cells, called extra-thymic Aire-expressing cells (eTAC) (35, 36). Growing evidence also suggests that such TSA expression may play a role in the maintenance of immunologic tolerance, and that self-antigen expression in the periphery may complement self-antigen expression in the thymus, providing a safety net to eliminate potentially dangerous autoreactive T lymphocytes that evade thymic negative selection (37).

Antigen tolerance based therapies for treatment of multiple sclerosis

Breakdown of self tolerance may result in development of autoimmune diseases. Multiple sclerosis (MS) is an example when immune system responds to self-antigens present in the CNS. MS is a chronic, progressive inflammatory disorder of the brain and spinal cord. The inflammatory plaque, whether observed in human tissue or in experimental models of disease (EAE), is the pathological hallmark of MS (38). Studies demonstrating the presence of inflammatory cells and their products in the brain lesions of MS patients, and in animal models of disease (EAE), has led to the generally accepted hypothesis that disease is mediated by pathogenic CD4+ Th1/Th17 cell mediated responses against myelin antigens, followed by a broader neurodegenerative process (38, 39).

Treatment modalities for MS are limited, with the most common treatments being steroids or anti-mitotic drugs acting nonspecifically on the immune system resulting in general immunosuppression accompanied by many severe side effects (40). Other drugs such as copaxone or IFN-β have therapeutic effect only in some patients. Thus numerous efforts have been made to develop a treatment able to control the autoimmune response in an antigen specific way.

One approach is based on the induction of antigen-specific tolerance. There are currently four different methods employed for inducing peptide-specific immune tolerance: altered peptide ligand induced tolerance, mucosal (oral-nasal)-induced tolerance, soluble-peptide-induced tolerance and tolerance induced via injection of ethylene carbodiimide peptide-coupled cells (41). Of these, mucosal tolerance has received most attention. This protocol relies on mucosal deposition of an antigen. Oral tolerance has been studied in many experimental disease models, including EAE, where it was found that animals fed with MBP were protected from disease (42, 43). Other studies showed that orally-induced Treg cells secreted anti-inflammatory cytokines, such as TGF-β, IL-4 and IL-10 (44). Promising achievements in the field of mucosal tolerance in EAE encouraged clinicians to treat MS patients by feeding them bovine MBP daily to suppress disease. In MS patients, MBP and PLP-specific TGF-β-secreting Th3-type cells have been observed in the peripheral blood of patients treated orally with bovine myelin preparation and not in patients who received placebo (45). Despite these promising observations, clinical trials failed to show any therapeutic benefit of bovine MBP feeding beyond the placebo effect (46, 47).

Skin induced tolerance

Both the skin and mucosa are constantly exposed to many antigens and play a crucial role in protecting the body from different pathogens present in the external world. While development of immune responses to pathogens is of vital importance to the macroorganism, responses to innocuous antigens are, at best, not helpful and often lead to harmful allergy. It is well known that immunization with an antigen via the digestive tract or nasal mucosa leads both to a local immune response and a state of profound immunosuppression in the periphery (48-50). This suppression seems to play an important role in avoiding the development of immune responses to non-pathogenic antigens.

Although the skin is considered an organ where immune responses are easily induced (51-53), little attention has been given to skin induced tolerance (54). Because skin and mucosa play similar function in our bodies (i.e., as a barrier to external pathogens) it is possible that epicutaneous (EC) application of antigen (Ag), apart from inducing a strong immune response, may also induce peripheral tolerance. Wang et al. showed that EC application of the protein antigen ovalbumin (OVA) resulted in allergic dermatitis accompanied with the appearance of IL-4 secreting T lymphocytes (55, 56). Additionally, Herric et al. showed that EC immunization with protein antigen induced a Th2 mediated model of asthma (57). These data suggested to us that, similar to mucosal immunization, deposition of protein antigens on the skin may also induce T cells producing anti-inflammatory cytokines that would suppress Th1-mediated responses causing their suppression.

To verify our hypothesis, we developed an experimental model where mice were EC exposed to hapten-conjugated protein (TNP conjugated mouse immunoglobulin (TNP-Ig) in the form of gauze patch prior to hapten TNP-CI sensitization that induces Th1 mediated contact sensitivity (CS) (Fig. 1). Indeed, we found that skin patch immunization with TNP-Ig before active sensitization with TNP-CI caused reduction of CS by 90% when compared to positive control (animals patched with PBS and then sensitized with hapten) (58, 59).

Observed suppression is transferable in vivo by TCRβ-β CD4+ CD8+ double positive lymphocytes harvested from lymphoid organs of skin patched animals and is not major histocompatibility complex-restricted nor antigen specific. Both CD25- and CD25- CD4+ CD8+ T cells are able to suppress adoptive transfer of Th1 effector cells mediating cutaneous contact sensitivity. In vivo treatment with monoclonal antibodies showed that the cytokines interleukin (IL)-4, IL-10 and transforming growth factor-β (TGF-β) are involved in the induction of the Ts cells. Additionally, using IL-10-/- mice we found that IL-10 is involved in skin induced tolerance. Further in vitro experiments showed that lymph node cells of skin tolerized mice non-specifically suppress [H]thymidine incorporation by
antigen-stimulated immune cells and this effect can be abolished by adding anti-TGF-β, but not anti-IL-4 nor anti-IL-10 antibodies. These studies indicate the crucial role of TGF-β in skin induced tolerance due to non-antigen-specific Ts cells and also show that IL-4, IL-10 and TGF-β play an important role in the induction of EC induced Ts cell suppression (59). Other factors such as NO and prostaglandins might be involved in described immunoregulation (60, 61). This subject is currently under investigation.

Additionally, we found that EC immunization with protein antigen suppresses Tc1 dependent immune response employing CD8 Tc1 mediated contact sensitivity and an animal model of skin graft rejection (62, 63).

These findings raised the possibility that EC immunization with the proper antigens could induce Ts cells that would protect from animal model of multiple sclerosis - EAE, similar to their protection from CS. Indeed, we have found that EC immunization with MBP prior to induction of EAE resulted in protection from developing EAE, as incidence was reduced by 50% (Fig. 2). In addition, disease severity was also significantly reduced in those tolerant mice that did develop disease (64, 65). Further experiments showed that EC immunization induced a population of Ts cells that function in an antigen non-specific manner. We also made similar observations in animal model of rheumatoid arthritis (Szczepanik et al.).

Employing adoptive cell transfer experiments and experiments with knock out mice lacking selected T cell populations, we found that protection from disease is mediated by TCRαβ+ CD4+ CD8+ double positive T suppressor (Ts) lymphocytes (66). Next, we showed that EC treatment of EAE mice with MBP after first signs of disease significantly ameliorated ongoing disease. Finally, similar to oral tolerance, we found that Ts cells induced via EC immunization in EAE model mediated inhibition both *in vitro* and *in vivo* by the production of TGF-β. Therefore, the *in vivo* mechanism of skin-induced, non-Ag-specific suppression in mice is most likely through the production of TGF-β by Ts cells (Fig. 3).

Maneuver of EC immunization seems to bear fruit as we found that myelin peptides applied EC to MS patients activated dendritic Langerhans cells in the skin at the site of immunization and induced a unique population of granular dendritic cells in local lymph nodes. In the periphery, epicutaneous immunization with myelin peptides resulted in the generation of type 1, IL-10 producing regulatory T cells, suppression of specific autoreactive proliferative responses and suppression of IFN-γ and TGF-β production. We demonstrate for the first time that EC immunization with myelin peptides generates tolerogenic responses and attenuates autoimmunity in MS patients (67).

To summarize, our data suggest that EC exposure to an antigen, similar to mucosal immunization, results in the induction of Ts cells. The inhibitory mechanisms are also much the same, emphasizing the similarities between these two tissues. The ease by which Ts cells can be generated through EC immunization may have important implications for designing therapeutic schemes aimed at modulating immune responses to self antigens involved in autoimmune diseases.
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