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Molecular Mechanisms of T cell Receptor and Costimulatory Molecule Ligation/Blockade in Autoimmune Disease Therapy

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Abstract

Pro-inflammatory CD4⁺ T cell mediated autoimmune diseases, such as multiple sclerosis and type 1 diabetes, are hypothesized to be initiated and maintained by activated antigen presenting cells (APCs) presenting self-antigen to self-reactive interferon-gamma (IFN- γ) and interleukin-17 (IL-17) producing CD4⁺ Th1/17 cells. To date, the majority of FDA approved therapies for autoimmune disease primarily focus on the global inhibition of immune inflammatory activity. The goal of ongoing research in this field is to develop both therapies that inhibit/eliminate activated autoreactive cells as well as antigen-specific treatments which allow for the directed blockade of the deleterious effects of self-reactive immune cell function. According to the two-signal hypothesis, activation of a naïve antigen-specific CD4⁺ T cell requires both stimulation of the T cell receptor (TCR) (signal 1), and stimulation of costimulatory molecules (signal 2). There also exists a balance between pro-inflammatory and anti-inflammatory immune cell activity, which is regulated by the type and strength of the activating signal as well as the local cytokine milieu in which the naïve CD4⁺ T cell is activated. To this end, the majority of ongoing research is focused on the delivery of suboptimal TCR stimulation in the absence of costimulatory molecule stimulation, or potential blockade of stimulatory accessory molecules. Therefore, the signaling pathways involved in the induction of CD4⁺ T cell anergy, as apposed to activation, are topics of intense interest.

Introduction

An important goal of current research in autoimmune diseases such as multiple sclerosis (MS) and type-1 diabetes (T1D) is to develop new therapies to specifically tolerize self-reactive immune cells. The preferred targets alter T cell receptor (TCR) and costimulatory molecule signaling and their respective intracellular signaling pathways. Multiple sclerosis is characterized by perivascular CD4⁺ T cell and mononuclear cell infiltration in the central nervous system (CNS) with subsequent primary demyelination of axonal tracks leading to progressive paralysis (1). The requirement of naïve T cells to receive two signals to become activated was first proposed by Lafferty and Cunningham (2). This two-signal hypothesis has become the basis for many potential therapies currently under development. The molecular mechanisms by which these therapies alter autoreactive CD4⁺ T cell function will be the focus of the current review and potential therapies that target components of the intracellular signaling pathways in CD4⁺ T cells will also be discussed. The first signal received by a naïve CD4⁺ T cell is from the Ag-specific TCR interacting with an antigenic peptide presented in the context of major histocompatibility complex II (MHC II) on the surface of antigen presenting cells (APCs). The second set of signals are delivered via costimulatory molecules that are expressed on the cell surface of activated APCs, and cytokines that are either produced

by the APC and/or by the activated CD4⁺ T cell itself. Classically, B7-1 (CD80) and B7-2 (CD86) expressed on the surface of the APC interact with the co-receptor CD28 that is constitutively expressed on the surface of CD4⁺ T cells (3,4). The overall effect of CD28 ligation is to increase the level of proliferation and cytokine production, promote cell survival, and enhance expression of CD40 ligand (CD40L) and adhesion molecules necessary for trafficking, such as very late antigen-4 (VLA-4) ($\alpha 4\beta 1$ integrin) (5–7). The costimulatory molecule pairs, CD28-CD80/CD86 and CD40-CD40L, and cellular adhesion molecules, such as VLA-4, represent putative therapeutic targets for blockade of autoreactive CD4⁺ T cell activation and trafficking to inflammatory sites. All of these potential therapeutic targets have been tested for the ability to decrease and/or inhibit disease to one extent or another, and will be discussed in detail below.

In addition to cell surface expressed costimulatory molecules, the presence or absence of secreted cytokines may affect disease outcome. For example, the production of IFN- γ or IL-4 by activated CD4⁺ T cells, or IL-12 by APCs directs the local population of naïve CD4⁺ T cells to differentiate toward the IFN- γ -producing Th1 cell or IL-4-producing Th2 cell phenotype, respectively (7). Recently, a third population of CD4⁺ effector T cells has been identified that secrete IL-17. The Th17 cell secretes IL-17, IL-6, and TNF- α , and is hypothesized to differentiate from a naïve CD4⁺ T cell precursor cell that has been activated in the presence of TGF- β and IL-6, and IL-17 secretion and/or Th17 cell survival is maintained by APC-secreted IL-23 (8–10). Th17 cells are critical for the development and maintenance of experimental autoimmune encephalomyelitis (EAE), the major animal model of MS (10,11). Recently published data show that the presence of IL-17 secreting CD4⁺ T cells are critical for the induction of EAE. This current hypothesis runs counter to the historical hypothesis that EAE is a Th1 cell-mediated disease. For example, in the absence of IFN- γ or IFN- γ receptor expression there is an exacerbation of disease. However, the data show that no disease occurs in IL-12 knockout mice and is decreased in the presence of anti-IL-12 mAb (12–15), but this may partly be explained by the decrease in the level of IL-17 produced and the survival of Th17 cells due to an absence of IL-23 (16). During immune homeostasis there is a balance between the activity of pro-inflammatory and anti-inflammatory T cells such that immune surveillance is maintained, while autoimmunity is avoided. Evidence has emerged that TGF- β is a critical differentiation factor that regulates this balance dependent upon the absence or presence of IL-6. The cytokine TGF- β is a critical differentiation factor for the generation of regulatory T cells in the presence of IL-2. On the opposing side of this balance, if the naïve CD4⁺ T cells are activated in the presence of both TGF- β and IL-6 the resulting cells differentiate into a Th17 cell phenotype. Therefore, the development of an immune-mediated therapy may work through one of three possible mechanisms either alone or in combination: 1) induction of anergy in self-reactive CD4⁺ T cells; 2) deletion of self-reactive CD4⁺ T cells by apoptosis; and/or 3) immune deviation.

MS is an autoimmune disease characterized by T cell responses to a variety of myelin proteins including myelin basic protein (MBP), myelin proteolipid protein (PLP), and/or myelin-oligodendrocyte glycoprotein (MOG) (17). There are four general courses of clinical disease in MS: 1) relapsing-remitting, 2) secondary-progressive, 3) primary-progressive, and 4) progressive-relapsing. Correspondingly, there are relapsing-remitting and chronic mouse EAE models of MS, as illustrated in Figure 1. Relapsing-remitting EAE (R-EAE) is characterized by transient ascending hind limb paralysis, perivascular mononuclear-cell infiltration, and fibrin deposition in the brain and spinal cord with adjacent areas of acute and chronic demyelination (18). Given that the inducing antigen (Ag) in MS has not been identified and the probability that CD4⁺ T cell responses to multiple epitopes on a number of myelin proteins are responsible for chronic disease progression, due to de novo activation of responses to endogenously released CNS epitopes via a process termed epitope spreading (19), the use of antigen-specific tolerance-based immunotherapies for MS is somewhat problematic.

Characterization of the cellular and molecular basis of epitope spreading in various chronic immune-mediated diseases and disease models is necessary for the effective design of Ag-specific treatments for human autoimmune diseases. However, a pathological role for epitope spreading is difficult to verify in human MS because the initiating antigen is not known. In contrast, animal models have the advantage that the initiating antigen is known, genetically identical animals are used, and the immune response is analyzed over a specified time course. The role of epitope spreading in relapsing-remitting EAE in the SJL mouse primed with the immunodominant PLP epitope, PLP₁₃₉₋₁₅₁, has been characterized (20). Following priming of SJL mice with PLP₁₃₉₋₁₅₁ peptide in complete Freund's adjuvant (CFA), peripheral PLP₁₃₉₋₁₅₁-specific CD4⁺ T cell reactivity is maintained throughout the disease. Prior to the first relapse, PLP₁₇₈₋₁₉₁-specific CD4⁺ T cell reactivity arises (intramolecular epitope spreading), and during the second relapse T cells specific for a myelin basic protein epitope, MBP₈₄₋₁₀₄, (intermolecular epitope spreading) are activated as illustrated in Figure 1. The development of these responses correlates with the extent of myelin destruction during the acute disease phase, and T cells isolated from the CNS of sick mice proliferate to the spread epitopes PLP₁₇₈₋₁₉₁ and MBP₈₄₋₁₀₄. These T cells can also transfer disease to naïve recipients. More convincingly, peptide-specific tolerance to the relapse-associated epitopes during remission blocks disease progression. Furthermore, while Ag-specific tolerance can be induced in this experimental model and the self-peptides have been well characterized, this is not the case in human disease. For example, in MS considerable effort has been made to identify a commonly shared autoreactive epitope(s). To date CD4⁺ T cells reactive to MBP₁₃₋₃₂, MBP₈₃₋₉₉, MBP₁₁₁₋₁₂₉, and MBP₁₄₆₋₁₇₀, MOG₁₋₂₀, MBP₃₅₋₅₅, and PLP₁₃₉₋₁₅₄ have been identified (21). While the development of more efficacious and focused antigen non-specific immunosuppressive therapies is currently favored, a clinical trial is currently ongoing to determine if peptide-specific CD4⁺ T cell tolerance can be induced to a cocktail of the aforementioned epitopes and effectively used as a therapeutic approach in MS patients.

In the NOD mouse model of T1D, mice develop a spontaneous autoimmune diabetes that resembles the human disease state in that it is characterized by the infiltration and destruction of pancreatic islets by autoreactive CD4⁺ and CD8⁺ T cells (22,23). It is the eventual destruction of insulin-producing pancreatic β cells by infiltrating autoreactive T cells that underlies the emergence of clinical symptoms in diabetes. While incomplete, efforts persist to identify the epitope targets of these infiltrating T cells in both the NOD mouse model and human diabetes. The initiating antigen is believed to be an insulin B chain peptide (InsB₉₋₂₃) (24) with other autoreactive CD4⁺ T cell specificities arising due to release of cryptic epitopes secondary to initial tissue destruction contributing to disease progression (S.D. Miller, et al., in preparation). Reactivity against the multiple autoantigens include islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), pro-insulin, the insulin B chain, and the 65 kDa isoform of glutamic acid decarboxylase (GAD65) is now believed to contribute to diabetes pathogenesis (25–30). These findings have already been translated into potential tools to predict an individual's susceptibility to diabetes such that the presence of either autoreactive T cells and/or autoantibodies to these epitopes being used as early predictors of subsequent disease onset (31–34). Due to the central role of both auto-reactive CD4⁺ and CD8⁺ T cells in pancreatic islet destruction, there has been a significant effort to use immunoregulatory strategies to treat diabetes. However, as in the treatment of MS most current techniques produce a general state of immunosuppression via the physical deletion/inactivation of entire subsets of T cells or nonspecific inhibition of antigen presentation, pro-inflammatory cytokine production or T cell trafficking creating the potential for unopposed opportunistic infection.

Antigen-Specific CD4⁺ T cell Tolerance

While CD4⁺ T cells can discriminate between specific peptide antigens, the TCR is not intrinsically able to distinguish self- from non-self-peptides. Therefore, during thymic CD4⁺

T cell selection, the majority of self-reactive T cells are clonally deleted subsequent to presentation of self-antigens on thymic antigen-presenting cells (APC) (35,36). Self-reactive CD4⁺ T cells that escape thymic negative selection maintain the capability to respond to self-antigen presented by activated peripheral APCs. The ability to control peripheral activation of self reactive T cells is dependent on the level of costimulatory molecules expressed on the surface of APCs. In turn, the level of costimulatory molecule expression and cytokine production of APCs is regulated by the presence or absence of inflammation, infectious agents, and other pathologic conditions. Thus, self tolerance and tissue homeostasis in the periphery is maintained, in part, by presentation of self peptides on immature APCs which lack expression of costimulatory molecules resulting in anergy induction in self-reactive CD4⁺ T cells (37). Apoptotic cells maintain membrane integrity until later stages of the process thereby allowing for the controlled breakdown and phagocytosis of apoptotic cellular contents by macrophages in a non-inflammatory manner. In contrast, necrotic cell death is characterized by organellar and cellular swelling leading to an immediate disruption of the plasma membrane and release of intracellular contents, thereby triggering phagocytosis of cellular contents in a presumed inflammatory environment (38). Therefore, if the autoreactive CD4⁺ T cells are activated in the absence of costimulation, the autoreactive CD4⁺ T cells would be anergized. Costimulation blockade thus represents a putative therapeutic strategy for treatment of established autoimmune diseases as a means to re-establish self tolerance. This topic will be elaborated upon in a following section.

As mentioned above, while the vast majority of autoimmunity therapies are antigen non-specific, a variety of current autoimmune therapies using antigen-specific approaches are under development. Following up on a report from our laboratory which showed that the intravenous injection of protein/peptide-pulsed splenic APCs (Ag-SP) which had been chemically ‘fixed’ with ethylene carbodiimide (ECDI) was a powerful method to induce to T cell tolerance *in vivo* (39), Jenkins, *et al.* demonstrated that Th1 clones encountering nominal antigen/MHC complexes in the absence of appropriate costimulation on chemically-fixed APCs were anergized (37). Anergy induction was shown by the failure of these T cells to proliferate and produce IL-2 in response to costimulatory competent APCs and their rescue by the addition of exogenous IL-2 (37,40,41). We have shown that the peripheral tolerance induced by the intravenous administration of syngeneic, neuroantigen-pulsed, MHC class II-bearing splenocytes fixed with ECDI to inhibit costimulatory signals (42,43), is a powerful and safe method for inducing antigen-specific tolerance specifically in the Th1 cells (39,44). The intravenous injection of Ag-SP is an efficient method of promoting clonal anergy and activation of Treg cells (39,45–48), and thereby T cell tolerance in many animal models of autoimmune and inflammatory disease including experimental autoimmune thyroiditis (49), uveitis (50), and neuritis (51), the NOD model of diabetes (S.D. Miller, *et al.*, in preparation), as well as transplant survival (52). In EAE, Ag-SP induces a long-lasting antigen-specific tolerance in both the active-priming and adoptive transfer models of EAE regardless of whether the treatment is administered at times prior to or following disease initiation (42,47,53,54). Ag-SP also appears to be nontoxic and well tolerated by treated animals at all stages of disease unlike intravenous tolerance using soluble peptides which depending on the antigen can induce severe anaphylactic responses resulting in death of treated animals (55,56). As Ag-SP can induce long-lasting Ag-specific tolerance in CD4⁺ T cells in the absence of any negative side-effects, Ag-SP possess significant therapeutic potential, and is currently being tested in a clinical trial for the treatment of MS.

The mechanism of Ag-SP-induced self-tolerance is not completely understood, but the two-signal hypothesis is presumed to play an active role in the induction of CD4⁺ T cell tolerance. Syngeneic donor spleen cells are fixed with ECDI in the presence of antigen, thereby cross-linking the free amino and carboxyl groups of the peptides to the donor cell surface proteins producing peptide-coated cells that function as potent tolerance-inducing carriers. The

mechanisms of Ag-SP-induced tolerance was initially hypothesized to be mediated by direct interactions between MHC II-peptide complexes and the TCR expressed by target CD4⁺ T cells (signal 1) (57,58). Furthermore, the level of costimulatory molecule expression by the donor cells (signal 2) is also believed to be an important factor in the ability of Ag-SP to render cells anergic (46). For example, LPS-pre-activated coupled cells with high CD80/CD86 expression are not capable of inducing tolerance suggesting that successful tolerance induction is dependent upon the lack of costimulatory signals coming from the APC (57,58). CTLA-4 ligation during secondary antigen encounter also appears to be important for the maintenance of the tolerized state (57,58) while a role for both PD-1/PD-L1 signaling as well as the activation of antigen-specific Tregs appear to be required for tolerance induction (59,60). Alternative mechanisms may also contribute to the induction of functional tolerance by Ag-SP as in addition to peptide antigens, both whole protein and mouse spinal cord homogenate (MSCH) also efficiently induce tolerance in CD4⁺ T cells when coupled to ECDI-fixed spleen cells (43,61,62). Ag-SP is also effective when multiple encephalitogenic peptides are coupled to cells allowing the simultaneous targeting of multiple myelin-associated antigens (63) allowing the effective blockade of possible spread epitopes. The efficiency of Ag-SP is independent of *de novo* antigen processing by the donor coupled cells since the inclusion of antigen processing inhibitors during fixation do not inhibit tolerance induction (64), although the antigen must be physically attached to the donor cells for tolerance induction to occur. Ag-SP tolerance in this case is hypothesized to occur through the reprocessing of the apoptotic donor coupled cells by host APCs that then re-present antigen to host T cells in a non-inflammatory manner. There is still much to be learned about the mechanism of coupled cell tolerance induction however, taken together, the findings suggest that Ag-SP is an efficient method to restore Ag-specific self-tolerance during autoimmune disease. Ag-SP also lacks many of the safety concerns that accompany other methods of tolerance induction such as the anaphylactic responses associated with intravenous soluble peptide tolerance. In light of these findings, the use of peptide-coupled APCs holds significant therapeutic promise as a potential therapy for MS and other autoimmune diseases.

Targets of Currently Approved Therapies in MS

The majority current FDA approved therapies for MS focus on immune deviation or non-specific immunosuppression. For example, the currently FDA approved therapies for MS patients are all aimed at the general suppression of immune cell function by the use of interferon β , random tetramers of amino acids (Copaxone), novantrone, or an antibody which blocks CNS trafficking of activated T cells (Tysabri). There also are a number of other monoclonal antibody-based drugs at various stages of FDA approval (see Table 1). The administration of interferon- β is used to decrease the severity and frequency of disease relapses. Secondly, systemic or mucosal administration of antigens or altered peptide ligands have been tested with mixed success. Copaxone (Glatiramer Acetate) is a random mixture of peptides composed of glutamine, lysine, alanine and tyrosine of various lengths, which is administered via daily subcutaneous injection for the treatment of relapsing-remitting MS. The mechanism of action is believed to be the elicitation of suboptimal TCR signaling in the absence of costimulatory molecule signaling (signal 1 in the absence of signal 2). Thus treatment with Copaxone is hypothesized to induce immune deviation toward a Th2 regulatory cell phenotype (disease-regulatory) as compared to Th1/Th17 phenotypes (disease-promoting) by inducing a low level of TCR stimulation. Copaxone may also block antigen presentation by competitive binding to MHC molecules. Besides the tolerogen-based therapies for the treatment of autoimmune diseases in humans (65), adhesion molecule and costimulatory molecule blockade are currently being tested. For example, the use of Tysabri, a monoclonal antibody to block the interaction of the adhesion molecule VLA-4 with its target ligand, VCAM-1 expressed by endothelial cells has been re-approved for the treatment of patients who have inadequate responses to other

approved MS therapies. Myelin-reactive T cells must migrate from the periphery into the CNS to mediate the demyelinating pathology associated with MS and EAE.

As mentioned above, CNS infiltration of inflammatory cells is a hallmark of MS. Activated immune cells adhere to activated endothelial cells, via adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1), leukocyte function-associated antigen-1 (LFA-1) (CD11a/CD18), Mac-1 (CD11b/CD18), VLA-4, and vascular cell adhesion molecule-1 (VCAM-1) prior to extravasation across the blood–brain barrier. Elevated levels of ICAM-1 and VCAM-1 have been identified on endothelial cells within both acute and chronically-active MS lesions, and their corresponding ligands (LFA-1 and VLA-4, respectively) are expressed on perivascular inflammatory cells within MS lesions (66,67). Furthermore, ICAM-1 positive astrocytes are found both within and around active MS lesions, but not in normal brain (68). In addition to the possible role in inflammatory cell migration, it has been proposed that glial cell expression of adhesion molecules may play a role in antigen presentation and T cell costimulation, as well as glial-extracellular matrix interactions (69). Interactions between these adhesion molecules and their ligands have also been implicated in the pathogenesis of EAE (70–73). Given the aforementioned findings, LFA-1 and VLA-4 are hypothesized to have important roles in endothelial-leukocyte interactions and in leukocyte extravasation, and antagonists of these molecules are currently being tested in clinical trials for their ability to block inflammatory cell migration into the CNS (74).

Beside the blockade of the adhesion molecules necessary for the inflammatory cell entry into the CNS, blockade of secretory chemoattractants is also under investigation. Chemokines enhance T cell and monocyte migration through direct chemoattraction and by activating leukocyte-expressed integrins to bind their adhesion receptors on endothelial cells. For example, blockade of the chemokine macrophage inflammatory protein-1 α (MIP-1 α) inhibits initiation of EAE, whereas blockade of monocyte chemoattractant protein-1 (MCP-1) inhibits disease relapse (75). Consistent with this observation, immunohistochemical analysis of MS CNS tissue shows that astrocytes, but not perivascular or parenchymal microglia, express MCP-1 in both actively demyelinating and chronic lesions (76). There is also increased expression of RANTES (chemotactic for lymphocytes and monocytes) by activated perivascular T cells in MS lesions (77), and levels of interferon-inducible protein 10 (IP-10) (chemotactic for activated T cells) are increased in the cerebral spinal fluid (CSF) of MS patients compared to controls. Correlating with the increase in chemokine expression, there is an increase in the frequency of CD4⁺ and CD8⁺ T cells in the CSF expressing the IP-10 receptor, CXCR3, in MS patients. CCR5, the RANTES receptor, is also detected on lymphocytes, macrophages and microglia in actively demyelinating lesions. Recent data also indicate that some of the chemokines and chemokine receptors that have been implicated in both MS and EAE are preferentially chemotactic for pro-inflammatory Th1 T cells (78). While, the extent to which chemokines and their receptors contribute to MS pathogenesis is unclear, the above findings collectively suggest that the IP-10/CXCR3 and RANTES/CCR5 pathways have selective roles in MS pathogenesis and may eventually serve as therapeutic targets.

Alteration of TCR Signaling as a Potential Therapy for Autoimmunity

Previously tested immunotherapeutic strategies have been shown to work at least in part, through the alteration of signal 1 and/or inhibition of costimulatory molecule stimulation (signal 2). In this manner CD4⁺ T cell anergy is hypothesized to be induced in peptide-specific T cells undergoing activation at the time of treatment via short-term blockade of CD28-CD80/CD86 interactions. CD28-CD80/CD86 inhibitory reagents are currently being tested in phase I/II clinical trials in various autoimmune diseases. The goal for treatment of autoimmune diseases, such as MS, is to re-establish tolerance to self-antigens. The difficulty in the development of these therapies lies in maintaining the ability of the patient to normally

recognize and react to non-self-antigens. The mechanism by which these potential therapies inhibit disease will be the focus of the following section. Several groups have investigated the therapeutic potential of anti-CD3 mAb treatment in the absence of costimulatory signals in the treatment of various autoimmune diseases (79–82). However, treatment with an unaltered anti-CD3 mAb is potentially a double-edged sword. While treatment eliminates pathogenic autoreactive CD4⁺ T cells, thereby ameliorating autoimmune disease progression, this therapy may also induce serious non-specific side effects through bystander activation of T cells. For example, the induction of general immunosuppression increases the patient's susceptibility to opportunistic infection and the common occurrence of high-dose syndrome in which treatment recipients suffer severe side-effects due to the non-specific production of inflammatory cytokines such as TNF- α (83). Furthermore, cross-linking of CD3 in some cases initiates a signal of sufficient strength that eliminates the need for a costimulatory molecule-induced reduction in the signal threshold required for T cell activation.

Due to the aforementioned complications for the use of an unaltered anti-CD3 mAb, modifications to the anti-CD3 mAb have been made for treatment so that the deleterious side effects are avoided by reducing/eliminating the ability of the antibody to bind to Fc receptors and thus the ability to efficiently cross-link the TCR. The regulatory properties of non-mitogenic anti-CD3 mAb treatment is believed to be due to the lower levels of TCR-mediated signaling, which favor T cell differentiation into a Th2 cell phenotype and the development of regulatory T cells (83,84). Therefore, the possibility exists that treatments induce immune deviation. In this scenario, the T cell-mediated immune response is changed from a Th1/Th17 cell-like (disease-promoting) response, to a Th2 cell-like (disease-regulating) response. In support of this hypothesis, findings from numerous studies suggest that cytokines mediate a protective effect in non-mitogenic anti-CD3 mAb treatment (85,86). However, there is currently debate concerning the exact contribution of cytokines to the underlying mechanisms of treatment. Furthermore, activated Th1 cells, but not naïve CD4⁺ T cells, appear to become unresponsive to subsequent restimulation following treatment with non-mitogenic anti-CD3 mAb (87). To gain a better understanding of the potential Th cell subset specificity of non-mitogenic anti-CD3 mAb treatment, the efficacy of non-mitogenic anti-CD3 mAb treatment to induce tolerance in Th1 and Th2 cells was compared. In this study when both Th1 cells and Th2 cells were treated with non-mitogenic anti-CD3 mAb there were similar decreases in the level of intracellular signaling, *i.e.*, CD3 ζ , ZAP-70, and MAP kinase. However, the data show that treatment of Th1 cells with non-mitogenic anti-CD3 mAb decreases the level of proliferation, IL-2 production and IFN- γ production. In contrast, treatment of Th2 cells induced an increase in the level of cellular proliferation and the level of IL-4 produced (88). These findings lend support to the theory that non-mitogenic anti-CD3 mAb may specifically down regulate Th1 cell function. As mentioned above, more recently proposed hypotheses assert that MS and EAE are mediated by the balance of Th17 cell activity versus regulatory T cell activity. This remains to be defined/studied following non-mitogenic anti-CD3 mAb treatment. Current findings suggest that non-mitogenic anti-CD3 mAb does not induce an alteration in the number or trafficking of regulatory T cells, however, the level of Th17 cells and the level of IL-17 produced still remain to be defined. In summation treatment of disease with a non-mitogenic anti-CD3 mAb provides a therapy that potentially blocks or induces a suboptimal signal 1 in the absence of costimulatory signals (signal 2). This therapy is hypothesized to represent a treatment in which only activated immune cells at the time of treatment are affected, allowing the maintenance of host defense against non-self-antigens at times post non-mitogenic anti-CD3 treatment.

Signaling Pathways and Potential Therapeutic Targets Associated with the TCR

The subsequent signaling pathways activated following TCR stimulation serve as both therapeutic readout and potential therapeutic target. While recognition of peptide complexed

with MHC II on the surface of the APC by the TCR is necessary for T cell activation (signal 1), the cytoplasmic tails of the alpha/beta chains of the TCR do not have inherent kinase activity. Therefore, signaling through the TCR is achieved by the association of the ligand-binding chains with accessory proteins that contain immunoreceptor tyrosine-based activation motifs (ITAMs). An intracellular signaling cascade is initiated by phosphorylation of ITAM tyrosines by Src family kinases following TCR cross-linking. While events that induce the association of Src family kinases with TCR remain undetermined, specialized cholesterol- and sphingolipid-rich membrane domains known as lipid rafts appear to function as a platform by which the accessory molecules associate in proximity with the TCR (89). Due to the biochemical properties of cholesterol and sphingolipids, the lipids are allowed to tightly pack together and to include specific membrane associated proteins while excluding others. It is currently hypothesized that Src family kinases preferentially associate with lipid rafts, and that upon the recognition of antigenic peptide the TCR translocates into the lipid rafts where the Src family kinases phosphorylate ITAMs on the cytoplasmic tail of the TCR (90). Therefore, TCR signaling may be regulated by the ability of the TCR to associate with lipid rafts upon cross-linking.

Based on the ability of lipid rafts to exclude or include specific proteins, lipid raft associated proteins are modified to allow for inclusion. For example, the Src family kinases Fyn, Lyn, and Lck, which initiate TCR ITAM phosphorylation, are myristoylated and palmitoylated (91,92). Another transmembrane protein involved in T cell activation following TCR cross-linking is LAT, which is palmitoylated upon CD4⁺ T cell activation (93). The function of LAT as it pertains to T cell activation vs. anergy is discussed in a following section. The overall physical outcome of ligating the TCR is formation of the immune synapse. T cell receptor signaling has not been studied following the exposure of Ag-specific CD4⁺ T cells to peptide coupled Ag-SP. To begin to identify the molecular mechanism by which non-mitogenic anti-CD3 mAb alters CD4⁺ T cell activity, the level of Ca²⁺-flux was found to be equivalent to mitogenic anti-CD3 mAb. However, non-mitogenic anti-CD3 mAb did not induce equivalent levels of CD4⁺ T cells proliferation and expression and CD69 (94). This finding suggests that while non-mitogenic anti-CD3 mAb does induce an intracellular signal, the intracellular signaling pathways activated by non-mitogenic anti-CD3 mAb and mitogenic anti-CD3 mAb differ. Since the TCR-APC immune synapse is a highly ordered membrane structure in which the TCR, the associated signaling proteins, the cytoskeleton, and cellular adhesion molecules concentrate to allow for sufficient intercellular protein-protein interaction (95). The effect of non-mitogenic anti-CD3 mAb treatment on the generation/organization of the synapse remains to be determined. For example, the TCR-APC immune synapse contains a central cluster of TCRs ringed by adhesion molecules, and on the cytoplasmic side, signaling molecules including Src family kinases, protein kinase C, and the integrin-associated cytoskeleton proteins including talin (96,97). Following cross-linking of the TCR, the immune synapse persists for more than an hour in a cytoskeleton-dependent manner, thereby allowing the TCR to be stimulated multiple times (95). By mutating LAT such that it cannot be palmitoylated, LAT is not able to associate with lipid rafts thereby altering TCR signaling (93). Stimulation of CD28 has also been shown to enhance the recruitment of lipid rafts to the immune synapse (98). In this manner the organization of the T cell plasma membrane during T cell-APC interaction contributes not only to the inclusion of the necessary signaling molecules but also allows for sufficient and sustained TCR signaling. Therapies that deliver low levels of TCR stimulation in the absence of costimulation may not lead to complete organization of the lipid rafts into the immune synapse.

In addition to the regulation by transcription factors, components of the TCR signaling complex are also implicated in the regulation of CD4⁺ T cell anergy. For example, the adaptor molecule linker for activated T cells (LAT) is a transmembrane protein that facilitates the formation of a multi-subunit signaling complex with other signaling molecules such as phospholipase C 1,

Gads-SLP-76, Grb2, and PI(3)K (99) (see Figure 2). LAT is essential for TCR signaling, and phosphorylation of LAT is necessary for activation of MAPK cascades, Ca^{2+} -flux, and activation of the transcription factor AP-1 following TCR stimulation. As mentioned in the previous section, the signaling cascade activated by the costimulatory molecule CD28 also activates AP-1. In this way TCR and CD28 signaling coordinate to increase the level of AP-1 present within the T cell allowing for the regulation of activation associated genes by the NFAT/AP-1 heterodimer. In the absence of AP-1, NFAT homodimerizes and regulates the expression of a cohort of ubiquitin E3 ligases, including Cbl-b, Itch, and GRAIL (100,101). The therapeutic targeting of NFAT will be addressed in a later section. Although Cbl-b is upregulated at both the mRNA and protein level in ionomycin-energized cells, ionomycin-treated Cbl-b-deficient CD4^{+} T cells are also defective in LAT phosphorylation. Since there is an increased steady-state level of LAT protein present in the cellular lysates of Cbl-b^{-/-} T cells, it is possible that Cbl-b regulates LAT steady-state protein amounts and thus more ionomycin is required to overcome this enlarged pool of LAT. To determine if the TCR signaling pathway is altered in anergic CD4^{+} T cells, anergized antigen-specific transgenic T cells were compared to control transgenic T cells. While the immediate phosphorylation of CD3 ζ and ZAP-70 is normal in anergized T cells, the adaptor protein LAT and its downstream target, PLC 1, are hypo-phosphorylated and the kinetics of both LAT and ZAP-70 activation is decreased in anergic T cells due to decreased recruitment of the p85 regulatory subunit of PI(3)K by LAT (102). Interestingly, normal activation of the CD28 pathway was noted in ionomycin-energized T cells upon restimulation, demonstrating that the costimulatory cascade, which itself contributes to LAT phosphorylation, was unaltered. Inhibition of LAT activation may thus serve as a viable target for the induction of CD4^{+} T cell anergy.

Alteration of B7/CD28/CTLA-4 Signaling Pathways as a Potential Therapy for Autoimmunity

The presence of activated peripheral myelin-reactive T cells appears to distinguish MS patients from normal subjects, in whom circulating myelin-reactive T cells do not express activation markers. Two genetic alleles have been identified that correlate with increased risk of MS development. The first is the HLA DR2. HLA DR2⁺ individuals have an increased probability that naïve self-reactive CD4^{+} T cells will receive the necessary TCR stimulus to become activated due to the ability of DR2 to present MBP and other myelin protein epitopes. An increased percentage of MS patients also express an allelic variant of CTLA-4 (103). In addition to the positive regulating CD28 costimulatory molecule, activated CD4^{+} T cells upregulate surface expression of CTLA-4, a CD28 homologue, which also binds CD80/86 (104), and serves as a negative regulator of T cell activation. Blockade of CTLA-4 interaction with CD80/CD86 using an intact anti-CD80 mAb has been shown to promote increased production of IFN- γ by Th1 cells (105). CD80 is expressed by all cell types in the CNS-infiltrating mononuclear cells during EAE, including CD4^{+} T cells (106). Furthermore, data from our laboratory show that anti-CD80 mAb binds to both APCs and to peptide-specific CD4^{+} T cells within the CNS following treatment. Similar observations have been made in MS suggesting a preferential role for CD80 in the disease process. Immunohistochemical staining of MS plaques and inflammatory stroke lesions demonstrated that while CD86 expression was expressed in both types of lesions, CD80 was uniquely associated with the MS plaques (107). Thus, CD80 appears to be the predominant costimulatory molecule in established CNS disease, as supported by experiments demonstrating that blockade of CD80/CD28 costimulation using an anti-CD80 Fab specifically inhibited the activation of T cells to endogenously presented myelin epitopes (epitope spreading) in relapsing EAE (108).

Clinical trials are ongoing to study the therapeutic effect of CD28-CD80/CD86 blockade by the use of the extracellular portion of CTLA-4 linked to the Fc portion of an immunoglobulin molecule (CTLA-4 Ig) under the trade name of abatacept. The use of abatacept has been

approved for use in rheumatoid arthritis (109,110) and is being evaluated for use in MS patients (111). CTLA-4 Ig treatment is thought to block CD28-CD80/CD86 interactions as CTLA-4 Ig binds with high affinity to CD80/CD86 expressed on activated APCs. In this manner the autoreactive CD4⁺ T cell would receive signal 1 in the absence of signal 2. CD80 and CD86 have been shown to have differential roles in T cell activation and differentiation (112). To illustrate this point, conflicting results have been obtained using anti-CD80 and anti-CD86 mAbs to regulate autoimmune disease. *In vivo* down-regulation of T cell effector function is controlled by T cell-expressed B7 following ligation with CTLA-4 which underscores the potential importance of T cell-T cell interactions as an immune regulatory control mechanism (113,114). Treatment with anti-CD80 mAb surrounding autoantigen priming blocks EAE development induced with suboptimal concentrations of PLP₁₃₉₋₁₅₁ or MBP₈₄₋₁₀₄ in SJL mice, whereas anti-CD86 mAb treatment exacerbates disease (115) or has no effect (116). In contrast, treatment with intact anti-CD86 mAb initiated during the remission following acute disease, does not affect disease progression (relapses) in PLP₁₃₉₋₁₅₁-induced R-EAE (108). Treatment with monovalent, non-cross-linking anti-CD80 Fab fragments during EAE remission blocked clinical relapses and epitope spreading to the PLP₁₇₈₋₁₉₁ epitope (108), whereas treatment with intact anti-CD80 mAb led to a profound exacerbation of disease relapses concomitant with accelerated epitope spreading (117). Likewise, treatment of mice with a small molecule inhibitor of CD28 during disease decreased disease severity and proliferation of myelin-specific CD4⁺ T cells upon *ex vivo* activation, and increased CD4⁺ T cell apoptosis (118). These findings suggest that intact anti-CD80 mAb may cross-link and induce a direct signal through CD80 expressed on either APCs and/or activated T cells, whereas anti-CD80 Fab may block CD80/CD28 costimulatory signals required for the activation of spread epitope-specific CD4⁺ T cells. Thus, short-term blockade of CD28/CD80 interactions may prove to have therapeutic efficacy, which would predominantly target activated T cells during the treatment period allowing maintenance of host defense against infection.

The aforementioned genetic analyses of MS patients support the hypothesis that CD80 expressed by autoreactive CD4⁺ T cells may contribute to the disease exacerbation. There is a positive correlation between the expression of allelic variants of CTLA-4 that have an increased affinity for binding to CD80 in MS patients (103). Beside the anti-CD80 mAb-induced increase in the level of IFN- γ , ongoing studies in our laboratory have begun to focus on the effect anti-CD80 mAb treatment has on the level of IL-17 produced by encephalitogenic CD4⁺ T cells in *ex vivo* recall responses, and naïve CD4⁺ T cell differentiation into Th17 cells. In this scenario, the expression of CTLA-4 might have the opposite effect of the “classic” negative regulatory role CTLA-4 has on CD4⁺ T cell activity (119–121). Instead, CTLA-4, with its increased affinity for CD80, might act as a cross-linker for CD80 sending a positive signal to the autoreactive T cell during T cell-T cell interactions. When taking the *in vitro* and *in vivo* data into consideration, a putative mechanism is suggested to explain why the timing of anti-CD80 mAb treatment has differing effects on disease outcome in R-EAE. As mentioned above, treatment of mice with anti-CD80 mAb during the onset of disease exhibited decreased disease severity (115), while treatment of mice during remission with an intact anti-CD80 mAb exhibited increased disease severity (108). In contrast, treatment with an anti-CD80 Fab fragment led to significantly decreased disease severity and relapse frequency when administered during disease remission. Both the *in vitro* and *in vivo* data support the hypothesis that the initial steps of activation of naïve CD4⁺ T cells are inhibited by anti-CD80 due to a lack of costimulation through CD28 interacting with CD80 expressed on APCs. For example, the addition of either an intact anti-CD80 mAb, anti-CD80 Fab, or CTLA4-Ig at the time of naïve CD4⁺ T cell activation in the presence of antigenic peptide and APCs blocks T cell activation (122,123). The time of treatment may be equivalent to the treatment regimen in which anti-CD80 mAb is administered to the mice at the time of priming with encephalitogenic CD4⁺ T cell epitopes. In contrast, if the intact anti-CD80 mAb is added to culture following activation, this more closely mimics the *in vivo* treatment regimen wherein anti-CD80

treatment is initiated during ongoing disease. Therefore, these results support the conclusion that cross-linking CD80 on effector CD4⁺ T cells induces increased production of IFN- γ leading to enhanced tissue destruction and increased epitope spreading by increasing CD4⁺ T cell effector function. The effect of anti-CD80 mAb stimulation on the induction of IL-17 produced by naïve CD4⁺ T cells activated in the presence of Th17 cell-promoting conditions is currently under investigation. Recent findings also strongly suggest that extreme caution must be taken when designing treatment regimens for autoimmune diseases such as MS based on antibody-mediated blockade of costimulatory molecules. Any treatment that has the potential to cross-link CD80 must be analyzed with the utmost care in that not only can blocking and/or cross-linking of CD80 on an APC affect cellular activity (124), but these same regimens may also directly upregulate autoreactive CD4⁺ T cell effector functions, thereby exacerbating disease.

Signaling Pathways and Potential Therapeutic Targets Associated with B7/CD28/CTLA-4

The structural mechanism of CD80/CD86 mediated costimulation is not completely understood and even less is known about the significance of the unique features of these two costimulatory molecules. Structural and cell-based FRET studies have demonstrated that CD80 and CD86 differ in their cell surface organization and suggest that these differences may play a functional role (125). Signaling via CD80 and CD86 in dendritic cells involves the expression of both CD80 and CD86, suggesting that the homo-oligomeric and hetero-oligomeric states may be important for APC function (126). Both CD80 and CD86 are type 1 transmembrane proteins with a membrane distal IgV and a membrane proximal IgC domain. They share approximately 25% sequence homology and interact with the same receptors, CD28 and CTLA-4. However, CD86 appears to be a weaker ligand as compared to CD80. For example, CD80 binds CTLA-4 and CD28 with equilibrium dissociation constants (K_d) of 0.2 and 4 μ M, respectively. In contrast, CD86 exhibits 5–10-fold lower affinities with K_d values of 2.6 μ M for CTLA-4 and 20 μ M for CD28 (127). A possible explanation for the difference in the K_d values between CD80 and CD86 may be that CD80 has been observed to exist as a dimer. In contrast to CD80, the structure of CD86 alone showed it to be a monomer in the crystalline state (128). However, both CD80 and CD86 could exist as dimers in their complexes with CTLA-4 suggesting the possibility that CD86 can undergo receptor-induced dimerization (129). Photobleaching-based FRET on a single cell basis suggest that CD80 is present predominantly as a dimer whereas CD86 is present as a monomer on the cell surface. Recent studies have also demonstrated that CD80 and CD86 exhibit different preferential binding to their receptors, CD28 and CTLA-4. Using APCs deficient in either CD80 or CD86 and imaging the sequestration of either CD28 or CTLA-4 in the immunological synapse, it has been shown that while CD80 favors binding to CTLA-4, CD86 shows a preference for CD28 (130). This *ex vivo* demonstration of preferential interaction of CD80 with CTLA-4 and CD86 with CD28 may appear to be only partially consistent with biochemically determined K_d values. *In vitro* CD80 and CTLA-4 exhibit a relatively strong binding interaction and this association is also favored *ex vivo*. On the other hand, the CD86-CD28 interaction is relatively weak *in vitro* and yet is favored when these molecules are expressed on the cell surface.

The cell surface organization and the preferred interaction of CD80 and CD86 with the respective ligands may in part regulate the activation of intracellular signaling pathways induced in the CD80/CD86 expressing cell. Over the past several years there has been an increase in the number of findings that suggest the ability of two of the B7 family members, *i.e.*, CD80 and CD86, to induce intracellular signals directly to the cell that the molecule is expressed on. For example, cross-linking CD80 on the surface of B cells activated in the presence of IFN- γ increases the level of IgG_{2a} produced, as well as increases pro-apoptotic genes (124). Likewise, dendritic cells activated in the presence of soluble CD28 increase the

expression of express IL-6 and IFN- γ in a CD80-, CD86-, and p38 MAP kinase-dependent manner (126). Considerable progress has been made towards determining the signaling pathway activated following cross-linking CD86. Cross-linking CD86 on the surface of B cells activated in the presence of IL-4 increases the rate of mature IgG₁ transcript produced via PI3K-dependent activation of the NF- κ B subunits p65 and p50. Likewise, activation of these same NF- κ B subunits is induced in macrophage cell lines following the cross-linking of CD80 and CD86 (131). Findings from our laboratory suggest that the CD80-induced increase in IFN- γ is similarly PI3K-dependent and a Ca²⁺-flux is induced following CD80 cross-linking (132) (see Figure 3). Taken together, it is tempting to conclude that the same intracellular signaling pathway is activated following both CD80 and CD86 cross-linking. However, cross-linking CD80 on B cells induces pro-apoptotic gene expression, while cross-linking CD86 induces anti-apoptotic gene expression (124). Therefore, while there may be common intracellular signaling intermediates activated by both CD80 and CD86, receptor-specific intracellular signaling intermediates must also be activated.

The high affinity interaction between CD4⁺ T cell-expressed CD80 and CD4⁺ T cell-expressed CTLA-4 is hypothesized to play a regulatory role during T cell-T cell interactions. It has been observed that Th2 cells express higher levels of CTLA-4 than Th1 cells. This finding was correlated with data showing that Th2 cells, but not Th1 cells, show variations in the organization of the immunological synapse dependent on B7 expression by the APC (133). CD80 and CTLA-4 are co-expressed by a small percentage of the CD4⁺ T cells by 48 h after activation allowing for CD80-CTLA-4 interaction. Cross-linking CTLA-4 inhibits CD4⁺ T cell activity by decreasing tyrosine phosphorylation of Fyn and ZAP-70 as well as Ca²⁺ mobilization following TCR stimulation (134–137). Since studies in human T cell lines suggest that an increase in intracellular Ca²⁺ would increase the level of CTLA-4 expressed (138, 139), we are currently investigating if cross-linking CD80 also alters the level of CTLA-4 expressed. These data may point to a putative T cell-T cell regulatory mechanism by which CD80 interaction with its ligand CTLA-4 would induce a temporal increase in cytokine production and survival signals for CD4⁺ T cells expressing CD80 during the early stages of an immune response. This hypothesis is supported by the finding that transfer of CD80/CD86^{-/-} T cells resulted in a significantly increased severity of GVHD as compared to transferred wildtype T cells, while transfer of T cells over-expressing CD86 initiated decreased GVHD as compared to wildtype T cells (140). This finding suggests that CD80/CD86 expression on T cells down-regulated allogeneic responses through CTLA-4 ligation. In contrast, CD4⁺ T cell-expressed CD80 has been shown to play a critical role in CD4⁺ T cell-mediated anti-tumor activity suggesting that inflammatory CD4⁺ T cell responses may be increased by CD80 expressed on tumor-infiltrating CD4⁺ T cells (141,142). However, since cross-linking CD80 may then also increase the level of CTLA-4 expressed by the activated CD4⁺ T cells, this would eventually result in down-regulation of the response.

Nuclear Factor of Activated T cells (NFAT): Regulation of T cell Activation, Anergy, and Potential Therapeutic Targets

The previous sections focused on defining the known immune mechanisms involved in an autoimmune disease and pointed out possible therapeutic targets. The following will discuss the signaling pathways that may play a putative role in the induction of self-reactive CD4⁺ T cell anergy following therapeutic intervention by Ag-SP, non-mitogenic anti-CD3 mAb, or CD80 blockade treatment. First and foremost it is probable that several forms of anergy exist that have yet to be completely characterized biochemically. Part of the confusion may arise from the multiple costimulatory molecules that modulate T cell responses following stimulation of the TCR. This discussion will focus on anergy induced by blocking cell cycle progression. The most consistent property of an anergic CD4⁺ T cells is the decreased production of IL-2 and decreased proliferation (143). Anergy has also been defined as an

unresponsive state, which is reversible by IL-2 (37,40,144). Therefore, anergy is only a relative measure of an immune response. For example, although substantial decreases in responsiveness can be achieved *in vitro*, that level of responsiveness may cause significant effects *in vivo*. The initial characterization of anergy *in vitro* in which TCR engagement (signal 1) occurred without costimulation (signal 2), demonstrated that T cell clones were unable to proliferate or produce IL-2 under these conditions. These studies initiated a flurry of investigation into proposed intrinsic signaling defects that suggested that a myriad of deficiencies, such as a lack of mitogen-activated protein kinase (MAPK) signaling, Ras activation, or the upregulation of dominant “anergic” factors, gave rise to the anergy phenotype (143). As a result, a coherent model for the molecular mechanism of anergy induction was difficult to develop, in part due to the varied model systems used to induce T cell anergy, including oral administration of soluble peptide or superantigen treatment *in vivo*, and cross-linking of the CD3 complex in the absence of costimulation *in vitro*. A more recent model system for induced T cell unresponsiveness utilizes prolonged nuclear factor of activated T cells (NFAT) occupancy of anergy-associated gene promoters in the absence of MAPK signaling induced by treatment with the potent Ca^{2+} ionophore, ionomycin. This causes the upregulation of a unique set of genes responsible for the induction of this form of T cell tolerance by the NFAT/NFAT homodimer. (145) Anergy induction upregulates the expression of several ubiquitin E3 ligases, including Cbl-b (Casitas B-lineage lymphoma B), Itch, and GRAIL (gene related to anergy in lymphocytes), leading to degradation of key signaling proteins in T cell activation (100). Cbl-b promotes the conjugation of ubiquitin to phosphatidylinositol 3-kinase (PI3 kinase) and modulates its recruitment to CD28 and TCR–CD3 complexes, thereby regulating the activation of Vav (see Figure 2). In support of this, the increased tyrosine phosphorylation of Vav in Cbl-b^{-/-} T cells was reversed by PI3 kinase inhibitors, as was the enhanced T cell proliferation and IL-2 production (146). While controversy still exists as to whether or not PI3 kinase plays a crucial role in T cell activation, particularly in CD28-mediated signaling (147), recent data show that ligation of CD28 induces the formation of a grb-2-associated binder 2 (grb-2)/SRC homology phosphatase-2/PI3 kinase complex (148). Therefore, the induction of CD4⁺ T cell tolerance is suggested to be regulated by altered NFAT transcriptional activity leading to expression of anergy-associated, rather than activation-associated, genes.

Of the multiple signaling pathways that are upregulated during T cell activation, Ca^{2+} signaling is a critical for the first step of anergy induction. Lack of CD4⁺ T cell costimulation through the interaction of CD28 with CD80/CD86 expressed on the surface of the APC correlates with an unbalanced partial form of signaling in which TCR-mediated Ca^{2+} influx predominates. While CD28 ligation is not directly coupled to Ca^{2+} mobilization, CD28 signaling potentiates TCR signals that do not involve Ca^{2+} influx. Experimentally, this is shown by the fact that anergy induced in CD4⁺ T cells activated with Ca^{2+} ionophores is closely related to that induced by the absence of CD28 costimulation following TCR stimulation. Likewise, the timing of the Ca^{2+} -flux may regulate the overall outcome, in that CD80-induced Ca^{2+} -flux does not induce CD4⁺ T cell anergy. As mentioned above, Ca^{2+} -induced anergy is mediated primarily by NFAT. NFAT is a transcription factor regulated by the protein phosphatase calcineurin, and both NFAT activation and anergy induction are blocked by calcineurin inhibitors, such as cyclosporin A (CsA) (149). NFAT was initially identified as an inducible nuclear factor that could bind the IL-2 promoter in activated T cells (150). However, when all of the proteins of the known NFAT family were isolated and molecularly characterized, it became clear that their expression is not limited to T cells. NFAT proteins can also form transcriptional complexes with other partners, and can even be transcriptionally active by themselves, has introduced the possibility of defining new roles for NFAT proteins in T cells (100). In the two-signal hypothesis for T cell activation, stimulation of the TCR (signal one) and costimulatory molecule stimulation, *i.e.*, CD28-CD80/CD86 (signal two), are required for full activation, where as signaling through the TCR only induces T cell anergy. In the absence of costimulatory

molecule-enhanced AP-1 and the CD28-induced stabilization of the immune synapse, NFAT regulates transcription of a specific program of genes involved in the negative regulation of TCR signaling (see Figure 2). In this model, costimulatory signals push the TCR-induced signaling above the threshold level allowing for cellular activation. For example, in the absence of the CD28-induced signaling pathway there is an unbalanced activation of the CD4⁺ T cell resulting in an altered set of transcription factors present in the nucleus, due to the decreased activation of RAS–MAPK, protein kinase C (PKC) or IKKs (inhibitor of NF-κB (IκB) kinases) signaling (151). To illustrate the dependence of anergy-associated gene expression on NFAT, *Nfat1*^{−/−} T cells and wildtype CD4⁺ T cells treated with the calcineurin inhibitor, CsA, thereby inhibiting the activation of NFAT, block the expression of these anergy associated genes and impair induction of anergy in treated cells (151).

Since NFAT proteins control two opposing aspects of T cell function, activation vs. anergy, it is likely that the availability of transcriptional partners in response to activating or energizing stimuli determines which set of genes is activated. Among the proteins expressed by anergic T cells are a group E3 ubiquitin ligases, *i.e.*, Itch, Cbl-b, and GRAIL (100,101,152,153). Alterations in the molecules that negatively regulate TCR signaling, such as Cbl-b have been shown to be involved in the initiation of autoimmune disease (154,155). For example, the loss of Cbl-b expression allows for the hyper-reactive signaling through the TCR. A characteristic feature of T cells from Cbl-b^{−/−} mice is a lower threshold of activation following signaling through the TCR resulting in hypersensitivity following TCR engagement, and activation of downstream signaling pathways without the normal requirement for co-receptor stimulation (154). These two signaling pathway intermediates represent two putative candidates by which Ag-SP-induced tolerance of CD4⁺ T cells occurs. Recently, several small organic molecules were identified that specifically inhibit calcineurin-induced NFAT activation, blocking NFAT-dependent cytokine production by T cells (156). While the current small molecule inhibitors are efficient at blocking NFAT-dependent transcription and are able to potentiate CsA effects, these molecules still act upstream of calcineurin (157). Therefore, the same non-specific side effects as CsA and FK506 may exist for *in vivo* use. The ability of small molecules to selectively inhibit calcineurin and NFAT protein–protein interactions points to the possibility of using them to modulate specific NFAT-regulated functions. Differential interactions between various NFAT family members and specific transcriptional partners might underlie the ability of NFAT to integrate multiple signaling pathways and control diverse cellular functions. If the protein–protein contact surfaces are specific for different interactions, molecules could be designed to block NFAT-regulated functions without affecting other calcineurin-regulated functions. Such molecules will most likely be therapeutically useful, with notably improved specificity and greatly reduced toxicity.

Conclusions

In general, the goal of future research in MS is to develop therapies which target the activation or trafficking of myelin-specific autoreactive T cells in an antigen-specific fashion and to combine these therapies with others which will promote myelin and axonal repair. It is clear that the T cell repertoire in an autoimmune response, such as peptide-induced relapsing-remitting EAE is dynamic in that CD4⁺ T cell responses to the initiating epitope, which play the dominant pathologic role during the acute disease episode, do not play a major role in disease relapses. Understanding of the mechanisms underlying spontaneous disease remission are critical to the ultimate design of therapeutic modalities. Current therapies for the re-establishment of self-tolerance in autoimmune disease focuses on the inhibition of signal 1 and/or signal 2. For example, blockade and/or provision of sub-threshold levels of signal 1 in an antigen-specific therapy includes ECDI-antigen coupled APC treatment, and non-specific signaling to activated T cells using non-mitogenic anti-CD3 mAb treatment. Blockade of signal 2 is currently used therapeutically in the ongoing clinical trials using CTLA-4 Ig to block CD28-

CD80/CD86 interactions. Some of the outstanding issues in the development of efficacious therapies in autoimmune disease are the development and testing of antigen-specific tolerance therapies which specifically target the activation and effector functions of myelin-specific autoreactive T cells without compromising the ability of the immune system to fight infections or enhancing the susceptibility of patients to development of cancers. This will require a more complete understanding of the immune mechanisms involved in the induction and progression of the disease. Therapies must also be developed that specifically inhibit the trafficking of myelin-specific autoreactive T cells to the CNS. Beside inhibiting immune cell function, the regeneration of functional neuronal activity must also be achieved. Therefore, therapies (drugs or use of oligodendroglial and neural progenitor cells) which will promote the repair of myelin and nerve axons damaged by the immune response must be developed. Such strategies will require a much more complete understanding of the complex processes involved in oligodendrocyte progenitor cell growth, differentiation and homing in the adult CNS. Ideally, these therapies will combine antigen-specific immunoregulation with myelin repair and axonal protection strategies.

The molecular regulation of tolerance induction is an emerging area of study in which alterations in intracellular signaling pathways are beginning to be identified. As presented in this review, the regulation of T cell activation appears to be controlled by NFAT through its interaction with AP-1 (158). Besides the positive regulation of transcription when dimerized with AP-1, NFAT also forms homodimers, as well as, complexes with other transcription factors and directly regulates transcription of anergy-associated genes (100). For example, alterations in the molecules that negatively regulate TCR signaling, such as Cbl-b have been shown to be involved in the initiation of autoimmune disease by allowing hyperactive TCR signaling (154,155). A characteristic feature of T cells from Cbl-b^{-/-} mice is a lower threshold of activation following signaling through the TCR resulting in hypersensitivity following TCR engagement, and activation of downstream signaling pathways without the normal requirement for co-receptor stimulation (154). Furthermore, the dysregulation of TCR signaling cascade associated with T cell survival, such as PI3 kinase pathway, is associated with the loss of self-tolerance and the development of autoimmune disease (159).

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Figure 1. Clinical Disease Courses in MS AND EAE

The clinical disease course of MS is classified according to the characteristics and severity of disease progression over time. The most common disease course of MS is **Relapsing Remitting MS (RRMS)**. This disease course is characterized by a defined acute attack (increase in disability), which is followed by a full recovery and subsequent attacks over time. **Secondary Progressive MS (SPMS)** is similar to RRMS, but instead of full recovery during remission, residual deficit is maintained. SPMS is characterized by less recovery during remission following attacks and fewer attacks as the disease course switches from a relapsing remitting disease course to a more progressive disease course. **Primary Progressive MS (PPMS)** is a disease course characterized by a progressive increase in disability over time in the absence of well-defined relapses and/or remissions. **Progressive Relapsing MS (PRMS)** is the least common of the disease courses characterized by a progressive disability from the onset of disease, but contains clear relapses in disease severity in the absence or presence of full recovery. While the initiating antigen/epitope is not known in MS, a number of commonly shared CD4⁺ T cell epitopes have been identified, *i.e.*, MBP₁₃₋₃₂, MBP₈₃₋₉₉, MBP₁₁₁₋₁₂₉, and MBP₁₄₆₋₁₇₀, MOG₁₋₂₀, MBP₃₅₋₅₅, and PLP₁₃₉₋₁₅₄. In contrast, animal models of MS have helped to identify putative mechanisms by which epitope spreading occurs. In R-EAE, the activation of the autoreactive CD4⁺ T cells that are specific for the initiating antigen epitope occurs in the draining lymph node. Also during the acute phase of disease the destruction of myelin allows for the release of both PLP and MBP. Due to antigen availability and CD4⁺ T cell precursor frequency, the activation of the secondary population of CD4⁺ T cell specific for PLP₁₇₈₋₁₉₁ occurs during the primary relapse, *e.g.*, intramolecular spread epitope. In the case of R-EAE the activation of the spread epitope-specific CD4⁺ T cells is

believed to occur within the CNS. During the secondary relapse, CD4⁺ T cells specific for MBP_{84–104} are activated, *e.g.*, intermolecular epitope spreading.

Figure 2. TCR Signal Transduction Pathways Involved in T-cell Activation and Anergy

Signals delivered by the engagement of the T-cell receptor (TCR; Signal 1) and co-stimulatory molecules (CD28; Signal 2) induce different signaling pathways that result in the activation of multiple transcription factors. Ligation of the TCR by peptide–MHC complexes triggers the recruitment of signaling molecules, such as phospholipase C-1 (PLC), which induces the Ca^{2+} influx, and nuclear factor of activated T-cells (NFAT) and protein kinase C- θ (PKC θ) that regulate the nuclear factor- κ B (NF- κ B) and activator protein-1 (AP1) pathway, respectively, which control nuclear transcriptional and gene activation. In the nucleus, NFAT cooperates with AP1 and other transcription factors to induce a program of gene expression, leading to interleukin-2 (IL-2) production. TCR engagement in the absence of co-stimulation results in the induction of NFAT proteins without concomitant AP1 activation. In the absence of cooperative binding to AP1 (FOS and JUN), NFAT regulates the transcription of a distinct set of anergy-inducing genes, such as Casitas B-lineage lymphoma B (CBL-B). Anergy-associated factors inhibit T-cell function at different levels leading to T-cell unresponsiveness.

Figure 3. Putative CD80 Signaling Pathway Involved in IFN- γ Production

Signals delivered by the engagement of the TCR (signal 1) and costimulatory molecules (CD28; signal 2) induce different signaling pathways that result in the activation of multiple transcription factors. Ligation of the TCR by peptide–MHC on an APC triggers the recruitment of the TCR and signaling elements, *e.g.* phospholipase C-1 (PLC-1) for the Ca²⁺ influx–nuclear factor of activated T cells (NFAT) pathway and PKC- for the NF- κ B and AP-1 pathway, which control nuclear transcriptional and gene activation to lipid rafts. To induce differentiation to an IFN- γ producing Th1 cell, STAT4 activation coupled with NFAT activated by the TCR induces the expression of T-bet. In turn T-bet and NFAT induce the expression of IFN- γ . Current findings suggest that inhibition of PI3K, PKC, and PLC (in red) block CD80 induced increase in IFN- γ production. This supports the hypothesis that stimulation via CD80 on CD4⁺ T cells positively regulates effector Th1 function. Cross-linking CD80 on T cells induces an increase in the tyrosine phosphorylation of multiple intracellular proteins, and increases the level of IFN- γ and T-bet transcription. Findings indicate that cross-linking CD80 on CD4⁺ T cells activated in Th1-promoting conditions increases the level of IFN- γ produced by increasing the rate of IFN- γ transcription via the activation of a Ca²⁺-dependent signaling pathway.

Table 1

FDA Approved Treatments for MS and Immunoglobulin-Derived Therapeutics.

Brand Name	Chemical Name	Manufacturer/Distributor	Year of FDA Approval	Indication
Avonex	Interferon β -1a	Biogen Idec	1996	Treatment of relapsing forms of MS (once a week via intramuscular injection)
Rebif	Interferon β -1a	Serono, Inc.	2002	Treatment of relapsing forms of MS (three times a week via subcutaneous injection)
Betaseron	Interferon β -1b	Berlex Laboratories, Inc.	1993	Treatment of relapsing forms of MS (daily via subcutaneous injection)
Copaxone	Glatiramer Acetate	TEVA Neuroscience, Inc.	1996	Treatment of relapsing remitting MS (daily via subcutaneous injection)
Novantrone	Mitoxantrone	Serono, Inc.	2000	Treatment of worsening relapsing remitting, progressive relapsing, and secondary progressive MS (four time a year via intravenous infusion)
Tysabri	Anit-VLA-4 mAb	Biogen Idec and Elan Pharmaceuticals	Withdrawn in Feb. 2005 (currently reapproved by the FDA)	Treatment of relapsing forms of MS (every four weeks via intravenous infusion)
Orencia (abatacept)	CTLA-4 Ig	Bristol-Myers-Squibb	2005	Treatment of rheumatoid arthritis
Rituxan (Rituximab)	Anti-CD20 mAb	Genentech	1997	Treatment of non-Hodgkin's lymphoma, also used to treat rheumatoid arthritis

* The above table is a summary of compiled information by the National Multiple Sclerosis Society, for further information see www.nationalmssociety.org/treatments.asp.