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COSTIMULATORY BLOCKADE BY CD28 PEPTIDE MIMICS: SUPPRESSION OF
EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

Mythily Srinivasan

The Ohio State University

2001

Dissertation Committee:

Professor Pravin T.P. Kaumaya, Adviser

Professor Caroline C. Whitacre, Co-Adviser

Professor Richard F. Mortensen

Professor Neil R. Baker

Approved by

A handwritten signature in black ink, appearing to read 'Pravin T.P. Kaumaya', written over a horizontal line.

Adviser

Department of Microbiology

Graduate Program

UMI Number: 9999444

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ABSTRACT

Experimental autoimmune encephalomyelitis (EAE), induced in mice by the injection of myelin basic protein and pertussis toxin, is a T cell mediated autoimmune disorder that serves as an animal model for the human disease, multiple sclerosis. Complete activation of T cells requires two signals; the first signal is antigen specific and is delivered via the T cell receptor (TCR) by the peptide-MHC complex on the antigen presenting cells (APC). The second costimulatory signal is provided largely via the CD28 molecule on T cells. CD28 on T cell surface binds B7 ligands on APC transducing signals that lead to transcriptional activation of several cytokine genes, principally IL-2, and upregulation of anti-apoptotic molecules such as Bcl-x_L. CTLA-4, a structural homologue of CD28, is expressed on activated T-cells binds the same ligands on the APC transmitting a negative signal to T cells terminating the immune response. The critical role played by B7/CD28:CTLA-4 costimulatory interactions in determining the fate of immune responses make it an attractive target for therapeutic immunomodulation. Previous studies have shown that B7 blockade can either ameliorate experimental autoimmune encephalomyelitis (EAE) by interfering with CD28 signaling or exacerbate the disease by concomitant blockade of CTLA-4 interaction. Therefore, we developed a functional CD28 mimic to selectively block B7:CD28 interactions. The rationale for the peptide therapy is based on the differences in the kinetics of interaction of CD28 and CTLA-4 with B7 ligands. CTLA-4

has been shown to bind the B7- ligands with ~ 20 to 50 fold higher avidity than CD28. We hypothesized that a peptide derived from the ligand binding CDR-3 like region of CD28 will selectively block B7:CD28 interactions by providing a steric hindrance for cell surface CD28 without affecting the high affinity B7:CTLA-4 interactions. The results of the mutagenesis studies and molecular modeling were integrated in designing a CD28 peptide mimic that constituted the highly conserved polyproline motif 'MYPPPY' and designated adjacent residues. Both the end group blocked parent CD28 peptide (EL-CD28) and its retroinverso isomer (RI-CD28) exhibited similar kinetics as the extracellular domain of CD28 for binding B7-1 and competed efficiently with the latter for binding B7-1. Structural characterization by circular dichroism showed that the synthetic CD28 peptides fold into a polyproline type II (PPII) helix, an extended structure with three residues per turn. Typically, PP II helices are commonly seen in regions of globular proteins involved in transient protein-protein interactions. The localization of the polyproline motif in the solvent exposed loop at the amino terminus, the binding kinetics and the presence of a PP II helical structure suggest that the synthetic CD28 peptides represent the ligand binding epitope. In vitro biological assays showed that the synthetic CD28 peptide mimics inhibited proliferation of encephalitogenic T cells. Remarkably, a single administration of EL-CD28 or RI-CD28 peptide significantly reduced disease severity in EAE. More importantly, the synthetic CD28 peptide mimic administered during acute disease dramatically improved clinical signs of EAE suppressing ongoing disease. Characterization of the mechanism of action showed that the observed protection is presumably a result of peripheral deletion of antigen specific CD4⁺ T cells following in vivo blockade of CD28 with synthetic CD28 peptides.

Dedicated to my family

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VITA

1986-1990 ----- B.D.S., Government Dental College and Hospital, Nagpur, India.

1992-1994 ----- M.D.S., Government Dental College and Hospital, Nagpur, India.

1994-1996 ----- Instructor, Oral Pathology, Ragas Dental College and Hospital, Madras, India.

PUBLICATIONS:

1. Srinivasan, M., Jewell, S. (2000) Evaluation of TGF- α And EGFR Expression In Oral Leukoplakia And Oral Submucous Fibrosis By Quantitative Immunohistochemistry. "Oral Oncology"
2. Srinivasan, M., Wardrop, R.M., Whitacre, C.C., Kaumaya, P.T.P. (1999) A CD28 CDR3 peptide analog inhibits CD4+ Tcell proliferation in vitro. In Fields, G.B., Tam, J.P., Baraby, G. (eds) Peptides for the New Millennium. Kluwer, Netherlands.
3. Srinivasan, M., Jewell, S. Quantitative estimation of PCNA, c-myc, EGFR and TGF- α in oral submucous fibrosis – An immunohistochemical study. (Submitted to "Oncology")
4. Srinivasan, M., Wardrop, R.M., Gienapp, I.E., Stuckman, S.S., Whitacre, C.C., Kaumaya, P.T.P. A retro-inverso peptide mimic of CD28 encompassing the 'MYPPPY' motif adopts a PP II helix and inhibits encephalitogenic T cells in vitro. (Manuscript in revision, Journal of Immunology).
5. Srinivasan, M., Gienapp, I.E., Stuckman, S.S., Kaumaya, P.T.P., Rogers, C., Whitacre, C.C. Suppression of experimental autoimmune encephalomyelitis by a retro-inverso CD28 peptide mimic. (Submitted to Journal of Experimental Medicine)

FILELDS OF STUDY

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TABLE OF CONTENTS

	<u>Page</u>
Abstract	ii
Dedication	iv
Acknowledgments	v
Vita	vi
Table of Contents	vii
List of Tables	x
List of Figures	xi
 Chapters	
1. Introduction	1
1.1. Multiple Sclerosis	1
1.2. Experimental autoimmune encephalomyelitis.....	5
1.3. Immunotherapy of MS and EAE	7
1.4. The B7: CD28/CTLA-4 pathway	19
1.5. The B7:CD28/CTLA-4 pathway and EAE.....	29
2. Development of CD28 Peptide Mimics	37
2.1. Can protein epitopes be mimicked?	37

2.2	Identification of the ligand binding epitope of CD28	50
2.3	Design of CD28 peptide mimic	53
3.	Materials and Methods	57
4.	Peptide mimics of CD28 encompassing the ‘MYPPPY’ motif adopt a PP II helix and inhibit encephalitogenic T cells in vitro.....	71
4.1.	Structural characterization of the CD28 peptides by circular dichroism...71	
4.2.	The CD28 peptide mimics bind B7-1 with fast kinetics	74
4.3.	The CD28 peptide mimics compete with CD28-Ig to bind B7-1	75
4.4.	CD28 peptides inhibit activation of TCR transgenic T cells specific for MBP-NAc1-11	76
4.5.	CD28 peptides suppress cytokine production by TCR transgenic T cells specific for MBP-NAc1-11.....	77
4.6.	Expression of activation markers following in-vitro stimulation of T-cells bearing TCR V α 4 V β 8.2 specific for MBP-NAc1-11.....	78
4.7.	CD28 peptide induce apoptosis in antigen specific T cells in vitro	79
4.8.	Discussion	81
5.	Suppression of experimental autoimmune encephalomyelitis using Peptide mimics of CD28	115
5.1	Costimulatory blockade in vivo by CD28 peptides inhibit	

	incidence of EAE in B10.PL mice.....	115
5.2.	Synthetic CD28 peptide treatment ameliorates established EAE	116
5.3.	Multiple treatments with synthetic CD28 peptide mimics during the preclinical phase exacerbates EAE.....	117
5.4.	CD28 peptides suppress antigen specific proliferation of MBP Primed T cells	117
5.5.	Effect of treatment with synthetic CD28 peptide mimics on cytokine production.....	119
5.6.	In vivo blockade of CD28 costimulation induced apoptosis in antigen specific T cells.....	120
5.7.	Treatment with CD28 peptide downregulates B7-1 expression on lymph node cells and splenocytes.....	121
5.8.	Discussion	123
6.	Future Directions	142
7.	Bibliography	145

LIST OF TABLES

<u>Tables</u>	<u>Page</u>
2.1 Peptides used in the present study.....	55
4.1 Calculated parameters for EI-CD28 and RI-CD28 peptides binding to B7-1Ig by direct kinetic analysis	99
4.2 Competitive kinetics between CD28-Ig and CD28 peptides for binding B7- 1Ig.....	103
5.1 Clinical effects of CD28 peptide treatment.....	131

LIST OF FIGURES

<u>Figure</u>	<u>Page</u>
2.1 Molecular model of the extracellular domain of CD28.....	56
2.1 Schematic representation of the retro-inverso modification.....	56
4.1 CD spectrum of free and end group blocked hexapeptide.....	88
4.2 CD spectrum of L-CD28, EL-CD28 and RI-CD28 peptides at 25°C.....	89
4.3 Effect of temperature on the CD spectrum of EL-CD28 and RI-CD28 Peptides	90
4.4 Effect of CaCl ₂ on the CD spectrum of EL-CD28 and RI-CD28 peptide.....	91
4.5 CD spectrum of RL-CD28 and CTLA-4 peptide	92
4.6 Direct immobilization of CD80-Ig to the CM-5 chip.....	93
4.7 Affinity and kinetics of L-CD28 binding to CD80-Ig	94
4.8 Affinity and kinetics of EL-CD28 binding to CD80-Ig.....	95
4.9 Affinity and kinetics of RI-CD28 binding to CD80-Ig.....	96
4.10 Binding of control RL-CD28 and D-CD28 peptides to CD80-Ig.....	98
4.11 Affinity and kinetics of CD28-Ig binding to CD80-Ig	100
4.12 Competitive kinetics between CD28-Ig and CD28 Peptides.....	102.

4.13	Purity of V β 8.2 CD4+ T-cells.....	104
4.14	MBP-NAc1-11 specific proliferative responses of LNC and splenocytes from MBP peptide specific TCR transgenic mice that carry a V α 4 V β 8.2 TCR Cultured with CD28 peptides	105
4.15	MBP specific proliferative responses of LNC and splenocytes from MBP peptide specific TCR transgenic mice that carry a V α 4 V β 8.2 TCR cultured with CD28 peptides.....	106
4.16	Fewer cells secrete IL-2 when stimulated in the presence of CD28 peptides	107
4.17	Expression of cell surface markers on antigen activated LNC	108
4.17	C and D: representative histogram of CD28 and FasL expression on antigen activated LNC at different time points	109
4.18	Expression of cell surface markers on antigen activated spleen cells	110
4.19	Expression of cell surface markers on APC's.....	111
4.20	Treatment with CD28 peptides induce apoptosis in CD4+ LNC	112
4.21	Treatment with CD28 peptides induce apoptosis in CD4+ splenocytes.....	113
4.22	Representative histogram showing apoptosis of CD4+ LNC after treatment with CD 28 peptides	114
5.1	CD28 peptide treatment inhibits EAE	128
5.2	CD28 peptide mimics attenuate established EAE.....	129
5.3	Effect of multiple injections of CD28 peptide mimics on clinical EAE.....	130
5.4	Antigen specific proliferative responses are reduced in mice treated in vivo	

	with CD28 peptides	133
5.5	Mice treated with CD28 peptides secrete reduced levels of IL-2.....	135
5.6	CD28 peptide treatment in vivo induces apoptosis in encephalitogenic T cells.....	136
5.7	Injection of CD28 peptides during antigen priming prevents upregulation of B7-1 on CD4+ LNC and splenocytes following induction of EAE.....	138
5.8	Expression of CD28 and CD152 on CD4+ LNC and splenocytes from mice treated with CD28 peptides following induction of EAE.....	139
5.9	Decreased B7-1/B7-2 ratio on CD4+ splenocytes following treatment wit CD28 peptide during acute EAE.....	141

Chapter 1

Introduction

1.1 Multiple Sclerosis: Multiple Sclerosis (MS) is an inflammatory demyelinating disease of the white matter of the central nervous system (CNS). Primarily a disease of the Western Hemisphere, an estimated 350,000 individuals in the United States have MS and about twice that number in Europe. It is a major cause of neurological disability in young adults between the ages of 20 and 40 yrs (1).

Clinical course of MS: MS is usually classified according to one of four standardized clinical courses: relapsing-remitting, primary-progressive, secondary-progressive or progressive-relapsing (2). In the relapsing remitting form, which accounts for two thirds of cases at the onset of the disease, neurologic attacks occur randomly over many years. The extent of recovery among patients and from attack to attack is variable but permanent deficits can accrue with each exacerbation, especially later in the course of the disease. This neurologic deterioration punctuated by intermittent acute attacks evolves over many years to a more chronic form of disease classified as secondary-progressive MS. The primary-progressive MS and progressive-secondary MS typically involve a gradual clinical decline without clear-cut exacerbations (3, 4). MS may affect almost any part of

the CNS, producing a wide range of clinical signs and symptoms. Most common are vision disturbances, nystagmus, dysarthria, diminished perception of vibration and position sense, ataxia and intention tremor, weakness or paralysis of one or more limbs, spasticity and bladder disorders. Neurologic abnormalities that are highly suggestive of MS are optic neuritis, internuclear ophthalmoplegia, and Lhermitte's sign, an electrical sensation that passes down the back and into the legs when the neck is flexed (5). Magnetic resonance imaging (MRI) provides the most accurate assessment of disease burden, identifying multifocal cerebral white matter lesions in more than 90% of people with MS (6).

Etiopathogenesis of MS: Although MS has been known for over 100 years, its etiology remains largely unknown. Epidemiologic studies suggest that both environmental and genetic factors play a role in the etiology of MS. Considerable body of evidence support an autoimmune pathology that develops early in life in the genetically susceptible individuals after a triggering event that initiates a T-cell mediated immune response (7). The proposed mechanisms for the initial activation of myelin-reactive T cells are molecular mimicry during bacterial or viral infection, dual TCR expression, stimulation with a superantigen or exposure to high local concentrations of cytokines secreted in the course of unrelated inflammatory reactions and loss of self tolerance due to a change in autoantigen expression or breakdown of an anatomical barrier (8). Potential autoantigens in MS include proteins expressed on the myelin sheath [myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP), myelin associated glycoprotein (MAG) and non-myelin constituents of the CNS (S-100b, heat

shock proteins (HSP-60, HSP-70 and ab crystalline) transaldolase (rate limiting enzyme of pentose phosphate pathway) and CNP protein (2', 3' cyclic nucleotide 3' phosphodiesterase within oligodendrocyte myelin membranes)] (9). Factors that favor an autoimmune etiology for MS are:

- a. ***Gender predilection:*** Like most autoimmune diseases, MS affects women twice as often as men (10).
- b. ***Elevated levels of immunoglobulins in the cerebrospinal fluid (CSF):*** Increased production of immunoglobulins (predominantly IgG and to a lesser extent IgA and IgM in some patients) in the CSF is a diagnostic feature in MS patients and presumably reflects a defect in immune regulation. This is supported by the observation that the increased production of immunoglobulins by peripheral blood lymphocytes (PBL) from MS patients can be corrected with suppressor factors for T cells indicating that the overactivity of B cells is related to impaired suppression. Abnormal function of CD8+ T cells and/or a reduction in the subset of CD4+ suppressor cells have been implicated for this defective immune regulation (7).
- c. ***Association with HLA genes:*** Analogous to other autoimmune disease, susceptibility to MS is strongly associated with human leukocyte antigen (HLA) class II genes. Different HLA-DR specificities are associated with MS in different ethnic groups. In addition to HLA-DR2 (DRB1*1501-DRB5*1101-DQA1*0102-DQB1*0602), higher risk is conferred by HLA-DQW6 amongst North American and Caucasian MS populations. Association with HLA-DR3 and HLA-DR4 provide some risk incidence in ethnic groups with a lower prevalence of MS. Although circulating myelin antigen reactive HLA-DR restricted T cells are present in both healthy

individuals and MS patients, the auto-antigen specific cells are in an activated state only in MS patients. Immunodominant MBP peptides have been shown to bind a number of HLA-DR alleles due to the presence of appropriate peptide-binding motifs. The encephalitogenic peptides of PLP preferentially bind HLA-DR4 alleles (5, 7, 11).

d. *Autoantigen-specific T cell response:* Additional support for autoimmune hypothesis comes from the studies showing significant increase in the precursor frequencies of MBP or PLP encephalitogenic peptide specific T cells in the blood and CSF of MS patients (12, 13). MBP specific T cells from MS patients preferentially use TCR V genes, although the genes used vary among patients. Thus, genes within the TCR α ($V\alpha$ 3.1 or $V\alpha$ 8.2) and β chain complex ($V\beta$ 17, $V\beta$ 12) may confer risks that are independent from HLA-genes (14). Furthermore, limited heterogeneity in $V\alpha$ and $V\beta$ chain usage has also been observed in the brain tissues derived from MS patients (15).

e. Collectively, these observations suggest that myelin antigen specific T cells expressing a specific V chain that recognize the encephalitogenic peptide in the context of a HLA-DR allele are involved in the pathogenesis of MS.

MS pathology: MS is a descriptive term characterizing the gross appearance of indurated or sclerotic plaques within the white matter seen in MS brains. The pathological hallmark of MS is the presence of CNS-confined perivascular inflammatory infiltrates. The T cells committed against CNS components provide the organ specificity of the pathogenic process. These myelin-specific T cells release proinflammatory cytokines such as $\text{INF-}\gamma$ that activates macrophages and other T cells in a non-antigen specific manner which in

turn act as effector cells either by directly killing oligodendrocytes or by releasing more inflammatory cytokines resulting in demyelination. MS is therefore considered as an organ-specific inflammatory demyelinating disease of the CNS, a localized immune reaction triggered by an hypothetical event igniting a inflammatory cascade leading to the activation of specific immunity which indefinitely perpetuates the inflammatory process (7, 16).

1.2. Experimental autoimmune encephalomyelitis: Experimental autoimmune encephalomyelitis (EAE) is an inflammatory demyelinating disease of the CNS that can be induced in susceptible strains of laboratory animals by appropriate immunization with CNS antigens (5). Historically, EAE evolved a century ago from studies of postvaccinal encephalomyelitis in humans, when it was reported that a small percentage of patients receiving Pasteur's rabies vaccine developed neuromyolytic signs. Subsequently Rivers and his colleagues reported that repeated intramuscular injection of extracts of normal rabbit brain induced pathological changes in the brains of monkeys that resembled the PVE in humans indicating that the neurological side effects of rabies vaccine were related to the contaminating CNS antigens (7). A search for the relevant antigen responsible for the neurologic activity of CNS extracts led to the identification of myelin proteins (MBP, PLP and MOG) as major encephalitogens. With the use of Freund's adjuvant, it became possible to reproducibly elicit EAE after a single injection of CNS extract or purified myelin antigen or its encephalitogenic peptide emulsified in the adjuvant material.

Different animal species including rats, mice, guinea pig and monkeys have been used leading to the description of various EAE models. Susceptibility to disease and the clinical, pathological and immunological picture of autoimmune demyelination depends on the mode of sensitization, the nature of the immunogen and the genetic background of each species and strain. The observation that EAE can be passively transferred to naive susceptible animals by the injection of activated encephalitogenic T cells obtained from sensitized animals strongly suggested a T cell mediated immune response for the pathogenesis of EAE (17).

EAE as an animal model for MS and as a preclinical ground for MS therapeutics:

The most useful model of MS should reflect the relapsing-remitting disease developing initial acute attacks with specific neurologic deficits such as paralysis and later chronic neurologic diseases with relapsing episodes of paralysis followed by clinical remission and ultimately permanent deficits. Multiple schemes for immunization including inoculation with T cell clones specific for myelin proteins and injection of various antigens with different combination of adjuvants have been used to produce chronic relapsing EAE that closely resembles human MS (18).

Of the EAE models, murine EAE is more often used as a preclinical ground to prove the efficacy of potential therapeutic agents for MS. Like MS, susceptibility to EAE in mice is closely linked to MHC haplotype. Thus, certain strains of mice readily develop the disease [B10.PL (IA^u, IE^u), PL/J (IA^u), SJL/J (IA^s)] whereas others are highly resistant to EAE [J (Ia^a), BALB C mice and C57BL] (5, 19). The clinical course of murine EAE can be quite variable, some models develop an acute monophasic disease, and others develop

a chronic relapsing remitting disease. These clinical variations of EAE serve as models for the different clinical courses observed in individual patients with MS (17). Encephalitogenic T cells can be retrieved from the blood of immunized as well as naïve animals, supporting the concept that autoaggressive lymphocytes are part of the natural immune repertoire as in humans. Pathology in chronic murine EAE is characterized by perivascular lymphocytic and monocytic infiltrates, demyelination and limited remyelination, reminiscent of the pathology seen in the CNS of MS patients (7, 20). In addition, EAE reflects many of the pathophysiological steps of MS such as increased expression of adhesion molecules on the endothelial cells and on infiltrating leukocytes, increased expression of costimulatory APC's, elevated level within the CNS of cytokines such as INF- γ and TNF- α and increased levels of metalloproteinases that participate in penetrating the blood brain barrier. One of the approved treatments for MS to come out of the EAE model is the Copoxane (Copolymer-1), a synthetic polymer analog of MBP that has been shown to reduce relapsing rate in MS (21). However, there are several discrepancies between the pathology of EAE and MS. For example, while systemic administration of INF- γ protected from paralysis in EAE, treatment with INF- γ exacerbated disease in MS (22). Similarly, while blockade of TNF- α with monoclonal antibodies (mAbs) or soluble TNF- α receptor suppressed EAE, treatment with these agents worsened MS (23, 24). Thus, extrapolations must be made with caution when predicting what might happen in MS, based on results obtained in the EAE model.

1.3. Immunotherapy of MS: Conventional immunosuppressive agents such as cyclophosphamide, corticosteroids and corticotrophin hormone have been used in the

treatment of MS. However, their effects are temporary and can best be termed palliative. In addition, the ensuing severe side-effects limit their long-term therapeutic value (25). Recently, focus is directed towards developing novel agents to manipulate immunological processes with biotechnological products that mimic, inhibit or otherwise interact with naturally occurring proteins or polynucleotides. The underlying principle is to induce or reinduce a lasting state of tolerance that does not require long-term immunosuppression (8). The following is an overview of immunotherapeutic approaches in the treatment of EAE and MS.

1.3.1. Therapies targeting inflammatory cytokines: Cytokines provide the necessary signals to turn on or off autoreactive T cells that could be inadvertently activated during the course of common infections and thus play a direct role to balance effector immune responses with avoidance of autoimmunity. At the peak of disease severity in EAE, the perivascular infiltrates have increased expressions of IL-2 and INF- γ . High levels of TNF- α detected in the CSF of MS patients suggest a pathogenic role for this cytokine in MS (26).

i. ***Type-I interferons.*** There is some evidence suggesting that infection by a virus or molecular mimicry following viral infection triggers the autoimmune process in MS (27). Hence, Type I interferons, recombinant INF- α and IFN- β proteins, were tried in MS for their antiviral and antiproliferative activities. In relapsing-remitting MS, INF- β reduced the frequency of clinical exacerbations and the progression of disease activity. The ability of the INF- β to inhibit MHC class II expression presumably contributed to

the observed beneficial effect in MS. However, the effect was partial and not curative (28).

ii. ***TNF- α antagonists:*** A synthetic immunomodulator, linomide (quinoline-3-carboxamide), suppressed chronic EAE in mice by acting as a TNF- α antagonist and inhibiting early T-cell activation. In a double blind placebo-controlled study, linomide significantly reduced the disease activity in MS patients (29). Biological inhibitors of TNF- α that have shown promising results in EAE models are mAbs to TNF and soluble TNF receptor proteins. However, administration of humanized TNF- α antibody or the recombinant TNF- receptor worsened the disease in MS patients (24).

iii. ***IL-2 antagonists:*** Treatment with mAb to IL-2 at the time of adoptive transfer of MBP-specific T cells drastically reduced symptoms of EAE (30).

iv. ***Strategies for immune deviation:*** In general, Th1-inducing cytokines can directly upregulate T cell autoimmunity whereas cytokines that promotes Th2 differentiation and downregulate Th1 autoimmune responses are associated with protection from autoimmune disease (7, 26). Therapeutic application of regulatory cytokines to shift the spectrum to Th2 cytokines is an attractive strategy for the treatment of MS. Administration of recombinant TGF- β , IL-4, IL-10 have been used to induce immune deviation in EAE and MS (31-33). Suppression of EAE clinical scores, a decrease in demyelination and decrease in message for INF- γ , TNF- α and IL-2 has been reported following IL-4 therapy (32).

v. ***Limitations of cytokine therapies:*** Redundancy and the pleiotropic nature of cytokines and the fact that their pro or anti-inflammatory effects may be dependant on the stage and nature of cellular interactions has placed limits on their use in the clinic.

For example, protection against EAE is provided by treatment with INF- γ and is also demonstrated in INF- γ knock out mice. However, in humans INF- γ enhanced MS exacerbations (22). Production of neutralizing antibodies that attenuates the treatment effect and recurrence of disease after stopping treatment have been observed with INF- β therapy (34). Furthermore, no characteristic pattern of expression of cytokines can be found in MS autopsy brain compared with other inflammatory and non-inflammatory afflictions of the brain (35, 36).

1.3.2. Therapies targeting the MHC-TCR complex: Interaction between a T cell receptor binding an antigenic peptide within a MHC molecule on an APC forming a trimolecular complex (TMC) is the basis for specific immune reaction. In principle, each component of the TMC can be targeted for immunomodulation.

*i. **MHC inhibitors:*** Antibodies directed against the MHC molecule presenting the autoantigen or the MHC class II complexed with autoantigenic peptides has been shown to prevent EAE. Vaccination with peptides derived from the MHC class II beta chain hypervariable region resulted in allele-specific suppression of EAE presumably due to generation of auto-anti-MHC class II antibodies that interfere with T-cell sensitization (37).

*ii. **Antigen specific unresponsiveness:***

a. Oral tolerance: Proteins passing through the gastrointestinal tract generate antigen-specific hyporesponsiveness called oral tolerance. The primary mechanisms mediating oral tolerance include deletion, anergy of antigen-specific T cells and active cellular suppression, the primary determining factor being the dose of fed antigen. Oral

administration of MBP or MBP peptides suppressed EAE. Low doses favor active suppression, whereas high doses favor deletion and anergy (38, 39). However, although some improvement has been noted in some patients with relapsing-remitting MS, broad ranging clinical improvement has not yet been observed. A more accurate choice of antigens, as well as more precise dosing and timing of antigen-administration might lead to better results in the future (40, 41).

b. Peripheral tolerance induced via intravenous, intranasal and intraperitoneal administration of MBP or MBP peptides also suppressed EAE (42-44).

c. Altered peptide ligands: Analogs of immunogenic peptides with direct modification of MHC-anchor position or modified TCR contact sites have been formulated as alternate peptide ligands (APL). Injecting an altered form of MBP into mice with EAE generate anti-inflammatory immune responses and ameliorate the symptoms of EAE. Early safety studies in humans indicated that APL was relatively well tolerated (45). However, two studies on APL therapy for relapsing-remitting had differing results. Although both studies confirmed that an altered form of a myelin protein might induce immune response in MS, one study indicated that the APL can induce anti-inflammatory protective response while the second study suggested that the APL may have triggered the observed exacerbations of MS (46, 47). Modified peptide analogs of the encephalitogenic epitope of human MBP prevented the induction of EAE presumably due to the delivery of a negative signal by the antagonist which overcomes the agonist response possibly through the activation of antigen specific regulatory T cells (48).

d. Copolymer -1: Copolymer-1 (Copoxone or glatiramer acetate) is a synthetic random polymer of L-alanine, L-glutamic acid, L-lysine and L-tyrosine in a molar residue ratio of 6.1/ 1.9/4.7/1/0 that is effective in the treatment of early relapsing-remitting MS and provides a successful example of clinical extension of a therapeutic agent identified in EAE (49). Copolymer-1 is approved by the FDA as a drug against MS. In a six-year follow-up study, glatiramer acetate showed a continued ability to reduce the relapse rate and slow the accumulation of disability in relapsing-remitting MS (50). The suppressive effect of copolymer-1 is limited to EAE and MS having no effect on other autoimmune diseases. It has been proposed that copolymer-1 functions as a MHC-inhibitor by competing with MBP, PLP or MOG for binding to class II molecules on APC. Copolymer-1 does not require internalization or processing by APC to displace MBP peptides from the groove of class II molecules (51). However, a D-amino acid analog of copolymer-1 also binds as efficiently to the groove of MHC class II but does not inhibit EAE suggesting that MHC binding is a necessary step but not sufficient and must be followed by more specific steps such as induction of regulatory T cells or TCR antagonism to inhibit EAE (52). Recently, oral Copolymer-1 has been shown to delay the onset and decrease the disease activity in EAE (53, 54). Strategies to develop mimics of the MBP epitope peptides that will maintain the immunomodulatory activity while being stable will be an attractive alternative approach for therapeutic application in MS.

e. **Limitations of MHC based therapies:** Success of MHC and antigen based therapies is dependent upon identification of the autoantigen and its specific

encephalitogenic epitope responsible for the ongoing immune response for each haplotype and develop customized therapeutic agents (46).

iii. **Targeting autoantigen-reactive T cells:** Potentially autoaggressive T cells in healthy individuals are suppressed by counter-regulatory mechanisms. In autoimmune diseases this equilibrium is lost. Stimulation of counter-regulatory mechanism may reestablish the balance and attenuate autoimmunity (55).

a. T cell vaccination: In human clinical trials, MS patients were vaccinated with irradiated MBP-reactive T cells cloned from their blood. The treatment was well tolerated and the frequency of MBP-specific T cells remained undetectable over a period of 1-3 years in majority of the patients (56, 57). The immunization induced CD8+ regulatory T cells capable of lysing only the immunizing clones. A shift in the T-cell repertoire to other determinants of MBP was observed indicated by the appearance of different MBP reactive T-cells in the blood of treated patients suggesting that T-cell vaccination provides only temporary protection against MS (58).

b. TCR peptide vaccination: There is some evidence that vaccination with the TCR CDR2 peptides slows progression of MS and decreases T cell responses to myelin basic protein (discussed in chapter 2).

c. **Limitations of T-cell based therapies:** Success of T cell vaccination and TCR peptide vaccination is limited by the complexity of human T cell response to MBP and other encephalitogens in MS. Although certain MBP sequences are more frequently recognized, T-cell lines selected for reacting against MBP recognize many different antigenic determinants. In addition, the TCR used by MBP-specific T cells

are remarkably heterogeneous even in the same individual. Furthermore, MBP is not the only encephalitogen associated with MS (8, 47).

1.3.3. Therapies targeting adhesion and costimulatory molecules: CNS is normally separated from the peripheral immune system by the blood brain barrier (BBB) constituted by the tight endothelial junctions of brain vasculature that limit access of plasma proteins and blood derived cells. T-cells activated in the periphery, however, readily cross the BBB and are reactivated locally in the CNS by specific CNS antigens. Transendothelial migration of leucocytes across the BBB is a complex process mediated by adhesion molecules, costimulatory receptor-ligand interactions as well as chemokines and chemokine receptors (59). Based on their structure, adhesion molecules are classified into several groups including selectins, cadherins, integrin and immunoglobulin superfamily members (60).

a. ***Integrin antagonists:*** Through the action of selectins, the T cells establish a loose reversible contact with endothelial cells. This initial rolling is followed by a firm irreversible adhesion mediated by integrins $\alpha_4\beta_1$ (VLA-4), $\alpha_L\beta_2$ (LFA-1), $\alpha_v\beta_3$ (vitronectin receptor) and CD2 (LFA-2) on activated T cells (61). Monoclonal antibodies against VLA-4 suppressed actively induced EAE. Antibodies to LFA-1 delayed the onset and reduced the clinical severity of EAE (62, 63). Short-term treatment with monoclonal antibody VLA-4 integrin (Antegren) resulted in a significant reduction in the number of new active lesions on MRI in MS patients (64). A number of peptides, cyclic peptides and peptidomimetics that function as VLA-4 antagonists are in preliminary stages of development (65).

b. ***ICAM-1, VCAM-1 and MAdCAM-1 antagonists:*** The endothelial cells of the blood vessels of the spinal cord and brain express low levels of ICAM-1 and VCAM-1 constitutively (61). However, in acute EAE and during clinical relapse there is upregulation of ICAM-1 and VCAM-1 on the endothelial cells that promote leukocytic infiltration into the CNS by binding LFA-1/MAC-1 and VLA-4 respectively. Administration of monoclonal antibodies against ICAM-1 prevented induction of EAE (66). Blocking of interaction of CD2, expressed on activated T cells, with LFA-1 suppressed both actively and passively induced EAE (67). An antibody directed against MAdCAM-1, the preferential ligand for $\alpha 4\beta 7$, effectively prevented the development of a progressive, non-remitting, form of EAE. Combined treatment with antibodies against ICAM-1, VCAM-1 and MAdCAM-1 exhibited rapid remission as compared any of the antibody alone (68).

c. ***Limitations of adhesion molecule therapy:*** Depending on the type of epitope of the adhesion molecule recognized, mAb to LFA-1 can either provoke or prevent EAE. Treatment with anti-ICAM-1 can lead to severe nephrotoxicity. Furthermore, therapeutic manipulation of adhesion molecules could affect important tissue reactions such as wound repair, homeostasis and fertility (66). Hence, the widely distributed adhesion receptors are less attractive targets for immunotherapy than are the costimulatory molecules expressed at the immunological synapse (discussed below).

1.3.4. Chemokine antagonists: Chemokines are chemotactic cytokines that interact with their respective cell surface receptors to mediate the recruitment of specific leukocyte subpopulations to sites of inflammation. In EAE locally released chemokines in the CNS

bind endothelial glycoproteins, increase integrin adhesiveness and induce directed movement of T cells and monocytes. Chemokines thought to be involved in EAE and MS are monocyte chemoattractant protein-1 (MCP-1), monocyte inhibitory protein- α (MIP- α), IL-8, IP-10, and RANTES (69). Administration of mAb to MIP-1 suppressed EAE (70)

1.3.5. Therapies targeting T cell differentiation molecules: The pathoanatomical hallmark of MS is the presence of perivascular infiltration chiefly of CD4⁺ T cells in areas of demyelination in the CNS. There is a significant increase in the number of circulating CD4 + T cells in the blood and CSF of MS patients (5)

- a. CD52 inhibitors: MS patients treated with humanized antibody against CD52 (expressed on all lymphocytes) developed lymphopenia that lasted for over a year, however, developed clinical exacerbations subsequently (71)
- b. CD4 antagonists: Anti-CD4 antibody inhibits both actively and passively induced EAE (72). Infusion of anti-CD4 antibody in MS patients suppressed in vitro measures of T cell response. However, repeated infusions resulted in the generation of anti-mouse antibodies in MS patients. Treatment with a mouse-human chimeric anti CD4 antibody had conflicting results with one study reporting long-lasting reduction of circulating CD4 + T cells and a second study observing no beneficial effects of treatment (73, 74). In an attempt to overcome the side-effects of anti-CD4 therapy while maintaining the beneficial effects of blocking CD4 costimulation, peptide analogs of the binding domains of CD4 have been developed. Conformationally constrained

synthetic peptide analogs derived from the critical regions of the extracellular domain of CD4 prevented and suppressed EAE (75).

c. **Limitations:** Clearly blockade of widely expressed CD52 and CD4 molecules is more likely to result in global immunosuppression. Hence, they are less attractive targets for selective immunotherapy (8).

1.3.6. Therapies targeting T-cell costimulatory molecules: Activation of autoreactive T-cells is required for successful passive transfer of disease in EAE (7). Similarly, the presence of activated myelin reactive T cells distinguishes MS patients from normal individuals in whom the circulating myelin-reactive clones are not activated (13). Efficient activation of naïve T cells is dependent upon two signals, an antigen specific signal delivered via TCR and a second costimulatory signal. A number of costimulatory molecules thought to be crucial for the function of T-cells have been identified. These include CD80, CD86, CD134 L and CD40 expressed on a wide variety of antigen presenting cells and CD28, CD152, CD134 and CD154 (CTLA-4) expressed on T cells. Activation of T cells in the absence of costimulation can lead to clonal anergy or deletion (76, 77). The critical role played by these costimulatory molecules in shaping the immune response makes them an ideal target for therapeutic immunomodulation. Several of these T cell costimulatory receptor-ligand interactions have been targeted for therapeutic intervention in EAE.

a. **Blockade of OX40:OX 40L interactions:** OX 40 (CD134), a member of the tumor necrosis factor receptor (TNF-R) family, is expressed exclusively on activated MBP specific CD4⁺ T cells in EAE. Anti-OX40 stimulates proliferation and IL12 production

by these cells. Administration of OX40-Ig fusion protein or anti-OX40-immunotoxin at the time of onset decreased the severity of disease in EAE (78).

b. **CD40:CD154 antagonists:** The binding of CD40 to CD154 plays an important role in the development of humoral immunity and in the regulation of T-cell mediated effector responses. Enhancement of T cell responses by binding of CD154 to CD40 is mediated via upregulation of MHC class II and costimulatory molecules CD80 and CD86 on APC's increasing their ability to activate naïve T cells via CD28 (79). Furthermore, CD154:CD40 interactions induces the secretion of IL-12 by APC's which is critical for the development of Th1 type immune response and EAE (80). CD 154^{-/-} mice are resistant to EAE following adoptive transfer of MBP-specific TCR transgenic T cells. Treatment with anti-CD154 mAb prevented and suppressed EAE (81).

c. **Inhibitors of B7/CD28: CTLA-4 pathway:** While B7:CD28 interactions provide positive costimulation for T cell activation, ligation of CTLA-4 delivers downregulatory signals terminating the immune response. Considerable evidence suggests that B7: CD28/CTLA4 pathway plays critical roles in the pathogenesis of EAE and MS. Administration of monoclonal antibodies to B7-1 and CTLA-4 Ig fusion protein, both of which abrogate B7: CD28 interactions prevented EAE (82).

d. **Limitations of costimulatory molecule inhibitors:** Short-term treatment with a humanized anti-CD154 antibody decreased severity of clinical disease in MS. However, patients developed severe thromboembolic events limiting its use for sustained clinical improvement (83). Administration of CTLA-4 Ig was ineffective in ongoing disease and treatment with anti B7-2 mAb and anti-CTLA-4 mAb exacerbated the disease in EAE (84).

Despite considerable evidence implying critical roles for costimulatory molecules in the pathogenesis of EAE and MS, the varied results observed with the use of blocking agents suggest a more complex regulatory mechanism for preferential effector responses directed against invading pathogens while avoiding autoimmunity. In addition, T cell costimulatory receptors may bind the same ligands on the APC compounding the effects of blockade of intermolecular interaction. For example, the conflicting outcomes of the blockade of CD28 signaling in EAE may be a result of inadvertent blockade of the downregulatory B7/CTLA-4 interactions. Notwithstanding these perceived difficulties in selective manipulation of T cell costimulation, the potential therapeutic advantages that can be achieved by directed and controlled manipulation of the B7/CD28:CTLA-4 interactions justifies every effort to identify strategies for developing efficient immunomodulatory agent with minimal adverse effects. Targeting this pathway in vivo represents a method for selective immunosuppression of antigen specific T cells while majority of the T cells are not affected. The most attractive feature is the possibility of achieving permanent antigen-specific tolerance by a short course of treatment. While any of the molecules involved in the B7/CD28: CTLA-4 complex may be considered a candidate therapeutic agent for immunomodulation, CD28 expressed on T cells is arguably the molecule of choice for developing immunosuppressive agents.

1.4. The B7/CD28:CTLA-4 pathway:

1.4.1. Expression of B7 costimulatory ligands. The family of B7 molecules consists of three members identified until today including B7-1 (CD80), B7-2 (CD86) and B7-h (4-1BB) (85). Of these both CD80 and CD86 are ligands for CD28 and CTLA-4. CD 86 is a

70-kDa glycoprotein that is constitutively expressed at low levels on resting B cells and the monocytes. CD 80 is a 60 kDa inducible glycoprotein. Two molecules of CD80/CD86 bind to a single CD28 or CTLA-4 molecule. The cell surface expression of CD80 and CD86 differs both qualitatively and quantitatively on the APCs. They exist as oligomerized molecules. CD80 can be expressed on blood monocytes only after INF- γ stimulation (86, 87). Following immune activation, the expressions of both CD80 and CD86 are upregulated on professional APCs such as dendritic cells, Langerhan's cells, macrophages, activated B cells and activated T cells. Levels of both CD80 and CD86 are rapidly elevated by GM-CSF and CD40L:CD40 signaling on dendritic cells. Expression is also upregulated by IL-2, IL-2R, IL-5, TNF- α and TNF- β and is downregulated by IL-10, TGF- β and cross-linking of low affinity IgG receptor (88). B7-1 and B7-2 are also expressed on T cells. Interestingly, it has been shown that B7-2 expressed on murine T cells is able to interact only with CTLA-4 and not with CD28 (89).

1.4.2. CD28 expression and signaling: CD28 is constitutively expressed on the surface of all human CD4⁺ T cells and 50% of CD8⁺ T cells and virtually on all murine T cells. It has a molecular mass of 23kDa and the mature protein migrates by SDS page at approximately 44kDa as a result of glycosylation at five sites of the native polypeptide (87). In general, CD28 exists a disulfide linked homodimeric glycoprotein, although monomeric forms are also known (76). Binding of B7 to CD28, in conjunction with a TCR signal, has multiple effects including upregulation of IL-2 receptor (IL-2R) α , β , and γ chains, increased IL-2 mRNA transcription, chemokine secretion, and increased transcription of anti apoptotic factors such as Bcl_{x-L} thereby contributing to the capacity for T-cell clonal expansion (90, 91). CD28 cross-linking in the presence of anti-CD3

further upregulates expression of G₁ cell cycle kinases and the degradation of the cell cycle inhibitor p27Kip in T cells, leading to the transition from G₀ to S phase of the cell cycle. In addition, B7/CD28 interactions stimulate CD40 expression permitting T-cell help for B cells (88).

1.4.2.a. Signal transduction via CD28: Support for the role of CD28 as the major T cell costimulatory molecule comes from the demonstration that CD 28 ligation is capable of reversing antigen-specific unresponsiveness in CD4⁺ T cell clones (76). CD28 triggering can couple to several signal transduction pathways depending on the valency of stimulation and on the activation state of the T cells. In contrast to the signal delivered via TCR, signaling via CD28 is generally resistant to cyclosporin A (92). The cytoplasmic domain of CD28 contains the sequence YMN¹M (tyr-met-asn-met) (ITAM), a consensus binding site for the SH2 domain containing signaling proteins (87)]. Simultaneous ligation of TCR and CD28 results in the aggregation of these molecules in the T:APC contact site so that the TCR-CD3 ϵ and CD28 become components of a supramolecular complex resulting in sufficient tyrosine phosphorylation of the ITAM and successful initiation of the TCR-associated downstream signaling events (93, 94).

1.4.3. Expression of CTLA-4 and signaling: Most CTLA-4 is found in intracellular compartment and is not expressed at detectable levels on the surface of resting T cells; rather CTLA-4 surface expression is induced by activation of the T cell (95). CTLA-4 expression on the surface of both murine and human T cells is detectable by 24 hours after activation and peaks by day two following stimulation. CTLA-4 is also expressed as disulfide linked homodimer or monomer and has a molecule mass of 20kDa with one

glycosylation site (77). Unlike CD28, CTLA-4 engagement delivers downregulatory signals. CTLA-4 ligation inhibits CD3/CD28 induced IL-2 mRNA accumulation by inhibiting IL-2 transcription, in part by inhibiting NF-AT nuclear translocation. It inhibits progression through the cell cycle by inhibiting upregulation of cell cycle proteins cyclin D3 and cyclin dependent kinase (cdk)-4 and inhibiting degradation of p27kip. In addition, CTLA-4 cross-linking augments TGF- β secretion that is known to suppress a number of cellular responses, including T cell proliferation and cytokine production (96, 97).

1.4.3.a. Signal transduction via CTLA-4: Unlike CD28, the consensus PI3K binding site YXXM in the cytoplasmic domain of CTLA-4 is embedded within a larger intracellular localization motif (TTGVYVKMPPT) that may target CTLA-4 to intracellular membranes. Intracellular CTLA-4 traffics to the plasma membrane at the site of TCR-APC interactions and is rapidly endocytosed. Upon binding the B7 ligands, the tyrosine in the YXXM motif is phosphorylated by protein kinase p56 lck and recruits PI3K and src homology-2 domain containing tyrosine phosphatases (SHP-1) and SHP-2 to this motif. Thus, CTLA-4 presumably attenuates TCR signaling by recruiting tyrosine phosphatases (87, 98).

1.4.4. Role of CD80:CD86/CD28:CTLA-4 pathway in the induction of productive immunity and termination of immune responses: Productive signaling depends upon receptor occupancy by ligand and the transduction time required to engage the signal transduction machinery. Receptor occupancy is determined by the receptor and ligand expression levels and their kinetic and equilibrium binding properties (99).

- i. Binding kinetics of B7 molecules: CD80 and CD86 display a restricted pattern of expression on APC and depending on the type of APC can be induced by a variety of stimuli. In general, following activation of APCs, CD86 is more rapidly induced than CD80. Both the molecules have very fast on and off rates of binding, but their binding kinetics to CD28 and CTLA-4 are distinct with CD80 binding and dissociating more slowly than CD86 (76, 100). Furthermore, the signal transduction pathways recruited by CD80 and CD86 ligation of CD28 differ quantitatively (101).
- ii. Binding kinetics of CD28: CTLA-4 receptors: CTLA-4 and CD28 have a receptor structure consisting of pre-existing covalent homodimers with two equivalent binding sites. CD28 is a low avidity receptor for both CD80 and CD86 whereas CTLA-4 is a high avidity receptor (86). Both CD28 and CTLA-4 have extremely fast off-rates of binding to CD80 and CD86 ($t_{1/2}$, ~ 10 sec). The high avidity of CTLA-4 binding is primarily due to its very fast on-rate (102). Only the most extensive cross-linking of CD28 induces responsiveness to IL-2 and expression of IL2R α chain by the resting T cells. Following binding to the oligomerized B7 molecules on the APC, CD28 receptors cluster at the T cell: APC contact site (receptor capping) and increase the local concentration of CD28. The rapid dissociation kinetics of the CD28: B7 complex leads to sequential engagement of more than one CD28 receptor by a B7 molecule which over time results in quasi-simultaneous occupancy of many CD28 receptors by a few B7 molecules, an effect termed “virtual receptor occupancy”. This would essentially cross-link different receptor molecules. CD28 receptor can deliver qualitatively different signals depending on its degree of aggregation (99).

Collectively, these observations suggest that the differences in the pattern, levels and kinetics of expression of B7 molecules in conjunction with the differences in the strength and quality of the signals delivered upon binding to CD28 and CTLA-4 determines the outcome of an immune response. When B7 ligands are expressed at low levels, the B7 costimulators may preferentially bind the high affinity receptor CTLA-4. However, CD28 signals would dominate when antigen is presented by APC with higher B7 levels such as upon encounter with immunogenic antigen or adjuvants, enhancing T cell proliferation and differentiation. Once activated, the increased levels of CTLA-4 expression would again be able to out compete CD28 for ligand and terminate T cell proliferation. As B7 expression declines to low levels, all the available B7 ligands may be engaged by the high affinity CTLA-4, favoring downregulation of the T-cell response. Thus, the differential pattern of expression and distinct avidities of the interacting molecules indicate that B7:CD28 and B7:CTLA-4 interactions predominate at distinct stages of immune response (88, 95).

1.4.5. Effects of blockade of B7/CD28:CTLA-4 interactions: The agents that have been used to block this costimulatory pathway include

1. hCTLA-4 Ig: Genetically engineered fusion protein between human CTLA-4 and C2 domains of murine IgG1.
2. mCTLA-4 Ig: Genetically engineered fusion protein between mouse CTLA-4 and C2 domains of murine IgG1.
3. Fab fragments of monoclonal antibody against CD28.
4. Monoclonal antibody against CD80.
5. Monoclonal antibody against CD86

6. Monoclonal antibody against CTLA-4.

1.4.5.a. Immune responses in CD80^{-/-}, CD86^{-/-}, CD28^{-/-} and CTLA-4^{-/-} mice:

i. CD80/CD86^{-/-} [B7 double knock out (B7 d/KO)] mice: The observation that CD80^{-/-} mice are capable of mounting an effective humoral and cell mediated response to nominal antigens lead to the identification and cloning of CD86 (76). Unlike the CD80 knock out mice, CD86^{-/-} mice are severely immunocompromised. Purified DO11.10 TCR (specific for the ova peptide 323-322 presented in the context of I-A^d) Tg T cells stimulated with B7 d/KO APC in the presence of optimal or supraoptimal concentration of antigenic peptide proliferated and produced IL-2 at reduced levels supporting the in vitro observation that B7 costimulation is important but not essential for T cell proliferation and IL-2 production. However, IL-4 production was significantly reduced from DO11.10 TCR Tg T cells stimulated with B7 d/KO APCs. T cells stimulated with wild type APCs, then restimulated with B7 d/KO APCs, produced relatively normal levels of IL-4 and INF- γ , while IL-2 production was significantly reduced (103). If B7 d/KO APCs were used in both the primary and secondary stimulation, there was essentially no IL-4 produced, and decreased proliferation as well as IL-2 and INF- γ production (104). Thus, the lack of costimulatory signals affected differentially the proliferation and cytokine production of T cells depending on the time the T cells were exposed to TCR signal in the absence of costimulation. Although B7 d/KO mice developed normal levels of IgM, they were unable to generate antigen-specific IgG1 and IgG2 α responses and lacked germinal centers (88).

ii. CTLA-4^{-/-} mice: The CTLA4 knock out mice manifest a massive lymphoproliferative disorder and multiorgan tissue destruction that results in death in 3-4 weeks of age.

Therefore, CTLA-4 mediated regulation of autoreactive T cells appear to be critical for the maintenance of immunological homeostasis (105, 106).

iii. CD28^{-/-} mice: Although CD28^{-/-} mice exhibited normal T and B cell development; selective defects in their immune responses were observed. In vitro T-cell responses to concanavalin A and anti-CD3 were reduced, but measurable (77). The T cell proliferative response to alloantigen is reduced in magnitude in CD28^{-/-} mice compared to wild type mice, but the response levels are not zero. TCR Tg CD4⁺ T cells from CD28^{-/-} mice mounted proliferative responses at earlier time points similar to that of normal CD28^{+/+} control animals (107). However, compared to the later, the former T cells produced small amounts of IL-2 and failed to maintain the proliferative responses. CD28^{-/-} mice are also unable to mount an effective memory or recall T-cell response. In addition, germinal center formation was defective and helper T cell activity was significantly reduced with low titers of IgG1 and IgG2b but increased IgG2a and normal IgM and IgG3 in CD28^{-/-} mice (108). Antibody production and immunoglobulin class switch after are variably affected in CD28^{-/-} mice depending on the nature of the antigenic stimulus (88). Thus, though the CD28^{-/-} mice confirmed the critical role of CD28 in immune responses, the relatively mild phenotype suggests that in the absence of CD28, other costimulatory molecules may functionally compensate.

1.4.5.b. In vivo regulation of immune responses by manipulation of the CD28/B7 pathway:

- i. ***Activation of immune responses:*** The inability of tumors to induce immune responses has been, in some cases, correlated with a lack of expression of costimulatory

molecules on the tumor cells. The expression of either CD80 or CD86 on a cell that lacks costimulatory molecule could convert a non-functional APC into a functional one. Based on this rationale, it has been observed that introduction of CD80 or CD86 into tumor cells enhanced the anti-tumor response provided the tumor specific antigens were presented in the context of MHC class I or MHC class II (77, 93, 109)]. Until now, all studies have concerned murine tumor models. In humans, the anatomical location of, the tumor mass, the compromised immune system and the long tumor/host relationship may make this approach less effective.

ii. ***Suppression of immune responses:*** The interruption of the B7:CD28 pathway can lead to the development of antigen-specific hyporesponsiveness, which can be exploited for suppression of immune responses in various disease models.

1. Transplantation: The importance of CD28/B7 interactions in vivo was first established in the transplant setting demonstrating that treatment with hCTLA-4 Ig results in long-lasting tolerance to human xenoantigen in mice. Although hCTLA-4 Ig prolonged graft survival in a fully mismatched rat cardiac allograft model, eventually all grafts were rejected (92). Combined infusions of anti B7-1 mAb and anti-B7-2 mAb or infusion of CTLA-4 Ig reduced the incidence of lethal graft versus host disease in bone marrow transplant recipients who were mismatched at both MHC class I and MHC class II with the donors (77).

2. Humoral responses: Primary antibody responses to soluble proteins such as the key limpet hemocyanin or cell bound antigens such as sheep red blood cells were completely inhibited by CTLA-4 Ig in a dose-dependent fashion in mice. Human CTLA-4Ig therapy during the primary response reduced secondary and tertiary

responses to the nominal antigen. The amount of reduction correlated with the concentration of hCTLA-4Ig used in the initial treatment, suggesting that the immunosuppression lasted only for the duration of circulating serum levels (77, 92).

3. Autoimmune diseases:

- i. Diabetes: Non-obese diabetes (NOD) mouse is a well-studied model that shares many features of human insulin dependent diabetes mellitus (IDDM). hCTLA-4 Ig or anti B7-2 mAb treatment of young NOD mice dramatically reduced the incidence of disease. Administration of anti-CD80 mAb, however, worsened the disease (110).
- ii. Systemic Lupus Erythematosus (SLE): In the murine model for SLE, NZB/NZW F1 mice (B/W) spontaneously develop lupus-like autoimmune disease characterized by the production of autoantibodies to self molecules such as dsDNA. The treatment of these mice with murine CTLA-4Ig prior to the detection of autoantibodies significantly inhibited autoantibody production and disease progression, even after cessation of treatment (111).
- iii. Allergic asthma: Allergic asthma, a disease characterized by airway hyperresponsiveness, eosinophilic inflammation and elevated IgE levels is mediated by CD4⁺ T lymphocytes producing the Th2 cytokines, IL-4 and IL-5. Administration of CTLA-Ig before antigen sensitization in A/J mice (a murine model of ova-induced airway hyperresponsiveness) or before pulmonary antigen challenge abolished antigen induced airway hyperresponsiveness and pulmonary eosinophilia, decreased the levels of IL-4 and Ag-specific antibody isotypes IgG1 and IgE (112).
- iv. Experimental autoimmune glomerulonephritis (EAG): EAG is an experimental model that can be induced in susceptible strains of rat by immunization with

preparations of rat glomerular basement membranes in adjuvant and shares multiple features with human Goodpasture's disease. Administration of CTLA-Ig ameliorated EAG reduced antibody production, the numbers of infiltrating T cells and macrophages and prevented target organ injury (113).

v. Psoriasis: Intravenous infusions of the soluble chimeric protein CTLA4Ig in patients with psoriasis vulgaris resulted in clinical improvement associated with reduced activation of lesional T cells, keratinocytes, dendritic cells (DCs), and vascular endothelium. Expression of CD40, CD54, and major histocompatibility complex (MHC) class II HLA-DR antigens by lesional keratinocytes was markedly reduced. The decreased number of lesional T cells has been attributed to inhibition of T- cell proliferation, T-cell recruitment, and/or apoptosis of antigen- specific T cells at extralesional sites (114, 115).

1.5: The B7/CD28:CTLA-4 pathway and EAE:

1.5.1. Role of B7:CD28 pathway in the pathogenesis of EAE and MS: Considerable amount of evidence suggests that the B7/CD28:CTLA-4 pathway plays a critical role in regulating T cell function at several stages of EAE. These include the initial activation, expansion and differentiation of T cells within peripheral lymphoid tissues, homing and entry into the CNS and reactivation of autoreactive T cells within the CNS that mediate tissue injury (1, 84, 116).

While nearly all MBP specific PL/J TCR transgenic mice (that express a V α 4 V β 8 TCR on T cells) in a RAG-1^{-/-} background develop spontaneous EAE, CD28^{-/-} Tg MBP+

RAG-1^{-/-} mice are free of disease providing a genetic evidence for the importance of CD28 costimulation in the initiation of spontaneous EAE. The T cells from CD28^{-/-} RAG-1^{-/-} MBP TCR Tg mice could proliferate and produce IL-2 in response to MBP in vitro, excluding clonal anergy as the mechanism of protection against spontaneous EAE in these mice (117). Although C57BL/6 mice that lack B7-1 or B7-2 ^{-/-} alone developed EAE comparably to wild type mice, deficiency of both B7-1/B7-1 ^{-/-} or CD28^{-/-} rendered these mice highly resistant to MOG peptide induced EAE with none developing clinical signs of EAE during the 35-day observation period. The lack of clinical disease was accompanied by a decrease in the number of inflammatory foci in the CNS parenchyma. Since the peripheral T cells from these mice produced increased levels of INF- γ and TNF- α , the resistance to EAE was presumably not due to lack of Th1 cytokines but may be due to reduced expansion of MOG-specific T cells. Significantly, the clinical disease and the inflammatory histopathology were markedly reduced in B7-1/B7-2 ^{-/-} mice after adoptive transfer of MOG-specific wild-type T cells implying a critical role for B7:CD28 interactions in the effector phases of EAE (118). In contrast, Grivin et al reported that in the B7-2^{-/-} NOD mice immunized with PLP peptide the clinical severity of EAE was markedly reduced as compared to wild type controls. The differences in these studies may be due to the differences in the myelin antigen used and/or the genetic background of the mice reflecting differences in the levels of expression of the B7 costimulatory molecules (119). Chromosomal regions encoding CD28/CTLA-4 and B7-1/B7-2 as susceptibility loci in the induction of EAE have been identified. It has been speculated that polymorphisms within these loci may regulate the differential expression of B7 costimulatory molecules in individual mouse strains (120).

Immunohistochemical and flow cytometric analyses of the *in vivo* expression of the B7 costimulatory molecules and their receptors during the course of acute disease, remission and relapse have demonstrated that the expression of these molecules are differentially regulated at different times during the course of EAE. Using T cell clones specific for PLP or MBP Prabhu Das et al (121) have shown that while resting murine T cells express B7-2 and little or no B7-1, following activation expression of B7-2 was downregulated with a concomitant increase in the expression of B7-1. Karandikar et al (82) studied the pattern of expression of B7-1 and B7-2 during various stages of PLP induced EAE in SJL mice. Flow cytometric analysis revealed a temporal upregulation of surface expression of B7-1 relative to B7-2 on splenic B cells, T cells and macrophages as well as on CNS infiltrating cells during acute disease in both actively and passively induced EAE. In contrast, Issazedah et al (122) showed by immunoperoxidase staining that in MBP induced EAE in (PLJ XSJL/J) F1 mice; B7-2 was upregulated on the cellular infiltrates within the CNS during the preclinical and acute phase of the disease and during relapses. Although B7-1 was upregulated on the splenocytes during acute phase of EAE, the CNS infiltrates did not express detectable levels during this stage. Significantly, B7-1 was upregulated on the CNS infiltrates and neurons during clinical remission. CD28 expression upregulated in parallel with B7-2 and correlated with the increase in Th1 cytokine expression in the CNS. Furthermore, CTLA-4 expression upregulated during acute disease peaked during remission and persisted during relapses. In a different system, it has been shown that two days after intranasal administration of the encephalitogenic peptide MBP NAc 1-9 to mice transgenic for a TCR specific for the I-

A^u restricted N-terminal peptide of MBP, MBPAc1-9, there is a maximal upregulation of CD28 and CTLA-4 on splenocytes and cervical lymph node cells. Furthermore, an increased proportion of CD4⁺ T cells expressed CTLA-4 even seven days after intranasal administration of the encephalitogenic peptide, at which time point the CD28 expression had returned to the basal level (123). T cell –activation by splenic and CNS APC's derived from mice with R-EAE was blocked most effectively by anti-B7-1, whereas anti B7-2 blocked proliferation more effectively when naïve splenocytes or lymph node cells were used as APC's substantiating a predominant role for costimulation via B7-1 relative to B7-2 in mice with ongoing R-EAE (124). In EAE induced in SJL mice by adoptive transfer of MBP specific T cells Cross et al (89) have demonstrated that a higher proportion of CD3⁺ T cells within the CNS express B7-1 more than B7-2 and the proportion of B7-1⁺ and B7-2⁺ T cells within the CNS is far greater than in the periphery. It has been suggested that B7-1⁺ T cells either selectively entered the CNS or were induced to express these molecules following entry into the CNS.

Experimental evidence has shown that CNS is constantly patrolled by the immune system. The demonstration that the monocytes and activated T cells slowly traffic through the normal CNS and that the CNS microenvironment is able to regulate immune responses has shed light on the mechanism underlying immune surveillance and active maintenance of immune privilege in the CNS. The differential expression of B7-1 molecule in the CNS compartment selectively triggered under local pathological conditions might induce regulatory autoreactive T cells to become pathogenic autoreactive T cells. Collectively, these studies indicate that B7/CD28:CTLA-4 mediated

costimulation is an important factor in determining encephalitogenicity and autoimmune demyelination in EAE (125).

1.5.2. Studies on treatment of EAE by blocking B7/CD28/CTLA-4 interactions: Recent insights on the role of costimulatory molecules in the pathogenesis of EAE have been used to inhibit the activation of naïve T cells recognizing autoantigens as well as to manipulate pathogenic responses of recently activated self-reactive T cells to control autoimmune diseases. In different models of EAE, several intriguing observations have been made.

A single injection of hCTLA-4Ig during immunization later suppressed MBP induced EAE in Lewis rats (126). In chronic relapsing EAE induced by transfer of MBP specific T cell, administration of hCTLA-4 Ig during immunization and during in vitro activation resulted in the amelioration of clinical disease. However, the effect of systemic administration of hCTLA-4 Ig appears to be schedule dependent in PLP induced EAE in SJL mice. These mice were protected with a single injection of hCTLA-4 Ig administered two days after immunization, but exhibited clinical exacerbations following hCTLA-4Ig treatment during acute disease (127). Specific blockade of CTLA-4 with an anti-CTLA-4 mAb during immunization with PLP peptide resulted in more severe disease progression in SJL mice (128). Systemic injection of APC's pulsed with the encephalitogenic peptide in the presence of CTLA-4 Ig administered before or during immunization with MBP in CFA protected Lewis rats from EAE presumably by inducing regulatory T cells that are capable of preventing priming or down-regulating other primed T cells that may have

encountered normal APCs capable of providing a costimulatory signal (129). Treatments with reagents targeting B7-1 or B7-2 have led to varying results in different animal models. In SJL mice, administration of anti B7-1 mAb during the induction of relapsing-remitting EAE or two days later protected the mice from disease. However, stopping the treatment during the late effector phase resulted in the reappearance of disease. In contrast, treatment with anti B7-2 mAb or both anti-B7-1 mAb and anti B7-2 mAb at the time of immunization exacerbated the disease severity (130). Treatment with anti B7-1 mAb also skewed the auto-antigen reactive T cells towards a Th2 (anti-inflammatory) phenotype while blockade of B7-2 was correlated with skewing towards a Th1 (proinflammatory) phenotype. Furthermore, transfer of antigen-specific Th2 clones generated by blocking the B7-1 molecule both prevented EAE and abrogated established disease. In addition, administration of Fab fragments of anti-B7-1 mAb during the first remission after the acute phase of EAE blocked disease relapses by inhibiting epitope spreading that mediates clinical relapses in diseased animals (131). In PLP₁₃₉₋₁₅₁ induced R-EAE, treatment with anti B7-1 (Fab) or mCTLA-4Ig inhibited development of the relapse associated PLP₁₇₈₋₁₉₁-specific response, and in MBP₈₄₋₁₀₄ induced R-EAE blocked development of PLP₁₃₉₋₁₅₁ responses. In contrast treatment with anti B7-1 mAb, anti-B7-2 mAb or hCTLA-4Ig during disease remission exacerbated the disease (132). Thus, most studies have supported the role of B7-1 as the functionally dominant costimulatory molecule in mice with ongoing relapsing remitting EAE. A significant percentage of CNS specimens from patients with MS also expressed increased levels of B7-1 but not B7-2 on lymphocytes localized to the perivenular inflammatory cuffs of the brain (125). The protective effect of anti-B7-1 mAb administered during immunization may be a

result of blocking signals delivered via B7-1 or due to blockade of B7/CD28:CTLA-4 interactions. Evidence that the blockade of CD28 signaling is actually responsible for reducing clinical disease following costimulatory blockade comes from the most recent studies by Perrin et al, demonstrating that treatment with Fab fragments of CD28 that does not stimulate CD28 signaling both prevented and suppressed EAE. The MBP specific T cells from these mice were less encephalitogenic when subsequently transferred to naïve recipients (133).

Thus, the potential advantages of in vivo manipulation of B7/CD28:CTLA-4 costimulatory axis are evident from many studies in EAE. However, failure of selective interference with specific intermolecular interactions at appropriate stages of the disease has led to conflicting results. Since multiple injections of CTLA-4Ig enhanced the disease it has been speculated that the constant presence of CTLA-4Ig prevented the delivery of a subsequent negative signal via B7 ligands interfering with the induction of regulatory cells (127). In addition, the disease potentiating effect of CTLA-4 Ig administered during remission may be a result of blocking the downregulatory B7:CTLA-4 interactions. So also, treatment with anti-B7-2 presumably blocks the high avidity interactions between B7-2 molecule on the APCs and the CTLA-4 on activated T cells that play critical roles in restricting the expansion of immunogenic T cells both during the preclinical stage and in the late effector phases of EAE. Furthermore, none of the agents used other than the recently reported Fab fragments of anti-CD28 mAb have been shown to be able to block an ongoing autoimmune pathology.

1.6. Objectives: Careful analysis of the above mentioned studies indicate that targeted immunomodulation by an agent that would selectively interfere with the interactions between the highly expressed B7-1 molecule on the APC's and the T cell activation costimulatory molecule, CD28, is more likely to ameliorate autoimmune disease progression. Based on these observations, we hypothesized that a small molecule mimic of CD28 will have maximum therapeutic potential in EAE. The rationale for CD28 therapeutics is based on the differences in the kinetics of interaction of CD28 and CTLA-4 with the B7 ligands. The CTLA-4 molecule binds the B7 ligands with 20-50 fold higher affinity than the CD28. We proposed that a mimic of the ligand binding epitope of CD28 could have binding properties equivalent to the native CD28 and selectively block B7:CD28 interactions by competing with cell surface CD28 for binding the B7 ligands. The cell surface CTLA4 can overcome this competition due to its higher affinity for B7 ligands. The objectives of the present study are as follows: 1) Identify the ligand binding epitope of CD28 that interacts with B7 molecule. 2) Transfer of the ligand binding epitope of CD28 onto a short peptide. 3) Design and synthesis of mimics of CD28 peptide to increase the in vivo stability. 4) Structural characterization of CD28 peptide and the peptide mimics. 5) Determination of the kinetics of interaction of CD28 peptide mimics with B7-1 molecule. 6) Functional characterization of CD28 peptide mimics in vitro. 7) Evaluation of the therapeutic potential of CD28 peptide mimics in vivo in EAE. 8) Functional characterization of the mechanisms of action of CD28 peptide mimics in vivo.

Chapter 2

Development of CD28 peptide mimics

2.1. Can protein epitopes be mimicked?

The non-palindromic nature of peptide and proteins is the basis for the synthesis of mimics or analogs of natural bioactive molecules. Intermolecular recognition essential for many specific and efficacious biochemical processes involves interaction between complementary molecular surfaces. Hence, identification and characterization of relevant molecular topology is central to rational drug design (134). There are several examples in the literature that show successful transfer of a protein binding epitope onto a smaller molecule. The basic premise is that the key residues of the binding epitope, in particular side-chain functional groups responsible for a significant portion of the binding affinity to a given receptor/ ligand, can be transferred to a much smaller molecule with the contributions to binding largely intact (135). Both peptide and non-peptide entities have been developed as small molecule mimics of protein binding epitopes. In many cases, the secondary structure of a peptide or protein serves primarily as a template or scaffold for presentation of specific side-chain groups in three dimensional shape for molecular recognition and biological activity such as signal transduction. The polypeptide backbone offers many advantages as a scaffold because it possesses thermodynamically and kinetically accessible pathways for transformation from one secondary structure to

another and these three-dimensional changes are required for many biological processes (136).

Peptide Antagonists of IgSF proteins

Immunoglobulin supergene family (IgSF) includes a large number of proteins that exhibit wide heterogeneity in terms of tissue and species distribution and biological functions. In the human immune system, members of this family are critically involved in the initiation, propagation and termination of immune responses. Nearly 40% of proteins on human leukocytes belong to the IgSF and act as cell adhesion or cell surface recognition molecules that trigger upon binding a subsequent functional event (60) While normally they are involved in generating protective immune responses, under certain conditions, such as continued presence of an infectious agent, the levels of expression of IgSF proteins are upregulated and may lead to the development of autoimmune diseases. In addition, many IgSF proteins also serve as receptors for viruses facilitating viral entry and survival within the host. Targeting the cell surface IgSF proteins is thus an attractive strategy for blocking aberrant immune responses and preventing viral infections.

Protein-protein interactions are generally quite specific and much can be learned about their functions from genetic, biochemical and structural studies. Recent analyses of human growth hormone-receptor complex and erythropoietin receptor complex indicated that only a subset of protein residues constituting the binding epitope make energetically significant contacts in the complex (137, 138). This raises the intriguing possibility that mimics of the few critical residues presented in appropriate spatial orientation for optimal

interaction representing the binding determinants of a receptor or a ligand may be sufficient to block large protein-protein interactions (139). With the advent of combinatorial peptide libraries and phage display, numerous peptides selected to bind other protein receptors have been found to target and block the binding sites of natural ligands supporting the concept that most receptors contain preferred binding sites on their surfaces. These sites have evolved to bind natural ligands but are competent for binding other ligands as well. Therefore, knowledge about the molecular topology of the interacting surface of the ligand and receptor can be capitalized for rational drug design of agonist/antagonist of such interactions (140).

The IgSF structure:

The IgSF domain is evolutionarily ancient. Both extracellular and intracellular proteins of *C. elegans* have been shown to possess IgSF domains. It has been suggested that the function of the IgSF domain in antigen-specific recognition by antibodies and TCRs is an evolutionarily recent adaptation of vertebrates (141). IgSF members have one or more Ig-like domains. Each domain is 110 amino acids in length and is characterized by certain conserved residues around the two cysteines found in every domain. The alternating hydrophobic and hydrophilic amino acids give rise to two closely packed β sheets consisting of anti-parallel β strands containing 5-10 amino acids. The β strands are lettered A through G, in the order in which they appear in the amino acid sequence. Strands located in opposite sheets are linked by connecting peptide loops called complementarity-determining regions (CDR). The loops in IgSF domains are named after the strands that they connect, and the ends of the domain formed by the loops will be

termed the top and the bottom, for the ends that contain the N- terminus and C-terminus of the domain sequence respectively. The loops at the top of the V domain are the BC, C'C'', DE and FG loops. The BC, C'C'' and FG loops constitute the CDRs (142). An important feature of the Ig domain structure is the mutual complementarity that allows strong interdomain non-covalent interactions. The CDRs possess a solvent-accessible active site or binding pocket, which is spatially separated from the rigid core that acts as a biomolecular scaffold to bring together exposed loops. Despite sharing a common structural scaffold, members of IgSF show distinct structural properties and engage in intermolecular interactions in many different ways (143). Similar to the observation that antibodies binding to antigens are mediated via residues in CDR, it has been shown that IgSF members also utilize loops at the top of domains to recognize distinct ligands (139).

TCR Peptide Therapy for Autoimmune diseases.

The most abundant TCRs are heterodimers consisting of transmembrane α and β chains, each with a variable and constant domain. Several studies have demonstrated that the self-reactive T lymphocytes in EAE preferentially use TCR β chain 8.2 V region gene and treatment with a V β 8.2 specific mAb significantly reduces the severity EAE (144). Subsequently, it was shown that immunization with synthetic peptides derived from the hypervariable CDR-2 region of the TCR V β prior to disease induction prevented EAE (145). In parallel with studies in EAE, it was shown that the MS patients showed a biased representation of TCR V β 6 chain genes in their cerebrospinal fluid (CSF) T cells (146). As a logical progress towards possible therapeutic application for human MS, a peptide vaccine from the TCR V β 6 CDR2 region (39-LGQPEFLTYFQNEAQLEKS-58) was

shown to be immunogenic in patients with multiple sclerosis expressing high CSF levels of V β 6 T cells. There is some evidence that vaccination with the TCR CDR2 peptides slows progression of MS and decreases T cell responses to myelin basic protein (147).

Restricted TCR usage has also been shown to play a critical role in the pathogenesis of autoimmune rheumatoid arthritis. Parenteral administration of a combination of three TCR peptides (V β 3, V β 14 and V β 17) resulted in significant clinical improvement in patients with rheumatoid arthritis (148).

TCR peptide therapy for retrovirus infection:

The immunoglobulin CDR regions have individual characteristic shapes that can be recognized by the appropriate antibodies as idiotypic determinants. Exogenous agents through idiotypic network connections may stimulate self-reactive lymphocytes. An early immune response is increased production of autoantibodies to TCR V β induced by superantigens in retrovirus infected mice. Multiple cycles of mutation in the retroviral superantigen gene leads to progressive expansion/depletion of some TCR α/β CD4⁺ T cells, polyclonal activation of T and B cells, aberrant cytokine production and subsequent immunodeficiency (149). During the normal course of retroviral infection, the levels of autoantibodies to TCR V β produced may not be sufficient to prevent downregulation of the immune responses. Hence, it was hypothesized that neutralization of the autoantibodies with specific peptide antigens may slow down the process of selective expansion or deletion of T-cells. Synthetic peptides derived from the CDR1 region of TCR V β 8.1 (CKPISGHNSLFWYRQT) and V β 5.2 (CSPKSGHDTVSWYQQA) significantly prevented retrovirus induced suppression of immune responses and largely

maintained normal cytokine production presumably by stimulating a natural immunoregulatory network that induces tolerance to T-cells expressing the targeted TCR V gene product. The protective effect was not significantly increased by the combined use of the two peptides suggesting that treatment with one TCR peptide likely stops aberrant activation of other T-cell subsets and hence, may be sufficient to prevent chronic viral infections (150).

Cell surface receptor antagonists:

Structurally, most accessory cell surface molecules expressed on T cell surface belong to the IgSF (141). Typically, these molecules have multiple domains with a propensity to form locally driven isolated structural elements arranged in tandem along the polypeptide chain. The minimal sequence that retains intrinsic biological activity (i.e. the bioactive core) has been identified for some of the cell surface molecules. Recently there has been a great enthusiasm in adapting this knowledge to develop specific peptide agonists and antagonists for immunomodulation (135, 151).

CD4 antagonists: While normally involved in protective immune responses against foreign antigens, under certain circumstances CD4⁺ T cells are triggered to act cells presenting self-antigens resulting the development of autoimmune diseases or causing transplant rejection. Hence, development of drugs that regulate CD4⁺ T cells may have potential therapeutic application.

Structurally, CD4 consists of four (D1-D4) external Ig like domains and binds via its D1 domain the non-polymorphic site of MHC class II. The amino terminal D1 domain has

three complementarity determining like regions (CDR). For T cell activation the CD4 molecule functions by dimerizing via its CDR-3 like region and an extended loop in the D1 and D4 domains. These observations provided the premises for designing CD4 peptide antagonists derived from the CDR like regions of CD4 ECD. Peptide mapping, molecular modeling and solution structure of CD4 ECD have identified residues that are critical for its interaction with MHC class II. Conformationally constrained analogues of a linear peptide derived from CDR-3 like region of murine L3T4 (86-104 C-C) and human CD4 (84-100 C-C) were shown to inhibit CD4 dependent T cell responses such as T cell proliferation and IL-2 production (152). Remodeling the peptide by the insertion of a “forced turn” using a P-G-P motif (CELENRKEEPGPC) to closely mimic the biologically active surface of the CD4 molecule increased the inhibitory potential of the peptide mimic (153). More significantly, a retro-inverso analogue of the modified peptide not only inhibited the clinical symptoms of experimental autoimmune encephalomyelitis but also reduced the severity of the established disease (154).

Like many protein-protein complexes the interface between CD4 and MHC class II involves a large surface area. Epitope mapping experiments in conjunction with computer-aided structural analysis identified additional residues in an adjacent protruding loop of CD4 ECD as also critical for its interaction with MHC class II. A cyclic heptapeptide (CNSNQIC) derived by main-chain cyclization was designed to closely mimic the conformational features of β turn adopted by the native loop in the human CD4 molecule. The resulting CD4 peptide mimic exhibited remarkable immunosuppressive effects in vivo in three different CD4⁺ T cell dependent disease

models including EAE, graft versus host disease across a haplomismatch MHC difference and skin allograft rejection by inhibiting the CD4: MHC class II interaction (155).

Inhibitors of CD4:gp120 interaction:

The pathogenesis of HIV induced disease involves the receptor-mediated actions of the external envelope glycoprotein gp 120, which binds endogenous receptors to alter normal ligand signaling. In addition to its role as an accessory molecule for T cell activation, CD4 molecule is also the primary receptor for human immunodeficiency virus. Peptide mapping and X-ray structural analysis revealed that 22 residues spanning amino acids 25-64 in the D1 domain and centered on the CDR-2 like region of CD4 molecule interact with gp120 as the virus gains entry into the CD4+ Tcell. Linear peptides encompassing the critical residues failed to inhibit the CD4:gp120 interaction and prevent viral infection. However, a pentapeptide from the CD4 CDR-2 like region (QGSFLT) derived as a trifluoroacetate salt closely mimicked the b turn at the Gln40 –Phe43 abrogated the binding of human T-lymphotropic virus type IIIB gp120 to CD4+ cells and reduced syncytium formation (156). Recently, a novel strategy has been developed to design a CD4 mimetic to inhibit CD4:gp120 interaction. Nine critical residues from the CDR-2 like region of CD4 (K35, G38, Q40-F43, T45, G47, R59) involved in the binding HIV-1 gp120 were transferred onto a structurally homologous scaffold such as the β hairpin motif of the scorpion toxin, scyllatoxin, such that the peptide mimetic adopted a conformation similar to that of the parent CD4 molecule. The CD4 peptide mimetic retained substantial gp120 binding activity and prevented infection of CD4+ cells by different viral isolates. Significantly, the scaffold or the miniprotein can be manipulated

by incorporating sequence changes as suggested by the structural and functional analyses to develop mimetics with higher inhibitory potential (157). Thus, the use of structurally homologous scaffolds to reproduce the core regions of protein-protein interface in small functional units that are amenable to modifications offers a powerful strategy to develop biologically stable antagonists of protein-protein interactions.

gp120 antagonists: Structural analyses have implicated V1/V2 and V3 regions of the viral outer envelope protein gp 120 as mediating binding to host cell surface receptors such as CD4 or chemokine CCR5, although only V2 peptides have so far been shown to be potent gp120 signaling antagonists. The gp120/receptor interactions are mediated by a discrete core pentapeptide sequence within the variable (V2) domain of the envelope glycoproteins. These pentapeptides are potent chemoattractants and gp 120 antagonists. [D-Ala1] peptide T-NH₂ (DAPTA) (d-ASTTTNYT-NH₂) has been shown to exhibit both agonistic chemotactic activity and antagonistic gp120 activity, thus acting as a mixed agonist/antagonist. Thus, DAPTA may specifically inhibit M-tropic CCR5 utilizing isolates of gp 120. The therapeutic implication of DAPTA relates to the prevention of early infection via macrophage-tropic HIV isolates utilizing CCR5 receptor as well as infection of the brain occurring via CCR5 expressing microglia (158).

CD8 inhibitors: CD8 is a costimulatory molecule expressed on the surface of a subset of mature T cells that have specificity for antigens presented by MHC class I proteins on the APC. CD8⁺ T cells function as cytolytic T cells directly involved in the killing of virus-infected cells, tumor cells and allogenic graft cells. However, CD8⁺T cells are primarily

responsible for deleterious responses associated with cellular and organ transplantation reactions. Therefore, development of CD8 inhibitory peptides has potential therapeutic application. CD8 functions as a α - α homodimer or α - β heterodimer. Structurally both α and β chains consist of an amino terminal IgV like ECD, a transmembrane domain and an intracellular cytoplasmic tail. The crystal structure of homodimeric human CD8 α has been recently solved. Like all members of the IgSF family CD8 ECD consists of highly solvent exposed three CDRs that constitute the active sites of interaction with MHC class I. Mutagenesis and peptide mapping studies have demonstrated the involvement of CDR-2 like region of CD8 α in the recognition of MHC class I molecule. Peptide analogues designed from a structural model of the mouse CD8 α molecule were assessed for inhibitory potential. A cyclic octameric peptide derived from the CDR-2 like region of mouse CD8 α molecule (CSSHNKPC) has been shown to be inhibitory during both the generation and effector stages of cytolytic T cell responses. Significantly, the CD28-CDR-2 peptide analogue was shown to prolong skin allograft survival in vivo (159). Theoretical analysis and the crystal structure of CD8-MHC class I complex suggested that residues in the CD8 α DE loop region is also involved in binding to MHC class I molecules and the activation of cytolytic T cells. Significantly a conformationally restricted peptide derived from the DE loop of human CD8 molecule (CKRLGDTFVC) has been shown to specifically inhibit CD8-MHC class I binding and inhibit CD8 $^{+}$ T cell action in vitro (160).

Antagonists of cell-cell or cell-matrix adhesion.

During the course of an immune response T cells interact with a variety of other cells and extracellular matrix (ECM) components. Cell-cell and cell-matrix adhesion are mediated

by a class of proteins that constitute a subset of the IgSF proteins that include the intercellular adhesion molecules (ICAM-1, ICAM-2 ICAM-3, ICAM-4 and ICAM-5), vascular adhesion molecule-1 (VCAM-1) and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) (60).

ICAM-1 peptide antagonist: ICAM-1 is a cell adhesion glycoprotein constitutively expressed on a variety of cell types including vascular endothelial cells, leukocytes, dendritic cells and mucosal epithelial cells. There are two counter-receptors for ICAM-1: lymphocyte function activating antigen that is expressed by all leukocytes and Macrophage function activating antigen (MAC-1) expressed by monocytes and macrophages. The levels of ICAM-1 expression are upregulated dramatically during inflammation. In addition to providing adhesion between vascular endothelial cells and leukocytes facilitating transendothelial migration, ICAM-1 also provides some costimulation for T-cell activation. ICAM-1 is also a receptor for human rhinoviruses. An antagonist of ICAM-1/LFA-1 can have multiple therapeutic applications (60).

Structurally, ICAM-1 consists of five IgSF domains. Crystal structure of ICAM-1 of the first two domains of ICAM-1 has been recently solved. In contrast to the above-mentioned examples of recognition of IgSF proteins located in the loops at the top of the domain, ICAM-1 binds LFA-1 through a bottom loop and edge of the domain. In common with other IgSF-integrin interactions, an acidic residue, Glu 34, has been shown to be the key residue in the binding of ICAM-1 with LFA-1 and is present on a relatively flat rather than a protruded surface (60). Evaluation of overlapping peptides spanning the first two domains of the ICAM-1 molecule resulted in the identification three linear peptides that inhibited adherence of T lymphocyte like cells to endothelial cells

(QTSVSPSKVILPRGGSVLVT, KELLPGNNRKV and REPAVGEP AETTTV). Cyclicization increased the inhibitory potential of the synthetic ICAM-1 peptides. The cyclicized peptide as determined by circular dichroism, NMR and molecular dynamics exhibited structural similarity with ICMA-1 (161). Significantly the inhibition of binding has been shown to be mediated via internalization of the ICAM-1 peptide by LFA-1 that can be utilized as a method of targeted drug delivery to leukocytes for the treatment of leukocyte related diseases (162)

Characterization of a peptide mimic of VCAM-1 binding epitope: Vascular cell adhesion molecule (VCAM-1) is a mononuclear leukocyte selective adhesion molecule that is expressed on vascular endothelial cells. Like ICAM-1, VCAM-1 also functions in transendothelial emigration of leukocytes at sites of inflammation. The expression of VCAM-1 is also upregulated by inflammatory cytokines and it acts by binding the integrin very late antigen-4 (VLA-4) to promote selective leukocyte emigration at these sites. Structurally VCAM-1 consists of 7 IgSF domains. The solution structure of the first two domains has been solved. The first domain has the binding site for interaction with VLA-4 and the second domain is thought to be important for orienting the recognition surface on the first domain. As in other IgSF-integrin interactions, mutagenesis studies have substantiated the importance of an acidic residue, Asp 40, in the binding of VCAM-1 to VLA-4. A segment from Thr37 to Asp 40 forms a β turn in the CD loop of domain 1, which is similar to a protruded loop other than the CDRs present in the CD4 molecule. The key residue Asp40 is located on the protruded loop and its main chain conformation is restricted by a pair of hydrogen bonds around it with Thr37 and Ser 41 (60). These

observations were adopted to design peptide inhibitors intended to mimic the conformation of the protein-binding loop. A cyclic but not a linear peptide C-Glu-Ile-Asp-Ser-Pro-Cys inhibited binding of $\alpha_4\beta_1$ integrin-bearing cells binding to VCAM-1(163). Other cyclicized octapeptides containing the TQIDSP sequence from VCAM-1 followed by a two-residue linker (NG) exhibited a high degree of similarity to the structure of VCAM-1 binding epitope competitively inhibited binding of $\alpha_4\beta_1$ with VCAM-1 even at subnanomolar concentrations indicating that the peptide and the protein were binding at a common receptor site. Furthermore, the activity of the cyclic peptides with specific substitutions correlated with the activity of VCAM mutants indicated that the peptides and the VCAM bind in a similar way. Significantly, substitutions that increased inhibitory potency of the peptide suggested new VCAM mutations with improved affinity for its interaction with $\alpha_4\beta_1$ (164). Thus, in addition to therapeutic application, synthetic peptide antagonists can also be used as tools in the study of complex protein-protein interactions.

Conclusions:

By definition, a versatile protein scaffold should constitute a conformational stable folding entity that is able to display a multitude of loop structures or amino acid sequences in a localized surface region (143). In all IgSF proteins the conserved backbone folding pattern (Ig-like fold) is thought to function as a stable biomolecular scaffold for presenting specific functional determinants on the faces of the β -sheets or at the connecting peptide loops. The loops that bridge the β strands show distinct variability in both sequence and structure with regard to helices and β sheets as a result of long term

molecular evolution (141). Thus, the variability of loops amongst the IgSF proteins largely contributes to their diverse functions. However, a common structural theme within the diversity is that the binding determinants for efficient biomolecular interactions are often located in these loops or CDRs. In this context, it is interesting to note that the surface binding pocket consists of either CDR3 or CC' loops for most of the IgSF protein-protein interactions discussed in this review. Hence, it is possible that similar strategies of peptide design can be extended to the studies of other IgSF protein-protein interactions and biological functions.

2.2. Identification of the ligand binding epitope of CD28 that interacts with B7 molecule.

The CD28 gene: In humans, the CD28 gene maps to chromosome 2 band q33 and in mouse to chromosome 1 band C. CTLA-4, a structural homologue of CD28 belonging to the same family, is also localized to the same chromosome in both the mouse and the human (165). The CD28 cDNA encodes a long open reading frame of 220 residues with typical features of an integral membrane protein. Removal of the N-terminal signal sequence gives a mature CD28 protein that consists of 202 amino acids comprising an extracellular domain (ECD) with five potential N-linked glycosylation sites (N-X-S/T), a 27 amino acid transmembrane domain, a 41 amino acid intracellular domain and has a molecular weight of 25066 Daltons (166).

CD28 – a member of the IgSF: Comparison of the amino acid sequence of the ECD of human CD28 revealed substantial homology with the mouse and rabbit immunoglobulin

heavy chain variable (IgV) regions. Within this domain two cysteine residues in the homology blocks Leu- (Ser or Thr)-Cys and Tyr- (Tyr or Phe)- Cys are shared by CD28, CD4 and CD8, immunoglobulin heavy chain and light chain variable sequences, and related molecules with approximately the same spacing (166). Thus, structurally CD28 belongs to the immunoglobulin supergene family (IgSF) with a single IgV like domain. Furthermore, amino acid sequences of the human CD28 ECD show a high degree of cross-species conservation with that of the mouse, rat, dog, rabbit and chicken ranging from 50%-90% amino acid identities. In both human and mouse, the cross molecule conservation between CD28 and CTLA-4 is about 27% within the putative cognate recognition V-like domain and 29% within the intracellular domains. Significantly, these molecules display a high degree of conservation within a region analogous to the CDR-3 region of the immunoglobulins where, in a stretch of 16 residues, there are 11 identities, four conservative or semiconservative substitutions and only one residue showing significant difference between human CD28 and CTLA-4. This region also includes the highly conserved non-IgSF consensus sequence MYPPPY that shows no cross-species or intermolecular evolutionary flexibility, allowing not even conservative substitutions (167).

Molecular model of CD28: Alignment of the CD28 sequences from different species with consensus IgV region sequences identified nine β strands with three complementarity determining regions (CDR) like regions and one non-CDR loop (DE-loop) in the membrane distal region of the molecule (167). Three-dimensional structures of many IgSF molecules have been determined by X-ray crystallography and nuclear

magnetic resonance (NMR) techniques. The common structural features of IgSF sometimes permit comparative molecular modeling studies where known 3D structures are used as templates to build models of related proteins with unknown structure (99). The energy minimized average NMR coordinates of monomeric CTLA-4 was used as the template for CD28 modeling. The fully conserved 'MYPPPY' sequence localized to the FG loop of the CDR-3 like region. The seven residue BC loop or the CDR-1 like regions shares the same length with CTLA-4 but has no sequence similarity. In the CD28 model all five potential N-linked glycosylation sites map to exposed positions (168).

Prediction of the ligand binding epitope of CD28: There is considerable evidence suggesting that similar to the CDR regions on antibody, the CDR- like regions in other IgSF molecules is critical for intermolecular recognition (139). Truncation of 99MYPPPY104 in CD28/CTLA-4 or its substitution by a flexible stretch of irrelevant residues caused total loss of ligand binding without loss of surface expression and with the retention of some antibody epitopes. Loss of reactivity was also observed when two downstream residues of 'MYPPPY' motif were mutated. Thus, the surface patch extending the 'MYPPPY' region is involved in CD80/CD86 binding to CD28 (167, 169). Other residues adjacent to this site that are conserved only in CD28 such as the F90 and D 106 may modulate ligand binding. Similar to CTLA-4, the FG loop in CD28 was modeled as a β bulge. However, the presence of a highly conserved N- linked glycosylation site at N111 while being accommodated by the β bulge in CTLA-4 will weaken the G strand hydrogen bonding in CD28 (168). Hence, it has been predicted that the G strand at the edge of the β sheet may be more flexible in CD28 than in CTLA-4

although it is also possible that the region may adopt a different conformation in CD28. Based on these observations we identified a 20-residue peptide comprising the highly conserved hydrophobic motif and the conserved flanking residues from the CDR-3 like region of CD28 as the ligand binding epitope (Fig 1).

2.3. Design of CD28 peptide mimics.

The aim of peptide modification is to overcome the problem of swift metabolism by proteolytic degradation while retaining the selected receptor antagonist activity. Modifications can be made at any point in a peptide and modifications can be applied in any combination: amino acids can be changed, deleted or added, backbone peptide bonds can be replaced with surrogates or with novel scaffold. The ultimate goal is to mimic the putative bioactive topology without loss of functional efficacy (170). Cyclicized peptides (Cys-Xaa-Xaa-Cys), peptides with reduced peptide bonds ($-\text{CH}_2\text{NH}-$), peptides incorporating D or unnatural amino acids, partially or completely modified retro-inverso peptides are some of the modifications that have been developed in the recent years to synthesize functional peptide mimics (134). Mueller et al have performed a series of experiments to demonstrate the functional efficiency of the modified peptides, peptides with reduced backbone or retro-inverso peptides, in the generation of neutralizing antibodies similar to that of the native immunodominant peptide suggesting that the modified peptides shared structural similarity with the native peptide. We propose to adopt a retro inverso modification in the design a CD28 peptide mimic.

Definition of retro-inverso peptide: A retro-inverso peptide is an isomer of a linear peptide in which the direction of the amino acid sequence is reversed and the chirality of each amino acid is inverted. This gives a peptide that has topochemical equivalence to the parent peptide with similar side chain orientation (171). An important aspect of this modification that has to be considered in terms of biological activity of CD28 peptide is ‘will the reversal of the standard peptide bond by the retro-bond alter the interaction of the receptor with its ligands?’ Work on the peptidomimetics in general suggests that in the context of peptide-hormone receptor binding, the peptide backbone largely serve to position the essential side chains. Use of D amino acid residues in place of the native L amino acid residues will give a good mimicry of the side chain topology (170).

The retro inversion and enantiomerization of the linear CD28 peptide produces an isomer topochemically complimentary to the parent CD28 peptide, but with noncomplimentary end groups. The chain termini have to be modified to mimic the parent peptide. The simplest modification to achieve close mimicry is to block the end-group charges by amidating the carboxy terminus and acetylating the amino terminus of the CD28 peptide.

Since the presence of formal charges at the α -NH₃⁺ and α -COO⁻ termini of a short peptide are likely to destabilize the secondary structure, the end groups of the parent peptide were also blocked. This also resulted in a closer mimic of the ligand binding epitope in vivo.

As a control to underlie the importance of the sequence specificity of the CD28 peptide mimic, we designed a reverse L-CD28 peptide and a scrambled CD28 peptide. The reverse L-CD28 peptide was synthesized using the L amino acids with the amino acids sequence in the reverse order of the parent peptide while the scrambled CD28 peptide

was synthesized with the amino acid sequence jumbled with respect to the parent peptide. As a control for the side-chain orientation of the amino acids in the peptide mimic, we also designed a D-retro peptide made up of D amino acids in the same order as that of the parent peptide. The parent L-peptide and the retro-inverso analogs are represented schematically in Fig 2. The peptide sequences used in this study are given in Table –1.1

Table :1. Amino acid sequences of synthetic CD28 peptides and control peptides.

<i>CD28 Peptide</i>	<i>Sequence</i>	<i>Abbreviation</i>
Analogue		
Free hexapeptide	NH ₂ MYPPPYCOOH	MYPPPY
End group blocked hexapeptide	CH ₃ COMYPPPYCONH ₂	EL-MYPPPY
CD28 Free peptide	NH ₂ KIEFMYPYPYLDNERSNGTICOOH	L CD28
End group blocked CD28	CH ₃ COKIEFMYPYPYLDNERSNGTICONH ₂	EL CD28
End group blocked Retro-Inverso CD28	CH ₃ COD[ITGNSRENDLYPPPYMFEIK]CONH ₂	RI CD28
End group blocked D CD28	CH ₃ COD[ITGNSRENDLYPPPYMFEIK]CONH ₂	D CD28
End group blocked reverse L CD28	CH ₃ COITGNSRENDLYPPPYMFEIKCONH ₂	RL CD28
End group blocked scrambled CD28	CH ₃ COTMEGYDPIKINPRSYNFEPLCONH ₂	SL CD28
End group blocked CTLA-4 peptide	CH ₃ COKICKVELMYPYPYLGIGNGACONH ₂	CTLA-4

D refers to D amino acid residue.

Fig 2.1. Molecular model of the extracellular domain of the CD28 molecule showing the three complementarity determining regions (CDR) and the DE loop.

Fig 2.2: Schematic representation of the retro-inverso modification of a parent L- peptide showing reversal of peptide bond direction and inversion of side chain chirality.

Chapter 3

Materials and Methods

3.1.1 Peptide synthesis and purification: Peptides of the CD28 CDR3 like region were synthesized by solid phase peptide synthesis following Fmoc/DCC/HOBt methodology on a fully automated peptide synthesizer (Model 396-5000 Multiple Peptide Synthesizer, Advanced Chemtech, Louisville, KY) as described (172). The free L CD28 peptide was assembled on 4-methylbenzhydrylamine resin (0.5mmol/g substitution) with 4-(hydroxymethyl) phenoxyacetic acid as the linker. The end group blocked peptides were assembled on an Fmoc'-2, 4-dimethyloxy-4'- (carboxymethyloxy)-benzylhydramine (Rink amide, 0.6mmol substitution) resin (Advanced Chemtech, Louisville, KY) as peptide amides. Coupling reactions utilized 6 equivalents of each amino acid with 1-hydroxybenzotriazole (HOBt), 2-(1H-benzotriazole-1-yl)-1,1,3,3, -tetramethyluronium tetrafluoroborate and distilled diisopropylethylamine (DIEA). Deprotection was accomplished with 30% piperidine in DMF. Immediately after the final deprotection step, the free NH₂ group of the terminal amino acid residue was acetylated with 3mmol acetimidazole in DMF (50 ml). The completion of acetylation was confirmed by a negative Kaiser Ninhydrin test. With respect to the parent peptide, the retro-inverso

peptide was assembled in a reverse order of aminoacids with Fmoc-D-aminoacid derivatives. The peptides were cleaved from the resin support with simultaneous side-chain deprotection by acidolysis using TFA with 5% phenol, 5% thioanisole and 2.5% ethanedithiol as scavengers. The crude peptides were purified by semi-preparative reverse phase high performance liquid chromatography (RP-HPLC) using a C₁₈ column (10mm by 25cm) (Vydac, Hesperia, CA) at a temperature of 32.5° C and a flow rate of 5ml/min. Peptides (5-10mg per run) were loaded in 0.1M acetic acid and chromatographed for 30 min with linear gradient of 10-60% of acetonitrile in water containing 0.1% trifluoroacetic acid (TFA). The separations were monitored at 230 and 280 nm.

Analytical HPLC was run using a VIDAC C₁₈ column (4.6 mm by 25 cm) using the same gradient as stated above. Eluants were monitored at 214 and 254 nm. Purified peptides were obtained in greater than 95% purity as assessed by reverse -phase HPLC. The identity of peptides was finally confirmed by matrix-assisted laser desorption/ionization time of flight mass spectrometry.

3.1.2 Circular dichroism:

Circular dichroism were recorded at room temperature on an AVIV Model 62A DS spectrometer equipped with a thermostatic temperature controller and interfaced to an IBM microcomputer as described (172). CD spectra were recorded in a quartz cell of 0.1cm pathlength over a range of temperatures from 5° C- 90°C. Each spectrum was obtained by averaging one nm/sec in the 190 to 270 nm wavelength range, using a bandwidth of 1.0nm and a response time of 1s. CD28 peptides dissolved in PBS, pH7.4

or in 50% trifluoroethanol (TFE) or 6mM CaCl₂ at 400μM concentration were used for CD measurements. Raw CD signals (in mill degrees) were converted to mean residue ellipticity (MRW) in deg.cm²/dmol using the formula $[\Theta]_{MRW} = [\Theta]_{obs}/10lcN$ where Θ_{obs} is the observed ellipticity, *l* is the path-length in cm, *c* is the molar concentration of peptide and *N* is the number of residues in peptide.

3.1.3 SPR Experiments:

Binding experiments were performed by SPR on a BIAcore instrument from Pharmacia Biosensor (Uppasala, Sweden). All experiments were performed at 37°C using HBS-EP buffer (25mM Hepes, pH7.4, 150nM NaCl, 3.4mM EDTA and, 005% surfactant P20) supplied by Pharmacia Biosensor.

CD80-Ig (extracellular domain of CD80 fused with the constant region of mouse IgG1 heavy chain, a gift from Dr. Jay Fine, Scheiring-Plough Institute, Kenilworth, NJ, USA) at 35μg/ml in 10mM sodium acetate buffer, pH 4.2, was coupled using the standard amine coupling procedure on a research grade CM 5 sensor chip with the following modifications (173). To reduce the immobilization level of the ligand the surface was activated for 3 (instead of 7) minutes with N-hydroxysuccinamide and N-hydroxymethyl-N- (3-diethylaminopropyl) carbodiimide. This typically resulted in 1422-1660 RU of CD80-Ig being immobilized to the sensor chip. Following coupling noncovalently bound ligand was removed by washing twice 5 mM NaOH.

Kinetic analysis was performed by injecting the analytes (L-CD28, EL-CD28, RI CD 28, RL CD28 and D CD28 peptides) at 0.38μM to 6μM concentrations for 300s with a flow

rate of 10 μ l/min in HBSS-EP, pH7.4, for 300s with a flow rate of 10 μ l/min. The analytes were also injected at the same concentration and injection times over an empty flow cell with nothing immobilized.

Competitive kinetic analysis.

Competitive kinetic analysis was performed by the sandwich method (174). Purified anti-mouse IgG1 Fc (31437zz) (Pierce, IL, USA) at 22 μ g/ml in 10mM acetate buffer was immobilized to the sensor chip using the same injection times as above. Unbound mAb was removed by washing twice with 0.1M glycine HCl for 2 mins. This resulted in 1182.2 RU of mAb bound to the sensor chip surface. After obtaining a stable base line CD80-Ig at 18 μ g/ml in HBS, pH 7.4, was injected as the first analyte for 180s with a flow rate of 10 μ l/min. Dissociation in buffers was followed for 60s. This resulted in the 286 – 342 RU of CD80-Ig bound to the ligand. To determine the binding kinetics of CD28-Ig, initial experiments were performed by injecting a range of CD28-Ig concentration (187 nM to 12 μ M) over immobilized CD80-Ig (324 RU) for 5 min at 10 μ l/min. CD80-Ig was regenerated using a 3min injection of 5mM NaOH. For competitive kinetic analysis 100 μ l of a 3.2 μ M CD28-Ig solution was mixed with 100 μ l of EL CD28 or RI CD28 peptides (6 μ M to 500 μ M in HBS-EP) and injected over the surface of CD80-Ig as secondary analyte for 5min with a flow rate of 10 μ l/min. The CD80-Ig surface was regenerated between injections by washing for 3 min with 5mM NaOH. As control the mixtures were also injected on an empty flow cell with no protein immobilized.

Data analysis was performed with BIAevaluation software version 2.1(BIAcore) (175).

The binding as measured in response units (RU) in BIAcore and the binding rate, dR/dt , can be used to evaluate the kinetics of the synthetic CD28 peptides - CD80-Ig interaction.

Prior to kinetic analysis data were adjusted to zero baseline level by subtracting the background responses obtained by injection of the analytes through a control flow cell with nothing immobilized. Data from direct kinetic analysis were analyzed as follows.

First the dissociation rate constant, k_d , (units: s^{-1}) was determined, by fitting experimental data from the buffer flow part of the sensogram to the equation

$$R(t) = R_1 * e^{-k_d * (t - t_1)} \dots\dots\dots(1)$$

where t_0 is the injection time, R_1 is the response level at the start of dissociation time t_1 and k_d is the dissociation rate constant.

The k_d value obtained was used as a constant during the analysis of the injection phase data. Binding data above the noise level was selected by converting the sensogram to a plot of the logarithm of the binding rate vs time (dR/dt vs. time). The k_a was determined by nonlinear curve fitting of the following equation

$$R(t) = R_{eq} * \{ 1 - e^{-(k_a * C + k_d) * t} \} \dots\dots\dots(2)$$

where $R(t)$ is the response at time t , R_{eq} is the steady state response level, k_a is the association rate constant (units: $M^{-1}s^{-1}$), k_d is the dissociation rate constant and C is the concentration of the injected peptide analyte. An offset was added to account for the refractive index differences between the analyte and the running buffer.

In competitive kinetic experiments the observed response R is the sum of the contributions R_1 and R_2 from the two analytes. The binding data was analyzed using the equation

$$R(t) = R_{CD28-Ig} + R_{CD28-P} \dots \dots \dots (3)$$

where

$$R_{CD28-Ig} = R_{max} k_{aCD28-Ig} C_{CD28-Ig} / k_f - k_s \{ k_{CD28-Ig} (k_f - k_s) / k_f * k_s + k_{CD28-Ig} - k_f / k_f * e^{-k_f(t-t_0)} - k_{CD28-Ig} - k_s / k_s * e^{-k_s(t-t_0)} \}$$

$$R_{CD28-P} = R_{max} MW_{CD28-Ig} k_{aCD28-P} C_{CD28-P} / MW_{CD28-P} * k_f - k_s \{ k_{CD28-P} (k_f - k_s) \} / k_f * k_s + (k_{CD28-P} - k_f / k_f) * e^{-k_f(t-t_0)} - (k_{CD28-P} - k_s / k_s) * e^{-k_s(t-t_0)} \}$$

$$k_f = 0.5 \{ k_{tCD28-Ig} + k_{CD28-P} + \sqrt{(k_{tCD28-Ig} - k_{CD28-P})^2 + 4k_{fCD28-Ig} k_{CD28-P} C_{CD28-Ig} C_{CD28-P}} \}$$

$$k_s = 0.5 \{ k_{tCD28-Ig} + k_{CD28-P} - \sqrt{(k_{tCD28-Ig} - k_{CD28-P})^2 + 4k_{CD28-Ig} k_{CD28-P} C_{CD28-Ig} C_{CD28-P}} \}$$

$$k_{tCD28-Ig} = k_{aCD28-Ig} C + k_{dCD28-Ig}$$

$$k_{tCD28-P} = k_{aCD28-APR} C_{CD28-P} + k_{dCD28-P}$$

When competitive data were analyzed k_a , k_d and R_{max} values obtained for the interaction between CD80-Ig and CD28-Ig were used as non-floating parameters and the rate constants for CD28 peptides were calculated from the injection phase data using the expanded equation 3.

After analyzing sensograms obtained from injections of increasing concentrations of peptide analytes or CD28-Ig, the experimental data was analyzed using the BIA simulation software 2.1 A plot of logarithm of (dR/dt) against time was calculated from the sensograms, and then the slopes at different concentrations were plotted against the concentrations of the peptide analyte. Different angles obtained of the linear lines

represent the variation of affinities, which gave the association rate constant (k_a) from the equation

$$\ln dR/dt = \ln(k_a C R_{\max}) - K_d C + k_d t \dots \dots \dots (4)$$

where dR/dt is the rate of change of SPR signal, C is the concentration of the analyte, $R_{\max}$ is the maximum analyte binding capacity in RU and R is the SPR signal in RU at time t . The dissociation constant was obtained from the equation

$$K_d = k_d/k_a \dots \dots \dots (5)$$

3.1.4 Antigens: Purified guinea pig MBP was kindly provided by Ingrid Gienapp of Dr. Whitacre's laboratory. The encephalitogenic peptide NAc1-11 (Ac-A-S-Q-K-R-P-S-Q-R-H-G-COOH) was synthesized as described above.

3.1.5 Magnetic Associated Cell Sorting for CD4+ T cells: CD4+ T cells were purified from pooled peripheral lymph nodes (inguinal, axillary, brachial, cervical, popliteal), and mesenteric lymph nodes and spleen of 6-8 wk old V α 4/V β 8.2 TCR transgenic mice by positive selection as described (176). Single cell suspensions were prepared by lympholyte M gradient, washed in separation buffer and counted. For every 1×10^7 cells 10 μ l of CD4+ direct microbeads and 90 μ l of labeling buffer were added. The cells were incubated at 6° – 12° C for 15 mins, washed by adding 10 x the volume of the separation buffer and resuspended in 500 μ l of separation buffer. The MiniMAcs column was set appropriately, washed with 500 μ l of separation buffer and then the labeled cells were allowed to pass through the column. The effluent is collected as the negative fraction.

The column is washed thrice to wash out the negative cells. Finally the column is removed from the magnet, placed on a new collection tube and the positive fraction was collected in 1 ml of buffer.

The purity of the cell population for CD4+, V β 8.2 and for the absence of B cell contamination (CD19) was assessed by flow cytometry. 1×10^6 lymph node cells and spleen cells from non-separated pool, CD4+ fraction and CD4 depleted or negative fraction were incubated with 25 μ g of antibodies for 30 minutes at 4°C. Three tubes per sample including isotype, CD4+(PE) and V β 8.2+(FITC) and CD19+(PE) and CL II + (FITC) were used. The control isotypes used were mouse IgG2A (FITC) and rat IgG2A (PE). The cells were washed and resuspended in 400 ml of PBS and fixed with 1% paraformaldehyde and stored for analysis. For each sample 10^4 events were collected and the data analyzed with a Coulter flow cytometer. The separated positive fraction was on an average 98% positive for CD4 and V β 8.2 (specific TCR transgene) (Fig 13). During the selection process T cells maintain a naïve phenotype with no evidence for T cell activation as measured by proliferation in culture.

3.1.6 In-vitro proliferation assays: Purified CD4 + T-cells (5×10^4 cells/well) were cultured together with wild type splenocytes from B10.PL mice as APC's (1×10^5 cells/well) in RPMI 1640 containing 10% FCS, 25mM HEPES, 2mM L-glutamine, 50 U/ml penicillin, 50 μ g/ml streptomycin, and 5×10^{-5} M 2-ME in round-bottom 96-well plates and MBP (40 μ g/ml) or MBP NAc1-11 (10 μ g/ml) or media only in the presence or

absence of synthetic CD28 peptide analogues at different concentrations in triplicate wells for 72 h, including a final 18-h pulse with [3 H] thymidine. Cultures were harvested onto glass-fiber mats using a Skatron harvester (Skatron, Sterling, VA) and were counted by liquid scintillation on LKB Betaplate (LKB, Rockville, MD). The means of the triplicate were determined and the results are expressed as delta cpm (mean counts per minute of cultures with Ag-mean counts per minute of cultures with medium alone) +/- SE.

Vehicle, synthetic CD28 peptides and control peptides treated mice were sacrificed on day 10 and day 26 after immunization and proliferation analysis was performed. Single cell suspensions prepared from spleens, peripheral lymph nodes (inguinal, axillary, brachial, cervical, popliteal), and mesenteric lymph nodes were cultured in the RPMI medium and stimulated with MBP (40 μ g/ml) or media only. Cultures were harvested and counted as above.

3.1.7 Elispot analysis for cytokine producing responder cells:

ELISPOT assays were performed using 96 well unfilter plates (Polyfiltronics, Rockland, MD) as described (40). The plates were coated overnight at 4°C with rat anti-mouse IL-2 (Clone JES6-1A12) at 2 μ g/ml or anti-mouse INF- γ (Clone R46A2) (Pharmingen, CA) at 4 μ g/ml. The plates were then washed 4 X with sterile PBS and blocked with 1% BSA in DMEM (Life Technologies, Gaithersburg, MD) for 2h at room temperature. Single cell suspensions of CD4 $^+$ lymph node cells and splenocytes from transgenic mice bearing V α 4 V β 8.2TCR specific for MBP-NAc 1-11 or lymph node cells and splenocytes

isolated from vehicle, synthetic CD28 peptides and control peptides treated mice and sacrificed on day 10 and day 26 after immunization, were washed and resuspended in HL-1 medium (BioWhittaker, Walkersville, MD) supplemented with 1% L-glutamine and 50 µg/ml gentamicin (Life Technologies, Grand Island, NY). LNC (5×10^6 cells/ml) and splenocytes (10×10^6 cells/ml) were added to the plates in triplicate with 40 µg/ml MBP or 10 µg/ml MBPNAc1-11 or with medium alone in the presence of CD28 peptides or control peptides at 25 µM, 50 µM, 75 µM and 120 µM concentrations under the conditions as specified for the proliferation assays. After 24h of culture in the incubator at 37°C, the plates were washed 3X with PBS and 4X with PBS containing Tween (1:2000) (PBST). Biotinylated anti mouse-IL-2 (Clone JES6-5H4) 2 µg/ml or anti-mouse INF-γ (Clone XMG1.2) (Pharmingen, CA) 2 µg/ml was then added and the plates were incubated at 4°C overnight. After washing 3X with PBST and 3X with PBS, the plates were incubated with goat anti- biotin antibody conjugated to alkaline phosphatase (Vector Laboratories Inc, Burlingame, CA) diluted to 1:1000 in PBST containing 1% BSA for 2 h at room temperature. The spots were visualized by adding BCIP/NBT phosphatase substrate (Kirkeguard & Perry Laboratories, Gaithersburg, MD). Image analysis of ELISA spot assays was performed on a Series 1 Immunospot Image Analyzer (Zeiss) customized for analyzing spots to meet objective criteria for size, chromatic density, shape and color. Frequencies are expressed as the number of MBP-responsive cells per million +/- SEM.

3.1.8 Detection of Cell surface expression of activation markers by Flow Cytometric

Analysis:

Cell surface expression of activation markers after in vitro stimulation: Pooled lymph nodes from TCR transgenic mice (V α 4/V β 8.2 specific for MBP NAc1-11) were cultured in RPMI 1640 in a 96 well round-bottom plate under the conditions specified for proliferation assays. Cells were harvested at the end of 48 hours and counted. 1×10^6 lymph node cells or spleen cells were incubated with 1 μ g of directly labeled monoclonal antibodies, FITC conjugated anti-V β 8.2 (Clone MR5-2) and PE conjugated anti-CD25 (clone 3C7), anti-CD69 (clone H1.2F3), anti-CD28 (clone 37.51), anti-CD152 (clone UC10-4F10-11), anti-Fas (clone Jo2) and anti-Fas-L (clone MFL-3) for 30 minutes at 4°C. The isotype control mAbs used were FITC conjugated mouse IgG2A (clone: G155-178), PE conjugated rat IgG2a (clone R35-95) and PE conjugated ham IgG (clone: A19-3). The cells were washed and resuspended in 400 μ l of PBS/1% paraformaldehyde and stored for analysis. For each sample 10^4 events were collected and the data analyzed on a Ziess flow cytometer (Coulter Corp.).

3.1.9 Detection of Apoptosis following In vitro stimulation:

CD4⁺ T cells from pooled lymph nodes and spleen from B10.PL TCR V β 8.2 V α 4 transgenic mice were cultured in RPMI 1640 in a 96 well round-bottom plate under the conditions specified for proliferation assays as described (177). Cells were harvested at the end of 48 hours and apoptotic cells among TCR V β 8.2⁺ CD4⁺ T cells were detected by the TUNEL method. Briefly, after washing twice with PBS containing 1% rat serum,

the cells were stained with PE labeled anti-mouseV β 8.1,8.2 TCR (clone MR5-2) (Pharmingen, San Diego, CA) for 30 min at room temperature and washed. Subsequently the cells were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. Then the cells were washed and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min at 4°C. After washing, the DNA strand breaks in the cells were detected by incubating the cells with fluorescein labeled TUNEL mix (terminal deoxynucleotidyl transferase (Tdt)) for 60 min at 37°C using an in situ cell death detection kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's recommendations. As a positive control cells were incubated in DNase (1 μ g/ml) for 10 minutes at room temperature prior to incubation with TdT. Negative control consisted of cells incubated without TdT. Cells were then washed with PBS and analyzed by flow cytometry.

Detection of apoptosis after in vivo priming: Vehicle and synthetic CD28 peptides or control peptide treated mice were sacrificed on day 10 or day 16 following GP-MBP immunization. A total of 1x10⁶ lymph node cells and splenocytes were stained with PE labeled anti-mouse CD4 (L3T4) (Clone RM 4-4) (Pharmingen, San Diego, CA) for 30 minute at 4°C. The cells were subsequently fixed, stained for DNA strand breaks and analyzed as above.

3.2.1 Mice:

Female B10.PL mice (6-8 wk old) were obtained from The Jackson laboratory (Bar Harbor, ME) and housed at The Ohio State University, (Columbus, OH). TCR transgenic

mice (B10.PL background, H-2^u) which express a TCR $\alpha\beta$ (V α 4/V β 8) specific for MBP peptide NAc 1-11 were generated as described previously. The transgenic mice were inbred and maintained under specific pathogen-free conditions. All mice were housed at Ohio State University (Columbus, OH).

3.2.2 Induction of EAE and CD28 peptide treatment:

Mice were injected subcutaneously over four sites on the flank with an emulsion containing 200 μ g of guinea-pig-MBP in CFA containing 200 μ g heat killed *Mycobacterium tuberculosis*, Jamaica strain. Pertussis toxin (List Biological, Campbell, CA) at 150ng in 0.2ml of PBS was given i.p. at the time of immunization and 48 hours later. Animals were observed daily for clinical signs and scored as follows: 1, limp tail or waddling gait with tail tonic; 2, waddling gait with limp tail (ataxia); 2.5, ataxia with partial paralysis of one limb; 3, partial hind-limb paralysis; 3.5, full paralysis of one limb with partial paralysis of the second limb; 4, full paralysis of two limbs; 4.5, moribund and 5, death.

For treatment of EAE, mice were injected intravenously with CD28 peptide reconstituted in sterile PBS (pH=7.4) at 500 μ g in 0.25ml on day 0 or day14 following immunization with MBP.

3.2.3 Detection of expression of costimulatory molecules following CD28 peptide treatment by Flow Cytometry: Vehicle and CD28 peptides treated mice were sacrificed on day 10 or day 16 following GP-MBP immunization. Single cell suspension of pooled

lymph nodes and spleen were prepared as before. Aliquots of 0.5×10^6 cells were blocked with PBS containing 2% rat serum and 0.1% sodium azide. The cells were then incubated with 1 μ g of directly labeled monoclonal antibodies, PE-conjugated anti-CD80 (Clone 16-10A1), anti-CD86 (Clone GL-1), anti-CD28 (Clone 37.51) and anti-Fas (Clone Jo2) in the dark for 30 min at 4°C. For the detection of FasL expression, the cells were blocked with anti-mouse CD16/CD32 (Clone 2.4G2) prior to incubation in biotinylated anti-mouse FasL (Clone MFL3). A three step staining procedure was followed for the detection of CD152. After blocking with anti-mouse CD16/CD32, the cells were incubated with purified anti-mouse CD152 (Clone 9H10) followed by incubation in biotinylated mouse anti-hamster IgG. Both FasL and CD152 were visualized by staining with PE conjugated streptavidin. Nonspecific background staining was determined by incubating cells with isotype control mAbs. All mAbs were purchased from Pharmingen (Sandiego, CA). Finally the cells were washed and resuspended in 500 μ l of PBS/1% paraformaldehyde. The samples were then analyzed by two or three color flow cytometry on a Ziess flow cytometer (Coulter Corp.).

Statistical Analysis: For proliferation ELISPOT and TUNEL analyses, a one-way ANOVA with Tukey's post-hoc was performed to determine the differences between groups.

Chapter 4

Peptide mimics of CD28 encompassing the “MYPPPY” motif adopt a PP II helix and inhibit encephalitogenic T cells *in vitro*.

4.1. Structural characterization of the CD 28 peptide mimics by circular dichroism.

Circular dichroism spectroscopy is exquisitely sensitive to the secondary structure of proteins. The technique is a variation of electronic absorption spectroscopy that uses circularly polarized light rather than normal isotropic light. It can be applied to molecules in solution, so that proteins or peptides can be studied in the aqueous environment. The technique thus makes it easier to monitor the changes in conformation of proteins or peptides that result from changes in temperature, pH, salt, solvent composition, ligand binding and quaternary structure. Normal absorption spectroscopy of electronic transitions is measured as the absorbance A , which depends on the concentration of the material, c , and the pathlength of the cell l . The constant of the proportionality between the measurement and the variables is a characteristic of the molecule called the extinction coefficient, ϵ , which varies with the wavelength, λ . CD of a molecule is defined as the difference between the extinction coefficients for left and right circularly polarized light. The CD bands can be positive or negative depending on which type of light is absorbed more strongly, and occur only at wavelengths for which there are normal absorption bands.

The three major secondary structures recognized in proteins are α -helices, β -sheets and β -turns. The CD spectra of α -helices show two characteristic helical minima one each near 220 nm and 208 nm and a maximum near 190 nm. Most β sheet CD spectra show a negative band in the 210-220 nm region and a positive band in the 190-200 nm region. The turns, with different sets of ϕ and ψ , give different classes of CD spectra. In addition to these conformations, a fourth conformational state designated ‘polyproline (PP) helix’ is identified in certain sequences of globular proteins. There are two types of polyproline helices differing substantially in the physical and spectroscopic properties. The peptide bonds in form I are in a cis disposition ($\omega = 180^\circ$), the helix being right handed with 3.3 residues per turn. The peptide bonds in form II are trans ($\omega = 0^\circ$); the helix is left-handed with 3 residues per turn. The PP II structure is biologically relevant and is more often encountered as a locally driven form where sequential proline residues are present. Typically, the CD spectrum of a PP II helix exhibits a weak positive band near 226nm and a strong negative band near 202nm.

4.1 a. CD spectrum of CD28 peptide mimics: The CD spectrum of the six residue free peptide and the end group blocked peptide comprising the “MYPPPY” motif alone at 25°C showed a weak minimum of $\theta = -9 \times 10^1 \text{ deg cm}^2.\text{dmol}^{-1}$ @ 200nm and $-13 \times 10^1 \text{ deg cm}^2.\text{dmol}^{-1}$ @ 199 nm respectively (Fig 4.1 A and B). The CD spectrum of free L CD28 peptide showed a large minimum at 202nm ($\theta = -62.73 \times 10^3 \text{ deg.cm}^2.\text{dmol}^{-1}$) and a slight maximum at 221nm ($\theta = -3.5 \times 10^3 \text{ deg cm}^2.\text{dmol}^{-1}$) at 25°C (Fig 4.2 A). The CD

spectrum of EL CD28 peptide presented a strong mean residue molar ellipticity minimum at 205nm ($\theta = -69 \times 10^3 \text{ deg.cm}^2.\text{dmol}^{-1}$) and a weak maximum at 214 nm ($\theta = -17 \times 10^3 \text{ deg cm}^2. \text{dmol}^{-1}$) although still in the negative ellipticity region at 25⁰ C (Fig 4.2 B). These observations are consistent with the CD spectrum of a PP II type helical structure. Compared to the free L CD28 spectrum, the intensity of ellipticity near 200nm was considerably enhanced in the EL CD28 peptide, suggesting stabilization of the helical secondary structure. When the temperature was raised, there was a gradual decrease in the mean residue molar ellipticity and a shift to the right. The mean residue ellipticity at 40°C was $\theta = -65 \times 10^3 \text{ deg cm}^2.\text{dmol}^{-1}$ @ 209nm, at 60°C was $-63.4 \times 10^3 \text{ deg cm}^2.\text{dmol}^{-1}$ @ 211nm, at 70°C was $-62.2 \times 10^3 \text{ deg cm}^2.\text{dmol}^{-1}$ @ 211nm and at 90°C was $-59.3 \times 10^3 \text{ deg cm}^2.\text{dmol}^{-1}$ at 214nm (Fig 4.3 A). Similar variation with increasing temperature has been observed in proline-rich peptides, due to a transition from the PP II helical structure to a disordered random coil conformation. The CD spectrum of EL-CD28 peptide in 6M CaCl₂ at 25⁰C exhibited a dramatic decrease in the intensity of the mean residue ellipticity minimum at 205nm ($\theta = -28 \times 10^3 \text{ deg cm}^2. \text{dmol}^{-1}$) suggesting complete disruption of the helical structure (Fig 4.4 A).

RI CD28 peptide in 50% TFE at 25⁰ C presented a mean residue ellipticity maximum at 205nm ($\theta = 57 \times 10^3 \text{ deg cm}^2. \text{dmol}^{-1}$), a weak minimum at 215 nm ($\theta = 1.14 \times 10^4 \text{ deg cm}^2. \text{dmol}^{-1}$) and a weak maximum at 223 nm ($\theta = 18.3 \times 10^3 \text{ deg cm}^2. \text{dmol}^{-1}$) (Fig 4.2 C). By increasing the temperature, the molar ellipticity maximum markedly decreased in intensity and shifted to a longer wavelength. The mean residue ellipticity maximum at 40°C was $\theta = 31 \text{ deg cm}^2. \text{dmol}^{-1}$ @ 212 nm, at 60°C was $\theta = 29 \text{ deg cm}^2. \text{dmol}^{-1}$ @ 215

nm and at 90°C was $\theta = 17.2.9 \times 10^3 \text{ deg cm}^2 \text{ dmol}^{-1}$ at 211nm (Fig 4.3 A). The CD spectrum of the retro-inverso CD28 peptide in 6M CaCl₂ showed a dramatic decrease in molar ellipticity maximum at 205nm ($\theta = 1.10 \times 10^4 \text{ deg cm}^2 \text{ dmol}^{-1}$), complete loss of both the molar ellipticity minimum at 215nm and the maximum at 223nm (Fig 4.4 B). Collectively, these features once again are characteristic of a typical PP II helix CD spectrum.

The CD spectrum of control peptides RL-CD28 and CTLA-4 peptides showed a mean residue ellipticity minimum at 25°C of $\theta = -16.4 \times 10^3 \text{ deg cm}^2 \text{ dmol}^{-1}$ and $\theta = -17.8 \times 10^3 \text{ deg cm}^2 \text{ dmol}^{-1}$ respectively (Fig 4.5 A and B).

4.2. The CD28 peptide mimics bind B7-1 with fast kinetics:

In the direct binding assay, B7-1Ig was immobilized to the sensor chip by the amine coupling method. The 1490RU difference between the report points 1 and 4 corresponds to the immobilized B7-1Ig and is equivalent to a surface concentration of approximately 1.49 ng/mm² (Fig 4.6). CD28 peptides at varying concentrations (0.375μM to 18.5μM) were injected as analytes. Sensograms from direct kinetic analysis of L-CD28, EL-CD28 and RI-CD28 peptide at different concentration from one experiment are represented in Figs 4.7- 4.9. All three peptides reached equilibrium binding very rapidly (between 12s to 20s) and in the washing phase dissociated even faster (between 10s to 12s). The background responses following injection of EL-CD28 and RI-CD28 peptides over an empty flow cell with no protein immobilized are equivalent (Figs 4.8 B and 4.9 B). The maximum response units obtained for binding to B7-1Ig were 378 RU, 667.5 RU and 444.2RU for L-CD28, EL-CD28 and RI-CD28 peptides respectively. Dissociation of L-

CD28, EL-CD28 and RI-CD28 peptides from bound B7-1Ig was analyzed from the buffer flow phase of the sensogram and the dissociation rate constant (k_d) for each curve was determined using equation (1). The injection phase of the sensogram was analyzed using equation (2) by nonlinear curve fitting incorporating the calculated k_d value to obtain the k_a value for each curve (Fig 4.8 C and 4.9 C). The calculated parameter values with standard error are presented in Table 4.1. Data for both k_a and k_d are quite consistent over the entire range of concentrations used. χ^2 estimates the difference between experimental and calculated data. In EL-CD28 and RI-CD28 peptides the difference is very low, more so in the later peptide, being close to the noise level of the instrument. A linear regression plot of the rate of change in the response against response units was plotted using these values of k_a and k_d for each CD28 peptide analyte. The slope of this plot was then plotted against the concentration of the peptide to yield a K_d of 2.24 μ M, 2.34 μ M and 2.53 μ M L-CD28, EL-CD28 and RI-CD28 peptides respectively for binding to B7-1Ig (Fig 4.7 C, 4.8 E, 4.9 E). The responses obtained following injections of control peptides, RL-CD28, D-CD28 and CTLA-4 peptides, were very similar to the background responses obtained following injection over an empty flow cell (Figs 4.10 A, B, and C)

4.3 CD28 peptide mimics competitive with CD28-Ig to bind B7-1:

In the sandwich method used for competitive kinetic analysis, indirect immobilization resulted in 286-342RU of B7-1Ig bound to the sensor chip surface via anti mouse IgG1Fc mAb. Direct kinetic analysis for the binding of CD28-Ig to B7-1Ig yielded an affinity constant of 3.17 μ M (Fig 4.11). Binding curves obtained from the competitive

experiments using the CD28-Ig at constant concentration and EL-CD28 or RI-CD28 peptides at increasing concentrations are depicted in Fig 4.12. The top curve is the interaction of CD28-Ig alone with the B7-1Ig. The response level gradually goes down as the concentration of CD28 peptide increases since the peptide competes with CD28-Ig for binding B7-1Ig (Fig 4.12 A and C). The background responses following injection of mixtures of CD28-Ig and EL-CD28 or RI-CD28 peptides over an empty flow cell with no protein immobilized are very low and equivalent (Figs 4.12 B and D). The values obtained from the kinetic analysis for the interaction of CD28-Ig (MW=50,264.36Da) were used as invariant parameters in competitive kinetic analysis with EL CD28 and RI-CD28 (MW= 2425.25 Da) peptides and values for the association and dissociation rate for competing CD28 peptide analytes were floated while fitting the injection phase of the curves to the expanded equation (3). The calculated parameter values are given in Table 3. The average dissociation constant for EL-CD28 binding to CD80-Ig ($K_d = 2.79\mu\text{M} \pm 1.32$) compares well with the value obtained with direct binding of the peptide to B7-1Ig ($K_d = 2.34\mu\text{M}$). However, the dissociation constant varied with varying concentration of EL CD28 peptide (Table 4.2). The average value of dissociation constant for RI-CD28 binding to B7-1Ig ($K_d = 1.75\mu\text{M} \pm 0.67$) is a little lower than the value obtained with direct binding of the peptide to CD80-Ig ($K_d = 2.53\mu\text{M}$). The values of dissociation constant obtained with varying concentrations of RI CD28 peptides were more consistent (Table 4.2).

4.4 Effect of CD28 blockade on activation of TCR transgenic T cells specific for MBP NAc1-11.

CD28 ligation acts in concert with TCR signaling to induce active proliferation of naïve T cells and individually to sustain the late proliferative response and long-term survival of activated T cells. Blockade of the B7: CD28 interaction is known to reduce proliferative responses of both naïve and activated T cells. To investigate the inhibitory potential of CD28 peptide analogues we performed T cell proliferation assays *in-vitro*. The magnitude of MBP specific proliferative responses of CD4⁺ lymph node cells and splenocytes from transgenic mice bearing the V β 8.2 V α 4 TCR specific for MBP Ac 1-11 were determined *in vitro* in the presence of varying concentrations of CD28 peptide analogues. Fig 4.14 shows significant decrease in the proliferative responses of CD4⁺ LNC and splenocytes to MBP Ac1-11 when treated with CD28 peptide analogues and the effect is not dose dependent. Maximum inhibition was observed in CD4⁺ LNC at 120 μ M concentrations of L-CD28 peptide (59.5%) followed by EL-CD28 peptide (47.6%) and RI-CD28 peptide (45.7%) (Fig 4.14 A). The proliferative responses of CD4⁺ splenocytes also decreased but to a lesser extent with the observed maximum inhibition of 50.2%, 38.2% and 42% with L-CD28, EL-CD28 and RI-CD28 peptide respectively (Fig 4.14 B). A similar decrease in the proliferative responses to MBP was also observed (Figs 4.15 A and B). The control RL-CD28 and D-CD28 peptides did not show any inhibition (Figs 4.14 and 4.15). These results suggest that synthetic CD28 peptide analogue block the CD28 signaling required for sustained T-cell proliferation *in vitro*.

4.5. Effect of CD28 blockade on IL-2 production by TCR transgenic T cells specific for MBP NAc1-11.

Augmentation of T cell proliferation following CD28 ligation is a result of its ability to increase the synthesis of IL-2. To determine whether the observed reduction in proliferation is reflected in the IL-2 secretion, we performed the ELISA spot assay. Fig 4.16 shows that MBP stimulated CD4⁺ LNC bearing V α 4 V β 8.2 TCR exhibited significantly reduced frequency of IL-2 secreting cells in the presence of 75 μ M and 100 μ M concentration of EL-CD28 peptide or RI-CD28 peptide when compared to untreated cells.

4.6 Expression of activation markers following in vitro stimulation on lymph node cells and splenocytes from B10.PL TCR transgenic (TCR V α 4 V β 8.2) mice:

To determine the appropriate schedule for treatment with CD28 peptide mimics for obtaining maximum inhibitory effect, we assayed the expression levels of activation markers on the CD4⁺LNC and splenocytes of B10.PL TCR transgenic (TCR V α 4 V β 8.2) mice at various time points after stimulation in vitro with MBP or NAc 1-11. Several interesting findings were observed (Figs 4.17 and 4.18). The resting CD4⁺ LNC and splenocytes exhibited constitutive levels of CD28 and Fas and no FasL or CD152. The expression of early activation marker, CD69, increased following antigen stimulation from 6 hrs through 12 hours and later dropped at 24 hrs and was upregulated again at 48 hrs on both CD4⁺ LNC and splenocytes stimulated with MBP or its encephalitogenic peptide. The CD25 expression was upregulated from 12hrs through 72 hrs with a slight decrease at 24 hrs. CD28 was upregulated above the constitutive levels at 12 hrs and exhibited a dramatic downregulation at 24 hours after stimulation with MBP or NAc1-11, more characteristically observed on CD4⁺ LNC. However, elevated levels of the CD28

were observed at later time points of 48hrs and 72 hrs. A similar pattern of initial upregulation of Fas at 12 hrs followed by a transient downregulation at 24 hrs and resurgence of elevated levels at 48 hrs was observed. Interestingly, the expression of FasL on CD4⁺ LNC and splenocytes was dramatically upregulated at 24 hrs and was back to undetectable levels at 48 hrs after antigen stimulation. Thus, the FasL expression on CD4⁺ LNC was upregulated at a time point when CD28 expression was downregulated. This effect of antigen induced transient downregulation of CD28 and transient upregulation of FasL was more significantly observed on LNC following stimulation with NAc 1-11 (Fig 4.17 A). Figs 4.17 C and D are representative histograms showing CD28 and FasL expression at various time points. The expression of CD152 was not detectable until 24 hours following antigen stimulation, after which the level of CD152 increased on both CD4⁺ LNC and splenocytes.

The expressions of all activation markers although slightly elevated at 24 hrs or 48hrs after stimulation with MBP or its encephalitogenic peptide respectively, was similar to the isotype controls at all other time points (Figs 4.19 A and B).

4.7 Blockade of B7:CD28 interaction induces apoptosis in antigen specific T cells in vitro.

Activation of naïve T cells in the absence of costimulation has been shown to induce programmed cell death (PCD). We examined whether the observed decrease in proliferative responses following treatment with CD28 peptide is a result of cell loss by enzymatic *in-situ* labeling. Apoptotic cells among MBP NAc1-11 stimulated TCR V β 8.2⁺ CD4⁺ T cells were detected by staining with FITC labeled Tdt (terminal

deoxynucleotidyl transferase). Cells incubated in DNase served as positive control. Treatment with EL-CD28 peptide or RI-CD28 peptide at the time of antigen stimulation resulted in apoptosis in a higher percentage of antigen specific CD4⁺ LNC and splenocytes (Fig 4.20 and 4.21). Maximum apoptosis of CD4⁺Vβ8.2⁺ LNC was observed in cultures harvested at 72 hrs following treatment with EL-CD28 peptide @75μM (66%) or RI-CD28 peptide @120μM (60%) when compared to untreated cells (25.6%) (Fig 4.20 A). CD4⁺Vβ8.2⁺ splenocytes also exhibited similar increase in apoptosis when treated with 75 μM of EL-CD28 peptide (46.5%) or RI-CD28 peptide (46.8%) when compared to untreated cells (16.4%) (Fig 4.21 A). Fig 4.22 is a representative histogram of two different experiments showing increased apoptosis of the CD4⁺ LNC and splenocytes cultured in the presence of indicated CD28 peptide analogue. Control cells cultured in the absence of antigen were 88% apoptotic. A separate group of cells were harvested 72 hours after culture. While the CD28 peptide treated CD4⁺Vβ8.2⁺ LNC and splenocytes exhibited increased apoptosis at this time point also, the degree of increase was not significantly higher than that observed at the earlier time point (Fig 4.20 B and 4.21 B).

Since our results on the pattern of expression of CD28 at various time points following antigen stimulation revealed that there is an initial downregulation of CD28 expression between 6 and 24 hrs of stimulation followed by an upregulation at 48 hrs after stimulation, we speculated that blocking the CD28 costimulation at a later time point may result in greater apoptosis. Quantitation of DNA strand breaks was carried out among antigen stimulated CD4⁺Vβ8.2⁺ LNC and splenocytes treated with CD28 peptide mimics 24 hours after stimulation and harvested 48 hours or 72 hours later. Although an increase

in apoptosis in CD4⁺Vβ8.2⁺ LNC was observed, the effect was lost in CD4⁺Vβ8.2⁺ splenocytes was comparable irrespective of the time of treatment with CD28 peptide mimics. At neither time points the increased apoptosis among CD4⁺Vβ8.2⁺ LNC was statistically significant (Figs 4.20 C and 4.21 C and data not shown).

4.8 Discussion:

The production of an immune response and maintenance of T cell homeostasis is largely determined by the balance of costimulation provided by the interacting B7:CD28/CTLA-4 molecules. Previous studies have exploited the fundamental importance of this interaction for modulation of T cell responses in autoimmune diseases or transplant rejection. In this study we directly targeted T cell CD28 costimulation by using a peptide mimetic of its ligand binding epitope. For the first time, we demonstrate that chemically synthesized T cell costimulatory receptor peptides incorporating the “MYPPPY” sequence fold into a PPII helical structure and effectively blocked the expansion of encephalitogenic T cells in vitro suggesting the potential for a therapeutic application.

4.8.1. The importance of the polyproline motif and the PP II helix in protein-protein interaction domains:

The importance of the polyproline motif in the CDR-3 like region of CD 28 for binding B7 ligands is well documented by mutagenesis (167, 169). However, the results of a previous study and our observations have shown that a synthetic peptide consisting of the “MYPPPY” motif alone does not constitute a minimal ligand binding epitope (178). In general, a “functional epitope” defined by mutagenesis is much smaller than the

“structural epitope” seen in the three-dimensional structures. This is particularly true of alanine scanning mutagenesis where the replacement of buried residues with alanine results in the loss of Van der Waals interactions involved in protein-protein interactions (179). Although Bajorath et al modeled the 99MYPPPYLDN104 motif in CD28 as β bulge by introducing an insertion to maintain the structural resemblance to the CDR-3 like region of CTLA-4, the presence of an N-linked glycosylation site at N107 precluded another insertion and hence the authors speculated that the region in CD28 may be more flexible and adopt a different conformation (168).

Proline rich regions (PRR) in proteins have been postulated to be constitute interaction domains. The PRRs have been classified into a, repetitive short PRRS that are important both structurally and in binding and signaling, b, tandemly repeated PRRs such as the proline rich glycoproteins in human saliva and c, nonrepetitive PRRs such as the proteins with SH3 domains or the WW domain where the proline rich motifs RPLPPP (Src) or PPXY (MHC Class I) form the consensus binding sequence for these domains respectively. PRRs are commonly believed to adopt the left-handed PP II helical conformation (180, 181).

Generally speaking, the PP II structure is encountered in proteins where sequential proline residues are present. Formation of PP II helix formation is driven by interactions between proline residues adjacent in sequence and by the conformational restrictions imposed by proline upon the preceding residue- longer range interactions do not play a significant role. PP II helix formation can thus be said to be a local event. The carbonyl

oxygens of each proline residue are solvent exposed, allowing for hydrogen bonding by solvent, a potentially stabilizing interaction for the PP II conformation. Other than proline, glutamine, leucine and positively charged residues are favored within the PP II helix. Residues within the PPII helices expose 60% more polar surface area and 50% more nonpolar surface area to solvent (180, 181). Thus, the PP II helices present an easily accessible hydrophobic surface, and a good hydrogen-bonding site (182). The accessibility of PP II helices are greatly enhanced by the fact that in proteins or known structure, they are frequently found at the amino or carboxy terminus where they form extended structures described as 'sticky arms' (151).

The 20-residue synthetic CD28 peptide analogues comprising the "MYPPPY" motif and designated flanking residues exhibited a typical CD spectrum of PP II helix. CD studies of a synthetic hexapeptide consisting of the "MYPPPY" motif alone did not exhibit a helical conformation. This suggests that the length of the CD28 peptide and interactions with flanking residues play a role in the formation of a PPII helix. To our knowledge, this is the first report of a PPII helical conformation in the ligand-binding region of a T cell receptor protein involved in signal transduction. The localization of the polyproline motif in the solvent exposed CDR3 like region together with the demonstration of a PP II helical structure strongly suggests that the identified synthetic CD28 peptide constitutes its ligand binding epitope.

The relative rigidity of the polyproline stretches in globular proteins means that they lose little conformational entropy upon binding, are favored over other exposed peptide

sequences and being solvent exposed have very fast on and off rates of binding (182). Hence, PRR are more commonly found in situations requiring rapid recruitment or interchange of proteins such as during signaling cascades and cytoskeletal rearrangements (151). Furthermore, it has been suggested that members of the IgSF, to which CD28 belongs, share a conserved backbone-folding pattern that likely provides a common scaffold for efficient protein-protein interactions (139). A surface binding pocket consisting of CC' or CDR-3 loops have been shown to mediate the binding of CD4 to MHC class II and of CD2 to lymphocyte function associated antigen-3 (LFA-3) (183). We evaluated the kinetics of binding of synthetic CD28 peptide to B7 ligands. Both the covalently closed analogue of CD28 peptide (2.34 μ M) and its retro-inverso isomer (2.53 μ M) interacted with CD80-Ig with an affinity constant that is slightly lower than that of CD28-Ig (3.17 μ M). Comparable binding with fast kinetics and low affinities for B7-1: CD28 interaction has been previously reported (102). Similar low affinity binding has also been reported for other members of IgSF, such as between CD2 and LFA-3 (184). More significantly, synthetic CD28 peptides competed efficiently with CD28-Ig to bind B7-1. Competition between CD28 peptides and CD28-Ig for binding CD80-Ig was monitored directly by real time competitive kinetic analysis. A similar approach for kinetic analysis of low molecular weight peptides has been previously reported. The kinetic data obtained from competition experiments for B7-1: CD28 peptides interactions are very similar to that obtained using direct kinetic analysis. These results demonstrate that CD28 peptides form complexes with B7-1 and represent a ligand binding epitope. Although the linear peptide derived from the conserved regions of human CTLA-4 molecule shared the 'MYPPPY' motif it lacked the PPII helical

conformation. This may be due to the presence of multiple glycines distal to the polyproline motif in the CTLA-4 molecule that precludes the formation of PP II helix. This is in accord with CD studies of glycine/proline co-polymers showing that the presence of glycine in polyproline peptides reduces the apparent PP II helical content (185). The absence of a PP II helical structure may account for the inability of this peptide to bind B7 ligands.

4.8.2 Functional Characterization of CD28 peptide mimics:

Prevention of CD28 activation with Fab fragments of anti-CD28 or CTLA-4 Ig is known to induce antigen-specific hyporesponsiveness in T cells (92, 186). Previous studies have shown reduction in the expansion of encephalitogenic cells following CD28 blockade by anti-B7-1 antibody or CTLA4-Ig (109, 177, 187). This difference in binding kinetics of T cell CD28 and CTLA-4 to their cognate ligands B7-1 and B7-2 on APC has been used to selectively block B7: CD28 interaction in the present study. The synthetic CD28 peptides with binding kinetics similar to cell surface CD28 provide a steric hindrance for the later to bind B7 ligands. This leads to a quantitative reduction in CD28 receptor occupancy and decreases the probability of signaling. CTLA-4, expressed on activated T cells, can override the competition from both CD28 peptides and cell surface CD28 due to its higher affinity for B7-ligands and downregulate T cell responses. Significantly, synthetic CD28 peptides inhibited proliferation of encephalitogenic CD4⁺ T cells in vitro. The lack of inhibition with the use of the synthetic hexapeptide consisting of the “MYPPPY” motif only substantiates the importance of interactions with flanking residues for the formation of a functional conformation.

Yashiro et al have demonstrated that anti-CD3 triggered naïve T cells exhibited comparable early proliferative responses when cultured in the presence of anti-CD28 mAb or antibodies against other T cell costimulatory molecules, However, unlike CD28 costimulation which induced high levels of IL-2 production and progressive T cell-proliferation, costimulation by other molecules was followed by apoptosis. These results indicate that a fundamental difference exists in the nature of costimulation between CD28 and other costimulatory molecules, which can be evaluated by the level of IL-2 production (188). Blockade of CD28 signaling has been shown to decrease IL-2 secretion in vitro (127). Consistent with this finding we also observed that treatment with synthetic CD28 peptide analogues significantly reduced IL-2 secretion by antigen activated T cells.

It has been shown that in cultures of 2c transgenic T cells (predominantly CD8+ T cells restricted for a class I restricted alloantigen) with low numbers of antigen-bearing splenocytes in the presence of CTLA-4Ig, simulating minimal TCR ligation and no CD28 ligation, increasing the concentration of the antigen increased the level of TCR mediated early T cell proliferation, but did not sustain the proliferative response. Despite the initial activation, the cells underwent apoptosis after 48 hours of culture (77, 109). Thus, CD28 signaling is required for sustained proliferation of both CD4+ and CD8+ T cells. Collette et al showed that depending on its engagement as a single signal or as a costimulus together with TCR, CD28 could either induce a dose-dependent death signal or protect from cell death respectively. CD28 cosignal can both upregulate the survival factor, Bcl-x_L and prevent FasL expression (189). Addition of CTLA-4Ig resulted in decreased

survival of activated T cells in vitro due to lack of Bcl-X_L (109). Consistent with this finding, we observed that the number of dead cells was significantly higher in cultures treated with EL CD28 peptide than in untreated or control peptide-treated cells stimulated with MBP Acl-11.

Current strategies for immunomodulation of autoreactive T cell responses include the use of monoclonal antibodies to block the critical molecules involved in T cell activation. However, the value of these antibodies as effective therapeutic agents is reduced by their inherent immunogenicity. In comparison, small peptide based therapeutics are less likely to be immunogenic with the potential for use over longer periods of time. Data presented here demonstrate that a peptide mimetic of the ligand-binding region of T cell CD28 molecule inhibits expansion of encephalitogenic CD4⁺ T cells in vitro by increasing apoptosis in the antigen receptor activated T cells. The characterization of mechanism of inhibition by synthetic CD28 peptide has been achieved in the absence of a cross linking agent to any molecules on the T cell surface. Thus, in addition to being a novel approach to develop new therapeutic agents, synthesis of bioactive peptides designed to mimic the surface epitopes involved in protein-protein interaction offers a powerful technique for characterization of the mechanisms involved in immune responses.

Fig 4.1: CD Spectrum of free (A) and end-groups blocked (B) hexapeptide “MYPPPY”
@ 400 μM in PBS 50% TFE at 25°C

Fig 4.2: CD spectrum of L-CD28 peptide (A), EL-CD28 peptide (B) and RI-CD28 peptide (C) at 412 μM in PBS 50% TFE at 25°C

Fig 4.3: Temperature sensitivity of CD 28 peptides: CD spectra of EL-CD28 peptide (A) and RI-CD28 peptide (B) @ 412 μ M in PBS 50% TFE at specified temperatures.

Fig 4.4: (A) CD spectra of end group blocked L CD28 peptide and (B) retro-inverso CD28 peptide @ 412 μ M in PBS 50% TFE @ 25°C, @ 90°C and in 6M CaCl₂. Mean residue ellipticity (θ) is expressed in degrees cm². dmol⁻¹.

Fig 4.5: CD spectrum of RL-CD28 peptide (A) and CTLA-4 peptide (B) at 412mM in PBS 50% TFE at 25°C.

B7-1 Ig Immobilization

Fig 4.6. Immobilization of B7-1Ig to the sensor chip. The beginning and the end of B7-1Ig injections are indicated by arrows. (1) EDC/NHS activation of the carboxymethylated dextran surface, (2) injection of 35mg/ml of CD80-Ig in 10mM acetate buffer, pH 4.2, (3) inactivation of unreacted esters by ethanolamine (4) washing off the noncovalently bound protein with 5mM NaOH giving the final immobilized level of CD80-Ig (1490 RU).

Fig 4.7: Affinity and kinetics of L-CD28 peptide binding to B7-1Ig.

(A) A range of L-CD28 peptide concentrations (375nM to 18 μ M) were injected for 300s at 10 μ l/min through a flow cell with B7-1Ig (1418 RU) or no protein immobilized (Control) (data not shown). The binding at each concentration as measured in response units is shown. (B) Injection phase analysis of a representative binding curve and the residual following injection of L-CD28 peptide (6 μ M) over B7-1Ig immobilized. (C) A plot of (dR/dt) against RU was calculated from the sensograms (data not shown) and the slope of this plot was plotted against concentration of L-CD28 peptide. The association rate constant of the interaction between L-CD28 and B7-1Ig as measured from the slope of this plot is indicated. The dissociation rate constant is obtained from the intercept. The data yields a K_d of 2.24 μ M for the interaction between L-CD28 peptide and B7-1Ig.

Fig 4.8: Affinity and kinetics of EL CD28 peptide binding to B7-1Ig.

(A) A range of EL CD28 peptide concentrations (375nM to 18 μ M) were injected for 300 s at 10 μ l/min through a flow cell with CD80-Ig (1488 RU) or (B) no protein (Control) immobilized. The binding at each concentration as measured in response units is shown. (C) Injection phase analysis of a representative binding curve and the residual following injection of EL-CD28 peptide (6 μ M) over B7-1Ig immobilized. (D) A plot of (dR/dt) against RU was calculated from the sensograms (data not shown) and the slope of this plot was plotted against concentration of EL-CD28 peptide. The association rate constant of the interaction between EL-CD28 and B7-1Ig as measured from the slope of this plot is indicated. The dissociation rate constant is obtained from the intercept. The data yields a K_d of 2.34 μ M for the interaction between EL-CD28 peptide and B7-1Ig.

Fig 4.9: Affinity and kinetics of RI CD28 peptide binding to B7-1Ig.

(A) A range of RI-CD28 peptide concentrations (375nM to 18 μ M) were injected for 300 s at 10 μ l/min through a flow cell with B7-1Ig (1512 RU) or (B) no protein (Control) immobilized. The binding at each concentration as measured in response units is shown. (C) Injection phase analysis of a representative binding curve and the residual following injection of RI-CD28 peptide (6 μ M) over B7-1Ig immobilized. (D) A plot of (dR/dt) against RU was calculated from the sensograms (data not shown) and the slope of this plot was plotted against concentration of

RI-CD28 peptide. The association rate constant of the interaction between RI-CD28 and B7-1Ig as measured from the slope of this plot is indicated. The dissociation rate constant is obtained from the intercept. The data yields a K_d of 2.53 μ M for the interaction between RI-CD28 peptide and B7-1Ig.

Fig 4.10. Background responses following injection of increasing concentrations of control peptides, viz., RL-CD28 (A), D-CD28 (B) and CTLA-4 (C) respectively through a flow cell with B7-1Ig immobilized (1428RU).

CD28 Peptide	C (μ M)	k_a	SE k_a	Chi ²	k_d	SE k_d
EL CD28	6	1.11×10^5	7.96×10^3	0.407	0.273	0.0252
	3	1.37×10^5	1.1×10^4	0.702	0.214	0.0283
	1.5	1.15×10^5	9.33×10^3	0.022	0.242	0.0933
	0.75	1.11×10^5	1.23×10^4	0.0292	0.274	0.05
	0.38	2.46×10^5	5.63×10^4	0.204	0.399	0.0249
RI CD28	6	1.12×10^5	9.05×10^3	0.000474	0.228	0.0347
	3	1.22×10^5	2.31×10^4	0.00396	0.146	0.0167
	1.5	1.41×10^5	5.14×10^4	0.1	0.183	0.0279
	1	1.71×10^5	5.77×10^4	0.0112	0.205	0.0309

Table 4.1. Calculated parameters for EL-CD28 and RI-CD28 peptides binding B7-1Ig by direct kinetic analysis. k_a is the association rate constant, k_d is the dissociation rate constant and SE is the standard error

Fig 4.11: Affinity and kinetics of CD28-Ig binding to B7-1Ig.

(A) A range of CD28-Ig peptide concentrations (10 μ M-60 μ M) were injected for 300 s at 10 μ l/min through a flow cell with B7-1Ig (1012 RU) or (B) no protein (Control) immobilized. The binding at each concentration as measured in response units is shown. (C and D) Injection phase analysis of a representative binding curve and the residual following injection of CD28-Ig (10 μ M) over B7-1Ig immobilized is shown. (E) A plot of (dR/dt) against RU was calculated from the sensograms (data not shown) and the slope of this plot was plotted against concentration of CD28-Ig. The association rate constant of the interaction between CD28-Ig and B7-1Ig is measured from the slope of this plot. The dissociation rate constant is obtained from the intercept. The data yields a K_d of 3.17 μ M for the interaction between CD28-Ig and B7-1Ig.

Fig 4.12: Competitive kinetics between CD28-Ig fusion protein and CD28 peptides for binding immobilized CD80-Ig fusion protein. CD28-Ig @3.2mM concentration mixed with the specified concentrations of EL-CD28 (A) or RI-CD28 (C) peptides were injected through a flow cell with CD80-Ig fusion protein immobilized indirectly via anti-mouse IgG1 Fc (326RU and 348RU respectively) or no protein immobilized as controls (B and D). The response of CD28-Ig binding goes down with increasing concentration of the specified CD28 peptide indicating that the later compete for binding CD80-Ig.

Table 4.2. Competitive kinetics between CD28 peptides and CD28-Ig for binding immobilized B7-1Ig

CD28 Peptide	Concentration(nM)	k_a	SE k_a	k_d	SE k_d	Chi ²	K_d (μ M)
EL CD28	3X10 ³	2.36x10 ⁵	9.29x10 ³	6.89	2.66	1.35	2.92
	2X10 ⁵	5.51x10 ⁴	1.68x10 ³	0.0862	0.0278	0.125	1.56
	1X10 ⁵	3.16x10 ⁴	2.38x10 ³	0.0803	0.0115	0.841	2.54
	0.5x10 ⁵	6.32x10 ⁴	5.3x10 ³	0.316	0.0231	1.24	5
	0.25x10 ⁵	3.36x10 ³	4.3x10 ³	0.0195	0.0565	0.0947	3.36
	0.125x10 ⁵	7.34x10 ⁴	4.61x10 ³	0.101	0.0255	1.89	1.38
RI CD28	3X10 ³	1.07x10 ⁴	8.3x10 ²	0.0206	0.0144	1.61	1.93
	2X10 ³	2.35x10 ⁴	1.76x10 ³	0.0245	0.0252	1.41	1.04
	1X10 ⁵	9.47x10 ⁴	5.72x10 ²	0.142	0.0112	0.359	1.5
	0.5x10 ⁵	9.24x10 ⁴	2.32x10 ³	0.275	0.0252	0.0578	2.98
	0.25x10 ⁵	5.44x10 ⁴	3.38x10 ³	0.0738	0.0114	0.685	1.36
	0.125x10 ⁵	1.05x10 ⁵	8.17x10 ³	0.177	0.0271	0.53	1.69

Calculated parameters for EL CD28 and RI CD28 peptides for binding B7-1Ig by competitive kinetic analysis are shown. Purified anti-mouse IgG1 Fc (31437zz) (Pierce, IL, USA) was used to indirectly immobilize B7-1Ig (286 – 342 RU). Mixtures of 100 μ l of a 3.2 μ M CD28-Ig and 100 μ l of EL CD28 or RI CD28 peptides (6.18 μ M to 503 μ M in HBS-EP) were sequentially injected as analytes. The values obtained from the kinetic analysis for the interaction of CD28-Ig (MW=50,264.36Da, $k_a = 1.38 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$, $k_d = 0.563 \text{ s}^{-1}$, data not shown) were used as invariant parameters. The values for the association and dissociation rate for competing CD28 peptide analytes were floated while fitting the injection phase of the curves to the expanded equation (3) ($R_t = R_1 + R_2$). k_a is the associate rate constant, SE k_a is the standard error of k_a , k_d is the dissociation rate constant, SE k_d is the standard error of k_d , Chi² is the difference between the observed and the experimental value and K_d is the dissociation constant. The average dissociation constant for EL-CD28 binding B7-1Ig is 2.79 μ M $\pm$ 1.32 and that for RI-CD28 binding B7-1Ig is 1.75 μ M $\pm$ 0.67.

Fig 4.13. Purity of V β 8.2+ CD4+ T cells isolated for in vitro analysis. Single cell suspensions of CD4+ T cells from the spleen (A and B) and pooled lymph nodes (C and D) of the TCR V α 2 V β 8.2 TCR transgenic B10.PL mice were incubated with FITC conjugated monoclonal antibodies; anti-mouse V β 8.2 (A and C), anti-mouse CD19 (B and D) and PE labeled anti-mouse CD4 mAb. The purity of CD4+ V β 8.2 + LNC is 92% and that of CD4+ V β 8.2+ splenocytes is 88%. The CD19+ B cell contamination is negligible in both LNC (2.5%) and splenocytes (5.5%).

Fig 4.14: Antigen specific proliferative responses of LNC and splenocytes from MBP peptide specific TCR transgenic mice that carry a V α 4 V β 8.2 TCR cultured with CD28 peptides. Single cell suspensions of CD4⁺ T cells isolated from the lymph nodes (A) and (B) spleen were stimulated with the encephalitogenic peptide of MBP, NAc1-11 and cultured for a total of 72 hrs (including an 18hr pulse with H³ thymidine) in the presence of varying concentrations of the specified CD28 peptide analogues as shown. Data represents mean thymidine uptake and is plotted as Δ cpm $\pm$ SE. Results are the mean of three different experiments. Proliferative responses of CD4⁺LNC (A) and spleen cells (B) treated with L-CD28, end group blocked CD28 and retro-inverso CD28 peptides at all concentrations used were significantly less than untreated and control peptide (reverse L-CD28 and D-CD28) treated cells. * = $p < 0.01$ and ^ = $p < 0.05$ by ANOVA.

Fig 4.15: Antigen specific proliferative responses of LNC and splenocytes from MBP peptide specific TCR transgenic mice that carry a V α 4 V β 8.2 TCR cultured with CD28 peptides. Single cell suspensions of CD4⁺ T cells isolated from the lymph nodes (A) and (B) spleen were stimulated with MBP and cultured for a total of 72 hrs (including an 18hr pulse with H3 thymidine) in the presence of varying concentrations of the specified CD28 peptide analogues as shown. Data represents mean thymidine uptake and is plotted as Δ cpm $\pm$ SE. Results are the mean of three different experiments. Proliferative responses of CD4⁺LNC (A) and spleen cells (B) treated with L-CD28, end group blocked CD28 and retro-inverso CD28 peptides at all concentrations used were significantly less than untreated and control peptide (reverse L-CD28 and D-CD28) treated cells. ^ = $p < 0.01$ and * = $p < 0.05$ by ANOVA.

Fig 4.16: Fewer cells secrete IL-2 when stimulated in the presence of CD28 peptides.

CD4⁺T cells were isolated from the pooled lymph nodes of Vα4 Vβ8.2 TCR transgenic mice and stimulated with 10μg/ml of NAc1-11 peptide of MBP either alone or in the presence of the specified concentrations of CD28 peptides and assayed by ELISPOT. A significant decrease in the number of MBP NAc1-11 responders were observed following treatment with EL-CD28 or RI-CD28 peptide @ 75μM and 100 μM concentrations.

Results are representative of two experiments. * = $p < 0.05$ and ^ = $p < 0.01$.

Fig 4.17. Expression of cell surface markers on antigen activated LNC. Single cell suspensions of CD4⁺ LNC were stimulated with MBP (B) or MBP NAc1-11 (A) as specified in Fig 4.15. The cells were harvested at the indicated hours and incubated with directly labeled specified mouse monoclonal antibodies. The levels of activation markers were measured by flow cytometry. The expressions of early activation marker, CD69 and the IL-2 receptor, CD25 were elevated at all time points. The expressions of CD28 and Fas were transiently downregulated at 24 hours after MBP NAc 1-11 stimulation, at which time point the expression of Fas L was transiently upregulated (A).

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109

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Fig 4.18. Expression of cell surface markers on antigen activated spleen cells. Single cell suspensions of CD4⁺ spleen cells were stimulated with MBP (B) or MBP NAc1-11 (A) as specified in Fig 4.15. The cells were harvested at the indicated hours and incubated with directly labeled specified mouse monoclonal antibodies. The levels of activation markers were measured by flow cytometry. The expressions of early activation marker, CD69 and the IL-2 receptor, CD25 were elevated at all time points. The expressions of CD28 and Fas were transiently downregulated at 24 hours after stimulation (A).

Fig 4.19: Expression of cell surface markers on APC's: Single cell suspensions isolated from the spleen of B10.PL mice were stimulated with MBP (B) or MBP NAc 1-11 (A) and harvested at the indicated time points. Surface expression of activation markers were measured by flow cytometry after incubation with the specified directly labeled mouse monoclonal antibody. In general the expression of all activation markers are marginally above the isotype control at a given time point.

Fig 4.20: Treatment with CD28 peptides induced apoptosis in activated CD4 +T cells. Single cell suspensions of CD4+ LNC were stimulated with MBP NAC1-11 in the presence of the indicated CD28 peptide at the specified concentration and cultured as in Fig 4.15. The CD28 peptides were added either at the beginning of antigen stimulation (A and B) or 24 hours later (C). The cells were harvested at the end of 48 hours (A) or 72 hours (B and C) and the DNA strand breaks were measured by the TUNEL assay. A significant increase in percent apoptosis was observed in the CD4+LNC treated with the CD28 peptides at either time points and harvested at 48 hours or 72 hours after antigen stimulation (A and B). The increase in apoptosis among CD4+LNC treated with CD28 peptide at 24 hours after antigen stimulation and harvested at 72 hours was not statistically significant (C). ^ = $p < 0.01$ and * = $p < 0.05$ by ANOVA.

Fig 4.21: Treatment with CD28 peptides induced apoptosis in activated CD4⁺T cells. Single cell suspensions of CD4⁺ spleen cells were stimulated with MBP-NAC1-11 in the presence of the indicated CD28 peptide at the specified concentration and cultured as in Fig 4.15. The CD28 peptides were added either at the beginning of antigen stimulation (A and B) or 24 hours later (C). The cells were harvested at the end of 48 hours (A) or 72 hours (B and C) and the DNA strand breaks were quantitated by the TUNEL assay. A significant increase in percent apoptosis was observed in the CD4⁺ spleen cells treated with the CD28 peptides at either time points and harvested at 48 hours or 72 hours after antigen stimulation (A and B). The increase in apoptosis among CD4⁺ spleen cells treated with CD28 peptide at 24 hours after antigen stimulation and harvested at 72 hours was not statistically significant (C). ^ = $p < 0.01$ and * = $p < 0.05$ by ANOVA.

Fig 4.22: CD28 synthetic peptide treatment increases apoptosis of CD4⁺ T cells in vitro. 5×10^5 CD4⁺ T cells were isolated from the pooled lymph nodes of Vβ8.2 Vα 4 TCR transgenic mice and stimulated with 10 μg/ml of NAc 1-11 peptide of MBP either alone or in the presence of the specified concentrations CD28 peptide. Lymph node cells were harvested after 48 hrs and apoptotic cells among Vβ8.2⁺ cells were detected by the TUNEL methods and analyzed by flow cytometry. Representative histograms showing increase in FITC conjugated TUNEL positive cells in cultures treated with 120 μM EL-CD28 or RI-CD28 are shown in (A) and (B) respectively.

Chapter 5

Suppression of Experimental Autoimmune Encephalomyelitis using Peptide Mimics of CD28.

5.1 Costimulatory blockade in vivo by CD28 peptides inhibit incidence of EAE in B10.PL mice

Blockade of B7: CD28/CTLA-4 interaction had varying effects on disease induction in different animal models of EAE. Treatment with B7-1mAb ameliorated disease whereas monoclonal antibodies to B7-2 and CTLA-4 exacerbated the disease. Our approach differs from the other methods of costimulatory blockade in that we have used a chemically synthesized CD28 peptide mimic to selectively block B7:CD28 interaction. CTLA-4 expressed on activated T cells can override the competition from the synthetic CD 28 peptide much the same way it would the competition from the cell surface CD28 due to its higher avidity for binding the B7 ligands. Our initial data showed that antigenic stimulation in the presence of CD28 peptides inhibited expansion of encephalitogenic T cells *in vitro*. In this study, we have evaluated the biological activity of synthetic CD28 peptide mimics during antigen priming *in vivo* in EAE. B10.PL mice immunized with GP-MBP (200µg) in CFA were injected intravenously 500µg of EL-CD28 or RI-CD28 or control CD28 peptides or 0.25ml of sterile PBS on the day of immunization. There was a

delay in the onset of clinical disease in the RI-CD28 peptide treated mice as compared to vehicle or control peptide treated mice (Fig 5.1 C and Table 5.1A). The vehicle treated and control peptides viz: L-CD28, RL-CD28 and D-CD28 peptide treated mice had maximum disease incidence of 100%, 100%, 91.5% and 91.5% and maximum mean cumulative score of 50.23, 47.26, 44.9 and 52.25 respectively. In contrast, significant inhibition of EAE was observed with mean maximum incidence of 70% and 60% and a maximum mean cumulative score of 29.2 and 19.39 in mice treated with EL CD28 and RI CD28 peptide respectively. The effect of RI CD28 peptide injection was more significant and lasted for the duration of observation (37 days) in one experiment (Figs 5.1 A and B) (Table 5.1 A).

5.2 Synthetic CD28 peptide treatment ameliorates established EAE.

Most studies on costimulatory blockade for therapeutic intervention in EAE have focused on the suppression of T cell responses during antigen priming and disease induction. We therefore assessed the ability of CD28 peptide mimic to suppress ongoing clinical disease. All mice immunized with MBP (200µg) in CFA were allowed to develop clinical disease for 14 days following GP-MBP immunization at which time most of the mice exhibited signs of early paralysis or complete hind limb paralysis. They were then distributed randomly into six groups with mean clinical score of each group approximately the same. Groups of mice were left untreated or injected intravenously 500µg of EL CD28 or RI CD28 or control CD28 peptides. The mean clinical score of untreated mice continued to increase reaching a maximum of 3.4 on day 20. Similarly the disease continued to progress in mice treated with control CD28 peptide, viz: LCD28,

RLCD28 and DCD28 reaching mean maximal clinical score of 3.8 (day 18), 3.4 (day 16) and 3.7(day 16) respectively. In contrast, mice treated with EL CD28 and RI CD28 peptide continued to show clinical improvement from day 16 throughout period of observation (26 days) post-immunization (Figs 5.2 A and B) (Table 5.1 C). This suggests that short-term blockade of CD28 costimulation is capable of attenuating ongoing disease progression in EAE.

5.3 Multiple treatments with synthetic CD28 peptide mimics between the initiation and preclinical phase exacerbates EAE:

Based on our initial observation that treatment with CD28 peptide mimics inhibits EAE induction we hypothesized that multiple treatments may have a greater inhibitory effect. In order to determine the effect of in vivo blockade of CD28 costimulation prior to activation and at multiple times during the preclinical phase, groups of mice were treated with 100 µg of EL-CD28 peptide or RI-CD 28 peptide, control peptides or sterile PBS on days -1, +1, +4, +7, and +9 with reference to MBP immunization. All mice developed EAE with no significant difference in onset. The maximum mean cumulative score of EL-CD28 peptide treated mice was the lowest, 19.2. There was no significant difference in the maximum mean cumulative scores of mice treated with RI-CD28 peptide (35.8) and control peptide treated mice (RL-CD28 peptide, 35.7; D-CD28 peptide, 30.4; L-CD28 peptide, 40.3) or vehicle treated mice (38) (Figs 5.3 A and B) (Table 5.1 B).

5.4 CD28 peptides suppress antigen specific proliferation of MBP primed T cells and cytokine production.

Previous studies have shown that treatment with a cyclized peptide of the murine CD4 molecule or CTLA-4 Ig can prevent antigen specific T cell priming *in vivo*. To determine the effect of costimulatory blockade *in vivo* by CD28 peptides, we assessed the proliferation of MBP-primed T cells upon restimulation *in vitro*. Mice immunized with GP-MBP (200mg) in CFA were injected intravenously either 500 μ g of EL CD28 or RICD28 or control CD28 peptide analogues or vehicle (PBS, pH 7.4). Draining lymph node cells and splenocytes were collected on day 10 post-immunization and cultured *in vitro* for 72 hrs in the presence of MBP (40 μ g/ml). There was a marked suppression of proliferation in LNC and splenocytes from mice treated with EL CD28 peptide (Δ cpm = 4603 $\pm$ 373 and 16524 $\pm$ 269) or RI CD28 peptide (Δ cpm = 2140 $\pm$ 438 and 10562 $\pm$ 324) as compared to vehicle treated mice (Δ cpm= 6061 $\pm$ 574 and 24169 $\pm$ 818) ($p<0.05$) (Figs 5.4 A and B). Splenocytes from mice treated with a single dose of 500 μ g of RI CD28 after clinical disease development on day 14 and collected two days later also exhibited reduced proliferation in response to MBP (Δ cpm = 4307 $\pm$ 2320) as compared to control peptide (Δ cpm = 12557.8 $\pm$ 1223) or vehicle treated mice (Δ cpm = 14549 $\pm$ 4202) (Fig 5.4 D).

The effect of costimulatory blockade *in vivo* could be either specific to T cells receiving signal 1 or have a much broader effect on the entire T cell compartment. To investigate this effect, splenocytes were retrieved on day 26 from vehicle treated and mice treated

with CD28 peptides on the day of immunization. The splenocytes from mice treated with RI-CD28 peptide continued to exhibit reduced proliferation in response to MBP (Fig 5.4 C). However, the splenocytes from all mice exhibited equivalent proliferative responses to stimulation with 5 μ g/ml of tetanus toxoid (Fig 5.4 E). Thus, CD28 peptides were able to specifically inhibit encephalitogenic T cells without affecting the primary *in vitro* responses to an unexposed antigen.

Draining lymph node cells and splenocytes from mice administered multiple treatments of CD28 peptides, control peptides or PBS were collected on day 10 or day 26 post-immunization and cultured *in vitro* for 72 hrs in the presence of MBP. There was no significant difference in the proliferation of LNC and splenocytes from mice treated with EL CD28 peptide (Δ cpm = 6037.3 $\pm$ 1501 and 10602.5 $\pm$ 531.1) or RI CD28 peptide (Δ cpm = 5438.35 $\pm$ 4885 and 10730.6 $\pm$ 2772.9) as compared to vehicle treated mice (Δ cpm= 8914.2 $\pm$ 8113 and 17090.2 $\pm$ 213.6) or control peptide treated mice (Fig 5.4 F and data not shown).

5.5 Effect of treatment with synthetic CD28 peptide mimics on cytokine production:

MBP reactive T cells that induce EAE are known to display a Th₁ phenotype secreting the proinflammatory cytokines IL-2, INF- γ and TNF- β . CD28 costimulation has been shown to increase IL-2 production by activating gene transcription and prolonging the IL-2 mRNA half-life. Cytokine production upon restimulation *in vitro* by *in vivo* MBP-primed lymph node cells and splenocytes was assessed by ELISPOT assay. Mice were treated as above on day 0 of immunization. Draining lymph node cells and splenocytes

were collected on day 10 post-immunization and cultured *in vitro* for 24 hrs in the presence of MBP. The frequency of IL-2 secreting lymph node cells decreased significantly EL CD28 (2776 +/- 53.5; $p < 0.05$) and RI CD28 (1753 +/- 37.7; $p < 0.01$) treated mice as compared to vehicle (4846 +/- 14.3) and control RL CD28 (3386 +/- 23.3) or D CD28 (2675 +/- 14.2) peptide treated mice (Fig 5.5 A). The frequency of INF- γ secreting lymph node cells and splenocytes at this time were equivalent in all cultures (Fig 5.5 B). Perrin *et al.*, have also reported similar findings of decreased IL-2 and unaltered INF- γ production after costimulatory blockade of MBP specific T cells.

5.6 In-vivo blockade of CD28 costimulation induced apoptosis in antigen specific T cells.

Either anergy or deletion of autoreactive T cells can explain the amelioration of clinical disease observed by blocking B7:CD28/CTLA-4 costimulation in several animal models of autoimmune diseases. Based on the observation of selective apoptosis of specific autoreactive T cells it has been suggested that the resolution of inflammation in EAE is a result of activation induced death of previously activated T cells interacting with non professional APC's that fail to deliver costimulatory signals. Our *in-vitro* experiments demonstrated that MBP NAc1-11 specific V β 8.2+ T cells undergo apoptosis when stimulated in the presence of CD28 peptides (Figs 4.20 and 4.22). To determine whether the protective effect of CD28 peptides in EAE was mediated through apoptosis, the DNA strand breaks in CD4+ lymphocytes were detected by enzymatic labeling of nicked ends. Cells incubated in DNase served as positive control. A significantly higher percentage of

CD4⁺ T cells were apoptotic in mice treated with RI CD28 peptide (16%) on the day of immunization as compared to vehicle treated mice (8.7%) or control peptide treated mice (10.2%) (Fig 5.6 A).

Although studies in genetically deficient mice have demonstrated a critical role for B7 costimulation in the effector phases of EAE, CTLA-4 Ig treatment has had limited effects on established EAE. Our observation of attenuation of established EAE following selective blockade of B7:CD28 interaction supports the observation of Perrin *et al.*, that short term CD28 blockade may be sufficient to control autoreactive effector T cells. As stated above RI CD28 peptides protected against EAE by peripheral deletion of CD4⁺ T cells. To determine whether decrease in severity of established disease is also a result of cell death, apoptosis among CD4⁺ T cells of mice treated with RI CD28 peptide after disease development was assessed by detecting FITC labeled DNA strand breaks by flow cytometry. Increase in mean percent apoptosis was observed in RICD28 peptide treated mice (8.3%) as compared to untreated mice (6%) and control peptide treated mice (2.6%) (Fig 5.6 B).

Fig 5.6 C and D are representative histograms showing increased apoptosis of LNC from mice treated with RI CD28 peptide on day 0 and day 14 respectively. This suggests that CD28 peptide mimic engages the B7-ligands on APC and effectively blocks the costimulation via cell surface CD28 receptor required for sustained activation and long-term survival of CD4⁺ T-cells.

5.7 Treatment with CD28 peptides downregulate CD80 expression in lymph node cells and splenocytes.

Previous studies on the *in-vivo* expression of the B7 molecules during the course of EAE have shown that the expression of CD80 is upregulated in the periphery correlating with the clinical disease. In one model of EAE, immunization of SJL mice with proteolipid protein peptide resulted in a significantly greater B7.1: B7.2 ratio during the preclinical phase. Furthermore, B7-1^{-/-}B7-2^{-/-} or CD28^{-/-} were shown to be highly resistant to MOG induced EAE. To investigate whether the observed protection following treatment with CD28 peptides is a result of reduced costimulation, expression of CD80, CD86 CD28 and CD152 in the peripheral lymphoid organs was analyzed by flow cytometry. Mice were sacrificed on day 10 or 26 following disease induction and CD28 peptide treatment.

Consistent with previous reports lymph node cells and splenocytes from naïve B10.PL mice expressed higher levels of CD86 than CD80. Immunization with GP-MBP in CFA resulted in a relative upregulation of CD80 in both splenocytes and lymph node cells of vehicle treated mice as compared to naïve animals. On the other hand, CD86 expression was downregulated on splenocytes from untreated and control peptide treated B10.PL mice as compared to naïve animals. There was only a mild increase in the expression of CD80 in lymph node cells and splenocytes of mice treated with RI CD28 peptide that expressed CD86 at levels similar to that in unimmunized animals. The difference in the expression of CD80 was less marked in EL CD28 peptide treated mice. Thus, the CD80/CD86 ratio was significantly greater in splenocytes and lymph node cells of untreated B10.PL mice as compared to RICD28 peptide treated mice (Figs 5.7 A, B, C

and D). The expression of CD28 on LNC and splenocytes from RI CD28 treated mice was decreased as compared to vehicle treated mice consistent with their inactive state as evidenced by decreased levels of CD25 (Fig 5.8 A, B and E). The expression of CD152 on the splenocytes in RI-CD28 peptide treated mice was slightly upregulated as compared to vehicle treated mice (Fig 32 D). In addition, CD4⁺ LNC from RI CD28 peptide treated mice exhibited reduced expression of Fas and FasL as compared to vehicle treated mice (Figs 5.8 F and G).

A similar pattern of elevated CD80 expression and decreased CD86 expression was observed in splenocytes of untreated mice sacrificed on day 26 postimmunization as compared to EL CD28 and RI CD28 peptide treated mice (data not shown). In general, CD28 expression was lower in CD4⁺ lymph node cells and splenocytes of mice treated with CD28 peptides as compared to vehicle treated mice assayed during the period of active disease development or later (data not shown). Since the proliferation of splenocytes in response to a previously unexposed antigen, tetanus toxoid, was equivalent in all groups of mice, the observed upregulation of CD80, CD86 and CD28 in untreated mice on day 26 following immunization probably reflects the beginning of a relapse and not global immunosuppression.

Analysis of expression of CD80 and CD86 in the peripheral lymphoid organs of mice treated with RI- CD28 peptide during acute EAE and sacrificed on day 16 post-immunization exhibited a similar trend of reduced CD80 expression on CD4⁺ T cells and a decreased CD80/CD86 ratio as compared to control peptide or vehicle treated mice (Fig

5.9 A and B). While the expressions of CD28 and Fas was slightly lower on the CD4+ LNC from RI_CD28 peptide treated mice as compared to vehicle treated mice, the levels of CD152 and Fas L were comparable between the two groups of mice (data not shown).

5.8 Discussion:

In this study, we evaluated the ability of two synthetic peptides derived from the ligand-binding region of CD28 to selectively block B7:CD28 interactions and alter the clinical course of EAE. A retro-inverso CD28 peptide mimic was demonstrated to optimally prevent and suppress clinical EAE.

5.8.1. Therapeutic potential of CD28 peptide mimics:

Treatment with synthetic CD28 peptide mimic at the time of immunization protects B10.PL mice from EAE. The retro-inverso isomer (RI-CD28) inhibited EAE development more effectively, with the treated mice exhibiting lower disease incidence and significantly decreased clinical severity than mice treated with the end group blocked parent peptide. This difference in activity may be attributed to a decreased susceptibility of a peptide composed of D-amino acids to proteases in vivo.

Importantly, a single administration of synthetic CD28 peptide mimics ameliorated ongoing disease in B10.PL mice with dramatic improvement in the clinical signs of EAE within 24-48 hrs after administration of RI-CD28 or EL-CD28 peptide. All of the mice treated with the CD28 peptide mimics showed a decrease in clinical score throughout the period of observation, but there was not complete clinical recovery. Previously, treatment

with Fab fragments of CD28 mAb during the first disease episode has been shown to attenuate established EAE in PL X SJL F₁ mice (133). Whereas systemic administration of CTLA-4Ig had minimal effect, local CNS delivery of CTL4-Ig using a nonreplicative adenoviral vector suppressed EAE (127, 190). Taken together, our data and those of others suggest that short-term CD28 blockade may provide a means to ameliorate an established autoimmune disease.

Multiple administrations of CD28 peptide mimics failed to protect the mice against EAE. There can be two plausible reasons for this failure. It is likely that multiple injections of the CD28-peptide may act as a booster facilitating the development of antibodies. However, anti-peptide antibodies were not detected in the serum of RI-CD28 peptide treated animals. It is likely that we were blocking the B7:CTLA-4 interactions that occur during the initial phases of T-cell activation. This is supported by our observation that 24hrs following antigen stimulation there is a transient downregulation of CD28 at which time point there is upregulation of CTLA-4. Hence, treatment with CD28 peptide mimics 24 hours after immunization may have paradoxically stimulated the antigen specific T cells by blocking CTLA-4 signaling. Furthermore, FasL is also known to be upregulated during this time point contributing to the protection of the activated cells from apoptosis (189).

If CD80 and CD86 are able to direct T cell differentiation and activation, the outcome of an immune response will depend upon the level of expression of the two molecules on the APC. Resting mouse T cells have been shown to express B7-2 with little or no B7-1.

Following activation, B7-2 is downregulated with a concomitant increase in B7-1 expression (82, 122). Our observation of increased expression of B7-1 relative to B7-2 on splenocytes in mice immunized for EAE is consistent with the studies of Karandikar et al., who reported similar findings in PLP peptide induced EAE (82). However, in contrast to their studies, we observed a similar trend of increased B7-1 expression in peripheral LNC as well. This difference may be attributed to different genetic backgrounds of the mice or the method of EAE induction (PLP versus MBP). Our results showed that splenocytes and LNC of mice treated with RI-CD28 peptide exhibited significantly lower B7-1 expression relative to B7-2 during both the initial and late effector phases of EAE. These data, together with the significant decrease in the frequency of IL-2 secreting cells and cell surface expression of CD25, suggest that there are fewer activated encephalitogenic T cells in the periphery of animals treated with RI-CD28 peptide. Collectively, our findings can be viewed as supportive of the hypothesis proposed by Cross et al., (89) that T cells expressing B7-1 preferentially migrate to the CNS during acute EAE.

Naive T cells undergo apoptosis unless provided with a TCR-mediated stimulus and CD28-mediated costimulation (191). Our observation of increased apoptosis of CD4⁺ T-cells from the lymph nodes of mice treated with RI-CD28 peptide following EAE induction suggests clonal deletion as the mechanism of protection against EAE following *in vivo* blockade of CD28 costimulation. This is supported by the absence of upregulation of CD25 and CD28 on CD4⁺ T-cells from the peripheral lymphoid organs of peptide treated mice as compared to the activated phenotype of CD4⁺ T-cells from vehicle

treated mice. Previously, Kearney et al., (192) showed that in vivo treatment with a combination of anti B7-1 and anti B7-2 mAbs blocked clonal expansion with subsequent loss of T cells.

The therapeutic potential of CD 28 blockade in autoimmune diseases is exemplified by the fact that within a decade of demonstration of its potent immunosuppressive effects, CTLA4-Ig has entered phase I clinical trials for the treatment of psoriasis (114). Multiple studies in EAE have pointed to the development of agents to selectively block CD28 costimulation for maximum therapeutic efficacy. Data presented here and the studies by Perrin et al., (133) show that selective blockade of CD28 can both prevent and suppress clinical EAE. In contrast to treatment with monoclonal antibodies, small peptide-based therapeutics has the advantage of inherent lack of immunogenicity and with a substantially lower molecular weight have potentially greater accessibility to tissues, including the central nervous system.

In conclusion, we have integrated the results of mutagenesis experiments, binding kinetics, molecular modeling and structural characterization of T-cell costimulatory molecules together with peptidomimetics in the design of a biologically active peptide mimic that selectively blocks CD28 costimulation in vivo. The potential therapeutic application of this peptide can be extended to most T-cell mediated autoimmune disorders and graft-versus host disease where the aim is to downregulate T cell responses and accelerate recovery.

Figure 5.1. CD28 peptide treatments inhibit EAE. B10.PL mice were immunized with MBP in CFA and injected with pertussis toxin i.p on the day of immunization and 2 days later. Animals received a single i.v. injection of 500 μ g of EL-CD28, RI-CD28 peptide, control (L-CD28, RL-CD28 and D-CD28) peptides in sterile PBS, or sterile PBS alone on the day of immunization. Animals were observed daily for signs of clinical disease for 26 days. (A) The data are presented as the mean clinical score in each group over time. (B) The severity of EAE as measured by the mean cumulative score divided by the number of days observed is significantly reduced in EL-CD28 and RI-CD28 peptide treated mice ($+p < 0.05$, $*p < 0.01$ by ANOVA) as compared to control mice. No significant differences were found between vehicle treated and control CD28 peptide treated mice. (C) A delay in the onset of disease in RI-CD28 peptide treated mice as compared to controls was also observed. Data shown represents pooled values from two experiments.

Figure 5.2. CD28 peptide mimics attenuate established EAE. B10.PL mice were immunized for EAE as in Fig 5.1. Fourteen days post-immunization, the animals were divided randomly into six groups with the mean clinical score of each group approximately the same. Animals then received a single i.v. injection of 500 μ g EL-CD28, RI-CD28, control CD28 peptides (in sterile PBS) or sterile PBS alone. (A). Mice that received EL- CD28 or RI-CD28 peptide showed significant clinical improvement throughout the period of observation. (B) The mean clinical severity was significantly reduced in EL-CD28 and RI-CD28 (*= p <0.05 by ANOVA) treated mice as compared to vehicle treated mice. There were no significant differences between control peptide treated and vehicle treated mice. Data shown represents pooled values from two experiments.

Fig 5.3: Effect of multiple injections of synthetic CD28 peptide analogues on clinical EAE. B10.PL mice were immunized with GP-MBP in CFA and injected pertussis toxin i.p on day 0 and 2. Animals received i.v. injections of 100 μ g of EL-CD28 or RI-CD28 peptide analogues or control (L-CD28, RL-CD28 and D-CD28) peptides in 0.25ml of sterile PBS, pH7.4 or sterile PBS alone on days -1, +1, +4 +7 and +9 with respect to the day of immunization. Animals were observed daily for signs of clinical disease for 26 days (A). The data are presented as the mean clinical score in each group on different days of observation. (B) No significant difference in the severity of EAE between the different groups of mice was observed.

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131,132

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Figure 5.4. Antigen specific proliferative responses are reduced in mice treated in vivo with CD28 peptides. Groups of mice were immunized with MBP and injected with

500µg/ mouse of CD28 or control peptides or sterile PBS either on the day of immunization or during acute disease (14 days post-immunization). LNC and splenocytes were collected 10 days or 26 days post-immunization (16 days post-immunization from mice treated during acute disease) and stimulated in triplicate with MBP or tetanus toxoid. Proliferation was measured after a 72 hr culture (A) LNC and (B and C) splenocytes from RI-CD28 and EL-CD28 peptide treated mice showed significant inhibition of proliferation in response to MBP as compared to cells from vehicle or control peptide treated mice. ++ = $p < 0.05$, * = $p < 0.01$ compared to vehicle treated and # = $p < 0.01$ compared to control peptide treated mice by ANOVA. (D) Splenocytes from mice treated with RI-CD28 peptide during acute disease showed significant inhibition of proliferation in response to MBP compared to cells from vehicle or control peptide treated mice. (E) Splenocytes from peptide treated mice in Panel A showed no differences in the response to tetanus toxoid. (F) Proliferative responses of splenocytes from mice given five i.v. injections 100µg each of the specified CD28 peptide analogues during the preclinical phase of the disease and collected 10 days post-immunization did not differ significantly between different groups of mice when stimulated in vitro with MBP.

Figure 5.5. Effect of synthetic CD28 peptide treatment on cytokine secretion. Groups of mice were immunized with MBP and injected with 500 μ g/ mouse of CD28 or control peptides or sterile PBS either on the day of immunization. LNC were collected 10 days post-immunization, stimulated in triplicate with MBP and IL-2 and INF- γ secretion was measured by ELISPOT assay. (A) The frequency of IL-2 secreting cells is significantly reduced in EL-CD28 and RI-CD28 treated mice as compared to vehicle treated mice. (B) No significant difference is observed in the frequency of INF- γ secreting cells between the different groups of mice. [^]=p<0.01 and ^{*}=p<0.05 by ANOVA.

Figure 5.6. CD28 peptide treatment in vivo induces apoptosis in encephalitogenic T cells. Mice immunized with MBP were injected intravenously with 500 μ g of RI-CD28 peptide, Control peptide or PBS on the day of immunization. LNC were collected 10 days post-immunization. Apoptosis among CD4⁺ cells was assayed by TUNEL using flow cytometry. (A) A higher percentage of CD4⁺ cells were apoptotic in RI-CD28 peptide treated mice (*= p <0.05 student t test) vs vehicle treated mice. (B) A greater percent apoptosis was also observed in CD4⁺ LNC collected 16 days post-immunization from mice treated with RI-CD28 peptide after clinical disease development, 14 days after immunization. (C) and (D) are representative histograms showing an increase in TUNEL positive cells in mice treated with RI-CD28 peptide on day 0 (C) or on day 14 (D).

Fig 5.7: Injection of CD28 peptides during antigen priming prevents upregulation of B7-1 on CD4+ LNC and splenocytes following induction of EAE.

B10.PL mice immunized with GP-MBP were injected with 500 μ g/mouse of CD28 peptide analogues or control peptides or sterile PBS (pH 7.4) on the day of immunization. LNC and splenocytes were collected on day 10 post-immunization and analyzed for B7-1, B7-2, CD28, Fas, and FasL expression. The data is represented as the mean of relative B7-1: B7-2 ratio in LNC (A) and splenocytes (B) each group of mice. A significant decrease in the ratio is observed in splenocytes of RI CD28 peptide treated mice as compared to vehicle treated mice (*= $p < 0.05$ by students t test). Representative histograms showing B7-1 and B7-2 expression in splenocytes collected on day 10 post-immunization from vehicle (C) or a mouse treated with 500 μ g RI CD28 peptide (D) on the day of immunization with GP-MBP are shown. The relative B7-1: B7-2 ratio and the percentage of positive staining cells above control staining are indicated.

Figure 5.8. Expression of CD28 and CD152 on CD4⁺ LNC and splenocytes from mice treated with CD28 peptides following induction of EAE. B10.PL mice immunized with MBP were injected with 500µg EL-CD28, RI-CD28, control peptides or sterile PBS on

the day of immunization. LNC and splenocytes were collected 10 days post-immunization and analyzed for CD28, CD152, Fas and FasL expressions. The data is represented as the mean percent positive expression of CD28 and CD152 on CD4+ LNC (A and C respectively) and splenocytes (B and D respectively) from each group of mice. There is a slight decrease in the expression of CD28 (A and B) and a slight increase in the expression of CD152 (C and D) in the RI-CD28 peptide treated mice. The expression of CD25 was significantly decreased on CD4+LNC from mice treated with RI-CD28 as compared to vehicle treated mice (E). No difference is observed in the expressions of Fas (F) and FasL (G) on CD4+ LNC between different groups of mice.

Figure 5.9. CD80/CD86 ratio was decreased on CD4⁺ LNC following treatment with CD28 peptide during acute EAE. B10.PL mice immunized with MBP were injected i.v. 500 μ g RI-CD28 or sterile PBS during acute EAE (14 days post-immunization). Splenocytes were collected on day 16 post-immunization and analyzed for CD80 and CD86 expression. Representative histograms showing CD80 (A) and CD86 (B) expression from vehicle or a mouse treated with 500 μ g RI-CD28 peptide are shown. Percentage of positive staining cells above the isotype control (not shown) is indicated. The CD80/CD86 ratio in the RI-CD28 peptide treated mice and vehicle treated mice is indicated.

Chapter 6

Future Perspectives

Detailed Structural Characterization and Rational design of CD 28 peptide mimics:

In terms of structural characterization of the CD28 peptide mimics the presence of a PP II helix can be unambiguously confirmed by determining the solution structure of synthetic CD28 peptides by nuclear magnetic resonance (NMR) spectroscopy. Initial analysis of the two dimensional NMR spectroscopy indicates that the prolines in the synthetic CD28 peptides are in a trans conformation consistent with similar observation among the PPII helices of other globular proteins. The design of the peptide mimics can then be modified by incorporating appropriate changes in the flanking residues to optimize the stability of its secondary structure both in vitro and in vivo.

Determination of optimum dosage and route of administration of CD28 peptide mimics:

Since like EAE, MS is a relapsing-remitting type of autoimmune disease, it is important to know whether synthetic CD28 peptide treatment can prevent or decrease the number of relapsing episodes. Although there was continued protection against EAE after CD28

peptide treatment in one set of experiment, it is not clear whether retro-inverso CD28 peptide can induce a long-term resistance to EAE. To answer this question, the ability of synthetic CD28 peptide administered at the time of disease induction to protect against EAE upon rechallenge after complete recovery has to be assessed.

The inhibitory effect was observed following a single administration of 500µg of synthetic CD28 peptides. The logical step would be to determine the optimum dosage of CD28 peptides for maximum therapeutic efficiency with minimum toxicity. This will help in establishing the full range of CD28 peptide induced inhibition. Furthermore, the initial therapeutic effects were observed following intravenous administration of CD28 peptides, which is the least preferred route for prospective human clinical trials. For best patient compliance it is essential to determine whether synthetic CD28 peptides are compatible with oral administration. Hence, it would be better to compare and test the bioavailability and clinical efficacy of synthetic CD28 peptides following oral, subcutaneous and intravenous administration.

A concern regarding the use of relatively stable D amino acid peptides is the long term effect of inhibition of CD28 signaling on antigen-specific CD4⁺ T cells responses in vivo. In other words, it is imperative to determine whether retro-inverso CD28 peptide will persist in vivo for long periods such that subsequent immune responses will be impaired. This can be addressed by challenging the retro-inverso CD28 peptide treated mice with an unrelated antigen at various time points following peptide administration.

Characterize in detail the mechanism of action of the inhibitory retro-inverso CD28 peptide in EAE.

Our observations of reduced clinical severity and antigen induced apoptosis in CD4+ T cells suggest that retro-inverso CD28 mediated protection may be due to fewer T cells infiltrating the CNS. This can be assessed determining the histopathology of the perfused and preserved tissues of the brain, spinal cord, peripheral lymph nodes and spleen of the retro-inverso CD28 peptide treated mice. Conclusive evidence for the therapeutic ability of CD28 peptide mimics in EAE will require demonstration of localization of the synthetic peptide in the target tissue within the central nervous system.

BIBLIOGRAPHY

1. Bar-Or, A., E. M. Oliveira, D. E. Anderson, and D. A. Hafler. 1999. Molecular pathogenesis of multiple sclerosis. *J Neuroimmunol* 100:252.
2. Lublin, F. D., and S. C. Reingold. 1996. Defining the clinical course of multiple sclerosis: results of an international survey. National Multiple Sclerosis Society (USA) Advisory Committee on Clinical Trials of New Agents in Multiple Sclerosis. *Neurology* 46:907.
3. Weinshenker, B. G., B. Bass, G. P. Rice, J. Noseworthy, W. Carriere, J. Baskerville, and G. C. Ebers. 1989. The natural history of multiple sclerosis: a geographically based study. I. Clinical course and disability. *Brain* 112:133.
4. Weinshenker, B. G., B. Bass, G. P. Rice, J. Noseworthy, W. Carriere, J. Baskerville, and G. C. Ebers. 1989. The natural history of multiple sclerosis: a geographically based study. 2. Predictive value of the early clinical course. *Brain* 112:1419.
5. Martin, R., and H. F. McFarland. 1995. Immunological aspects of experimental allergic encephalomyelitis and multiple sclerosis. *Crit Rev Clin Lab Sci* 32:121.
6. Miller, D. H. 1994. Magnetic resonance in monitoring the treatment of multiple sclerosis. *Ann Neurol* 36:S91.
7. Martin, R., H. F. McFarland, and D. E. McFarlin. 1992. Immunological aspects of demyelinating diseases. *Annu Rev Immunol* 10:153.
8. Hohlfeld, R. 1997. Biotechnological agents for the immunotherapy of multiple sclerosis. Principles, problems and perspectives. *Brain* 120:865.

9. Ristori, G., Montesperelli C., Buttinelli C., Battistini L., Cannoni S., Borsellino G., Bompreszi R., Perna A., Salvetti M. 1999. Non-myelin Antigen Autoreactivity in Multiple Sclerosis. In *From basic immunology to immune-mediated demyelination*, Vol. 1. G. Martino, Adorini, L., ed. Springer, New York, p. 162.
10. Bebo, B. F., Jr., K. Adlard, J. C. Schuster, L. Unsicker, A. A. Vandenbark, and H. Offner. 1999. Gender differences in protection from EAE induced by oral tolerance with a peptide analogue of MBP-Ac1-11. *J Neurosci Res* 55:432.
11. Adorini, L. 1999. Pathogenetic mechanisms of autoimmunity. In *From basic immunology to immune-mediated demyelination*, Vol. 1. G. A. Martino, L., ed. Springer, New York, p. 26.
12. Pette, M., K. Fujita, B. Kitze, J. N. Whitaker, E. Albert, L. Kappos, and H. Wekerle. 1990. Myelin basic protein-specific T lymphocyte lines from MS patients and healthy individuals. *Neurology* 40:1770.
13. Lovett-Racke, A. E., J. L. Trotter, J. Lauber, P. J. Perrin, C. H. June, and M. K. Racke. 1998. Decreased dependence of myelin basic protein-reactive T cells on CD28-mediated costimulation in multiple sclerosis patients. A marker of activated/memory T cells [published erratum appears in *J Clin Invest* 1998 Apr 1;101(7):1542]. *J Clin Invest* 101:725.
14. Acha-Orbea, H., D. J. Mitchell, L. Timmermann, D. C. Wraith, G. S. Tausch, M. K. Waldor, S. S. Zamvil, H. O. McDevitt, and L. Steinman. 1988. Limited heterogeneity of T cell receptors from lymphocytes mediating autoimmune encephalomyelitis allows specific immune intervention. *Cell* 54:263.
15. Ben-Nun, A., R. S. Liblau, L. Cohen, D. Lehmann, E. Tournier-Lasserre, A. Rosenzweig, J. W. Zhang, J. C. Raus, and M. A. Bach. 1991. Restricted T-cell receptor V beta gene usage by myelin basic protein-specific T-cell clones in multiple sclerosis: predominant genes vary in individuals. *Proc Natl Acad Sci U S A* 88:2466.
16. Martino, G., Furlan, R., Poliani, P. L. 1999. Inflammation and Multiple Sclerosis: a close interplay. In *From basic immunology to immune-mediated demyelination*, Vol. 1. G. A. Martino, L., ed. Springer, New York, p. 185.

17. Tsunoda, I., and R. S. Fujinami. 1996. Two models for multiple sclerosis: experimental allergic encephalomyelitis and Theiler's murine encephalomyelitis virus. *J Neuropathol Exp Neurol* 55:673.
18. Steinman, L. 1999. Assessment of animal models for MS and demyelinating disease in the design of rational therapy. *Neuron* 24:511.
19. Uccelli, A. 1999. Animal models of demyelination of the central nervous system. In *From basic immunology to immune-mediated demyelination.*, Vol. 1. G. A. Martino, L., ed. Springer, New York, p. 232.
20. Chou, Y. K., D. N. Bourdette, H. Offner, R. Whitham, R. Y. Wang, G. A. Hashim, and A. A. Vandenbark. 1992. Frequency of T cells specific for myelin basic protein and myelin proteolipid protein in blood and cerebrospinal fluid in multiple sclerosis. *J Neuroimmunol* 38:105.
21. Arnon, R. 1996. The development of Cop 1 (Copaxone), an innovative drug for the treatment of multiple sclerosis: personal reflections. *Immunol Lett* 50:1.
22. Panitch, H. S., R. L. Hirsch, J. Schindler, and K. P. Johnson. 1987. Treatment of multiple sclerosis with gamma interferon: exacerbations associated with activation of the immune system. *Neurology* 37:1097.
23. Baker, D., D. Butler, B. J. Scallon, J. K. O'Neill, J. L. Turk, and M. Feldmann. 1994. Control of established experimental allergic encephalomyelitis by inhibition of tumor necrosis factor (TNF) activity within the central nervous system using monoclonal antibodies and TNF receptor-immunoglobulin fusion proteins. *Eur J Immunol* 24:2040.
24. Klinkert, W. E., K. Kojima, W. Lesslauer, W. Rinner, H. Lassmann, and H. Wekerle. 1997. TNF-alpha receptor fusion protein prevents experimental auto-immune encephalomyelitis and demyelination in Lewis rats: an overview. *J Neuroimmunol* 72:163.
25. Thompson, A. J., and J. H. Noseworthy. 1996. New treatments for multiple sclerosis: a clinical perspective. *Curr Opin Neurol* 9:187.

26. Navikas, V., and H. Link. 1996. Review: cytokines and the pathogenesis of multiple sclerosis. *J Neurosci Res* 45:322.
27. Hafler, D. A. 1999. The distinction blurs between an autoimmune versus microbial hypothesis in multiple sclerosis [comment]. *J Clin Invest* 104:527.
28. Study, M. 1995. Interferon beta-1b in the treatment of multiple sclerosis: final outcome of the randomized controlled trial. The IFNB Multiple Sclerosis Study Group and The University of British Columbia MS/MRI Analysis Group [see comments]. *Neurology* 45:1277.
29. Karussis, D. M., Z. Meiner, D. Lehmann, J. M. Gormi, A. Schwarz, A. Linde, and O. Abramsky. 1996. Treatment of secondary progressive multiple sclerosis with the immunomodulator linomide: a double-blind, placebo-controlled pilot study with monthly magnetic resonance imaging evaluation. *Neurology* 47:341.
30. Duong, T. T., J. St. Louis, J. J. Gilbert, F. D. Finkelman, and G. H. Strejan. 1992. Effect of anti-interferon-gamma and anti-interleukin-2 monoclonal antibody treatment on the development of actively and passively induced experimental allergic encephalomyelitis in the SJL/J mouse. *J Neuroimmunol* 36:105.
31. Racke, M. K., S. Dhib-Jalbut, B. Cannella, P. S. Albert, C. S. Raine, and D. E. McFarlin. 1991. Prevention and treatment of chronic relapsing experimental allergic encephalomyelitis by transforming growth factor-beta 1. *J Immunol* 146:3012.
32. Racke, M. K., A. Bonomo, D. E. Scott, B. Cannella, A. Levine, C. S. Raine, E. M. Shevach, and M. Rocken. 1994. Cytokine-induced immune deviation as a therapy for inflammatory autoimmune disease. *J Exp Med* 180:1961.
33. Crisi, G. M., L. Santambrogio, G. M. Hochwald, S. R. Smith, J. A. Carlino, and G. J. Thorbecke. 1995. Staphylococcal enterotoxin B and tumor-necrosis factor-alpha-induced relapses of experimental allergic encephalomyelitis: protection by transforming growth factor-beta and interleukin-10. *Eur J Immunol* 25:3035.
34. Rice, G. P., B. Paszner, J. Oger, J. Lesaux, D. Paty, and G. Ebers. 1999. The evolution of neutralizing antibodies in multiple sclerosis patients treated with interferon beta-1b. *Neurology* 52:1277.

35. Genain, C. P., K. Abel, N. Belmar, F. Villinger, D. P. Rosenberg, C. Linington, C. S. Raine, and S. L. Hauser. 1996. Late complications of immune deviation therapy in a nonhuman primate [see comments]. *Science* 274:2054.
36. Woodroffe, M. N., and M. L. Cuzner. 1993. Cytokine mRNA expression in inflammatory multiple sclerosis lesions: detection by non-radioactive in situ hybridization. *Cytokine* 5:583.
37. Guery, J. C., and L. Adorini. 1993. Selective immunosuppression of class II-restricted T cells by MHC class II-binding peptides. *Crit Rev Immunol* 13:195.
38. Marino, M., A. Ippolito, and G. Fassina. 1999. Inhibition of experimental autoimmune encephalomyelitis in SJL mice by oral administration of retro-inverso derivative of encephalitogenic epitope P87-99. *Eur J Immunol* 29:2560.
39. Whitacre, C. C., I. E. Gienapp, A. Meyer, K. L. Cox, and N. Javed. 1996. Treatment of autoimmune disease by oral tolerance to autoantigens. *Clin Immunol Immunopathol* 80:S31.
40. Benson, J. M., S. S. Stuckman, K. L. Cox, R. M. Wardrop, I. E. Gienapp, A. H. Cross, J. L. Trotter, and C. C. Whitacre. 1999. Oral administration of myelin basic protein is superior to myelin in suppressing established relapsing experimental autoimmune encephalomyelitis. *J Immunol* 162:6247.
41. Weiner, H. L. 1997. Oral tolerance for the treatment of autoimmune diseases. *Annu Rev Med* 48:341.
42. Bai, X. F., F. D. Shi, B. G. Xiao, H. L. Li, P. H. van der Meide, and H. Link. 1997. Nasal administration of myelin basic protein prevents relapsing experimental autoimmune encephalomyelitis in DA rats by activating regulatory cells expressing IL-4 and TGF-beta mRNA. *J Neuroimmunol* 80:65.
43. Elliott, E. A., H. I. McFarland, S. H. Nye, R. Cofield, T. M. Wilson, J. A. Wilkins, S. P. Squinto, L. A. Matis, and J. P. Mueller. 1996. Treatment of experimental encephalomyelitis with a novel chimeric fusion protein of myelin basic protein and proteolipid protein. *J Clin Invest* 98:1602.

44. Gaur, A., B. Wiers, A. Liu, J. Rothbard, and C. G. Fathman. 1992. Amelioration of autoimmune encephalomyelitis by myelin basic protein synthetic peptide-induced anergy. *Science* 258:1491.
45. Smilek, D. E., D. C. Wraith, S. Hodgkinson, S. Dwivedy, L. Steinman, and H. O. McDevitt. 1991. A single amino acid change in a myelin basic protein peptide confers the capacity to prevent rather than induce experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci U S A* 88:9633.
46. Kappos, L., G. Comi, H. Panitch, J. Oger, J. Antel, P. Conlon, L. Steinman, A. Rae-Grant, J. Castaldo, N. Eckert, J. B. Guarnaccia, P. Mills, G. Johnson, P. A. Calabresi, C. Pozzilli, S. Bastianello, E. Giugni, T. Witjas, P. Cozzone, J. Pelletier, D. Pohlau, and H. Przuntek. 2000. Induction of a non-encephalitogenic type 2 T helper-cell autoimmune response in multiple sclerosis after administration of an altered peptide ligand in a placebo-controlled, randomized phase II trial [In Process Citation]. *Nat Med* 6:1176.
47. Bielekova, B., B. Goodwin, N. Richert, I. Cortese, T. Kondo, G. Afshar, B. Gran, J. Eaton, J. Antel, J. A. Frank, H. F. McFarland, and R. Martin. 2000. Encephalitogenic potential of the myelin basic protein peptide (amino acids 83-99) in multiple sclerosis: results of a phase II clinical trial with an altered peptide ligand [In Process Citation]. *Nat Med* 6:1167.
48. Crowe, P. D., Y. Qin, P. J. Conlon, and J. P. Antel. 2000. NBI-5788, an altered MBP83-99 peptide, induces a T-helper 2-like immune response in multiple sclerosis patients [In Process Citation]. *Ann Neurol* 48:758.
49. Teitelbaum, D., R. Arnon, and M. Sela. 1997. Copolymer 1: from basic research to clinical application. *Cell Mol Life Sci* 53:24.
50. Johnson, K. P., B. R. Brooks, J. A. Cohen, C. C. Ford, J. Goldstein, R. P. Lisak, L. W. Myers, H. S. Panitch, J. W. Rose, R. B. Schiffer, T. Vollmer, L. P. Weiner, and J. S. Wolinsky. 1998. Extended use of glatiramer acetate (Copaxone) is well tolerated and maintains its clinical effect on multiple sclerosis relapse rate and degree of disability. Copolymer 1 Multiple Sclerosis Study Group [see comments]. *Neurology* 50:701.
51. Aharoni, R., D. Teitelbaum, R. Arnon, and M. Sela. 1999. Copolymer 1 acts against the immunodominant epitope 82-100 of myelin basic protein by T cell receptor

antagonism in addition to major histocompatibility complex blocking. *Proc Natl Acad Sci U S A* 96:634.

52.Sela, M., and E. Zisman. 1997. Different roles of D-amino acids in immune phenomena. *Faseb J* 11:449.

53.Teitelbaum, D., R. Arnon, and M. Sela. 1999. Immunomodulation of experimental autoimmune encephalomyelitis by oral administration of copolymer 1 [see comments]. *Proc Natl Acad Sci U S A* 96:3842.

54.de Seze, J., G. Edan, M. Labalette, J. P. Dessaint, and P. Vermersch. 2000. Effect of glatiramer acetate (Copaxone) given orally in human patients: interleukin-10 production during a phase I trial [letter]. *Ann Neurol* 47:686.

55.Ben-Nun, A., H. Wekerle, and I. R. Cohen. 1981. Vaccination against autoimmune encephalomyelitis with T-lymphocyte line cells reactive against myelin basic protein. *Nature* 292:60.

56.Medaer, R., P. Stinissen, L. Truyen, J. Raus, and J. Zhang. 1995. Depletion of myelin-basic-protein autoreactive T cells by T-cell vaccination: pilot trial in multiple sclerosis. *Lancet* 346:807.

57.Correale, J., B. Lund, M. McMillan, D. Y. Ko, K. McCarthy, and L. P. Weiner. 2000. T cell vaccination in secondary progressive multiple sclerosis. *J Neuroimmunol* 107:130.

58.Zhang, J., and J. Raus. 1995. Clonal depletion of human myelin basic protein-reactive T-cells by T-cell vaccination. *Ann N Y Acad Sci* 756:323.

59.Merrill, J. E., and S. P. Murphy. 1997. Inflammatory events at the blood brain barrier: regulation of adhesion molecules, cytokines, and chemokines by reactive nitrogen and oxygen species. *Brain Behav Immun* 11:245.

60.Wang, J., and T. A. Springer. 1998. Structural specializations of immunoglobulin superfamily members for adhesion to integrins and viruses. *Immunol Rev* 163:197.

61. Shimizu, Y., G. A. van Seventer, K. J. Horgan, and S. Shaw. 1990. Roles of adhesion molecules in T-cell recognition: fundamental similarities between four integrins on resting human T cells (LFA-1, VLA-4, VLA-5, VLA-6) in expression, binding, and costimulation. *Immunol Rev* 114:109.
62. Yednock, T. A., C. Cannon, L. C. Fritz, F. Sanchez-Madrid, L. Steinman, and N. Karin. 1992. Prevention of experimental autoimmune encephalomyelitis by antibodies against alpha 4 beta 1 integrin. *Nature* 356:63.
63. Gordon, E. J., K. J. Myers, J. P. Dougherty, H. Rosen, and Y. Ron. 1995. Both anti-CD11a (LFA-1) and anti-CD11b (MAC-1) therapy delay the onset and diminish the severity of experimental autoimmune encephalomyelitis. *J Neuroimmunol* 62:153.
64. Tubridy, N., P. O. Behan, R. Capildeo, A. Chaudhuri, R. Forbes, C. P. Hawkins, R. A. Hughes, J. Palace, B. Sharrack, R. Swingler, C. Young, I. F. Moseley, D. G. MacManus, S. Donoghue, and D. H. Miller. 1999. The effect of anti-alpha4 integrin antibody on brain lesion activity in MS. The UK Antegren Study Group [see comments]. *Neurology* 53:466.
65. Lin, K. C., and A. C. Castro. 1998. Very late antigen 4 (VLA4) antagonists as anti-inflammatory agents. *Curr Opin Chem Biol* 2:453.
66. Archelos, J. J., S. Jung, M. Maurer, M. Schmied, H. Lassmann, T. Tamatani, M. Miyasaka, K. V. Toyka, and H. P. Hartung. 1993. Inhibition of experimental autoimmune encephalomyelitis by an antibody to the intercellular adhesion molecule ICAM-1. *Ann Neurol* 34:145.
67. Leger, O. J., T. A. Yednock, L. Tanner, H. C. Horner, D. K. Hines, S. Keen, J. Saldanha, S. T. Jones, L. C. Fritz, and M. M. Bendig. 1997. Humanization of a mouse antibody against human alpha-4 integrin: a potential therapeutic for the treatment of multiple sclerosis. *Hum Antibodies* 8:3.
68. Kanwar, J. R., R. K. Kanwar, D. Wang, and G. W. Krissansen. 2000. Prevention of a chronic progressive form of experimental autoimmune encephalomyelitis by an antibody against mucosal addressin cell adhesion molecule-1, given early in the course of disease progression.[In Process Citation]. *Immunol Cell Biol* 78:641.

69. DeVries, M. E., L. Ran, and D. J. Kelvin. 1999. On the edge: the physiological and pathophysiological role of chemokines during inflammatory and immunological responses. *Semin Immunol* 11:95.
70. Karpus, W. J., N. W. Lukacs, B. L. McRae, R. M. Strieter, S. L. Kunkel, and S. D. Miller. 1995. An important role for the chemokine macrophage inflammatory protein-1 alpha in the pathogenesis of the T cell-mediated autoimmune disease, experimental autoimmune encephalomyelitis. *J Immunol* 155:5003.
71. Moreau, T., A. Coles, M. Wing, J. Thorpe, D. Miller, I. Moseley, J. Issacs, G. Hale, D. Clayton, N. Scolding, H. Waldmann, and A. Compston. 1996. CAMPATH-1H in multiple sclerosis. *Mult Scler* 1:357.
72. Waldor, M. K., S. Sriram, R. Hardy, L. A. Herzenberg, L. Lanier, M. Lim, and L. Steinman. 1985. Reversal of experimental allergic encephalomyelitis with monoclonal antibody to a T-cell subset marker. *Science* 227:415.
73. Lindsey, J. W., S. Hodgkinson, R. Mehta, D. Mitchell, D. Enzmann, and L. Steinman. 1994. Repeated treatment with chimeric anti-CD4 antibody in multiple sclerosis. *Ann Neurol* 36:183.
74. van Oosten, B. W., M. Lai, F. Barkhof, D. H. Miller, I. F. Moseley, A. J. Thompson, S. Hodgkinson, and C. H. Polman. 1996. A phase II trial of anti-CD4 antibodies in the treatment of multiple sclerosis. *Mult Scler* 1:339.
75. Marini, J. C., B. A. Jameson, F. D. Lublin, and R. Korngold. 1996. A CD4-CDR3 peptide analog inhibits both primary and secondary autoreactive CD4+ T cell responses in experimental allergic encephalomyelitis. *J Immunol* 157:3706.
76. Lenschow, D. J., T. L. Walunas, and J. A. Bluestone. 1996. CD28/B7 system of T cell costimulation. *Annu Rev Immunol* 14:233.
77. Sperling, A. I., and J. A. Bluestone. 1996. The complexities of T-cell co-stimulation: CD28 and beyond. *Immunol Rev* 153:155.

78. Weinberg, A. D., K. W. Wegmann, C. Funatake, and R. H. Whitham. 1999. Blocking OX-40/OX-40 ligand interaction in vitro and in vivo leads to decreased T cell function and amelioration of experimental allergic encephalomyelitis. *J Immunol* 162:1818.
79. Grewal, I. S., and R. A. Flavell. 1998. CD40 and CD154 in cell-mediated immunity. *Annu Rev Immunol* 16:111.
80. Grewal, I. S., H. G. Foellmer, K. D. Grewal, J. Xu, F. Hardardottir, J. L. Baron, C. A. Janeway, Jr., and R. A. Flavell. 1996. Requirement for CD40 ligand in costimulation induction, T cell activation, and experimental allergic encephalomyelitis. *Science* 273:1864.
81. Howard, L. M., A. J. Miga, C. L. Vanderlugt, M. C. Dal Canto, J. D. Laman, R. J. Noelle, and S. D. Miller. 1999. Mechanisms of immunotherapeutic intervention by anti-CD40L (CD154) antibody in an animal model of multiple sclerosis. *J Clin Invest* 103:281.
82. Karandikar, N. J., C. L. Vanderlugt, J. A. Bluestone, and S. D. Miller. 1998. Targeting the B7/CD28:CTLA-4 costimulatory system in CNS autoimmune disease. *J Neuroimmunol* 89:10.
83. Illei, G. G., and P. E. Lipsky. 2000. Novel, non-antigen-specific therapeutic approaches to autoimmune/inflammatory diseases [In Process Citation]. *Curr Opin Immunol* 12:712.
84. Anderson, D. E., A. H. Sharpe, and D. A. Hafler. 1999. The B7-CD28/CTLA-4 costimulatory pathways in autoimmune disease of the central nervous system. *Curr Opin Immunol* 11:677.
85. Abbas, A. K., and A. H. Sharpe. 1999. T-cell stimulation: an abundance of B7s [news; comment]. *Nat Med* 5:1345.
86. Greene, J. L., G. M. Leytze, J. Emswiler, R. Peach, J. Bajorath, W. Cosand, and P. S. Linsley. 1996. Covalent dimerization of CD28/CTLA-4 and oligomerization of CD80/CD86 regulate T cell costimulatory interactions. *J Biol Chem* 271:26762.

- 87.Slavik, J. M., J. E. Hutchcroft, and B. E. Bierer. 1999. CD28/CTLA-4 and CD80/CD86 families: signaling and function. *Immunol Res* 19:1.
- 88.McAdam, A. J., A. N. Schweitzer, and A. H. Sharpe. 1998. The role of B7 co-stimulation in activation and differentiation of CD4+ and CD8+ T cells. *Immunol Rev* 165:231.
- 89.Cross, A. H., J. A. Lyons, M. San, R. M. Keeling, G. Ku, and M. K. Racke. 1999. T cells are the main cell type expressing B7-1 and B7-2 in the central nervous system during acute, relapsing and chronic experimental autoimmune encephalomyelitis. *Eur J Immunol* 29:3140.
- 90.Chambers, C. A., and J. P. Allison. 1999. Costimulatory regulation of T cell function. *Curr Opin Cell Biol* 11:203.
- 91.Boise, L. H., A. J. Minn, P. J. Noel, C. H. June, M. A. Accavitti, T. Lindsten, and C. B. Thompson. 1995. CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-XL. *Immunity* 3:87.
- 92.Van Gool, S. W., P. Vandenberghe, M. de Boer, and J. L. Ceuppens. 1996. CD80, CD86 and CD40 provide accessory signals in a multiple-step T-cell activation model. *Immunol Rev* 153:47.
- 93.Boussiotis, V. A., G. J. Freeman, J. G. Gribben, and L. M. Nadler. 1996. The role of B7-1/B7-2:CD28/CTLA-4 pathways in the prevention of anergy, induction of productive immunity and down-regulation of the immune response. *Immunol Rev* 153:5.
- 94.Ward, S. G. 1999. The complexities of CD28 and CTLA-4 signalling: PI3K and beyond. *Arch Immunol Ther Exp* 47:69.
- 95.Chambers, C. A., M. F. Krummel, B. Boitel, A. Hurwitz, T. J. Sullivan, S. Fournier, D. Cassell, M. Brunner, and J. P. Allison. 1996. The role of CTLA-4 in the regulation and initiation of T-cell responses. *Immunol Rev* 153:27.
- 96.Bluestone, J. A. 1997. Is CTLA-4 a master switch for peripheral T cell tolerance? *J Immunol* 158:1989.

- 97.Perkins, D., Z. Wang, C. Donovan, H. He, D. Mark, G. Guan, Y. Wang, T. Walunas, J. Bluestone, J. Listman, and et al. 1996. Regulation of CTLA-4 expression during T cell activation. *J Immunol* 156:4154.
- 98.Lee, K. M., E. Chuang, M. Griffin, R. Khattri, D. K. Hong, W. Zhang, D. Straus, L. E. Samelson, C. B. Thompson, and J. A. Bluestone. 1998. Molecular basis of T cell inactivation by CTLA-4. *Science* 282:2263.
- 99.Linsley, P. S., J. Ledbetter, R. Peach, and J. Bajorath. 1995. CD28/CTLA-4 receptor structure, binding stoichiometry and aggregation during T-cell activation. *Res Immunol* 146:130.
- 100.Lenschow, D. J., A. I. Sperling, M. P. Cooke, G. Freeman, L. Rhee, D. C. Decker, G. Gray, L. M. Nadler, C. C. Goodnow, and J. A. Bluestone. 1994. Differential up-regulation of the B7-1 and B7-2 costimulatory molecules after Ig receptor engagement by antigen. *J Immunol* 153:1990.
- 101.Slavik, J. M., J. E. Hutchcroft, and B. E. Bierer. 1999. CD80 and CD86 are not equivalent in their ability to induce the tyrosine phosphorylation of CD28. *J Biol Chem* 274:3116.
- 102.van der Merwe, P. A., D. L. Bodian, S. Daenke, P. Linsley, and S. J. Davis. 1997. CD80 (B7-1) binds both CD28 and CTLA-4 with a low affinity and very fast kinetics. *J Exp Med* 185:393.
103. Schweitzer, A. N., F. Borriello, R. C. Wong, A. K. Abbas, and A. H. Sharpe. 1997. Role of costimulators in T cell differentiation: studies using antigen-presenting cells lacking expression of CD80 or CD86. *J Immunol* 158:2713.
- 104.Schweitzer, A. N., and A. H. Sharpe. 1998. Studies using antigen-presenting cells lacking expression of both B7-1 (CD80) and B7-2 (CD86) show distinct requirements for B7 molecules during priming versus restimulation of Th2 but not Th1 cytokine production. *J Immunol* 161:2762.

105. Tivol, E. A., F. Borriello, A. N. Schweitzer, W. P. Lynch, J. A. Bluestone, and A. H. Sharpe. 1995. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* 3:541.
106. Waterhouse, P., J. M. Penninger, E. Timms, A. Wakeham, A. Shahinian, K. P. Lee, C. B. Thompson, H. Griesser, and T. W. Mak. 1995. Lymphoproliferative disorders with early lethality in mice deficient in *Ctla-4* [see comments]. *Science* 270:985.
107. Lucas, P. J., I. Negishi, K. Nakayama, L. E. Fields, and D. Y. Loh. 1995. Naive CD28-deficient T cells can initiate but not sustain an in vitro antigen-specific immune response. *J Immunol* 154:5757.
108. Ferguson, S. E., S. Han, G. Kelsoe, and C. B. Thompson. 1996. CD28 is required for germinal center formation. *J Immunol* 156:4576.
109. Sperling, A. I., J. A. Auger, B. D. Ehst, I. C. Rulifson, C. B. Thompson, and J. A. Bluestone. 1996. CD28/B7 interactions deliver a unique signal to naive T cells that regulates cell survival but not early proliferation. *J Immunol* 157:3909.
110. Herold, K. G., D. J. Lenschow, and J. A. Bluestone. 1997. CD28/B7 regulation of autoimmune diabetes. *Immunol Res* 16:71.
111. Finck, B. K., P. S. Linsley, and D. Wofsy. 1994. Treatment of murine lupus with CTLA4Ig. *Science* 265:1225.
112. Keane-Myers, A., W. C. Gause, P. S. Linsley, S. J. Chen, and M. Wills-Karp. 1997. B7-CD28/CTLA-4 costimulatory pathways are required for the development of T helper cell 2-mediated allergic airway responses to inhaled antigens. *J Immunol* 158:2042.
113. Reynolds, J., F. W. Tam, A. Chandraker, J. Smith, A. M. Karkar, J. Cross, R. Peach, M. H. Sayegh, and C. D. Pusey. 2000. CD28-B7 blockade prevents the development of experimental autoimmune glomerulonephritis. *J Clin Invest* 105:643.
114. Abrams, J. R., M. G. Lebowitz, C. A. Guzzo, B. V. Jegasothy, M. T. Goldfarb, B. S. Goffe, A. Menter, N. J. Lowe, G. Krueger, M. J. Brown, R. S. Weiner, M. J. Birkhofer, G. L. Warner, K. K. Berry, P. S. Linsley, J. G. Krueger, H. D. Ochs, S. L. Kelley, and S.

Kang. 1999. CTLA4Ig-mediated blockade of T-cell costimulation in patients with psoriasis vulgaris. *J Clin Invest* 103:1243.

115. Abrams, J. R., S. L. Kelley, E. Hayes, T. Kikuchi, M. J. Brown, S. Kang, M. G. Lebowitz, C. A. Guzzo, B. V. Jegasothy, P. S. Linsley, and J. G. Krueger. 2000. Blockade of T lymphocyte costimulation with cytotoxic T lymphocyte-associated antigen 4-immunoglobulin (CTLA4Ig) reverses the cellular pathology of psoriatic plaques, including the activation of keratinocytes, dendritic cells, and endothelial cells. *J Exp Med* 192:681.

116. Racke, M. K., R. B. Ratts, L. Arredondo, P. J. Perrin, and A. Lovett-Racke. 2000. The role of costimulation in autoimmune demyelination. *J Neuroimmunol* 107:205.

117. Oliveira-dos-Santos, A. J., A. Ho, Y. Tada, J. J. Lafaille, S. Tonegawa, T. W. Mak, and J. M. Penninger. 1999. CD28 costimulation is crucial for the development of spontaneous autoimmune encephalomyelitis. *J Immunol* 162:4490.

118. Chang, T. T., C. Jabs, R. A. Sobel, V. K. Kuchroo, and A. H. Sharpe. 1999. Studies in B7-deficient mice reveal a critical role for B7 costimulation in both induction and effector phases of experimental autoimmune encephalomyelitis. *J Exp Med* 190:733.

119. Girvin, A. M., M. C. Dal Canto, L. Rhee, B. Salomon, A. Sharpe, J. A. Bluestone, and S. D. Miller. 2000. A critical role for B7/CD28 costimulation in experimental autoimmune encephalomyelitis: a comparative study using costimulatory molecule-deficient mice and monoclonal antibody blockade. *J Immunol* 164:136.

120. Encinas, J. A., M. B. Lees, R. A. Sobel, C. Symonowicz, J. M. Greer, C. L. Shovlin, H. L. Weiner, C. E. Seidman, J. G. Seidman, and V. K. Kuchroo. 1996. Genetic analysis of susceptibility to experimental autoimmune encephalomyelitis in a cross between SJL/J and B10.S mice. *J Immunol* 157:2186.

121. Prabhu Das, M. R., S. S. Zamvil, F. Borriello, H. L. Weiner, A. H. Sharpe, and V. K. Kuchroo. 1995. Reciprocal expression of co-stimulatory molecules, B7-1 and B7-2, on murine T cells following activation. *Eur J Immunol* 25:207.

122. Issazadeh, S., V. Navikas, M. Schaub, M. Sayegh, and S. Khoury. 1998. Kinetics of expression of costimulatory molecules and their ligands in murine relapsing experimental autoimmune encephalomyelitis in vivo. *J Immunol* 161:1104.

123. Metzler, B., C. Burkhart, and D. C. Wraith. 1999. Phenotypic analysis of CTLA-4 and CD28 expression during transient peptide-induced T cell activation in vivo. *Int Immunol* 11:667.
124. Vanderlugt, C. L., W. S. Begolka, K. L. Neville, Y. Katz-Levy, L. M. Howard, T. N. Eagar, J. A. Bluestone, and S. D. Miller. 1998. The functional significance of epitope spreading and its regulation by co-stimulatory molecules. *Immunol Rev* 164:63.
125. Windhagen, A., J. Newcombe, F. Dangond, C. Strand, M. N. Woodroffe, M. L. Cuzner, and D. A. Hafler. 1995. Expression of costimulatory molecules B7-1 (CD80), B7-2 (CD86), and interleukin 12 cytokine in multiple sclerosis lesions. *J Exp Med* 182:1985.
126. Khoury, S. J., E. Akalin, A. Chandraker, L. A. Turka, P. S. Linsley, M. H. Sayegh, and W. W. Hancock. 1995. CD28-B7 costimulatory blockade by CTLA4Ig prevents actively induced experimental autoimmune encephalomyelitis and inhibits Th1 but spares Th2 cytokines in the central nervous system. *J Immunol* 155:4521.
127. Perrin, P. J., D. Scott, L. Quigley, P. S. Albert, O. Feder, G. S. Gray, R. Abe, C. H. June, and M. K. Racke. 1995. Role of B7:CD28/CTLA-4 in the induction of chronic relapsing experimental allergic encephalomyelitis. *J Immunol* 154:1481.
128. Hurwitz, A. A., T. J. Sullivan, M. F. Krummel, R. A. Sobel, and J. P. Allison. 1997. Specific blockade of CTLA-4/B7 interactions results in exacerbated clinical and histologic disease in an actively-induced model of experimental allergic encephalomyelitis. *J Neuroimmunol* 73:57.
129. Khoury, S. J., L. Gallon, R. R. Verburg, A. Chandraker, R. Peach, P. S. Linsley, L. A. Turka, W. W. Hancock, and M. H. Sayegh. 1996. Ex vivo treatment of antigen-presenting cells with CTLA4Ig and encephalitogenic peptide prevents experimental autoimmune encephalomyelitis in the Lewis rat. *J Immunol* 157:3700.
130. Perrin, P. J., D. Scott, C. H. June, and M. K. Racke. 1995. B7-mediated costimulation can either provoke or prevent clinical manifestations of experimental allergic encephalomyelitis. *Immunol Res* 14:189.

131. Kuchroo, V. K., M. P. Das, J. A. Brown, A. M. Ranger, S. S. Zamvil, R. A. Sobel, H. L. Weiner, N. Nabavi, and L. H. Glimcher. 1995. B7-1 and B7-2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways: application to autoimmune disease therapy. *Cell* 80:707.
132. Miller, S. D., C. L. Vanderlugt, D. J. Lenschow, J. G. Pope, N. J. Karandikar, M. C. Dal Canto, and J. A. Bluestone. 1995. Blockade of CD28/B7-1 interaction prevents epitope spreading and clinical relapses of murine EAE. *Immunity* 3:739.
133. Perrin, P. J., C. H. June, J. H. Maldonado, R. B. Ratts, and M. K. Racke. 1999. Blockade of CD28 during in vitro activation of encephalitogenic T cells or after disease onset ameliorates experimental autoimmune encephalomyelitis. *J Immunol* 163:1704.
134. Chorev, M., and M. Goodman. 1995. Recent developments in retro peptides and proteins--an ongoing topochemical exploration. *Trends Biotechnol* 13:438.
135. Cochran, A. G. 2000. Antagonists of protein-protein interactions. *Chem Biol* 7:R85.
136. Hruby, V. J. 1993. Conformational and topographical considerations in the design of biologically active peptides. *Biopolymers* 33:1073.
137. Clackson, T., and J. A. Wells. 1995. A hot spot of binding energy in a hormone-receptor interface. *Science* 267:383.
138. Livnah, O., E. A. Stura, D. L. Johnson, S. A. Middleton, L. S. Mulcahy, N. C. Wrighton, W. J. Dower, L. K. Jolliffe, and I. A. Wilson. 1996. Functional mimicry of a protein hormone by a peptide agonist: the EPO receptor complex at 2.8 Å [see comments]. *Science* 273:464.
139. Huang, Z., S. Li, and R. Korngold. 1997. Immunoglobulin superfamily proteins: structure, mechanisms, and drug discovery. *Biopolymers* 43:367.
140. Cunningham, B. C., and J. A. Wells. 1997. Minimized proteins. *Curr Opin Struct Biol* 7:457.

141. Chothia, C., and E. Y. Jones. 1997. The molecular structure of cell adhesion molecules. *Annu Rev Biochem* 66:823.
142. Williams, A. F., and A. N. Barclay. 1988. The immunoglobulin superfamily--domains for cell surface recognition. *Annu Rev Immunol* 6:381.
143. Skerra, A. 2000. Engineered protein scaffolds for molecular recognition. *J Mol Recognit* 13:167.
144. Imrich, H., C. Kugler, N. Torres-Nagel, R. Dorries, and T. Hunig. 1995. Prevention and treatment of Lewis rat experimental allergic encephalomyelitis with a monoclonal antibody to the T cell receptor V beta 8.2 segment. *Eur J Immunol* 25:1960.
145. Vandembark, A. A., G. Hashim, and H. Offner. 1989. Immunization with a synthetic T-cell receptor V-region peptide protects against experimental autoimmune encephalomyelitis. *Nature* 341:541.
146. Kotzin, B. L., S. Karuturi, Y. K. Chou, J. Lafferty, J. M. Forrester, M. Better, G. E. Nedwin, H. Offner, and A. A. Vandembark. 1991. Preferential T-cell receptor beta-chain variable gene use in myelin basic protein-reactive T-cell clones from patients with multiple sclerosis. *Proc Natl Acad Sci U S A* 88:9161.
147. Gold, D. P., R. A. Smith, A. B. Golding, E. E. Morgan, T. Dafashy, J. Nelson, L. Smith, J. Diveley, J. A. Laxer, S. P. Richieri, D. J. Carlo, S. W. Brostoff, and D. B. Wilson. 1997. Results of a phase I clinical trial of a T-cell receptor vaccine in patients with multiple sclerosis. II. Comparative analysis of TCR utilization in CSF T-cell populations before and after vaccination with a TCRV beta 6 CDR2 peptide. *J Neuroimmunol* 76:29.
148. Moreland, L. W., E. E. Morgan, T. C. Adamson, 3rd, Z. Fronek, L. H. Calabrese, J. M. Cash, J. A. Markenson, A. K. Matsumoto, J. Bathon, E. L. Matteson, K. M. Uramoto, C. M. Weyand, W. J. Koopman, L. W. Heck, V. Strand, J. P. Diveley, D. J. Carlo, C. J. Nardo, S. P. Richieri, and S. W. Brostoff. 1998. T cell receptor peptide vaccination in rheumatoid arthritis: a placebo- controlled trial using a combination of Vbeta3, Vbeta14, and Vbeta17 peptides [see comments]. *Arthritis Rheum* 41 :1919.

149. Liang, B., Z. Zhang, M. Araghiniknam, C. Eskelson, and R. R. Watson. 1997. Prevention of retrovirus-induced aberrant cytokine secretion, excessive lipid peroxidation, and tissue vitamin E deficiency by T cell receptor peptide treatments in C57BL/6 mice. *Proc Soc Exp Biol Med* 214:87.
150. Watson, R. R., J. Y. Wang, K. Dehghanpisheh, D. S. Huang, S. Wood, S. K. Ardestani, B. Liang, and J. J. Marchalonis. 1995. T cell receptor V beta complementarity-determining region 1 peptide administration moderates immune dysfunction and cytokine dysregulation induced by murine retrovirus infection. *J Immunol* 155:2282.
151. Kay, B. K., M. P. Williamson, and M. Sudol. 2000. The importance of being proline: the interaction of proline-rich motifs in signaling proteins with their cognate domains. *Faseb J* 14:231.
152. McDonnell, J. M., K. J. Blank, P. E. Rao, and B. A. Jameson. 1992. Direct involvement of the CDR3-like domain of CD4 in T helper cell activation. *J Immunol* 149:1626.
153. Friedman, T. M., A. P. Reddy, R. Wassell, B. A. Jameson, and R. Korngold. 1996. Identification of a human CD4-CDR3-like surface involved in CD4+ T cell function. *J Biol Chem* 271:22635.
154. Jameson, B. A., J. M. McDonnell, J. C. Marini, and R. Korngold. 1994. A rationally designed CD4 analogue inhibits experimental allergic encephalomyelitis [see comments]. *Nature* 368:744.
155. Satoh, T., J. M. Aramini, S. Li, T. M. Friedman, J. Gao, A. E. Edling, R. Townsend, U. Koch, S. Choksi, M. W. Germann, R. Korngold, and Z. Huang. 1997. Bioactive peptide design based on protein surface epitopes. A cyclic heptapeptide mimics CD4 domain 1 CC' loop and inhibits CD4 biological function. *J Biol Chem* 272:12175.
156. Chen, S., R. A. Chrusciel, H. Nakanishi, A. Raktabut, M. E. Johnson, A. Sato, D. Weiner, J. Hoxie, H. U. Saragovi, M. I. Greene, and et al. 1992. Design and synthesis of a CD4 beta-turn mimetic that inhibits human immunodeficiency virus envelope glycoprotein gp120 binding and infection of human lymphocytes. *Proc Natl Acad Sci U S A* 89:5872.

157. Vita, C., E. Drakopoulou, J. Vizzavona, S. Rochette, L. Martin, A. Menez, C. Roumestand, Y. S. Yang, L. Ylisastigui, A. Benjouad, and J. C. Gluckman. 1999. Rational engineering of a miniprotein that reproduces the core of the CD4 site interacting with HIV-1 envelope glycoprotein. *Proc Natl Acad Sci U S A* 96:13091.
158. Redwine, L. S., C. B. Pert, J. D. Rone, R. Nixon, M. Vance, B. Sandler, M. D. Lumpkin, D. J. Dieter, and M. R. Ruff. 1999. Peptide T blocks GP120/CCR5 chemokine receptor-mediated chemotaxis. *Clin Immunol* 93:124.
159. Choksi, S., B. A. Jameson, and R. Korngold. 1998. A structure-based approach to designing synthetic CD8alpha peptides that can inhibit cytotoxic T-lymphocyte responses. *Nat Med* 4:309.
160. Li, S., S. Choksi, S. Shan, X. Hu, J. Gao, R. Korngold, and Z. Huang. 1998. Identification of the CD8 DE loop as a surface functional epitope. Implications for major histocompatibility complex class I binding and CD8 inhibitor design. *J Biol Chem* 273:16442.
161. Gursoy, R. N., D. S. Jois, and T. J. Siahaan. 1999. Structural recognition of an ICAM-1 peptide by its receptor on the surface of T cells: conformational studies of cyclo (1, 12)-Pen-Pro-Arg- Gly-Gly-Ser-Val-Leu-Val-Thr-Gly-Cys-OH. *J Pept Res* 53:422.
162. Gursoy, R. N., and T. J. Siahaan. 1999. Binding and internalization of an ICAM-1 peptide by the surface receptors of T cells. *J Pept Res* 53:414.
163. Wang, J. H., R. B. Pepinsky, T. Stehle, J. H. Liu, M. Karpusas, B. Browning, and L. Osborn. 1995. The crystal structure of an N-terminal two-domain fragment of vascular cell adhesion molecule 1 (VCAM-1): a cyclic peptide based on the domain 1 C-D loop can inhibit VCAM-1-alpha 4 integrin interaction. *Proc Natl Acad Sci U S A* 92:5714.
164. Quan, C., N. J. Skelton, K. Clark, D. Y. Jackson, M. E. Renz, H. H. Chiu, S. M. Keating, M. H. Beresini, S. Fong, and D. R. Artis. 1998. Transfer of a protein binding epitope to a minimal designed peptide. *Biopolymers* 47:265.
165. Harper, K., C. Balzano, E. Rouvier, M. G. Mattei, M. F. Luciani, and P. Golstein. 1991. CTLA-4 and CD28 activated lymphocyte molecules are closely related in both

mouse and human as to sequence, message expression, gene structure, and chromosomal location. *J Immunol* 147:1037.

166.Aruffo, A., and B. Seed. 1987. Molecular cloning of a CD28 cDNA by a high-efficiency COS cell expression system. *Proc Natl Acad Sci U S A* 84:8573.

167.Truneh, A., M. Reddy, P. Ryan, S. D. Lyn, C. Eichman, D. Couez, M. R. Hurle, R. P. Sekaly, D. Olive, and R. Sweet. 1996. Differential recognition by CD28 of its cognate counter receptors CD80 (B7.1) and B70 (B7.2): analysis by site directed mutagenesis. *Mol Immunol* 33:321.

168.Bajorath, J., W. J. Metzler, and P. S. Linsley. 1997. Molecular modeling of CD28 and three-dimensional analysis of residue conservation in the CD28/CD152 family. *J Mol Graph Model* 15:135.

169.Peach, R. J., J. Bajorath, W. Brady, G. Leytze, J. Greene, J. Naemura, and P. S. Linsley. 1994. Complementarity determining region 1 (CDR1)- and CDR3-analogous regions in CTLA-4 and CD28 determine the binding to B7-1. *J Exp Med* 180:2049.

170.Chorev, M., and M. Goodman. 1993. A dozen years of retro-inverso peptidomimetics. *Acc. Chem. Res* 26:266.

171.Goodman, M., and M. Chorev. 1979. On the concept of linear modified retro-peptide structures. *Accounts in chemical research* 12:1.

172.Lairmore, M. D., A. M. DiGeorge, S. F. Conrad, A. V. Trevino, R. B. Lal, and P. T. Kaumaya. 1995. Human T-lymphotropic virus type 1 peptides in chimeric and multivalent constructs with promiscuous T-cell epitopes enhance immunogenicity and overcome genetic restriction. *J Virol* 69:6077.

173.Johnsson, B., S. Lofas, and G. Lindquist. 1991. Immobilization of proteins to a carboxymethyl-dextran-modified gold surface for biospecific interaction analysis in surface plasmon resonance sensors. *Anal Biochem* 198:268.

- 174.Karlsson, R. 1994. Real-time competitive kinetic analysis of interactions between low- molecular-weight ligands in solution and surface-immobilized receptors. *Anal Biochem* 221:142.
- 175.Karlsson, R., L. Jendeberg, B. Nilsson, J. Nilsson, and P. A. Nygren. 1995. Direct and competitive kinetic analysis of the interaction between human IgG1 and a one domain analogue of protein A. *J Immunol Methods* 183:43.
176. Wardrop, R. M., 3rd, and C. C. Whitacre. 1999. Oral tolerance in the treatment of inflammatory autoimmune diseases. *Inflamm Res* 48:106.
- 177.Inaba, M., K. Kurasawa, M. Mamura, K. Kumano, Y. Saito, and I. Iwamoto. 1999. Primed T cells are more resistant to Fas-mediated activation-induced cell death than naive T cells. *J Immunol* 163:1315.
- 178.Ellis, J. H., M. N. Burden, D. V. Vinogradov, C. Linge, and J. S. Crowe. 1996. Interactions of CD80 and CD86 with CD28 and CTLA4. *J Immunol* 156:2700.
- 179.Conte, L. L., C. Chothia, and J. Janin. 1999. The atomic structure of protein-protein recognition sites. *J Mol Biol* 285:2177.
- 180.Creamer, T. P. 1998. Left-handed polyproline II helix formation is (very) locally driven. *Proteins* 33:218.
- 181.Stapley, B. J., and T. P. Creamer. 1999. A survey of left-handed polyproline II helices. *Protein Sci* 8:587.
- 182.Adzhubei, A. A., and M. J. Sternberg. 1993. Left-handed polyproline II helices commonly occur in globular proteins. *J Mol Biol* 229:472.
- 183.Jones, E. Y., S. J. Davis, A. F. Williams, K. Harlos, and D. I. Stuart. 1992. Crystal structure at 2.8 Å resolution of a soluble form of the cell adhesion molecule CD2. *Nature* 360:232.

184. Weng, Z., and C. DeLisi. 1998. Toward a predictive understanding of molecular recognition. *Immunol Rev* 163:251.
185. Petrella, E. C., L. M. Machesky, D. A. Kaiser, and T. D. Pollard. 1996. Structural requirements and thermodynamics of the interaction of proline peptides with profilin. *Biochemistry* 35:16535.
186. Harding, F. A., J. G. McArthur, J. A. Gross, D. H. Raulet, and J. P. Allison. 1992. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature* 356:607.
187. Walunas, T. L., A. I. Sperling, R. Khattri, C. B. Thompson, and J. A. Bluestone. 1996. CD28 expression is not essential for positive and negative selection of thymocytes or peripheral T cell tolerance. *J Immunol* 156:1006.
188. Yashiro, Y., X. G. Tai, K. Toyo-oka, C. S. Park, R. Abe, T. Hamaoka, M. Kobayashi, S. Neben, and H. Fujiwara. 1998. A fundamental difference in the capacity to induce proliferation of naive T cells between CD28 and other co-stimulatory molecules. *Eur J Immunol* 28:926.
189. Collette, Y., A. Benziene, D. Razanajaona, and D. Olive. 1998. Distinct regulation of T-cell death by CD28 depending on both its aggregation and T-cell receptor triggering: a role for Fas-FasL. *Blood* 92:1350.
190. Croxford, J. L., J. K. O'Neill, R. R. Ali, K. Browne, A. P. Byrnes, M. J. Dallman, M. J. Wood, M. Fedlmann, and D. Baker. 1998. Local gene therapy with CTLA4-immunoglobulin fusion protein in experimental allergic encephalomyelitis. *Eur J Immunol* 28:3904.
191. Van Parijs, L., A. Ibraghimov, and A. K. Abbas. 1996. The roles of costimulation and Fas in T cell apoptosis and peripheral tolerance. *Immunity* 4:321.
192. Kearney, E. R., T. L. Walunas, R. W. Karr, P. A. Morton, D. Y. Loh, J. A. Bluestone, and M. K. Jenkins. 1995. Antigen-dependent clonal expansion of a trace population of antigen-specific CD4⁺ T cells in vivo is dependent on CD28 costimulation and inhibited by CTLA-4. *J Immunol* 155:1032.