T-CELL SIGNALING IN RESPONSE TO ALTERED MYELIN BASIC PROTEIN PEPTIDES

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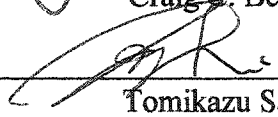

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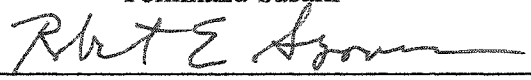

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Abstract

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Experimental autoimmune encephalomyelitis (EAE) can be induced in H-2k mice with the N-terminal peptide of myelin basic protein (MBP). Helper T-cells type 1 (T_H1) specific to the MBP Ac(1-11) peptide mediate the autoimmune response and it has been previously shown that the progression of EAE can be inhibited by altered peptide ligands (APLs) that apparently target these T cells. However, there have not been any strong correlations between the *in vitro* reactivity of a given APL and its efficacy in blocking disease. We hypothesized that if a large number of APLs are evaluated, they will segregate into subsets defined by their activity as measured by different assays for *in vitro* T-cell activation. We suggest that those peptides with therapeutic efficacy will be primarily contained within one subset. Computer models

were used to design altered MBP Ac(1-11) peptides that incorporated both natural and non-natural amino acids. The peptides were screened with various cell-based assays against a panel of monoclonal MBP Ac(1-11)-specific T-cell clones derived from B10.A mice that had been primed with MBP protein. A number of peptides with antagonist or partial agonist activities were identified. A hierarchy of activities was defined based on the ability of a peptide to induce early signaling, proliferation, cytokine production, and antagonism. In addition, a more extensive study of responses by one of the T-cell clones showed a hierarchy of T-cell *effector* functions. Among the peptides identified as antagonists, the most potent also demonstrated partial agonist activities. The *in vitro* reactivity of the altered peptides within this group is similar to the activities of peptides previously shown to be effective in blocking EAE.

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LIST OF ABBREVIATIONS

ABC Drugs.	Avonex, Betaseron, and Copaxone
Ac.	Acylated
APC.	Antigen-Presenting Cell
APL.	Altered Peptide Ligand
CD.	Clusters of Differentiation
CFA.	Complete Freund's Adjuvant
CNS.	Central Nervous System
CTL.	Cytotoxic T Lymphocyte
CTLA-4.	Cytotoxic T Lymphocyte-associated Antigen-4
EAE.	Experimental Autoimmune Encephalomyelitis
FMOC.	Flourenylmethoxycarbonyl
GAD.	Glutamic Acid Decarboxylase
Hb.	Hemoglobin
HEL.	Hen Egg white Lysozyme
HIV.	Human Immunodeficiency Virus
HPLC.	High Performance Liquid Chromatography
ICAM.	Intercellular Adhesion Molecules
IDDM.	Insulin-Dependent Diabetes Mellitus
IFA.	Incomplete Freund's Adjuvant
IL.	Interleukin

INF.	Interferon
ITAM.	Immunoreceptor Tyrosine-based Activation Motif
k_a or k_{on}.	Association (On)-rate
k_d or k_{off}.	Dissociation (Off)-rate
K_{eq}.	Equilibrium Constant
L.	Ligand
LFA.	Lymphocyte Function-associated Antigen
MAPK.	Mitogen Activating Protein Kinase
MBP.	Myelin Basic Protein
MHC.	Major Histocompatibility Complex
MOG.	Myelin Oligodendrocyte Glycoprotein
MS.	Multiple Sclerosis
NFAT.	Nuclear Factor of Activated T cells
NMP.	N-Methyl-2-pyrrolidinone
NOD mice.	Non-Obese Diabetic mice
PLP.	Proteolipid Protein
R.	Receptor
TCR.	T-Cell Receptor
TFA.	Triflouroacetic Acid
TGF-β.	Transforming Growth Factor - Beta
T_H1, 2, or 3.	Helper T Cell Type 1, 2, or 3
T_r.	Regulatory T Cell

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DEDICATION

I wish to dedicate this thesis to my grandmother, Dorothy Shearer (October 1905 to September 1998), who passed away as I was just beginning my second year in graduate school. Her love of life and determination to pursue her passions was and always will be my inspiration. She taught me to shoot for the stars, and although she is gone, I look to the sky and know that she is smiling down on me.

Chapter 1: Introduction

A Tale of Two Signals: A Journey Into Our Complex Immune System

PROLOGUE

The immune system is composed of a complex series of cells and molecules that exist for the sole purpose of protecting the body from foreign pathogens. However, like any complex system, problems and tribulations arise that can overcome even the strongest of systems. Devastating diseases like cancer and autoimmune diseases occur due to problems arising within this extremely intricate system. The most common autoimmune disease of the central nervous system (CNS) is Multiple Sclerosis (MS), which afflicts about 300,000 individuals in the United States and about one million worldwide (1,2,3,4). The cause of this disease is unknown, but several treatments exist today that help patients cope with the disease and reduce the relapse of symptoms by less than 50% (5). Nevertheless, even though discoveries have been made to improve MS patients' standard of living, a "cure" is the ultimate goal. By investigating the various molecules and participants in the immune system, the mysteries surrounding the etiology of MS can start to become unraveled and, hopefully, answers can be found.

THE JOURNEY BEGINS.....

Act I: The Immune System

The immune system consists of a wide variety of cells and molecules that are capable of eliminating foreign pathogens (6,7). Generation of an effective cellular immune response involves two major groups of cells: lymphocytes and antigen-presenting cells (APCs). T lymphocytes begin as progenitor cells formed during hematopoiesis that enter the thymus gland as immature thymocytes. Thymocytes that originally have both CD4 and CD8 surface molecules and express the αβ T-cell receptor (TCR)-CD3 complex develop into either CD4+ or CD8+ lymphocytes (6,7). This differentiation is dependent on their avidity for self-peptides bound to proteins of the major histocompatibility complex (MHC) (class I or class II) in the thymus. If the thymocyte interacts weakly with a self-peptide/MHC complex, it is positively selected (8). If the thymocyte interacts strongly with the self-peptide/MHC complex, it is negatively selected and induced to undergo apoptosis (programmed cell death) (8). The positively selected T cells differentiate into CD4+ helper T cells if they were selected with a peptide/MHC class II complex or CD8+ cytotoxic T cells if they interact with a peptide/MHC class I complex. The fully developed naive T cells then enter the periphery where they remain in a resting state until they encounter an antigen-peptide/MHC complex. For CD4+ helper T cells, the peptide/MHC class II complex is normally presented on the surface of specialized antigen-presenting cells such as macrophages or dendritic cells (Figure 1.1). In contrast, CD8+ cytotoxic T cells react with peptide/MHC class I complexes presented on all cells. Upon

recognition of the T cell's cognate antigen, a cascade of signaling events that lead to activation will occur. Early signaling events include zeta (ζ)-chain phosphorylation, intracellular calcium flux, and extracellular acid release. Late markers include cytokine production and T-cell proliferation (9). The late markers are used to define when a T cell has been activated (Figure 1.1). Activated cytotoxic T cells kill altered self-cells (*i.e.*, virus-infected cells) and activated helper T cells secrete cytokines that regulate the activity of other cells in the immune response.

Act II: Helper T-Cell Activation and Signaling

In order for helper T cells to become activated, the antigen must be presented in the context of the MHC class II protein. The structural basis of TCR recognition of peptide-MHC class II complexes is only partly understood. Crystal structures of peptide-MHC class II complexes reveal that peptides lie within an MHC protein groove, anchored by a series of hydrogen bonds between the peptide backbone and the MHC (10,11,12,13). Peptide side chains, also, interact with MHC protein residues (Figure 1.2). Bound peptide has a polyproline twist conformation within the groove of the MHC. Some of the peptide side chains are anchored within pockets of the MHC where small polymorphic changes have substantial effects on binding of various amino acid side chains in the pocket (14). Other side chains protrude out of the groove and are available to the TCR.

When the TCR comes into contact with the peptide/MHC complex, a series of signaling events occur leading to transcription of second messengers and other

proteins important in controlling the immune system (Figure 1.3). Initially, adhesion proteins like ICAM molecules on APCs bind to their ligands like LFA-1 on T-cells to generate a nanometer scale interface between a T cell and the APC that is referred to as the immunological synapse (15). The CD4+ T-cell receptors screen the peptide/MHC class II complexes, and if recognition occurs, this initial low affinity interaction becomes a high affinity interaction (16). This high affinity interaction causes signaling through the TCR/CD3 complex by some as of yet unknown mechanism. Since the TCR itself has a short cytoplasmic domain inside the cell, it requires the accessory chains; CD3 γ, δ, ε; and the large cytoplasmic homodimer, CD3-associated ζ-chains in order to propagate signals to the nucleus (reviewed in ref. 6,7). The CD3-associated-ζ-chains have a total of six immunoreceptor tyrosine-based activation motifs (ITAMS), and the kinases, Lck and Fyn, phosphorylate these tyrosines upon receptor activation. The other CD3 chains have one ITAM each. These ITAMS also become phosphorylated by the protein tyrosine kinases, Lck and Fyn (17). Lck is associated with the CD4 co-receptor, which is thought to stabilize the tri-molecular complex (TCR/peptide/MHC). Once the ITAMS are phosphorylated, another tyrosine kinase, ZAP-70, binds to two phosphotyrosines on the CD3-ζ-chain (18,19), and is itself activated by Lck.

Once phosphorylation occurs, the signal at the cell membrane can be propagated to the nucleus by several different pathways (6,7,22). ZAP-70 activates the phospholipase C-γ pathway, which eventually results in an increase in calcium within the cell and activation of the transcription factors, NFκB and NFAT (20).

Another major pathway triggered is the mitogen activating protein kinase (MAPK) cascade leading to more transcription factors, like Fos and Jun (20). These transcription factors lead to gene transcription in the nucleus resulting in proliferation and effector functions (Figure 1.3). These activated cells are effector T cells. After the pathogen is removed from the immune system, a subset of these effector T cells will remain as memory T cells, which are more easily activated the next time the same pathogen enters the body.

Other receptor signals are also necessary for activation of the naive T-cell (6,7), which has not encountered its specific antigen in the periphery. This "second-signal" is achieved by ligation of the constitutively expressed CD28 co-receptor on T cells to upregulated B7 molecules on activated APCs (Figure 1.3). Effector and memory T cells are less dependent on costimulatory signals to become activated. When the T-cell becomes activated, another co-receptor is upregulated, cytotoxic T lymphocyte-associated antigen-4 (CTLA-4). This receptor binds to CD28 with a higher affinity and sends an inhibitory signal that can limit the proliferative response of the T cells to antigen and B7 molecules. Without CTLA-4, mice die from a fatal disorder due to overproliferation of lymphocytes (21). CTLA-4 is an important immunoregulatory co-receptor on T cells (22), and it plays a role in autoimmunity (23). Other inhibitory mechanisms are present such as phosphatases that help regulate the activation of the T cell by turning off activated receptors.

When complete T-cell proliferation and cytokine production occurs, then the ligand (peptide/MHC) is called an agonist ligand. However, it has been shown by

many studies that small modifications in the peptide/MHC complex can alter the region that is recognized by the TCR. In fact, T cells can have substantial changes in their functional response due to small structural changes in their ligand (24). A small change in the peptide's amino acid sequence, especially at TCR contact residues, has been shown to block proliferation or give an intermediate or incomplete response. Peptides that do not induce proliferation but can block agonist stimulation are called antagonists. Peptides that induce some proliferation or cytokine production are called partial agonists (24). One can imagine that these altered peptide ligands (APLs) that block an agonist peptide could potentially be used as immunotherapeutic drugs to block the body's attack of itself in an autoimmune disease.

Act III: Helper T-cell differentiation

Research in functional T-cell responses has grown exponentially over the last decade. In the mid 1980's, Mosmann and coworkers first reported that cloned murine helper (CD4+) T lymphocytes could be divided into two functional subsets on the basis of the immunoregulatory cytokines that these clones produced (25). Naive T cells differentiate into effector cells upon activation. These effector cells can become either T_H1 or T_H2 cells depending on the cytokines present (Figure 1.4). T_H1 cells develop in the presence of Interleukin (IL)-12 and Interferon (INF)-γ during infections by such pathogens as intracellular bacteria and viruses. They produce cytokines like INF-γ, IL-2, and Tumor Necrosis Factor (TNF)-β, which can activate macrophages and induce cell-mediated immunity. T_H2 cells differentiate in the presence of IL-4,

which occurs during infestations with complex parasites (*i.e.*, worms) or environmental allergens. T_H2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, and Transforming Growth Factor (TGF)-β, which cause strong antibody responses, favor eosinophil differentiation and activation, and induce humoral immunity (6,7,26). Other cells of the immune system probably produce the different cytokines that trigger differentiation into two T-cell subsets. Besides the presence of these cytokines (INF-γ, IL-12, and IL-4) other events can affect the differentiation of helper T cells like the type of pathogen (*i.e.*, virus versus parasite), nature of the peptide (*i.e.*, agonist versus partial agonist), and the amount of peptide present (6,7). T_H1-like and T_H2-like cells are also seen in humans, but the distinction between the two subsets is not as clearly separated as in the animal models (27,28). These helper subsets have implications in autoimmune disease that will be discussed later in this chapter.

Act IV: Kinetics of T-cell Activation

In the past, an affinity/avidity or equilibrium model was used to described T-cell receptor (TCR) activation (29,30). In this model, the number of receptors bound versus unbound (as determined by the equilibrium constant, K_{eq}) for any given ligand determines the output of the signal. This model predicts that agonist ligands would have a higher affinity for the TCR than partial agonist and antagonist ligands. However, the differences between the affinities of ligands for the TCR are small (on the order of micromolar) and only span a few orders of magnitude (31,32,33). These small changes in affinities are enough to cause significant changes in the T-cell

response, and, thus, it seems unlikely that T-cell activation can be described entirely by an affinity model. In addition, ligands with different affinities can cause the same T-cell response (34) as well as ligands that have similar affinities can cause different responses (35). Hence, there are many observations that need to be incorporated into a model of T-cell activation that are not easily explained by a simple equilibrium or avidity model (29,36).

One such observation is TCR antagonism, which was thought to be due to competition for the receptor (37). However, since a single peptide/MHC complex can serially trigger approximately 200 TCRs (38) and the number of MHC molecules available on the MHC is large, antagonist ligands could never completely compete for the agonist ligand. In addition, low concentrations of antagonist ligands have been shown to block agonist ligands present at much higher concentrations even when the total number of ligands is small with respect to the number of receptors (39). Most antagonist ligands actually have weaker affinities than agonist ligands (32,33). Also, superantigen activated T cells were antagonized by APLs (40). Since superantigens and peptide/MHC ligands bind to different places on the TCR, then TCR antagonism cannot be due to competitive inhibition. A new model for T-cell activation must be able to incorporate all of these observations, which are not easily explained by an affinity model.

An alternative model is the Kinetic Discrimination Model (41), which is basically a revision of McKiethan's Kinetic Proofreading Model (42). Both models state that the length of time the ligand is bound to receptor determines the T-cell

response rather than the ratio of bound to unbound ligands (41,42). The Kinetic Discrimination Model elaborates on the Proofreading Model by more adequately explaining TCR antagonism (41). The Kinetic Discrimination Model is also based on the length of time the ligand is bound to receptor and the kinetics of this reaction (41). It has been shown that the on-rates (k_a) of ligands for the TCR differ very little, but the off-rates (k_d) seem to correlate to T-cell responsiveness (33). When ligand (L, peptide/MHC) binds to a receptor (R, TCR), an equilibrium (K_{eq}) exists between the bound and unbound states (Figure 1.5 and ref. 41). If the ligand and receptor bind (LR), then the first intracellular event, possibly phosphorylation of the ITAMs associated with the CD3 complex (9), can occur and the receptor is modified (LRP_1). If the ligand and receptor are bound for even a longer period of time, then the receptor is modified further (LRP_2) and maybe even further (LRP_3). When the ligand does eventually dissociate from the receptor, a modified TCR remains ($RP_{1,\ 2,\ or\ 3}$), and the ligand goes on to bind to another receptor (serial triggering; ref. 38). If the ligand (L) were an antagonist or partial agonist peptide, the modified receptor, RP_1 or RP_2, would only be partially activated. However, if the receptor and ligand are bound for optimal (longer) period of time as in agonist ligands, then this could give rise to a fully activated TCR (RP_3).

In the Kinetic Discrimination Model, all ligands send both positive and negative signals (41). Partly activated receptors (*i.e.*, RP_1 and RP_2 in Figure 1.5) generate negative or incomplete signals that inhibit the formation of positive or complete signals. Agonist ligands have slow off-rates and would generate mostly

positive signals. Pure antagonist ligands have fast off-rates and generate mostly negative signals. A partial agonist would generate a mixture of positive and negative signals. In other words, a partial agonist would inhibit its own signaling. It has been shown that ligands that are antagonists at low concentration and partial agonists at higher concentrations exist (39 and D. Beaudoin, unpublished results). As the number of bound moderate-affinity ligands increases (*i.e.*, higher concentration), then there is a greater probability that a ligand can rebind a partly activated receptor and thereby complete the activation.

The duration of TCR ligation determines the completeness of the response in these kinetic models of TCR activation. It has been shown that TCR-ligand dissociation rates correlate with T-cell activation better than TCR-ligand affinities do (33). Agonist ligands have been shown to have small TCR dissociation-rates, while antagonist ligands have much larger TCR dissociation-rates (33). A complete T-cell response includes all activation events, while a partial response includes only a portion of the activation events or a reduced intensity of the activation events. Early T-cell activation events have been shown to include phosphorylation of the CD3 ζ-chain, increase in calcium flux, and release of extracellular acid. Later activation events include production of cytokines and proliferation (Figure 1.6 and ref. 9). Rabinowitz, *et. al.,* described a hierarchy of events in T-cell activation that appear to correlate with TCR-ligand kinetics (9). Phosphorylation of the ITAMS of the CD3 complex is the earliest known event to occur in T-cell activation and occurs even with some antagonist peptides that bind for the shortest period of time (43). In fact, the pattern of

ζ-chain phosphorylation has been correlated with agonist, partial agonist, and antagonist ligands (43,44,45,46,47) giving further evidence that these ligands affect T-cell recognition of the peptide/MHC rather than simply corresponding to the affinity of ligand for receptor. Proliferation is the latest event to occur and occurs with agonist peptides and some partial agonist peptides. Partial agonist peptides, with intermediate off-rates, give an intermediate response like calcium flux without proliferation or a reduced amount of proliferation. Cytokines were not originally placed on this plot (9), but depending on the altered peptide ligand and the cytokine produced, cytokine production could occur before full proliferation as well as afterwards (Figure 1.6). This difference may be due to the cells need for growth factors like IL-2 to proliferate, which would lead to less stringent thresholds for growth cytokines whereas effector cytokines like IL-4 and IL-10 could require a higher threshold of activation (see Chapter 3 for further discussion). Figure 1.6 does not mean to suggest that there is a stepwise relationship between all of these events, but, rather, that each event has its own threshold of activation.

Act V: Autoimmune Disease – T-Cell Activation due to Self-Antigens

An autoimmune disease occurs when the immune system becomes activated by self-antigens instead of foreign pathogens. In the case of Multiple Sclerosis (MS), the candidate autoantigens are apparently present in proteins of the central nervous system (CNS) myelin (48). The myelin sheath in the CNS is formed from the cell membrane of oligodendrocytes that has been concentrically wrapped around the axon of nerve

cells (49). The CNS myelin is composed of both lipids (75-80%) and proteins (20-25%) (48). An electrical signal is transmitted along the axons and this nerve impulse is propagated more rapidly with myelinated axons. Myelin Basic Protein (MBP) accounts for about 25-30% of the protein of the myelin sheath and is found in the cerebral spinal fluid of humans (48). MBP-specific T cells have been isolated from MS patients for *in vitro* studies (2,68) and it has attracted attention as a candidate for an MS autoantigen (48). Other myelin antigens include proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG). The symptoms of MS arise primarily from demyelination of axons in the CNS due to T-cell mediated inflammation.

The cause of MS onset is not entirely understood. The disease is thought to be mediated by T_H1 cells specific for the myelin-based-antigens. These helper T cells are presumably activated by peptide/MHC, cross the blood-brain barrier, and recruit other cells of the immune system, which causes an inflammatory response in the CNS, specifically in the white matter of the brain (2,48). The general hypothesis is that MS is an autoimmune disease for genetically susceptible persons, especially women (2,50). People with certain MHC proteins, primarily human leukocyte antigen (HLA)-DR, as well as twins and other family members seem to have a higher occurrence of this disease (48). In addition, the varying prevalency rates due to geography or ethnicity seem to suggest an environmental role for this disease (48). Even though a specific pathogen has not been determined, it has been shown that various microbes have sequence similarities to myelin peptide epitopes (1,51,52,53) and can activate

myelin-specific T cells (1,53). Even without knowledge of the cause for disease onset, some treatments have been developed in the last twenty years.

At the present moment, the "ABC"s of drug treatments for MS include several drugs that are primarily immunomodulatory or anti-inflammatory (4,5,54,55,56). For the treatment of early stages of MS, drugs include the interferon-beta (INF-β) drugs like Avonex (INF-β 1a, **A** drug) and Betaseron (INF-β 1b, **B** drug) and a polypeptide-based drug, Copaxone (**C** drug). Copaxone, or glatiramer acetate, has been shown to be crossreactive with MBP-specific T cells and induce suppressor T cells that can block a rodent model of MS, experimental autoimmune encephalomyelitis (EAE), caused by both MBP and PLP encephalogenic epitopes (57). Other drugs for the treatment of worsening stages of MS include the chemotherapeutic drug, Novantrone, or the general immunosuppressants like methotrexate or cyclophosphamide, which only have moderate benefit on chronic stages of the disease (54,55). Even though immunosuppressive drugs have been shown to have some benefit to MS patients, all these drugs just alleviate the symptoms and help patients live with the disease. Not to mention, most of these drugs require patients to get several injections per week (55). Research for new drugs is focusing on ameliorating the symptoms more substantially.

An established murine model for MS, EAE, has proven to be an asset for studying this debilitating disease (58). Both diseases cause paralysis. However, MS occurs spontaneously in humans, but EAE is induced in mice by injecting an immunogen. Immunizing with whole myelin, isolated myelin antigens like MBP, PLP, MOG, or synthetic peptides that contain one of the defined T-cell epitopes, can

induce EAE (59). These epitopes include MBP (1-9), MBP (89-101), and PLP (139-151). The EAE model provides a useful research tool since the sequences of the peptides presented to the T cells are known and EAE is mediated by activation of helper T cells (T_H1), which is also thought to occur in MS (59). Once the myelin-based proteins are introduced into the animal, the peptide bound to MHC class II is presented to helper T cells in the lymph nodes (Figure 1.7). The disease is induced in rodents when helper T cells are activated by antigen and cross the blood-brain barrier (60). These T cells instigate an inflammatory response in the brain (61). They secrete cytokines that direct other cells in the immune system to destroy the myelin sheath around nerve cells. When cytotoxic (CD8+) T cells are presented myelin peptides on MHC class I, they lyse the cells (with myelin) of the nervous system (60,62). Eventually the rodent will develop clinical signs of disease. Altered peptide ligands antagonize the helper T cell's signal to destroy myelin and thereby block the progression of EAE, which suggests that APLs could be used as immunotherapeutic drugs (63,64,65).

Act VI: Altered Peptide Ligands - The Potential Hero

Altered peptide ligands (APLs) bound to MHC proteins have been shown to produce a spectrum of T-cell responses. As mentioned before, peptides can cause full proliferation and cytokine production (agonists), block the response caused by agonist ligands (antagonists), or cause a subset of responses (partial agonists, Figure 1.8). Allen and coworkers discovered APLs that blocked full proliferation of murine T cells

and some that were partial agonists. They first identified TCR and MHC contact residues of Hemoglobin (64-76) [Hb 64-76] using single amino acid substitutions in their peptides (66). One APL, which substituted serine for alanine-70, partly activated an Hb-specific murine T-cell clone causing upregulated expression of IL-2 receptors, LFA-1, and increased cell volume, but not cytokine production or proliferation (67). T-cell clones first cultured with APC and the Hb S70 analogue were completely unresponsive, or anergic, to the wild-type peptide. Activation of the T cell was not a matter of competition for TCR sites because the analogue peptide was removed before stimulation with wild-type peptide. Allen and coworkers extended their findings by ascertaining the relationship between partial agonists and antagonists in the hemoglobin model (40). The APLs previously identified as partial agonists for some helper T-cell clones [*i.e.*, Hb (64-76), S70] could also antagonize proliferative responses of other helper T-cell clones. In addition, different clones responded differently to the same peptide. Altered peptide ligands have been shown *in vitro* for human T-cell clones as well (68). Thus, different altered peptide ligands can cause various responses in one T-cell clone and the same APL can cause different responses in different clones.

The T-cell response *in vivo* is heteroclitic, which is observed by the fact that any monoclonal population within the polyclonal population will recognize the antigen in a different way. The fact that APLs can behave as an antagonist for one clone and an agonist for another clone (40) has raised potential concerns in designing immunotherapeutic peptides. The autoimmune disease can be worsened rather than

improved by such treatment. In fact, Wraith and coworkers created antagonist peptides by substituting amino acids into important TCR and MHC contact sites of MBP (1-9) (69). Proline-6 was replaced by one of the following amino acids: glycine, threonine, or valine. In addition, lysine-4 was replaced by tyrosine in all three of the antagonist peptides to increase binding to the MHC class II. These *in vitro* antagonist peptides unexpectedly induced EAE in mice. The peptides were antagonists for a monoclonal population of T cells, but they were agonists for the polyclonal population. These results show the dangers involved in using a limited number of T-cell clones for *in vitro* work. Nevertheless, Steinman and coworkers have shown that some peptides identified as antagonists for a single clone can block the progression of EAE in the Lewis rat (63). TCR contact sites of MBP (87-99) were altered to alanine and studied for their ability to antagonize a T-cell clone and a T-cell line. Three *in vitro* antagonists were identified, but only one, MBP K91A, prevented EAE in an epitope-specific manner. In addition, Kuchroo and coworkers have shown that an analogue of the encephalitogenic myelin PLP (139-151) peptide, W144L/H147R, is a powerful antagonist for antigen-specific T-cell clones *in vitro* (64). This peptide can also protect animals from the induction of EAE. In later studies, Wraith and coworkers discovered antagonist peptides of MBP (1-9) that did block EAE progression (65). Both MBP 3I4Yand 3K4Y (altered from 3Q4K) antagonized T cell activation *in vitro* and blocked EAE induction *in vivo*. These results demonstrate that the therapeutic use of analog peptides remains an achievable objective, but it requires considerable attention to attain the desired *in vivo* outcome (65).

Act VII: Inducing Tolerance to Self - Why may APL therapy work?

If the same peptide can cause different responses in different clones, then how do APLs block the progression of disease as in the examples listed above? In order to understand the mechanism by which this may occur, the idea of tolerance needs to be defined (70). Tolerance is the failure to respond to an antigen; for example, the immune system is mostly tolerant of self-antigens. When tolerance is lost, the immune system can destroy self-tissues, as in autoimmune diseases such as EAE and MS (6). Self-reactive T cells are usually deleted in the thymus in a process called negative selection or clonal deletion (8). Unfortunately, some of these cells escape negative selection and are released into the periphery. Thus, the immune system has developed mechanisms for peripheral tolerance, such as clonal deletion, anergy, antagonism, immune deviation, and bystander effects. Clonal deletion usually occurs in the thymus during the selection process, but has been shown to occur in the periphery as well (70). The cells are triggered to undergo apoptosis (programmed cell death). The other mechanisms of tolerance occur mostly in the periphery. When a lymphocyte no longer responds to activation, the cell is in a state of anergy. Anergy can be induced when T cells fail to receive a second signal during activation with APLs or high doses of agonist ligand (71,72). However, one of the most important mechanisms of peripheral tolerance is change in the local cytokine environment.

Specific cytokines have been implicated in various autoimmune diseases. Immune responses are dominated by one of the T-helper subtypes (T_H1 and T_H2) and each subtype can inhibit the activity of the other. Therefore, autoimmune diseases,

allergies, and infections could potentially be cured or at least improved by altering cytokine profiles. Kuchroo and coworkers showed that EAE (which is T_H1 mediated) induced with the native PLP (139-151) peptide could be inhibited in SJL mice with the altered peptide ligand, PLP W144Q. The APL induces T cells that are cross-reactive with the wild-type peptide and produce T_H2 (IL-4 and IL-10) and T_H0 (INF-γ and IL-10) cytokines (73). Steinman and coworkers showed that the MBP (87-99) K91A (see above) blocks the development of EAE in SJL mice by inducing cytokine shifts from T_H1 to T_H2 in the target T cells (63,74). Further modifications of the MBP K91A peptide led to an APL (D-Ala83-Lys84-Leu89-Ala-91-MBP 83-99) that was used in phase II clinical trials with MS patients (75). This APL induced a T_H2-type T-cell response that was cross-reactive with the native peptide. In MS patients completing the four-month study, the volume and number of enhancing lesions within the central nervous system decreased. Unfortunately, the trial was suspended when 9% of the patients developed hypersensitivity reactions (*i.e.*, itching, rashes, hives, and abdominal pain), which can probably be associated with the T_H2 response (75). More recently, other subtypes of CD4+ T cells have been discovered that also change the local cytokine environment. These cells are collectively called regulatory T cells, T_{reg}, or suppressor T cells. Regulatory T cells can presumably suppress or regulate the immune response of other cells (76,77,78,79,80). These bystander effects have been shown to occur in systems where the PLP(139-151) peptide could induce bystander suppression of T cells responsive to itself as well as to MBP (1-9) 4Y and MBP (89-101). PLP (139-151) could suppress EAE caused by two non-linked T-cell epitopes

(MBP peptides), showing that its regulatory effects had to be bystander suppression (81). Regulatory T cells are found in both animals and humans (79).

Categorizing regulatory T cells has been somewhat confusing due to the differences in the experimental contexts that these cells were evaluated. Therefore, various definitions or subtypes of these suppressor T cells have surfaced over the last few years (77,78,79,80,82). Regulatory T cells that are both CD4+ and CD25+ (the IL-2-receptor α chain) comprise one of the major subtypes found in mice (83,84) and humans (82,85). (CD25 expression is normally upregulated when a naive T cell becomes activated.) The CD4+CD25+ regulatory T cells seem to depend on a cell contact-dependent mechanism rather than the secretion of anti-inflammatory cytokines like IL-4, IL-10, and TGF-β (80). However, some CD4+ CD25+ T cells secrete anti-inflammatory cytokines like TGF-β or IL-10 in order to regulate the immune system (79,86). Regulatory T cells are critical to the maintenance of self-tolerance, which can be demonstrated by eliminating CD4+CD25+ T cells from the organism. A wide spectrum of autoimmune diseases can develop (87). In addition, these CD4+CD25+ regulatory T cells also constitutively express CTLA-4 (86,88,89). Naive T cells express CD28 on their surface, which delivers a costimulatory signal to the T cell upon binding to B7 molecules on the surface of APCs. This provides the second signal needed to activate the T cell. Expression of CTLA-4, another molecule specific for B7 molecules, is upregulated on the T cell's surface upon activation. CTLA-4 binds with a higher affinity to B7 molecules and inhibits the proliferative response of

T cells (6). Constitutive CTLA-4 expression among CD4+ T cells is primarily limited to regulatory T cells, which suggests CTLA-4's involvement in their regulation of the immune system (86). However, the suppressor function of CD4+CD25+ T cells *in vitro* seems to be independent of interactions of CD28 and CTLA-4 (90). Basically the role of CTLA-4, if any, in CD4+CD25+ T cells' mechanism of suppression is not known.

Another important subtype of regulatory T cells that have been shown to suppress the immune response are the anti-inflammatory cytokine producers, which are seen in both humans and animals (79). These T cells will usually produce TGF-β, IL-10, IL-4, or a combination of these, which have various suppressive effects on the immune response as shown in Figure 1.9. These other regulatory T-cells have been called T_H3 (91) and T_r1 cells (92).

Regulatory T cells, also called T_r1 and T_H3 cells, have been mistaken for T_H2 cells in the past because of their cytokine profiles. Weiner and coworkers showed that MBP–specific T-cell clones from the lymph node of orally tolerized mice secreted large amounts of TGF-β with varying amounts of IL-4 and IL-10. Through two-color flow cytometric analysis, they were able to show that clones, which stained high for TGF-β were usually low in IL-4 and IL-10 (76). These experiments suggest that these T cells could be a unique subset. The action of TGF-β is critical for prevention of autoimmunity, as shown by mice engineered to express a dominant-negative form of TGF-β receptor II subunit (which blocks the function of all isoforms of TGF-β)

specifically in T cells. These mice developed a spontaneous autoimmune disease that included inflammatory infiltrations in several organs and the production of autoantibodies (93). T_r1 cells have also been shown to inhibit a T_H2 response, which suggests that regulatory T cells may play a role not only in autoimmune disorders (often associated with a T_H1 response) but also in allergic responses associated with T_H2 cells (94).

The source of these regulatory T cells is not entirely known (95). Are these regulatory T cells a subtype of CD4+ T cells as in the T_H1 and T_H2 cells or are they a feature of predetermined lineage that differentiate in the thymus? If regulatory T cells originated only in the thymus, then designing therapeutic drugs to generate the production of these cells in the periphery would be a problem. However, induction of these cells has been shown to occur in the periphery. The non-obese diabetic (NOD) mouse is an established murine model for insulin-dependent diabetes mellitus (IDDM), which results from the T-cell-mediated destruction of the insulin-producing pancreatic β-cells. NOD mice spontaneously develop insulitus at about 4 weeks of age, which eventually progresses into diabetes. The disease is mediated by T_H1 cells specific for glutamic acid decarboxylase (GAD65). Kaufman and coworkers showed that administering GAD65 in a mode that promotes a T_H2 response [*i.e.*, GAD65 with Incomplete Freund's Adjuvant (IFA)] could inhibit disease progression. NOD mice have been reported to have deficiencies in T_H2 development (96). Therefore, they were not sure that administration of GAD65 in IFA would induce tolerance as it does in EAE models. Serreze and coworkers were able to show that immunization of NOD

mice with GAD65 peptides after autoimmunity had developed was epitope specific, and that only certain epitopes (217-236 and 290-309) were able to prevent progression of insulitus and overt IDDM. The peptide epitopes differed in their efficacy to induce sufficient T_H2 cell activity (97). Even if some regulatory T cells (CD4+CD25+ possibly) do differentiate in the thymus, induction of other regulatory T cells (like the suppressive cytokine producers) in the periphery does appear to be possible. Hence, induction of these regulatory T cells and/or T_H2 cells can be a probable immunotherapy for autoimmune diseases.

Act VIII: The Tale Continues.....

Even though APLs have been shown to work in rodent models of MS, we still do not have a systematic way of determining, *in vitro*, the peptides that will block disease. Hence, the goal for this project was to determine whether there was a correlation between *in vitro* reactivity of APLs and their efficacy in blocking EAE. One of the best-characterized autoantigenic epitopes in mice is the N-terminal peptide of myelin basic protein (MBP) (Figure 1.2B). Both TCR and MHC contacts are well defined for this peptide. APLs of MBP Ac(1-11) were tested *in vitro* using many different T-cell-based assays. In addition, kinetics of TCR/ligand interactions are discussed as it relates to later signaling events like proliferation and cytokine production due to APLs. And finally, preliminary results describing early and late signaling events of three monoclonal T-cell clones is shown. The impact that the

heteroclitic nature of T cells has on designing immunotherapeutic drugs will be discussed as well.

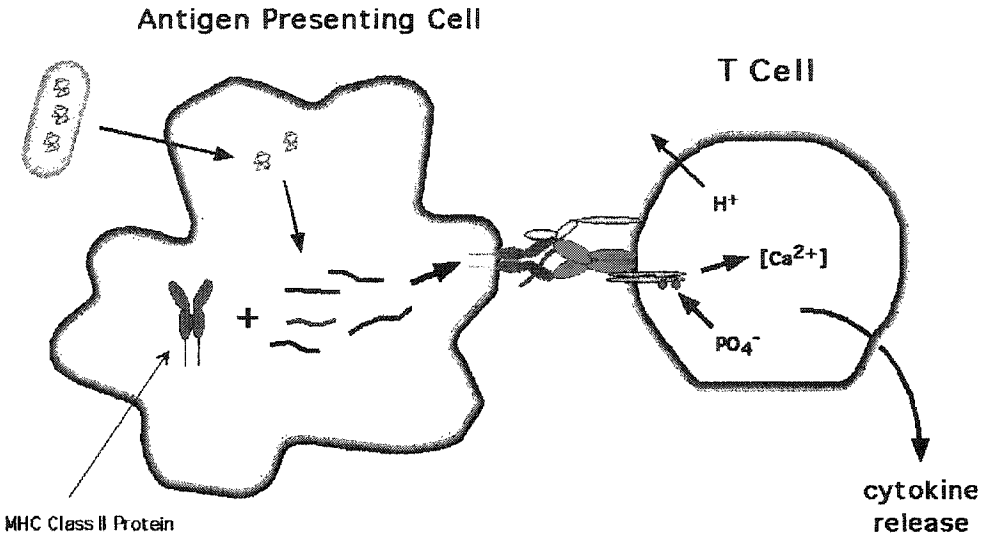

Figure 1.1: Helper T-cell activation by peptide/MHC class II complex. Exogenous antigen is ingested by endocytosis and degraded into small peptides. The peptides are presented with MHC class II proteins on the surface of the APC to CD4+ helper T cells. The peptide/MHC complex causes a cascade of signaling events: zeta-chain phosphorylation, calcium flux, extracellular acid release, and cytokine production. (Figure compliments of C. Beeson).

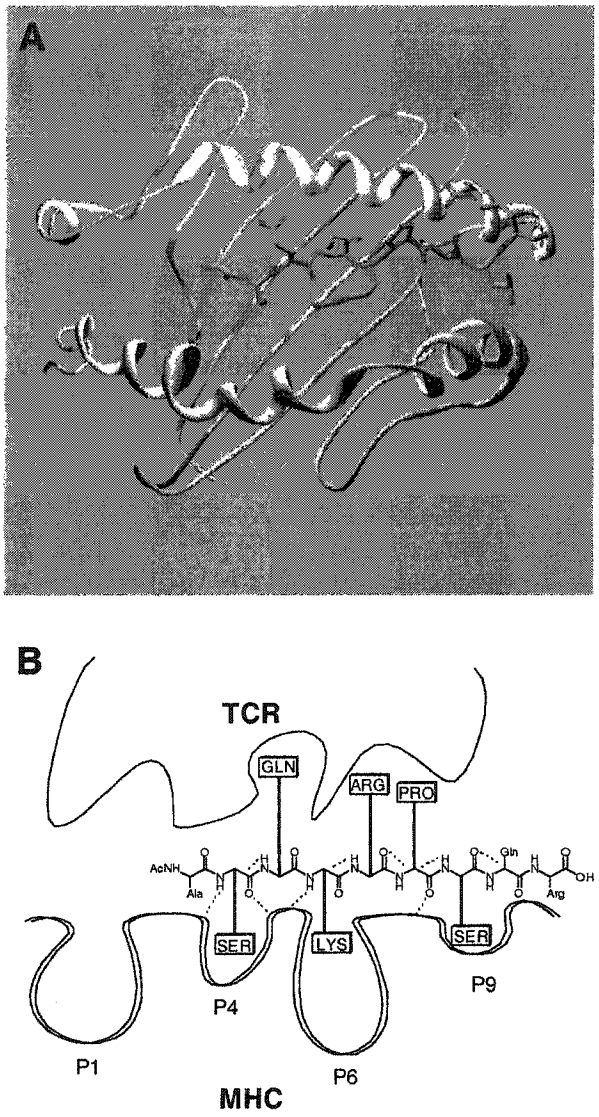

Figure 1.2: MBP peptide is presented to helper T cells by MHC class II protein. A.) Ribbon diagram of N-terminal MBP with the MHC class II protein (I-A^u). (Adapted from ref.14). B.) The N-terminal MBP wild-type peptide has a weak affinity for the MHC class II proteins, I-A^u or I-A^k. Mutations at residue 4 to more hydrophobic side chains increase the affinity for the MHC (Lysine-4 to alanine or tyrosine). Residues serine-2, lysine-4, and serine-7 bind in the P-4, 6,9 pockets of the MHC, respectively. The aliphatic portion of arginine-5 binds in the P7 pocket. Glutamine-3, arginine-5, and proline-6 are TCR contact sites. (Figure complements of C. Beeson).

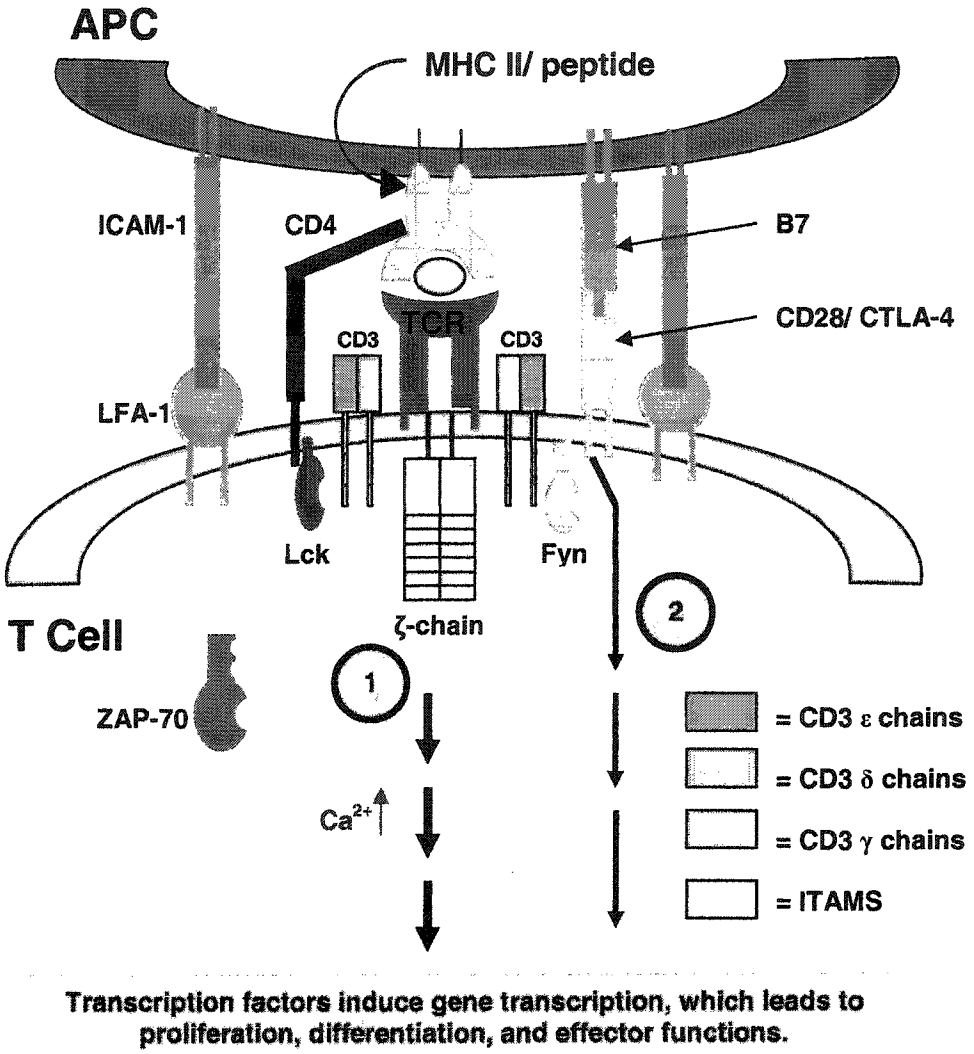

Figure 1.3: Naive T-cell activation requires two signals. Initially, adhesion molecules like ICAM-1 bind to their ligands, LFA-1, on T-cells in a low affinity conformation. The CD4+ T cell screens the peptide/MHC class II complex, and if recognition occurs, this low affinity interaction becomes a high affinity interaction due to a conformational change in LFA-1. This high affinity interaction leads to signals sent through the TCR/CD3 complex. CD4 is recruited and binds to the MHC stabilizing this reaction and recruiting the kinase, Lck, to the immunological synapse. The TCR on the helper T cell binds to its cognate antigen bound to the peptide MHC class II protein causing a series of phosphorylation events of various tyrosines in ITAMS by kinases (Lck and Fyn). Lck and Fyn are, themselves, activated by a phosphatase (not shown). ZAP-70 binds to the phosphorylated ITAMS and starts a cascade of signaling events that lead both to an increase in intracellular calcium and induction of transcription factors. For naive T cells, a second signal is needed to activate the cell. Constitutively expressed CD28 binds to B7 on antigen-presenting cells and provides the second signal. Another ligand for B7 molecules is CTLA-4 and has been shown to be a negative signaling molecule that helps inhibit T-cell activation to prevent over-proliferation. Effector and memory T cells are less dependent on this second signal than naive T cells. (Adapted from ref. 6 and 7).

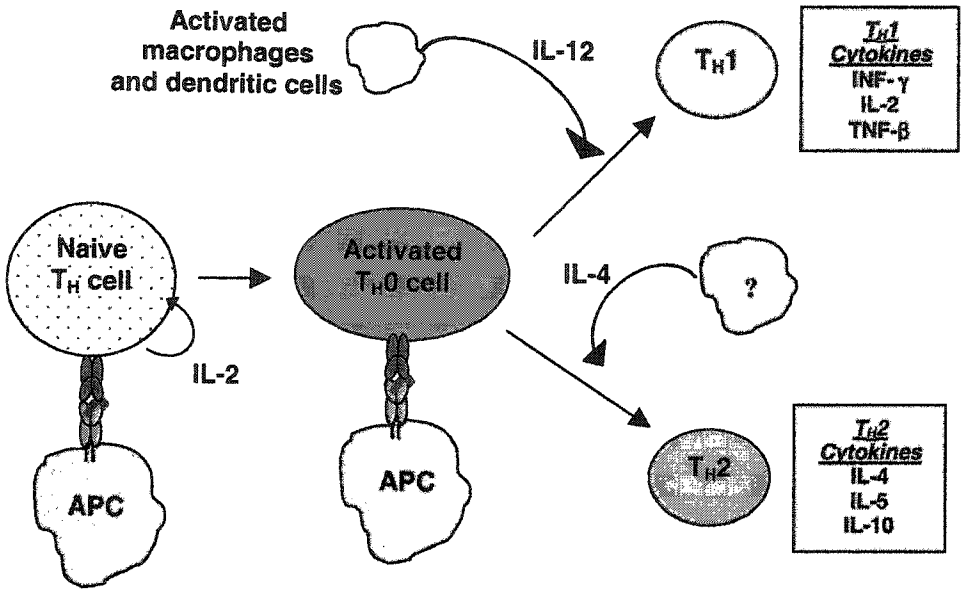

Figure 1.4: Activated CD4+ T cells can develop into two subsets, T_H1 and T_H2. Naive CD4+ T cells in the presence of a peptide/MHC class II complex are activated and become immature effector T cells, or T_H0 cells. These effector T cells differentiate into two subsets depending on the cytokines present. T_H1 cells develop in the presence of IL-12 and INF-γ during infections by such pathogens as intracellular bacteria and viruses that are phagocytosed by macrophages or dendritic cells. They produce cytokines like INF-γ, IL-2, and TNF-β, which can activate macrophages and induce cell-mediated immunity. T_H2 cells differentiate in the presence of IL-4, which occurs during infestations with complex parasites (*i.e.* worms). The source of IL-4 is speculated to be from the T cells themselves as well as from other sources. T_H2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, and TGF-β, which cause strong antibody responses, favor eosinophil differentiation and activation, and induce humoral immunity. (Adapted from ref. 7).

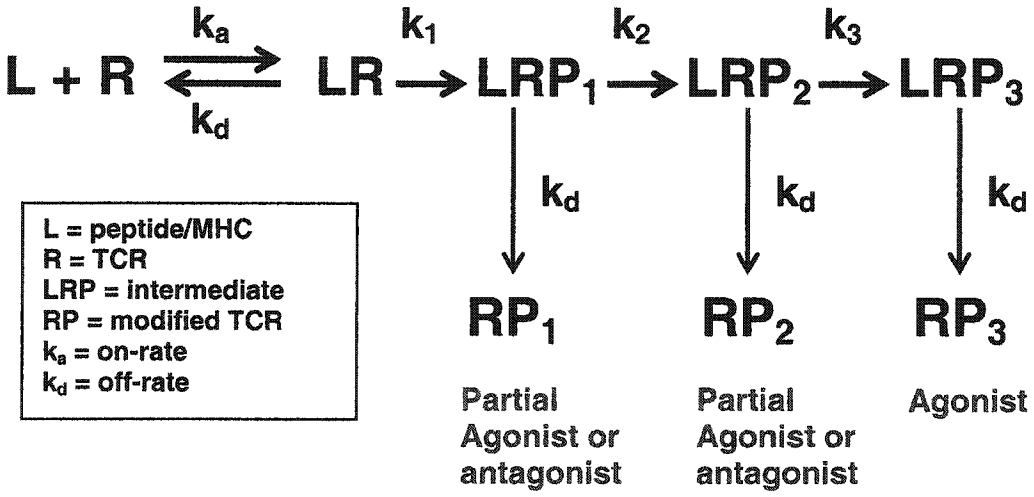

Figure 1.5: Kinetic Discrimination Model of T-cell activation. When ligand (or peptide/MHC) binds to receptor, an equilibrium exists between bound and unbound forms. If the ligand and TCR bind, then the first intracellular event occurs and the receptor is modified forming LRP_1. Depending on the off-rates of the ligand, the ligand can dissociate ($RP_{1,2,3}$) or continue to further modify the receptor. Agonist ligands are bound longer than the antagonist or partial agonist ligands. All ligands form both positive (complete) and negative (incomplete) signals. The ligand that remains bound the longest gives the most positive signals and a more complete response. (Adapted from ref. 41).

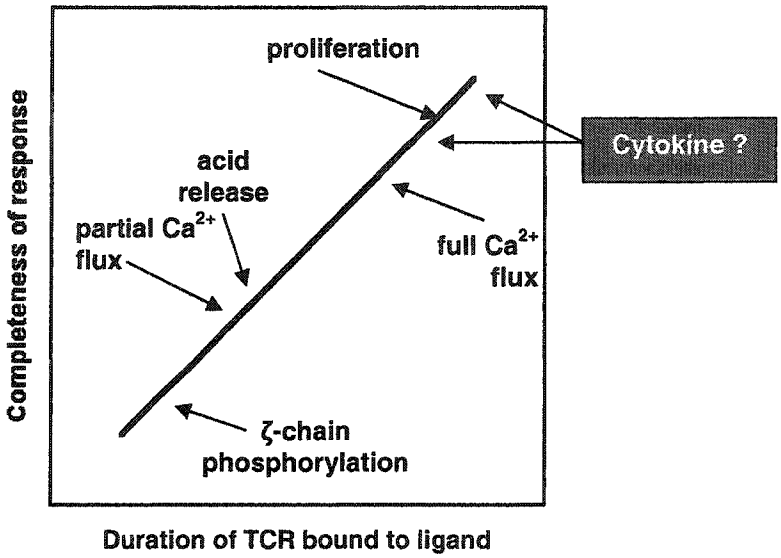

Figure 1.6: The duration of TCR ligation corresponds to the completeness of the response. The plot depicts the relationship between the duration of the TCR ligation and the completeness of the response. (Adapted from ref. 9). Arrows show where responses might be placed on the plot. I have hypothetically placed cytokine production before and after proliferation depending on the T cell (naive vs. effector) and the cytokine (IL-2 vs. IL-4 and IL-10). For example, IL-2, a growth factor, may be synthesized slightly before full proliferation, but effector functions like IL-4 and IL-10 may have a slightly higher threshold of activation (see discussion in Chapter 3).

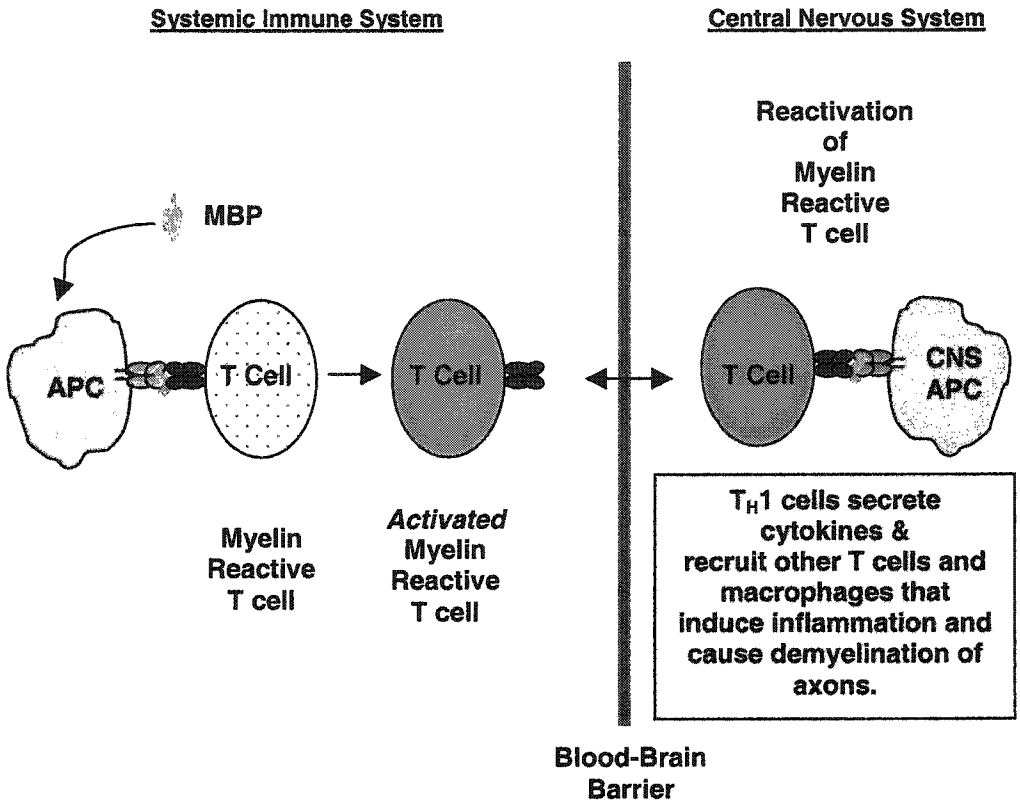

Figure 1.7: EAE is a murine model for the human autoimmune disease, MS. Once the myelin-based proteins are introduced into the animal, the peptide bound to MHC class II is presented to helper T cells in the lymph nodes. The disease is induced when helper T cells are activated by antigen and cross the blood-brain barrier. These T cells instigate an inflammatory response in the brain. They secrete cytokines that direct other cells in the immune system to destroy the myelin sheath around nerve cells. When cytotoxic T cells are presented myelin on MHC class I, they lyse the cells (with myelin) of the nervous system. Eventually the rodent will develop clinical signs of this debilitating disease including paralysis. (Adapted from ref. 98).

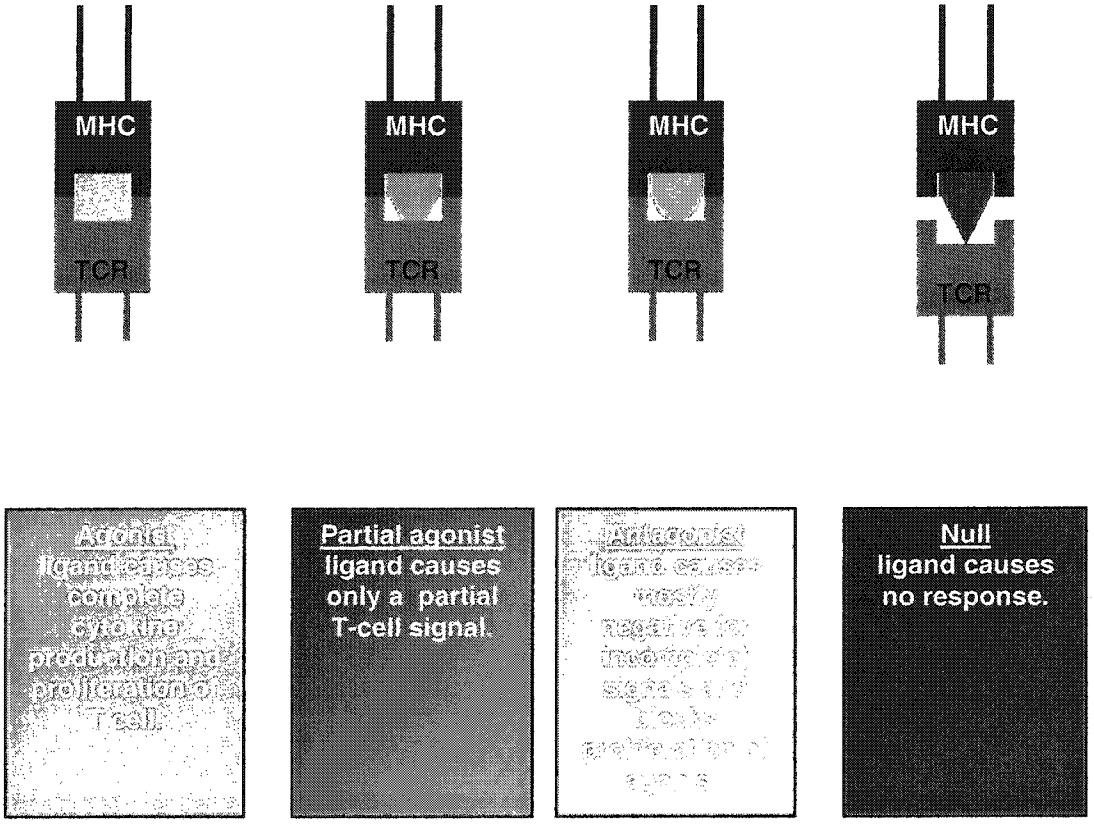

Figure 1.8: APLs can cause a spectrum of responses. When the wild-type ligand is modified to an APL, the T-cell can have a variety of responses ranging from full agonist all the way down to null peptide. (Adapted from ref. 99).

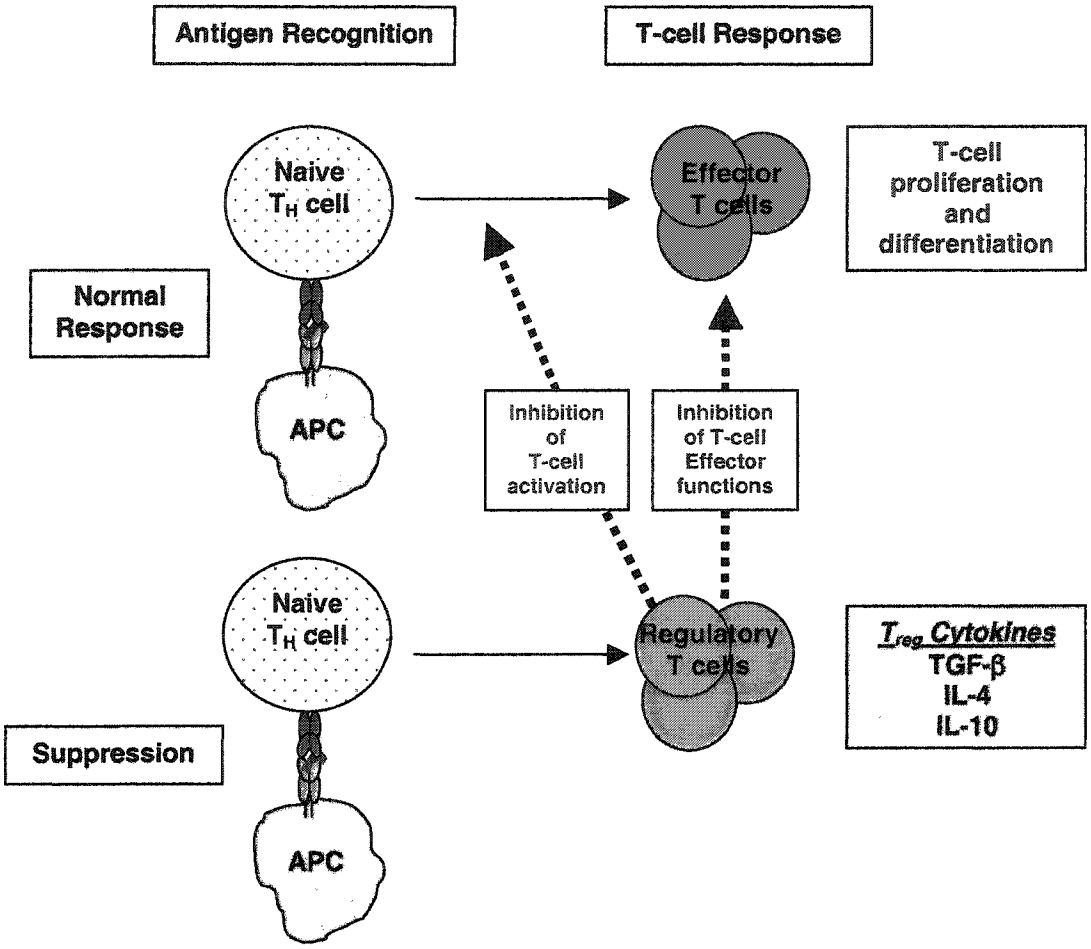

Figure 1.9: Regulatory or suppressor T cells can have bystander effects. Naive helper T cells differentiate into T_H1 cells when APCs, such as macrophages, present peptide-MHC class II complexes to the TCR. In the presence of IL-12 (secreted by the APC), the T_H1 cells release INF-γ, which can activate more macrophages. Regulatory T cells can secrete various cytokines, TGF-β, IL-10, and IL-4, that can suppress the immune response either by acting on APCs or T cells. These bystander effects can inhibit the effects of T_H1 cells that are beneficial in autoimmune diseases mediated by these T cells. (Adapted from ref. 7).

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Chapter 2

Hierarchical Signaling in T-Cell Responses to Altered MBP Ac(1-11) Peptides

ABSTRACT

Experimental autoimmune encephalomyelitis (EAE) can be induced in H-2k mice with the N-terminal peptide of myelin basic protein (MBP). Altered peptide ligands (APLs) of myelin protein epitopes have been previously shown to block the progression of EAE by inhibiting the effector functions of the T_H1 cells that mediate the disease. However, strong correlations between the *in vitro* reactivity of a given APL and its efficacy in blocking disease have not been demonstrated. We hypothesized that if a large number of APLs are evaluated they will segregate into subsets defined by their activity as measured by different assays for *in vitro* T-cell activation. We suggest that those peptides with therapeutic efficacy will be primarily located within one subset. Using computer models as a guide, altered MBP Ac(1-11) peptides were designed incorporating both natural and non-natural amino acids. A monoclonal MBP Ac(1-11)-specific T-cell line derived from B10.A mice (B10.AE3 T-cell clones) was used to screen these APLs using various cell-based assays. A number of peptides with antagonist or partial agonist activities were identified using the B10.AE3 T-cell clones. A hierarchy of activities was defined for this T-cell clone based on the ability of a peptide to induce proliferation, cytokine production, and

antagonism. Among the peptides identified as antagonists, the most potent also demonstrated partial agonist activities. The *in vitro* reactivity of the altered peptides within this group is similar to the activities of peptides previously shown to be effective in blocking EAE.

INTRODUCTION

The immune system generates a wide variety of cells and molecules that respond to and eliminate foreign pathogens (1,2). Generation of an effective cellular immune response involves two major groups of cells: lymphocytes and antigen-presenting cells (APCs). T lymphocytes begin as progenitor cells formed during hematopoiesis that enters the thymus gland as immature thymocytes. Thymocytes that express an αβ T-cell receptor (TCR)-CD3 complex can develop into either CD4+ or CD8+ lymphocytes. This differentiation is due to their avidity for self-peptides bound to proteins of the major histocompatibility complex (MHC) (class I or class II) in the thymus. If the thymocyte interacts weakly with a self-peptide, it is positively selected. The positively selected T cells further differentiate into CD4+ helper T cells if they interact with a peptide/MHC class II complex or CD8+ cytotoxic T cells if they interact with a peptide/MHC class I complex. These mature T cells remain in a resting state until they encounter a peptide/MHC complex in the periphery. For CD4+ helper T cells, the peptide/MHC class II complex must be presented on the surface of specialized APCs. When a helper T cell comes into contact with its cognate antigen presented on an MHC class II protein, a cascade of signaling events that leads to

proliferation, or cell growth, occurs. Early signaling events include CD3 zeta-chain phosphorylation, intracellular calcium flux, and extracellular acid release. Late markers include cytokine production and T-cell proliferation (3). The late markers are used to define when a T cell has been activated, and activated helper T cells secrete cytokines that regulate the activity of other cells in the immune response.

As mentioned previously, in order for the CD4+ (or helper) T cell to be activated, the peptide must be presented by MHC class II proteins on the surface of APCs. Crystal structures of peptide/MHC class II complexes reveal that peptides lie within an MHC protein groove, anchored by a series of hydrogen bonds between the peptide backbone and the MHC (4,5,6,7). Some of the peptide side chains are anchored within pockets of the MHC where small polymorphic changes can have substantial effects on binding of various amino acid side chains in the pocket (8). Other side chains protrude out of the groove and are available to the TCR. Small modifications in the peptide/MHC complex can, therefore, alter the region that is recognized by the TCR (9). Hence, a small change in the peptide's amino acid sequence, especially at TCR contact residues, has been shown to sometimes block proliferation (antagonism) or give an intermediate or incomplete response (partial agonism; 9,10,11,12).

The T-cell response can be measured using different markers for cellular events that lead to activation. These events include protein phosphorylation, extracellular acid-release, calcium flux, cytokine production, and proliferation (3). Agonist peptides can cause all these events to occur where partial agonists can cause

only a subset to occur (9). For instance, production of only one of several cytokines or reaching a lower maximum amount of proliferation would classify an antigen as a partial agonist. Indeed, binding of TCR to a peptide/MHC complex does not even necessarily lead to activation. For example, antagonist ligands bind TCRs and inhibit the T cell's signal received by agonist ligand (13), which suggests a possible therapy for autoimmune disease. By modifying the signal caused by an autoantigen, one may be able to 'cure' or at least improve an autoimmune disease by blocking or changing the T-cell's autoreactive signal (21,22).

Even though mechanisms exist to prevent T cells from becoming activated towards self-antigens *(i.e.*, clonal deletion (14), induction of anergy (14,15,16), and bystander suppression (17,18)), expansion of T cells specific for self-antigens can still occur and result in an autoimmune disease. An established murine model for the human autoimmune disease multiple sclerosis (MS) is experimental autoimmune encephalomyelitis (EAE), which can be induced by immunizing with whole myelin, isolated myelin antigens like myelin basic protein (MBP) or proteolipid protein (PLP), or synthetic peptides that contain one of the defined T-cell epitopes (reviewed in ref. 19). Once the myelin-based proteins are introduced into the animal, the myelin-derived peptide bound to MHC class II protein is presented to helper T cells in the lymph nodes. During the immunization, Complete Freund's Adjuvant (CFA) and pertussis toxin are also injected to assure a vigorous activation of helper T cells (a T_H1 response). The disease is induced when the helper T cells activated by the injected antigen cross the blood-brain barrier where they recruit other cells of the immune

system to cause inflammation (20). Clinical signs of disease include paralysis and even death.

Since EAE and MS are mediated by helper T cells, blocking this response by modifying the agonist antigen to create altered peptide ligands (APLs) that inhibit this response is an area of research for disease therapy (21,22). As mentioned before, altered peptides bound to MHC proteins have been shown to produce a spectrum of T-cell responses. Agonist peptides can cause full proliferation and cytokine production (agonists), block the response caused by agonist ligands (antagonists), or cause a subset of responses (partial agonists) (9). In addition to different APLs affecting the response within one T cell, different T cells specific for the same antigen can have completely different responses due to the same APLs. For example, the same peptide can behave as an antagonist for one clone and an agonist for another clone (12). This raises a potential concern in designing immunotherapeutic peptides since the autoimmune disease can be worsened rather than improved by such treatment. Nevertheless, Steinman and coworkers have shown that some peptides identified as antagonists for a single T-cell clone can block the progression of EAE in the Lewis rat model (23). The TCR contact sites of MBP (87-99) were altered to alanine and studied for their ability to antagonize a T-cell clone and a T-cell line. Three *in vitro* antagonists were identified, but only one, MBP K91A, prevented EAE in an epitope-specific manner. In addition, Kuchroo and coworkers have shown that an analogue of the encephalitogenic myelin PLP (139-151) peptide, W144L/ H147R, is a powerful antagonist for antigen-specific T-cell clones *in vitro*, and can also protect animals from

the induction of EAE *in vivo* (24). In addition, T cells specific for this peptide produce T_H2/T_H0 cytokines, which makes it not only an antagonist, but also a partial agonist. Wraith and coworkers discovered antagonist peptides of MBP (1-9) that did block EAE progression (25). Both MBP 3I4Yand 3K4Y (altered from 3Q4K) antagonized T cell activation *in vitro* and blocked EAE induction *in vivo*. This peptide also induced proliferation of at least 2 T cell lines and also could be called a partial agonist. A few APLs have been used in phase II clinical trials for MS (26,34). Several groups have attempted to try to explain physiological basis for APL blockade of disease, but the exact mechanism probably depends a great deal on the system being studied (27,28,29,30,31).

Mechanisms of action have been suggested for why APLs work *in vivo* even in the presence of a diverse T cell population. Past experiments have shown that some antagonists can block T-cell activation even in the presence of excess agonist ligand suggesting that antagonists don't function by competition for the MHC (56,58). If MHC competition was the mechanism, then TCR antagonists would be able to block activation *in vitro*, but not necessarily *in vivo* due to the diverse population of T cells (38). Nevertheless, APLs have been shown to block disease in some cases (23,24,25), which implies a different mechanism of inducing tolerance for self-peptides within individuals with autoimmune diseases. Some of the mechanisms of tolerance induction suggested most recently include release of immunosuppressive cytokines like TGF-β (32,33), shifting the local cytokine environment from T_H1 to T_H2 (23,27,34), and/or suppression by regulatory T cells (17,35,36,37). These bystander

effects have been shown to occur in systems where the APL could induce bystander suppression of self-reactive T cells (38). The bottom line is that APLs have been shown to block EAE, but the difficulty lies in establishing a good method of determining which APLs will block disease from *in vitro* data.

Altered peptide ligands can antagonize the helper T cell's signal to destroy myelin blocking the progression of EAE, suggesting that they could be used as immunotherapeutic drugs (23,24,25). However, a correlation between peptides that block disease *in vivo* and their *in vitro* characteristics has not been established. We examined various T-cell responses to a large series of peptides to determine whether there is a correlation between *in vitro* reactivity of APLs and their potential efficacy in blocking EAE *in vivo*. One of the best-characterized autoantigenic epitopes in mice is the N-terminal peptide of MBP. Both TCR and MHC contacts are well defined for this peptide (8,46,47). Using both natural and non-natural amino acids, the MBP Ac(1-11) peptide was modified by changing TCR and MHC contact sites. The amino acids were selected based on their steric and electronic features as well as their location on the peptide. After the peptides were synthesized, they were assayed for the extent to which they activated T cells using various cell-based assays that determine both early and late activation signals as well as antagonism. Among the peptides identified as antagonists, the most potent also demonstrated partial agonist activities. We conclude that it is this partial agonist activity that will make these peptides interesting *in vivo*, which has also been seen in other systems as described above (23,24,25).

MATERIALS AND METHODS

Animals: Female B10.A-H2/SgSnJ (B10.A) mice (5-6 weeks old) were received from the Jackson Laboratory (Bar Harbor, ME). The B10.A mice were kept in the animal facilities of the University of Washington (Seattle, WA) and the Medical University of South Carolina (Charleston, SC). All experiments were conducted in accordance with the animal use guidelines of these facilities.

Peptides: Myelin Basic Protein 1-11 (Ac-ASQKRPSQRHG) and altered peptides were synthesized using standard Flourenylmethoxycarbonyl (FMOC) chemistry with an Applied Biosystems 431A peptide synthesizer. The peptides were N-acetylated before being cleaved from the rink-amide resin (Novabiochem, San Diego, CA) using a ten-fold molar excess of acetic anhydride (Aldrich, Milwaukee, WI) and pyridine (Aldrich) in N-Methyl-2-pyrrolidinone (NMP) (Advanced ChemTech, Louisville, KY) on the synthesizer. Then, the peptides were cleaved with 92% triflouroacetic acid (TFA) (Aldrich), 3% thioanisole (Aldrich), and 5% water and precipitated using t-butyl methyl ether (Aldrich). Cleaved peptides were purified (C_{18}-reverse phase) using a water/acetonitrile gradient that contains 0.1% TFA. The HPLC system used was a Waters™ (Milford, MA) 2487 Dual λ Absorbance Detector with a Prep LC 25 mm Module. Fractions were characterized by high-resolution mass spectrometry (Bruker Esquire Ion Trap LC-Mass Spectrometer, Billerica, MA) using an electrospray source. The sequence and structures of non-natural amino acids of MBP

Ac(1-11) and the other APLs used in the following experiments are listed in Table 2.1 and Table 2.2.

Aza-peptides: Other peptides used in the following experiments were synthesized as part of research done into peptide mimetics known as aza-peptides by Michael Hart (unpublished data). The alpha-carbon of an amino acid residue is replaced with nitrogen in aza-peptides. MBP (1-9) was extended on the N-terminus with either four glycine residues (Ac-G4) or four aza-glycine (Aza-G4) residues as well as extended with one aza-glycine on both ends (Aza-G1). The aza-peptide, 4Aza-Ala, is analogous to MBP Ac(1-7) 4A peptide. The 4A, 6Aza-Pro peptide is similar to the MBP Ac(1-9) 4A6Pro peptide (also called 4A). The sequences are listed in Table I.

Origin of T-cell clones: B10.A mice were immunized subcutaneously at the base of the tail with 200 μg of MBP Ac(1-11) peptide in Complete Freund's Adjuvant as described (39,40). After 8 days, lymph node cells were harvested and restimulated with 40 μM MBP Ac(1-11) and irradiated B10.A spleen cells three times (8 to 10 day intervals) in order to expand the population into a T-cell line. The T-cell line was subcloned by limiting dilution. The monoclonal populations obtained were named with a letter and number description (*i.e.,* B10.AE3 and B10.AF2, abbreviated as E3 and F2 here). The T-cell clones were maintained as described below.

Maintenance of T-cell clones: B10.A clones ($1x10^6$ cells/mL) are maintained by restimulation every 10-12 days using γ-irradiated (3000 rad) splenocytes from B10.A mice (Jackson Laboratories) and the wild-type peptide, MBP Ac(1-11) 4K, (60 μM).

Clones are given 150 U/mL of recombinant mouse IL-2 (Pharmingen, San Diego, CA) with another dose (100 U/mL) given 48 hours later. The media used for the clones was RPMI-1640 (GibcoBRL, Grand Island, NY) with 10 mM HEPES (Sigma St. Louis, MO), 2.0 g/L sodium bicarbonate (Sigma), 0.05 mM 2-mercaptoethanol, 100 U/mL penicillin (Sigma), and 100 μg/mL streptomycin (Sigma). In addition, 10% heat-inactivated fetal calf serum (GibcoBRL, Australian source) was added to media when needed.

Proliferation Assays: T-cell clones (7.5 to 9.6 $x10^4$ per well) were stimulated with γ-irradiated (3000 rad) B10.A splenocytes (6-10 APCs: T cell) and various MBP Ac(1-11) peptides at 37°C and 5% CO_2 overnight (225 μL final volume). The supernatant (50 to 60 μL) was removed at 6 hours for the detection of IL-2. In addition, the supernatant (100 μL) was collected after 18 to 19 h from each well for detection of all other cytokines. The plates containing supernatant were frozen at –80°C until used in ELISA assays (see below). Then, 1 μC_i of H^3-thymidine (American Radiolabeled Chemicals, Inc., St. Louis, MO) was added to each well of the plates that contained the T cells and splenocytes (39). After 21 to 24 h, the DNA was collected on filter mats by using a SKATRON Combi cell harvester (SKATRON, Sterling, VA). The samples on the filter mats were placed in biovials and 3 mL of Ecoscint scintillant (National Diagnostics, Atlanta, GA) was added. The radioactivity in decays per minute (dpm) was measured on a Beckman LS 6500 scintillation counter (Beckman, Fullerton, CA).

Antagonist Assays: Antigen-presenting cells (5.25 to 9.6 x10^5 B10.A splenocytes per well) were incubated for 3 to 4 hours with MBP Ac(1-11) 4E (3 μM) or 4A (120 μM) peptide. The cells were washed to remove unbound peptide and incubated with various concentrations of MBP Ac(1-11) APLs and T-cell clones (7.5 to 9.6 x10^4 per well) in 96 well plates. Removal of supernatant, addition of ^{3}H-thymidine, and harvesting of cells was done as in the proliferation assay.

ELISA Assays: OptEIA™ ELISA kits (Pharmingen, San Diego, CA) were used to detect mouse cytokines: IL-2, IL-4, IL-10, IL-12, and INF-γ. As mentioned above, the supernatant was collected from the proliferation assays and frozen at -80°C. The directions were followed as described in the kits. Briefly, ELISA 96 well plates (Nunc ProBind; Nalge Nunc, Rochester, NY) were first incubated with the capture antibody (purified monoclonal antibody) overnight at 4°C. In order to help prevent non-specific binding of proteins, the plates were then washed with phosphate buffered saline (PBS, Sigma) containing 0.05% TWEEN-20 (Sigma) and blocked with PBS containing 10% heat-inactivated FCS for 1 h at room temperature. The plates were washed and incubated with samples and standards for 2 h at room temperature. Unbound sample was removed and detection antibody (biotinylated monoclonal antibody) plus avidin-horse radish peroxidase conjugate was added for 1 h. The samples were given one last wash and TMB Substrate Reagent (Pharmingen) was added. The substrate is a 50:50 mixture of hydrogen peroxide in a buffered solution and 3,3',5,5' tetramethyl benzidine (TMB) in dimethyl sulfoxide and methanol. When

mixed together, TMB substrate solution reacts with peroxidase-labeled conjugates to develop a blue color. The reaction was quenched after half an hour with concentrated sulfuric acid, which changes the color from blue to yellow. The absorbance was measured on a Molecular Devices $Thermo_{max}$ plate reader (Molecular Devices, Sunnyvale, CA) at 450 nm (minus 570 nm for wavelength correction) within 30 min of stopping the reaction. A standard curve was produced and used to convert the absorbance data for the samples into concentration of cytokine (pg/mL). In the case of IL-2, the concentration and volume of media in the well at the time of collection (0.225 mL) were multiplied.

RESULTS AND DISCUSSION

Altered MBP peptide design

Binding of the peptide to the MHC complex is crucial to T-cell activation. In crystal structures of peptide/MHC class II complexes (4,5,6), the peptide backbone shows a similar extended conformation in the MHC protein's binding cleft. Individual side chains of the peptide "fit" into pockets (P1 to P9) of the MHC and are called MHC contact residues (Figure 1.2), which results in stable binding and dissociation half times of tens to hundreds of hours (41). In contrast, MBP Ac(1-11) binds to its MHC class II protein (I-A^u and I-A^k) leaving the N-terminal portion of the cleft empty (7,8). The half-times for MBP Ac(1-11) 4K dissociating from MHC proteins are <3 minutes for I-A^k and ~15 minutes for I-A^u (42).

The MHC class II proteins, I-A^k and I-A^u, are very similar MHC proteins and differ primarily in which amino acids line the side-chain-binding pockets (43). MBP Ac(1-11) bound to the I-A^u MHC protein has been shown to bind only half of the MHC binding cleft, so that the first MHC pocket (P1) is not occupied (7,8, see Figure 1.2 as well). In both MHC proteins, the P6 pocket is a deep anchoring pocket. In I-A^u, the β9 residue lining the P6 pocket is a valine, but in I-A^k, it is a histidine that would be protonated at endosomal pH (8). Changing lysine-4 in MBP Ac(1-11) to a more hydrophobic residue like alanine increases peptide binding to both MHC proteins. MBP Ac(1-11) 4A dissociates from the MHC class II, I-A^k, with a dissociation half time of ~30 minutes (44) and from I-A^u with a dissociation half time range of 10-30 minutes (45). Since the MBP peptide 4A has a higher affinity for its class II MHC, it is often used as the "core" peptide (42). For this reason, all MBP Ac(1-11) peptides were synthesized on the 4A, rather than 4K, background. In addition, changing residue 4 to a glutamic acid (4E) increases the binding to I-A^k by >20-fold over wild-type and ~2-fold over MBP Ac(1-11) 4A (8, 40). However, the 4E peptide decreases the binding to I-A^u by at least 10-fold (8). The increase in binding to I-A^k of the 4E peptide over the 4A peptide was taken advantage of in the antagonism assays, which will be discussed later. The increase in binding of 4E over 4A can be explained by the histidine-valine polymorphism in the P6 pocket (8), where histidine in the P6 pocket of I-A^k would be positively charged at endosomal pH attracting the negative charge of the glutamic acid. Besides the wild-type peptide's lysine-4 in the P6 pocket, other MHC contact residues that have less effect on binding

to the MHC are serine-2 and serine-7 which bind in the P4 and P9 pockets of the MHC, respectively (7,8).

We substituted the major TCR contact sites with natural and non-natural amino acids based on their steric and electronic features. Glutamine-3, arginine-5, and proline-6 are the major TCR contact sites (7,8,46,47). TCR contact sites were altered using MBP Ac(1-11) 4A as the "core" peptide for reasons described above. Arginine-5 has been defined as a major TCR contact residue of the MBP peptide (7,8,46,47). Not only does arginine-5 contact the TCR, the aliphatic portion of arginine-5 binds in the P7 pocket of the MHC (8,46). One substitution made at this position is citrulline (Table 2.2). Citrulline is isosteric with arginine, but is not positively charged at physiological pH. Other TCR contact sites are glutamine-3 and proline-6. Table 2.2 shows other mutations made at position 3. Several of the substituted residues at this position are more hydrophobic amino acids like alanine that lack the hydrogen bond donor and acceptor properties of glutamine. These substitutions will explore the steric considerations of adding extra methylene and methyl groups (Table 2.2). Other amino acids, like histidine, maintain some of the hydrogen bond donor and acceptor properties, but vary in size. Glutamic acid is negatively charged at physiological conditions, while glutamine is isosteric but neutral. The third TCR contact residue, proline-6, has a significant effect on the conformation of the peptide backbone because it is a conformationally rigid amino acid. Therefore, we must be careful when substituting at residue 6 because altering the peptide conformation can disrupt T-cell recognition at arginine-5. Substituting proline-6 with hydroxyproline maintains the

proline's peptide backbone constraints while still affecting T-cell recognition (see Table 2.2).

The four aza-peptides listed in Table 2.1 are part of research into peptide mimetics by a coworker, Michael Hart (55 and unpublished data). Aza-peptides substitute the alpha carbon for nitrogen in the amino acid (see Figure 2.1) and the substitution forms a monoalkyl-diacyl hydrazine (55). These aza-peptides also seem to be more resistant to amino- and carboxy- peptidases making them more resilient *in vivo* than natural peptides (48), which is a benefit for peptide-based drug design. The Spartan molecular modeling program was used to calculate *ab initio* energies for the energetics of rotation about the ϕ dihedral angle. Through molecular modeling, aza-peptides appear to adopt a conformation similar to that of the type-II polyproline helix found in peptides properly bound to an MHC protein (48). For our experiments, an aza-alanine and an aza-proline were substituted in MBP Ac(1-7) or Ac(1-9) for alanine and proline in the peptides, 4Aza-Ala and 4A6Aza-Pro (Table 2.1). The length of the peptide is irrelevant here because only the first 6 residues are required for MHC binding and T-cell activation (46). The Aza-G1, Ac-G4, and Aza-G4 peptides (see Table 2.1) are MBP (1-9) that has been extended at the N-terminus with glycine or aza-glycine residues. It has been shown that extending the MBP (1-14) 4Y peptide on the N-terminus with Ova (323-328) residues can increase the binding to the MHC, and the extended peptide can still trigger cytokine production by an MBP-specific T-cell clone (8). The E3 T-cell clone's responses to these aza-peptides are summarized in Table 2.3.

Measuring T-cell proliferation using ^{3}H-Thymidine incorporation

The peptides mentioned above were initially screened against the B10.AE3 T-cell clones using a proliferation assay. T-cell clones were stimulated with γ-irradiated B10.A splenocytes and various MBP peptides for 16 to 20 h followed by a 21 to 24 h dose of 1μC_i of ^{3}H-thymidine per well. The radioactivity due to ^{3}H-thymidine incorporated into the DNA of proliferating cells was measured with a scintillation counter. Figure 2.2 shows the proliferation of E3 T cell clones due to only the agonist and partial agonist peptides. The MBP Ac(1-11) peptides (4A, 4E, 4A6Hyp, 4A6Sar, and 4A6MeAla) shown in plot A of Figure 2.2 are defined as agonist peptides (see also Table 2.3). These peptides cause full (100%) proliferation (at least) as compared with the known powerful agonist peptide, 4A (49). The peptides in plot B (Figure 2.2) are a combination of weak and partial agonists. The weak agonists (*i.e.,* 4K) reach the same %maximum of proliferation as the agonists, but with a much higher peptide concentration. The partial agonists (*i.e.,* 4A6Pip) reach a %maximum of proliferation that is significantly lower than the agonist peptides, even at high peptide concentrations. Both the Ac-G4 and Aza-G4 are categorized as partial agonists. Both of their cytokine production was different from the 4A peptide, even though Ac-G4's maximum proliferation was similar to the 4A peptide. Other peptides were screened using the B10.AE3 clones and these are summarized in Table 2.3. The peptides 4A6Pip, 4A6Sar, and 4A6MeAla are also agonists or partial agonists for other B10.A T cell clones (D. Beaudoin, Chapter 4).

Determination of antagonist peptides using prepulse assays

Prepulse assays were used to measure antagonism (13). The 4E peptide was used rather than the 4A peptide for the prepulse assays since the 4E peptide binds to I-A^k ~2-fold better than 4A (8,40). Nevertheless, the 4A was still used as the positive control since all other APLs were based on the 4A background. Under prepulse conditions, only a small number of MHC molecules will be occupied by 4E peptide, which leaves a large number of MHC molecules on the APC capable of interacting with other peptides (50, 51). The 4E prepulsed APCs were incubated with APLs at varying concentrations and E3 T-cell clones (Figure 2.3A). At 0 μM APL (shown as 1 x 10^{-5} μM concentration in Figure 2.3B due to log axis), 100% proliferation represents the proliferation of E3 T cell clones due to the 4E prepulse only. Notice this is only a suboptimal proliferation since increasing concentrations of agonist peptide (4A) causes further proliferation (Figure 2.3B). The negative control was the 4A5K peptide, which does not cause proliferation or cytokine production in E3 clones (Table 2.3). Prepulsing with agonist peptide should eliminate MHC competition since the amount of agonist peptide occupies only a fraction of the number of MHCs present on the APC. The null 4A5K peptide should show simply the same as no APL (100% proliferation) even at high concentrations. However, we did see a small drop with the 4A5K peptide, which implies that either there is MHC competition or the 4A5K peptide is a weak antagonist. In order to investigate this further, we used an epitope of hen egg white lysozyme (HEL (46-61), NTDGSTDYGILQINSR) that forms stable complexes with I-A^k proteins (45) and will not stimulate or antagonize MBP-specific

T cell clones. The proliferation measured with the HEL peptide also dropped a little at high peptide concentration (data not shown), which cannot be due to the antagonism of the E3 T cell clone suggesting some small amount of MHC competition or toxicity at very high peptide concentrations. Thus, the APL must decrease the %proliferation significantly below that of the 4A5K in order to be classified as an antagonist.

Cytokine Production by T-cell clones due to APLs

Cytokine release was also determined using BD Pharmingen OptEIA^{TM} ELISA kits as described by the manufacturer's instructions. The optimum time at which IL-2, IL-4 and IL-10 should be collected was determined by collecting supernatant at 6, 19, and 28 hours (Figure 2.4). The time at which IL-2 production reached a maximum was at 6 hours. The IL-4 and IL-10 concentrations were measured after 18 to 19 hours. IL-2 production was not detectable at 18 hours for any of the peptides, which suggests the IL-2 is being utilized almost immediately by the clones (results not shown). IL-2 production for some representative peptides is shown in Figure 2.5. The agonists and weak agonists produce the most cytokine while the other peptides produce little to no IL-2 (see Table 2.3).

The E3 clones produce significant amounts of IL-4 and IL-10 with no IL-12 or INF-γ, which defines them as T_H2 T cells. The dose response curves for IL-4 and IL-10 production are shown in Figure 2.6. In general, the E3's produce more IL-10 than IL-4 for most of the peptides. In addition, the strong agonists seem to produce the most of these two cytokines, but only at concentrations well beyond the maximal amount of proliferation (compare Figures 2.2 and 2.6). In addition, the APL 4A6Sar

and not the 4A agonist produced the most IL-4 and IL-10 while the wild-type 4K produced very little IL-4 and a respectable amount of IL-10. It appears that the signaling threshold for cytokine production is higher than the threshold for proliferation, at least for the E3 clones (see Chapter 3).

The MBP peptides were divided into subsets depending on their half-maximum and maximum of proliferation as well as their cytokine production. The E3 T-cell clone's responses to these peptides are summarized in Table 2.3. The responses to APL were compared to the 4A peptide and rated accordingly.

CONCLUSIONS

In general, the B10.AE3 clones are T_H2 based on their ability to produce only the cytokines, IL-4 and IL-10, which agrees with preliminary data on the clones when they were subcloned (K. Tate, unpublished results). The trend in cytokine production is agonists>weak agonists>partial agonists> null peptide (Figure 2.6 and Table 2.3), which is similar to the proliferation trend. However, the dose response curves for IL-4 and IL-10 (Figure 2.6) have their maximum cytokine production shifted to higher concentrations where the proliferation of E3 has started to decline (D. Beaudoin, Chapter 3). In addition, the APLs were not able to induce a cytokine shift from T_H2 to T_H1, which suggests that once differentiated, the E3 T cell clone cannot change from type 2 to type 1, which has been reported in the literature for other T cells (52).

One of the first observations that immediately came to our attention is that the E3 T-cell clones show a fair amount of flexibility in their response to mutations at

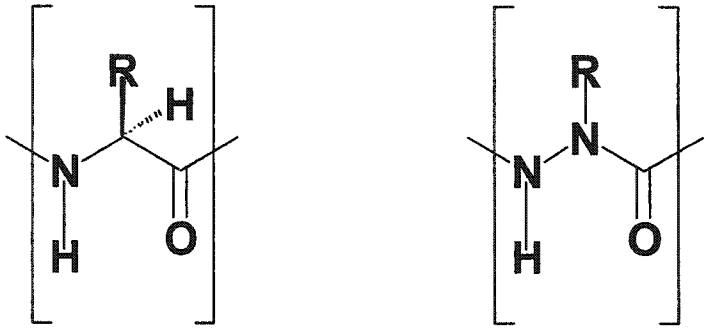
proline-6 of the MBP Ac(1-11) peptide as shown by the proliferation assays (Figure 2.2 and Table 2.3), which would suggest that this is a secondary TCR contact for this clone as defined by Paul Allen (9). Almost all the substitutions, except for dipropyl glycine (Dpg), in position-6 have produced peptides that fit into the agonist or partial agonist subset. Dipropyl glycine may be sterically too hindered. After all, it is the only APL in the group that has two alkyl substituents at the alpha-carbon. In addition, Dpg does not have a methyl or methylene substituent on the nitrogen like the other peptides 4A6Pro (4A peptide), 4A6Hyp, 4A6Sar, 4A6MeAla, and 4A6Pip. The N-alkyl amino acids have been shown to increase binding activity for some proline-favoring proteins and they confer some protease resistance (53,54). Responses to substitutions at arginine-5 (only a few) and glutamine-3 are less flexible. Substitution of glutamine-3 with homoserine produces a partial agonist/antagonist. Others, 3A, 3Nva, 3L, and 3S, do cause some proliferation at extremely high concentrations, but only when the cells are healthy and more sensitive to activation. Most of these are also good antagonists. Although the four aza-peptides shown in Table 2.3 were not powerful inhibitors of proliferation, they did fall into the weak agonist and partial agonist category, which gives support for using aza-peptides as peptide mimetics (55).

Besides proliferation and cytokine production, these peptides were screened for antagonism. Only about 40% of the peptides screened for antagonism were determined to be antagonist peptides for the E3 clone. Most of these were APLs substituted in the third position of the MBP Ac(1-11). Others and we have shown that APLs with single amino acid substitutions can antagonize responses to the antigenic

peptide. This antagonism is not due to competition for peptide binding to MHC and it is not due to competition for binding of peptide-MHC complexes to TCRs (56,58). One mechanism that accounts for T-cell antagonism is the Kinetic Discrimination Model (56). This model assumes that the peptide-MHC ligand binds to the TCR and the length of time that the receptor is bound to ligand determines the extent of receptor activation, which is the same assumption used in the Kinetic Proofreading Model (57). Both models assert that if the ligand remains bound long enough for complete receptor activation, then a complete signal is generated that leads to full proliferation and cytokine production (agonism). In the Kinetic Discrimination Model it was argued that if the ligand dissociates prior to complete receptor activation then the partly activated receptor will inhibit further activation of other TCRs on the T cell (antagonism).

The Kinetic Discrimination Model predicts that ligands whose average TCR dissociation rate is very fast will mostly do nothing short of generating an occasional inhibitory signal from the few that bind longer than average. These ligands would be weak antagonists. Ligands whose average dissociation rate is moderate will mostly generate inhibitory signals and an occasional activation signal due to the few ligands that bind longer than average. At very high concentrations these same peptides could rebind partly activated receptors and then generate complete signals. This unusual class of ligands would exhibit potent antagonism at low concentrations and partial agonism at higher concentrations. Indeed, we observed that the most powerful MBP antagonists are also partial agonists (*i.e.*, 4A3Hse and 4A6Pip). The duality of partial

agonism and antagonism has also appeared in the literature. For example, it has been shown that the human immunodeficiency virus (HIV) and hepatitis-B virus produce natural variants of antigen peptides that potently inhibit the cytotoxic T lymphocyte (CTL) response even though the peptides are also partial agonists at higher concentrations (58,59). Finally, with respect to using APLs to block autoimmune disease, Steinman and coworkers identified three antagonists *in vitro* but only one blocked disease (23). Careful examination of the published data describing this work shows that the one APL that did block disease was also a partial agonist *in vitro* and the other two were not (although this observation was not noted by the authors). Clearly, the phenomenon of partial agonism deserves special attention, and we suggest that this is likely to be a characteristic of APLs that block disease. Since it is relatively straightforward to screen partial agonism, these results are likely to enable efficient screening of APLs *in vitro* to select the most effective candidates for pre-clinical *in vivo* studies.



Peptide Aza-Peptide

Figure 2.1: Comparing the peptide structure with the aza-peptide structure. The alpha-carbon of the peptide is replaced with a nitrogen in the aza-peptide.

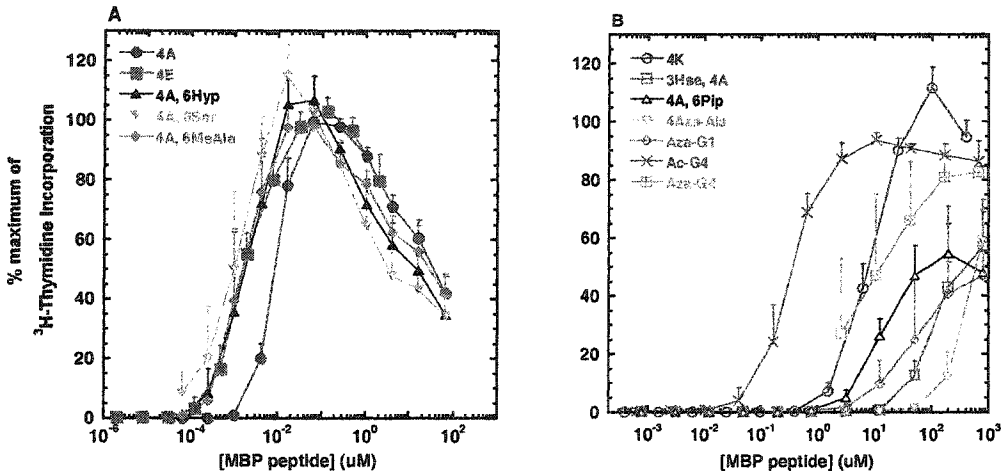


Figure 2.2: B10.AE3 proliferation by MBP altered peptide ligands. Plots A and B show the proliferation of B10.AE3 T-cell clones due to APLs in percent relative to the maximum proliferation of the 4A peptide measured as ^{3}H-thymidine uptake. The data shown are averages of 3-6 separate experiments. (The maximum average range was $28x10^{3}$ to $503x10^{3}$ decays per minute (dpm)). The background measurement (T cells and APCs in media) was less than 3% of the maximum dpm. The error bars represent the average deviation and only the top half is shown. The two graphs have been separated in order to see data for all 12 peptides clearly. Peptide sequences are listed in Table 2.1 and only agonists and partial agonists are shown. (Abbreviations – Hse, Homoserine; Hyp, Hydroxyproline; MeAla, N-Methyl Alanine; Pip, Pipecolic Acid; and Sar, Sarcosine).

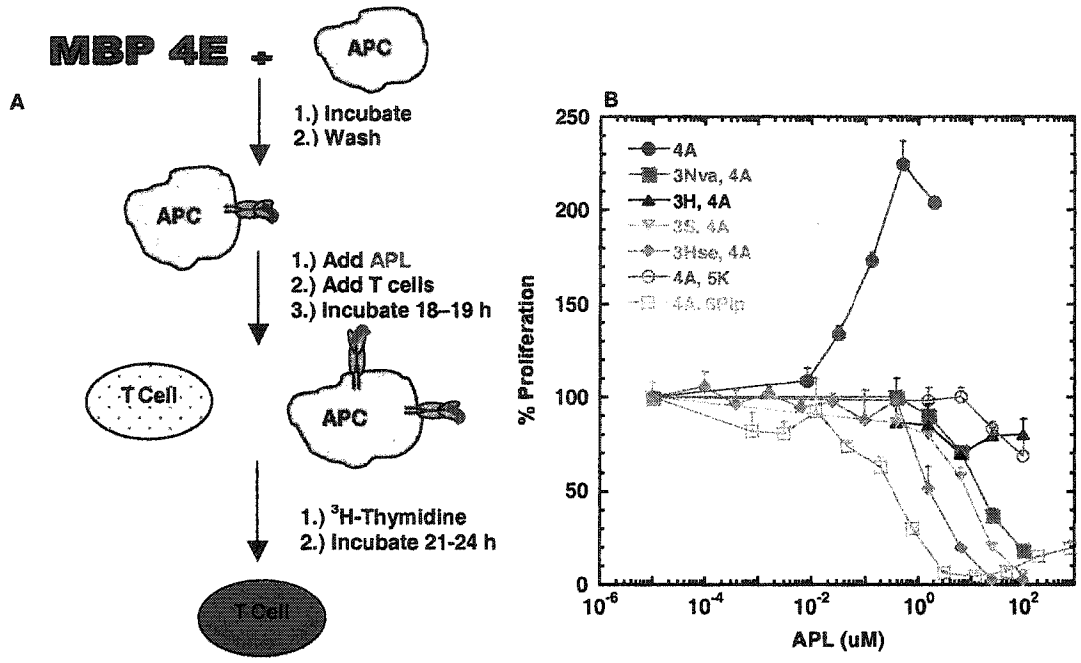


Figure 2.3: Antagonism of B10.AE3s by MBP altered peptide ligands. A.) B10.A splenocytes (APCs) were prepulsed with 3 uM 4E peptide for 3 to 4 h and washed. Then, prepulsed APCs and T cells were added to 96 well plates that already contained APLs at various concentrations and incubated overnight. Then, ^{3}H-Thymidine (1 μC_i/well) was added and incubated for 21 to 24 h. B.) The plot shows antagonism as a function of proliferation in the presence of APL relative to the proliferation of the 4E peptide without APL (shown as 10^{-5} μM APL rather than 0 μM because of log plot). The data shown is from one representative experiment out of at least three. (The average maximum due to just the 4E prepulse (at 0μM APL) is $110x10^3$ dpm). The error bars represent the average deviation of two trials and only the top half is shown. Peptide sequences are shown in Table 2.1, and not all peptides are shown for clarity. (Abbreviations – Hse, Homoserine; Nva, norvaline; and Pip, Pipecolic Acid).

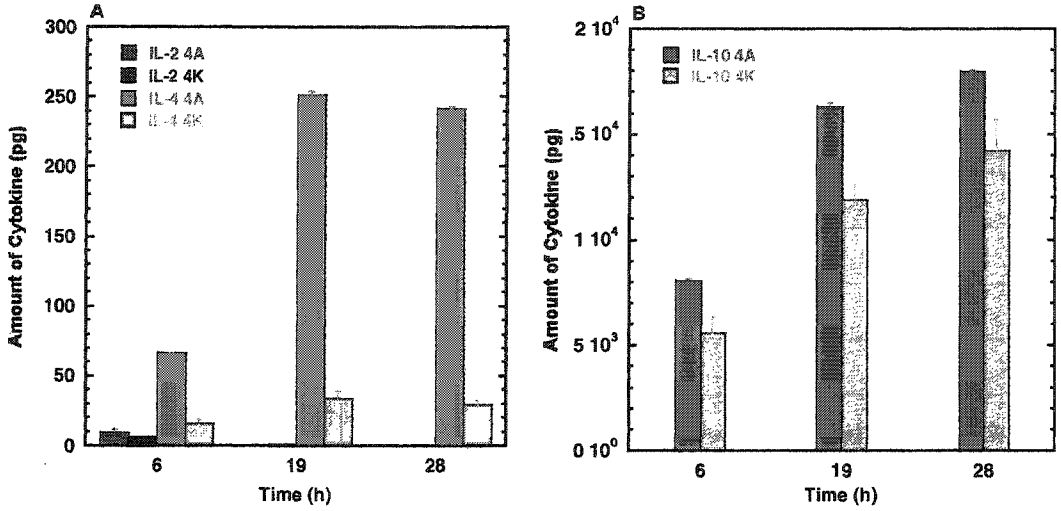


Figure 2.4: Determining the optimum time to collect B10.AE3 supernatant to measure cytokine production. The concentration of MBP Ac(1-11) 4A and 4K peptides was 67 μM and 800 μM, respectively. The concentration of peptide was chosen based on the highest concentration measured in proliferation assays that could give measurable amounts of cytokine. Supernatant from proliferation assays was removed after 6, 19, and 28 h. Cytokine production was measured as absorbance at 450 nm using BD Pharmingen (San Diego, CA) OptEIA^{TM} ELISA kits (Plot A: IL-2 and IL-4 and Plot B: IL-10). A standard curve was produced and used to convert the absorbance data for the samples into concentration of cytokine (pg/mL). Then, the concentration of cytokine (pg/mL) was multiplied by the volume (0.225 mL) in the well at the time the supernatant was collected to get the amount of cytokine (pg). The data shown represents one experiment with 2 trials for each time point. The error bars represent the average deviation. ELISA experiments were repeated at 6 h for IL-2 and 18-19 h for IL-4 and IL-10.

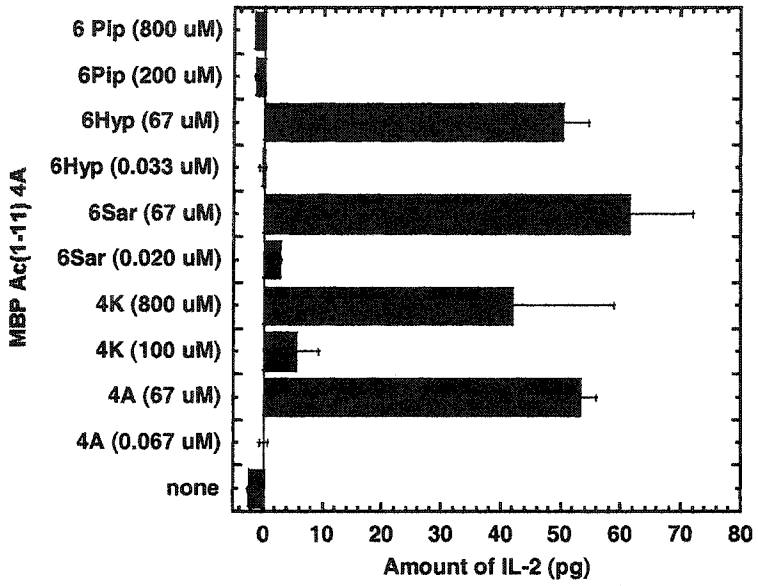


Figure 2.5: IL-2 production by B10.AE3s stimulated by APLs. B10.AE3 production of IL-2 was measured using an ELISA assay as described in the methods section. The concentration of peptide is located in parenthesis next to the APL above. Concentrations chosen for assay were based on the amount of peptide required to reach maximal proliferation as well as a significantly greater concentration that gives more cytokine response. After 6 h, 50-60 μL per well was removed and frozen at -80°C until ELISA assay could be performed. A standard curve was produced and used to convert the absorbance data for the samples into concentration of cytokine (pg/mL). The concentration of cytokine (pg/mL) was multiplied by the volume (0.225 mL) contained in the wells at the time the supernatant was collected. Data is shown as the amount of cytokine production in total pg per well of a few representative APLs. The data shown is from one representative experiment out of two. The error bars represent the average deviation of three trials. Peptide sequences are listed in Table 2.1. (Abbreviations – Hse, Homoserine; Hyp, Hydroxyproline; Nva, Norvaline; Pip, Pipecolic Acid; Sar, Sarcosine).

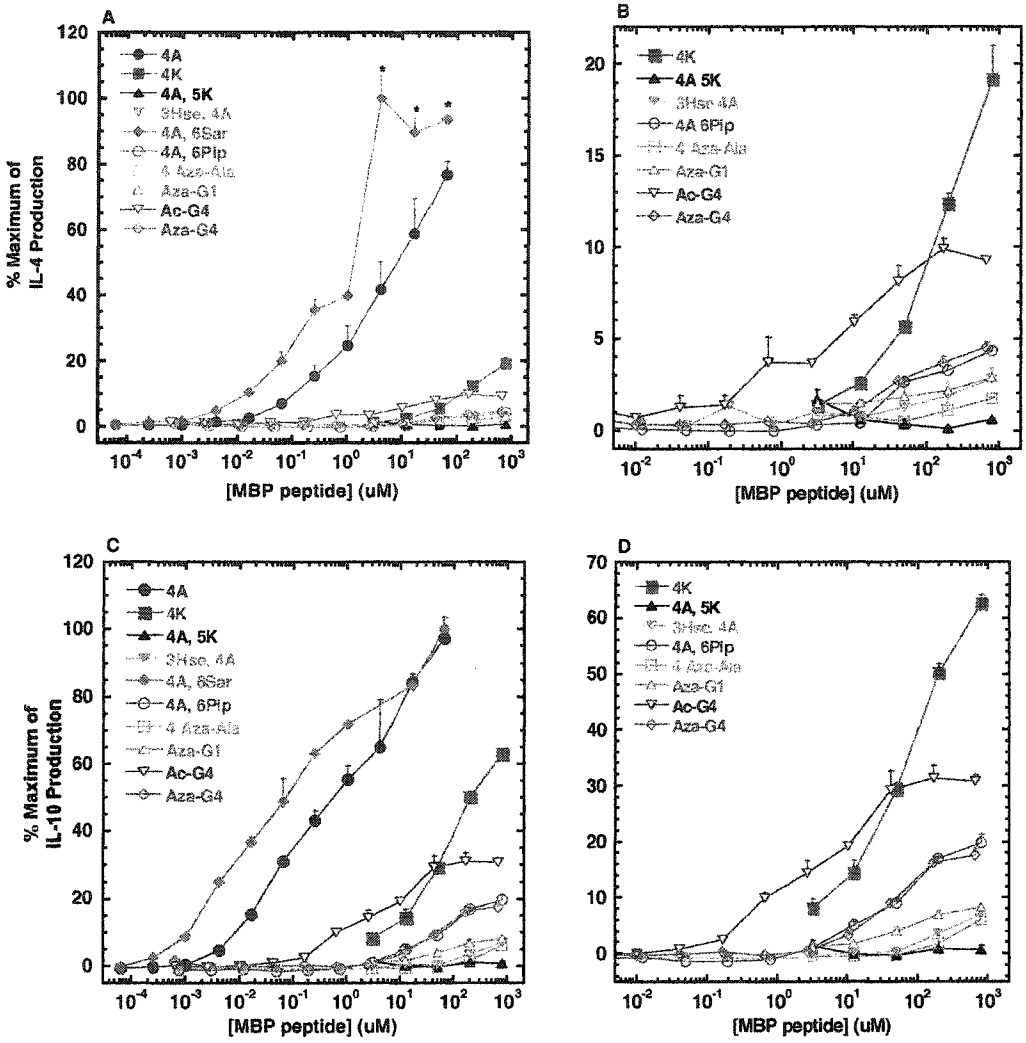


Figure 2.6: Cytokine production by B10.AE3 T-cell clones. Supernatant from proliferation assays was removed after 18 h before addition of ^{3}H-thymidine. Cytokine production was measured as absorbance at 450 nm using BD Pharmingen (San Diego, CA) OptEIA^{TM} ELISA kits (Plot A and B: IL-4 and Plot C and D: IL-10). A standard curve was produced and used to convert the absorbance data for the samples into concentration of cytokine (pg/mL). Data is shown as the maximum cytokine production in percent relative to the average of the cytokine production of the 4A6Sar peptide (at 4.2 μM for IL-4 and at 67 μM for IL-10). The averages at these concentrations are 580 pg/mL for IL4 and $58x10^3$ pg/mL for IL-10. Plots A and C show the dose response curve for IL-4 and IL-10, respectively. B and D show the cytokine production of the peptides that require significantly higher concentrations to produce less cytokine. The data shown is from one representative experiment out of at least two. The error bars represent the average deviation of two trials and only the top half is shown. The symbol, *, represents data points that were outside the linear range (at high concentrations) of the standard curve.

Table 2.1: Altered MBP Peptides. All MBP Ac(1-11) peptides were based on the N-terminal sequence of rat MBP. For the positions 3, 5, and 6 APLs, X denotes a residue that was replaced with either a natural or non-natural amino acid (see Table 2.2 and 2.3). The aza-peptide, 4Aza-Ala, was based on MBP Ac(1-7) and the last four peptides were based on MBP (1-9). Aza-amino acids are **underlined**. Michael Hart synthesized the aza-peptides (unpublished results). N-terminal acetylation is denoted as 'Ac'. All peptides were synthesized by standard FMOC chemistry.

Name	Sequence
4K	AcASQKRPSQRHG
4A	Ac-------A----------
4E	Ac-------E----------
Position 3 APLs (3X 4A)	Ac-----XA----------
Position 5 APLs (4A 5X)	Ac-------AX----------
Position 6 APLs (4A 6X)	Ac-------A--X---------
4Aza-Ala	Ac-------**A**------
4A 6Aza-Pro	Ac-------A--**P**------
Aza-G1	**G**-------A--------**G**
Ac-G4	AcGGGG-------A---------
Aza-G4	**GGGG**-------A---------

Table 2.2: Structures of amino acids substituted for TCR contact sites of MBP Ac(1-11) 4A. All amino acids used to substitute for residues in the wild-type peptide were L-amino acids. The stereochemistry is shown below as a general guide in order to simplify the rest of the structures. Abbreviations: Abu: 2-aminobutyric acid, Cit: Citrulline, Dpg: Dipropyl Glycine, Hse: Homoserine, Hyp: Hydroxyproline, MeAla: N-Methyl Alanine, Nle: Norleucine, Nva: Norvaline, Pip: Pipecolic Acid, Pra: Propargyl, Sar: Sarcosine, and Tle: Tert-Leucine.

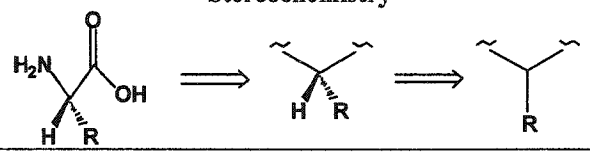
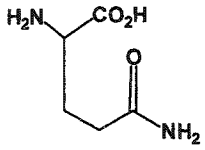
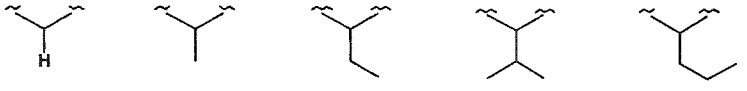
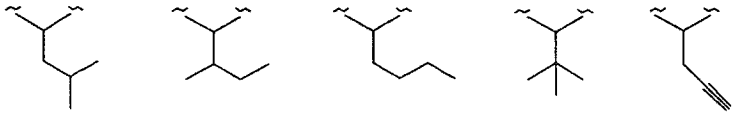
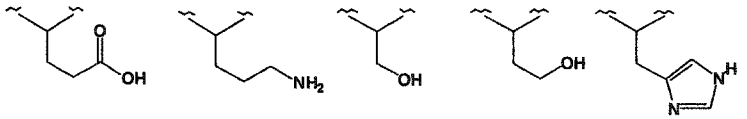
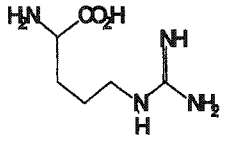
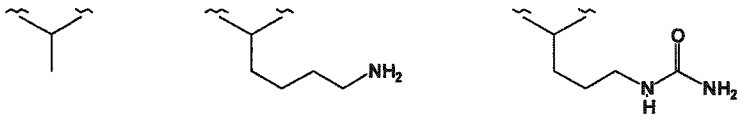
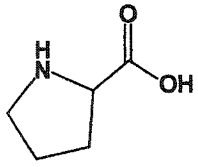
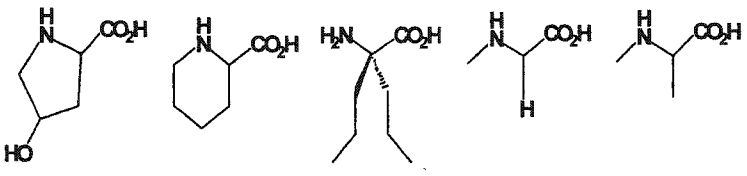
#	Wild-Type Residue	Substitutions for R
		Stereochemistry 
3	 Gln	 Gly Ala Abu Val Nva  Leu Ile Nle Tle Pra  Glu Orn Ser Hse His
5	 Arg	 Ala Lys Cit
6	 Pro	 Hyp Pip Dpg Sar MeAla

Table 2.3: Summary of responses given by B10.AE3 T cell clones. Proliferation was measured using ^{3}H-thymidine incorporation in three separate experiments except for 3I4A, 3Tle4A, 3Orn4A (two separate experiments) and 4A6Aza-Pro (one experiment). Antagonism was determined using both the 4A and 4E peptides to prepulse APCs and was repeated at least three times except for 3Tle4A, 3E4A, 5Cit4A, 6Dpg4A (twice) and 4Aza-Ala (once). Cytokine production was measured using OptEIA™ ELISA kits (BD Pharmingen). The cytokines; IL-2, IL-4, and IL-10; were measured at least twice for the peptides indicated in the table except for 3G4A, 3Abu4A, 3V4A, 3Pra4A, 3E4A, 3H4A, and 4A6Aza-Pro which were done only once. The cytokines, IL-12 and INF-gamma, were measured only once for the peptides indicated. The +/- in IL-12 column represents values slightly above background, and probably requires further studies. Responses not determined are represented by 'nd'. See the legend below for description of the symbols (+,-). The +/- indicates that there was little to no response or that the response was difficult to evaluate. Peptide sequences and abbreviations are located in Tables 2.1 and 2.2, and procedures are described in the Methods and Results sections.

Subsets	WT	APL (all 4A)	Proliferation Assay	Antagonism Assay	Cytokines				
					IL-2	IL-4	IL-10	INF-g	IL-12
Strong Agonists	K	4E	++++	nd	++++/+++++	++++	++++	nd	nd
	P	6Hyp	++++	-	++++	++++	++++	-	+/-
	P	6Sar	++++/+++++	-	+++++	++++/+++++	++++/+++++	-	+/-
	P	6MeAla	++++	-	+++/++++	+++/++++	++++	-	-
Agonist	K	4A	++++	-	++++	++++	++++	-	-
Wk. Agonists	K	4K	++++	nd	+++/++++	++	+++	-	+/-
Partial Agonists	Q	3Hse	++/+++	+++	-	+/-	+/-	-	nd
	A	4Aza-Ala	++/+++	-	-	+/-	+/-	nd	nd
	P	6Pip	++/+++	+++	+/-	+/-	+/++	-	-
	P	6Aza-Pro	++++	nd	nd	++/+++	+++	nd	nd
		Ac-G4	+++/++++	-	+/-	+/++	++	nd	nd
		Aza-G4	+++/++++	-	-	+/-	+/++	-	-
		Aza-G1	++/+++	+/-	-	+/-	+/-	nd	nd
Very Weak Partial Agonists	Q	3A	+/-	-	-	-	-	nd	nd
	Q	3Nva	+/-	++	+/-	-	-	nd	nd
	Q	3L	+/-	++	+/-	-	-	nd	nd
	Q	3S	+/-	++	+/-	-	-	nd	nd
Antagonists Only	Q	3Abu	-	+/++	nd	-	nd	-	nd
	Q	3V	-	+	nd	-	nd	-	nd
	Q	3I	-	+/++	nd	nd	nd	nd	nd
	Q	3Nle	-	+	nd	nd	nd	-	nd
	Q	3G	-	+/-	-	-	-	nd	nd
Null Peptides	Q	3Tle	-	-	nd	nd	nd	nd	nd
	Q	3Pra	-	-	nd	-	nd	-	nd
	Q	3K	-	-	nd	nd	nd	nd	nd
	Q	3E	-	-	nd	-	nd	-	nd
	Q	3Orn	-	-	nd	nd	nd	nd	nd
	Q	3H	-	-	nd	-	nd	-	nd
	R	5K	-	-	-	-	-	nd	nd
	R	5Cit	-	-	nd	-	nd	-	nd
	P	6Dpg	-	-	nd	nd	nd	nd	nd

LEGEND

Proliferation and Cytokines

+++++	Significantly better (>120%) than the Rat MBP (1-11) 4A peptide
++++	Gives maximum amount of proliferation (100-120%) compared to Rat MBP (1-11) 4A peptide
+++	Gives only 50 to 99% maximum proliferation
++	Gives only 5 to 49% maximum proliferation
+	Very weak response (< 5%)
-	No response

Antagonism

+++	<5 uM to cause 50% inhibition of 4A or 4E prepulse (EXCELLENT)
++	6<X<25 uM to cause 50% inhibition of 4A or 4E prepulse (AVERAGE)
+	26<X<50 uM to cause 50% inhibition of 4A or 4E prepulse (WEAK)
-	>50 uM to cause 50% inhibition of 4A or 4E prepulse (little to no antagonism)

NOTES TO CHAPTER 2

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Chapter 3

Dissociating Cytokine Production from T-Cell Proliferation

ABSTRACT

Helper T cells (or CD4+ T cells) are activated by cognate antigen bound to the major histocompatibility complex (MHC) class II proteins on the surface of antigen-presenting cells (APCs). Antigens can produce a full spectrum of T-cell responses ranging from no response (null ligand) to full activation (agonist ligands). Some ligands even block the response of agonist ligands and are called TCR antagonists. On the other hand, partial agonist peptides can elicit some effector functions and not others. It has been proposed that different effector functions (*i.e.*, cytokines or proliferation) have different thresholds of activation. The B10A.E3 T cell clones respond to MBP Ac(1-11) 4K peptide bound to the I-A^k MHC class II protein. Different signaling thresholds for effector functions of this MBP-specific-T-cell clone were identified using cell-based assays. Although the extent of response differs depending on the dose of the altered peptide ligand (APL), the hierarchy of response (proliferation<IL-10<IL-4) is the same. These results are discussed in the context of a kinetic model for T-cell activation.

INTRODUCTION

As discussed in Chapter 2, the immune system generates a wide variety of cells and molecules that are capable of eliminating foreign pathogens (1,2). Helper CD4+ T-cells are activated by antigen bound to the major histocompatibility complex (MHC) class II on the surface of antigen-presenting cells (APCs). In the presence of their cognate antigen, the T cells become activated and cause a cascade of signaling events that results in cytokine production and proliferation (3). When the peptide/MHC class II complex on the surface of antigen-presenting cells (APCs) binds to the T-cell receptor (TCR) on CD4+ T cells, the initial reaction (in seconds) appears to be tyrosine phosphorylation of the TCR-associated CD3 proteins by *src* family kinases (reviewed in ref. 4 and Chapter 1). Phosphorylation of the CD3 ζ-chain recruits the *syk*-family tyrosine kinase, ZAP-70 (5,6), which, when activated by tyrosine phosphorylation by Lck, can lead to activation of various pathways including phospholipase C-γ and the mitogen-activated protein kinase (MAPK) cascade (reviewed in ref. 4,7). The phospholipase C-γ pathway eventually leads to an increase in intracellular calcium. Both pathways lead to activation of transcription factors that initiate the synthesis of various cytokines and other proteins (8) producing second messengers important to the maintenance of the immune response. All of these signaling events will lead to proliferation of the T cell and other effector functions that control and regulate the immune system.

T cell activation is not merely an on/off switch, but a complex series of various pathways leading to transcription of important genes. By simply making conservative amino acid substitutions to T-cell contact residues of the peptide, the T-cell response can be varied over a large spectrum of different activities (9,10,11). Peptides that are varied only by a few amino acids are called altered peptide ligands (APLs). These APLs complexed with the proper MHC protein can modify the response of the T cell (12,13,14). As discussed in the previous chapter, agonists cause full proliferation and cytokine production and partial agonists can cause an intermediate response. TCR antagonists block the response caused by agonist ligands. Each of these effector functions activated by agonist or partial agonist peptides can stimulate either all or a subset of the T-cell's effector functions, which can modulate the immune response. Some of these early activation events include phosphorylation of the TCR-associated CD3 intracellular domains, an increase in intracellular calcium concentration, and increased acid extrusion (3). Late events include production of cytokines and proliferation.

Mechanisms to explain how the TCR can distinguish between agonist and antagonist ligands have been suggested. The simplest mechanism is that the affinity or avidity of the ligand for the T-cell receptor determines the extent of T-cell stimulation, that is, the higher the affinity, the more receptors are bound at a given ligand concentration and the greater the signal (15). However, characterizations of partial agonists and TCR antagonists started a revision of this mechanism (16). The

observation that two different antagonist peptides with similar activities had a ~3 fold difference in affinities is evidence against this equilibrium model (17). Second, the TCR dissociates from ligands with an off-rate of seconds (18,19) and antagonist peptide-MHC complexes have been shown to have faster off-rates than agonist ligands, which suggests that the lifetime of the TCR/peptide/MHC complex (the tri-molecular complex) is the determining factor for full or partial activation (17,20). For example, ligands with the same activity have been shown to have different affinities, but the same TCR off-rates (17). More importantly, since antagonist ligands have faster off-rates and, thus, lower affinities, than agonist, they would not be able to compete for TCRs (the ratio of TCRs to ligand is on the order of one thousand or higher).

The observations that the extent of T-cell activation correlates with the TCR-ligand off-rate has led to at least two kinetic mechanisms for T-cell signaling, the Kinetic Discrimination (19) and Kinetic Proofreading Models (18). Both models assume that the TCR has to remain bound to its peptide-MHC ligand for some period of time before the receptor is fully activated. One possible candidate for the time-dependent process is phosphorylation of the intracellular domain of the CD3 ζ-chain, which has twelve tyrosines that can be phosphorylated. Ligands can give different patterns of phosphorylation that correlate with the agonist or antagonist activity of the ligand (21,22,23). Complete phosphorylation leads to recruitment and activation of the tyrosine kinase ZAP-70. Partial phosphorylation is responsible for the failure to

activate ZAP-70 (21,22). In the Kinetic Discrimination Model it was suggested that the partly phosphorylated receptor is inhibitory to the activation of other TCRs. Thus, ligands that dissociate rapidly from the TCR would be antagonists.

Experimental data from Rabinowitz, *et al.*, showed that the extent of early T-cell activation signals, such as ζ-chain phosphorylation or calcium signaling, correlated with the duration of TCR binding (3), which supports the Kinetic Discrimination Model. Their results also suggested that the threshold for TCR signaling that produced a maximal level of downstream signals differed for each of the downstream signals. For example, almost all ligands, including some antagonists, caused an increase in acid extrusion while only ligands with moderate to long TCR-duration lifetimes caused complete calcium signaling. The concept of a threshold for signaling was clearly supported by results from a study reported by Wülfing, *et al.*, which demonstrated that there is a threshold for TCR signaling (*i.e.*, number of activated TCRs per unit time) that determined whether a T cell would flux calcium (42). It was proposed that the threshold of TCR signaling required for calcium signaling could be different than for other downstream signals or effector functions. Indeed, results from Vallitutti, *et al.*, suggested that the level of TCR signaling needed for cytotoxic T lymphocyte (CTL) killing is lower than for CTL proliferation (24).

In this chapter it is shown that the TCR signaling thresholds for cytokine production by the B10A.E3 MBP Ac(1-11)-specific T-cell line are different from the threshold for proliferation. Using both natural and non-natural amino acids, the MBP

Ac(1-11) peptide was modified by changing TCR and MHC contact sites. After the peptides were synthesized, they were assayed for the extent to which they activated T cells using cell-based assays that determine both cytokine production and proliferation. It is shown that for a series of APLs of MBP Ac(1-11) there is a hierarchy of signaling thresholds for effector functions. The threshold for proliferation is lower than the threshold for IL-4 and IL-10 production by the T-cell line. Within the cytokines IL-4 and IL-10, there is also a hierarchy of thresholds. The threshold of TCR signaling required for maximal IL-10 secretion appears to be lower than the threshold for IL-4 secretion. These results suggest that generally there may be a higher TCR-signaling threshold for effector functions than for proliferation, which is consistent with the idea that the immune system places a higher level of stringency on function that on proliferation.

MATERIALS AND METHODS

Animals: Female B10.A-H2/SgSnJ (B10.A) mice (5-6 weeks old) were received from the Jackson Laboratory (Bar Harbor, ME). The B10.A mice were kept in the animal facilities of the University of Washington (Seattle, WA) and the Medical University of South Carolina (Charleston, SC). All experiments were conducted in accordance with the animal use guidelines of these facilities.

Peptides: Myelin Basic Protein 1-11 (Ac-ASQKRPSQRHG) and altered peptides were synthesized using standard Flourenylmethoxy carbonyl (FMOC) chemistry with

an Applied Biosystems peptide synthesizer as discussed in Chapter 2. Peptides were purified (C_{18}-reverse phase) using a water/acetonitrile gradient that contains 0.1% TFA. The HPLC system used was a Waters™ (Milford, MA) 2487 Dual λ Absorbance Detector with a Prep LC 25 mm Module. Fractions were characterized by high-resolution mass spectrometry (Bruker Esquire Ion Trap LC-Mass Spectrometer, Billerica, MA) using an electrospray source. The sequence and structures of non-natural amino acids of MBP Ac(1-11) and the other APLs used in the following experiments are listed in Table 3.1.

Origin of T-cell clones: B10.A mice were immunized subcutaneously at the base of the tail with 200 μg of Rat MBP Ac(1-11) peptide in complete Freund's adjuvant as described (25,26). After 8 days, lymph node cells were harvested and restimulated with 40 μM MBP Ac(1-11) and irradiated B10.A spleen cells three times (8 to 10 day intervals) in order to expand the population into a T-cell line. The T-cell line was subcloned by limiting dilution. The monoclonal populations obtained were named with a letter and number description (*i.e.,* B10.AE3 and B10.AF2 or E3 and F2 for short). The T-cell clones were maintained as described below.

Maintenance of T-cell clones: B10.A clones ($1x10^6$ cells/mL) are maintained by restimulation every 10-12 days using γ-irradiated (3000 rad) splenocytes from B10.A mice (Jackson Laboratories) and the wild-type peptide, MBP Ac(1-11) 4K, (60 μM). Clones are given 150 U/mL of recombinant mouse IL-2 (Pharmingen, San Diego, CA) with another dose (100 U/mL) given 48 hours later. The media used for the clones

was RPMI-1640 (GibcoBRL, Grand Island, NY) with 10 mM HEPES (Sigma St. Louis, MO), 2.0 g/L sodium bicarbonate (Sigma), 0.05 mM 2-mercaptoethanol, 100 U/mL penicillin (Sigma), and 100 μg/mL streptomycin (Sigma). In addition, 10% heat-inactivated fetal calf serum (GibcoBRL, Australian source) was added to media when needed.

Proliferation Assays: T-cell clones (7.5 to 9.6 $x10^4$ per well) were stimulated with γ-irradiated (3000 rad) B10.A splenocytes (6-10 APCs: T cell) and various MBP Ac(1-11) peptides at 37°C and 5% CO_2 overnight (225 μL final volume). The supernatant (100 μL) was collected after 18 to 19 h from each well for detection of IL-4 and IL-10. The plates containing supernatant were frozen at –80°C until used in ELISA assays (see below). Then, 1 μC_i of H^3-thymidine (American Radiolabeled Chemicals, Inc., St. Louis, MO) was added to each well of the plates that contained the T cells and splenocytes (25). After 21 to 24 h, the DNA was collected on filter mats by using a SKATRON Combi cell harvester (SKATRON, Sterling, VA). The samples on the filter mats were placed in biovials and 3 mL of Ecoscint scintillant (National Diagnostics, Atlanta, GA) was added. The radioactivity in decays per minute (dpm) was measured on a Beckman LS 6500 scintillation counter (Beckman, Fullerton, CA).

ELISA Assays: OptEIA™ ELISA kits (BD Pharmingen, San Diego, CA) were used to detect mouse cytokines, IL-4 and IL-10. As mentioned above, the supernatant was collected from the proliferation assays and frozen at -80°C. The directions were followed as described in the kits and in Chapter 2. The absorbance was measured on a

Molecular Devices $Thermo_{max}$ plate reader (Molecular Devices, Sunnyvale, CA) at 450 nm (minus 570 nm for wavelength correction) within 30 min of stopping the reaction. A standard curve was produced and used to convert the absorbance data for the samples into concentration of cytokine (pg/mL).

RESULTS AND DISCUSSION

Altered MBP peptide design

MBP Ac(1-11) 4K peptide (wild-type) is an encephalogenic epitope in B10.A mice. As discussed in Chapter 2, binding of the peptide to the MHC complex is crucial to T-cell activation. In the following experiments, the MBP Ac(1-11) 4A, rather than the wild-type 4K, was used as the "core" peptide (27) due to the increased binding to the $I\text{-}A^k$ MHC class II protein (28). Major and minor TCR contact residues were altered to both natural and non-natural amino acids based on their steric and electronic features. Glutamine-3, arginine-5, and proline-6 are the major TCR contact sites (29,30,31,32). Arginine-5 has been defined as a major TCR contact residue of the MBP peptide. Not only does arginine-5 contact the TCR, the aliphatic portion of arginine-5 binds in the P7 pocket of the MHC (30,31). Replacement of arginine-5 with lysine creates a null peptide that we used as our negative control (C. Beeson, unpublished results). Peptide sequences are listed in Table 3.1.

Determining the optimum time to measure cytokine production by E3 T-cell clones

Cytokine release was determined using BD Pharmingen OptEIA™ ELISA kits as described by the manufacturer's instructions. The optimum time at which IL-4 and IL-10 should be collected was determined by collecting supernatant at 6, 19, and 28 h (Figure 3.1). The cytokines, IL-4 and IL-10, were produced at a maximum after 18 to 19 h. Since H^3-Thymidine was added at 18-19 h and 28 hours seem to produce about the same amount of cytokine, the 18-19 h time point was used to collect supernatant for IL-4 and IL-10 detection.

Comparing IL-4/IL-10 production by E3 T-cell clones with proliferation

Helper T-cells type 1 and 2, T_H1 and T_H2, are divided into two groups based on their cytokine production (33). Significant production of IL-12 and INF-γ are called T_H1 cells and production of IL-4 and IL-10 are called T_H2 cells. The E3 clones are T_H2 clones and the dose response curves of IL-4 and IL-10 production due to a few APLs are shown in Figures 3.2 and 3.3. For both of the strong agonists, 4A and 4A6Sar, the E3's do not even start producing IL-4 (Figure 3.2 and 3.3) until the concentration of peptide is higher than what is needed for maximum proliferation. The peptide concentration at which cytokine production has reached 50% of its maximum is about 2 to 3 logs higher than the concentration needed for 50% maximum proliferation, which demonstrates a hierarchy of TCR signaling between proliferation and cytokine production (Figure 3.2). Results are similar for IL-10 versus

proliferation except that the difference between the half maximum of cytokine production versus proliferation is not as great (only 1 to 2 logs difference, Figure 3.3). In addition, the APL, 4A6Sar, not the known 4A agonist, produced the most IL-4 and IL-10, which suggests that it may even be a 'super-agonist' peptide. In addition the threshold for IL-4 production is greater than IL-10 production.

IL-4 alone, but not IL-10, induces sub-optimal amount of proliferation in E3 T-cell clones

Since the cytokine production was increasing as the proliferation was decreasing, we needed to determine if the cause of T cell decline was due to the increase of IL-4 or IL-10. Therefore, the 4A peptide, γ-irradiated splenocytes and E3 T cell clones were incubated with either IL-4 or IL-10 to see if the cytokine causes cell death or decreased proliferation. Proliferation was measured as the amount of ^{3}H-thymidine incorporation as described in the Materials and Methods section. In Figure 3.4, IL-4 alone, but not IL-10, causes a small increase in proliferation of the E3 T cell-clones. However, the cytokine was added at a concentration that would give half maximum response in the linear range (absorbance versus amount of cytokine) determined from the standard curve in the OptEIA™ ELISA kits (BD Pharmingen). This concentration, 0.1 ng/mL IL-4 and 0.3 ng/mL IL-10, is a small fraction of the actual amount of cytokine that the E3's produce (Figures 3.2 and 3.3) and adding higher cytokine concentrations would give a more definitive answer. Nevertheless, in two separate and consecutive experiments, IL-4 without 4A peptide showed an

increase in proliferation above background (Figure 3.4). (One experiment (3 to 4 months before these two) showed no response to IL-4 alone (data not shown). IL-10 was not done at this time). IL-4 is a costimulatory growth factor for lymphocytes (34,35,36), which may mean that a small population of T-cell clones could have been expressing IL-4 receptor constitutively either due to long term culture or were not completely rested when stimulated in this experiment. Another observation from Figure 3.4 is that the proliferation due to 4A peptide and cytokines appears to go down compared to the 4A peptide only. The results from the 4A peptide were estimated from the dose response curve done on the same day, but the conditions were slightly different. (The volume of each well was 200 μL for the cytokines and 4A peptide experiment and 125 μL for the dose-response experiment.) The difference in volume may have increased the proliferation of the T cells with 4A alone (dose response) due to the decrease in volume and crowding of the T cells. Nevertheless, a small decrease in the presence of both 4A and either cytokine may be expected since both IL-4 (34) and IL-10 (37) are associated with growth of lymphocytes. In summary, further investigations are warranted.

CONCLUSIONS

Others have shown the differential effects of cytokine production and proliferation (38,39,40,41). Allen and coworkers have shown that an APL of murine hemoglobin (64-76) caused IL-4 production but no proliferation, where the wild-type

peptide caused both (39). They defined partial agonists as peptides that cause some, but not all effector functions. In addition, Crowe and coworkers showed a hierarchy of cytokine responses for a human T_H0 clone specific for MBP (83-99) (38). They concluded that at certain concentrations of antigen, selective cytokines would be produced where the T_H2/T_H1 cytokine ratio decreased as the peptide concentration increased. Here, it is shown that cytokine production and proliferation of the E3 T cells are dissociated at the level of peptide concentration. The threshold of TCR signaling needed for cytokine production is significantly greater than for proliferation (Figure 3.2 and 3.3). In addition, within cytokine production, the cytokines IL-4 and IL-10 have different threshold requirements, which suggest that even different cytokines are sensitive to differing amounts of TCR signaling. The hierarchy of signaling thresholds for the E3 T-cell clones is proliferation < IL-10 < IL-4. The stronger agonists, 4A and 4A6Sar, have significant shifts in their dose response (Figure 3.2 and 3.3). The gap between cytokine productions for the weaker agonists appears to be less pronounced but still follows the basic scheme of proliferation < IL-10 < IL-4. Since the 4K peptide is a weak agonist and concentrations were not taken beyond 800 μM, it is harder to tell when the cytokine production reaches maximum dose response. Nevertheless, the cytokine production still does not start to increase until the maximum proliferation has been reached (or, in some cases, just before proliferation has reached a maximum).

Different thresholds of signaling for different effector functions can be described by kinetic mechanisms (19). Studies have shown that the T-cell response is related to the lifetime of the TCR-ligand complex (17,20). Agonist ligands remain bound to the TCR for about 10-fold longer than to antagonist ligands (17). Rabinowitz, et al., demonstrated that a hierarchy of early events in T-cell activation could be correlated to the time ligand remains bound to receptor (3). They suggested a hierarchy of signaling events in the order of ζ-chain phosphorylation, partial calcium response, acid release, full calcium response, and, in the long-term, proliferation (ref. 3 and Figure 1.6). The positioning of cytokine production in that hierarchy was not discussed. Wülfing, *et al.*, reported an extensive study of T-cell calcium signaling induced by agonists, partial agonists and antagonists (42). They suggested that when a TCR is activated by ligand and it is phosphorylated, it begins to signal. However, the activated TCR is then rapidly 'turned-off' by sequestration of a phosphatase. Thus, in order to maintain a constant level of TCR signaling, many TCRs per unit time need to be activated by ligand. The number of TCRs activated per time during the initial signaling process is a threshold for calcium signaling and activation by ligands with low affinity for the TCR (*i.e.*, weak or partial agonists) can only achieve this threshold at very high ligand concentrations. The antagonist ligands that only rarely cause TCR activation can never induce signaling above threshold. There is no reason to think that the threshold of TCR signaling needed for maximal calcium flux is the same as what would be needed for cytokine production or proliferation.

The experiments presented in this chapter have compared the thresholds for later events for an established murine T_H2 T cell whose effector functions includes secretion of IL-4 and IL-10. The hierarchy of thresholds was found to be proliferation<IL-10<IL-4 (Figure 3.2 and 3.3). It can be postulated that the effector functions of a T cell are more stringently regulated than simply the activation and proliferation of the T cell. Thus, the immune system may have evolved to allow T cells that receive moderate signals to survive while requiring a much higher level of signaling for a response that will mobilize the rest of the immune system. This postulate would be consistent with the differential requirements for thymic selection, peripheral tolerance, and maintenance of memory T cells. It was also found that the gap between thresholds for cytokine production and proliferation was smaller for weak agonists than for agonists. These results are consistent with the Kinetic Discrimination Model because the faster off-rate of the weaker agonists means that they will not give sufficient signaling to induce proliferation unless presented at the very high concentrations that also come very close to those needed for an even higher threshold.

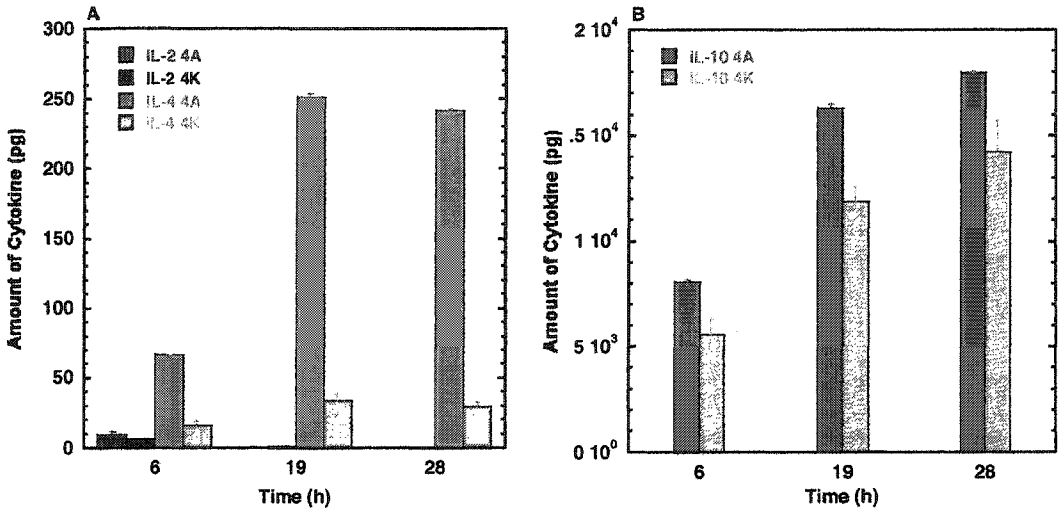

Figure 3.1: Determining the optimum time to collect B10.AE3 supernatant to measure cytokine production. The concentration of MBP Ac(1-11) 4A and 4K peptides was 67 μM and 800 μM, respectively. The concentration of peptide was chosen based on the highest concentration measured in proliferation assays that could give measurable amounts of cytokine. Supernatant from proliferation assays was removed after 6, 19, and 28 h. Cytokine production was measured as absorbance at 450 nm using BD Pharmingen (San Diego, CA) OptEIA^{TM} ELISA kits (Plot A: IL-2 and IL-4 and Plot B: IL-10). A standard curve was produced and used to convert the absorbance data for the samples into concentration of cytokine (pg/mL). Then, the concentration of cytokine (pg/mL) was multiplied by the volume (0.225 mL) in the well at the time the supernatant was collected to get the amount of cytokine (pg). The data shown represents one experiment with 2 trials for each time point. The error bars represent the average deviation. ELISA experiments were repeated at 6 h for IL-2 and 18-19 h for IL-4 and IL-10.

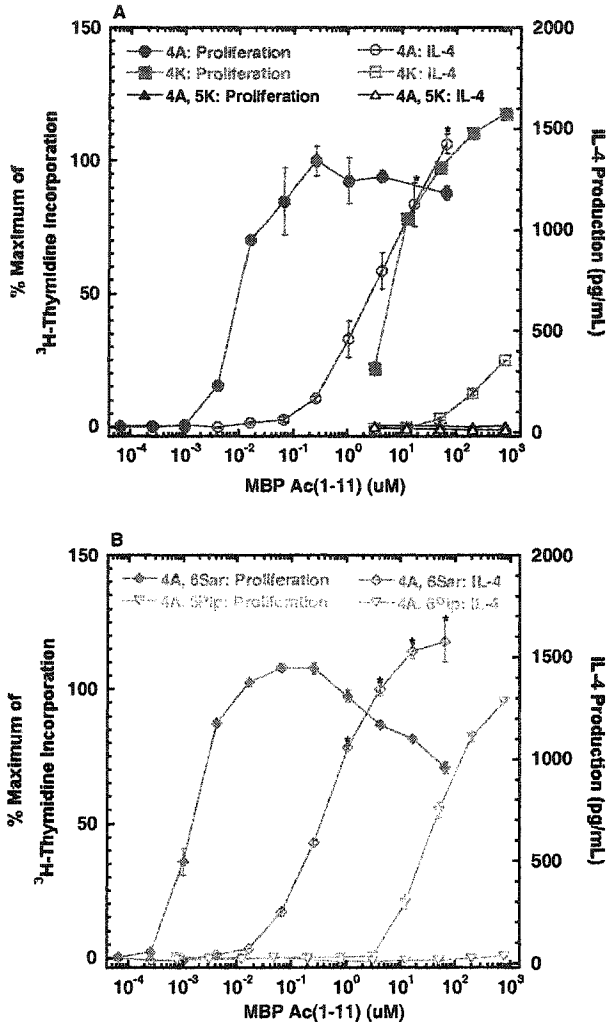

Figure 3.2: Comparing proliferation and IL-4 production by B10.AE3 T-cell clones. Plots A and B compare the proliferation of the B10.AE3 T-cell clones with IL-4 production. Plot A shows the positive (4A) and negative (4A5K) controls as well as the wild-type peptide (4K). Plot B shows examples of some APLs. The proliferation due to MBP peptides is represented as the percent relative to the maximum proliferation of the 4A peptide measured as ^{3}H-thymidine uptake. The proliferation data shown is from one representative experiment of at least three. (The maximum average for the 4A peptide was $183x10^3$ decays per minute (dpm). Background average (no peptide) was 271 dpm.) IL-4 production was measured as absorbance at 450 nm using BD Pharmingen (San Diego, CA) OptEIA^{TM} ELISA kits. A standard curve was produced and used to convert the absorbance data for the samples into concentration of cytokine (pg/ml). IL-4 data is one representative experiment of at least three, except for 4A5K was only done twice. The symbol (*) signifies data (both trials) that had absorbances above the linear range of the standard curve. The error bars represent the average deviation of two trials. Peptide sequences are listed in Table 3.1. (Abbreviations – Pip, Pipecolic Acid; and Sar, Sarcosine).

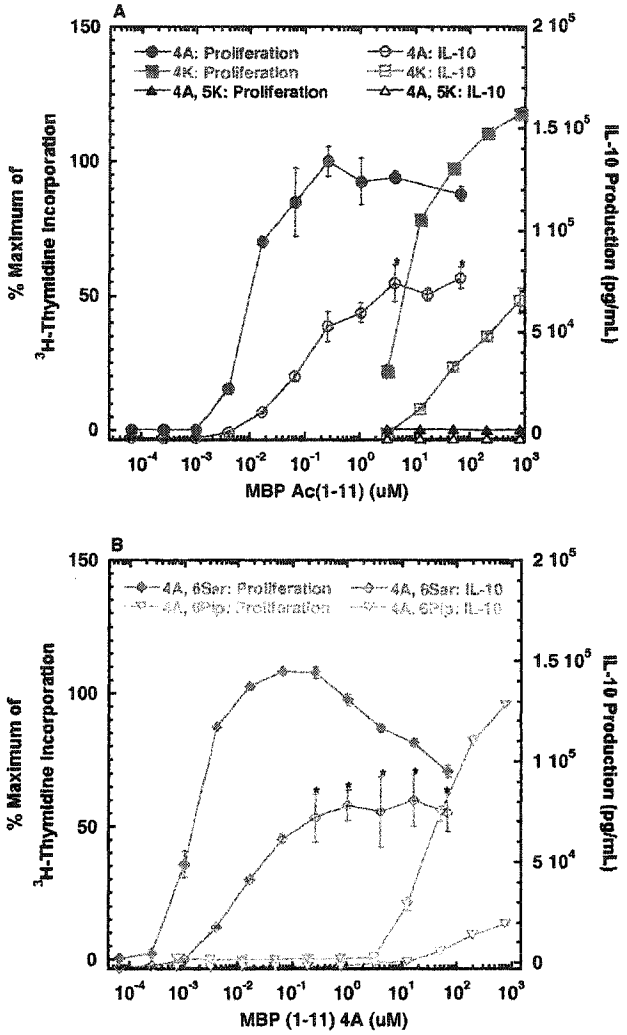

Figure 3.3: Comparing proliferation and IL-10 production by B10.AE3 T-cell clones. Plots A and B compare the proliferation of the B10.AE3 T-cell clones with IL-10 production. Plot A shows the positive (4A) and negative (4A5K) controls as well as the wild-type peptide (4K). Plot B shows examples of some APLs. The proliferation due to MBP peptides is represented as the percent relative to the maximum proliferation of the 4A peptide measured as ^{3}H-thymidine uptake. The proliferation data shown is from one representative experiment of at least three. (The maximum average for the 4A peptide is 183×10^{3} decays per minute (dpm). Background average (no peptide) was 271 dpm.) IL-10 production was measured as absorbance at 450 nm using BD Pharmingen (San Diego, CA) OptEIA™ ELISA kits. A standard curve was produced and used to convert the absorbance data for the samples into concentration of cytokine (pg/ml). IL-10 data is one representative experiment of at least three, except 4A5K was only done twice. The symbol (*) signifies data that had absorbances above the linear range of the standard curve. In this case, only one of two trials was out of the linear range, and the average absorbance was within the linear range. The error bars represent the average deviation. Peptide sequences are listed in Table 3.1. (Abbreviations – Pip, Pipecolic Acid; and Sar, Sarcosine).

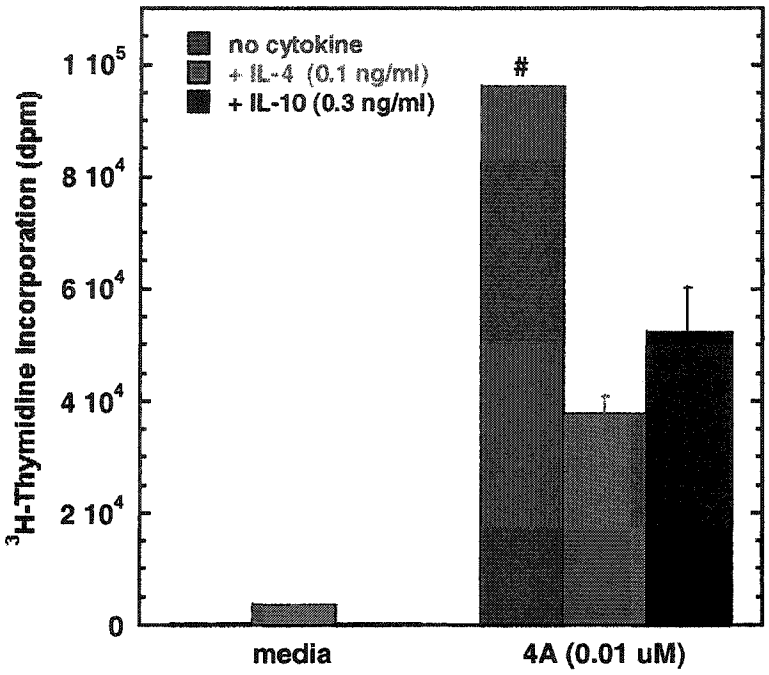

Figure 3.4: B10.AE3 proliferation due to IL-4 and IL-10 in the presence of MBP Ac(1-11) 4A peptide. Proliferation for B10A.E3 T-cell clones was measured as described in methods section. The cytokine concentrations were determined from the standard curve given in the OptEIA^{TM} ELISA kits (BD Pharmingen) and was the concentration at fifty percent of the maximum absorbance at 450 nm. The 4A concentration is the concentration at which we see the half maximum proliferation for most experiments. The proliferation of B10.AE3 due to IL4 or IL-10 in addition to 4A peptide was measured as ^{3}H-thymidine uptake. The symbol (#) designates data that was estimated from the dose response curve done on the same day, so no error bars are shown. The data shown in decays per minute (dpm) was from one representative experiment out of least two. One additional experiment for IL-4 addition showed no response to IL-4 only. The error bars represent the average deviation. Peptide sequences are listed in Table 3.1.

Table 3.1: Altered Peptides of MBP Ac(1-11). All MBP Ac(1-11) peptides were based on the N-terminal sequence of Rat MBP. For 4A6Sar and 4A6Pip, substitute X with the proper non-natural amino acid. N-terminal acetylation is denoted as 'Ac'. All peptides were synthesized by standard FMOC chemistry.

Name	Sequence	Subtype for E3s
4K (wild-type)	AcASQKRPSQRHG	weak agonist
4A	Ac------ A----------	agonist
4A5K	Ac------ AK----------	null
4A6Sar	Ac------ AX----------	strong agonist
4A6Pip	Ac------ AX----------	partial agonist/antagonist

NOTES TO CHAPTER 3

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Chapter 4

Taking a Look at Other MBP-Specific T-cell Clones

ABSTRACT

The T cells within one clonal population have a structurally distinct TCR than the receptor of another clonal population that responds to the same antigen. The T-cell response is said to be heteroclitic, which refers to the fact that the T cells 'see' the same antigen but they might 'see' it differently. For example, one T cell may focus on the peptide N-terminus while another might focus on the C-terminus. Therefore, in order to thoroughly evaluate the immunotherapeutic potential of a particular APL, one clonal T-cell line might not provide enough information. Hence, a panel of clonal T-cell lines is better suited to fully evaluate the complete repertoire of responses that might be elicited by the APL *in vivo*. In this chapter, preliminary results describing the responses of two additional MBP Ac(1-11)-specific B10.A T-cell lines to APLs are reported. The T-cell responses were evaluated using a series of cell-based assays as described in Chapter 2 and 3. It was found that there is a considerable variation in the T-cell's sensitivity to alterations of peptide structure- some T cells are relatively promiscuous while others exhibit high fidelity.

INTRODUCTION

The immune system generates a wide variety of cells and molecules that respond to and eliminate foreign pathogens (1,2). Even though mechanisms exist to prevent T cells from becoming activated towards self-antigens (*i.e.*, clonal deletion (3), induction of anergy (3,4,5), and bystander suppression (6,7)), expansion of T cells specific for self-antigens does still occur during autoimmune diseases (1,2). An example of an autoimmune disease is Multiple Sclerosis (MS) in humans and Experimental Autoimmune Encephalomyelitis (EAE), which is a MS model in rodents (8). Even though the cause of MS onset is not completely understood, studies on EAE have given further insight and hopes for treatment of this debilitating disease. EAE can be induced with epitopes found in the myelin sheath of nerve cells, such as proteolipid protein (PLP) and myelin basic protein (MBP), and it can be 'cured' in mice or Lewis rats with altered peptide ligands (APLs) (9,10,11). Peptide therapy could potentially provide better treatment for MS, while the current ones just help patients cope or live with the disease.

Altered peptide ligands bound to major histocompatibility complex (MHC) proteins have been shown to produce a spectrum of T-cell responses (12). As mentioned before, peptides can cause full proliferation and cytokine production (agonists), block the response caused by agonist ligands (antagonists), or cause a subset of responses (partial agonists). Allen and coworkers discovered APLs of the Hemoglobin (64-76) [Hb 64-76] peptide that blocked full proliferation of murine T cells and some that were partial agonists (13,14,15). They noticed that different T-

cells could respond differently to the same peptide (15). The APLs previously identified as partial agonists for some helper T-cell clones [*i.e.*, Hb (64-76), S70] could also antagonize proliferative responses of other helper T-cell clones. Thus, different altered peptide ligands can cause a spectrum of responses in one T-cell clone and the same APL can cause completely different responses in different clones.

The heteroclitic nature of the T-cell responses raises a potential concern in designing immunotherapeutic peptides. An autoimmune disease potentially would be worsened rather than improved if an APL produced an agonist response for some T cells. In fact, Wraith and coworkers created antagonist peptides by substituting amino acids at important T-cell receptor (TCR) and MHC contact sites of MBP (1-9) 4Y (16). Although these peptides were antagonists for a TCR-transgenic T-cell line, they also induced EAE when injected into mice with Freund's adjuvant and pertussis toxin. A polyclonal T-cell line generated from non-transgenic B10.PL mice proliferated in response to the APLs. Thus, a peptide that is an antagonist for one T cell is likely an agonist for another and it seems unlikely that APLs would ever be able to 'block' disease.

However, APL ligand therapy of EAE does work. Steinman and coworkers have shown that some peptides identified as antagonists for a clonal T-cell line can block the progression of EAE in the Lewis rat (9). The TCR contact sites of MBP (87-99) were altered to alanine and the peptides were studied for their ability to antagonize a clonal and polyclonal T-cell lines. Three *in vitro* antagonists were identified, but only one, MBP K91A, prevented EAE in an epitope-specific manner.

In addition, Kuchroo and coworkers have shown that an analogue of the encephalitogenic myelin PLP (139-151) peptide, W144L/H147R, is a powerful antagonist for antigen-specific T-cell clones *in vitro* (10). This peptide can also protect animals from the induction of EAE. In later studies, Wraith and coworkers, discovered antagonist peptides of MBP (1-9) that did block EAE progression (11). Both MBP 3I4Yand 3K4Y (altered from 3Q4K) antagonized T cell activation *in vitro* and blocked EAE induction *in vivo*. Indeed, research into APLs has led to promising results from phase II clinical trials with MS patients (17,52).

If the same peptide can cause different responses in different clones, then how do APLs block the progression of disease as in the examples listed above? In order to understand the mechanism by which this may occur, the idea of tolerance needs to be addressed. Tolerance is the failure to respond to an antigen; for example, the immune system is mostly tolerant of self-antigens. When tolerance is lost, the immune system can destroy self-tissues, as in the autoimmune diseases, EAE and MS (1). Self-reactive T cells are usually deleted in the thymus in a process called negative selection (1,2). Unfortunately, these cells sometimes escape negative selection and are released into the periphery. The immune system has developed mechanisms for peripheral tolerance, such as clonal deletion (3), anergy (3,4,5), immune deviation (10), and bystander effects (6,18). Thus, the T cells that mediate an autoimmune disease have somehow escaped negative selection and peripheral tolerance. Since all T cells are positively selected on self-peptides they are intrinsically self-reactive (at least partly) and the question then is what factors cause a release from peripheral tolerance? In

MS, the factor causing tolerance breakdown is not known. In EAE, the breakdown is induced myelin antigen, Freund's adjuvant, and pertussis toxin; the latter two promote the production of inflammatory cytokines.

Specific inflammatory cytokines play an important role in various autoimmune diseases (19). Immune responses are sometimes dominated by one of the T-helper subtypes, T_H1 or T_H2, (20), and each subtype can inhibit the activity of the other (1). Since autoimmune diseases primarily involve T_H1 T cells, the diseases potentially could be cured or at least attenuated by altering cytokine profiles to produce more T_H2 cells (19). Kuchroo and coworkers showed that EAE, which is T_H1 mediated, induced with the native PLP (139-151) peptide could be inhibited in SJL mice with the altered peptide ligand, PLP W144Q. The APL induces T cells that are cross-reactive with the wild-type peptide and produce T_H2 (IL-4 and IL-10) and T_H0 (INF-γ and IL-10) cytokines (21). A shift from a T_H1 to a T_H2 cytokine environment is an example of induction of tolerance, and the strategy of switching cytokine profiles has been efficacious in the treatment of other autoimmune diseases as well (19).

More recently, other 'suppressor' subtypes of CD4+ T cells (called regulatory T cells or T_{reg}) have been discovered and these have been referred to as T_r1 cells (22), T_H3 cells (23), and CD4+CD25+ regulatory T cells (24). All three release some variation of the immunosuppressive cytokines (IL-4, IL-10, and/or TGF-β) when activated, but the CD4+CD25+ T_{reg} cells are also thought to use a cell-contact dependent mechanism (24). All three are CD4+ cells and some constitutively express CTLA-4 and CD25. The T_{reg} cells apparently suppress or regulate the immune

response of other cells (6,18,25,26,27) and they are found in both animals and humans (6,28). Although reports of T_{reg} cell suppression varies, most reports suggest that when T_{reg} cells are activated by one antigen they secrete suppressor cytokines such as IL-10 and TGF-β that suppress any activated T cells. Thus, T cells activated by one antigen can be suppressed by the activation of T_{reg} cells by an unrelated antigen, which is referred to as a bystander effect. The possible role of T_{reg} cells in APL therapy is suggested by the report that the PLP (139-151) peptide could induce bystander suppression of T cells responsive to the MBP (1-9) 4Y and MBP (89-101) peptides (29). It is notable that if an APL induces T_{reg} cells then the fact that the APL might also be an agonist for other autoreactive T cells is irrelevant.

All of the results presented in the previous chapters were obtained with only one monoclonal T-cell line (E3s). The following chapter provides preliminary results describing the responses of two other MBP Ac(1-11)-specific T-cell clones (E2s and F2s). The data both support and expand on data already gathered when the cells were originally developed (K. Tate, unpublished results). It is shown that each one of these T-cell clones responds differently to each APL and that some T cells are much more tolerant of various substitutions and others are quite stringent. These results amplify the concern that if one is to identify therapeutic APLs, the choice of T cells used to screen the APLs can strongly bias the types of T cell responses that could be obtained.

MATERIALS AND METHODS

Animals: Female B10.A-H2/SgSnJ (B10.A) mice (5-6 weeks old) were received from the Jackson Laboratory (Bar Harbor, ME), and cared for as described previously in Chapters 2 and 3.

Peptides: Myelin Basic Protein 1-11 (Ac-ASQKRPSQRHG) and altered peptides were synthesized using standard Flourenylmethoxy carbonyl (FMOC) chemistry with an Applied Biosystems 431A peptide synthesizer as previously described in Chapter 2. Cleaved peptides were purified (C_{18}-reverse phase) using a water/acetonitrile gradient that contains 0.1% TFA using HPLC as described previously. Fractions were characterized by high-resolution mass spectrometry (Bruker Esquire Ion Trap LC-Mass Spectrometer, Billerica, MA) using an electrospray source. The sequence and structures of non-natural amino acids of MBP Ac(1-11) and other APLs used in the following experiments are listed in Tables 2.1 and 2.2.

Origin of T-cell clones: B10.A mice were immunized subcutaneously at the base of the tail with 200 μg of MBP Ac(1-11) peptide in Complete Freund's Adjuvant as described (30,31, and Chapter 2). The T-cell line was subcloned by limiting dilution. The monoclonal populations obtained were named with a letter and number description (*i.e.*, B10.AE3 and B10.AF2, abbreviated as E3 and F2 here). Preliminary assays were done on the T-cell clones and they were categorized as either T_H1 (F2 and E2 clones) or T_H2 (E3 clones) (K. Tate, unpublished results). The T-cell clones were maintained as described below.

Maintenance of T-cell clones: B10.AE3 clones ($1x10^6$ cells/mL) are maintained by restimulation every 10-12 days using γ-irradiated (3000 rad) splenocytes from B10.A mice (Jackson Laboratories) and the wild-type peptide, MBP Ac(1-11) 4K, (60 μM). T-cell clones are given 150 U/mL of recombinant mouse IL-2 (Pharmingen, San Diego, CA) with another dose (100 U/mL) given 48 h later. The media used for the clones was RPMI-1640 (GibcoBRL, Grand Island, NY) as described in Chapter 2. The F2s and E2s were cared for similarly, but with a few differences during the time the experiments with them were done. The F2 and E2 T-cell clones ($5\text{-}7x10^5$/mL) were maintained by restimulation every 6-11 days using splenocytes as above and MBP Ac(1-11) 4K (40-80 μM for F2s and 60 μM for E2s). F2s and E2s were given 50 U/mL of recombinant mouse IL-2 (Sigma) with another dose (25 U/mL, Sigma) 48 h later. The media used at this time was Dulbecco's modified eagle medium (DMEM) (GibcoBRL, Grand Island, NY) with 10 mM 3-(N-morpholino)propanesulfonic acid (MOPS) (Sigma), 1.85 g/L sodium bicarbonate (Sigma), 0.05 mM 2-mercaptoethanol, 100 U/mL penicillin (Sigma), and 100 μg/mL streptomycin (Sigma). In addition, the media was supplemented with extra amino acids (Sigma): 216 mg/L L-glutamine, 36 mg/L L-asparagine, 116 mg/L L-arginine, and 6 mg/L folic acid. 10% heat-inactivated fetal calf serum (GibcoBRL) was added to media when needed. The difference in protocol only reflects our fine tuning of the maintenance protocol over time. The F2s and E2s were available for experiments first, but died soon afterwards. The E3s came out of the freeze more slowly, but lasted much longer and more reliably

allowing the tissue culture protocol to become perfected. In addition, DMEM was substituted for RPMI-1640 since the clones were originally subcloned using this media, and we hoped that this might improve our tissue culture survival. IL-2 for E2s and F2s was bought from a different source (Sigma) than the IL-2 used for the E3s (Pharmingen) and had a different specific activity. Proper concentrations of IL-2 were determined after repetitive tissue culture and the health of the cells.

Maintenance of KK cells: The KK cells, an adherent murine L-cell transfected with I-A^k (32), were used as antigen-presenting cells during the Cytosensor® Microphysiometer experiments. The KK cells were harvested every 3 days with trypsin-EDTA (Sigma). The cells were pelleted to remove the trypsin-EDTA solution and resuspended at a cell density of 1-2 x10^4 cells/mL in fresh media. The media contains RPMI-1640 (GibcoBRL) with 10 mM HEPES (Sigma), 2 g/L sodium bicarbonate, 0.05 mM 2-mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin. 10% heat- inactivated fetal calf serum (FCS) is added to the media when needed. HAT media supplement (Sigma) is added in order to maintain the high level of MHC expression.

Proliferation Assays: T-cell clones (2.5 to 9.6 x10^4 per well) were stimulated with γ-irradiated (3000 rad) B10.A splenocytes (6-10 APCs: T cell) and various MBP Ac(1-11) peptides at 37°C and 5% CO_2 (225 μL final volume). The supernatant (100 μL) was collected from each well for detection of all cytokines after 18 to 19 h (E3 T-cell clones, except IL-2 was collected at 6 h for this clone) or 40 to 48 h (F2 and E2

clones). The plates containing supernatant were frozen at –80°C until used in ELISA assays (see below). Then, 1 μC_i of ^{3}H-thymidine (American Radiolabeled Chemicals, Inc., St. Louis, MO) was added to each well of the plates that contained the T cells and splenocytes (30). After 18 to 24 h, the DNA was collected on filter mats by using a SKATRON Combi cell harvester (SKATRON, Sterling, VA). The filter mats were washed with water for a total of 15-19 seconds for the F2s and 23 seconds for the E2s and E3s. The longer wash time (i.e., 23 seconds) minimized the signal to noise. The radioactive samples on the filter mats were placed in biovials and 3 mL of Ecoscint scintillant (National Diagnostics, Atlanta, GA) was added. The radioactivity in decays per minute (dpm) was measured on a Beckman LS 6500 scintillation counter (Beckman, Fullerton, CA).

ELISA Assays: OptEIA™ ELISA kits (Pharmingen, San Diego, CA) were used to detect mouse cytokines: IL-2, IL-4, IL-10, IL-12, and INF-γ. For the E3 T-cell clones, the times for collecting the supernatant were as follows: 6 h (IL-2), 18-19 h, (IL-4 and IL-10), and 40-48 h for IL-12 and INF-γ (from an antagonist assay). For the F2s and E2s, the supernatant was collected at 40-48 h for all cytokines measured. As mentioned above, the supernatant was collected from the proliferation assays and frozen at -80°C. The directions were followed as described in the ELISA kits. The absorbance was measured on a Molecular Devices $Thermo_{max}$ plate reader (Molecular Devices, Sunnyvale, CA) at 450 nm (minus 570 nm for wavelength correction). A

standard curve was produced and used to convert the absorbance data for the samples into concentration of cytokine (pg/mL).

Determining proper dilution factor for detecting IL-10 and INF-γ: The supernatant from the E3 and E2 T-cell clones stimulated with the 4A peptide was serially diluted. Supernatant (5 μL) from the original plate was added to 195 μL of PBS/10%FCS (1/40 dilution). Then, each well was diluted by half until 1/5120 dilution was reached. Sample was removed from each well to get a final volume of 100 μL. ELISA assays were done as described above.

Cytosensor® Microphysiometer: Acid release was measured as described in previous literature (30,33,34,35,36). In brief, T cells (2.4 to 8 x 10^6 total) were mixed with KK cells at a ratio of 10 to 40 T cells per APC and collected by centrifugation. The cells were resuspended in 45 μL DMEM medium and 15 μL of melted low-temperature-melting agarose (Molecular Devices) at 37°C. The agarose-cell mixture (7 μL) was spotted onto the membrane of 8 Cytosensor® cell capsules (Molecular Devices). After 10 min, the cell capsule was assembled according to standard Cytosensor® protocol and loaded in the Cytosensor® chamber maintained at 37°C. The chamber was perfused with Molecular Device's Modified RPMI-1640 (low buffer RPMI-1640 with 1 mM phosphate and no bicarbonate) with 1g/L endotoxin free bovine serum albumin (Calbiochem, LaJolla, CA) (pH 7.4). Extracellular acidification rates were determined using a 20 s potentiometric rate measurement after a 58 s pump cycle and a 10 second delay (90 s total cycle time). The response of the

APLs was normalized to the basal rate (cells perfused with buffer only) in Figure 4.4. The results were qualitatively evaluated from the rates (μV/s) as they compared to the MBP (1-11) 4A peptide at 10 uM and 1 μM and summarized in Table 4.1.

RESULTS AND DISCUSSION

Measuring T-cell proliferation using ^{3}H-Thymidine incorporation

T-cell proliferation was determined using two T_H1 (F2 and E2) clones and one T_H2 (E3) clone (Figure 1). E3 proliferation (Figure 4.1A) is a modified version of Figure 2.2 and ^{3}H-Thymidine uptake was measured after ~2days (^{3}H-thymidine was added after ~1 day and harvested the next; also called 1/2 day protocol here). Plots B-D are proliferation plots for F2s and E2s measured after ~3 days (^{3}H-thymidine was added after ~2 days and harvested the next; also called 2/3 days protocol here). The difference in time reflects changes in protocol over time. In the beginning, it was determined that adding ^{3}H-Thymidine after 2 days and harvesting DNA after three days (2/3 days protocol) was the optimum time for measuring proliferation and this may still be true for the F2s and E2s. However, most of the experiments done with the E3s followed the 1/2 days protocol. The optimum time reflects the rate of cell division (*i.e.*, DNA synthesis) and the rate can change as the cells are continuously restimulated. In addition, T-cell lines often became more efficient at recognizing antigen over time and this was most evident with the E3 T cells. The F2 T cells' proliferation was measured four times, but two of these had extremely high backgrounds (cells only) and poor signal-to-noise and the other two had fairly large

backgrounds. Also, the E2 responses were only measured at two APL concentrations, which made the subtype/rating more difficult for Table 4.1. In most cases, the rating of ++ or + for E2 responses was estimated from the two data points (Figure 4.1D).

Although the data for the E2 and F2 T cells are not as complete as for the E3 T cells, we can make general deductions about how the different monoclonal T cell populations respond to the various APLs. The 4A6Sar and 4A6MeAla peptides are strong agonists for the E3s but, they are weak or partial agonists for the F2s and E2s (Figure 4.1 and Table 4.1). However, the E2s and F2s differ in their response to substitutions at glutamine-3; the F2s exhibit little to no proliferation in response to peptides modified at this position but the E2s proliferate in response to almost all of the position-3 modified peptides (Table 4.1). The E3s are also sensitive to substitutions at glutamine-3 but they are very tolerant to substitutions at proline-6. The E2s are the most accommodating to changes in both positions and the F2s appear to be the most specific of the three clones at these positions. In general, all of the T cells are intolerant of APLs with arginine-5 substituted by other amino acids, which is also consistent with the argument that arginine-5 is the major T-cell contact (37,38,39,40).

Cytokine Production by T-cell clones due to APLs

The clones were already screened when they were originally developed and they were assigned as either T_H1 or T_H2 based on their cytokine profiles (K. Tate, unpublished results). The F2s and E2s were determined to be T_H1 because they produce IL-12 and INF-γ. The E3s were determined to be T_H2 because they produce

IL-4 and IL-10. The following data both confirms and expands on these results (Table 4.1). The cytokines produced by these T cells are consistent with the results when they were first subcloned, which confirms that these cells have not altered in some way due to long term cryogenic storage or repeated restimulation. In addition, the results demonstrate that these T-cell clones do not alter their cytokine profiles due to exposure to the APLs. This is consistent with the observation that T-cell lines generated by restimulation are usually fixed in phenotype and function (41).

INF-γ production initially was determined for both the F2s and E2s using only 50% dilution of supernatant, but the amount of IFN-γ produced was so high that variations of cytokine production were difficult to determine for Table 4.1. Therefore, caution should be used when looking at the IFN-γ data (Figure 4.2). Regardless, the data shows that these clones do produce INF-γ in response to some if not all APLs. In a different experiment with the E2 clones using a 1/80 dilution, it was still difficult to see differences in the responses to strong agonists. Despite these caveats, a hierarchy of E2 T cell responses to different APLs was evident (Figure 4.2B/C and Table 4.1). An IFN-γ dose response was not determined for the E2 T cells, except for the 4A and 4K peptides.

Originally, 50 μL of supernatant was used to measure IL-2 and IL-4 cytokines. However, 50 μL supernatant was too much for IL-10 production by E3s and INF-γ by F2s and, thus, ELISA experiments were done in order to determine the proper dilution factor for these clones (Figure 4.3). The supernatant from cells stimulated by the 4A

peptide was serially diluted and the absorbance at 450 nm (minus the absorbance at 570 nm for wavelength correction) was measured and plotted versus concentration of peptide. It was determined that the E2 (and presumably the F2) supernatant must be diluted by more than 1/640 in order to not exceed the maximum absorbance of the detector (3 to 4 absorbance units). However, before these experiments could be repeated further, the E2s suffered a sudden death and they never returned. The proper dilution to determine IL-10 production by E3 T-cell clones (1/80 dilution) was also determined in the same manner (Figure 4.3B).

Measuring Acid release by Cytosensor® Microphysiometer

Many different cell signaling events cause an increase or decrease in the rate of extracellular acid release that occurs in a matter of seconds, which makes it a good indicator of changes in metabolism and T-cell activation. Microphysiometry is an established method used to measure acid release rates for all kinds of cell lines including T-cell clones (30,33,35). The instrument uses a silicon-based pH sensor to measure changes in extracellular pH with high sensitivity (33,35). Both T cells and APCs (L-cells transfected with the I-A^k MHC class II protein (32)) were immobilized in agarose and low buffer RPMI media (Molecular Devices) was perfused over them. The cell's responses to peptides were measured after brief exposure of the cells to peptide. The results obtained in Figure 4.4 are the best representative experiments for each T-cell clone. The F2s were measured successfully (meaning measurable responses over response to no peptide) at least 4 times on the Cytosensor, and one representative experiment is shown (Figure 4.4B). On the other hand, the E2s were

measured once successfully (Figure 4.4C). Once protocol was worked out, E2s and F2s usually had acid response significantly above baseline. However, the E3s were measured several times, only three gave measurable results with APC and peptide. One of those E3 experiments is shown in Figure 4.4A and the other two experiments had either a large signal to noise, very little response, or both.

In Figure 4.4A, the E3 responses to 10 μM 4A6MeAla and 100 μM 4A6Pip were measured with cells that had previously been exposed to peptides that did not change the acid response from basal (<2%). The E3 response to 10 μM 4A6Hyp was measured after the cells had responded weakly to another peptide (~8% basal rate difference). The difference in acidification rate of added peptide is shown rather than the % from resting T cells. For the F2s, each peptide shown was added to resting cells (Figure 4.4B). For the E2's, the 10 μM 3L4A peptide was added after the 10 μM 4A5Cit peptide (shown Figure 4.4C) since the first peptide cause little to no acid release. In all cases, the first peptide was washed away with buffer before addition of the second peptide.

In general, changes in the acid release rate paralleled proliferation responses with few exceptions (Table 4.1). For example, the 3V4A and 3Nva4A peptides caused a weak acidification response for the E3s (~3% and 8% basal; 4A peptide had only 10% basal, data not shown), but they induced little to no proliferation (Table 4.1). This is consistent with a kinetic model that suggests that each APL causes both positive (complete) and negative (incomplete) signals (42) rather than being an on/off

switch. Rabinowitz, *et al.*, also observed similar results using the moth cytochrome-*c* peptide where an antagonist blocked proliferation but caused some increased acidification (43). Another exception is the 4A6Pip peptide, which causes partial proliferation for the E3 T-cell clones (Figure 2.2), partial cytokine production (Tables 2.3 and 4.1), and antagonizes proliferation of the 4E peptide (Figure 2.3), but it does not cause any increased release of acid (Figure 4.4A). These results are reminiscent of data reported by other groups that demonstrated cytokine production without some early signaling events (44,45). These observations support the notion that the signaling events may not necessarily be stepwise and are triggered by different pathways with different thresholds of activation (43). Even the early signaling events, acid release and intracellular calcium, are not necessarily triggered by the same pathway (46).

CONCLUSIONS

The responses of one clone are not necessarily enough to determine whether a particular peptide will be effective at blocking disease (16). In this chapter, preliminary results for the APL responses of additional T-cell clones were presented and Table 4.1 summarizes the results for all T cells studied. Although some of the results are incomplete, general trends can be seen from Table 4.1. Not only do the clones respond differently to different peptides as might be expected (15,16), a hierarchy of T-cell sensitivities to structural modifications is evident. For example, the E2 clones are the most tolerant of all the clones because these clones respond to

almost all (75%) of the APL tested (Table 4.1). The E3 clones, on the other hand, tolerate some substitutions at proline-6, but they have very little tolerance for APLs with substitutions at glutamine-3. The F2s appear to be more specific and respond to only a few random APLs. These results suggest that each clone has one primary TCR contact residue, arginine-5, and a secondary TCR contacts whose importance varies depending on the T-cell clone.

Even though the diverse repertoire of T cells *in vivo* potentially creates a "road block" to finding the perfect APL for immunotherapeutic purposes, APL therapy has been shown to work (9,10,11). The "perfect" peptide for inducing cytokine immune deviation or bystander effects may just need careful tweaking (47). In fact, one of the present therapies for relapsing-MS is a mixture of synthetic polypeptides composed of 4 amino acids called glatiramer acetate (or Copolymer 1 or Copaxone; Teva Marion Partners, Kansas City, Mo.) (48,49,50,51). The mixture of peptides may even be the key to working around the heteroclitic nature of the immune system. This and other clinical trials (17,52) of MBP APLs gives hope that maybe careful study will provide us with a good candidate for effectively treating disease. "Road blocks" are challenges to overcome, but not necessarily insurmountable given time and patience.

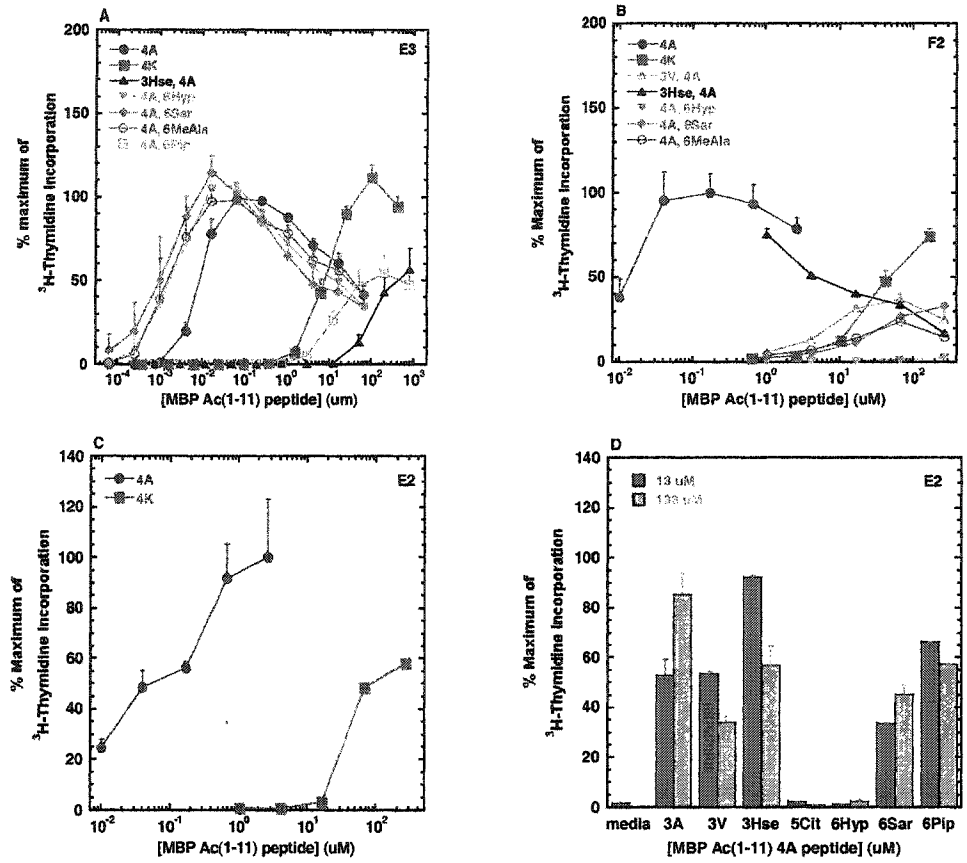

Figure 4.1: B10.A T-cell clones' proliferation due to MBP Ac(1-11) APLs. Plots A-D show the proliferation of B10.A T-cell clones due to APLs in percent relative to the maximum proliferation of the 4A peptide measured as ^{3}H-thymidine uptake. The error bars represent the average deviation and only the top half is shown. Peptide sequences are listed in Table 2.1. A.) E3 T-cell clones: The data shown is representative of 3-6 separate experiments each with two trials at each concentration. The maximum average range was $28x10^3$ to $503x10^3$ decays per minute (dpm). The background measurement (T cells and APCs in media) was less than 3% of the maximum dpm. (See Figure 2.2 as well). B.) F2 T-cell clones: The data shown is the best representative plot of four experiments. Three of the four had either high backgrounds, significant amounts of signal to noise, or both. The average maximum for this experiment was $77x10^3$ dpm at 0.17μM 4A with a background of less than 10%. C.) and D.) E2 T-cell clones: The data shown is the only experiment with counts above background for these clowns. Only two concentrations were done for the APLs, so they are represented in a bar graph (Plot D). The average maximum for this experiment was $17x10^3$ dpm with backgrounds less than 2% of the maximum (see media in plot D). Error bars are not shown for 4K (Plot C) and 4A6Pip (Plot D) because the two trials were harvested accidentally using the same row on the filter mat (~twice as much radioactivity per scintillation vial). The average for one trial was approximated by dividing the total dpm's by 2. (Abbreviations – Cit, Citrulline; Hse, Homoserine; Hyp, Hydroxyproline; MeAla, N-Methyl Alanine; Pip, Pipecolic Acid; and Sar, Sarcosine).

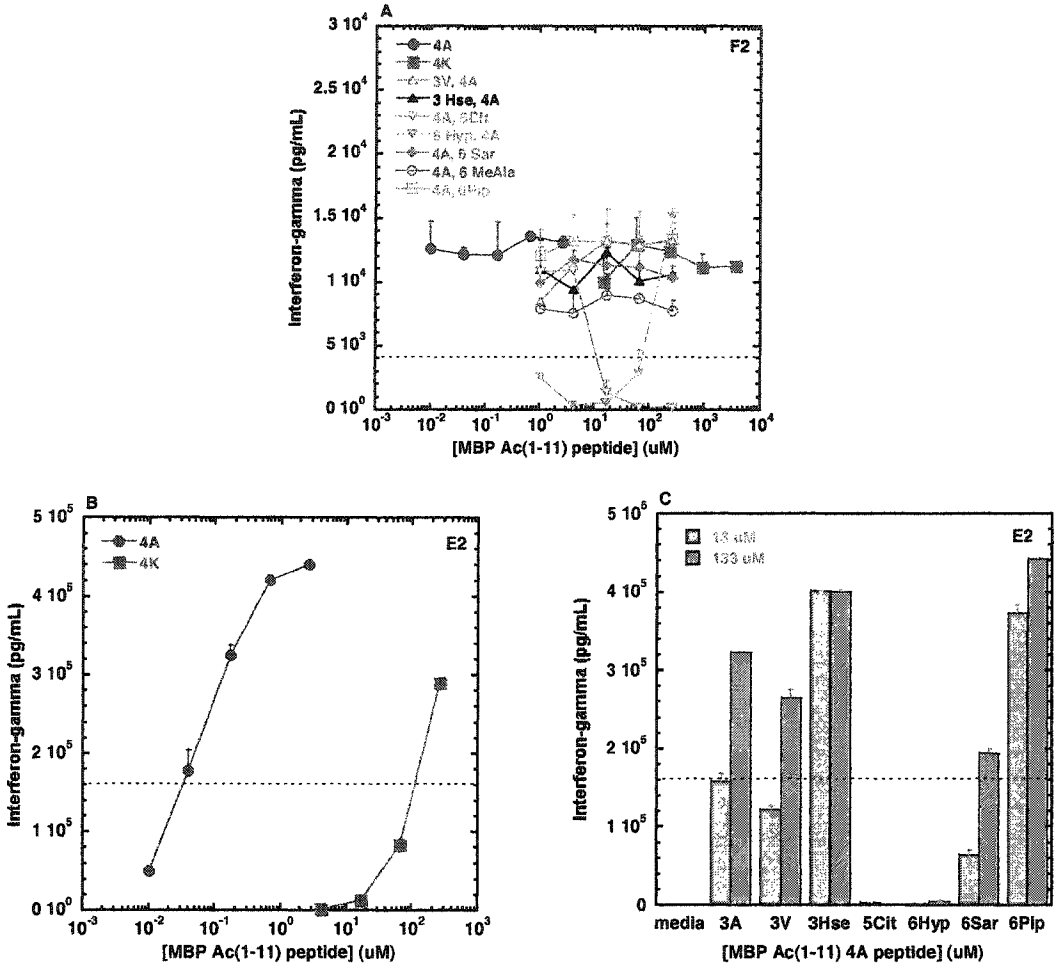

Figure 4.2: INF-γ production by B10.A T-cell clones. Supernatant from proliferation assays of the F2s and E2s was removed after 2 days (approximately 40 to 48 h) before addition of H^3-thymidine. Interferon-gamma (INF-γ) production was measured as described in the BD Pharmingen (San Diego, CA) OptEIA^{TM} ELISA kits. A standard curve was produced and used to convert the absorbance data for the samples into concentration of cytokine (pg/mL). The dotted line represents the upper limit of the standard curve taking into consideration the dilution factor of the sample (Plot A: $4x10^3$ pg/mL and Plot B and C: $1.6x10^5$). Data is shown in pg/mL of INF-γ and the top half of the average deviation of two trials is shown. A.) F2 T-cell clones: The data shown is from the only experiment done for these clones. The supernatant from proliferation assays was diluted by 1/2 with PBS/10%FCS, which does not dilute the sample enough. The average background (T cells and APCs in media only) was between $4x10^3$ and $14x10^3$ pg/mL, which was outside the linear range of the standard curve. B.) and C.) E2 T-cell clones: The data shown is from one representative experiment that diluted the supernatant from the proliferation assay by 1/80 (1.25 µL super /100 µL total). The background was less than 1500 pg/mL, but some of the maximum absorbances were still out of the linear range. This experiment was repeated 2-3 more times with the same supernatant, but at lower dilutions that gave high backgrounds. (Abbreviations – Cit, Citrulline; Hse, Homoserine; Hyp, Hydroxyproline; MeAla, N-Methyl Alanine; Pip, Pipecolic Acid; and Sar, Sarcosine).

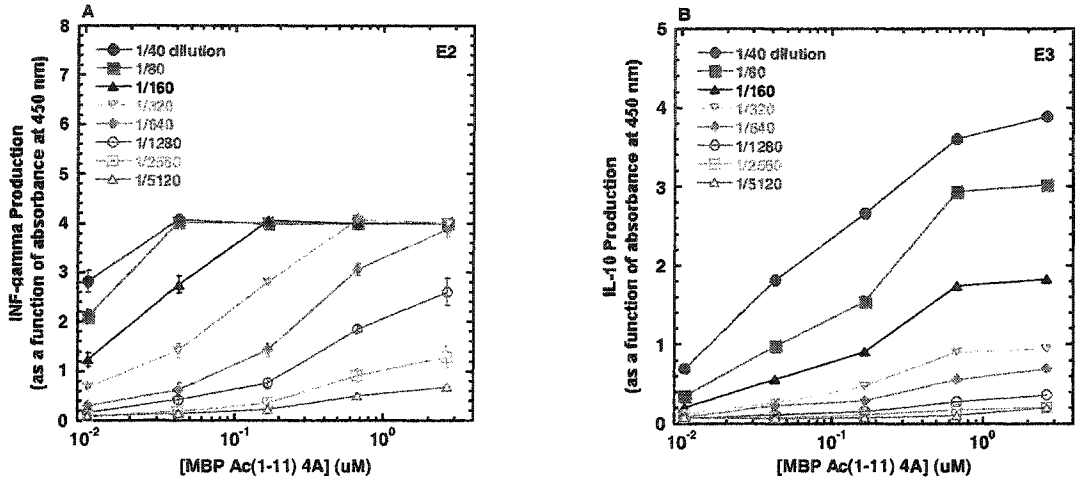

Figure 4.3: Determining the proper dilution factor for INF-γ and IL-10. Supernatant from E2 and E3 T-cell clones stimulated by 4A peptide was diluted by serial dilution in order to determine the proper dilution factor to measure INF-γ (E2) and IL-10 (E3) production by an ELISA assay. The data shown is the only experiment and is shown as a function of absorbance at 450 nm (minus absorbance at 570 nm for wavelength correction) vs. concentration of the 4A peptide for each dilution. A.) INF-γ production by E2 T-cell clones. B.) IL-10 production by E3 T-cell clones. This experiment was not done on F2 T-cell clones.

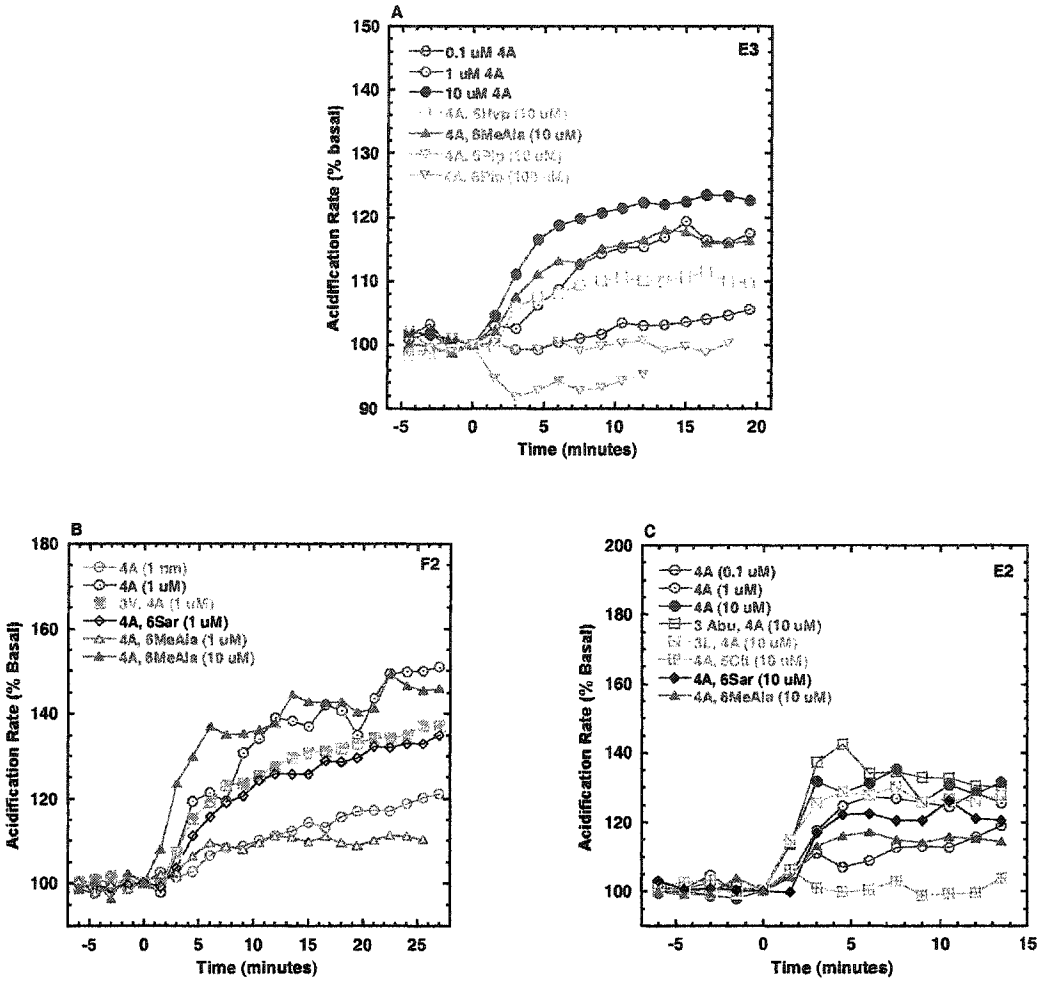

Figure 4.4: Acid release was determined by the Cytosensor® Microphysiometer. The acidification rate was measured on the Microphysiometer as told in the methods section. The data is representative of one experiment and shown as the % basal rate of acid release (acid release of T cells and APCs without peptide). At 0 minutes, the peptide, diluted to a certain concentration in modified RPMI buffer, was added and a response was measured for 10 to 30 minutes. The data shown is acid release as a result of constant flow of buffer with peptide over the cells. However, the T-cells did remain activated after peptide was removed (data not shown). A.) E3 T-cell clones: The data shown is the best representative of one of three experiments. The other two experiments had extremely large signal to noise. B.) F2 T-cell clones: three other experiments were performed on the F2s for various peptides (see Table 4.1 footnotes). C.) E2 T-cell clones: The data shown is from the only experiment. (Abbreviations – Abu, 2-Aminobutyric acid; Cit, Citrulline; Hse, Homoserine; Hyp, Hydroxyproline; MeAla, N-Methyl Alanine; Pip, Pipecolic Acid; and Sar, Sarcosine).

Table 4.1: Summary of responses given by B10.A T cell clones. – Procedures are described in the Methods section. All peptides use the MBP Ac(1-11) 4A as the 'core' peptide unless otherwise informed. Responses that were not determined are represented by 'nd'. See the Legend below each table for description of the symbols (+,-). The +/- indicates that there was little to no response or that it was hard to evaluate. Abbreviations for subtypes include: wk. (weak), p. (partial), and st. (strong). The symbol (?) represents data that was difficult to interpret. The null means that the peptide was determined to be null by C. Beeson (unpublished results). For E2s and F2s, subtypes are only "possible" ones since there wasn't enough data to be absolutely sure. Abbreviations: Abu: 2-aminobutyric acid, Cit: Citrulline, Dpg: Dipropyl Glycine, Hse: Homoserine, Hyp: Hydroxyproline, MeAla: N-Methyl Alanine, Nle: Norleucine, Nva: Norvaline, Pip: Pipecolic Acid, Pra: Propargyl, Sar: Sarcosine, and Tle: Tert-Leucine. A.) E3s B.) F2s C.) E2s

A.) *B10.AE3 T-cell Clones*[a]

Subtype for E3	WT	APL	*B10.AE3*						
			Proliferation Assay	Acid Release	Cytokines				
					IL-2	IL-4	IL-10	INF-g	IL-12
agonist	K	4A	++	++	++	++	++	-	-
wk. agonist	K	4K	++	nd	++	+	++	-	+/-
antagonist/null?	Q	3G	-	nd	-	-	-	nd	nd
wk. p. agonist	Q	3A	+/-	nd	-	-	-	nd	nd
antagonist	Q	3Abu	-	nd	nd	-	nd	-	nd
antagonist	Q	3V	-	+	nd	-	nd	-	nd
wk. p. agonist/antagonist	Q	3Nva	+/-	++	+/-	-	-	nd	nd
wk. p. agonist/antagonist	Q	3L	+/-	nd	+/-	-	-	nd	nd
antagonist	Q	3I	-	nd	nd	nd	nd	nd	nd
antagonist	Q	3Nle	-	nd	nd	nd	nd	-	nd
null	Q	3Tle	-	nd	nd	nd	nd	nd	nd
null	Q	3Pra	-	nd	nd	-	nd	-	nd
null	Q	3E	-	nd	nd	-	nd	-	nd
null	Q	3Orn	-	nd	nd	nd	nd	nd	nd
wk. p. agonist/antagonist	Q	3S	+/-	nd	+/-	-	-	nd	nd
p. agonist/antagonist	Q	3Hse	+/++	nd	-	+/-	+/-	-	nd
null	Q	3H	-	nd	nd	-	nd	-	nd
nd	R	4K, 5A	nd	nd	nd	nd	nd	nd	nd
null	R	5K	-	nd	-	-	-	nd	nd
null	R	5Cit	-	nd	nd	-	nd	-	nd
st. agonist	P	6Hyp	++	+/++	++	++	++	-	+/-
p. agonist/antagonist	P	6Pip	+/++	-	+/-	+/-	+	-	-
null	P	6Dpg	-	nd	nd	nd	nd	nd	nd
st. agonist	P	6Sar	++/+++	++	+++	++/+++	++/+++	-	+/-
st. agonist	P	6MeAla	++	+/++	++	++	++	-	-

LEGEND

+++	Significantly better (>120%) than the Rat MBP Ac(1-11) 4A peptide
++	Gives 50 to 120% maximum proliferation compared to 4A peptide
+	Gives 1 to 49% maximum proliferation compared to 4A peptide
-	No response

[a] E3 T-cell clones: This chart is a modified version of Table 2.3 in order to compare degrees of response (*i.e.*, + or -) with the other two T-cell clones. Notice that there are only three +++ and not five as in Table 2.3. The amount of acid released was determined by Cytosensor® and was done three times with 4A (10 and 1μM) and 4A6MeAla (10 μM), twice with 4A (0.1 μM), 4A6Hyp (10 μM), 4A6Pip (100 μM), and 4A6Sar (10 μM), and once with 3V4A (10 μM), 3Nva4A (10 μM), and 4A6Pip (10 μM) during a total of three separate experiments. Two of the three experiments had significant amounts of signal to noise and in all experiments the E3's responded with no more than 25% difference than basal. Proliferation was measured after approximately 2 days. The cytokines were determined after 6 h (IL-2) and 18-19 h (IL-4 and IL-10) at least twice. INF-γ and IL-12 were determined after 40-48 h only once. INF-γ was estimated from an antagonist assay supernatant rather than proliferation assay.

Table 4.1 (continued)
B.) *B10.AF2 T-cell Clones*[b]

Possible Subtype for F2	WT	APL	*B10.AF2*						
			Proliferation Assay	Acid Release	Cytokines				
					IL-2	IL-4	IL-10	INF-g	IL-12
agonist	K	4A	++	++	nd	-	nd	++	nd
wk. agonist	K	4K	++	+	nd	-	nd	++	nd
nd	Q	3G	nd	nd	nd	nd	nd	nd	nd
p. agonist	Q	3A	+/++	nd	nd	-	nd	++	nd
nd	Q	3Abu	nd	nd	nd	nd	nd	nd	nd
p. agonist	Q	3V	+	+/++	nd	-	nd	+/++	nd
p. agonist	Q	3Nva	+/-	nd	nd	-	nd	++	nd
p. agonist	Q	3L	+/-	nd	nd	-	nd	++	nd
p. agonist	Q	3I	+/-	nd	nd	-	nd	++	nd
nd	Q	3Nle	nd	nd	nd	nd	nd	nd	nd
nd	Q	3Tle	nd	nd	nd	nd	nd	nd	nd
nd	Q	3Pra	nd	nd	nd	nd	nd	nd	nd
p. agonist/null	Q	3E	+/-	?	nd	-	nd	+	nd
p. agonist	Q	3Orn	+/-	nd	nd	-	nd	++	nd
nd	Q	3S	nd	nd	nd	nd	nd	nd	nd
p. agonist	Q	3Hse	+/-	nd	nd	-	nd	++	nd
p. agonist	Q	3H	+/-	nd	nd	-	nd	++	nd
null	R	4K, 5A	-	nd	nd	-	nd	+/-	nd
null	R	5K	nd	nd	nd	nd	nd	nd	nd
null	R	5Cit	+/-	nd	nd	-	nd	+/-	nd
p. agonist/null	P	6Hyp	-	nd	nd	-	nd	+/++	nd
p. agonist	P	6Pip	+/-	+/++	nd	-	nd	++	nd
nd	P	6Dpg	nd	nd	nd	nd	nd	nd	nd
wk. or p. agonist	P	6Sar	+	+/++	nd	-	nd	++	nd
wk. or p. agonist	P	6MeAla	+	+	nd	-	nd	++	nd

LEGEND

+++	Significantly better (>120%) than the Rat MBP Ac(1-11) 4A peptide
++	Gives 50 to 120% maximum proliferation compared to 4A peptide
+	Gives 1 to 49% maximum proliferation compared to 4A peptide
-	No response

[b] F2 T-cell clones: For proliferation, each peptide was done at least once with successful results and twice for 4A, 4K, 3V4A, 3Hse4A, 4A6Hyp, 4A6Sar, and 4A6MeAla. The following peptides were done two more times with significantly high counts for cells only (background): 4A, 4K, 4A3V, 4A3Nva, 4A3L, 4A3I, 4A3E, 4A3H, 4A3Hse, 4A6Hyp, 4A6Pip, 4A5Cit, and 4A6Sar. The following peptides were done at least one more time without good results: 3Nva4A, 3A4A, and 5A4A. The proliferation was measured after 3 days. Acid release was determined four times for 4A (1 μM), three times for 4A (10 μM), twice for 4A (0.1 μM), and once for 4A (1 nm), 4K (7.5 and 75 μM), 3V4A (1 and 100 μM), 3E4A (100 μM), 6Pip (10 μM), 4A6Sar (0.1, 1, and 100 μM), and 4A6MeAla (0.1, 1, and 100 μM) during a total of four separate experiments. Cytokines, INF-γ and IL-4, were only determined by ELISA once from supernatant collected after 40-48 h. INF-γ should be repeated due to very high backgrounds by diluting the supernatant.

Table 4.1 (continued)
C.) *B10.AE2 T-cell Clones*[c]

Possible Subtype for E2	WT	APL	*B10.AE2*						
			Proliferation Assay	Acid Release	Cytokines				
					IL-2	IL-4	IL-10	INF-g	IL-12
agonist	K	4A	++	++	-	-	nd	++	nd
wk. agonist	K	4K	+/++	nd	-	-	nd	++	nd
nd	Q	3G	nd	nd	nd	nd	nd	nd	nd
wk. agonist	Q	3A	++	nd	-	-	nd	++	nd
agonist	Q	3Abu	++/+++	++	-	-	nd	++	nd
agonist	Q	3V	+/++	nd	-	-	nd	++	nd
agonist	Q	3Nva	++/+++	nd	-	-	nd	++	nd
agonist	Q	3L	++	++	-	-	nd	++	nd
agonist	Q	3I	+/++	nd	-	-	nd	++	nd
agonist	Q	3Nle	++	nd	-	-	nd	++	nd
agonist	Q	3Tle	++	nd	-	-	nd	++	nd
wk. or p. agonist	Q	3Pra	+/++	nd	-	-	nd	++	nd
null	Q	3E	-	nd	-	-	nd	-	nd
agonist	Q	3Orn	++/+++	nd	-	-	nd	++	nd
nd	Q	3S	nd	nd	nd	nd	nd	nd	nd
agonist	Q	3Hse	++/+++	nd	-	-	nd	++	nd
agonist	Q	3H	++	nd	-	-	nd	++	nd
nd	R	4K, 5A	nd	nd	nd	nd	nd	nd	nd
null	R	5K	nd	nd	nd	nd	nd	nd	nd
null	R	5Cit	-	-	-	-	nd	-	nd
null	P	6Hyp	-	nd	-	-	nd	-	nd
agonist	P	6Pip	++	nd	-	-	nd	++	nd
nd	P	6Dpg	nd	nd	nd	nd	nd	nd	nd
wk. agonist	P	6Sar	+	++	-	-	nd	+/++	nd
wk. agonist	P	6MeAla	+	+/++	-	-	nd	+/++	nd

LEGEND

+++	Significantly better (>120%) than the Rat MBP Ac(1-11) 4A peptide
++	Gives 50 to 120% maximum proliferation compared to 4A peptide
+	Gives 1 to 49% maximum proliferation compared to 4A peptide
-	No response

[c] E2 T-cell clones: For proliferation, each peptide was done once and measured after 3 days. Ranking proliferation was difficult due lack of dose response, but maximum was estimated from a scatter graph. Acid release was determined in one experiment for 4A (0.1, 1, 10 μM) and 10 μM each for 3Abu4A, 3L4A, 4A5Cit, 4A6Sar, and 4A6MeAla. INF-γ was determined from two separate pushes, but was repeated two to three times for each push due to the high backgrounds. Results for INF-γ are from the one ELISA experiment that was a 1/80 dilution and had low backgrounds (see Figure 4.2B/C). Again ranking was estimated due to the lack of a dose response curve. IL-2 and IL-4 were only done once. All supernatant for ELISA assays was collected after 40-48 h after stimulation of T cell, which might explain the lack of IL-2 production.

NOTES TO CHAPTER 4

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Chapter 5

Past, Present, and Future

SUMMARY

The previous four chapters have discussed the results of my research using altered peptide ligands (APLs) to activate myelin basic protein (MBP)-specific T-cell clones. One monoclonal T-cell line, B10.AE3s, was used to extensively evaluate their various responses to APLs (Chapter 2) and the different threshold of activation for different effector functions (Chapter 3). Preliminary data for two other monoclonal T-cell lines, B10.AF2s and B10.AE2s, were compared to the B10.AE3s in Chapter 4. In addition, the heteroclitic nature of the T-cell response and how this could affect the design of immunotherapeutic peptide drugs was reviewed (Chapter 4). In this final chapter, past and present research is briefly reviewed to highlight the future directions of this research.

PAST

In the past, activation of the T-cell receptor (TCR) was thought to be based on classic affinity/avidity models of receptor activation (1). This model predicts that the stronger response elicited by a ligand of a higher affinity is due to a higher proportion of occupied receptors. In this model, antagonism would occur by competitive inhibition of ligand for receptor (2) or the induction of an inhibitory receptor

conformation. Later results showed that these suppositions couldn't be entirely true. The T-cell response seems to correlate to the dissociation rate of the TCR for the ligand (peptide/major histocompatibility complex (MHC)) rather than purely the affinity (3,4,5,6). In addition, since antagonist ligands dissociate faster than agonist ligands, TCR antagonism cannot be explained simply by competitive inhibition (3,5). (For further discussion, please refer to Chapter 1). The discovery of APLs provided a tool for probing T-cell activation, especially the intricate network of pathways that lead to very specific effector functions.

Allen and coworkers showed that by altering only one or more amino acids in a peptide epitope, the T-cell response could be varied substantially (7,8,9). The T cell is not simply turned on or off, but can give a full spectrum of responses ranging from agonism, partial agonism, and antagonism (10). In addition, it has been shown that the response to the same peptide can vary between two different T-cell clones (9). A hierarchy of responses even exists for cytokine production without proliferation (11). This concept of T cells being cross-reactive to APLs with very few modified amino acids suggested that they could be used to block diseases mediated by specific T-cells.

PRESENT

APLs have been used to treat autoimmune diseases like experimental autoimmune encephalomyelitis (EAE, ref. 12,13,14) in rodents. In general, antagonist peptides are identified through various *in vitro* cell-based assays and then they are

tested *in vivo* using the appropriate rodent model (12,13,14). Not surprisingly, only a small fraction of these antagonist peptides actually work *in vivo*, presumably due to the heteroclitic nature of the T cell response. Since T cells 'see' the same ligand differently, careful attention must be paid to the different responses that a single APL can elicit from different clonal T cells.

Careful inspection of literature shows that the peptides that block EAE also seem to have some partial agonist activity, although this is not usually discussed (12,13,14). A systematic *in vitro* method of determining which peptide(s) will be most effective *in vivo* would make screening more efficient, which is where my project began. The previous three chapters discuss the design and synthesis of MBP Ac(1-11) APL peptides and the characterization of the full spectrum of responses of three MBP-specific-T-cell clones to this panel of APLs. We suggest that the partial agonist peptides, and not just the antagonist peptides, are the APLs that deserve special attention for *in vivo* studies. Even though the heteroclitic nature of the T-cell creates a potential barrier to the success of developing altered peptide therapy for autoimmune diseases, the reality is that APL therapy does work for animal models (12,13,14) and has shown some therapeutic efficacy in a clinical trial (15). Since the human immune system is inevitability more complex, crossing this barrier will inevitability take more studies.

FUTURE

The impact of APLs on immunological research cannot be denied. It has both provided a tool for studying T-cell activation as well as possible therapeutic strategies for the treatment of autoimmune diseases. Even though treatments using MBP peptides in clinical trials has not been entirely successful (15,16), the hope for the future is that proper "tweaking" of the peptide sequence or sequences will provide an APL that can completely block the progression of an autoimmune disease like MS (17). The research described in the previous four chapters provides useful *in vitro* data for the APLs described. Even though *in vivo* data is necessary to fully evaluate the therapeutic usefulness of these APLs, the literature (12,13,14) already suggests (although not directly addressed by authors) that partial agonists are the key to finding the effective peptide that can block disease. Therefore, APLs that are partial agonists and antagonists are the peptides that may be of interest *in vivo*.

What does this mean for preliminary drug design? The screening process of APLs could become more efficient making it easier to screen a much larger and more structurally diverse panel of APLs *in vivo* (Figure 5.1). Partial agonism towards T-cell proliferation segregates with maximal antagonism of MBP-induced proliferation, which is a characteristic of APLs that block disease (12,13,14). Since it is relatively straightforward to screen partial agonism, these results are likely to enable more efficient *in vitro* screening to select the most effective candidates for pre-clinical *in-vivo* studies. Studies *in vivo* are inevitably more time consuming and expensive than

in vitro assays like ^{3}H-thymidine incorporation, ELISA assays, antagonism assays, and many others. In addition, a large number of T-cell lines will also help evaluate the potential of the candidate (14). If an APL is a partial agonist/antagonist for more than one monoclonal T-cell population, then, presumably, although not necessarily, the probability that this APL is effective *in vivo* is higher.

Even though the number of *in vitro* assays may increase the workload in the lab, the number of peptides screened *in vivo* will be less due to eliminating more of the ineffective APLs. This can both give more time to tweaking the peptides and decrease the costs of *in vivo* studies. By subtyping the peptides, more information is already gathered about the peptides and may give some insight into their mechanism of action or at least a direction for further study. Finally and most importantly, the potential candidates can go onto toxicity studies and then clinical trials with Multiple Sclerosis patients.

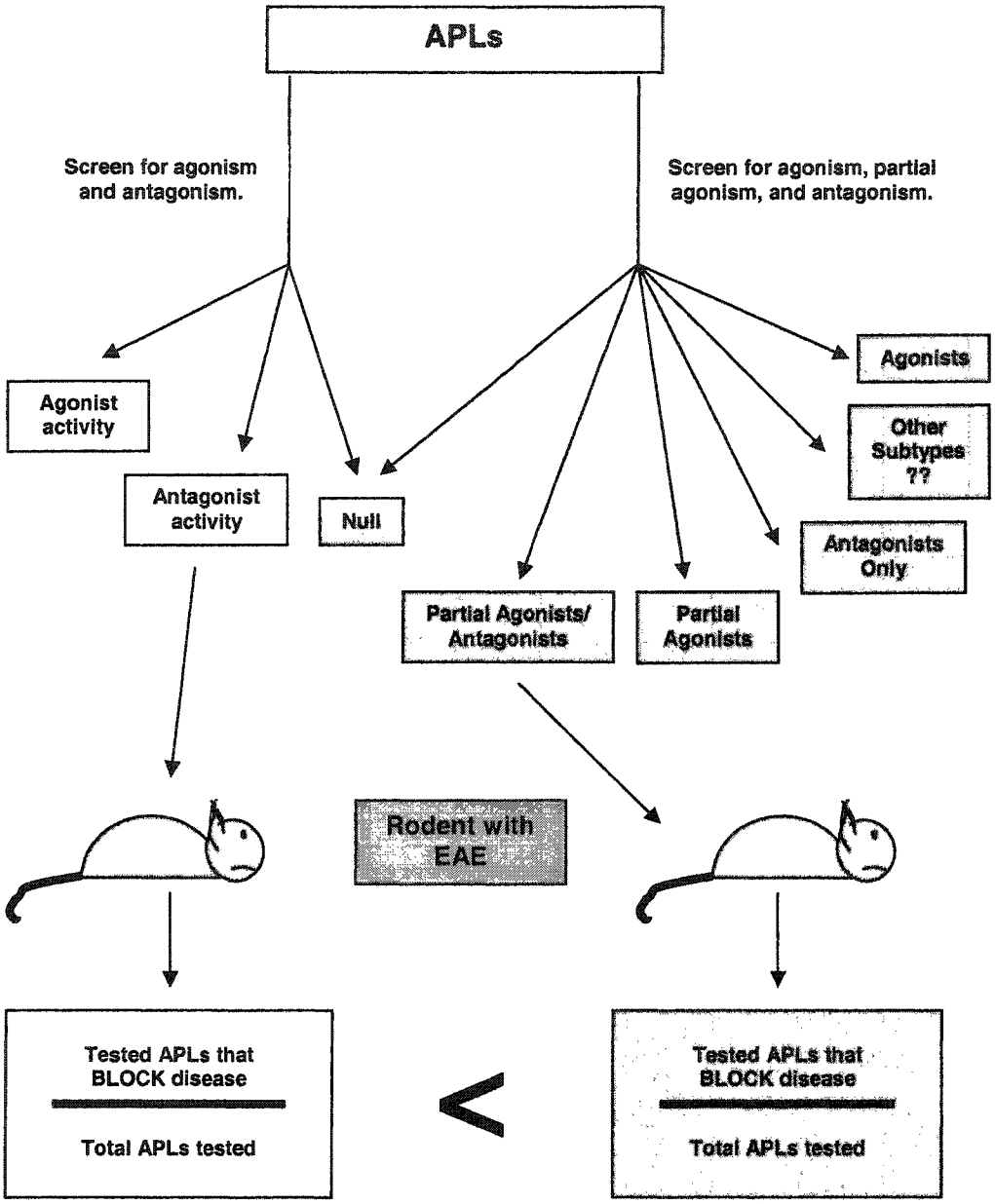

Figure 5.1: Choosing APLs systematically from a specific subtype will make *in vivo* studies more efficient. Presently, antagonism determines the APL candidacy for *in vivo* studies (white path on the left). We suggest that APLs effective at blocking disease will have a specific phenotype *in vitro* (shown here as antagonists/partial agonists). Choosing the APLs with this specific phenotype for *in vivo* studies (blue path on the right) could possibly increase the ratio of effective APLs to total peptides tested, which will make the APL screening process more efficient.

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VITA

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Danelle was born in Seattle, WA to Roy and Evelyn Dahlgren. Her parents encouraged Danelle to try everything and try hard. She grew up in Renton, WA and attended schools in the Issaquah School District. She was the valedictorian of Liberty High School in 1992. She then pursued both Chemistry and Biochemistry degrees at Seattle University graduating with honors in 1997. Wanting to further her career in science, she attended the University of Washington for her graduate studies in chemistry. While working on her Ph.D. project that incorporated both immunology and chemistry, she decided to pursue a career in Clinical Chemistry. In July 2003, Danelle will be starting the fellowship program in the Lab medicine department at the University of Washington. In her spare time, she enjoys SCUBA diving with her husband and friends. Other passions include taking care of her two miniature pinschers, Aurora and Mystra. The cutest little pups in the whole world.