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Therapeutic Blockade of T Cell Antigen Receptor Signal Transduction and Costimulation in Autoimmune Disease

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Abstract

CD4⁺ T cell-mediated autoimmune diseases are initiated and maintained by presentation of self-antigen by antigen-presenting cells (APCs) to self-reactive CD4⁺ T cells. According to the two-signal hypothesis, activation of a naïve antigen-specific CD4⁺ T cell requires stimulation of both the T cell antigen receptor (signal 1) and costimulatory molecules, such as CD28 (signal 2). To date, the majority of therapies for autoimmune disease approved by Food and Drug Administration primarily focus on the global inhibition of immune inflammatory activity. The goal of ongoing research in this field is to develop antigen-specific treatments which allow for the blockade of the deleterious effects of self-reactive immune cell function, while maintaining the ability of the immune system to clear non-self antigens. To this end, the signaling pathways involved in the induction of CD4⁺ T cell anergy, as apposed to activation, are a topic of intense interest. This Chapter will discuss components of CD4⁺ T cell activation pathway that may serve as therapeutic targets for the treatment of autoimmune disease.

Keywords

Antigen-presenting cell; APC; AP-1; autoimmune disease; CD4⁺ T cell; CD80 (B7-1); CD86 (B7-2); CD28; costimulation; cytotoxic T lymphocyte-associated antigen 4; CTLA-4; epitope spreading; experimental autoimmune encephalomyelitis; EAE; immune synapse; linker for activated T cells; LAT; lipid rafts; major histocompatibility complex II; MHC II; mitogen-activated protein kinase; MAPK; multiple sclerosis; MS; nuclear factor of activated T cells; NFAT; peptide-coupled cell; phosphatidylinositol 3-kinase; PI3 kinase; small molecule; T cell antigen receptor; TCR; two-signal hypothesis

Introduction

An important goal of current research is to develop new therapies for autoimmune diseases by specific inhibiting and/or tolerizing self-reactive immune cells. While the present Chapter will focus on the regulation of T cell antigen receptor (TCR) and costimulatory molecule signaling pathways in one particular autoimmune disease, multiple sclerosis (MS) and its mouse model, experimental autoimmune encephalomyelitis (EAE), similar approaches are ongoing in other autoimmune diseases, as well as in tissue transplantation. Approximately 350,000 people in the United States have MS, a T cell-mediated demyelinating disease hypothesized to be triggered by an initiating event, possibly infectious. In these subjects, myelin-specific autoreactive CD4⁺ T cells damage central nervous system (CNS) myelin. MS is characterized

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by perivascular CD4⁺ T cell and mononuclear cell infiltration with subsequent primary demyelination of axonal tracks leading to progressive paralysis¹. While CD4⁺ T cells can discriminate between specific peptide antigens in the context of MHC II in an antigen-specific and MHC II haplotype-restricted manner through use of the TCR², the TCR in and of itself is not intrinsically able to distinguish the difference between self- and 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 (APCs).^{3,4} 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 in the periphery is maintained, in part, by presentation of self-peptides on immature APCs that lack expression of costimulatory molecules resulting in anergy induction in self-reactive CD4⁺ T cells. Costimulation blockade also 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.

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).⁵ There are four courses of clinical disease in MS: 1) relapsing-remitting, 2) secondary progressive, 3) primary progressive, and 4) progressive-relapsing (Fig. 1). Correspondingly, there are relapsing-remitting and chronic mouse EAE models of MS. 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.⁶ Given that the inducing antigen (Ag) in MS has not been identified and that CD4⁺ T cell responses to multiple epitopes on a number of myelin proteins activated via epitope spreading are probably responsible for chronic disease progression, the use of antigen-specific tolerance-based immunotherapies is problematic at this time. Furthermore, in human MS, a pathological role for epitope spreading is difficult to verify because the initiating antigen is not known. In contrast, animal models, such as EAE, have the advantage that the initiating antigen is known. For example, in the SJL model of disease in which mice are primed with PLP₁₃₉₋₁₅₁ in complete Freund's adjuvant (CFA), peripheral PLP₁₃₉₋₁₅₁-specific CD4⁺ T cell reactivity is maintained throughout the disease. However, 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, arise due to intermolecular epitope spreading (Fig. 2). While Ag-specific tolerance can be induced in this experimental model and the self-peptides have been well characterized, this is not true for humans with MS. Therefore, the development of more efficacious and focused antigen non-specific immunosuppressive therapies is currently favored.

T cell activation: target for treatment of disease

As first proposed by Lafferty and Cunningham,⁷ activation of naïve T cells requires two signals. The first signal received by a naïve CD4⁺ T cell comes from the Ag-specific TCR interacting with an antigenic peptide presented in the context of MHC II on the APC surface. The second set of signals includes secretory products, such as cytokines, that are produced by either the APC or the activated CD4⁺ T cell itself, and costimulatory molecules that are expressed on the cell surface of activated APC. For example, CD80 (B7-1) and CD86 (B7-2) expressed on the APC surface interact with the coreceptor CD28 that is constitutively expressed on the surface of CD4⁺ T cells.^{8,9} 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 VLA-4.¹⁰⁻¹² The costimulatory molecule pairs, CD28-CD80/CD86 and CD40-CD40L, and cellular adhesion molecules represent putative therapeutic targets for blockade of autoreactive CD4⁺ T cell activation and trafficking to inflammatory sites. In addition to CD28 costimulation, the production of interferon-gamma (IFN- γ) or interleukin 4 (IL-4) by activated CD4⁺ T cells, or release of IL-12 by activated macrophages, dendritic or B cells, direct the local population of naïve CD4⁺ T cells to differentiate toward the IFN- γ -producing Th1 cell or IL-4-producing Th2 cell phenotypes, respectively.¹² Recently, a third population of CD4⁺ effector T cells that secrete IL-17 has been identified. The Th17 cell secretes IL-17, IL-6, and TNF- α , and is hypothesized to differentiate from a common naïve CD4⁺ T cell precursor cell that has been activated in the presence of TGF- β and IL-6. Furthermore, APC-secreted IL-23 is thought to maintain the survival of Th17 cells *in vivo*.^{13,14} and Th17 cells are critical for the development and maintenance of EAE.^{15,16} 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; or 3) immune deviation.

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 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. While these therapies are still under development, current therapies for MS approved by Food and Drug Administration (FDA) focus on immune deviation or non-specific immunosuppression. For example, 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 of various lengths composed of glutamine, lysine, alanine and tyrosine. This mixture 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). Treatment with Copaxone is hypothesized to induce a low level of TCR stimulation, thus inducing immune deviation toward a Th2 phenotype (disease-regulatory) as compared to Th1/Th17 phenotypes (disease-promoting). In an attempt to further test the two-signal hypothesis, several groups have investigated the therapeutic potential of anti-CD3 monoclonal antibody (mAb) treatment of various autoimmune diseases. However, treatment with an unaltered anti-CD3 mAb is potentially a double-edged sword such that 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 . Furthermore, cross-linking of CD3 may in some cases initiate 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 associated with the use of an unaltered anti-CD3 mAb, modifications to the anti-CD3 mAb have been made so that the deleterious side effects are avoided by reducing/eliminating the antibody ability to bind to Fc receptors and thus

decreasing the ability to efficiently crosslink 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, since the non-mitogenic anti-CD3 mAb is not able to be stabilized by Fc receptors on the surface of the APCs to allow for efficient TCR cross-linking. In this manner non-mitogenic anti-CD3 mAb is hypothesized to favor T cell differentiation into a Th2 cell phenotype and the development of regulatory T cells (Treg).^{17,18} Therefore, the possibility exists that treatments induce immune deviation. In this scenario, the T cell-mediated immune response is changed from a Th1/Th17-like (disease-promoting) response, to a Th2-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.^{19,20} 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.²¹ 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 has been compared in Th1 and Th2 cells revealing that tolerance is induced in Th1 cells as determined by proliferation and IL-2 production.²² In contrast, no effect on the Th2 cell phenotype and activity was seen. These findings lend support to the theory that non-mitogenic anti-CD3 mAb may specifically downregulate Th1 cell function. 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 by which only activated immune cells are affected at the time of treatment, thus allowing the maintenance of host defense against non-self-antigens at times post non-mitogenic anti-CD3 treatment.

Besides the administration of antigens in a tolerogenic form for the treatment of autoimmune diseases in humans,²³ adhesion molecule and costimulatory molecule blockade are currently being tested. For example, the use of Tysabri, a monoclonal antibody able 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. Clinical trials are also ongoing to study the therapeutic effect of CD28-CD80/CD86 blockade by the use of the extracellular portion of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) covalently linked to the Fc portion of an immunoglobulin molecule (CTLA-4-Ig). 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.²⁴ Thus, conflicting results have been obtained using anti-CD80 and anti-CD86 mAbs to regulate autoimmune disease. Treatment with anti-CD80 mAb surrounding autoantigen priming has been shown to block EAE development induced with suboptimal concentrations of PLP₁₃₉₋₁₅₁ or MBP₈₄₋₁₀₄ in SJL mice, whereas anti-CD86 mAb treatment has been reported to either exacerbate disease²⁵ or have no effect.²⁶ 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.²⁷ Treatment with monovalent, non-cross-linking anti-CD80 Fab fragments during EAE remission blocks clinical relapses and epitope spreading to the PLP₁₇₈₋₁₉₁ epitope,²⁷ whereas treatment with intact anti-CD80 mAb leads to a profound exacerbation of disease relapses concomitant with accelerated epitope spreading.²⁸ Likewise, treatment of mice with a small molecule inhibitor of CD28 during disease decreases disease severity and proliferation of myelin-specific CD4⁺ T cells upon *ex vivo* activation and increases CD4⁺ T cell apoptosis.²⁹ Thus, short-term blockade of CD28-CD80 interactions may represent a therapy which would predominantly target activated T cells during the treatment period, thus allowing maintenance of host defense against infection.

Coupled-cell tolerance: antigen-specific induction of tolerance to a self-antigen

While the therapeutic treatments mentioned above are antigen non-specific, a variety of current autoimmune therapies uses antigen-specific approaches and is currently under development. For example, the intravenous injection of ethylene carbodiimide (ECDI)-antigen-coupled splenocytes (Ag-SP) is an efficient method of promoting clonal anergy of antigen-specific CD4⁺ T cells both *in vivo* and *in vitro*.^{30–33} Ag-SP promotes T cell tolerance in many animal models of autoimmune and inflammatory disease including experimental autoimmune thyroiditis,³⁴ uveitis,³⁵ and neuritis,³⁶ as well as the non-obese diabetic (NOD) mouse model of diabetes (Kohm, AP, Miller SD, unpublished observations) and transplant survival.³⁷ 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.^{33,38–40} Ag-SP also appears to be nontoxic and well tolerated by treated animals at all stages of disease. In contrast, i.v. tolerance induced by intravenous administration of soluble peptides can induce severe anaphylactic responses and depending on the antigen result in death of treated animals.^{41,42} Since Ag-SP can induce long-lasting Ag-specific tolerance in CD4⁺ T cells in the absence of any negative side effects, Ag-SP possesses significant therapeutic potential for the future autoimmune therapy.

The mechanism of Ag-SP-induced self-tolerance is not completely understood. 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. ECDI fixes antigen to the cells via 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).^{43,44} 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.³² For example, lipopolysaccharide (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.^{43,44} CTLA-4 ligation during secondary antigen encounter also appears to be important for the maintenance of the tolerized state.^{43,44}

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.^{45–47} Ag-SP is also effective when multiple encephalitogenic peptides are coupled to cells allowing the simultaneous targeting of multiple myelin-associated antigens⁴⁸ and effectively blocking possible spread epitopes (Fig. 1 and 2). 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.⁴⁹ On the other hand, the antigen must be physically attached to the donor cells for tolerance induction to occur. Donor spleen cells from MHC II-deficient mice that are ECDI-coupled to myelin peptides are able to ameliorate clinical disease⁵⁰ but twice the number of MHC II-lacking Ag-SP donor cells is required to induce the level of protection equivalent to that of syngeneic derived donor cells. Tolerance in this case is hypothesized to occur through the reprocessing of the donor-coupled cells by host APCs that are then able to represent antigen to host T cells.⁵⁰ 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 tolerance induced by intravenous injection of soluble peptides. In light of these findings, the use of peptide-coupled APCs holds therapeutic promise as a potential therapy for MS and other autoimmune diseases.

Immune synapse: activation of the TCR in lipid rafts

As mentioned above, CD4⁺ T cells respond to their environment by the use of a multichain immune recognition receptor (MIRR), *i.e.*, the peptide-specific, MHC II-restricted TCR. While TCR-mediated recognition of peptide bound to the MHC II molecules on the surface of the APC is necessary for T cell activation (signal 1), the cytoplasmic tails of the TCR alpha/beta chains do not have inherent kinase activity. Signaling through the TCR is achieved through the associated accessory proteins that contain immunoreceptor tyrosine-based activation motifs (ITAMs). Following cross-linking of the TCR, the intracellular signaling cascade is initiated by phosphorylation of ITAM tyrosines by Src family kinases. 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 platforms for the interaction (Fig. 3).⁵¹ Due to the biochemical properties of cholesterol and sphingolipids, the lipids are tightly packed together, including specific membrane-associated proteins and 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.⁵² 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.^{53, 54} A list of the TCR signaling components that associate with lipid rafts is presented in Table 1. Another transmembrane protein involved in CD4⁺ T cell activation is LAT, which is palmitoylated upon TCR cross-linking.⁵⁵ LAT mutants that cannot be palmitoylated, are not able to associate with lipid rafts, thereby altering TCR signaling.⁵⁵ 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. The immune synapse is a highly ordered membrane structure in which the TCR, associated signaling proteins, cytoskeleton, and cellular adhesion molecules are concentrated to allow for sufficient intercellular protein-protein interactions.⁵⁶ The TCR-APC immune synapse is a dynamic structure, containing a central cluster of TCRs ringed by adhesion molecules. On the cytoplasmic side, it contains signaling molecules, such as Src family kinases and protein kinase C, and the integrin-associated cytoskeleton proteins including talin.^{57,58} 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.⁵⁶ Stimulation of CD28 has also been shown to enhance the recruitment of lipid rafts to the immune synapse.⁵⁹ In this manner the organization of the T cell plasma membrane during T cell-APC interaction not only contributes to the inclusion of the necessary signaling molecules but also allows for sufficient and sustained TCR signaling.

NFAT: Regulation of T cell activation and anergy

The previous sections were focused on defining the known immune mechanisms involved in an autoimmune disease and pointed out possible therapeutic targets. The following sections will discuss the signaling pathways that may play a putative role in the induction of self-reactive CD4⁺ T cell anergy. 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.⁶⁰ Anergy has also been defined as an unresponsive state, which is reversible by IL-2.^{61–63} By this definition, therefore, one can conclude that an anergic CD4⁺ T cell has been previously activated to the extent that it expresses the high-affinity IL-2 receptor and that the anergic T cell is unresponsive, but not non-viable. A critical point is that anergy is only a relative measure of the immune response. For example, although substantial decreases in responsiveness can be achieved *in vitro*, that level of responsiveness may cause significant effects *in vivo*.

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.⁶⁰ 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 Ca²⁺ 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.⁶⁴ 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.⁶⁵ 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 (Fig. 3). In support of this, the increased tyrosine phosphorylation of Vav in Cbl-b^{−/−} T cells and the enhanced T cell proliferation and IL-2 production have been shown to be reversed by PI3 kinase inhibitors.⁶⁶ While controversy still exists as to whether or not PI3 kinase plays a crucial role in T cell activation, particularly in CD28-mediated signaling,⁶⁷ 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.⁶⁸ Therefore, the induction of CD4⁺ T cell tolerance is suggested to be regulated by altered NFAT transcriptional activity leading to expression of anergy- rather than activation-associated, genes.

Of the multiple signaling pathways that are upregulated during T cell activation, Ca²⁺ 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 Ca²⁺ influx predominates. While CD28 ligation is not directly coupled to Ca²⁺ mobilization, CD28 signaling potentiates TCR signals that do not involve Ca²⁺ influx. Experimentally, this is shown by the fact that anergy induced in CD4⁺ T cells activated with Ca²⁺ ionophores is closely related to that induced in the absence of CD28 costimulation following TCR stimulation. As mentioned above, Ca²⁺-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 the calcineurin inhibitors, such as cyclosporin A (CsA).⁶⁹ NFAT was initially identified as an inducible nuclear factor that could bind the IL-2 promoter in activated T cells.⁷⁰ However, when all of the proteins of the known NFAT family were isolated and characterized, it became clear that their expression is not limited to T cells (Table 2). At least

one NFAT family member is expressed by almost every cell type that has been examined. The NFAT family consists of five members: NFAT1 (also known as NFATp or NFATc2), NFAT2 (also known as NFATc or NFATc1), NFAT3 (also known as NFATc4), NFAT4 (also known as NFATx or NFATc3), and NFAT5 (also known as TonEBP or OREBP). All NFAT proteins have a highly conserved DNA-binding domain that is structurally related to the DNA-binding domain of the REL family of transcription factors. Four of the NFAT family members are regulated by Ca^{2+} signaling, while NFAT1, 2, and 4 are the predominate family members expressed in CD4^+ T cells. Since Ca^{2+} is activated following TCR stimulation, the NFAT family members are activated within a CD4^+ T cell, but the outcome of NFAT activation depends on whether or not the CD4^+ T cell receives costimulatory signals.

The molecular regulation of tolerance induction is an emerging area of study in which the complexity of intracellular signaling is beginning to be identified. The regulation of T cell activation is regulated by NFAT through its interaction with AP-1.⁷¹ The discovery that NFAT proteins can also form transcriptional complexes with other partners and even be transcriptionally active by themselves, has introduced the possibility of defining new roles for NFAT proteins in T cells.⁶⁵ In the two-signal hypothesis for T cell activation, stimulation of the TCR (signal 1) and costimulatory molecule stimulation, *i.e.*, CD28-CD80/CD86 (signal 2), are both 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 (Fig. 3). 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 inhibitor of NF- κ B (I κ B) kinases (IKK) signaling.⁷² To illustrate the dependence of anergy-associated gene expression on NFAT, treatment of *Nfat1*^{-/-} T cells and wildtype CD4^+ T cells with the calcineurin inhibitor, CsA, inhibits the activation of NFAT, blocks the expression of these anergy associated genes and impairs induction of anergy in treated cells.⁷² Since NFAT proteins control two opposing aspects of T cell function, activation and 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, there is a group of E3 ubiquitin ligases, *i.e.*, Itch, Cbl-b, and GRAIL.^{65,73–75} 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.^{76,77} For example, the loss of Cbl-b expression allows for the hyper-reactive signaling through the TCR. Interestingly, T cells from Cbl-b^{-/-} mice are characterized by a lower threshold of activation following TCR-mediated signaling that results in hypersensitivity upon TCR engagement and activation of downstream signaling pathways without the normal requirement for coreceptor stimulation.⁷⁶ These two signaling pathway intermediates represent two putative candidates for mechanism by which Ag-SP-induced tolerance of CD4^+ T cells occurs.

NFAT inhibitors: putative therapeutics

Given the important role of NFAT proteins in the control of T cell activation, NFAT is considered to be an optimal target for therapeutic approaches aimed at regulating T cell-mediated immune responses. For example, inhibitors of calcineurin, such as CsA and FK506, block the downstream activation of NFAT. These compounds are extensively used as immunosuppressive agents.⁷⁸ While the mechanism of action of these inhibitors is through the blockade of calcineurin and the inhibition of NFAT activation, they are not specific for NFAT. Thus, the caveat exists that non-NFAT-associated effects on T cell function are involved in regulating T cell function.⁷⁹ To test this possibility, several studies have begun to identify the

protein-protein interaction site between NFAT and calcineurin. By developing an inhibitory peptide that blocks the interaction between NFAT and calcineurin, the specificity of the inhibitor can be significantly increased. For example, the interaction of NFAT proteins with calcineurin is mapped to their N-terminal regulatory domain, allowing for the development of a more specific NFAT inhibitor, *i.e.*, a high affinity calcineurin-binding peptide, MAGHPVIVITGPHEE.^{80,81} A cell-permeable version of the VIVIT peptide, which is able to selectively inhibit calcineurin-mediated NFAT dephosphorylation,⁸¹ has been successfully used to prolong graft survival in an experimental system of islet-cell transplantation in mice.⁸² While these peptides are much more selective than CsA, they maintain the ability to inhibit the interaction of calcineurin with other substrates that use similar PXIXIT motifs to interact with calcineurin, *e.g.* calcineurin-binding protein 1 (CABIN1) or A-kinase anchor protein (AKAP79).⁸³ Recently, two additional regions of calcineurin have been found to be necessary for NFAT binding.^{83,84} Therefore, it has been hypothesized that the amino acids flanking the PXIXIT motif may provide specific targets for future therapeutics.

The therapeutic use of peptide inhibitors is still limited by problems associated with route of administration and half-life/stability of the inhibitor *in vivo*. As an alternative, the use of small organic molecules may overcome the peptide inhibitor limitations, since limitless structural changes can be designed to improve the specificity, stability, delivery and distribution of these molecules. Recently, several small organic molecules were identified that specifically inhibit calcineurin-induced NFAT activation, blocking NFAT-dependent cytokine production by T cells.⁸⁵ While the current small molecule inhibitors are efficient at blocking NFAT-dependent transcription and able to potentiate CsA effects, these molecules still act upstream of calcineurin.⁸⁶ Therefore, for these agents, the same non-specific side effects as known for 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.

LAT: an Alternative component of TCR signaling

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 LAT is a transmembrane protein that facilitates the formation of a multisubunit signaling complex with other signaling molecules, such as phospholipase C γ 1, Gads-SLP-76, Grb2, and PI3 kinase⁸⁷ (Fig. 3). LAT is essential for TCR signaling, and upon TCR stimulation, phosphorylation of LAT is necessary for activation of MAPK cascades, Ca²⁺ flux, and activation of the transcription factor AP-1. 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 pathways are coordinated 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.^{65,73} Although Cbl-b is upregulated at both the mRNA and protein levels 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 the 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 ζ and ZAP-70 is normal in anergized T cells, the adaptor protein LAT and its downstream target, PLC γ 1, are hypophosphorylated. The kinetics of both LAT and ZAP-70 activation is also decreased in anergic T cells due to decreased recruitment of the p85 regulatory subunit of PI (3)K by LAT.⁸⁸ Interestingly, normal activation of the CD28 pathway was noted in ionomycin-anergized T cells upon restimulation, demonstrating that the costimulatory cascade, which itself contributes to LAT phosphorylation, is unaltered. Inhibition of LAT activation may thus serve as a viable target for the induction of CD4⁺ T cell anergy.

As discussed previously, a critical step in the activation of a naïve CD4⁺ T cell is the costimulatory signal provided by the APC, where duration and strength of signal parameters help determine the outcome of the T cell-APC interaction.⁸⁹ It has been demonstrated that although the number of conjugates between T cells and APCs is not altered, the percentage of synapses that contains LAT is markedly decreased in ionomycin-anergized T cells.⁸⁸ These results are consistent with previous work suggesting that ionomycin-anergized T cells form unstable immune synapses. In order for LAT to be phosphorylated by active ZAP-70, it must first be palmitoylated to facilitate trafficking to lipid rafts. In stimulated cells pretreated with ionomycin, there is diminished LAT localization to lipid rafts in addition to the lack of LAT phosphorylation compared to control cells stimulated without ionomycin treatment (Fig. 3). Because CD4 and Fyn localize to lipid rafts in both sets of stimulated cells, this argues that there is not a global defect in the constituents of lipid rafts. Based on these findings, many intriguing questions for future investigations into the control of T cell activation can be formulated. LAT is located at the crucial juncture between recognition of an incoming signal from the TCR and its dissemination into multiple intracellular signaling cascades and second messengers, and attenuation of LAT localization and phosphorylation severely cripples T cell activation.

Conclusions

It is clear that the T cell repertoire in an autoimmune response, such as peptide-induced relapsing-remitting EAE, is dynamic and CD4⁺ T cell responses to the initiating epitope play the dominant pathologic role during the acute disease episode but not in disease relapses. Understanding of the mechanisms underlying spontaneous disease remission is critical to the ultimate design of therapeutic modalities. Current therapies for the re-establishment of self-tolerance in autoimmune disease focus on the inhibition of signal 1 and/or signal 2. For example, blockade and/or provision of subthreshold levels of signal 1 in an antigen-specific therapy includes ECDI-antigen-coupled APC treatment and treatment by non-mitogenic anti-CD3 mAb to induce non-specific signaling in activated T cells. Blockade of signal 2 using CTLA-4-Ig to block CD28-CD80/CD86 interactions is currently used in the ongoing clinical trials. 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 Chapter, the regulation of T cell activation appears to be controlled by NFAT through its interaction with AP-1.⁷¹ Besides the positive regulation of transcription when dimerized with AP-1, NFAT also forms homodimers and complexes with other transcription factors, directly regulating transcription of anergy-associated genes.⁶⁵ 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 for hyperactive TCR signaling.^{76,77} A characteristic feature of T cells from Cbl-b^{-/-} mice is a lower threshold of the TCR-mediated activation, resulting in hypersensitivity following TCR engagement, and activation of downstream signaling pathways without the normal requirement for coreceptor stimulation.⁷⁶ 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.⁹⁰ This Chapter has illustrated that three potential points of intervention exist for the induction of CD4⁺ T cell anergy and may serve as a potential therapeutic targets for regulation of autoimmune disease: 1) increasing the frequency of NFAT/NFAT homodimers as opposed to NFAT/AP-1 heterodimers; 2) increasing anergy-associated signaling pathway intermediates, such as Cbl-B and GRIL; and 3) downregulation of the TCR signaling complex components, e.g. LAT.

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Figure 1. Clinical disease courses in multiple sclerosis (MS) and its mouse model, experimental autoimmune encephalomyelitis (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. PRMS contains clear relapses in disease severity in the absence or presence of full recovery. In the SJL mouse, relapsing-remitting model of MS, *i.e.*, R-EAE, the dominant spread epitope for each consecutive relapse is well characterized. During the acute phase of the disease the majority of activated CD4⁺ T cells are specific for the dominant proteolipid protein epitope, PLP₁₃₉₋₁₅₁, used to induce disease. The dominant epitope during the primary relapse is PLP₁₇₈₋₁₉₁ (intramolecular epitope spreading) and during the secondary relapse this epitope is MBP₈₄₋₁₀₄ (intermolecular epitope spreading). In contrast, epitope spreading in the chronic disease model has been suggested, but the consecutive dominant epitopes have not been identified.

Figure 2. Epitope spreading

Animal models of multiple sclerosis (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 (blue CD4⁺ T cell) occurs in the draining lymph node. Upon activation, the activated CD4⁺ T cells enter the circulation and extravasate into the central nervous system (CNS). Once in the CNS, the autoreactive CD4⁺ T cells initiate myelin destruction and activate resident and infiltrating APCs. The activated infiltrating immune cells secrete cytokines and chemokines that not only recruit immune cells into the CNS but also help to open the blood-brain barrier (BBB). Myelin antigens not only reactivate the CD4⁺ T cells that are specific for the initiating antigen, but are also released, phagocytized,

processed, and presented by APCs to CD4⁺ T cells. For example, in PLP₁₃₉₋₁₅₁-induced R-EAE in SJL mice, the initiating epitope is PLP₁₃₉₋₁₅₁, and this population of CD4⁺ T cells is responsible for the initial acute phase of the disease. During the acute phase of the disease, the destruction of myelin allows for the release of both PLP and MBP. Due to antigen availability, the activation of the secondary population of CD4⁺ T cells specific for PLP₁₇₈₋₁₉₁ (red CD4⁺ T cell) occurs prior to the primary relapse, *e.g.*, intramolecular spread epitope. In the case of R-EAE, the activation of the spread epitope-specific CD4⁺ T cells has been shown to occur within the CNS.⁹¹ During the secondary relapse, CD4⁺ T cells specific for MBP₈₄₋₁₀₄ are activated, *e.g.*, intermolecular epitope spreading.

Figure 3. Signal transduction pathways involved in T cell anergy

Signals delivered by the engagement of the TCR (signal 1) and costimulatory molecules, such as CD28, (signal 2) induce different signaling pathways that result in the activation of multiple transcription factors. Prior to crosslinking of the TCR, LAT and CD28 are located outside of lipid rafts (red portion of the lipid bilayer). 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 Ca^{2+} influx–nuclear factor of activated T cells (NFAT) pathway and PKC- θ for the NF- κB and AP-1 pathway, which control nuclear transcriptional and gene activation to the lipid rafts. Prior to crosslinking the TCR, the necessary components for TCR signaling are distributed throughout the T cell surface. Following crosslinking, the TCR and its accessory signaling proteins are recruited to lipid rafts. As a consequence, the TCR is a central component of the immune synapse in which sufficient TCR stimulation (signal 1) and costimulatory molecule stimulation (signal 2) occur. In the nucleus, NFAT cooperates with AP-1 and other transcription factors to induce a program of gene expression leading to IL-2 production. TCR engagement (signal 1) in the absence of costimulation (signal 2) results in induction NFAT proteins without concomitant AP-1 activation. In the absence of cooperative binding to AP-1 (FOS and JUN), NFAT transcriptionally regulates a distinct set of anergy-inducing genes, *e.g.* *Cbl-b*. Anergy-associated factors inhibit T cell function at different levels leading to a state of T cell unresponsiveness.

Table 1

Lipid raft-associated components of the TCR signaling complex

TCR-associated signaling molecules included in lipid rafts before TCR crosslinking
Lck
Fyn
Itk
Syk
Ras
Cbl-b
CD4
Actin
TCR-associated signaling molecules included in lipid rafts after TCR crosslinking
Zap-70
Slp-76
Vav
Grd-2
PLC- γ 1
PKC
LAT
TCR
TCR-associated signaling molecules excluded from lipid rafts after TCR crosslinking
CD45

Table 2

NFAT family members

NFAT Family Member	Alternative names	Regulation	Expression	Immune phenotype of knockout mice	Reference
NFAT1	NFATc2, NFATp	Ca ²⁺ /calcineurin	T cells; vascular endothelial cells; skeletal muscle cells; chondrocytes; adipocytes; pancreatic islet cells	<ul style="list-style-type: none"> Enhanced B- and T-cell responses; Th2 skewing with decreased IFN-γ production and increased IL-4 expression; Allergic responses; Suppression of chondrogenesis 	71,72,92-95
NFAT2	NFATc, NFATc1	Ca ²⁺ /calcineurin	T cells; cardiac muscle cells, skeletal muscle cells	<ul style="list-style-type: none"> Embryonic lethal due to defect in cardiac valve; in RAG complementation system: impaired Th2 cell responses and IL-4 production 	71,72,92-95
NFAT3	NFATc4	Ca ²⁺ /calcineurin	Perivascular tissue cells; adipocytes; cardiac muscle cells	Not reported yet	71,72,92-95
NFAT4	NFATc3, NFATx	Ca ²⁺ /calcineurin	T cells; skeletal muscle cells; keratinocytes	<ul style="list-style-type: none"> Decreased number of single-positive CD4 and CD8 cells due to increased apoptosis of double positive thymocytes 	71,72,92-95
NFAT5	TonEBP; OREBP	Osmotic stress	T cells; most cell types	<ul style="list-style-type: none"> Decreased cellularity in thymus and spleen; Impaired T cell function in hyperosmotic conditions. 	71,93