2009

Influencing T Helper Cell Bias in Autoimmunity

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INFLUENCING T HELPER CELL BIAS IN AUTOIMMUNITY

by

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A dissertation submitted to the faculty of the Medical School of South Carolina in partial fulfillment of the requirement for the degree of Doctor of Philosophy in the College of Graduate Studies.

Department of Molecular and Cellular Biology and Pathobiology

2009

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ACKNOWLEDGEMENTS

My success as a graduate and medical student at the Medical University of South Carolina would not be possible without the support of so many other people in my life. I would like to thank the members of the Banik and Beeson lab, who have helped me immensely develop my skills as a researcher and scientist. The Banik lab: Arabinda (your amazing skills as an experimenter), Kelly (hours of assistance with manuscripts and data), Denise (for keeping it all flowing and making everyone play nice), Supriti (your attention to detail), Amena (your help with the critters) and the many summer students. The Beeson Lab: Gyda (for keeping it all flowing and making everyone play nice), Jason (for your guidance in all things MD/PhD), Ryan (for all your help with chemistry and being a good friend, you helped put things in perspective all too soon), Renee (for your training and protocols), Josh (for getting the APLs going), Sean (for making the lab a fun place to be), and most recently Nicole (for taking over this project, good luck). Dr. Banik and Dr. Beeson; thank you for your guidance and wisdom and for allowing me the freedom to combine these projects in your labs. For the support from the MD/PhD program that makes this process all possible. For the support my family; my parents and in-laws for not thinking this was crazy.

Most importantly, my loving wife Jennifer. You have given me your support and graciously allowed me to toil in the lab for so many years. This process would never have been the same without you and I count myself blessed that you came along for the journey.

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ABBREVIATION

ALS	Amyotrophic Lateral Sclerosis
APC	Antigen Presenting Cell
APL	Altered Peptide Ligand
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium ion
CCL2	Chemokine Ligand 2 (formally MCP-1)
CFA	Complete Freund's Adjuvant
CMV	Cytomegalovirus
CNS	Central Nervous System
CSF	Cerebral Spinal Fluid
DMSO	Dimethylsulfoxide
EAE	Experimental Autoimmune Encephalomyelitis
FBS	Fetal Bovine Serum
GATA	"GATA" binding Transcription Factor
HLA	Human Leukocyte Adhesion
i.p.	Intraperitoneal
IL	Interleukin
INF	Interferon
LN	Lymph Node
MAG	Myelin-associated Glycoprotein
MBP	Myelin Basic Protein
MHC	Major Histocompatibility Complex
MOG	Myelin Oligodendrocyte Protein
MS	Multiple Sclerosis
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PLP	Proteolipid Protein
PML	Progressive Multifocal Leukoencephalopathy
PPMS	Primary Progressive Multiple Sclerosis
PRMS	Primary Relapsing Multiple Sclerosis
RA	Rheumatoid Arthritis
RRMS	Relapsing Remitting Multiple Sclerosis
s.c.	Subcutaneous
SEM	Standard Error of the Mean
SPMS	Secondary Progressive Multiple Sclerosis
STAT	Signal Transducer and Activator of Transcription
T-bet	T Box Transcription Factor
TCR	T cell Receptor
TGF	Transforming Growth Factor
Th	T helper Cell
TNF	Tumor Necrosis Factor
Treg	Regulatory T Cell
VCAM	Vascular Cell Adhesion Molecule

ABSTRACT

JONATHAN TY BUTLER. Influencing T Helper Cell Bias in Autoimmunity. (Under the direction of NAREN L. BANIK AND CRAIG C. BEESON). Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) that affects approximately 400,000 people in the United States every year and results in neurological deficits. Inflammatory events associated with MS include activation of auto-reactive T cells and migration of these and other immune cells into the CNS, leading to a coordinated attack upon oligodendrocytes and demyelination. Most current therapies only treat the symptoms of disease, not the cause, which is still in large part unknown. Therefore, the identification of the etiology of this complex disease and the development of new therapies is of great importance.

Targeting these T cells by several mechanisms may prove a valuable strategy for addressing the complex nature of MS. Improvement in clinical signs has occurred in experimental autoimmune encephalomyelitis (EAE), the animal model of MS, with the use altered peptide ligands (APLs) as well as calpain inhibitors. APLs produced hypersensitivity reactions in clinical trials at high doses so the hypothesis was formulated that altering these APLs with non-natural amino acids will improve bioavailability. Calpain is a calcium-mediated neutral protease involved in many normal physiological as well as pathological events. We hypothesize that calpain plays a role in several cellular processes involving T cells including migration as well as subtype bias. Three specific aims were designed to test these hypotheses. First, that aza-amino acid incorporation into APLs would increase protease resistance while preserving antigen

recognition. Next, that calpain is involved with T cell migration, specifically chemotaxis, as well as, T helper cell bias, by negatively regulating Th2 type cells. Finally, the combination of APL and calpain inhibitor treatment in an EAE model will result in a therapy that is more effective at reducing clinical signs at lower concentrations than either treatment alone. The data obtained from these experiments indicated: (1) aza-amino acids increase APL protease resistance, (2) calpain is involved with T cell chemotaxis, (3) calpain inhibition results in Th2 proliferation and potentiation and (4) combined calpain inhibitor/APL treatment is effective at reducing clinical signs of EAE; thus suggesting, that combined therapy is a viable strategy for developing MS treatments.

CHAPTER ONE

LITERATURE REVIEW AND SIGNIFICANCE

SIGNIFICANCE

Autoimmunity is a consequence of the inappropriate activation of the host immune system that results in a disease state caused by the body's own defense mechanisms. Autoimmune conditions are the third most costly diseases to treat in industrial nations of the world, behind only heart disease and cancer. The prevalence of autoimmunity is difficult to estimate due to the number and variety of conditions that the category encompasses; in Denmark alone, a country with a health registry, it is estimated that upwards of 5% of the population suffers from some disease burden as a result of an autoimmune condition.

One of these devastating diseases, thought to result from autoimmune processes, is multiple sclerosis (MS). The tragedy of MS is that it is a disease of younger individuals typically diagnosed between the ages of 20 and 50 and affecting upwards of 2.5 million individuals worldwide with about 400,000 cases in the USA. There are approximately 200 new cases diagnosed in the United States every week. Estimates suggest that MS results in $9.5 billion in medical costs and lost productivity each year.

Much has been discovered about MS since it was first identified 150 years ago, yet it remains a disease of unknowns. The exact cause of the disease, the initiating factors, who is susceptible and why the disease progresses are all areas of active investigation. Much has been learned about the immune system and central nervous system (CNS) by studying this disease but there is still a long way to go before MS is fully understood and an effective treatment is discovered that truly treats the disease's cause and symptoms.

Continued studies of the immune system and the mechanisms involved in initiation and continuation of disease is therefore required. The complexity and heterogeneity of MS itself allows for many potential areas of study including the cells that appear to be involved with pathology, the initiation of autoimmune inflammation, the resolution of disease symptoms, and the initiation and propagation of repair in damaged tissue. MS has been studied extensively in a collection of animal models called experimental autoimmune encephalomyelitis (EAE). The model is not a completely accurate recapitulation of the disease course of MS but it does enable focused study of the various components of the disease so long as the proper animal model is used and the question is properly restricted. The initiation of the disease is an important area of study. Thus far treatments based on the target antigens of the autoreactive immune cells have had mixed results and warrant improvement. One of the main targets is myelin basic protein (MBP) and therapies based upon its modification to initiate tolerance or alter the immune response have shown promise, though have yet to yield any approved therapies. The complex nature of the disease is still being uncovered but the calcium (Ca^{2+}) activated neutral protease calpain is thought to be involved with disease pathology as a degenerative protease as well as being involved in signaling and cellular mechanisms related to T cell activation and migration. The focus of this dissertation is to explore the pathogenesis of MS and EAE based on the following specific aims:

AIM 1: To examine the hypothesis that incorporating aza-amino acids into altered peptide ligands (APLs) of MBP will increase their protease resistance while still maintaining bioactivity.

AIM 2: To test the hypothesis that calpain contributes to disease pathology through its role in

A: Chemotaxis and Chemokinesis;

B: Regulating Th bias through proteolytic regulation of the STAT signaling pathways.

AIM 3: To examine the hypothesis that combined APL and calpain inhibitor treatment will be more effective at blocking experimental autoimmune encephalomyelitis than either treatment alone.

The exploration of these specific aims will add to the knowledge of MS and EAE by:

RESULT 1: Proving a possible peptide alternative that will aid in the development of improved therapeutics.

RESULT 2: Elucidating and demonstrating some of the mechanisms by which calpain inhibitors may be influencing T cells and how that relates to the observed therapeutic effect in EAE.

RESULT 3: Demonstrating that multi-compound therapeutic treatment targeting several pathogenic pathways is more effective at lower concentration than single compound therapy alone.

LITERATURE REVIEW

Autoimmunity

The mammalian immune system is composed of many organs, cell types, proteins and effector molecules working in concert to maintain the homeostasis and functionality of the entire organism. In order to effectively counteract foreign invaders and disease states the cells have to recognize and respond to a variety of challenges including microbial and viral proteins, parasites and other non-native antigens and mutated self molecules that lead to cancer. The immune system must accomplish this while being able to recognize itself and avoid triggering autoimmune reactions. The innate immune system (neutrophils, macrophages, dendritic cells) normally activates by pattern recognition through Toll-like receptors (Kawai and Akira 2009). The adaptive immune system (T cells and B cells) consists of cells that have randomly arranged receptors that must first pass self tolerance tests during development before being released into the vasculature (Hengartner et al. 1988; Kappler et al. 1987). Unfortunately, sometimes these self-recognizing cells escape, potentially leading to autoimmunity. During autoimmune responses multiple components of the immune system interact to target the host cells and systems. The innate immune system can initiate a response that will favor priming of the adaptive immune system to spread and carry forward an expanding self-directed response. Vice versa, the adaptive immune system can escape self tolerance by deletion or regulation and recognize its specific self-antigen and inappropriately initiate an

immune response using the innate immune system to reinforce that response (Lang et al. 2007).

Autoimmunity is a topic as broad as the number of components that make up a functioning immune system. This dissertation and literature review will focus on the autoimmune aspect of the disease of MS and its animal model EAE.

Multiple Sclerosis (MS)

Multiple Sclerosis (MS) is a heterogeneous autoimmune disease of unknown origin that affects about 400,000 people in the United States (Anderson et al. 1992). The disease derives its name from the sclerotic plaques observed in the CNS (Charcot 1868) that are the result of inflammatory responses and the hallmark demyelinated lesions. The disease typically follows one of at least four potential clinical courses. The most common form, affecting about 85% of the patient population, is a relapsing-remitting course (RRMS) that is characterized by attacks of neurological deficit followed by periods without symptoms or less severe symptoms. The majority of these patients eventually develop secondary progressive MS (SPMS) in which the neurological deficits will progress without improvement. Primary progressive MS (PPMS) affects about 15% of patients and results in a progressive neurological decline from onset of symptoms without relapses. Progressive relapsing MS (PRMS) features progression from onset with relapses later in the disease course (Keegan and Noseworthy 2002). MS is also classified by the clinical severity adding to the complexity of the classification of multiple sclerosis subtypes. These include benign MS,

diagnosed retrospectively, in which patients remain fully neurologically functional for 15 years after diagnosis (Rovaris et al. 2009) as well as malignant MS, or the Marburg variant, which is a rare but particularly aggressive form of the disease in which patients demonstrate a rapidly acute clinical course (Poser and Brinar 2004). Other variants have been identified based on various clinical manifestations and may include alternative immune mechanisms including Schilder's 1912 diffuse sclerosis, Balo's disease, Devic's disease and Bickerstaff's encephalitis (Poser and Brinar 2004).

The disease can also be classified by the pathology in the CNS lesions observed from biopsy or autopsy. In pattern I the inflammatory response is mostly T cells and macrophages. Pattern II is characterized by B cells and autoantibodies. Pattern III is thought to result from a primary oligodendrocytes dystrophy. Finally, pattern IV demonstrates considerable oligodendrocyte loss and is also suggestive of an oligodendrocyte pathology (Lucchinetti et al. 2000). Most of these patterns share a T cell and macrophage inflammatory response but the ultimate mechanism for injury seems to vary between individuals reinforcing the heterogeneity of MS. The pathogenesis and resulting paralysis observed in MS is thought to result from an inflammatory attack on the myelin sheath resulting in demyelinated axons and a loss of nervous conduction, axonal transection and eventually neuronal death. Continued immune insults and a failure of proper remyelination by the oligodendrocytes precursor cells leads to progressive symptoms of MS (Franklin and Ffrench-Constant 2008).

The etiology of the disease is still unknown but there are thought to be environmental components that favor autoimmune initiation in genetically susceptible individuals to initiate a MS clinical pathology. The geographical distribution of higher incidence in higher latitudes and temperate climates points to several possible etiologies including vitamin D levels (Ascherio and Munger 2007) and environmental pathogens (Lipton et al. 2007). No single genetic trait or mutation identified in MS patients that differs from non-diseased individuals has been identified; instead susceptibility appears to result from a multi-gene disease profile. The human leukocyte adhesion (HLA) domain is the strongest susceptibility locus for genetic predisposition to MS. However, a number of other common genes that have low odds ratios, but in concert may contribute to a susceptible genetic profile, have also been identified (The International Multiple Sclerosis Genetics 2007). The important genetic components consist of the cytokine domains and receptors IL-7/IL-7R and IL-2/IL-2R (Maier et al. 2009) as well as the CD58 (De Jager et al. 2009) genes that encode the LFA-3 co-stimulatory molecule important for enhanced Treg function which may play a role in immune response.

The initiation of the disease and the profile of susceptible individuals is still under active investigation but the pathological mechanisms of the disease are somewhat better characterized. The involvement of autoimmune myelin specific T cells is fairly well accepted (McFarland and Martin 2007). The mechanism by which these cells become primed and activated is again somewhat open to conjecture including genetic susceptibility in individuals with the disease, antigen

mimicry by an environmental pathogen of myelin protein (Lipton et al. 2007; Steiner and Sriram 2007) and the nature of the myelin antigen itself. Once the myelin specific T cells are primed and activated they enter the CNS and initiate and contribute to an autoimmune assault upon the myelin sheath ultimately resulting in demyelination. Autoreactive T cells appear to be key in the pathogenesis but there is also involvement of the innate immune system (O'Brien et al. 2008) as well as the B cell branch of the adaptive immune response and the complement system (Nikbin et al. 2007).

Most of the current approved therapies for MS attempt to decrease the inflammatory state (corticosteroid therapy, interferon-β) or target the T cells by decreasing their numbers (cyclophosphamide, mitoxantrone), modifying the T cell phenotype somehow (glatiramer acetate), or denying the cells entry to the CNS (natalizumab) (Wiendl et al. 2008). Glatiramer acetate is the only approved therapy that is thought to result in immune modulation by mimicking a variety of myelin antigens but the precise mechanism of action is still unknown (Schrempf and Ziemssen 2007). Improved targeting of therapeutics, reduction of side effects and development of therapeutics that treat the disease itself instead of just the symptoms are all desired goals. The complexity and heterogeneity of MS, the unknown etiology and the shortcoming involved with the current treatments all warrant further investigation. No spontaneous animal models exist for MS, hence making it difficult to study in a true recapitulation of the disease state, however one of the main tools utilized to study MS is the animal model of EAE. This induced model of immune attack upon the CNS has shed valuable

insight into the complex interaction of the CNS and the immune system as well as leading to several of the current approved therapies (glatiramer acetate, mitoxantrone, natalizumab) available for the treatment of MS (Steinman and Zamvil 2006).

Experimental Autoimmune Encephalomyelitis (EAE)

Probably the best animal model of MS is EAE even though it has shortcomings in accurately modeling human disease. The long history of this model began in the 1930s with monkeys in order to study vaccine associated paralysis (Rivers et al. 1933). Since its discovery, EAE has been extensively studied and has been demonstrated in various animals including marmosets, guinea pigs, primates, rats, mice, hamsters, dogs, sheep and rabbits (Baxter 2007). There are two methods to recapitulate the demyelinating pathology observed in MS; active EAE or passive EAE. The active model involves immunizing an animal with one of the following: brain or spinal cord homogenate, purified myelin, myelin proteins and most recently the antigenic sequence of the myelin proteins homogenized with complete Freud's adjuvant and mycobacterium (Gold et al. 2000). The mycobacterium acts as an adjuvant though multiple toll like receptors and plays a role in priming autoreactive T cells (Hansen et al. 2006) adding to the notion of the importance of the innate immune system's recognition of foreign agents in the pathogenesis of EAE and potentially MS. A pro-inflammatory immune response is then increased by injecting the animal with pertussis toxin several times (Munoz and Sewell 1984). The role of

pertussis in the model has been elucidated to include the initiation of the inflammatory cytokines IL-6, TGFβ, and IL-17 which prime the immune reaction toward an inflammatory bias (Gijbels et al. 1990; Hofstetter et al. 2007) as well as permeabilize the blood brain barrier (Linthicum et al. 1982).

The passive model of EAE involves the immunization of a syngenic donor animal and then the removal of antigenic cells, typically T cells. The cells are restimulated with antigen and adoptively transferred into a recipient animal. The recipient animal will demonstrate the clinical signs of EAE if the transferred cells are indeed pathogenic and involved with the disease process. This technique helped identify the role that T helper cells specific to myelin proteins play in the disease course (Ben-Nun et al. 1981). Depending on the species and genetic strain of the animals used, clinical signs develop after 9-20 days and involve spinal cord, brain, optic nerve or all of the CNS (Baxter 2007).

More recently it has been possible to identify EAE models that favor brain pathology. This pathology seems to be dependent upon the type of T cell causing the pathology (Abromson-Leeman et al. 2004) and the ratio of subtypes of T cells present (Stromnes et al. 2008). There have been many different EAE models to generate the various profiles of MS including acute monophasic models that only display the signs once and then are resistant to disease (Swanborg 2001b), relapsing remitting models that follow a similar course to RRMS (Brown and McFarlin 1981) and chronic models that display signs that peak and resolve somewhat but never return to a baseline level of no disease (Zamvil et al. 1985). The focus of EAE work has been with T cells due to demonstrated ability to

transfer EAE pathology, but it is important to recognize that autoantibodies also play a role in the pathology of MS since anti-myelin antibodies have been shown to exacerbate demyelination in EAE (Schluesener et al. 1987). The role of B cells may be restricted to antigen presentation to T cells to initiate disease and not initiate disease themselves with autoantibodies (Bettelli et al. 2006a; Krishnamoorthy et al. 2006; Ransohoff 2006). Needless to say, understanding MS is difficult when the animal model is not entirely understood and none of the identified EAE models perfectly recapitulates MS. Still, various questions can be addressed depending on the model that is chosen and which component of the disease is focused upon (Steinman and Zamvil 2006). In fact, variations on the model are under active development in order to accurately study the various parameters of MS. These models will allow for more relevant translation including the necessary pathogenic cells involved for localization (Stromnes et al. 2008) and the location of the lesions resulting from restricted inflammation (Zhao et al. 2008). T cells are still regarded as necessary for the initiation of the autoimmune response. Therefore no matter how autoimmunity begins or progresses, the study of the T cell is a focal point worth investigating.

T CELLS

Development and Subtypes

T cells are leukocytes that develop in the thymus and serve as an adaptive component of the immune system. These cells develop into a variety of

subtypes depending on the surface molecules that they display and the intended immune challenge that they will encounter. The first division is the associated molecules that aid the T cell receptor (TCR) and divide the cells into $CD8^+$ cytotoxic T cells and $CD4^+$ T helper cells. The $CD8^+$ cytotoxic T cells recognize antigen presented on major histocompatibility complex (MHC) class I molecules. The MHC class I molecules are present on all cells in the body, and allow the $CD8^+$ T cells to target intracellular pathogens. The $CD4^+$ T helper (Th) cells recognize antigen presented on MHC class II molecules. The MHC class II molecules are only present on antigen presenting cells (APCs) which allows the $CD4^+$ T cells to target extracellular pathogens by assisting other immune cells such as B cells and macrophages. Th cells also assist in innate immune responses through cytokine secretion and serve as memory cells to preserve the immune history and prepare for future challenges (Janeway et al. 1997). $CD4^+$ Th cells are further subdivided by the cytokines they secrete and role they play in the adaptive immune response and include Th1, Th2, Th17 and regulatory T (Treg) cells (Zhu and Paul 2008). The Th1 and Th2 subtypes were first identified by the cytokine profiles they produced when stimulated, their surface molecule pattern and their helper function (Mosmann et al. 1986). Subsequently it has been discovered that Th1 cells utilize interferon (IFN)-γ to signal though the signal transducer and activator of transcription (STAT) 1 (Lighvani et al. 2001) and induce the transcription factor T-bet (Szabo et al. 2000) during differentiation of the subtype. Following differentiation the Th1 response is amplified through interleukin (IL)-12 signaling which utilizes STAT4 to direct IFN-γ production and

increase inflammation without the TCR, and independent of the antigen (Thierfelder et al. 1996). The Th1 subtype favors inflammation and assists macrophages and other inflammatory cells. The Th2 subtype was found to be dependent upon IL-4 signaling though STAT6 (Zhu et al. 2001) and the transcription factor GATA-3 (Zheng and Flavell 1997). This pathway is required for differentiation and increases IL-4 production necessary for autocrine signaling. The Th2 subtype assists in B cell production of antibodies and also secretes cytokines that counteract the inflammatory subtypes (Mosmann et al. 2005). Recently several more distinct subtypes have been identified of which Th17 cells play a role in inflammation and autoimmunity once associated with Th1 subtypes (Harrington et al. 2005). The Th17 cell is differentiated by stimulation of TCR in the presence of IL-6 and TGFβ (Bettelli et al. 2006b; Mangan et al. 2006; Veldhoen et al. 2006) leading to production of IL-23 which acts through STAT3 (Mathur et al. 2007) to activate the transcription factor RORγt (Ivanov et al. 2006). Th17 cells remain a very active area of research. The other most recently identified subtype is the inducible Treg cells (iTreg) which serves to regulate the immune response by suppressing the immune system once the challenge has passed (Sakaguchi 2004). The iTreg cells utilize TGFβ and IL-2 to drive STAT5 (Yao et al. 2007) to activate the transcription factor Foxp3 (Fontenot et al. 2003) which reinforces the profile. Figure 1 summarizes the various subtypes by immune role, hallmark cytokine and transcription factors. During development, these cells are screened in the thymus against recognizing self antigen too strongly in order to prevent

autoimmune reactions (Kappler et al. 1987). This mechanism does not completely eliminate self-reactive T cells, which are found in both normal individuals as well as patients with autoimmunity (Van Parijs and Abbas 1998). When the cells do not properly gain self-tolerance the role of preventing autoimmunity falls to the iTreg cell (Sakaguchi 2004). The various subtypes play different roles in MS and EAE. While the complete picture is not yet understood, it is beneficial to review what is known at this point.

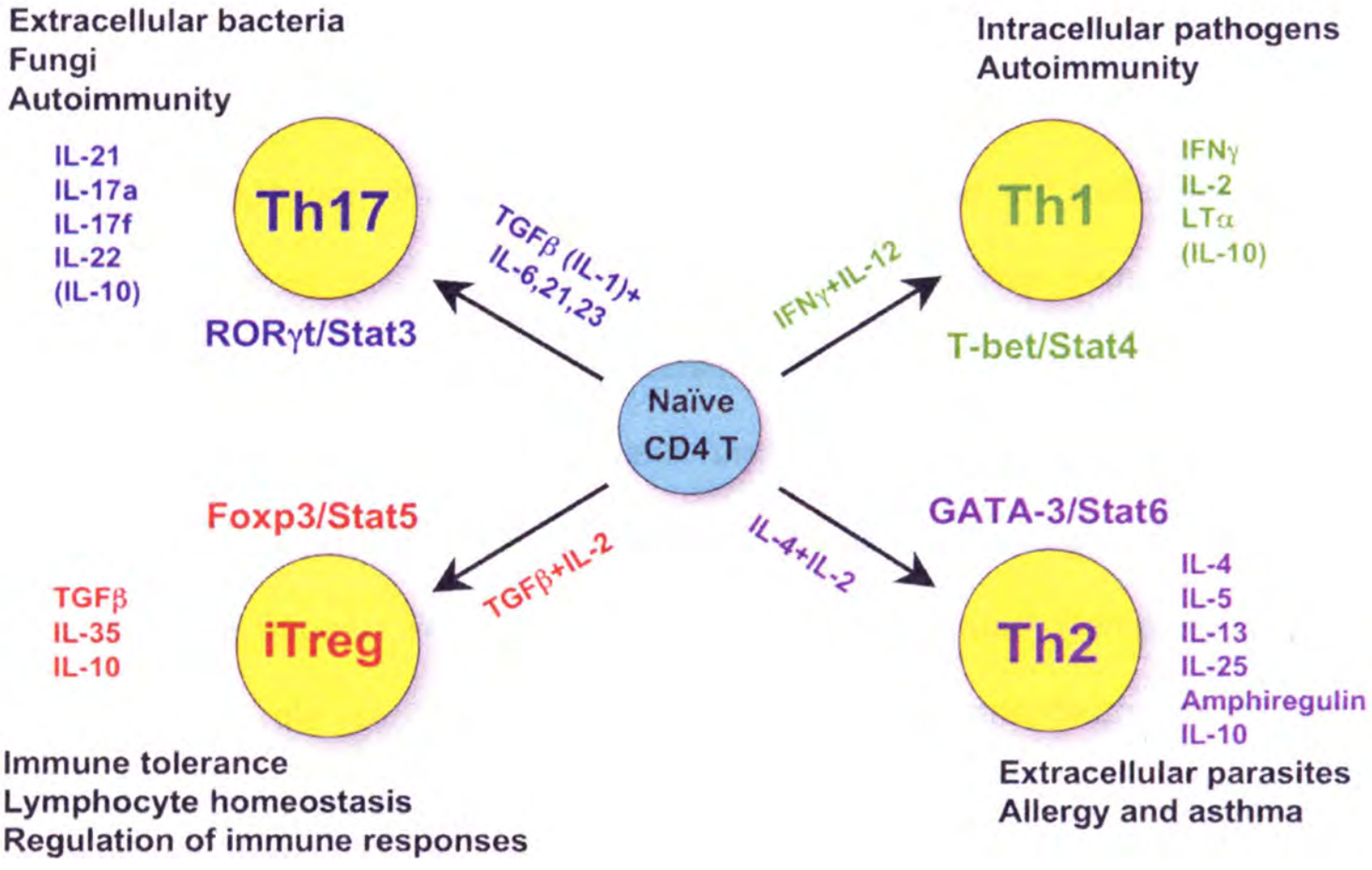


Figure 1: Th Cell Subtypes. The hallmark cytokines and transcription factors that bias the various subtypes of $CD4^+$ Th cells during development from a common precursor cell (Zhu and Paul 2008).

Role of T Cells in MS and EAE

The classic view of T cell involvement in MS and EAE was that inflammatory $CD4^+$ Th1 cells specific for MBP or other myelin proteins were improperly activated and resulted in the myelin attack and subsequence demyelination (Steinman et al. 1995). The identification of an IL-17 secreting CD4+ T cell subtype that was distinct from Th1 and Th2 subtypes (Harrington et al. 2005) has opened up a new avenue of study in autoimmunity. The Th17 subtype has been demonstrated to play a primary role in the pathogenesis of EAE (Komiyama et al. 2006; Langrish et al. 2005). Since then, a flurry of research papers on Th17 role in autoimmunity and its normal function of protection from extracellular bacteria and fungi has been published (Weaver et al. 2006). The role in EAE is quite well established but the origins of the Th17 cells in humans appears somewhat divergent from the mouse (Romagnani et al. 2009). Their role in MS is also questionable representing one of the shortcomings of translation from EAE. The actual identity of the pathogenic subtypes involved and how these autoreactive T cells become activated is a topic of much debate including theories of molecular mimicry (Wucherpfennig and Strominger 1995), ineffective priming and TCR avidity (McCue et al. 2004). The cause of the disease is disputed but the notion that there is an imbalance in the immune response is not.

Conventional theory held that increasing the Th2 cytokine profile would counteract the Th1 response and reduce the inflammatory events thereby, preventing the disease state or pushing the inflammatory state into remission

(Wei et al. 2007). With the discovery of iTreg cells, research switched focus to these cells as the mediators of controlling disregulated autoimmune responses (Costantino et al. 2008). Removing the iTreg cells results in onset of EAE (Sakaguchi 2000) demonstrating their importance for proper immune tolerance. Transfer of iTreg cells has also been shown to decrease other autoimmune diseases mediated by CD4+ T cells (Sakaguchi 2004). The entire role of iTreg cells in MS is not yet known but their proper function appears to be important for self-tolerance. The immunology of MS does not just involve CD4+ T cells but increasingly CD8+ T cells are being recognized for their role in the CNS pathology (Zozulya and Wiendl 2008) as well as their capability and involvement in EAE (Huseby et al. 2001). The T cells, irrespective of which subtype is initiating the reaction, enters the CNS and initiates or mediates an autoimmune attack upon the myelin sheath resulting in demyelination and reduction of action potential conduction down the axon. Figure 2 is a visualization of the pathological progression of autoreactive T cells into the CNS and demonstrates the attack upon the myelin sheath as well as the roles that other immune cells play in the pathogenesis targeting myelin. The various roles that the Th cell subtypes play in MS and EAE are still actively evolving as more and more properties and functions are uncovered. The various cytokines that these cells produce have an influence on the inflammatory environment locally and in the entire animal. Therefore the secreted cytokines are an important area to explore.

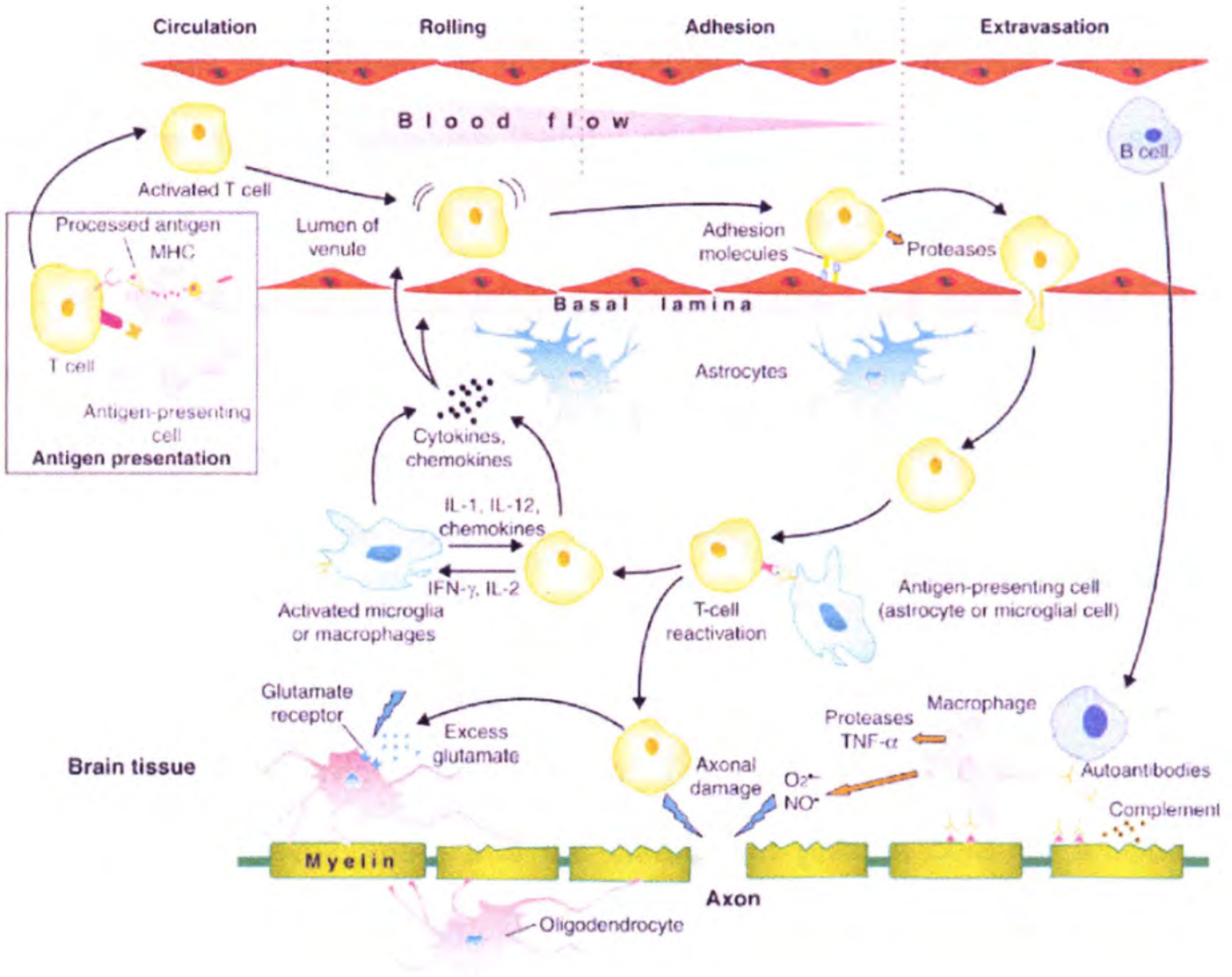


Figure 2: T Cells in MS. The multiple roles that T cells play in the initiation of the MS inflammatory cascade following CNS entry as well as the coordination of myelin degeneration (Baranzini and Hauser 2002).

Cytokines

Cytokines are proteins produced by various cell types including immune cells to reinforce the immune profile by autocrine/paracrine signaling. Among many other actions, cytokines either reinforce an immune response in similar subtype Th cells or suppress the activation state of Th cell subtypes that would counteract the immune response thereby reinforcing a specific immune state (Zhu and Paul 2008). The Th1 cytokines can counteract Th2 and vice versa. The Th17 cytokines can counteract Treg and vice versa. The subtypes of $CD4^+$ T helper cells were first identified by the different cytokine profiles that they produced and divided into Th1 and Th2 subtypes (Mosmann et al. 1986). There is some overlap in subtype production of cytokines, but of the 35 currently known interleukin cytokines, there are some important trends to point out.

Th1 cells tend to produce IFN-γ and IL-12 resulting in a proinflammatory profile and aid in cytotoxic cell proliferation. Th2 cells produce IL-4, IL-5 and IL-13 aiding B cell development and the creation of plasma cells for antibody production. More recently the cytokine profiles of Treg and Th17 cells have been elucidated. Tregs tend to produce IL-10 and TGFβ resulting in a true anti-inflammatory profile to induce anergy in the other T cells in the vicinity, thereby reducing the inflammatory cascade. Th17 cells produce IL-17 and IL-21, IL-23 and favor a proinflammatory profile thought to initiate the pathogenic process observed in EAE (Dittel 2008). The cytokines produced by these various T cell subtypes also have an effect on the other cells around them and can direct an immune response and inform the local cells' behavior.

IFN-γ from Th1 cells was thought necessary to initiate EAE because it upregulates MHC class II molecules as well as adhesion molecules on endothelial cells important for T cell migration, however IFN-γ knockout mice were actually more susceptible to EAE (Ferber et al. 1996) suggesting it may be involved with modulating the disease process. To further complicate matters, in a clinical trial of IFN-γ, half of the patients with MS experienced an exacerbation of their disease (Panitch et al. 1987). More recent studies have shown that T cell produced IFN-γ recognition by CNS cells may be important for localization of lesions (Lees et al. 2008). Needless to say, the elucidation of IFN-γ's role in MS is still evolving. The prototypic Th2 cytokines IL-4, IL-5, and IL-13 have been shown to decrease the EAE clinical disease (Chitnis and Khoury 2003) and repress the Th1 and Th17 phenotypes (Zhu and Paul 2008). Some of the pathogenesis ascribed to Th1 cells is know known to actually be mediated by Th17 cells that secrete IL-17, IL-6 and tumor necrosis factor alpha (TNFα) stimulating local cells to actively secrete chemokines and initiate a strong inflammatory response (Ouyang et al. 2008). This process normally provides protection against extracellular bacteria and fungi; however, in autoimmunity the system allows for activation of self-recognizing cells that coordinate an attack upon the host system. Finally, the major effector cytokine secreted by Treg cells is IL-10 (Taylor et al. 2006). IL-10 appears to function by decreasing antigen presentation and inflammatory cytokines. The true role of T cell subtypes in MS is still debated (Romagnani et al. 2009). As important as the profiles of the cells

present and the cytokines they produce is the actual mechanism by which they enter the CNS. Therefore, an exploration of T cell migration is warranted.

T Cell Migration

Unactivated, naïve and memory T cells normally reside in the lymphoid tissue and vasculature and upon antigen recognition initiate an inflammatory response and counteract foreign challenges. An activated T cells leaves the vasculature after it detects an attractant chemokine through receptor recognition. This migration occurs in several steps through integrins upregulation leading to rolling on the vasculature walls, adhesion and finally migration between the fibroblasts that compose the walls of the vessels. The CNS perivascular space normally has a relatively small number of T cells compared to other tissues (Norman and Hickey 2005). Inflammatory cell migration into the CNS appears necessary to initiate and continue the EAE and MS disease state but can occur without the classical interaction pattern of rolling or tumbling along the vascular wall (Vajkoczy et al. 2001). Normal trafficking of T cells through the CNS is greatly increased during an inflammatory event in MS or a relapse initiating the destructive cascade that leads to oligodendrocyte targeting and demyelination. The autoreactive T cells have to interact with their antigen in order for the cells to become primed and activated and initiate an autoimmune attack. The specific integrin, α4β1, was identified on T cells that binds to vascular cell adhesion molecule (VCAM)1. This interaction is specific for CNS vasculature and it was found that EAE was blocked by monoclonal antibodies to the α4 unit (Yednock et

al. 1992). This antibody was humanized and tested in EAE and MS patients before being approved by the FDA in 2004. Natalizumab is an α4β1-integrin blocking monoclonal antibody which functions to prevent T cells from binding to VCAM1 and blocks them from leaving the vasculature and entering the CNS (Ransohoff 2007). There have been problems with this therapy including progressive multifocal leukoencephalopathy (PML) which brings into question its safety profile (Koralnik 2006b). An association with increased cytomegalovirus (CMV) or Borna virus infection with natalizumab (Koralnik 2006b) but the treatment was associated with JC virus infection (Stuve et al. 2008). PML does not occur in animals that are used for EAE models so this was an unintended consequence. The drug has since been restored to the market albeit with stringent prescribing guidelines and monitoring requirement (Steinman and Zamvil 2006). Blocking migration of pathogenic cells is a logical strategy to decrease disease symptoms and other therapeutics that employ this strategy yet have fewer negative side effects may also prove beneficial. The problem with natalizumab is that it excludes all T cells from the CNS even though maintenance of non-autoreactive T cells or Tregs migration is probably beneficial. When autoreactive T cells do get into the CNS, their major pathological mechanism is a coordinated attack upon the myelin sheath (figure 2).

Myelin

Myelin is the compound that is the central target of attack in MS and EAE. Myelin is produced by oligodendrocytes in the CNS and Schwann cells in the

PNS (Waksman 1999). Oligodendrocyte cell membranes wrap around axons and undergo a process called compaction where the cytoplasm is squeezed out in order to produce myelin to provide insulation between the nodes of Ranvier and increases conduction velocity of action potentials (Martenson 1992). Myelin is composed of 80% lipids and 20% proteins, of which several are prominent antigenic targets in MS and EAE and targets of inflammatory assault. Due to the unknown etiology of MS and the heterogeneous clinical course, the various protein and lipid components of myelin have been extensively studied. Attempts were made to associate specific myelin proteins with the variety of clinical courses observed (Sospedra and Martin 2005). Myelin basic protein (MBP), is the most studied autoantigen in MS due to its abundance in the CNS in a specific isoform. Myelin oligodendrocyte protein (MOG) is a small protein that is a component of the outer lamella only found in the CNS and is the most antigenic of the myelin proteins. Proteolipid protein (PLP) is a myelin protein that is mildly antigenic in EAE and only a small percentage of MS patients react positively to it. Myelin-associated glycoprotein (MAG) requires very sensitive tests in order to detect it and the peptide sequences are only marginally immunogenic (Andersson et al. 2002). MS patients have been identified with specific T cells which react to MBP, PLP, MAG and MOG (Soderstrom et al. 1994) demonstrating the antigenicity of these various myelin components. The antigenic potential also extends to autoantibodies generation against MBP, MOG, PLP, and MAG (Baig et al. 1991; Berger et al. 2003; Warren and Catz 1994) which have all been identified in the cerebral spinal fluid (CSF) of MS

patients. The presence of autoantibodies to myelin proteins in normal individuals points to a more fundamental and elusive mechanism by which people with MS develop neurological deficits (Hedegaard et al. 2008). MBP is found in greater quantity in the CNS and also a different isoform exists in the CNS which raises the question of how the peripheral T cells that have escaped self-reactive deletion become activated in order to enter the CNS (Sospedra and Martin 2005) and contribute to pathology.

Myelin Basic Protein (MBP)

One of the major components of myelin is MBP and was one of the first protein targets of autoreactive T cells isolated from MS patients identified (Richert et al. 1983). Subsequently, a more complex repertoire of reactive T cells to MBP has been isolated from MS patients and EAE models and the numerous immunodominant regions which vary by species and strains were identified (Tabira and Kira 1992). Distinct sequences of the protein are immunodominant and produce higher levels of antigenicity than other sequences due to the binding kinetics of the sequences in susceptible human leukocyte antigen (HLA) domains (Li et al. 2000). The HLA is the human equivalent of the MHC domain in rodents which presents antigen to T cells. The antigenic sequences can begin with distinct sequences of the MBP protein but as the disease progresses the autoreactive T cells population has the potential to expand its recognition repertoire, termed epitope spreading, (Goebels et al. 2000) leading to greater disease burden and expanded inflammation. The interaction between the most

antigenic sequence and its corresponding MHC class II molecule is surprisingly weak (Nicholson et al. 2005). This suboptimal interaction may contribute to how autoreactive T cells escape deletion in the thymus during development. Studies have long shown that tolerance in MBP specific T cells can be induced by treating EAE with the antigenic sequence of MBP and this tolerance relates to the avidity of the TCR (McCue et al. 2004).

This weak interaction also allows for some degenerative potential for the sequence of MBP and for manipulation of this antigenic sequence to increase the binding kinetics of the peptide residues present in the antigenic sequence. These sequence changes result in an altered peptide ligand (APL) (Karin et al. 1994). Several therapeutics have been developed from MBP characteristics including glatiramer acetate (Schrempf and Ziemssen 2007), which is random polymer composed of the MBP amino acid molar ratio of L-glutamic acid, L-lysine, L-alanine and L-tyrosine, as well as several targeted antigens of MBP (K. G. Warren 2006) and various APLs of the antigenic sequence of MBP (Lutterotti et al. 2008). The goal of these therapeutics is to treat early in order to reduce the incidence of relapse, progression and epitope spreading of immunogenic cells.

Altered Peptide Ligands (APLs)

The T cell receptor (TCR) and major histocompatibility complex class II (MHC II) with a peptide displayed in the binding groove along with accessory binding molecules form the complex (immunological synapse) (Dustin 2008) necessary to activate a specific T cell. Inside of this complex lies the peptide, the

component of a protein processed inside the cell and bound to a specific MHC II molecule, for which a specific TCR will bind. This peptide is the antigenic sequence of a target protein. The sequence of this peptide can be altered to influence the binding kinetics of the TCR, either increasing or decreasing the affinity, and thereby alter the response from the target T cell (Karin et al. 1994). In fact, the TCR can recognize ligand peptides that have no sequence homology (Hemmer et al. 1998), thus opening the possibility for development of APLs with no potential for cross reaction. This altered response can result in immune modulation and change the default inflammatory response.

The concentration of antigen present also has an influence on the avidity of the TCR present in a population of T cells with less antigen resulting in a population with strong avidity for an antigen and more antigen resulting in a population with a low avidity TCR (Mazzanti et al. 2000). This observation demonstrates the importance of bioavailability in APL design and the population of reactive cells that may result. Antigen specific therapy has been attempted with a number of myelin protein sequences (Lutterotti et al. 2008). In fact, this strategy has been employed to alter the disease course of EAE and has also been attempted in MS patients with altered MBP peptide (Bielekova et al. 2000; Kappos et al. 2000). These clinical trials were promising but ultimately proved disappointing when unintended hypersensitivity reactions occurred. The hypersensitivity responses were theorized to be due to the development of an allergy to the APL caused by a shift to a Th2 bias after loss of self tolerance. Additionally, when the trial was performed again, this time with doses spaced

farther apart in order to decrease hypersensitivity reactions. no clinical benefit was seen above standard therapy (Steinman and Zamvil 2006)

Experiments have shown that it is possible to develop an allergic response to self antigens. These animals developed myelin autoreactive antibodies and an anaphylactic type response following repeated challenge with PLP antigen after resolution of an initial EAE disease course (Pedotti et al. 2001). The low bioavailability of the test APL and large frequent doses required for effective treatment possibly contributed to making the patients sensitized to the APL (Kappos et al. 2000). More recently, a phase 2 clinical trial utilizing the immunodominant sequence of MBP (MBP8298) as a therapy realized significant results in delayed progression of disease in a subgroup of HLA-DR2 or 4 patients (Warren et al. 2006). Trials are currently underway utilizing other antigen-directed approaches including a DNA vaccine of a plasmid encoding full length MBP (Garren et al. 2008) and trivalent TCR peptides (Garren et al. 2008) in order to increase Treg proliferation. The antigen directed approach is still viable and APLs are the most specific version of this strategy, despite the hypersensitivity side effects. The APL strategy is not without merit.

CALPAIN

Structure and Substrates

Calpain is a calcium (Ca^{2+}) activated neutral cysteine protease. The existence of a protease activated by Ca^{2+} was first identified in rat brain (Guroff

1964) and since then over 15 subtypes have been identified that are either ubiquitously expressed or are tissue specific (Suzuki et al. 2004). The two most commonly studied subtypes are μ-calpain and m-calpain (achieving half-maximal activation with μM or mM concentration of Ca^{2+} *in vitro* respectively). These two calpains are also referred to as calpain 1 and calpain 2, respectively (Ray and Banik 2002). Throughout this dissertation both will be collectively called calpain.

Calpain is required for normal function and development as it is an embryonic lethal knockout (Zimmerman et al. 2000). With regards to autoimmunity and MS/EAE, calpain activity has been detected in immune cells as well as in all cells in the CNS, demonstrating its importance for proper physiological function (Liu et al. 2008; Schaecher et al. 2002). The structure of μ-calpain and m-calpain exists as heterodimers composed of a catalytic 80kDa large subunit and a regulatory 30kDa subunit. The 30kDa subunit is identical for both isoforms (Suzuki et al. 2004). The large subunit has 3 potential Ca^{2+} binding motifs and the small subunit has 2 potential Ca^{2+} binding motifs (Ray and Banik 2002). The crystal structure of μ-calpain reveals that cooperative calcium binding is required to allow alignment and activation of the protease (Moldoveanu et al. 2002). Calpain has known signaling functions and is involved in a multitude of cellular processes including apoptosis (Zhivotovsky et al. 1997), activation (Penna et al. 1999; Schaecher et al. 2004), cell cycle regulation (Janossy et al. 2004) and migration (Butler et al. 2009; Franco and Huttenlocher 2005; Huttenlocher et al. 1997). Calpain activation state is controlled endogenously by intracellular Ca^{2+} levels and its associated endogenous inhibitor, calpastatin

(Emori et al. 1987). Increasing Ca^{2+} level leads to autocatalytic activation of the subunits for which calpastatin serves the purpose of counteracting in a regulatory manner (Takano and Murachi 1982). Calpastatin has proved useful in the study of calpain in regards to knockout animal techniques. In order to study the effects of a calpain knockout (embryonic lethal) without actually knocking out the protein, a transgenic was developed that overexpressed the endogenous inhibitor of calpain, calpastatin (Higuchi et al. 2005). This model has been used to demonstrate the importance of calpain in CNS inflammatory pathologies. Due to the many cellular functions that calpain participates in, effective exogenous inhibitors would prove useful.

Synthetic Inhibitors

Since the identification of calpain as an important protease involved with multiple signaling and effector functions in a variety of cell types the desire to develop effective and specific inhibitors has existed. The endogenous inhibitor calpastatin is too large to be an effect drug molecule. Therefore, two potential strategies were employed in the development of effective calpain inhibitors: (1) target the active site by covalently binding to the cysteine residue; (2) indirectly target the protease by preventing Ca^{2+} binding to inhibit the conformational changes that opens the active site. The first inhibitors were epoxides derived from aspergillus (Sugita et al. 1980) but had the problem of cross reactivity with papains and cathepsins. The peptidyl aldehyde type inhibitors were first isolated from actinomycetes as leupeptins but also inhibited serine proteases (Aoyagi et

al. 1969). Additionally, these early compounds had the problem of poor cellular permeability. The aldehydes were improved with the synthesis of the cell permeable inhibitor calpeptin (Tsujinaka et al. 1988). Unfortunately, these early peptidyl aldehydes have an extremely short half-life *in vivo* (Wang and Yuen 1997). Effort has been made to improve the bioavailability of the inhibitors by synthesizing non-peptide based compounds. Compounds based on quinolinecarboxamide scaffolds have demonstrated effective inhibition of μ-calpain (Graybill et al. 1995) as well as xanthene derived aldehydes, α-ketocarboxamides and fluoromethlketones (Chatterjee et al. 1996). Selective inhibitors that target the Ca^{2+} binding sights based on α-mercapto acrylic acid derivatives have been developed that are extremely selective (Wang et al. 1996). Historically, the largest obstacles for developing an effective calpain inhibitor as a therapeutic have been the solubility, effective half-life and membrane permeability. However, recent advances in the design of calpain inhibitors has increased all of these parameters to the point where an effective orally available inhibitor is now developed (Shirasaki et al. 2005; Shirasaki et al. 2006). This inhibitor based on a peptidyl α-ketoamide greatly reduces the side reactions that peptidyl aldehydes undergo while maintaining potency in the active site (Cuerrier et al. 2006).

Physiological and Pathological Function in MS/EAE

Calpain disregulation has the potential to either be the cause or consequence of pathological states given its regulatory role in cellular processes.

Increased calpain activity has been demonstrated in infiltrating monocytes into the CNS during the disease state of EAE (Schaecher et al. 2002) as well as increased activity observed the peripheral blood monocyte cells (PBMC) isolated from MS patients in a relapse state (Imam et al. 2007). Calpain degrades MBP (Banik et al. 1994) and PLP (Ray et al. 2003b) producing the antigenic fragments to which autoreactive T cells from EAE animals and MS patients react (Deshpande et al. 1995). The general inflammatory state in the CNS during a relapse favors calpain activation which drives a Th1 profile as well as increases in native glial cells leading to dysfunction and adding to the pathological state (Shields et al. 1999b). Calpain is upregulated in the inflammatory cells of the spleen before clinical signs of EAE adding evidence to its role in either T cell priming or initial activation (Shields et al. 1999a). Calpain seems to be correlated to the Th1 subtype and negatively correlated to the Th2 subtype (Imam et al. 2007). Calpain also plays a role in the pathogenesis of EAE as treatment with calpain inhibitors blocks the clinical signs of the disease (Guyton et al. 2006; Hassen et al. 2006). The role that calpain plays in the neurodegeneration observed during EAE (Hassen et al. 2008) has also been extensively studied in our lab.

CHAPTER TWO

AZAPEPTIDE INCORPORATION INTO ALTERED PEPTIDE LIGANDS

INTRODUCTION

$CD4^+$ T helper cell activation and response is a critical component of an effective mammalian immune system. Normal development of the $CD4^+$ T cell in the thymus requires recognition of antigen presented in the context of a MHC II molecule on the surface of an APC. Deletion of non-responsive, as well as hyper-responsive cells, contributes to preventing autoreactive T cells from entering the body, reducing the potential for self recognition and autoimmunity. While T cells that recognize self proteins do exist in the circulation of normal individuals (Mazzanti et al. 2000) autoimmunity due to inappropriate T cell activation does occur, either through antigen mimicry (Greene et al. 2008), low affinity antigen (McCue et al. 2004) or T cell escape (Kawamura et al. 2008). While the exact etiology of MS is unknown, it is thought that the pathology is largely due to activation of self-reactive T cells against myelin antigens and the initiation of an inflammatory response in the CNS resulting in demyelination (Keegan and Noseworthy 2002). The involvement of T cells responsive to myelin proteins is a mechanism elucidated with and more easily demonstrated in the animal model of MS, EAE (Baxter 2007).

Therapies based upon altering the inappropriate T cell response through targeting antigen presentation is possible (Kappos et al. 2000; Karin et al. 1994; Leadbetter et al. 1998). Changing the antigenic peptide's sequence to influence binding kinetics can alter the response of autoreactive T cells (Rabinowitz et al. 1997). These changes result in APLs. APLs of MBP have been effective at reducing the clinical disease of EAE (Karin et al. 1994; Smilek et al. 1991) but

trials in MS patients using the same methodology proved disappointing (Bielekova et al. 2000; Kappos et al. 2000). The high doses required to achieve clinical benefit proved problematic due to hypersensitivity responses in some of the test subjects. A subsequent trial was attempted with a reduced dose and reduced treatment frequency but no therapeutic benefit was observed (Steinman and Zamvil 2006). Optimization and improvements to the APL to increase bioavailability prior to human testing may have proved beneficial and resulted in a more favorable therapeutic outcome.

The TCR/MHC II complex is specific for the antigens that it will bind, but does demonstrate some degree of degenerative binding with regard to the peptide sequence that will interact within the binding groove (Smilek et al. 1991). Once the TCR and MHC II contact points have been identified, it is possible to perform logical substitutions to the native peptide sequence to alter the properties of the ligand in order to either increase or decrease binding (Matsui et al. 1994). Figure 3 is based on the molecular model (Lee et al. 1998) of the MBP Ac1-9 peptide binding to the I-A^u MHC II and gives an idea of where the residue/peptide contacts the TCR and MHC II. The contact points of the MBP Ac1-9 peptide to the I-A^u MHC II molecule and 172.10 TCR were confirmed a few years later by the crystal structure (He et al. 2002) (Figure 4). Structural modifications can be performed to also alter the peptide's biokinetic profile including solubility and protease resistance.

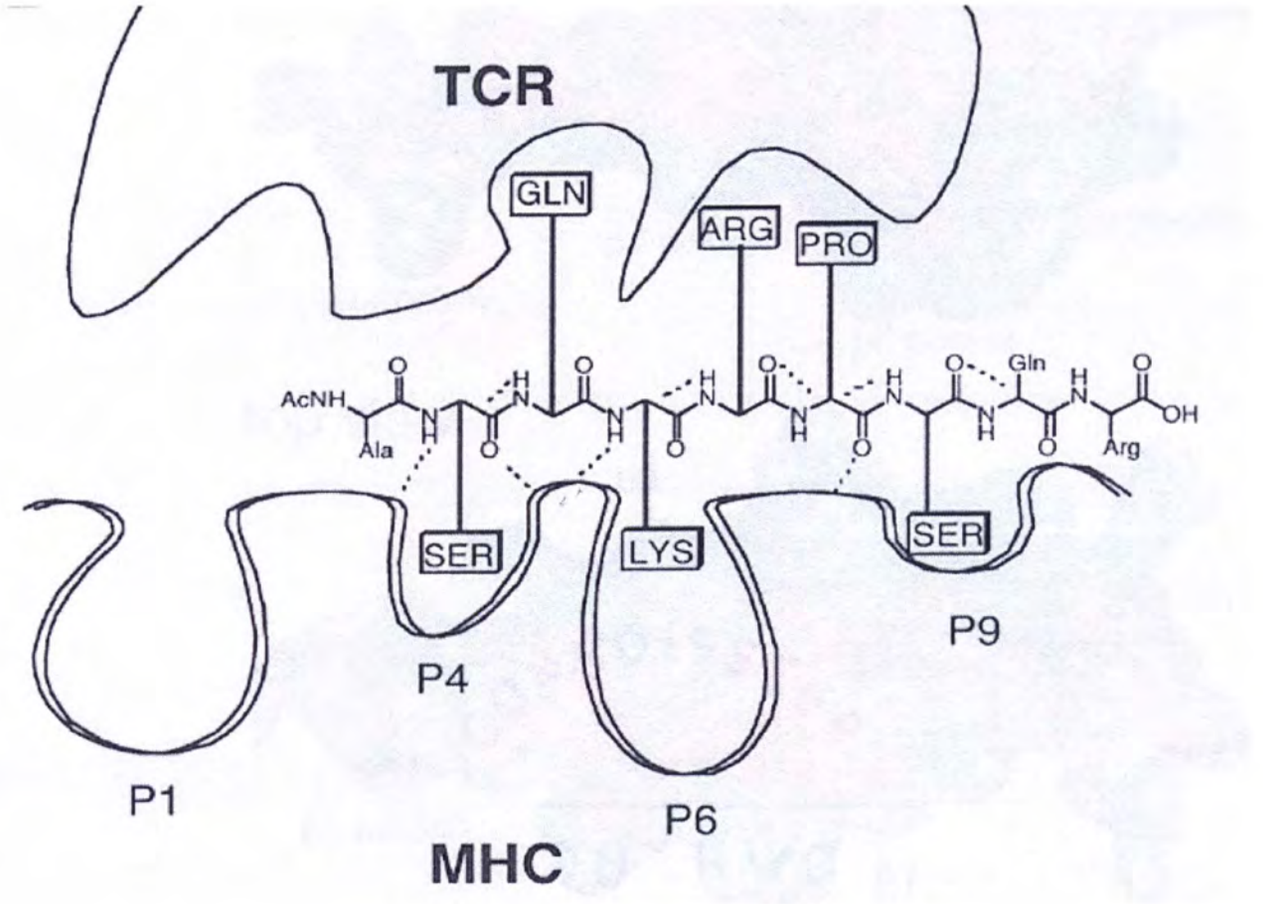


Figure 3: MBP Presented to a T cell. A representative drawing of the MBP Ac1-9 peptide binding to the I-A^u MHC II molecule and the 172.10 TCR.

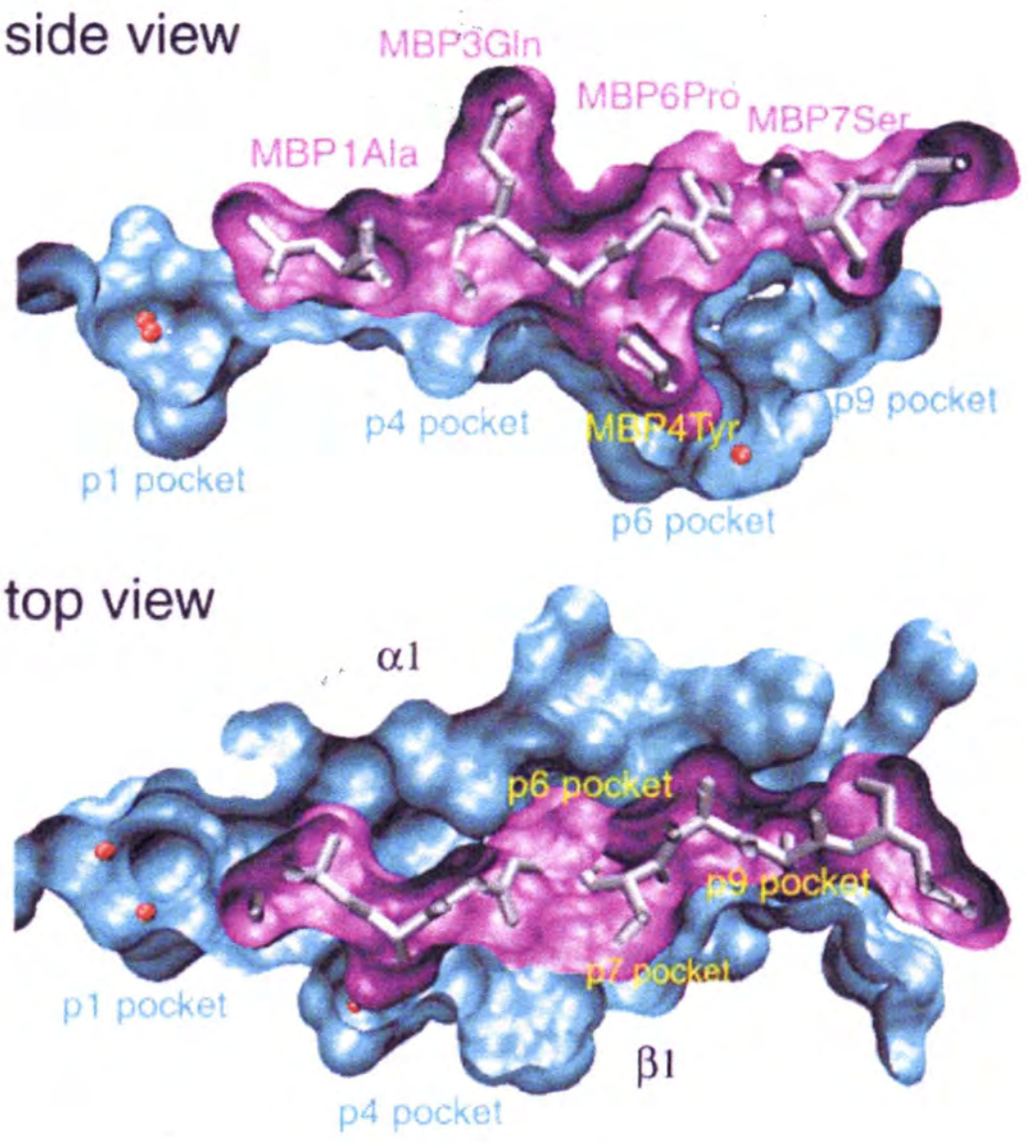


Figure 4: MBP Binding to MHC II. The crystal structure of MBP Ac1-11 binding to the I-A^u MHC II protein confirming the empty P1 pocket (He et al. 2002).

Peptides in the body, either native signaling peptides or peptides used as drugs, are subject to degradation by non-specific proteases on the cell surfaces (Boonen et al. 2009). Therefore, changes to a peptide sequence in order to create an APL with increased protease resistance may result in a peptide with increased bioactivity. The increased protease resistance may allow for a reduced dose while still maintaining an immunomodulatory effect ultimately resulting in an APL with less potential for hypersensitivity side reactions.

The following work sought to address some of the short comings of the bioavailability recognized in previous APL work (Zaliauskiene et al. 2002). Aza-amino acids, an amino acid with nitrogen substituted for the α-carbon, (Figure 5) were utilized to create APLs and some of the properties that contribute to bioavailability were tested. MBP and its interaction with the mouse I-A^u MHC II complex was examined in order to determine if substitution would result in a peptide that still bound to the specific antigen presentation cleft. Protease resistance compared to the native MBP Ac1-9 sequence was also tested. Finally, the recognition and initiation of an activation response though the TCR using aza-APLs as the stimulus was determined. Though this study utilizes MBP as the starting sequence to create APLs, the resulting substitutions with aza-peptides are applicable to any situations where immune modulating APLs are a potential therapeutic and improved bioavailability is desired.

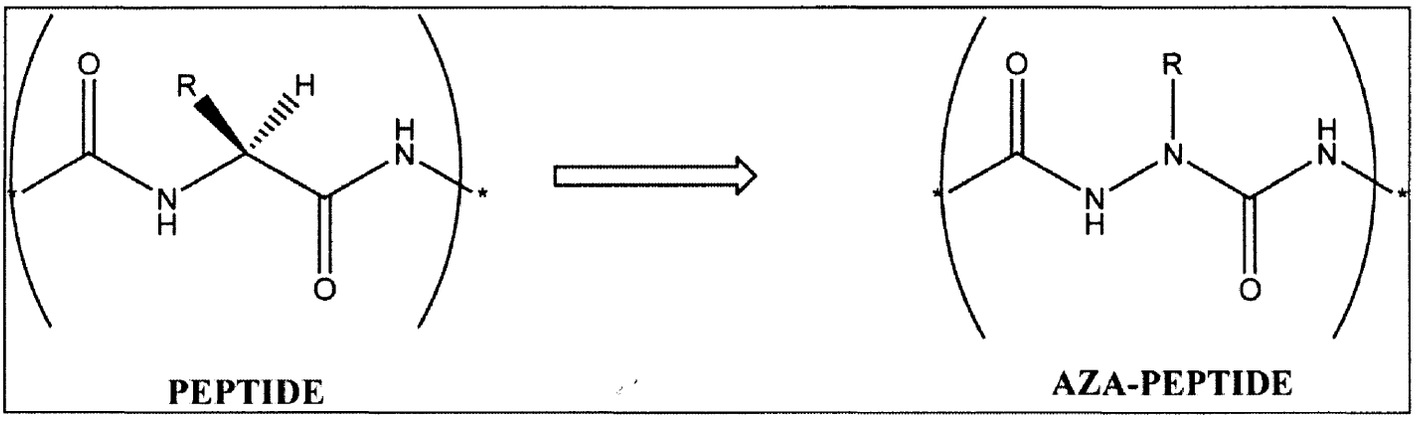


Figure 5: An Aza-Amino Acid. The substitution of the α-carbon in an amino acid for a nitrogen results in an aza-peptide. These aza-amino acids were chemically inserted into peptide sequence during synthesis of the APLs.

APL Sequences and the IL-2 Response

Name	MBP Peptide Sequence	IL-2 Response
MBP Ac1-9/4K/Native sequence	↓↑↓↑↑↓ Ac-ASQKRPSQR	++
MBP Ac1-9/5K/Null sequence	↓↑↓↑↑↓ Ac-ASQK**K**PSQR	-
MBP Ac1-9/4A/Strong agonist	↓↑↓↑↑↓ Ac-ASQ**A**RPSQR	+++
2azaG4A	↓ ↑↓↑↑↓ Ac-A**G$_{aza}$**Q**A**RPSQR	+
MBP Ac1-9/4Y/Agonist	↓↑↓↑↑↓ Ac-ASQ**Y**RPSQR	++
3azaG4Y	↓↑ ↓↑↑↓ Ac-AS**G$_{aza}$Y**RPSQR	-
4Y5azaG	↓↑↓↑ ↑↓ Ac-ASQ**YG$_{aza}$**PSQR	-
4F	↓↑↓↑↑↓ Ac-ASQ**F**RPSQR	+++
2azaG4F	↓ ↑↓↑↑↓ Ac-A**G$_{aza}$**Q**F**RPSQR	++

Table 1: A summary table of the aza-substitutions performed to the native MBP sequence. The arrows above the native sequence designate the contact points where down arrows correspond to MHC II molecule contacts and up arrows correspond to TCR contacts. The IL-2 response is the maximal response from the T cell activation assay for the various APLs relative to the native sequence.

MATERIAL AND METHODS

Cell Culture and Reagents

A mouse L929 line of fibroblast like cells that have been transfected to express I-A^u MHC II protein (Tate et al. 1995) and a T cell hybridoma (Goverman et al. 1993) that expresses the 172.10 TCR specific for rat MBP Ac1-11 peptide were used in the T cell activation studies. The L929 cells were maintained in Dulbecco's modified Eagle F12 media (Gibco, Grand Island, NY) supplemented with 100 U/ml penicillin and 1 µg/ml streptomycin (Gibco) and 10% FCS (Hyclone, Logan, UT) at 37^0C in a 5% CO_2 atmosphere (Thermo Fisher Scientific, Waltham, MA) and grown to desired confluency for each of the assays performed. The 172.10 cells were maintained the same way as the L929 cells except RPMI 1640 media (Gibco) was used instead of DMEM F12.

Peptide Synthesis and Labeling

Peptide synthesis grade reagents were purchased from Advanced Chemtech (Louisville, KY) and peptide components were obtained from Peptides International (Louisville, KY). The native and altered MBP peptides were synthesized using rink amide resin (Novabiochem, Darmstadt, Germany) solid phase Fmoc-HBTU/tert-butyl protection chemistry as previously described (Hart and Beeson 2001). After synthesis was complete the resin was divided. Half was deprotected and cleaved from the resin for the serum degradation and T cell activation assays and half was fluorescein-labeled before final deprotection and cleavage for the MHC II binding assay. Peptides were cleaved from the resin by mixing with 90% trifluoroacetic acid (Sigma-Aldrich Corp. St. Louis, MO), 4%

Tetraiodosilane (Sigma) and 6% water for 3 hours and then precipitated with diethyl ether (Sigma). The solvent was decanted and dried for 24 hrs under high vacuum. The peptides were then dissolved in dimethylsulfoxide (DMSO) and purified by reverse phase high performance liquid chromatography (RP-HPLC) on an acetonitrile/water gradient (Waters, Milford, MA). The peptides were characterized by electrospray ionization mass spectrometry (ES-MS) on a LCQ Advantage Max mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Aza-peptides were synthesized following the same methodology with the exception of aza-glycine insertion by chemical synthesis at the desired substitution points during elongation as described previously (Hart and Beeson 2001). Some of the sequences that were synthesized are represented in table 1.

MHC II Binding Assay

The various MBP Ac1-9 peptides were labeled with fluorescein for use in MHC II binding studies. Soluble I-A^u MHC II protein was isolated from BW5134 cell lysate (National Cell Culture, Minneapolis, MN) and purified by M115 antibody exchange column (Millipore, Billerica, MA). An excess of APL (5 μM) was incubated with the isolated MHC II protein (50-100 μl) overnight at 37^{0}C. The unlabeled peptide was then removed by Sephadex G50-SF (Sigma) spin column (~1 ml) purification. The complex was then incubated at 37^{0}C and samples (50 μl) were taken at time points determined as the experiment progressed. Sample composition was monitored with HPLC (Shimaduzu, Columbia, MD) on a size exclusion column (Phenomenex, Torrance, CA). The retention time of the MHC II/peptide complex was different than the labeled free

peptide which allows for semiquantification of the dissociation. The peak height for the complex is graphed versus time and the resulting curve is used to calculate the disassociation half time of labeled peptide from MHC II.

Serum Degradation Half Life Assay

The unlabeled APLs were tested to determine their resistance to protease degradation. The serum degradation experiments are a modification of published methods (Kokko and Dix 2002) utilizing matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry. The peptides were dissolved at 10mM in sterile H_20 and then 30 µl was added to 270 µl of rat serum (Sigma). A degradation resistant peptide composed of D-amino acids was used as an internal standard and was added to the serum at the same concentration. The mixture was then incubated at 37^0C and 20 µl samples were taken at specified time points. The time points vary with the protease resistance of the peptide and the optimum times were determined during testing. The 20 µl sample was added to 80 µl of a 3:1 methanol:ethanol mixture to precipitate the proteins from the serum and then spun in a microcentrifuge (Eppendorf, Westbury, NY) at 12,000 g for 10 minutes. Supernatant, which contained the peptide, was then combined with α-cyano-4-hydroxycinnamic acid matrix and spotted on a gold MALDI plate (Applied Biosciences, Foster City, CA). The samples were spotted in triplicate. The ratio between the internal standard peptide peak and the test peptide peak was then determined using a Voyager-DE STR Biospectrometry Workstation (Applied Biosciences) by MALDI-TOF MS.

The ratio was graphed against the sample times. The degradation half-life was then determined from the equation obtained from the natural log of the ratio graphed against sample times using Microsoft Excel (Microsoft, Bellevue, WA).

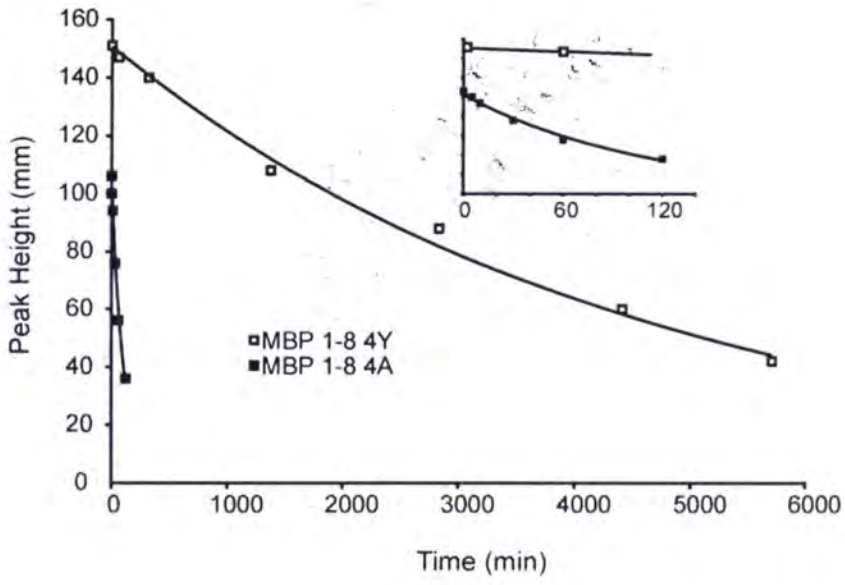
T cell Activation Assay

The T cell activation experiments used the L929 cells that have been transfected with I-A^u MHC II protein as an APC cell line (Tate et al. 1995) to present antigen to the 172.10 T cell hybridoma (Goverman et al. 1993) that has a TCR specific for rat MBP Ac1-11 peptide. IL-2 production by the 172.10 cells was used as an indication of the degree of stimulation through the TCR in response to the APL being tested. Aza-peptides from the binding experiment as well as unlabeled APLs with and without aza-amino acid substitutions were tested. Serial dilution of test peptides were added to 3 x 10^4 APC cells and 6.5 x 10^4 172.10 cells in 96 well plates (Corning, Corning, NY) and incubated at 37^{0}C for 48 hours. Supernatant samples were then taken and frozen at -80^{0}C until the cytokine analysis was performed. The IL-2 concentration was determined by sandwich ELISA (BD Bioscience, San Diego, CA) in a 96 well plate format following standard protocols. The absorbance of the ELISA plates was read with a Vmax absorbance plate reader (Molecular Devices, Sunnyvale, CA) at 450 nm. The samples were quantitated by comparing the absorbance readings to a standard curve prepared with known concentrations of IL-2.

MHC II/MBP Peptide Binding

In order for a $CD4^+$ T cell to respond to an antigen and for activation to take place, the TCR must interact with an antigen/MHC II molecule complex present on the surface of an APC (Zhu and Paul 2008). The TCR will interact with the antigen/MHC molecule even if there is some variation in the antigenic peptide's sequence allowing for a greater immunological range (Hemmer et al. 1998). Changes to this sequence due to amino acid substitution can increase or decrease the binding time and vary the resulting response from the T cell. The APLs were tested for binding to soluble $I\text{-}A^u$ MHC II to determine if the modification made maintained the peptide/MHC II interaction in order to predetermine potential usefulness in a cellular system. Figure 6A is the representative time plot MBP Ac1-11 peptide with an alanine substituted at position 4 (4A) and the MBP Ac1-11 with a tyrosine substituted at position 4 (4Y). The graph represents the peak height on a HPLC readout of the fluorescein labeled peptides after incubating at 37^0C. The formula of the best fit logarithmic curve of these graphs was used to calculate the dissociation half off time. Figure 6B is a bar graph of the calculated dissociation half times from $I\text{-}A^u$ MHC II molecule of the various labeled APLs.

A



B

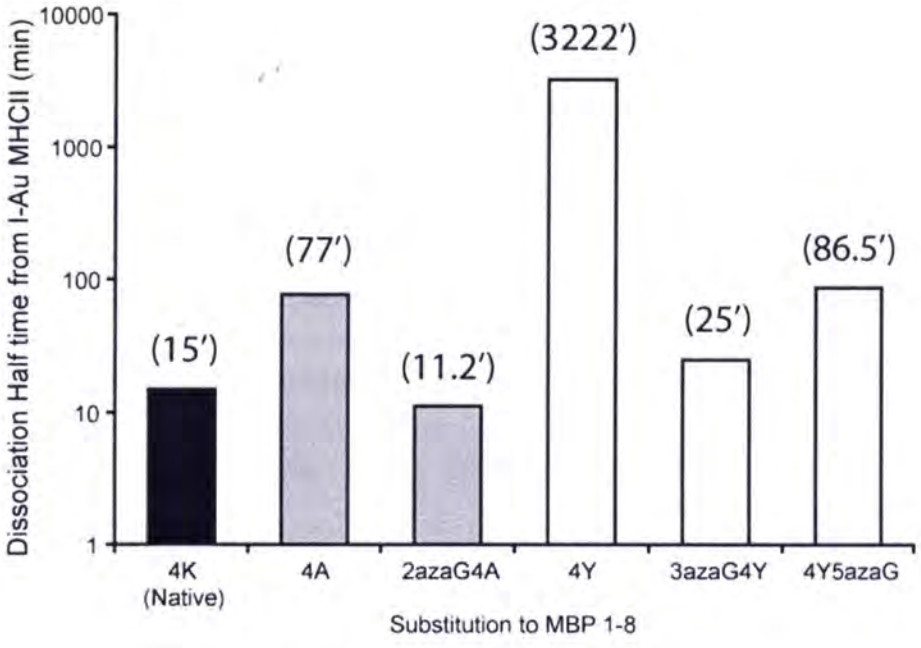


Figure 6: The Half Off Time of Modified MBP from the I-A^u Soluble MHC II Protein. A, The peak height of the APL/MHC II complex as measured by integration following HPLC size exclusion at various time points. The inset is the same data over a shorter time period. B, The calculated half off times of several aza-APLs compared to the native MBP Ac 1-9 (4K) peptide from the I-A^u soluble MHC II protein. The half off time in minutes is indicated above the bar.

The formulas for the kinetic graphs were used to calculate logarithmic half points which was taken as the dissociation off half times. Some substitutions resulted in stronger binding with increased half off binding times and some substitutions resulted in shorter binding times that were comparable to the native 4K MBP Ac1-9 peptide sequence. The aza-peptide substitutions altered the binding kinetics but the binding still occurred, resulting in dissociation half off times that ranged between the native MBP Ac1-9 peptide (4K) (15 minutes) and a known strong binding (4Y) (3222 minutes) sequence.

APL Degradation in Rat Serum

The degradation times of the peptides in rat serum were determined to assess if aza-amino acid substitution provided protease resistance. Rat serum was utilized because the native MBP sequence is the rat sequence. Figure 7A demonstrates representative curves obtained by graphing the ratio of an APL to a resistant internal standard at various time points following incubation in rat serum as described in the methods. The formula for the curve is used to calculate the degradation half-life of the test peptide. Figure 7B is a bar graph of the calculated half-lives of some of the APLs that were tested in order to compare the half-lives of the native MBP Ac1-9 peptide to other natural amino acid APLs as well as to aza-APLs.

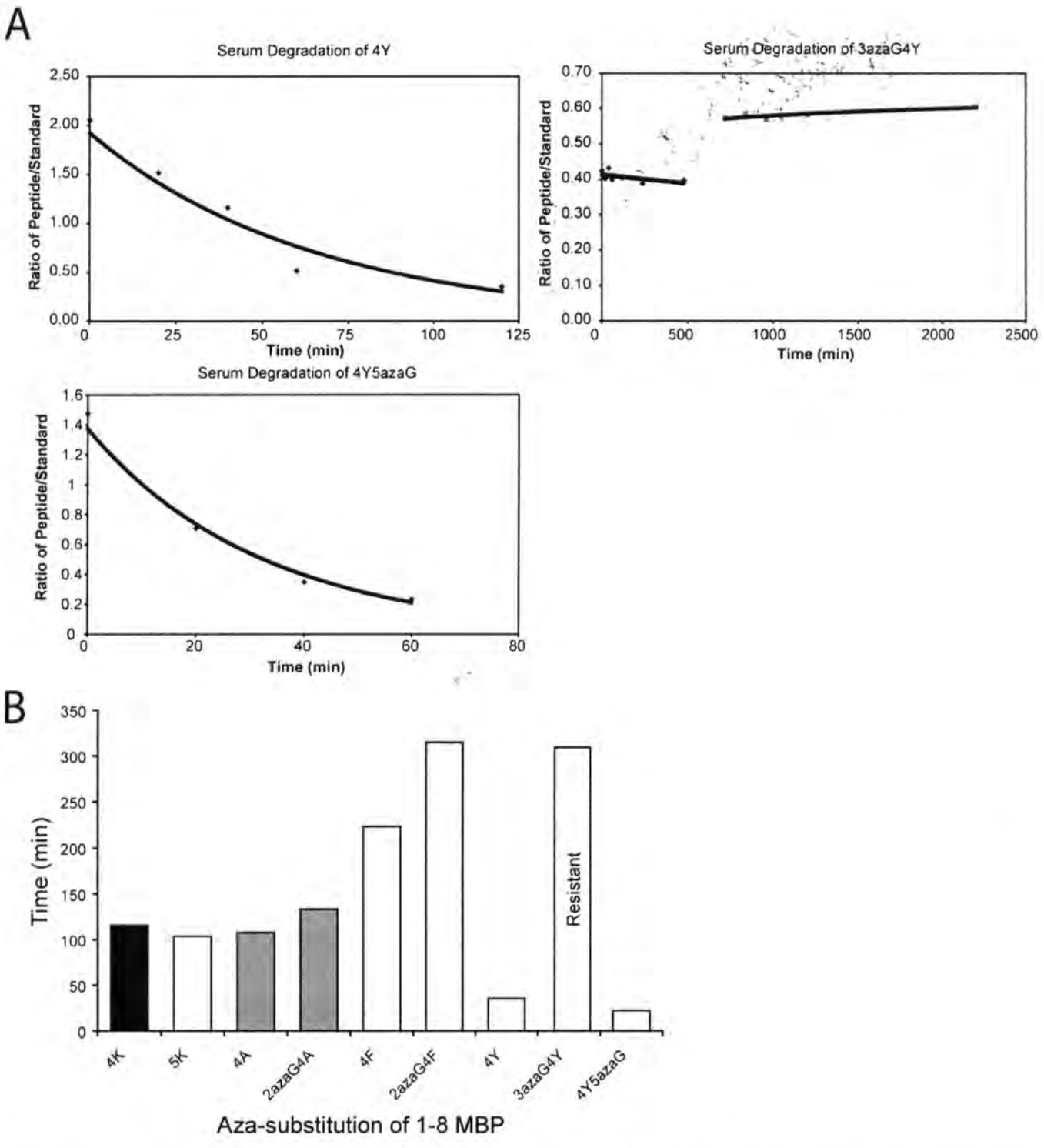


Figure 7: The Degradation Times of Various APLs in Rat Serum. A, the ratio of APL to standard non-degraded peptide control at various time points following incubation in rat serum at 37^0C. B, the calculated half-lives of various substituted APL compared to the native MBP sequence (4K).

Comparing the natural peptide APLs to the native sequence, the substitution of alanine at position 4 did not appreciably alter the half-life (6% difference). Alternatively, a phenylalanine substation (4F) markedly increased the half-life (48% increase) while a tyrosine substation (4Y) markedly decreased the half-life (69% decrease) compared to the native lysine (4K) at position 4. Substitution with aza-glycine at position 2 or 3 produced APLs that had longer half-lives than the native sequence. These aza-APLs also possessed half-lives that were longer than their corresponding native peptide APL. In fact, insertion of an aza-glycine at position 3 resulted in an APL that was completely resistant to degradation in rat serum. The substitution of aza-glycine at position 5 conversely results in an APL with a half-life that was less than the native sequence (81% less) and also less than the native 4Y APL (38% less).

Effect on T cell Activation

T cell activation assays were performed to assess if the changes made to the MBP sequence preserved their potential bioactivity. This assay demonstrated that modest changes to the MBP native sequence can still activate T cells through the TCR that recognizes MBP Ac1-11 (table 1). The bioactivity of these APLs with aza-amino acid substitutions is demonstrated in a cellular system by utilizing cytokine production as an end point. Table 1 displays some relative IL-2 responses for some of the peptides that were tested. Figure 8 is a bar graph that represents the maximal IL-2 produced by the 172.10 T cell hybridomas when stimulated with MBP APLs. The minimal concentration of APL

required to achieve that maximal IL-2 response is listed below the bar. The inset graph demonstrates that increasing the APL concentration used for stimulus results in a corresponding increased production of IL-2. The maximal response was taken as the point where the IL-2 increase reached a plateau. Figure 8 demonstrates that none of the aza-substituted APLs produced an IL-2 response comparable to the native peptide at any of the concentrations tested. In addition, no detectable IL-2 was produced when the 3azaG4Y APL or the 4Y5azaG APL were used as the stimulating peptide.

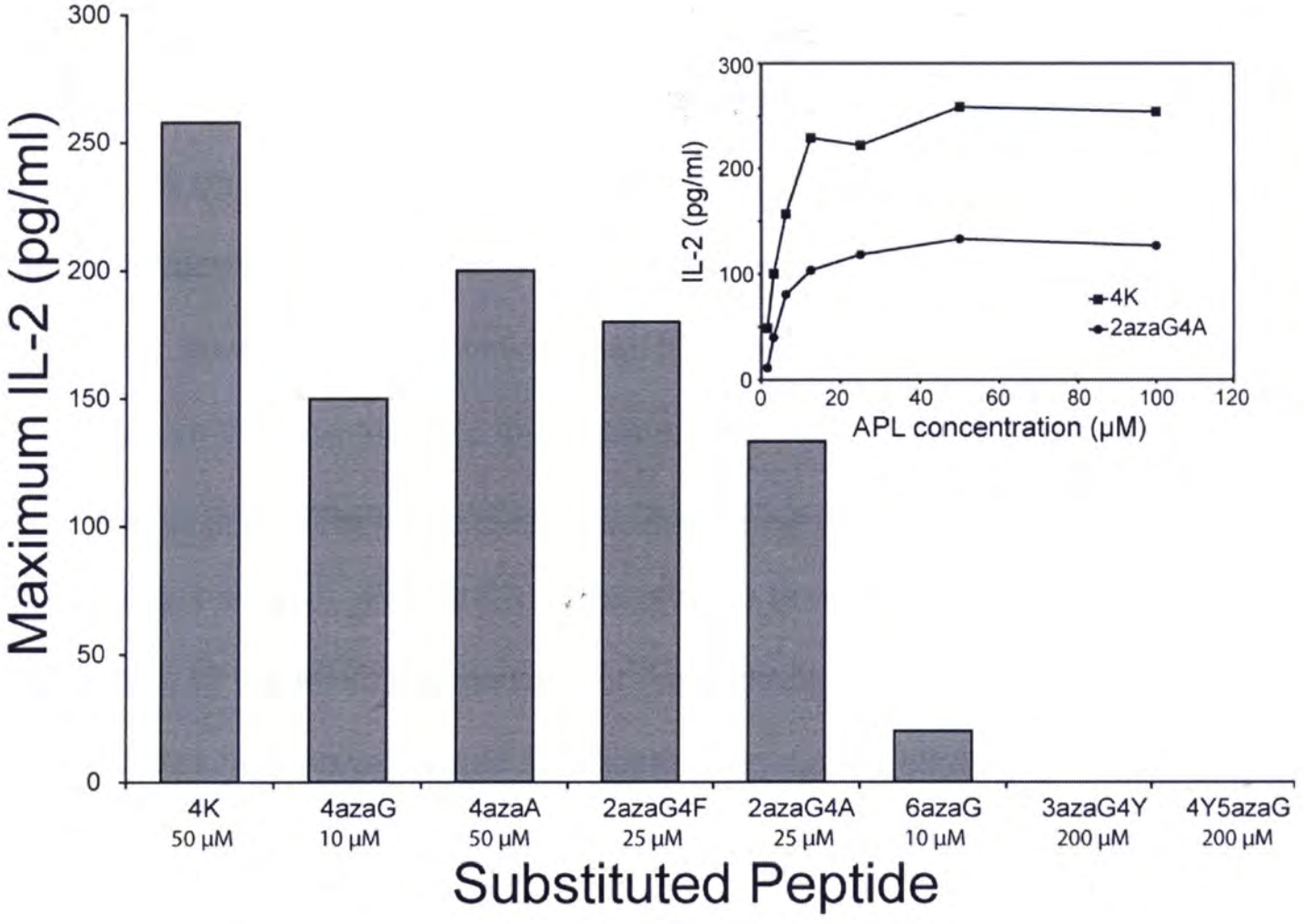


Figure 8: The Maximal IL-2 Production from 172.10 Cells Following Stimulation with Various APLs. The APL concentration required to give the maximal stimulation is below the respective bar. The inset is a plot of IL-2 concentration at various APL concentrations and the concentration of APL that produces the maximal IL-2 concentration was taken at the point at which the plot plateaus.

DISCUSSION

Utilization of aza-peptide substituted altered peptide ligands provides a novel solution to the problem of *in vivo* hypersensitivity response to peptide-based drugs through increased protease resistance of the peptide sequence thereby decreasing the required therapeutic dose.

This study demonstrated the possibility of making subtle changes to the native antigenic sequence while maintaining binding to an MHC II presentation molecule. Maintaining the binding to MHC II molecules is the first check point to any changes that are made to the peptide sequence. If the changes are so great that binding is no longer possible then the peptide will not be properly presented to the target T cells. Figure 6B demonstrates that aza-peptide insertion affects the binding to the MHC II molecule but that binding is still preserved. Even if the aza-peptides themselves would significantly reduce the binding, rational changes to the APL sequence, such as position four substitutions to create scaffold sequences with greatly increased binding. These scaffolds can then force the MHC II molecule to present an APL with an aza-glycine substitution. Overtly strong binding is not the only goal of APLs though as variable binding strengths lead to altered T cell responses which are difficult to predict based solely on the sequence modifications (Hart and Beeson 2001). Modification of the MHC anchors has been demonstrated previously as an effective method for creation of partial agonist APLs (Ryan et al. 2004). So no matter what logical substitutions are made to the sequence based on the contact points (figure 3) testing is still required.

Increased resistance to protease degradation through altering the native MBP antigenic sequence with aza-amino acids was also demonstrated. The utilization of APLs and peptides as drugs has the potential side effects of hypersensitization and allergic reactions (Bielekova et al. 2000; Kappos et al. 2000). The previously tested APLs that made it to clinical trials were stopped as a result of unintended allergic response potentially due to the high concentrations required. These high concentrations were required due to low bioavailability as well as inadequate optimization. There are various modifications to a peptide sequence which produce protease resistance. N-terminal acetylation increases protease resistance compared to an unacetylated terminus (data not shown). The native sequence of MBP Ac1-9 is n-terminal acetylated so the starting peptide has a higher protease resistance compared to a peptide sequences with a non-acetylated N-terminus. The strategy of using aza-peptides will potentially reduce the concentration required for clinical effectiveness of APLs though even more increased protease resistance. Aza-peptides are not naturally occurring so proteases have not had the evolutionary exposure to develop an active site that can cleave these (peptide) bonds. These aza-peptide substitutions produced a further decrease of protease degradation (figure 7B) compared to the native (4K) sequence. The aza-amino acid substitutions that were made were with aza-glycine and resulted in increased protease resistance (4A compared to 2azaG4A) and (4F compared to 2azaG4F). Multiple aza-amino acid substitution or aza-amino acids substitutions beside glycine are possible and may result in even greater resistance. Even though the APL binds to MHC II and

demonstrates increased resistance to degradation, it is still beneficial to test whether TCR activation is still possible.

Despite the changes that were made to the peptide, aza-peptide substitution can result in an APL that maintains bioactivity as measured by the T cell activation assay. This assay used a T cell reporter that has a TCR specific for the native peptide sequence, yet T cell activation and IL-2 production was still possible with an altered sequence. This demonstrates both the degenerative nature of the 172.10 TCR as well as the feasibility of using aza-peptides for APL creation. None of the aza-peptide substituted APLs (figure 8) were able to induce a maximal IL-2 production comparable to the native peptide sequence (4K) or to certain known strong agonist peptides, such as a 4A substitution, (table1). IL-2 production was still possible though and at APL concentrations (figure 8) comparable to the concentration required for the native peptide to achieve maximal stimulation. This may mean that the APLs are still effective T cell antigens and do not require higher concentrations to produce a biologically relevant effect even though the sequence differs from the native antigenic peptide. Further testing would be required in an animal system, after more thorough optimization of the sequence, in order to confirm the bioactivity of the effects observed in these experiments.

Aza-peptide inclusion in APLs is a viable technique for the creation of immune modulatory peptides that display properties of the native sequence yet possess increased resistance to protease cleavage. The utility of these aza-APLs potentially apply to any *in vivo* situation where peptide based therapies are

relevant and provides a potential strategy for decreasing the hypersensitivity side effects that have, to this point, plagued many of the clinical trials and applications as therapeutic treatments.

CHAPTER THREE

THE INVOLVEMENT OF CALPAIN IN THE PROCESS OF JURKAT T CELL CHEMOTAXIS

T cell involvement in the disease process of MS (Hickey 1991; McFarland and Martin 2007) and its animal model, EAE are well established (Swanborg 2001a; Waksman and Adams 1962). T cell infiltration of the CNS is greatly increased in the plaques of MS patients (Hickey 1991) and in the CNS of EAE animals immediately before the onset of pathological signs and during clinical disease (Waksman and Adams 1962). Increased activity and expression of the Ca^{2+}-activated protease calpain has been demonstrated in inflammatory cells (Shields et al. 1999a; Shields et al. 1998) during the disease state in EAE animals. Moreover, inhibition of calpain has been demonstrated to decrease the T cell infiltration during acute EAE (Hassen et al. 2006). Calpain has known signaling functions and is involved in a multitude of cellular processes including apoptosis (Zhivotovsky et al. 1997), activation (Penna et al. 1999; Schaecher et al. 2004), cell cycle (Janossy et al. 2004) and migration (Franco and Huttenlocher 2005; Huttenlocher et al. 1997).

T cell exclusion from the CNS is the strategy employed by the FDA approved α4β1-integrin blocking monoclonal antibody natalizumab (Ransohoff 2007). This drug has not been without problems including several patients on the medication developing PML (Koralnik 2006a) leading to the temporary withdrawal of the drug from the market. In addition, alternative targets for MS therapy are warranted due to the low effectiveness of many of the current standard therapies (Kleinschnitz et al. 2007). Targeted exclusion of T cells from the CNS would be of great benefit not only to MS but for many other autoimmune

diseases that have a T cell component of their pathology including rheumatoid arthritis (RA) (Cope et al. 2007) and amyotrophic lateral sclerosis (ALS) (Graves et al. 2004). Inflammatory cells entering the CNS in MS and EAE have been show to have elevated calpain levels (Shields et al. 1998). If calpain was indeed involved with T cell migration then calpain inhibition may provide an alternative method for reducing pathological T cell entry into the CNS.

Calpain has been shown to be involved in the migration of numerous cell types including CHO cells (Huttenlocher et al. 1997), fibroblasts (Shiraha et al. 2002), myoblasts (Leloup et al. 2006), and neutrophils (Lokuta et al. 2003). The chemokine CCL2 (previously known as monocyte chemoattractant protein-1) and its predominant receptor CCR2, a G protein-coupled receptor, have been extensively associated with inflammatory pathologies (Jee et al. 2002; Mahad et al. 2006). CCL2 has been extensively shown as a specific chemoattractant for monocytes and T cells (Carr et al. 1994; Sanders et al. 2000). Even though the initiating stimulus for migration is well established, the downstream mechanism of T cell chemotaxis is as of yet incomplete.

In this study, our aim was to gather evidence for the involvement of calpain in the process of T cell migration. This analysis utilized the Jurkat E6-1 cell line as a model for T cells as well as normal human PBMCs. Increased activity of calpain was observed in response to the CCL2 stimulation as well as the restriction of calpain's role to involvement in the directed migration of chemotaxis. This is in contrast to other immune cell types where calpain has been demonstrated to play a role in the negative regulation of chemokinesis

(Lokuta et al. 2003). The current study provides preliminary evidence for the involvement of calpain in T cell chemotaxis. Further examination of calpain's involvement in the chemotaxis of native T cells and pathological T cells from MS patients and EAE animals is supported by the findings of this study.

MATERIALS AND METHODS

Cell Culture and Reagents

The T cell type used was the Jurkat E6-1 line (ATCC, Manassas, VA) grown in RPMI 1640 (MediaTech, Inc., Herndon, VA) media supplemented with L-glutamine, 100 U/ml penicillin and 1 µg/ml streptomycin (Gibco, Grand Island, NY) and 10% FCS (Hyclone, Logan, UT) at 37^0C in a 5% CO_2 atmosphere (Thermo Fisher Scientific, Waltham, MA) to desired confluency for each of the assays performed. This cell type was selected due to the ease of use and quantities available. Fura-2 (Invitrogen, Molecular Probes, Carlsbad, CA) was used as the calcium indicator dye for the intracellular free calcium assays. Calcein AM (Invitrogen) was used in the migration assay as the cellular tracking dye. Calpeptin (Calbiochem, San Diego, CA) was used as the pan-calpain inhibitor and CCL2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was the chemokine used as the chemoattractant in the migration assays. Cytochalasin D (Sigma, St. Louis, MO) was also used in the migration assay. CMAC, *t*-BOC-Leu-Met A6520 (Invitrogen) was used in the calpain activity assay as the fluorescent calpain specific substrate.

Intracellular Free Calcium Assay

The level of intracellular free [Ca^{2+}] was measured in Jurkat cells using the ratiometric fluorescent calcium indicator fura-2 according to our described method (Das et al. 2005) with slight modification for cell type. Restated is the modified method for intracellular free [Ca^{2+}] determination (Grynkiewicz et al.

1985). Briefly, cells were grown to 5-6 x 10^5 cells/ml in supplemented RPMI 1640, pelleted by centrifugation, washed once with phosphate buffered saline (PBS) and resuspended in 1-2 ml Locke's Buffer (154 mM NaCl, 5.6 mM KCl, 3.4 mM $NaHCO_3$, 1.2 mM $MgCl_2$, 5 mM HEPES, 1 mM $CaCl_2$ and 5.6 mM glucose adjusted to pH 7.4). The cells were then counted on a hemocytometer and Locke's Buffer was added to adjust the concentration to 0.5 - 1 x 10^6 cells/ml for dye loading. Fura-2 AM was dissolved in DMSO and diluted in the cells to a final loading concentration of 5 µM. The cells were loaded with the dye at 37^0C for 30 minutes with shaking and protected from light. The cells were then washed 2 times with ice cold Locke's Buffer without Ca^{2+} and diluted with 4^0C Locke's Buffer to a final concentration of 0.5 x 10^6 cells/ml and stored on ice until the assay was performed but no longer than 1 hour. Assays were performed on 1 x 10^5 cells in 180 µl in a 96 well plate format using a Gemini XPS fluorescent plate reader (Molecular Devices, Sunnyvale, CA) and fluorescence was read using dual excitation and a fixed emission at ex. 340 nm / 380 nm and em. 510 nm. The plate was loaded with the appropriate number of cells and then incubated at 37^0C for 10 minutes to stabilize the temperature then a basal 2 minute kinetic reading was taken. The experimental test compound was then added to the cells in 20 µl and 5 minute kinetic reading was taken. The fluorescence at the maximum free [Ca^{2+}] was then determined by adding 25 µl of 250 µM digitonin (Fisher Scientific, Pittsburg, PA). The fluorescence at the minimum free [Ca^{2+}] was then determined by the addition 25 µl of 50 mM EGTA (Sigma). The intracellular free [Ca^{2+}] was calculated from these fluorescence values using the

equation: $[Ca^{2+}] = K_d (R-R_{min})/(R_{max}-R) \times (F^{380}_{max}/F^{380}_{min})$ The cell specific value of K_d was determined experimentally to be 127 nM for Jurkat cells by using the Calcium Calibration Buffer Concentrate Kit (Invitrogen). The value R is the ratio of fluorescence emission from the two excitation wavelengths following exposure to the test compound. The values R_{max} and R_{min} are the ratio of fluorescence emissions following exposure to digitonin and EGTA respectively. The value F^{380}_{max} is the resultant fluorescence emission that was measured by excitation at 380 nm when the free $[Ca^{2+}]$ was zero and was obtained during the EGTA exposure. The value F^{380}_{min} is the resultant fluorescence emission that was measured by excitation at 380 nm for saturating levels of free $[Ca^{2+}]$ and was obtained during the digitonin exposure.

Migration Assay

The migration assay was performed with a 96 well format Boyden Chamber (Neuroprobe, Gaithersburg, MD) following the described methods (Frevert et al. 1998) with modification for the Jurkat cell type and chemoattractant optimization. Specifically, Jurkat cells were grown to confluency as described above to a final concentration of 1×10^6 cells/ml. Cells were pelleted by centrifugation and resuspended in RPMI 1640 without phenol red (MediaTech, Inc.) supplemented with 0.1% bovine serum albumin (BSA) (Fisher Scientific). Calcein AM was dissolved in DMSO and added to the cells at a final concentration of 2 µM and incubated at 37^0C for 45 minutes with shaking. The cells were then washed 2 times with 37^0C RMPI 1640 without phenol red and

resuspended at a concentration of 4 x 10^6 cells/ml in preparation for loading the chamber. The chemokine, CCL2, was diluted to experimentally determined optimal concentration in RPMI 1640 without phenol red and added to a 30 µl 96 well plate (Neuroprobe). A polycarbonate filter with 5 µm pores (Neuroprobe) was then attached to the plate. The migration chamber was then assembled and cells in 50 µl were added to the top well. The top well volume was then filled with 150 µl of media or media containing an appropriate test compound. The whole chamber was incubated for 3 hours protected from light in a 37^0C humidified incubator with 5% CO_2 atmosphere. The chamber was then disassembled and the filter was gently scraped with a cell scraper (Fisher Scientific) and washed with PBS to remove any attached cells. The plate, with the filter still attached, was then centrifuged at 400 x g for 10 minutes (Eppendorf, Westbury, NY). The filter was then carefully detached and the plate read on the fluorescent plate reader at excitation 485 nm and emission 535 nm.

Calpain Activity Assay

The intracellular calpain activity was assessed following the method published by (Shao et al. 2006) with modification for cell type and instrumentation. Briefly, Jurkat cells were grown to desired confluency as described before and then washed into RPMI 1640 without phenol red and 0.1% BSA. The cells were placed in 35mm culture plates (MatTek Corp., Ashland, MA) with incorporated cover slip for live cell microscopic imaging and kept at 37^0C until labeling. The plates were then placed into a temperature and

atmospherically controlled stage of a Zeiss LSM 510 NLO laser scanning confocal/multiphoton microscope (Zeiss, Thornwood, NY) equipped with a Coherent Chameleon tunable femtosecond Ti-Sapphire laser (Coherent, Inc., Santa Clara, CA) with META spectral detection (Zeiss). The instrument was focused before labeling was performed. The amino acid conjugated coumarin based fluorescent dye CMAC, *t*-BOC-Leu-Met was then injected into the dish at a final concentration of 50 μM. Time lapse microscopic images were then captured every 15 seconds for 20 minutes. Various treatments including addition of chemoattractant and calpain inhibitor were added and then further time lapse captures were performed. Average spot density from cells in the field were then averaged and plotted against time to determine increasing fluorescence levels over time (dF/dt).

Statistical Analysis

All data are shown as means ± SEM. Data from various experiments was analyzed using SPSS software (SPSS, Inc., Chicago, IL). Statistical significance was determined by using either the unpaired Student's *t* test or one way ANOVA with Games- Howell post hoc test at a 95% confidence interval when multiple group comparisons were required. Statistical significance was obtained when p was ≤ 0.05.

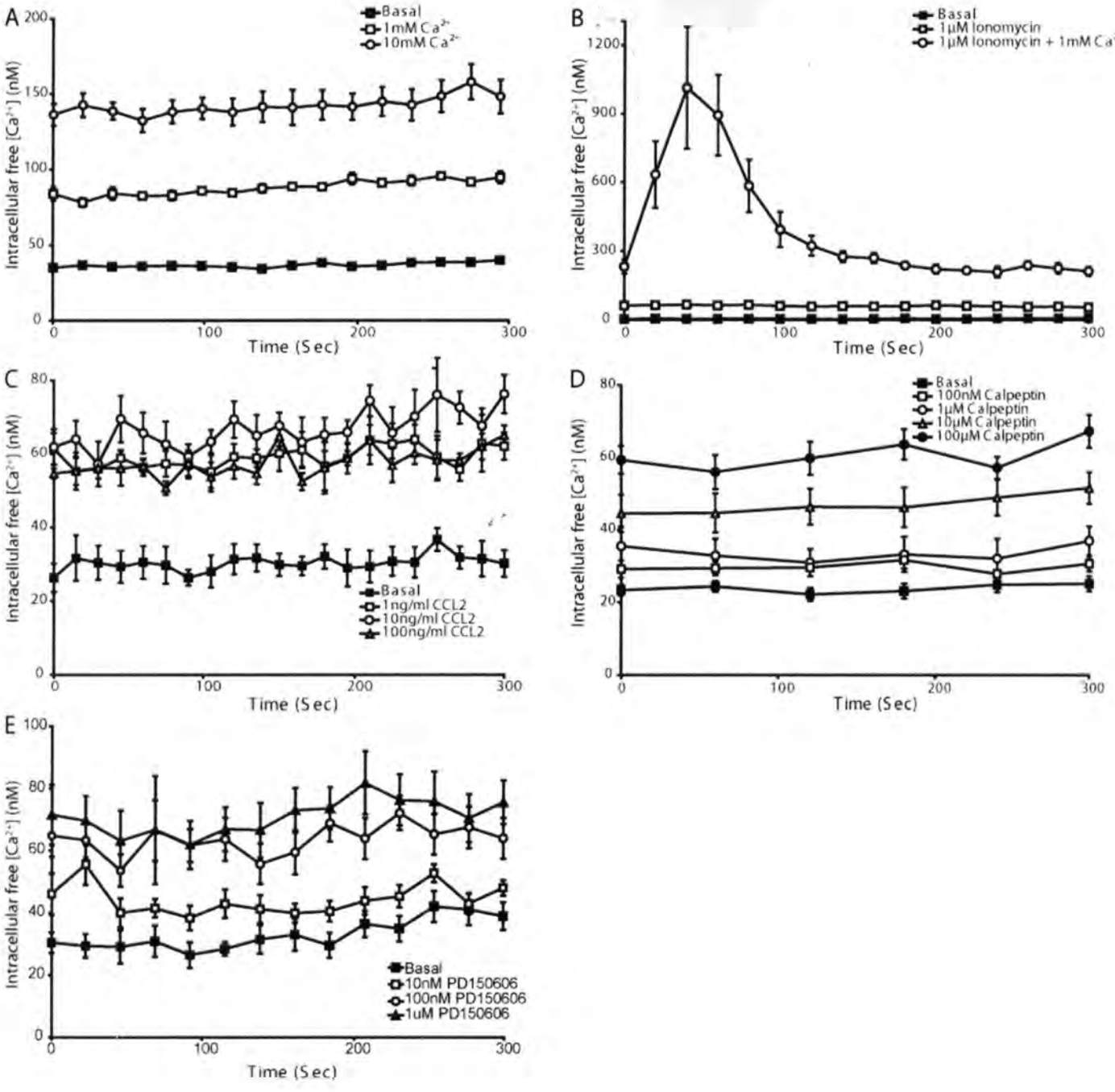
RESULTS

Intracellular Free [Ca^{2+}] Level in Response to Chemokine and Calpain Inhibitor

To investigate if an environment that was favorable for calpain activation was present in the Jurkat cells during exposure to chemoattractant the intracellular free calcium concentrations were explored. It has been reported that certain calpain inhibitors can block the elevation of intracellular free [Ca^{2+}] levels (Liu et al. 2002) and this effect was also examined. The Jurkat cells were first exposed to various extracellular calcium concentrations to determine the effects on intracellular free [Ca^{2+}] levels (figure 9A). Intracellular free [Ca^{2+}] increased corresponding to the concentration dependent increase in the extracellular [Ca^{2+}] levels. In order to confirm that our method for measuring intracellular free [Ca^{2+}] would measure calcium spikes, indicative of extracellular calcium signaling, 1 μM of ionomycin was added to the assay with and without 1 mM extracellular [Ca^{2+}] (figure 9B). This positive control demonstrated a large calcium spike in contrast to the low level steady state elevation. The chemokine CCL2 was observed to elevate intracellular [Ca^{2+}] levels when exposed to concentration of 1 ng/ml to 100 ng/ml (figure 9C). When the cells were exposed to sub ng/ml levels of CCL2, 0.01 ng/ml, the change in intracellular free [Ca^{2+}] level was no longer observed (data not shown). An intracellular free [Ca^{2+}] elevation in the Jurkat cells was also observed when exposed to various concentrations (100 nM to 100 μM) of the calpain inhibitor calpeptin (figure 9D). The observed trend was increased levels of intracellular free [Ca^{2+}] corresponding to increased

concentration of calpeptin. Elevation of intracellular free $[Ca^{2+}]$ was also observed in response to various concentrations (10 nM to 1μM) of the calpain inhibitor P150606 (figure 9E), which binds to the calcium binding site of calpain to prevent activation.

Figure 9: Intracellular Free [Ca^{2+}] in Response to Various Ionic Stimulus, Chemokine Exposure, and Protease Inhibition. Intracellular free [Ca^{2+}] changes were measured using the ratiometric intracellular fluorescent dye fura-2 loaded into Jurkat E6-1 cells and read on a fluorescent plate reader following exposure to experimental compounds. **A:** Intracellular free [Ca^{2+}] rise to a steady state in response to elevated extracellular [Ca^{2+}]. **B:** A calcium ionophore, ionomycin, effectively transports Ca^{2+} across the plasma membrane and creates an intracellular free [Ca^{2+}] spike in the cell. **C:** The chemokine CCL2 causes an elevation in intracellular free [Ca^{2+}] at an optimum concentration of 10 ng/mL. **D:** The calpain inhibitor, calpeptin, causes an elevation of intracellular free [Ca^{2+}] in a concentration dependent trend. **E:** The calpain inhibitor, PD150606, causes an elevation of intracellular free [Ca^{2+}] in a concentration dependent trend. Each time point represents the mean ± SEM (n = 6-8/time point).


A
Basal
1mM Ca2+
10mM Ca2+
Intracellular free [Ca2+] (nM)
Time (Sec)
B
Basal
1μM Ionomycin
1μM Ionomycin + 1mM Ca2+
Intracellular free [Ca2+] (nM)
Time (Sec)
C
Basal
1ng/ml CCL2
10ng/ml CCL2
100ng/ml CCL2
Intracellular free [Ca2+] (nM)
Time (Sec)
D
Basal
100nM Calpeptin
1μM Calpeptin
10μM Calpeptin
100μM Calpeptin
Intracellular free [Ca2+] (nM)
Time (Sec)
E
Basal
10nM PD150606
100nM PD150606
1uM PD150606
Intracellular free [Ca2+] (nM)
Time (Sec)

CCL2 Induced Jurkat Cell Migration is Largely Chemotactic

Cellular migration can be divided into increased random motion and increased directed motion. Chemokinesis is the increase in random motion caused by a cytokine, while chemotaxis is directed motion toward a chemokine. A standard method (Colvin et al. 2004; Frevert et al. 1998; Wilkinson 1998) of measuring cellular migration is through the use of a Boyden chamber which is composed of two compartments separated by a membrane with holes smaller than the resting diameter of the cell. In order for cells to move to the lower compartment and be detected by the assay, they must actively change their shape and migrate through a pore in the membrane. Jurkat cells placed into a Boyden chamber will eventually equilibrate over time as random migration of the cells carry them through the filter membrane (figure 10A) which over the 3 hours of the experiment appears as migration for the unstimulated group. The effects of various types of chemokine stimulus can be assessed by where in the chamber compartments the compounds are placed relative to the cells. CCL2 stimulation in both a chemokinetic fashion, chemokine above and below the filter, and in a chemotactic manner, chemokine below the filter, produced significant migration ($p < 0.05$) compared to the unstimulated group. The major difference that was observed in the chemotaxis stimulated group ($p < 0.05$) was significantly different than both the unstimulated (58% different) and chemokinetically stimulated (44% different) groups. Increased chemotaxis was observed as a concentration dependent response (figure 10A) toward higher concentrations of

CCL2 until the cells reached maximal chemotaxis at 50 ng/ml CCL2. Concentrations of CCL2 above 50 ng/ml did not increase chemotaxis.

Calpain Inhibition is Restricted to Reducing Chemotaxis in Jurkat Cells Stimulated with CCL2

Previous work on other immune cell types have reported that calpain is a negative regulator of cell migration and that calpain inhibition leads to increased chemokinesis (Lokuta et al. 2003). In order to determine if calpain plays a similar negative regulator role for chemokinesis in Jurkat T cells migration assays were performed with a calpeptin gradient without chemokine stimulation (figure 10B). In this unstimulated environment (no CCL2 present) cellular migration was not significantly different than control (no calpeptin) for any of the calpeptin concentrations tested. Jurkat T cells migration assays were also performed with chemokine stimulation in a chemokinetic environment (figure 10C). In this chemokinetically stimulated environment, CCL2 (50 ng/ml) above and below the membrane, cellular migration was also not significantly different than control (no calpeptin) for any of the calpeptin concentrations tested. In contrast, calpain inhibition did produce a significant ($p < 0.05$) concentration dependent reduction in chemotaxis toward CCL2 (50 ng/ml) (figure 10D). Chemotaxis was reduced by 31.1%, 39.5%, and 68.1% at 1 μM, 10 μM and 100 μM of calpeptin respectively.

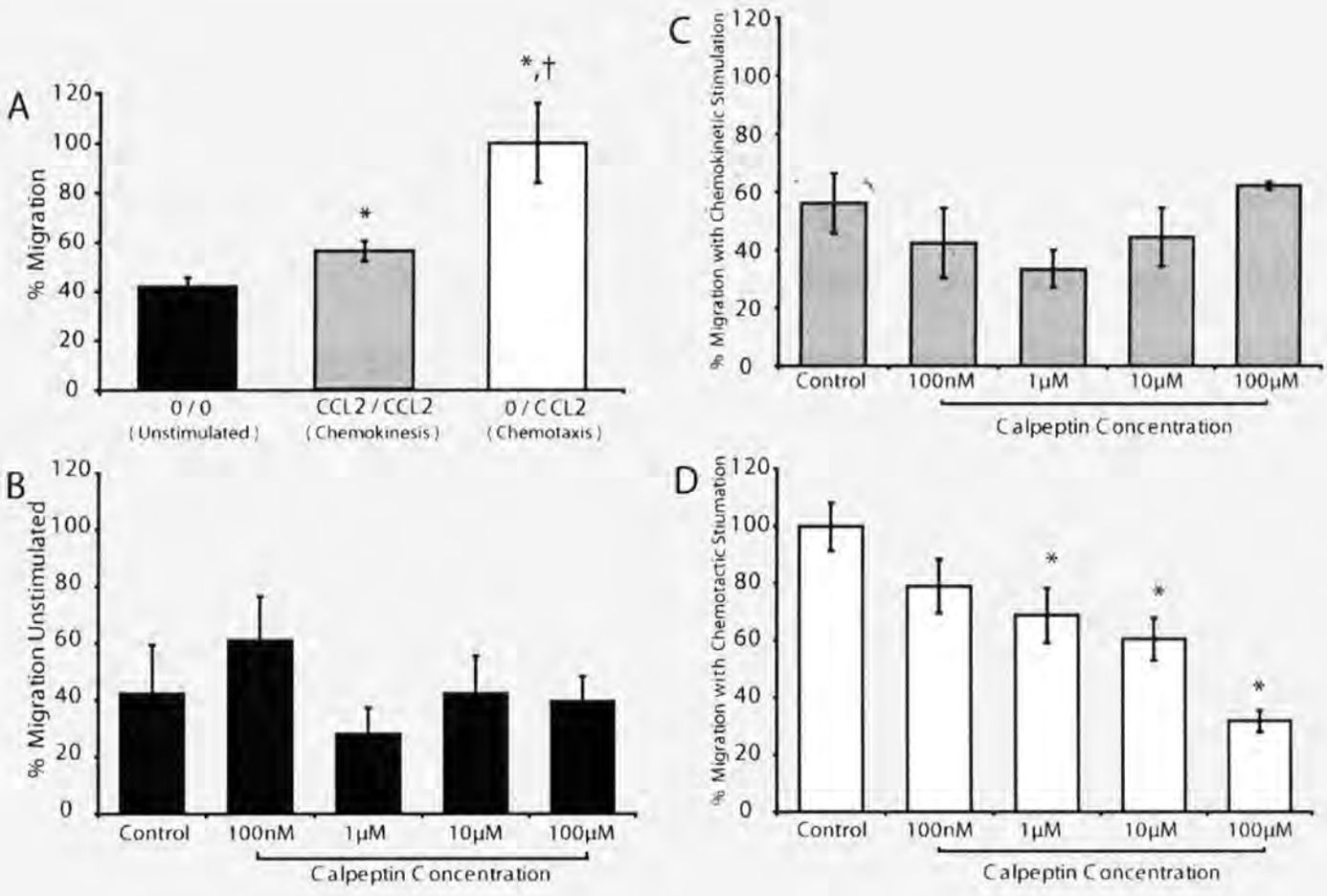


Figure 10: Effect of Calpain Inhibitor, Calpeptin, on CCL2-Induced Chemokinesis and Chemotaxis in Jurkat E6-1 Cells. Jurkat cells were loaded with the fluorescent dye calcein AM to aid in cell tracking and then loaded into a 96 well Boyden Chamber with various treatments to assess migration status. **A:** CCL2 (50 ng/ml) was placed both above and below the chamber's filter membrane to study the chemokinetic response or was only placed below the filter to study the chemotactic response. Mean ± SEM (n = 8). *, $p < 0.05$ vs unstimulated; †, $p < 0.05$ vs chemokinesis. **B:** Calpain inhibition with calpeptin in the absence of CCL2 did not significantly alter the levels of migration at any of the concentrations tested. **C:** Inhibition of calpain with calpeptin did not significantly alter the migration of Jurkat cells in response to a chemokinetic stimulation (50 ng/ml CCL2 above and below the filter membrane). **D:** Calpeptin caused a concentration dependent inhibition of migration of cells toward CCL2 (50 ng/ml). Mean ± SEM (n = 8). *, $p < 0.05$ vs vehicle (Set as 100% chemotaxis).

Calpain Inhibition also Reduces Chemotaxis in Human PBMCs Stimulated with CCL2

Migration assays were also performed on normal human PBMCs in response to chemotactic stimulation by CCL2 in the presence of the calpain inhibitor calpeptin. Calpain inhibition produced a significant ($p < 0.05$) concentration dependent reduction in chemotaxis toward CCL2 (100 ng/ml) (figure 11). Chemotaxis was reduced by 19.4%, 42.1%, and 41.7% at 1 μM, 10 μM and 100 μM of calpeptin respectively. Chemotaxis also trended toward reduced migration with the two lower concentrations of calpeptin of 10 nM and 100 nM.

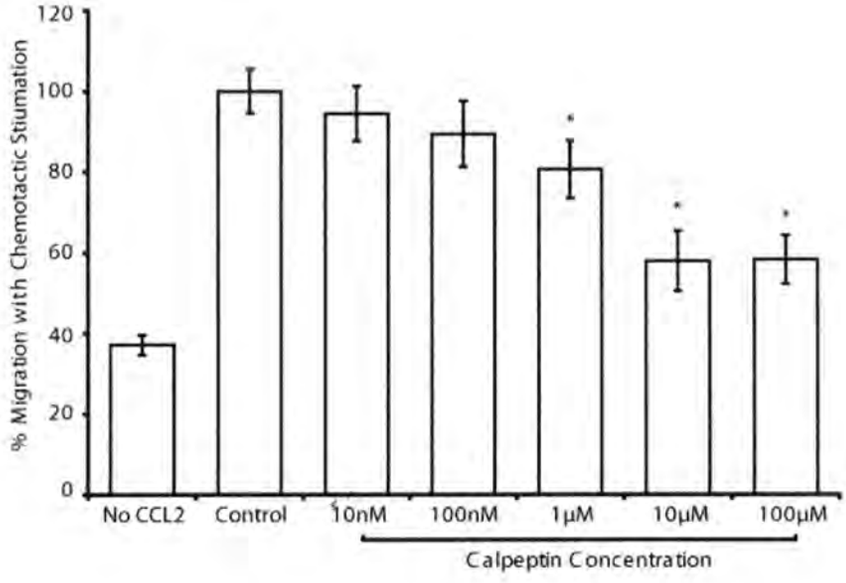


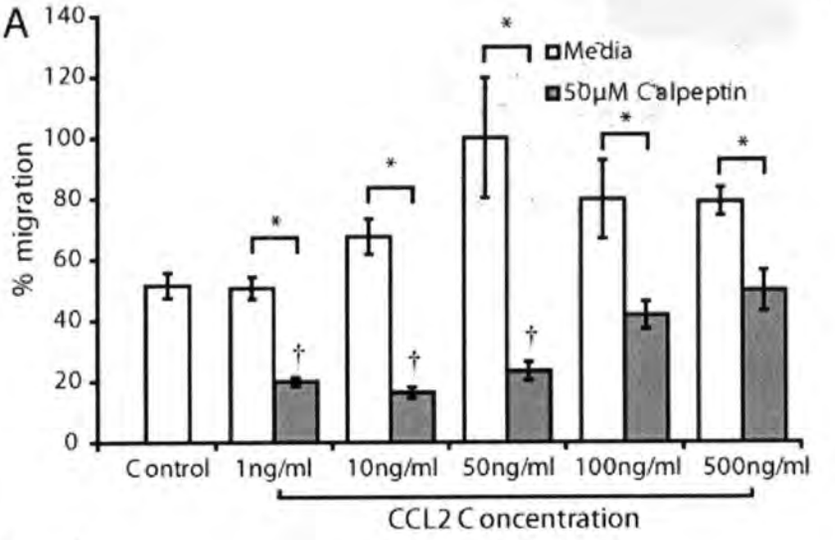
Figure 11: Effect of Calpain Inhibitor, Calpeptin, on CCL2-Induced Chemotaxis in Human PBMC Cells. PBMCs were loaded with the fluorescent dye calcein AM to aid in cell tracking and then loaded into a 96 well Boyden Chamber with various treatments to assess migration status. Calpeptin caused a concentration dependent inhibition of migration of cells toward CCL2 (100 ng/ml). Mean ± SEM (n = 3). *, $p < 0.05$ vs control (Set as 100% chemotaxis).

Calpain is Part of a Chemotaxis Signaling Cascade

The number of cells that undergo chemotaxis toward a signal is dependent upon the concentration of that signal. The optimal concentration of CCL2 to induce maximal chemotaxis was determined by performing the migration assay with a chemoattractant gradient (figure 12A). The highest level of chemotaxis was observed at 50 ng/ml of CCL2 even though the chemotactic effect appeared to plateau at concentrations greater the 50 ng/ml. Calpain inhibition with calpeptin at the experimentally derived ID_{50} for Jurkat cell chemotaxis toward CCL2 (50 μM) produced a significant inhibition of chemotaxis ($p < 0.05$) for all of the CCL2 concentrations tested compared to chemotaxis without calpain inhibition (figure 12A). At the lower concentrations of CCL2 (1 ng/ml – 50 ng/ml) there was significant inhibition ($p < 0.05$) of chemotaxis with calpeptin compared to control levels. This significance disappeared at the higher concentrations of CCL2 (100 ng/ml and 500 ng/ml). The polymerization of actin is a non-signaling mechanism that is required for migration to occur (Vicente-Manzanares et al. 2005). Inhibition of this process with cytochalasin D (25 μM) produced a significant inhibition ($p < 0.05$) of chemotaxis at CCL2 concentrations of 10ng/ml through 500 ng/ml (figure 12B). The percentages of chemotaxis in the cytochalasin D groups compared to control were not significantly different at any of the concentration of CCL2 tested. Cytochalasin D (25μM) and calpeptin (50 μM) were also tested together in order to determine if the migratory inhibition effect was additive by using both compounds (figure 12C). Inhibition of this process with cytochalasin D (25 μM) and calpeptin (50 μM) produced a

significant inhibition ($p < 0.05$) of chemotaxis at CCL2 concentrations of 10ng/ml through 500 ng/ml compared to the media only group. Significant difference between the cytochalasin D group (figure 12B) and the combined treatment group (figure 12C) was not observed though the combined group trends toward less chemotaxis.

Figure 12: Effect of Chemoattractant Concentration on Calpain Inhibitor-Induced Reduction in Chemotaxis: A Response Distinct from the Inhibition by Cytochalasin D. Chemoattractant was loaded in the lower wells of a 96 well Boyden chamber in increasing concentrations while Jurkat cells were loaded in the upper chamber. **A:** The chemotaxis of Jurkat cells in the increasing chemoattractant gradient is compared to chemotaxis in the same gradient when an IC_{50} concentration of calpeptin was added to the cells in the upper wells. Chemotaxis of Jurkat cells was highest at an optimum CCL2 concentration of 50 ng/ml. Calpeptin-induced reduction in chemotaxis was observed only up to 50 ng/mL of CCL2 and the effect was lost at higher CCL2 concentration. Mean ± SEM (n = 8). *, $p < 0.05$ between groups for media in upper chamber vs calpain inhibitor; †, $p < 0.05$ migration inhibition compared to control. **B:** The chemotaxis of Jurkat cells in the increasing chemoattractant gradient is compared to chemotaxis in the same gradient when the actin polymerization inhibitor, cytochalasin D was added to the cells in the upper wells. Cytochalasin D groups were not significantly different than control, however it could significantly attenuate the CCL2 induced chemotaxis. Mean ± SEM (n = 8). *, $p < 0.05$ between groups for media in upper chamber vs cytochalasin D. **C:** The chemotaxis of Jurkat cells in the increasing chemoattractant gradient is compared to chemotaxis in the same gradient when cytochalasin D and calpeptin was added to the upper wells. Combined treatment group displayed the same trend as cytochalasin D treatment alone. Mean ± SEM (n = 4). *, $p < 0.05$ between groups for media in upper chamber vs calpeptin and cytochalasin D.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50μM Calpeptin
Control
1ng/ml
10ng/ml
50ng/ml
100ng/ml
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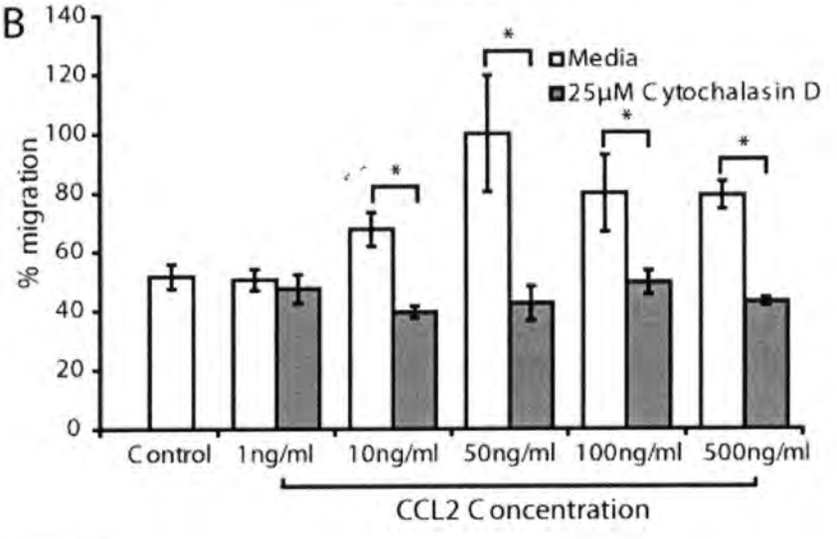
CCL2 Concentration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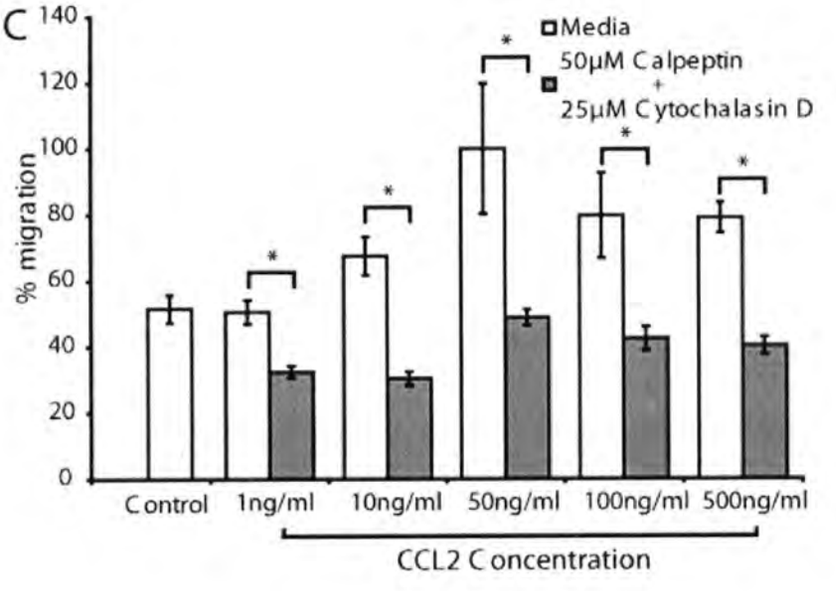
C
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500ng/ml
CCL2 Concentration

Direct Observation of Calpain Activity in Response to CCL2 Exposure

The effect of calpain on chemotaxis has been indirectly inferred in the previous experiments from effects observed with a biochemical inhibitor. In order to directly assess if calpain activity increased in response to CCL2 stimulation live cell imaging of a fluorescent dye coupled to a calpain substrate was used. The specificity of CMAC, *t*-BOC-Leu-Met as a calpain substrate and thereby indicator of calpain activity has been previously shown (Shao et al. 2006) in hepatocytes. Jurkat E6-1 cells used in this study displayed a basal level of calpain activity (figure 13A-D) so the protocol was modified accordingly for this cell type. Due to this basal activity simple fluorescence compared to non-fluorescence would not indicate altered activity. Sample micrographs from three groups of time lapse series (figure 13A) demonstrated the basal level of activity with increased fluorescence over time as well as increased fluorescent over time in the CCL2 (50 ng/ml) group. The CCL2 (50 ng/ml) + calpeptin (100 μM) series demonstrated the specificity of CMAC, *t*-BOC-Leu-Met as a calpain substrate. The masking examples (figure 13B) demonstrated the importance of choosing cells at random from the field of view due to the differential response in the culture between strong responders and weak responders. The same mask was used at each time point to track the same cells for the entire time of the experiment. The average spot density of the representative cells (10 for each field) were graphed against time for each of the exposures (figure 13C) revealing the differential response. Both the basal and the CCL2 groups revealed calpain activity while the CCL2 + calpeptin group did not show any activity. This data

was quantified by comparing the rate of increasing fluorescence (dF/dt) (figure 13D). The CCL2 stimulated group demonstrated a dF/dt that was significantly higher ($p < 0.05$) than the unstimulated basal group. The CCL2 + calpeptin group had a dF/dt significantly lower ($p < 0.05$) than both the basal and the CCL2 stimulated groups.

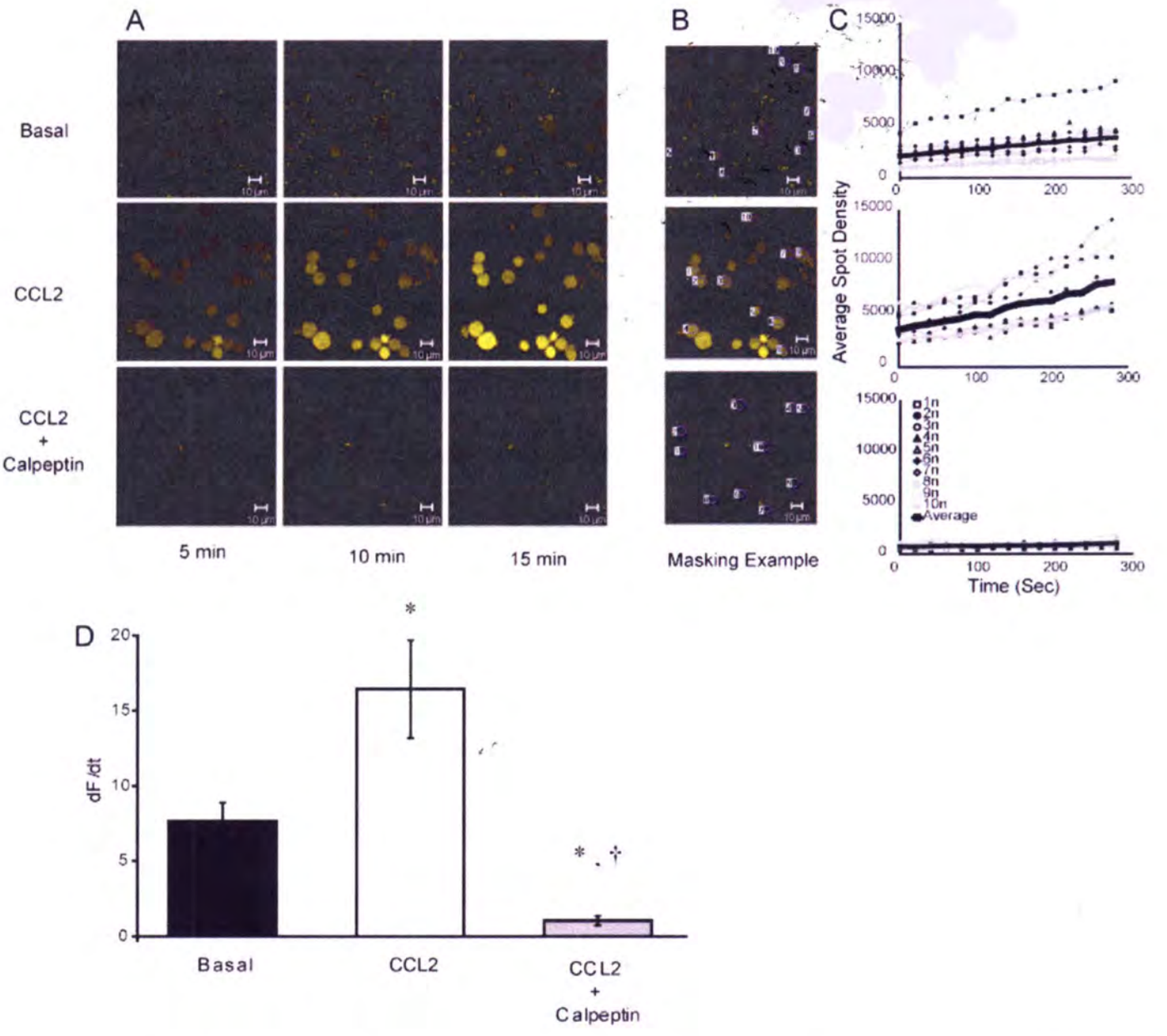


Figure 13: Intracellular Calpain Activity Increased in Response to the Chemokine CCL2. Increased calpain activity was imaged in live Jurkat E6-1 cells based on the increasing fluorescence of the calpain specific dye CMAC, *t*-BOC-Leu-Met following exposure to chemokine (50 ng/ml CCL2) or calpain inhibitor (100 µM calpeptin). **A:** Representative micrographs from a 15-min time lapse exposure measuring fluorescence of 50 µM CMAC at 370 nm. **B:** Representative images of spot density selections of random cells which was repeated for every time point. **C:** The average spot density of ten individual cells over the time lapse series for each exposure group. The mean of the ten cells is also included on each of the graphs. **D:** The change in average fluorescence density for the mean of each experimental group. Mean ± SEM (n = 10). *, $p < 0.05$ between the experimental groups and basal dF/dt; †, $p < 0.05$ between the CCL2 group and CCL2 + Calpeptin group.

DISCUSSION

Migration of the adaptive immune system's cellular components into pathological areas in response to chemokine signaling is important for organisms to fight infection. The existence of autoimmune disease is evidence that this vital immune response does not always function in a way that is beneficial to the organism as a whole. T cell migration into areas of inflammation is a critical step in their role in the pathology of the autoimmune conditions in which they participate. T cell infiltration into the CNS of MS patients and EAE animals has a strong correlation with the disease pathology observed. The infiltrating inflammatory cells and T cells have been shown to have increased calpain expression and activity (Shields et al. 1998). Even more important, treatment with calpain inhibitor has the ability to reduce the clinical score in EAE and also improve the morphology of the CNS tissue (Guyton et al. 2006; Hassen et al. 2006).

Calpain has been shown to have a role in the migration of various cell types but, as of yet, never in the adaptive immune cell type of T cells. Evidence for calpain's direct involvement in chemotaxis toward CCL2 was presented in these experiments and its differing role in cellular migration from the innate immune cell type of neutrophils (Lokuta et al. 2003). The intracellular environment in response to CCL2 was demonstrated as supportive for calpain activation (figure 9C). Other work (Bach et al. 2007) has also demonstrated an intracellular calcium elevation in response to the chemokine CXCL12 (SDF-1α) which provides evidence for the involvement of calpain in the chemotactic

response to various chemokines and is not limited to CCL2. Whenever intracellular calcium levels change to another steady state, calpain activity should be investigated. Our use of calpeptin also appears to cause a mild elevation in steady state intracellular free calcium levels (figure 9D). Previous work has demonstrated that certain calpain inhibitors have the ability to block further calcium uptake (Liu et al. 2002). Calpeptin is of a class of protease inhibitors that binds to the active site of calpain and not to the calcium binding site (Ray et al. 2003a; Wang 1990). This means that irrespective of the resulting intracellular calcium level in response to calpeptin treatment, calpain activity will still be inhibited. The intracellular free calcium levels were also measured in response to the calpain inhibitor PD150606 which inhibits calpain by binding to the calcium binding site (Wang et al. 1996) to prevent protease activation and a similar mild elevation of intracellular calcium levels was also observed (figure 9E). This mild elevation could be an inherent property of all calpain inhibitors due to homeostatic feedback control of basal calpain activity on intracellular free [Ca^{2+}] but it is small enough to go undetected in most studies and is not large enough to alter the calpain inhibition.

Our current work supports that of previous work with CCL2 in that it functions on T cells primarily as a chemotactic cytokine and has limited chemokinetic properties (Carr et al. 1994). Calpain inhibition appears directly concentration dependent with respect to chemotaxis reduction, in that the greater the inhibition the greater the reduction in chemotaxis. Our current work differs from previous reports (Lokuta et al. 2003) in that the demonstration of a

significant role for calpain in the process of chemokinesis was not possible but our results were primarily restricted to chemotaxis (figure 10). There are a number of possible reasons for this discrepancy including differing cell types, sensitivity of assays used, various migratory stimulation utilized and various calpain inhibitors used. While the difference is note worthy between previous studies, the disease relevance is more important for directed migration which would guide pathogenic T cells to the CNS in EAE and MS. The observation of this inhibitory effect in Jurkat T cells (figure 10D) as well as in normal human PBMCs (figure 11) adds increased evidence to its relevance as a disease relevant process. The exact role of calpain in chemokine induced chemotaxis is as of yet unknown, but evidence points to its involvement in a signaling pathway. The ability of an increasing concentration of CCL2 to overcome calpeptin induced chemotactic inhibition (figure 12A) lends evidence to a role in a signaling pathway. When a known downstream effect, polymerization of actin, of migration is blocked with a pharmacological agent, cytochalasin D, the inhibition and recovery effect observed with the calpain inhibitor is not reproduced, further strengthening the evidence for the specific involvement of a calpain-mediated mechanism in T cell chemotaxis. Chemotaxis was also examined with a combined treatment of calpeptin and cytochalasin D to determine if the compounds produced an additive effect. The combined treatment (figure 12C) produced a profile similar to cytochalasin D alone (figure 12B) but with a non-significant trend toward slightly greater inhibition. This profile provides further

evidence that the cytochalasin D acts downstream of calpain in the migration mechanism as the cytochalasin D profile alone (figure 12B) is reproduced.

The current work has been performed on Jurkat E6-1 cells to provide preliminary data in a T cell like cell before expansion into cells directly obtained from and derived from MS patients and EAE animals. In order to add more relevance to this work as a transition to work in more disease relevant models a similar observed effect in normal human PBMC was presented (figure 11). While the current work does not provide direct evidence for therapeutic intervention it does support the continued expansion into more disease relevant models. The current study also does not address the specific isoforms of calpain that may be involved with migration. The two major ubiquitous calpains μ-calpain and m-calpain are often expressed at different levels in various cell types which may also be the case of the Jurkat cells. The current study identifies a basal level of calpain activity (figure 13) and also calpain expression (data not shown). Calpain changes using western blot were also attempted, which would allow for identification of subtypes with various specific antibodies. Unfortunately, the changes were not large enough to detect differences between treatment groups. The short time frames in which migration occurs would tend to favor changes in activity over changes in protein expression. The lack of inhibitors specific for the subtypes is also a limitation for assessing protease subtype variations. Future studies are ongoing, with more disease relevant cell types, using RNAi to assess these more specific changes and narrow down the protease isotypes involved.

Current MS treatments are moderately effective at best (Kleinschnitz et al. 2007) and most treatments eventually loose effectiveness for many patients as the disease progresses. Therapies that target a mechanism selectively upregulated or active during the disease state, such as increased calpain activity in T cells from MS patients (Imam et al. 2007), provide a targeted approach that may prove an effective treatment. Calpain activity contributes to many cellular processes including apoptosis (Zhivotovsky et al. 1997), cell cycle regulation (Witkowski and Bryl 2004), T cell activation (Schaecher et al. 2001), and T cell chemotaxis (as shown in this investigation).

CHAPTER FOUR

THE INVOLVEMENT OF CALPAIN IN $CD4^+$ T HELPER CELL BIAS

INTRODUCTION

Multiple sclerosis (MS) is an autoimmune disease of the CNS that is thought in large part to be initiated and mediated by an inflammatory infiltration of the CNS by self reactive $CD4^+$ T helper cells (McFarland and Martin 2007). Different autoimmune diseases depend on the nature of the immune response and the proliferation of certain Th subtypes. MS is thought to be the result of the development of an inflammatory Th cell bias composed of overreactive Th1 and Th17 subtypes leading to a self directed inflammatory attack resulting in myelin degeneration and the pathological symptoms observed. T helper cell bias is controlled through the binding affinity of the antigenic peptide, the associated molecules present and the prevalent cytokine environment (Zhu and Paul 2008). The T cell differentiates through specific signaling pathways, including various STAT molecule subtypes which transmit the activation response to certain transcription factors upregulating specific cytokine loci and reinforce the T helper cell subtype. There is cross reactivity in the signaling pathways but taking a simplistic view Th1 subtype develops from IFN-γ and IL-12 signal through STAT1/T-bet to drive further cytokine expression and subtype reinforcement. Th2 subtype develops from IL-4 and IL-2 signaling through STAT6/GATA3. Th17 subtype develops through TGFβ, IL-1 and IL-6, IL-21, IL-23 signaling through STAT3/RORγt to develop and reinforce the subtype. Finally, the Treg subtype develops through TGFβ and IL-2 signaling though STAT5/Foxp3 to drive further regulatory cytokine secretion (Zhu and Paul 2008). This is a simplistic overview

of the signaling but gives a framework in which to identify some of the signaling components on which calpain may be acting.

Various STAT molecules are known substrates of the ubiquitously expressed calcium activated protease, calpain (Hendry and John 2004). Previous studies have shown that activated STAT6 is degraded in T cell hybridoma cells and this degradation can be prevented with calpain inhibitors (Zamorano et al. 2005). STAT6 which is known to contribute to the Th2 subtype bias through IL-4 (Zhu et al. 2001). The Th2 subtype also has the ability to down regulate the Th1 and Th17 type cells through cytokine secretion. Our lab and others have observed that EAE disease signs are reduced with calpain inhibitor treatment (Guyton et al. 2006; Hassen et al. 2006). The regulation of STAT6 through activation and its target transcription factors may be a mechanism through which calpain inhibition provides clinical benefit.

The lab has also demonstrated increased calpain activity in the infiltrating monocytes during acute EAE which tend to be biased toward the Th1 and Th17 subtypes (Shields et al. 1998). The correlation between calpain activity and T cell subtype may be circumstantial or may have real merit. From these observations, the hypothesis was formed that calpain activity is involved with T helper cell bias. This hypothesis was tested by utilizing primary MBP Ac1-11 specific T cell cultures biased toward the various T helper cell subtypes, as well as several MBP Ac1-11 specific T cell lines to perform proliferation assays, monitor cytokine profile, and perform western blotting for transcription and signaling molecules in the presence of various concentrations of several classes

of calpain inhibitors. These studies provide insight into the various roles calpain plays in Th cell bias and proliferation and increases our understanding of the role that T cells play in the pathophysiology of EAE and MS as well as the mechanisms involved by which calpain inhibitors decrease the disease signs of EAE.

MBP Specific T cell Culture

$CD4^+$ T helper cells (B10A.E3 or B10A.E2) were originally isolated from B10.A-$H2^a$ H2-$T18^a$/SgSnJ (Jackson Labs, Bar Harbor, ME) mice peritoneal lymph nodes (LN) following subcutaneous (s.c.) inoculation with MBP Ac1-11 in complete Freund's adjuvant (Rabinowitz et al. 1997). The cells were diluted to a single cell culture and stimulated with γ-irradiated (3000 rad) isogenic speloncytes, MBP Ac1-11 and rIL-2 (Invitrogen). The cells were grown in RPMI 1640 (MediaTech, Inc., Herndon, VA) supplemented with 10% FCS (Hyclone, Logan, UT), 10 mM HEPES (Sigma), 2 mM GlutaMAX (Invitrogen), and 50 μM β-mercaptoethanol (Gibco, Grand Island, NY). The cells were restimulated every 7-9 days with a 10 fold excess of γ-irradiated isogenic speloncytes, 50 μM MBP Ac1-11 and rIL-2 and maintained at 6-7 x 10^5 cells/ml. Media and rIL-2 was added during the first 72 hours and cells were split as needed to maintain the proper cellular concentration. At the time of restimulation the required cells were split off for assays. The MBP specific cell lines were utilized for the proliferation assay, cytokine profiling, western blotting, flow cytometry, subcellular localization microscopy and adoptive transfer of EAE.

Primary MBP Specific T cell Culture

Proliferation assay and cytokine profiling were performed on the various subtypes of helper T cells using cytokine biased primary culture. B10.PL mice (Jackson Labs) were injected s.c. with an emulsification of 400 μg of guinea pig

MBP in complete Freund's adjuvant (Difco, Lawrence, KS) at four locations over the back followed with 200 μg of pertussis toxin (Sigma) injected intraperitoneal (i.p.) at the time of inoculation and then again 48 hours later. Ten to twelve days later the draining LNs and the spleen were dissected out and fritted glass slides were used to grind the tissue into a single cell suspension and strained through a 70 μm cell filter (BD Biosciences) to remove the larger tissue debris. The LN cells were grown with a 10 fold excess of spleen cells and were stimulated with 50 μM MBP Ac1-11 in the same media as the MBP specific cell culture lines.

The various subtypes favored for proliferation with exogenous recombinant cytokines and neutralizing antibodies. For Th1 potentiation, IL-12 (10 ng/ml; R&D Systems, Minneapolis, MN) and anti-IL-4 (2 μg/ml; R&D Systems or Santa Cruz Biotechnology, Santa Cruz, CA). For Th2 potentiation, IL-4 (10 ng/ml; Cell Sciences, Canton, MA) and anti-IL-12 (2 μg/ml; R&D Systems). For Th17 potentiation, IL-1β and IL-23 (10 ng/ml; R&D Systems) and anti-IFN-γ and anti-IL-4 (10 μg/ml; R&D Systems). For Treg potentiation, IL-2 (150 U/ml; BD Biosciences) and $TGF\beta_1$ (1 ng/ml; R&D Systems) and anti-IFN-γ and anti-IL-4 (10 μg/ml; R&D Systems). The cytokines were chosen not to differentiate naïve T helper cells to the respective subtypes but rather to reinforce the proliferation of the differentiated subtypes already present in the culture. Supernatant samples were taken at 24 hours following plating and the media was replaced with fresh media and comparable cytokines and antibodies.

Proliferation Assay

The proliferation assay was set up on a restimulation day for the MBP specific T cell cultures or on the day of plating for the primary culture. The assay was performed in 96 well plates (Corning) with 8-9 x 10^4 MBP specific T cells per well and a 6-10 fold excess of γ-irradiated isogenic speloncytes as antigen presenting cells per well. Cells were activated with either the native MBP Ac1-11 peptide or a MBP Ac1-11 peptide with an alanine substitution at position 4 for a positive stimulation control. Various concentrations of calpain inhibitors were added to the wells. No rIL-2 was used in this assay during the stimulation. The primary culture had the appropriate cytokines and antibodies added to the media. After 24 hours 100 µl of supernatant was removed for cytokine profiling and 2 µCi of ^{3}H-thymidine in fresh media was added to each well. After 24 more hours the cells were lifted with trypsin EDTA (Invitrogen) treatment and harvested with a semi-automated cell harvester (Skatron Instruments, Sterling, VA) onto a filter paper membrane (Skatron). Each well's filter was added to a scintillation vial (Fisher Scientific) with 2 ml of Ecoscint A (National Diagnostics, Atlanta, GA) scintillation fluid. A TRI-CARB 2900TR Liquid scintillation analyzer (PerkinElmer, Shelton, CT) was utilized to determine the amount of ^{3}H-thymidine present in the cells as an indicator of DNA replication and proliferation.

Cytokine Profiling

Cells were grown to desired confluency and either plated in a 96 well plate for proliferation assay testing or in 6 well plates for large scale experiments. Cell

free supernatant was collected 24 hours after exposure to the specified treatments and frozen at -80^{0}C until the cytokines were assayed. OptIEA Sandwich ELISA (BD Biosciences) was utilized to test for individual cytokine levels in the various exposures following the standard protocol. A matched antibody pair (BD Biosciences) was utilized for IL-17A with a modified ELISA protocol based upon the BD kit instructions. The cytokines tested were IL-2, IFN-γ, IL-12p70, IL-4, IL-10 and IL-17A. The cytokine profile of the E3 cells and unbiased primary T cells were further explored using an inflammatory cytokine antibody array (Ray Biotech Inc, Norcross, GA) to more fully characterize the subtype profile. This array is an immunoblot array performed following the kit instructions and detected on a Fluochem FC2 Chemiluminescent CCD detection system (Alpha Innotech). AphaEaseFC (Alpha Innotech) software was used to calculate the densitometry of the various spots in the array and Excel (Microsoft) was used to graph the data.

Western Blotting

Briefly, the total protein samples were extracted following the lysis with ultrasonic homogenization of control and treated MBP specific T cells, quantitated spectrophotometrically, denatured in boiling water for 5 min, and loaded onto SDS-polyacrylamide gradient (4–20%) gel (Bio-Rad Laboratories, Hercules, CA). The proteins were resolved by electrophoresis in Laemmli buffer, and then electroblotted to ImmobilonTM-P membranes (Millipore, Bedford, MA, USA). For detection of each specific protein band on the blots, appropriate

dilutions of the primary IgG antibodies were used either 1:250 or 1:1000 depending on the supplier. STAT1, 4 and 6, T-bet and GATA3 primary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The blots were first incubated with the primary IgG antibody followed by incubation with the appropriate alkaline horseradish peroxidase (HRP)-conjugated secondary IgG antibody (ICN Pharmaceuticals, Aurora, OH). The ChemiGlow (Alpha Innotech, San Leandro, CA) H_2O_2 catalyzed substrate solution was added to the blots. The protein bands were then detected with a Fluochem FC2 Chemiluminescent CCD detection system (Alpha Innotech). The images were then processed using AphaEaseFC (Alpha Innotech) to quantify the OD units of the bands. β-Actin expression was used as a control for equal loading and to normalize the samples to one another.

Microscopy

The subcellular localization of transcription factors was visualized utilizing fluorescent immunohistochemistry with modification for cell type and instrumentation. Briefly, T cells were plated at standard concentration for 48 hours with designated experimental conditions. Cells were then lifted, washed twice with blocking buffer (BB), PBS with 1% goat serum (Hyclone) and 1% horse serum (Hyclone), and then fixed with -20^{0}C MeOH for 5 min. Cells were washed again with BB and then incubated for 40 min with 1:50 primary antibody at 4^{0}C. Cells were washed with BB then incubated for 30 min with 1:50 fluorescent secondary antibody at 4^{0}C protected from light. Cells were washed

again with BB and resuspended in a minimal amount of Vectashield with DAPI (Vector Labs, Burlingame, CA) and then mounted in 35 mm culture plates (MatTek Corp., Ashland, MA) with incorporated cover slip for microscopic imaging and kept at 4^0C until imaging. The plates were imaged with a Zeiss LSM 510 NLO laser scanning confocal/multiphoton microscope (Zeiss, Thornwood, NY) equipped with a Coherent Chameleon tunable femtosecond Ti-Sapphire laser (Coherent, Inc., Santa Clara, CA) with META spectral detection (Zeiss). The various fluorescent channels were imaged simultaneously and digitally overlaid.

Statistical Analysis

All data are shown at means ± SEM. Data from various experiments was analyzed using SPSS software (SPSS, Inc., Chicago, IL). Statistical significance was determined by using either the unpaired Student's *t* test or one way ANOVA with Games- Howell post hoc test at a 95% confidence interval when multiple group comparisons were required. Statistical significance was obtained when p was < 0.05.

Calpain Inhibition Increases Th2 Subtype Proliferation

The proliferation of several MBP Ac1-11 specific cell lines with various concentrations of several APLs was measured to demonstrate the proliferation potential related to antigenic stimulation. These studies were also performed to identify the proper stimulation to use for future studies. The APL (4A), MBP Ac1-11 with alanine at position 4, binds to MHC II much stronger than the native peptide (figure 5), produced increased proliferation compared to the proliferation that results from stimulation with the native antigen (4K). When the proliferation assay was performed on the E3 cells with various concentrations of the calpain inhibitor calpeptin, a concentration dependent proliferative response was observed. Figure 14A compares the ^{3}H-thymidine incorporation across the various stimulation and treatment groups. A more graded effect is observed with the native (4K) peptide stimulation. Figure 14B transforms the ^{3}H-thymidine incorporation into a percentage relative to each stimulation group's vehicle control set at 100%. This allows for a more direct comparison of the effect of calpeptin on proliferation. Figure 14B demonstrates that the maximum proliferation of the E3 cells is observed when 10nM of calpeptin is present in the culture. The proliferation of E3 is significantly greater than both controls when stimulated with the 4K peptide.

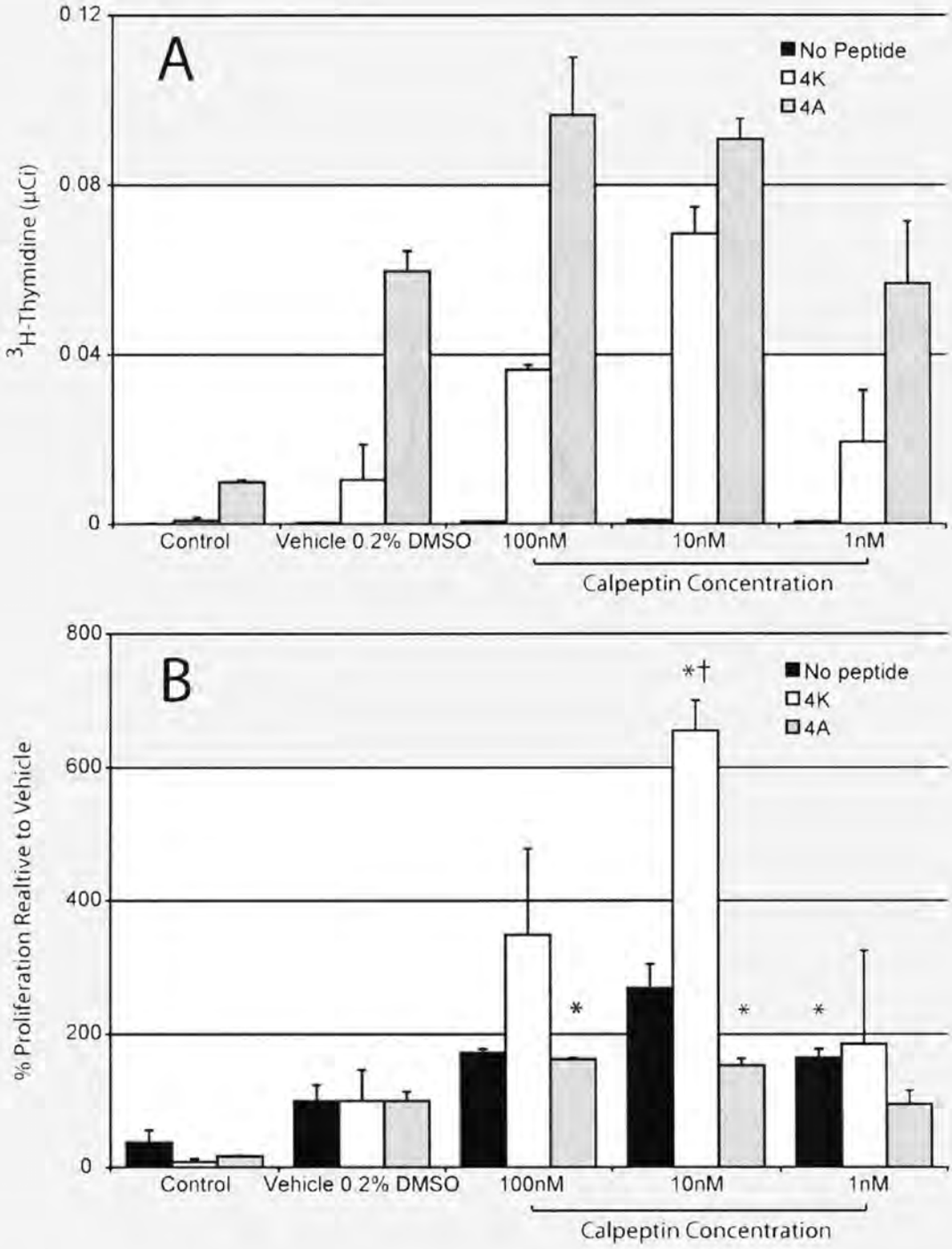


Figure 14: T Cell Proliferation with Various Stimuli. Proliferation of MBP Ac1-11 specific T cells either in the presence of no antigenic peptide, the native MBP Ac1-11 (4K) peptide, or a strong agonist APL MBP Ac1-11 with an alanine substitution at position 4 (4A). Cells were incubated in the presence of several concentration of the calpain inhibitor calpeptin. A, the raw ^{3}H-thymidine concentration B, ^{3}H-thymidine % relative to the vehicle group set at 100. Mean ± SEM (n = 3). *, $p < 0.05$ vs control; †, $p < 0.05$ vs vehicle group.

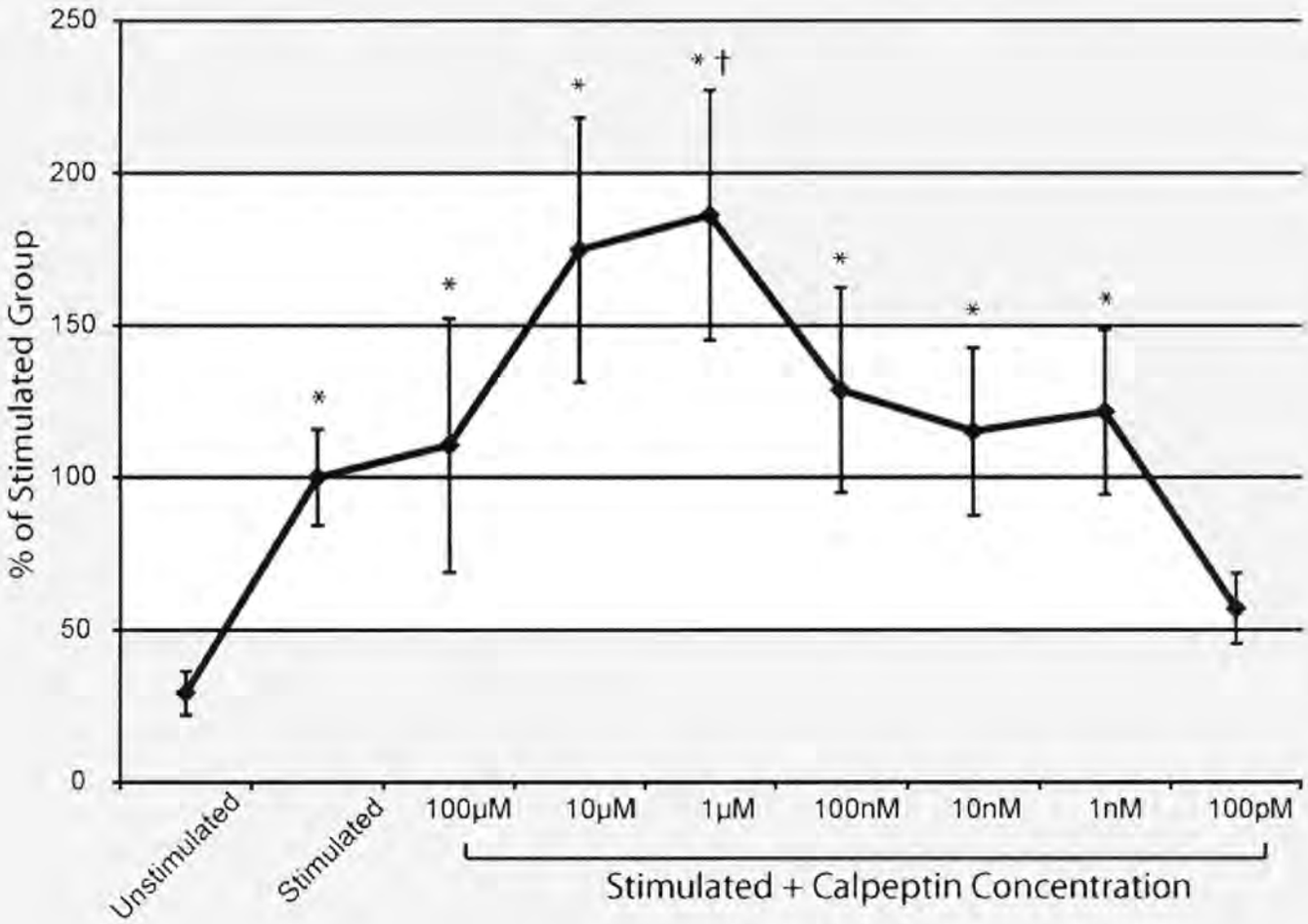


Figure 15: Calpain Concentration Dependent T Cell Proliferation. Proliferation of MBP Ac1-11 specific E3 T cells when stimulated with MBP Ac1-8 peptide in the presence of various concentrations of the calpain inhibitor calpeptin. Mean ± SEM (n = 4). *, $p < 0.05$ vs unstimulated control; †, $p < 0.05$ vs stimulated group.

From the results obtained in figure 14, the native 4K MBP peptide was chosen for further studies of the effect of calpain inhibition on proliferation due to the graded effect. Figure 15 shows the results of proliferation experiments performed with a wider range of concentrations of calpeptin used to determine if the proliferative effect was concentration dependent. The proliferation of all of the groups except the 100 pM calpeptin group were significantly greater than the unstimulated group. The groups trended toward increased proliferation at the midrange of calpeptin concentrations tested and returned to the calpeptin independent level of stimulation at the lower concentrations. The proliferation was significantly higher than the stimulated group when 1 μM of calpeptin was also present in the culture. The subtype of the cells used for figures 14 and 15 was suspected to be Th2 from cytokines studies present later in this chapter. In order to determine if the proliferative effect of calpain inhibition was restricted to a particular Th subtype the next set of experiments repeat the proliferation assays on the various subtypes of Th cells.

The effect of calpain inhibition on the various Th cell subtypes in the context of cellular proliferation potential was determined. Cells were isolated from draining LN and spleen from mice 10 days following MBP inoculation and restimulated with MBP Ac1-8 (4K) as described in the methods to favor the proliferation of the specific $CD4^+$ T helper cell subtypes. Figure 16 is the ^3H-thymidine incorporation into these cells when biased toward the various Th subtype profiles in the presence of various concentrations of the calpain inhibitor, calpeptin. Figure 16 gives an overview of all of the various subtypes relative to

each other. When the proliferation experiment is performed without biasing cytokines or antibodies present in the media (figure 17A) there is no significant effect on proliferation from the various concentrations of calpeptin compared to unstimulated or stimulated controls. When the cells are cultured in the presence of the cytokines favoring the proliferation of the Th1 or Th17 (figures 17B and 17D) subtypes the addition of calpeptin either, does not affect ^{3}H-thymidine uptake or actually decreases ^{3}H-thymidine uptake meaning that proliferation is either not affected or is decreased compare to the control cells. When the cells are cultured to favor Th2 subtype (figure 17C) the addition of calpeptin increases ^{3}H-thymidine uptake at the middle range of inhibitor concentration. The proliferation profile results in figure 17C more closely match the results observed in figure 15 with the E3 MBP specific cell culture line. The addition of calpeptin results in significantly increased ^{3}H-thymidine incorporation in the 10 μM, 1 μM, 100 nM groups compared to the stimulation group without calpain inhibitor. The proliferation increase peaks at 100 nM of calpeptin and decreases back to the control level of ^{3}H-thymidine incorporation in a concentration dependent manner.

Figure 18A-C compares the ^{3}H-thymidine levels between the various subtypes for each group and concentration of calpeptin tested. Figure 18A compares Th1 to Th2 and they are significantly different between every calpeptin group besides the highest two (10 μM and 1 μM). Figure 18B compares Th1 to Th17. There is significant difference between the subtypes at 10 nM and 1 pM of calpeptin. Figure 18C compares Th2 subtype to Th17. The proliferation of these two subtypes is significantly different in the unstimulated group and at 100 nM,

10 nM and 1 nM concentration of calpeptin. All of the subtypes were significantly different than the no cytokine bias group at all of the concentrations of calpeptin tested figure 16.

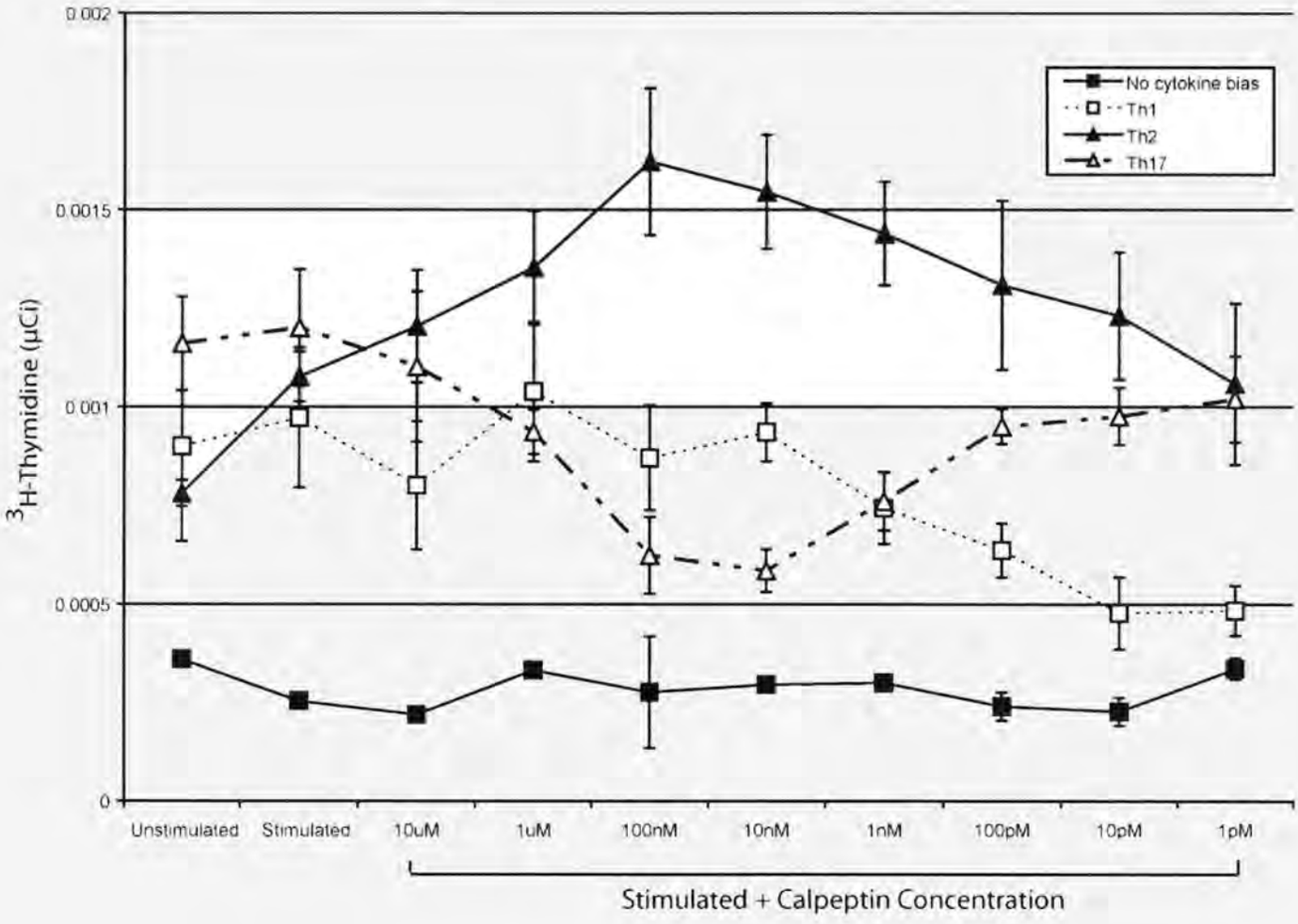


Figure 16: Primary T Cell Proliferation with Calpeptin. A comparison of the proliferation of all of the subtypes of Th cells responsive to MBP Ac1-8 as measured by ^{3}H-thymidine uptake when stimulated with MBP Ac1-8 peptide in the presence of various concentrations of the calpain inhibitor calpeptin. The various Th subtypes were potentiated by cytokines and antibodies present in the media. Mean ± SEM (n = 3).

Figure 17: Th Subtype Proliferation with Calpeptin. A comparison of the proliferation of all of the subtypes of Th cells responsive to MBP Ac1-8 as measured by ^{3}H-thymidine uptake when stimulated with MBP Ac1-8 peptide in the presence of various concentrations of the calpain inhibitor calpeptin divided by subtype. A, the primary LN and splenocyte cells that did not receive any cytokine or antibody bias in the media. B, the primary LN and splenocyte cells that were potentiated with rIL-12p70 and anti-IL-4. C, the primary LN and splenocyte cells that were potentiated with rIL-4 and anti-IL-12p70. D, the primary LN and splenocyte cells that were potentiated with rIL-1b, rIL-23 and anti-IFN-γ, anti-IL-4. Mean ± SEM (n = 3). *, $p < 0.05$ vs unstimulated control; †, $p < 0.05$ vs stimulated group.

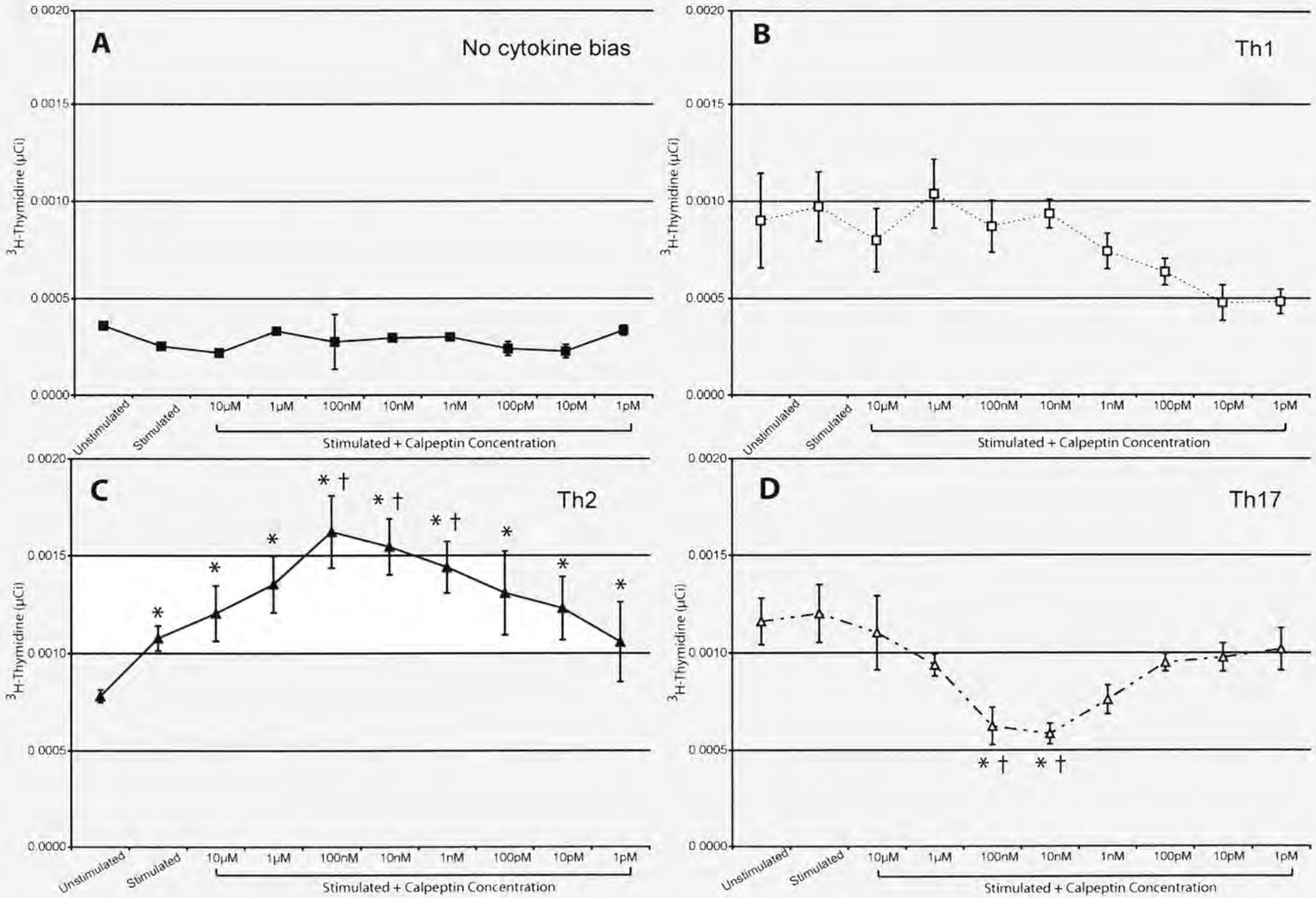
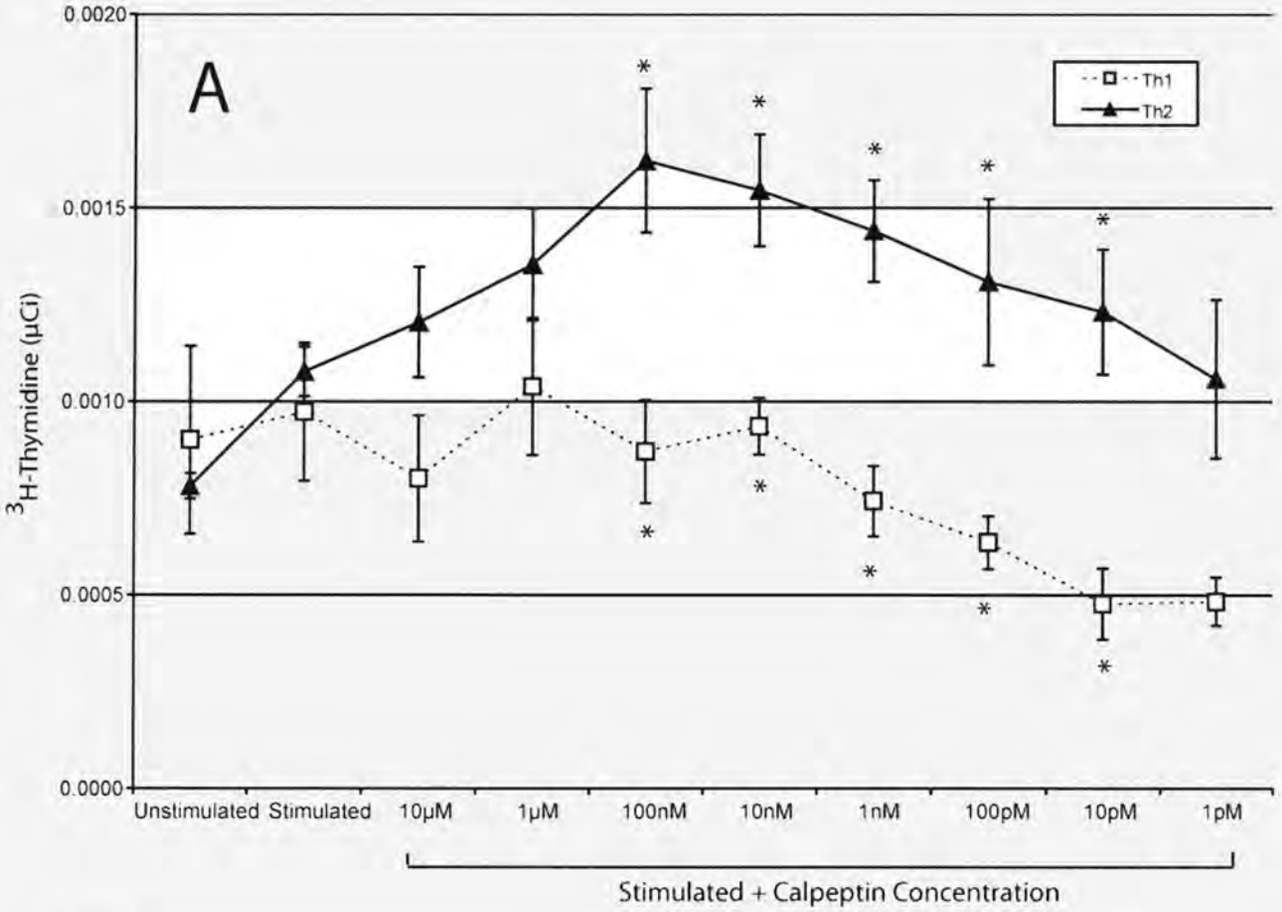
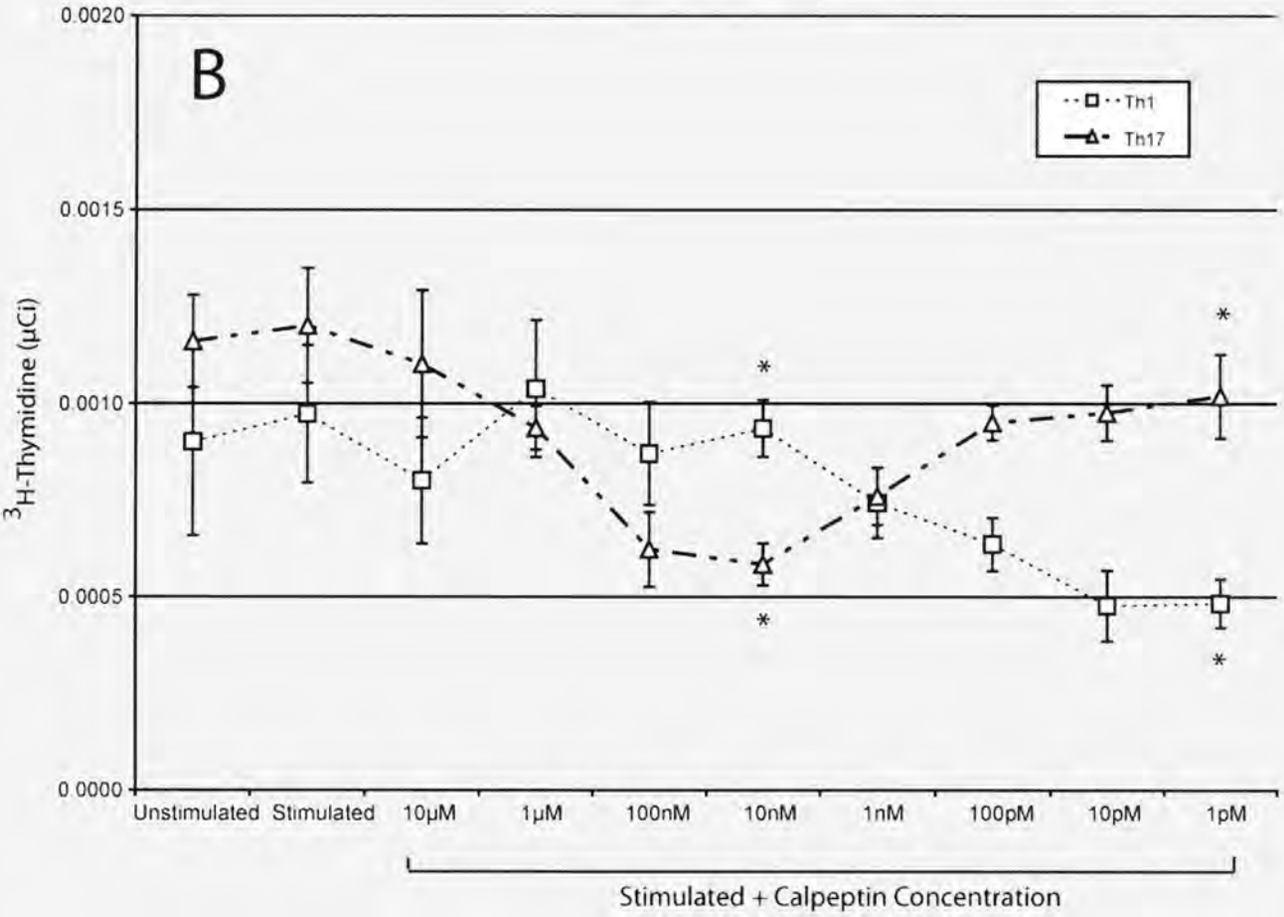

A
No cytokine bias
B
Th1
C
Th2
D
Th17
^{3}H-Thymidine (µCi)
0.0020
0.0015
0.0010
0.0005
0.0000
Unstimulated
Stimulated
10µM
1µM
100nM
10nM
1nM
100pM
10pM
1pM
Stimulated + Calpeptin Concentration
*
†

Figure 18: Th Subtype Proliferation with Calpeptin Compared to Each Other. A comparison of the proliferation of all of the subtypes of Th cells responsive to MBP Ac1-8 as measured by ^{3}H-thymidine uptake when stimulated with MBP Ac1-8 peptide in the presence of various concentrations of the calpain inhibitor calpeptin divided by subtype. A, compares the proliferation of Th1 biased cells to Th2 biased cells at each data point. B, compares the proliferation of Th1 biased cells to Th17 biased cells at each data point. C, compares the proliferation of Th2 biased cells to Th17 biased cells at each data point. Mean ± SEM (n = 3). *, $p < 0.05$ between groups at the same treatment point.


A
Th1
Th2
0.0020
0.0015
0.0010
0.0005
0.0000
^{3}H-Thymidine (µCi)
Unstimulated
Stimulated
10µM
1µM
100nM
10nM
1nM
100pM
10pM
1pM
Stimulated + Calpeptin Concentration


B
Th1
Th17
0.0020
0.0015
0.0010
0.0005
0.0000
^{3}H-Thymidine (µCi)
Unstimulated
Stimulated
10µM
1µM
100nM
10nM
1nM
100pM
10pM
1pM
Stimulated + Calpeptin Concentration

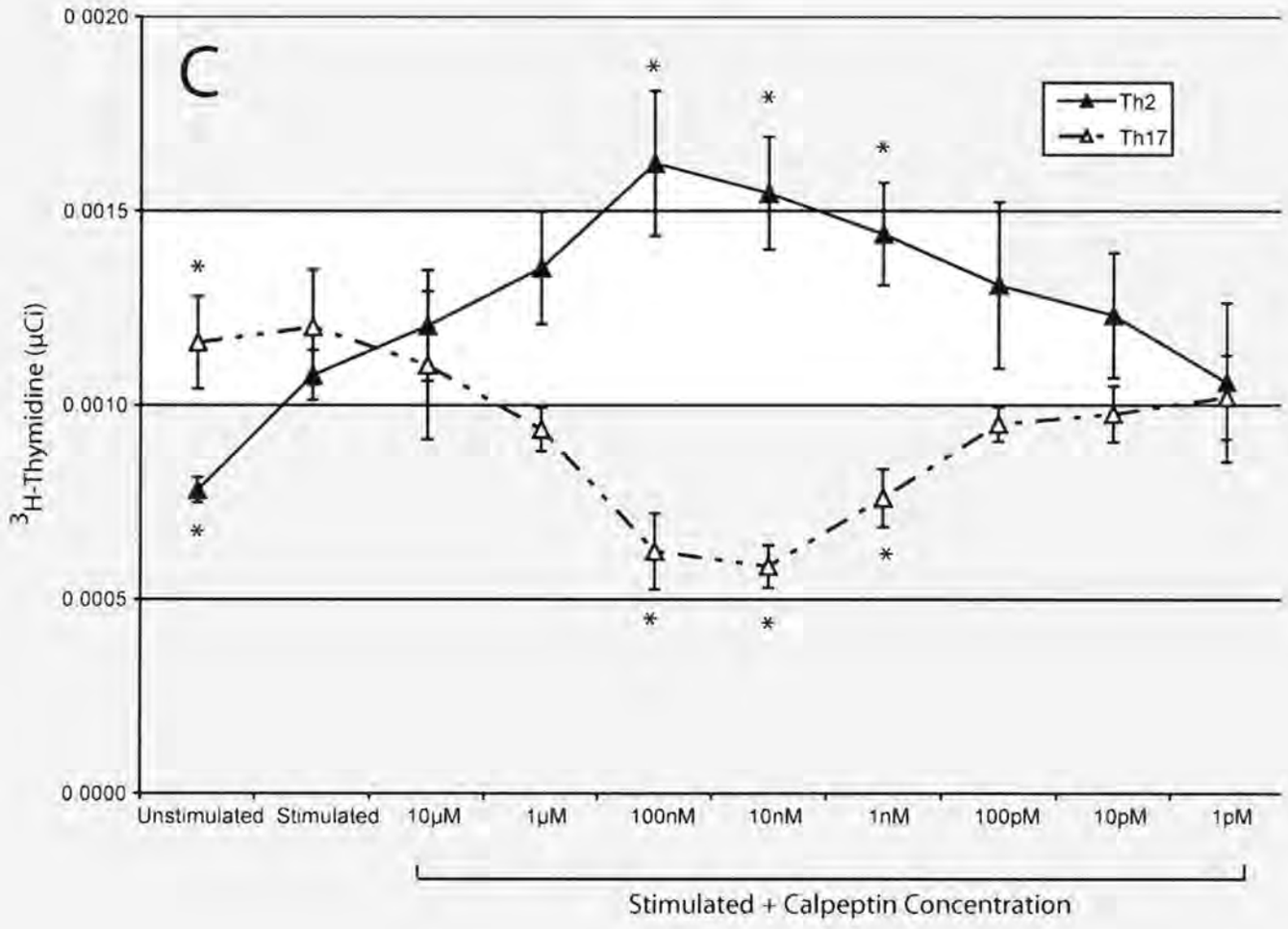
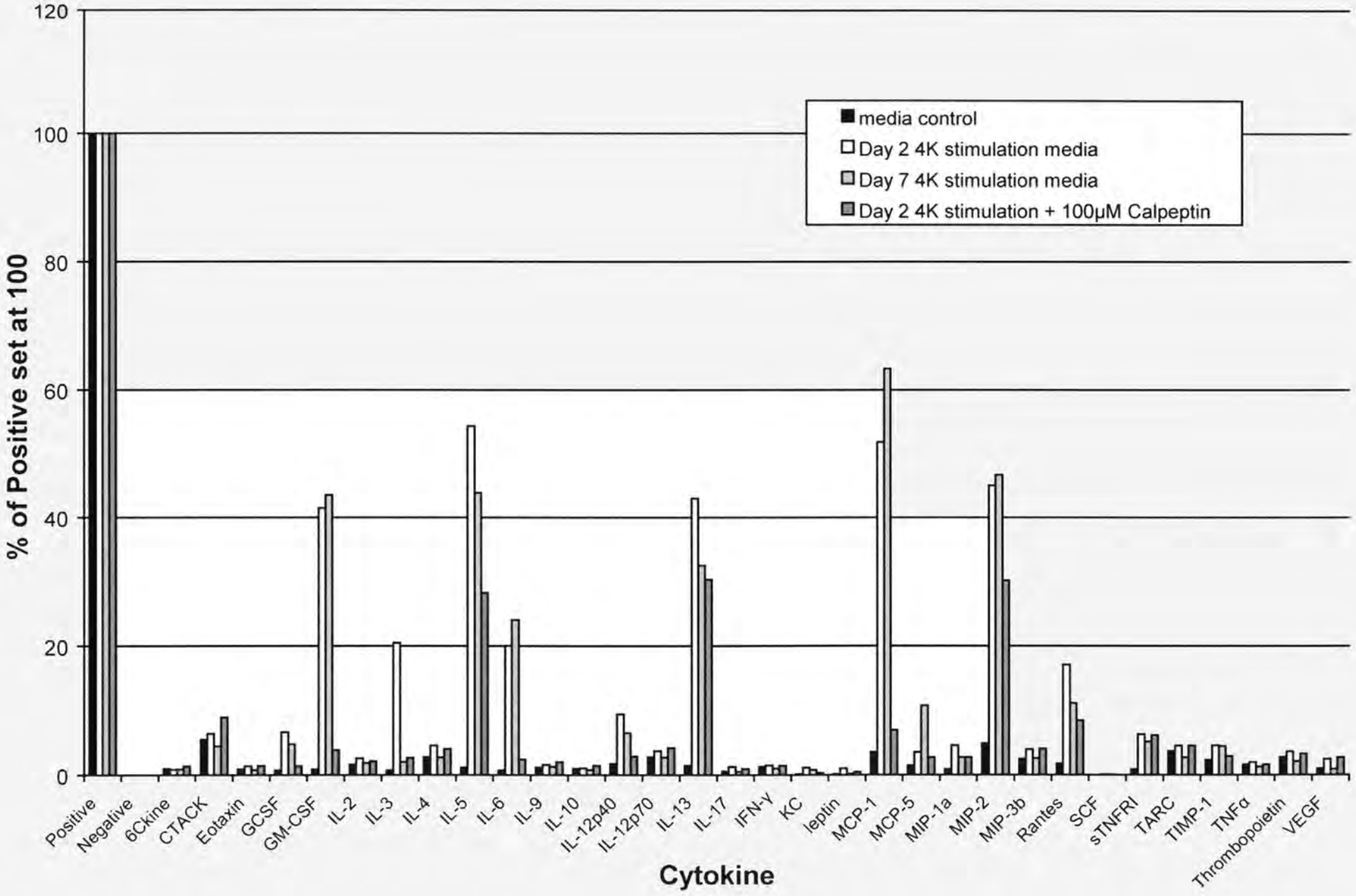


Figure 18: Th Subtype Proliferation with Calpeptin Compared to Each Other. A comparison of the proliferation of all of the subtypes of Th cells responsive to MBP Ac1-8 as measured by ^{3}H-thymidine uptake when stimulated with MBP Ac1-8 peptide in the presence of various concentrations of the calpain inhibitor calpeptin divided by subtype. A, compares the proliferation of Th1 biased cells to Th2 biased cells at each data point. B, compares the proliferation of Th1 biased cells to Th17 biased cells at each data point. C, compares the proliferation of Th2 biased cells to Th17 biased cells at each data point. Mean ± SEM (n = 3). *, $p < 0.05$ between groups at the same treatment point.

The Effect of Calpain Inhibition on Th Cell Cytokine Profile

A more extensive secreted cytokine profile of the E3 MBP specific cell culture stimulated with the native 4K MBP peptide is presented in figure 19. The cytokines were assessed with an immunoblot array to more specifically classify the T helper cell subtype profile that the E3 cells represent and also to determine if there was any alteration of the cytokines produced when calpeptin was present. Figure 19 is a comparison of the relative cytokine presence compared to the array's positive spots for a media control, a culture stimulated with MBP Ac1-8 after 2 days and 7 days. Figure 19 also compares the cytokines present 2 days after restimulation in the presence of 100 μM of calpeptin. As far as profiling the cytokines from E3 cells are concerned for T helper cell subtype categorization, the cells secrete GM-CSF, IL-3, IL-5, IL-6, IL-13, MCP-1, MIP-2 and RANTES. Comparing the Day 2 culture sample to the Day 7 culture sample the only real difference is with IL-3 which is decreased by Day 7 to almost control levels. The effect observed when comparing the 100μM calpeptin culture to the stimulated culture is mainly a decrease of secreted cytokines. The degree to which these cytokines are decreased is the major difference between them. The cytokines that are decreased 20% or less are IL-5, IL-13, MIP-2 and RANTES. The cytokines that are decreased 20% or greater are GM-CSF, IL-3, IL-6, MCP-1. The Th subtype that these cytokines most closely resemble is of a Th2 subtype. The cytokine profile does not fit a Th2 cytokine profile completely but due to the fact that this is a cell line and not a primary culture a slight variation is not surprising.

Figure 19: E3 Cell Cytokine Profile. The cytokine profile of MBP Ac1-11 specific T cells stimulated with the native MBP Ac1-11 (4K) peptide as measured by an immunoblot cytokine array. The cytokine levels are relative to a positive spot present on the immunoblot. The samples tested were a media control, the supernatant 2 days and 7 days after restimulation of the cells with 4K MBP Ac1-8, and the supernatant 2 days after restimulation in the presence of 100 μM of the calpain inhibitor calpeptin. The bars represent the mean of several spots for each cytokine.


120
100
80
60
40
20
0
% of Positive set at 100
media control
Day 2 4K stimulation media
Day 7 4K stimulation media
Day 2 4K stimulation + 100µM Calpeptin
Positive
Negative
6Ckine
CTACK
Eotaxin
GCSF
GM-CSF
IL-2
IL-3
IL-4
IL-5
IL-6
IL-9
IL-10
IL-12p40
IL-12p70
IL-13
IL-17
IFN-γ
KC
leptin
MCP-1
MCP-5
MIP-1a
MIP-2
MIP-3b
Rantes
SCF
sTNFRI
TARC
TIMP-1
TNFα
Thrombopoietin
VEGF
Cytokine

The cytokine profiles were also assessed using ELISA to focus on the specific cytokines of interest. Early experiments pointed in the direction of a Th2 type cytokine profile for the E3 cells so that became the focus. The supernatant from the proliferation assays was sampled at 24 hours after plating of the experiment as described in the methods section and these samples were used for the ELISA experiments. Figure 20 is the IL-4, 10, 12 and IFN-γ levels for the various groups comparing calpeptin concentrations to the unstimulated and stimulated groups without calpeptin exposure. Significant differences comparing the unstimulated and stimulated controls to the calpeptin groups are observed in the Th2 cytokines IL-4 and IL-10. All of the IL-10 levels were significantly different than the unstimulated control. The IL-10 level was significantly lower in the 100 μM and 10μM calpeptin groups compared to the stimulated group with no calpeptin. The Th1 cytokines, IFN-γ and IL-12, were extremely low for all of the groups tested and no significant difference between groups was observed.

The cytokine profile for the E3 MBP specific T cells with different stimulating peptides was also assessed in the context of calpain inhibition (figure 21A and 21B). The cytokine profile was assessed without stimulating with MBP to assess the effects of calpain inhibition in the absence of stimulation. The profile was also assessed when the cells were stimulated with the native MBP Ac1-8 peptide (4K) to determine the cytokine profile and the effects of several concentrations of calpain inhibitor on these cytokines. The cytokine profile was also measured when using a strong agonist, the MBP Ac1-8 with an alanine substitution at position 4 with several concentration of calpeptin. The cytokine

profiles for IL-4 and IL-10 for these various stimulation paradigms are presented in figure 21A and 21B. The only significant difference observed was between the 4K stimulated groups with 10 nM of calpeptin present and the control and vehicle groups.

Figure 22 displays the IL-4 and IL-10 levels produced by the E3 cells used in the proliferation experiment from figure 15, performed with a wider range of concentrations of calpeptin. The IL-10 levels were significantly higher for all groups compared to the unstimulated control. The IL-4 levels were significantly higher than the unstimulated control in the stimulated group and the 100 nM, 10 nM and 100 pM calpeptin groups. The IL-4 and IL-10 levels were significantly lower in the 100 μM calpeptin group compared to the stimulated group with no calpeptin.

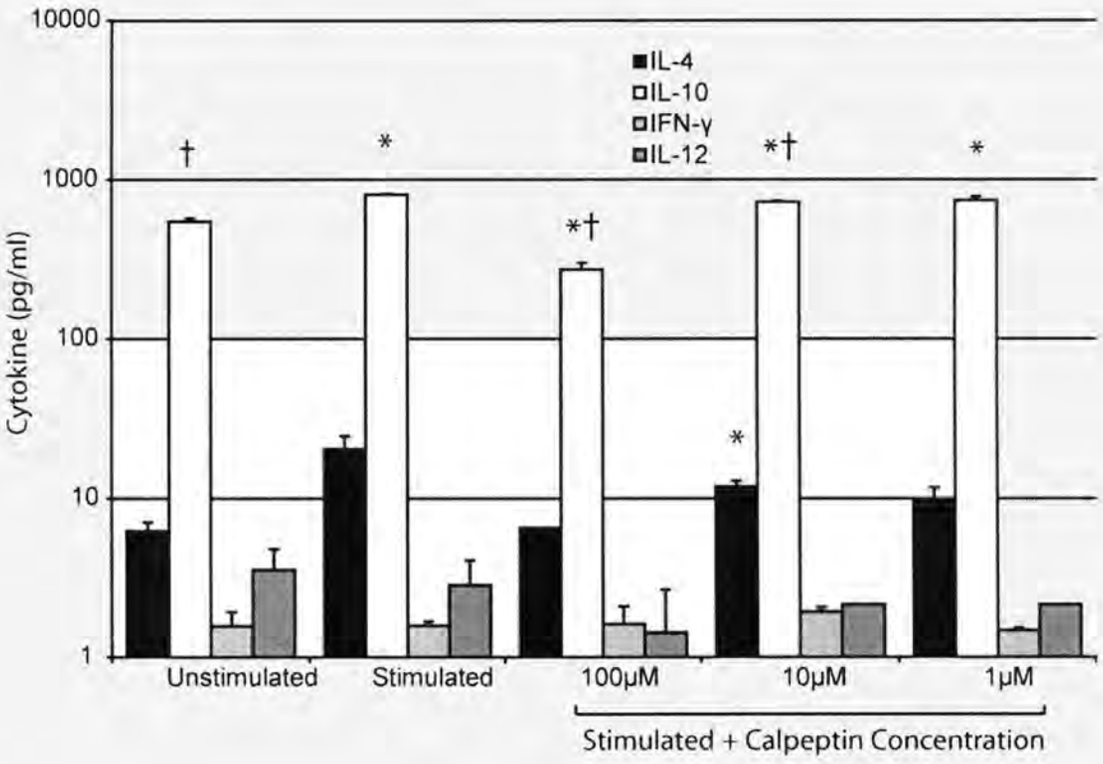


Figure 20: Calpeptin Alters T Cell Cytokines. Cytokines produced by MBP Ac1-11 specific T cells when stimulated with the native MBP Ac1-11 (4K) peptide in the presence of several concentration of the calpain inhibitor calpeptin. Mean ± SEM (n = 3). *, $p < 0.05$ vs unstimulated control; †, $p < 0.05$ vs stimulated control group.

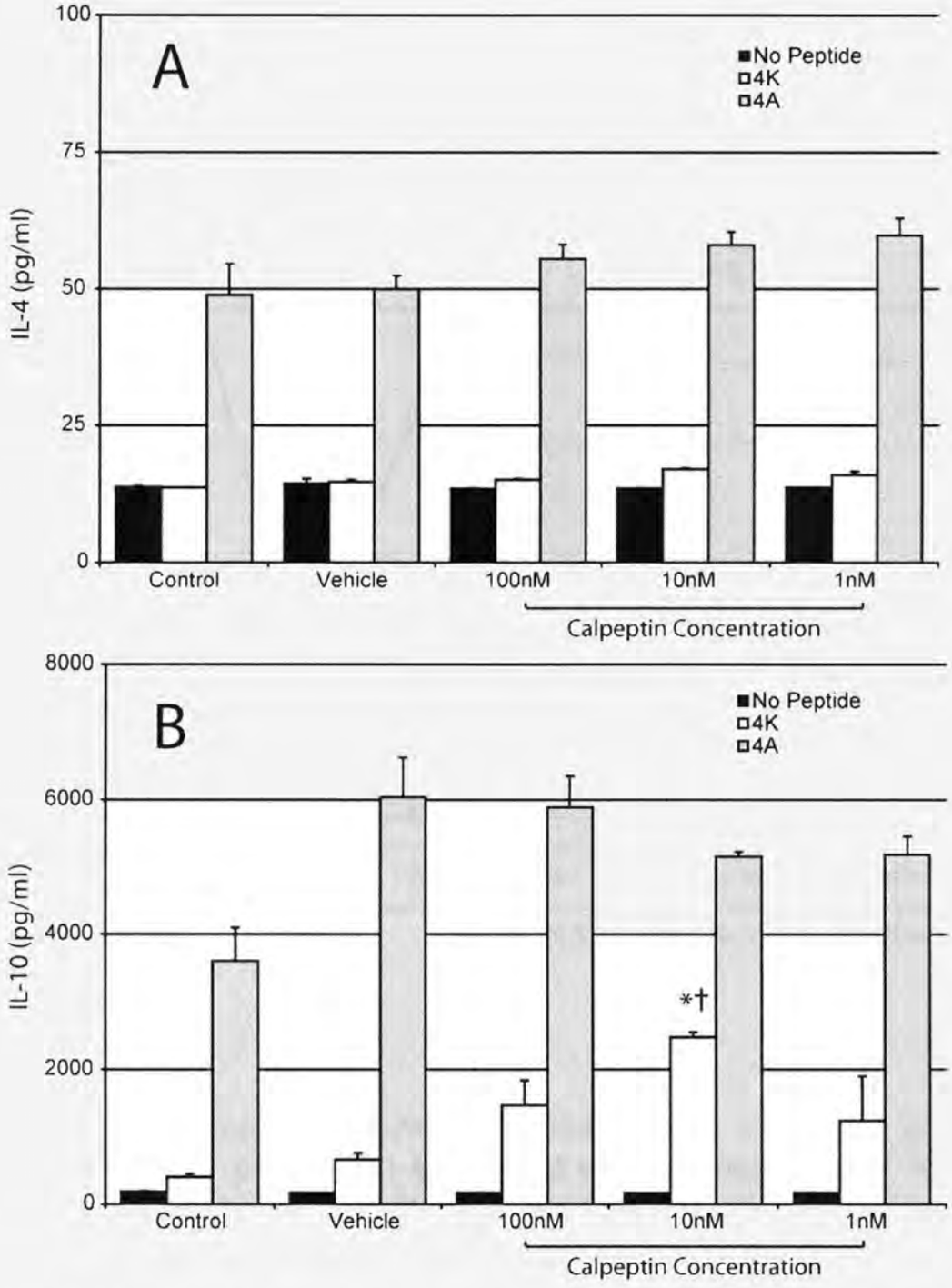


Figure 21: Th2 Cytokines with Various Stimuli. Cytokine production of MBP Ac1-11 specific T cells either in the presence of no antigenic peptide, the native MBP Ac1-11 (4K) peptide, or a strong agonist altered peptide ligand MBP Ac1-11 with an alanine substitution at position 4 (4A). Cells were incubated in the presence of several concentration of calpeptin. A, IL-4 concentration. B, IL-10 concentration. Mean ± SEM (n = 3). *, $p < 0.05$ vs control; †, $p < 0.05$ vs vehicle group.

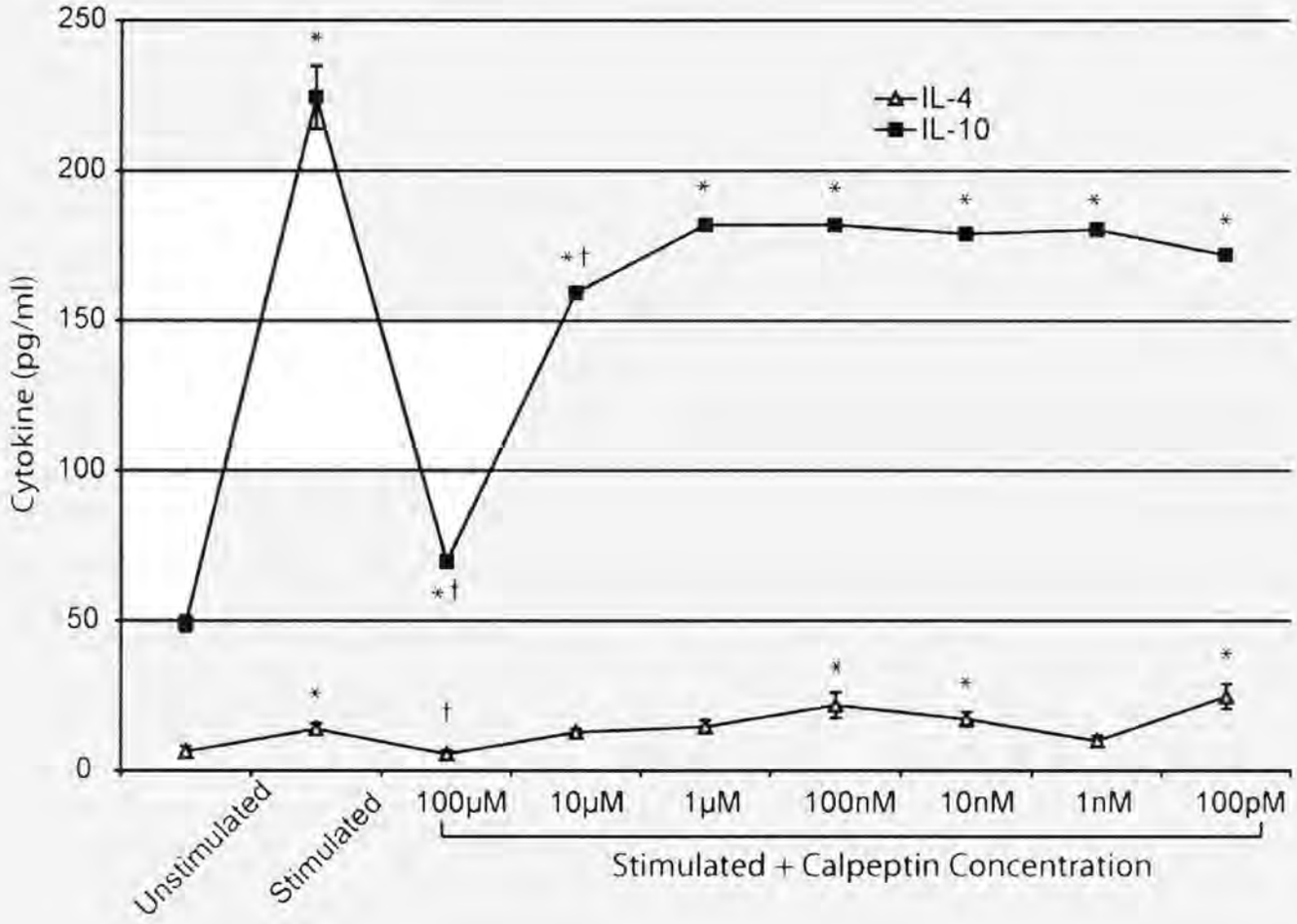


Figure 22: Th2 Cytokines in Response to Calpeptin. Cytokines produced by MBP Ac1-11 specific T cells when stimulated with the native MBP Ac1-11 (4K) peptide in the presence of a various concentrations of the calpain inhibitor calpeptin. Calpain inhibition significantly reduced the cytokine expression at the highest concentrations of calpeptin. Cytokine expression was significantly greater than the unstimulated control for all of the concentrations tested with respect to IL-10 and greater than unstimulated control for the 100 nM, 10 nM and 100 pM groups with respect to IL-4. Mean ± SEM (n = 4). *, $p < 0.05$ vs unstimulated control; †, $p < 0.05$ vs stimulated control without calpeptin.

The unbiased cytokine profile of the primary lymphocytes from MBP inoculated mice was measured with the immunoblot cytokine array and is displayed in figure 23. These are the cytokines secreted when the cells are stimulated with MBP Ac1-8 without being biased with exogenous cytokines or neutralizing antibodies. The hallmark cytokines present in relative high amounts are IL-6, IL-12p40 (a component of both IL-12 and IL-23) and IFN-γ. These cytokines mainly indicate that the cells are of an inflammatory profile.

The cytokine profiles of the primary biased cells were also determined from supernatant samples that were taken from the proliferation experiments. Figure 24A are the IL-2 levels of the various subtypes when exposed to a range of calpeptin concentrations. Figure 24B are the IFN-γ levels of the various subtypes when exposed to a range of calpeptin concentration. Figure 24C are the IL-10 levels of the various subtypes when exposed to a range of calpeptin concentration. Figure 24D are the IL-17A of the various subtypes when exposed to a range of calpeptin concentration. Several of the cytokines could not be accurately assessed from all of the subtype groups because that specific cytokine was exogenously added to favor the proliferation of one of the various subtypes, for instance IL-4. The unbiased group was assessed for IL-4 levels to determine if they changed in response to various concentration of calpeptin. The levels of IL-4 from the unbiased group of primary cells stimulated with MBP Ac1-8 when exposed to various concentrations of calpeptin was not significantly different than the controls (data not shown).

The unbiased cytokine profile of the primary lymphocytes from MBP inoculated mice was measured with the immunoblot cytokine array and is displayed in figure 23. These are the cytokines secreted when the cells are stimulated with MBP Ac1-8 without being biased with exogenous cytokines or neutralizing antibodies. The hallmark cytokines present in relative high amounts are IL-6, IL-12p40 (a component of both IL-12 and IL-23) and IFN-γ. These cytokines mainly indicate that the cells are of an inflammatory profile.

The cytokine profiles of the primary biased cells were also determined from supernatant samples that were taken from the proliferation experiments. Figure 24A are the IL-2 levels of the various subtypes when exposed to a range of calpeptin concentrations. Figure 24B are the IFN-γ levels of the various subtypes when exposed to a range of calpeptin concentration. Figure 24C are the IL-10 levels of the various subtypes when exposed to a range of calpeptin concentration. Figure 24D are the IL-17A of the various subtypes when exposed to a range of calpeptin concentration. Several of the cytokines could not be accurately assessed from all of the subtype groups because that specific cytokine was exogenously added to favor the proliferation of one of the various subtypes, for instance IL-4. The unbiased group was assessed for IL-4 levels to determine if they changed in response to various concentration of calpeptin. The levels of IL-4 from the unbiased group of primary cells stimulated with MBP Ac1-8 when exposed to various concentrations of calpeptin was not significantly different than the controls (data not shown).

Figure 23: Unbiased Lymphocyte Cytokine Profile. The various cytokine levels from primary lymphocytes obtained from a MBP inoculated animal and stimulated with MBP Ac1-8 (4K) for 48 hours. These primary cells were not potentiated with exogenous cytokines or neutralizing antibodies. The cytokine levels are relative to a positive spot present on the immunoblot. The primary cytokines of interest are IL-6, IL-12p40 and IFN-γ indicating the cell type present was primarily an inflammatory Th1 subtype. The bars represent the mean of several spots for each cytokine.

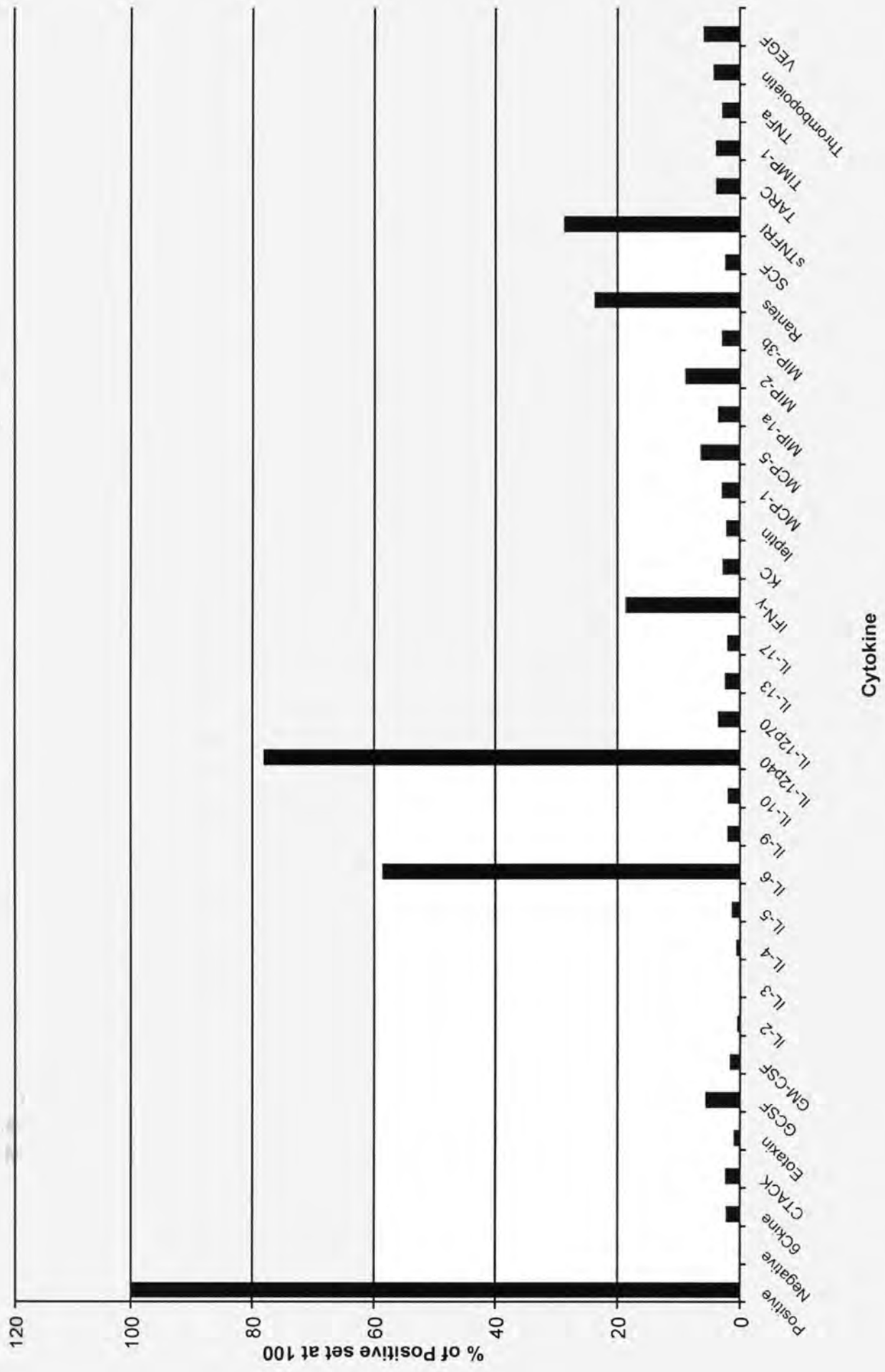
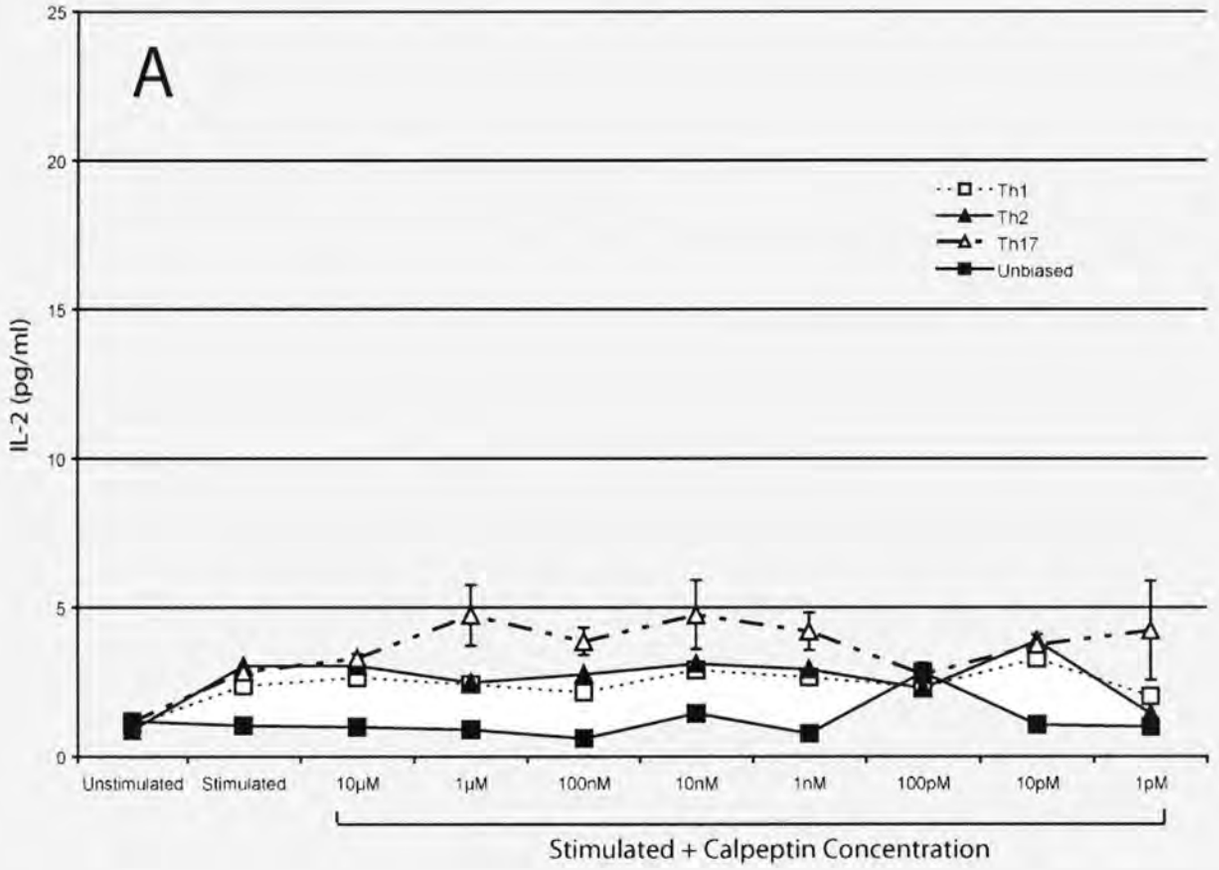

% of Positive set at 100
0
20
40
60
80
100
120
Cytokine
Positive
Negative
6Ckine
CTACK
Eotaxin
GCSF
GM-CSF
IL-2
IL-3
IL-4
IL-5
IL-6
IL-9
IL-10
IL-12P40
IL-12P70
IL-13
IL-17
IFN-γ
KC
leptin
MCP-1
MCP-5
MIP-1a
MIP-2
MIP-3b
Ranles
SCF
sTNFRI
TARC
TIMP-1
TNFa
Thrombopoietin
VEGF

Figure 24: Th subtype cytokines with calpeptin present. A comparison of the cytokine production of all of the subtypes of Th cells as measured by ELISA assay when stimulated with MBP Ac1-8 peptide in the presence of various concentrations of the calpain inhibitor calpeptin. The cytokine profiling was performed to determine if calpain inhibitor was having any effect at various concentrations on the cytokine production. A, compares the production of IL-2 for the various subtypes. B, compares the production of IFN-γ for the various subtypes. C, compares the production of IL-10 for the various subtypes. D, compares the production of IL-17A for the various subtypes. Mean ± SEM (n = 3).


A
IL-2 (pg/ml)
25
20
15
10
5
0
Th1
Th2
Th17
Unbiased
Unstimulated
Stimulated
10μM
1μM
100nM
10nM
1nM
100pM
10pM
1pM
Stimulated + Calpeptin Concentration

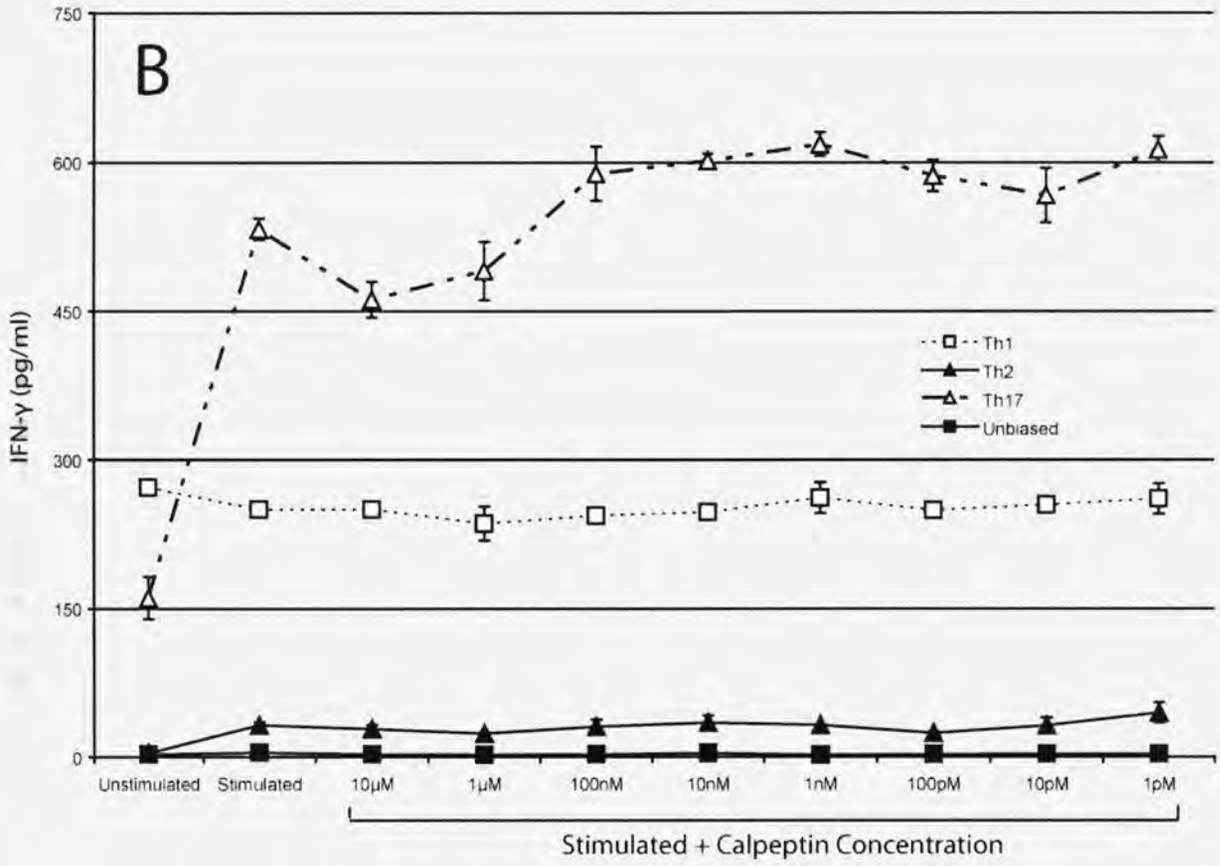
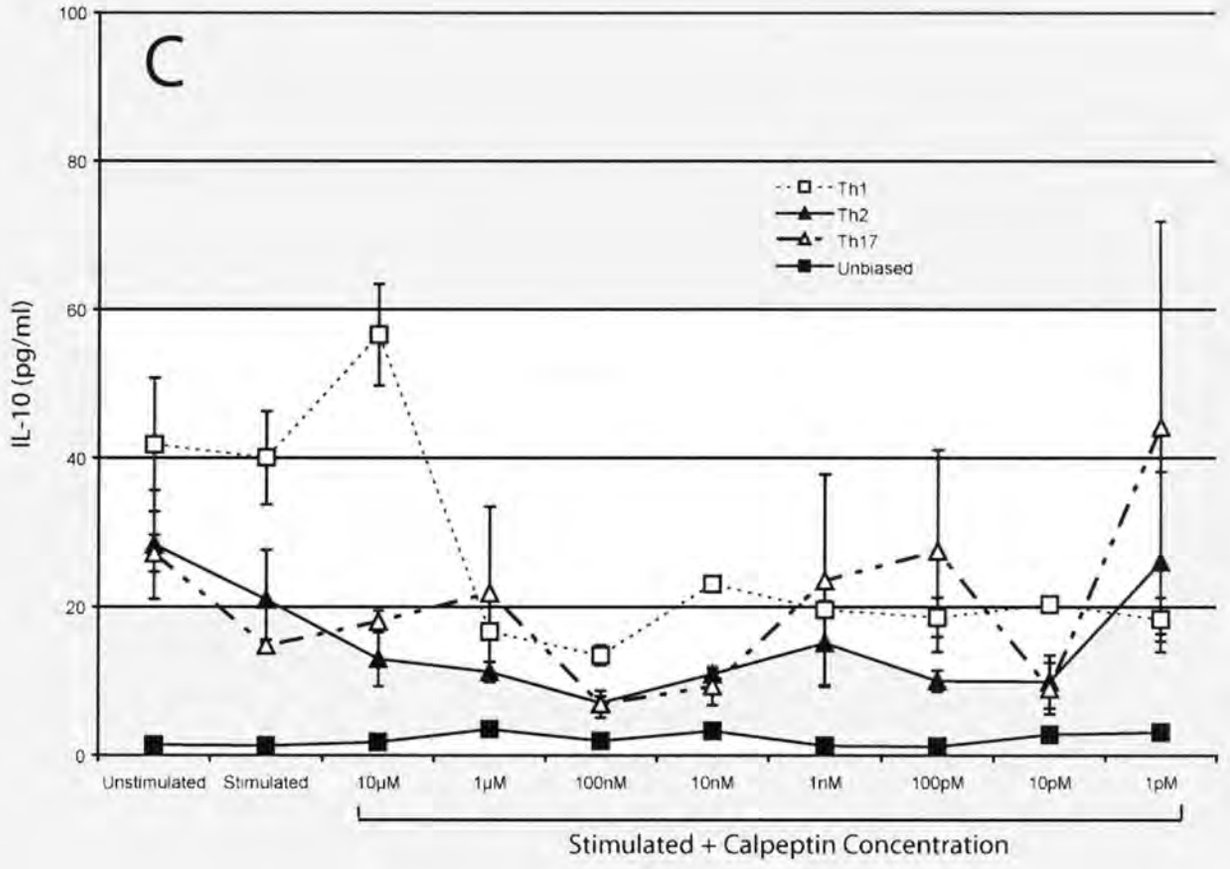
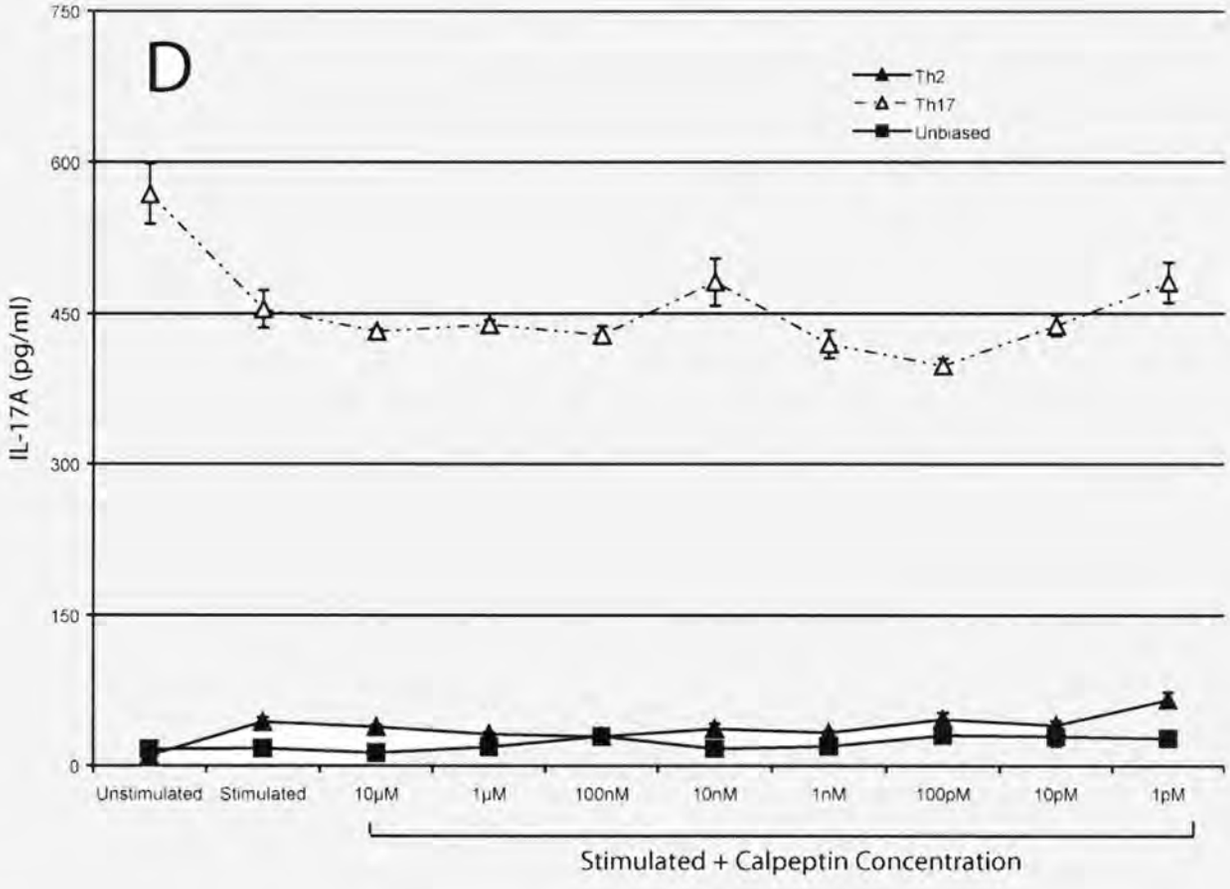

B
IFN-γ (pg/ml)
750
600
450
300
150
0
Th1
Th2
Th17
Unbiased
Unstimulated
Stimulated
10μM
1μM
100nM
10nM
1nM
100pM
10pM
1pM
Stimulated + Calpeptin Concentration

Figure 24: Th subtype cytokines with calpeptin present. A comparison of the cytokine production of all of the subtypes of Th cells as measured by ELISA assay when stimulated with MBP Ac1-8 peptide in the presence of various concentrations of the calpain inhibitor calpeptin. The cytokine profiling was performed to determine if calpain inhibitor was having any effect at various concentrations on the cytokine production. A, compares the production of IL-2 for the various subtypes. B, compares the production of IFN-γ for the various subtypes. C, compares the production of IL-10 for the various subtypes. D, compares the production of IL-17A for the various subtypes. Mean ± SEM (n = 3).


C
Th1
Th2
Th17
Unbiased
IL-10 (pg/ml)
100
80
60
40
20
0
Unstimulated
Stimulated
10µM
1µM
100nM
10nM
1nM
100pM
10pM
1pM
Stimulated + Calpeptin Concentration


D
Th2
Th17
Unbiased
IL-17A (pg/ml)
750
600
450
300
150
0
Unstimulated
Stimulated
10µM
1µM
100nM
10nM
1nM
100pM
10pM
1pM
Stimulated + Calpeptin Concentration

The Effect of Calpain Inhibition on STAT Protein Levels

Calpain does appear to play some role in the T helper cell bias but exactly where in the activation cascade is still unclear. In order to further determine where calpain may be playing a role through assessing the status of the various STAT signaling molecules which are known to play a role in the T helper cell subtype activation and proliferation by activating specific transcription factors that potentiate the various subtypes. This was assessed mainly though western blotting of the whole cell lysates of E3 cells following exposure to various activating stimulus and various concentrations and types of calpain inhibitors. STAT1 and STAT4 are more important for Th1 subtype activation and proliferation and STAT6 is more important for Th2 subtype activation. Figure 25A are representative blots the 91kD STAT1α, 84kD STAT1β, 89kD STAT4, 110kD STAT6 and 42kD β-actin proteins. The bar graph in figure 25B represents the mean densitometry of the bands relative to the control band for several experiments. There is no significant difference observed between the STAT1 and STAT4 protein levels for any of the groups tested. The STAT6 bands from the stimulated group and the stimulated groups with 10 μM and 1 μM of calpeptin are significantly higher than the unstimulated control group. The STAT6 protein level from the 100 μM calpeptin groups is significantly lower than the stimulated control group. This may be due to the high level of calpain inhibitor affect a cell cycle mechanism and arresting a majority of the cells in a phase with low levels of STAT6 protein.

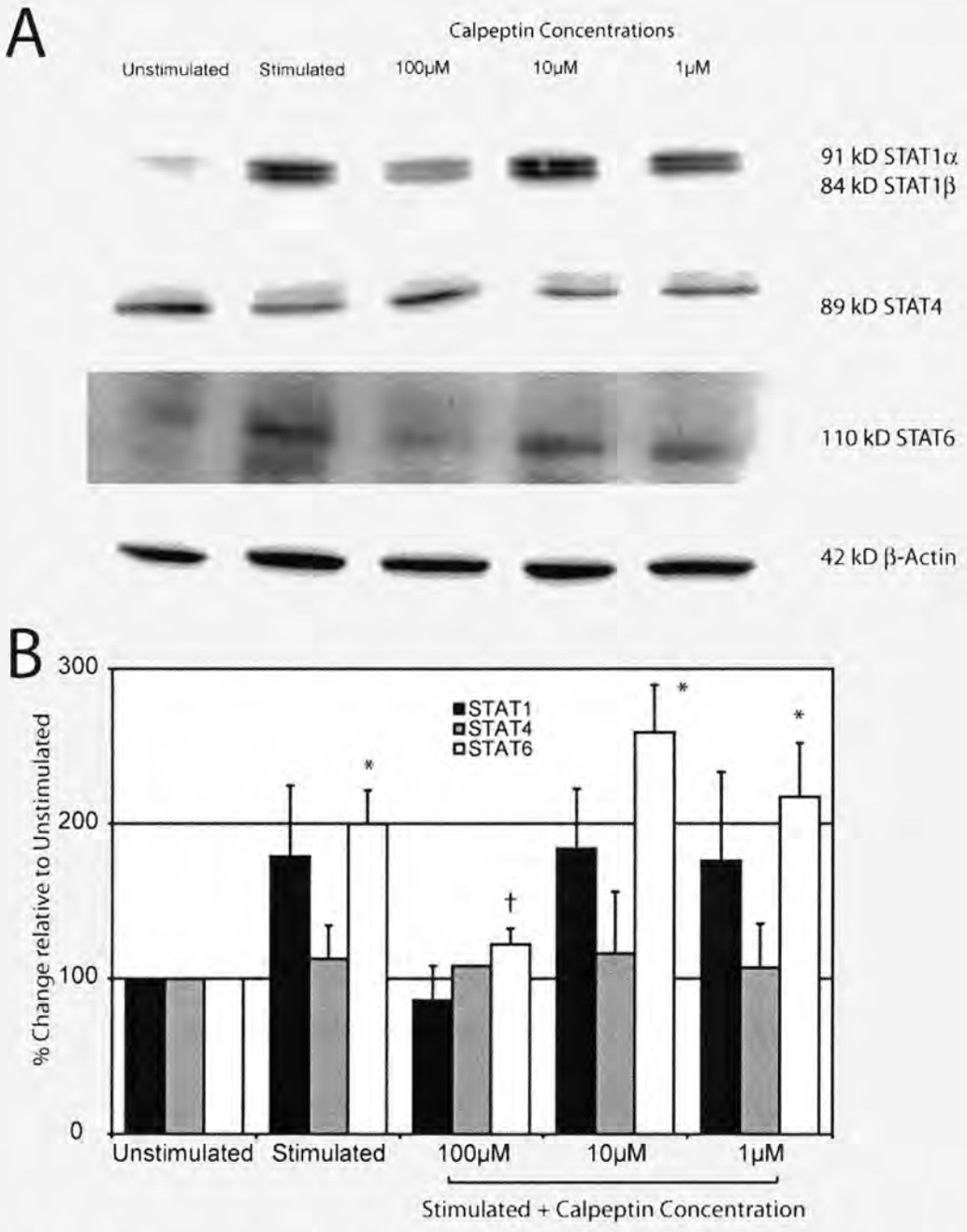


Figure 25: The Effect of Calpeptin on STAT Protein Levels. Changes in various STAT proteins in MBP Ac1-11 specific T cells following stimulation with MBP Ac1-8 and various concentration of the calpain inhibitor calpeptin. A, representative blots showing 91kD STAT1α, 84kD STAT1β, 89kD STAT4, 110kD STAT6 and 42kD β-actin. B, Densitometric analysis showing percent change in the various STAT proteins relative to the unstimulated control group set at 100. Mean ± SEM (n = 4). *, $p < 0.05$ vs unstimulated control; †, $p < 0.05$ vs stimulated group without calpeptin.

The Effect of Calpain Inhibition on Transcription Factor Protein Levels

In order to further assess the potential role of calpain in T helper cell subtype bias, the concentration of certain transcription factors that are activated by STAT molecules and transcribe the cytokine loci necessary for the ongoing proliferation of the various subtypes were examined. The main transcription factors that were measured in the E3 cells were the Th1 and Th2 transcription factors T-bet and GATA3 respectively by western blotting on whole cell lysates. Figure 26A are the representative blots of the 62kD T-bet, 50kD GATA3 proteins and the 42kD β-actin loading control. Figure 26B is the average densitometry of the protein bands from several of the experiment that were performed. The unstimulated and stimulated groups are not significantly different. The calpain stimulated group with Ca^{2+} and ionomycin and the stimulated groups with 10μM and 1μM calpeptin are also not significantly different than either of the unstimulated control or the stimulated group. The highest calpeptin group, 100μM, is significantly different than the other groups for both of the transcription factors measured.

The effect of calpain inhibitor on the transcription factors T-bet and GATA3 were also assessed on the Jurkat T cell lymphoma cell line. The Jurkat T cells were activated through the TCR by plate bound anti-CD3. Whole cell lysate was assessed by western blotting and figure 27A are the representative blots of the 62kD T-bet, 50kD GATA3 proteins and the 42kD β-actin loading control. Figure 27B is the densitometry of the protein bands relative to the unstimulated control. A group stimulated with Ca^{2+} and ionomycin was included in this experiment to

determine if increased calpain activity had any effect on transcription levels. Not enough repetitions were performed to achieve statistical significance but the important trend observed was a shift of more T-bet in the stimulated group to less T-bet in the stimulated group with calpeptin. At the same time, there is a shift from less GATA3 in the stimulated group to more GATA3 in the stimulated group with calpeptin included.

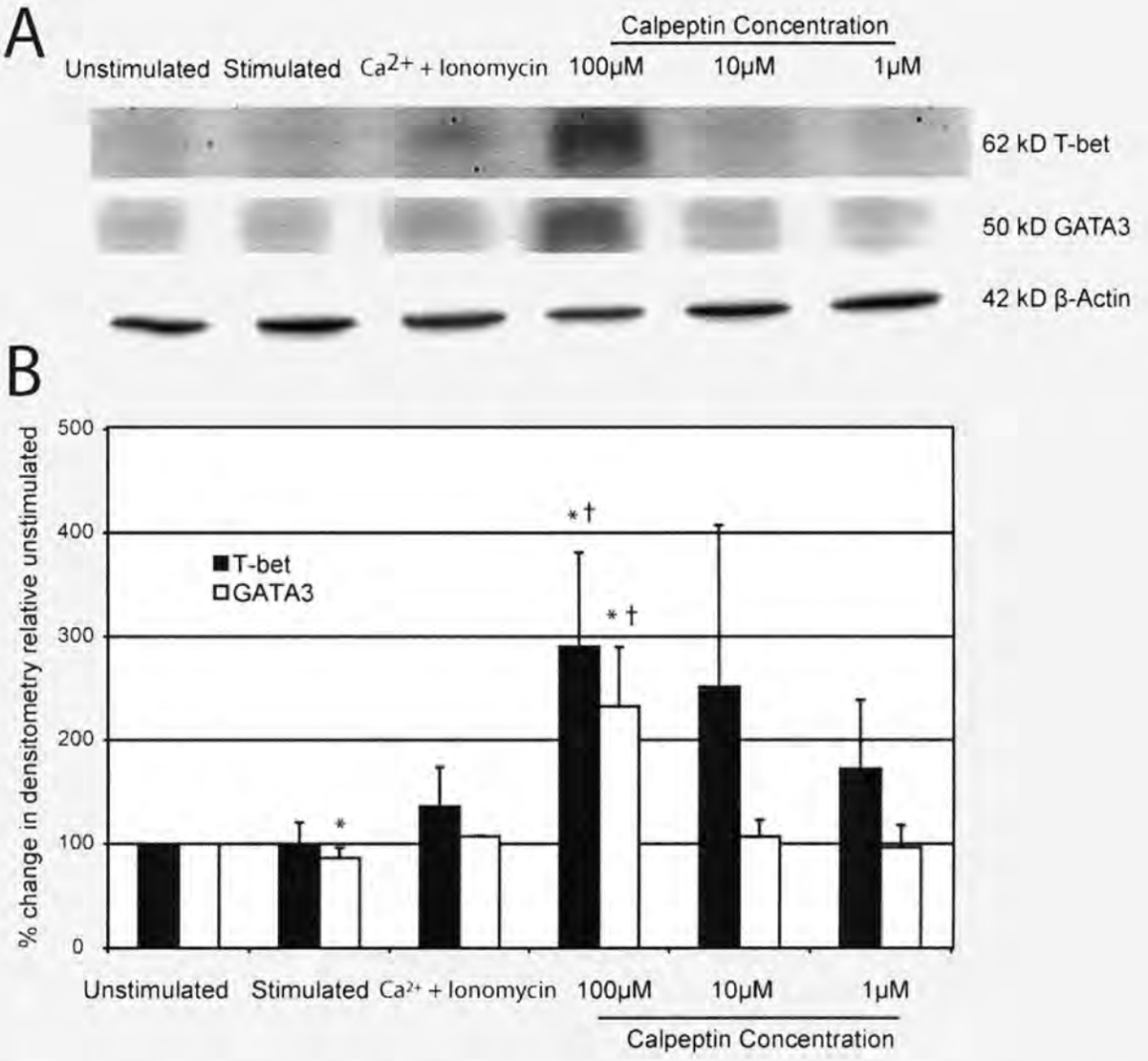


Figure 26: The Effect of Calpeptin on Transcription Factor Protein Levels. Changes in the T-bet and GATA3 transcription factors in MBP Ac1-11 specific T cells following stimulation with MBP Ac1-8 and various concentration of the calpain inhibitor calpeptin. The Ca^{2+} + Ionomycin group is a positive control group to contrast calpain activation to calpain inhibition. A, representative blots showing 62kD T-bet, 50kD GATA3 and 42kD β-actin. B, Densitometric analysis showing percent change in the T-bet and GATA3 proteins relative to the unstimulated control group set at 100. Mean ± SEM (n = 3). *, $p < 0.05$ vs unstimulated control; †, $p < 0.05$ vs stimulated group without calpeptin.

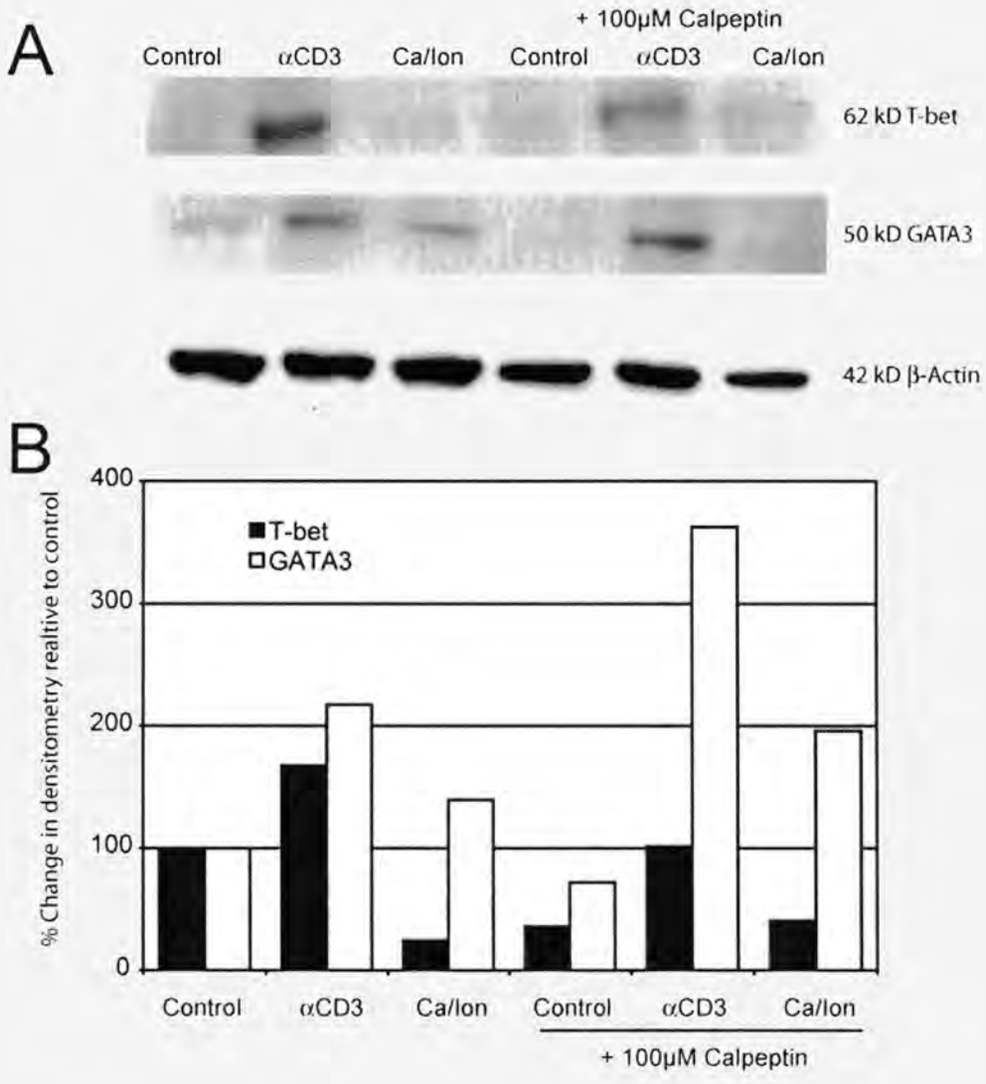


Figure 27: The Effect of Calpeptin on Transcription Factor Protein Levels in Jurkat T Cells. Changes in the T-bet and GATA3 transcription factors in Jurkat T cells following stimulation with α-CD3 or Ca^{2+} and Ionomycin (Ca/Ion) and in the presence of the calpain inhibitor calpeptin. A, representative blots showing 62kD T-bet, 50kD GATA3 and 42kD β-actin. B, Densitometric analysis showing percent change in the T-bet and GATA3 proteins relative to the unstimulated control group set at 100. Mean ± SEM.

The Effect of Calpain Inhibition on Transcription Factor Localization

The lack of a clear cut strong significant difference in the transcription factors and STAT molecules in the E3 cell line using western blotting lead us to attempt to determine if the proliferative effect observed in Th2 cells by calpain inhibition was not due to just changes in protein levels but due to localization of the proteins in the cell. The signal for proliferation and activation comes from surface receptors on the T cells and must travel to the nucleus in order to activate the necessary transcription factors. Fluorescent immunohistochemical staining was used to assess the subcellular localization of STAT1, STAT6, T-bet and GATA3 following stimulation with MBP Ac1-8 (4K). The localization was also assessed following exposure to several concentrations of calpain inhibitor. Figure 28A demonstrates the staining of STAT1 in green and T-bet in red for the various groups; control, stimulated, 10 μM calpeptin and 1 μM calpeptin. The nuclei of the cells are stained blue with DAPI to aid in determining cytoplasmic or nuclear localization. The cells demonstrated are typical of the staining observed in the whole culture. There does appear to be a difference between the staining of the control and stimulated cells. The stimulated cells display a more punctate staining pattern compared to the control cells which demonstrate a more diffuse staining pattern restricted to the cytoplasm. Both of the calpain groups demonstrate cells with increased T-bet staining that seems to either surround the nucleus or reside in the periphery of the nucleus. Figure 28B demonstrates the staining of STAT6 in green and GATA3 in red for the various groups; control, stimulated, 10 μM calpeptin and 1 μM calpeptin. The control and stimulated

groups appear to demonstrate similar staining patterns. Both of the calpain groups demonstrate cells with increased GATA3 staining that seems to either surround the nucleus or reside in the periphery of the nucleus.

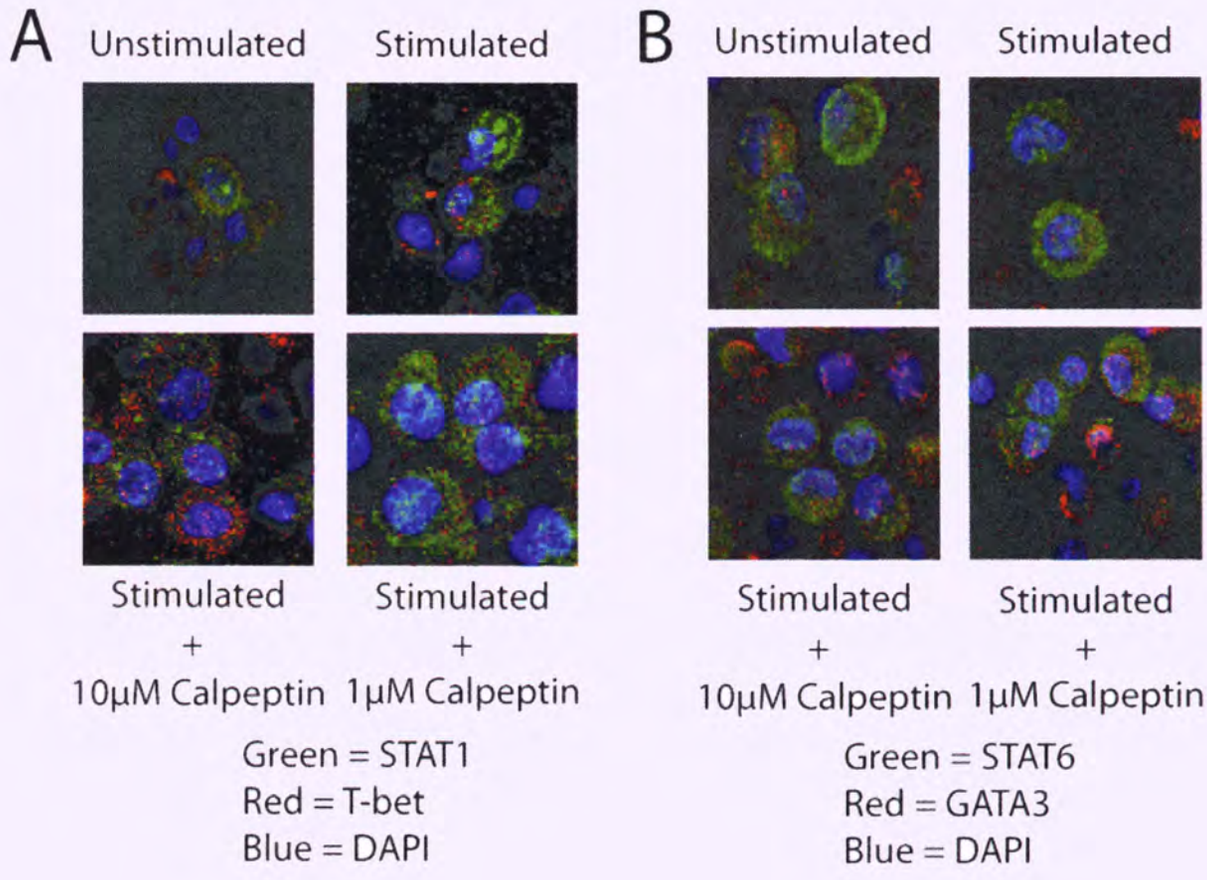


Figure 28: IHC Staining for Transcription Factor Localization. Representative images of MBP Ac1-11 specific T cells stimulated with MBP Ac1-8 peptide for 48 hours and then stained with immunohistochemical techniques for A, STAT1 and T-bet and B, STAT6 and GATA3. The nucleus of the T cells was stained with DAPI. In both staining paradigms the calpeptin treated groups demonstrate a larger percentage of cells staining positive for the transcription factors T-bet and GATA3 compared to untreated control and untreated stimulated group. n = 3 for all treatment and staining groups.

DISCUSSION

Calpain is a protease involved in a multitude of cellular processes in many cell types. Research in our lab on calpain inhibitor in the EAE model of MS has demonstrated a reduction of clinical signs and disease severity with treatment. The clinical signs and relapse are reduced in an adoptive transfer model of EAE in which the primed T cells were exposed to calpain inhibitor before transfer to the susceptible animals. The mechanism for this reduction is explored in the chapter. From the current study calpain appears to act as a negative regulator of Th2 cells through the cleavage of STAT6. Calpain is also known to be upregulated in activated Th1 cells before the onset of clinical disease (Shields et al. 1999a). Therefore the balance of calpain activity probably plays a role in Th cell bias in response to immune challenge contributing to the proper immune response.

The hypothesis is that calpain plays a role in Th subtype bias and potentiation of the Th2 subtype is tested in the following chapter through proliferation studies, cytokine profiling and protein level examination in MBP Ac1-11 specific T cells and in primary lymphocytes isolated from MBP primed animals.

The $CD4^+$ MBP Ac1-11 T cell line, as determined by flow cytometry (data not shown), demonstrated an increased proliferation when various concentrations of calpain inhibitor were included in the assay (figure 14 and 15). This proliferative potential was observed both in the presence and absence of stimulation in figure 14. The effect was not significant when the cells were not

stimulated with their antigen but the trend was apparent in figure 14B. A DMSO control was included in this experiment because T cells increase proliferation in response to sulfoxide compounds. The DMSO was the solvent for calpeptin so it was also included in any stimulated group at the same concentration to allow for an accurate comparison proliferative effects due solely to calpeptin. The effect of calpain inhibition on proliferation was more pronounced and significant during stimulation providing evidence that calpain signaling may act in the activation pathway of this type of Th subtype. This experiment with different methods for activating the MBP Ac1-11 specific T cells also demonstrated that overactivation can drown out the proliferative effect seen with the calpain inhibitor. The native peptide was used for stimulation even though the proliferation and cytokine section (figure 21) were more pronounced with the strong agonist MBP Ac1-11 (4A).

In order to confirm the proliferative effect observed with calpain inhibition, the proliferation assays were expanded to include a larger range of calpeptin concentrations (figure 15) to determine if the proliferation would come back to a stimulated baseline when the concentration of calpain inhibitor was greatly reduced. Figure 15 demonstrates a nice dose dependent proliferation response to the calpain inhibitor calpeptin. This assay was also performed with several other varieties of calpain inhibitors including SNJ6017 and PD150606 which target the active site and calcium binding site respectively. Proliferation was observed but the dose dependent response was not always readily reproducible (data not shown). This MBP Ac1-11 T cell line only one Th cell subtype, so to

test if the calpain inhibitor effect was also applicable in the other Th cell subtypes primary lymphocyte cells were utilized.

The same procedure is used to create the pathogenic T cells for the adoptive transfer EAE model and the primary cells utilized in this chapter. The primary cells did not demonstrate any alterations in properties with regards to calpain inhibitor exposure if they were just stimulated with antigen, therefore cytokines and neutralizing antibodies were utilized to enhance subtype proliferation and increase the effect observed with calpain inhibitor. Biasing was also utilized to isolate the effect in the specific subtypes. Figure 16 demonstrates the subtypes together and demonstrates the necessity of biasing treatment in order to detect proliferative changes with this assay. Figures 17 and 18 demonstrate the distinctly different proliferative profiles observed in the Th subtypes with various calpeptin concentrations. The profile of figure 17B is strikingly similar to figure 15 which increases the evidence that calpain plays a role in Th2 cell subtype as well as increasing the evidence that the MBP Ac1-11 specific cell line is of a Th2 subtype lineage. Recently the importance of Th17 cells in the pathogenesis of EAE has been demonstrated and the decrease in Th17 subtype proliferation (figure 17D) is another potential mechanism by which calpain inhibitor decreases EAE disease. The primary cell subtypes were compared to each other (figure 18) in order to demonstrate that the significance of calpain inhibition on proliferation varies by subtype. The proliferation is important for the cell to become the dominate subtype but cytokines also play a

role in the inflammatory environment present during an immune reaction, so they were also explored in the context of calpain inhibition.

In order to further characterize and identify the subtype of the E3 MBP Ac1-11 specific T cell a cytokine array was performed (figure 19). There are extraneous cytokines present but the specific cytokines IL-5, IL-6, and IL-13 for a Th2 subtype are present in the stimulated group. The media control was performed because the media contains FBS which is known to contain some cytokines. The calpeptin treated group does not have any cytokines that are upregulated compared to the stimulated control; in fact, every cytokine is reduced by a sizeable amount which is probably due to the fact that the calpeptin concentration is 100 μM. At this concentration there was an observed significant decrease of cytokines as measured by other assays as well (figure 20 and figure 22). This array does add evidence to the fact that E3 MBP Ac1-11 specific T cells are of a Th2 lineage and therefore place the proliferation assays into context with regard to calpain inhibition and their proliferative potential. Figure 20 demonstrates that the amount of Th1 cytokines, IFN-γ and IL-12 are extremely low with the E3 cells. This figure also shows that calpeptin produces a significant decrease of cytokines at higher concentration probably related to nonspecific cell cycle effects, reinforcing the importance of low dosing, as described in chapter 5. The supernatant from the proliferation assays that were performed on the MBP Ac1-11 specific T cells with various peptides (figure 14) was also assayed for cytokine concentration (figure 21). There was no significant difference observed with IL-4 and the only significant difference observed with IL-10 was with the

group exposed to 10nM calpeptin as seen in figure 21A and 21B. This lack of significant difference had more to do with the number of viable cells present in the experiment as significance is observed with the same cell type and for the same cytokines in figures 20 and 22. Again the graded response is observed with the native 4K MBP peptide stimulation (figure 21B) compared to the 4A strong agonist which results in a lot more cytokine but not a graded response. Figure 22 again demonstrates that calpeptin can significantly reduce the cytokine secretion at higher concentrations but does not significantly increase the secretion at the concentrations that increase proliferation. The pathogenicity of the E3 MBP Ac1-11 specific T cells was also tested *in vivo* by attempting to induce EAE following an adoptive transfer protocol on mice with an intact immune system as well as sublethally irradiated mice with an impaired immune system. In both instances the E3 cells failed to transfer an EAE disease profile (data not shown).

This cytokine observation was repeated in the primary cell cultures in which the cytokines were measured. Figure 24 demonstrates that the subtypes are in fact becoming the dominate subtype in their respective cultures by the profile measured. However, calpain inhibitor treatment does not produce any significant alteration at most of the concentrations tested. Several of the subtype cytokines that would have been interesting to test and were tested in the E3 cells, such at IL-4, could not be tested in the primary culture because exogenous cytokine was added as part of the assay. The biasing protocol was substantiated by figure 23 which demonstrates the cytokine profile of an unbiased culture and

contains IL-6, IL-12p40 and IFN-γ at higher amounts indicating a predominately inflammatory cell type or either Th1 or a priming to Th17. While these assays reveal that calpain is involved with Th cell bias they do not shed significant light on the possible mechanism. They do, however, demonstrate that cytokine secretion is preserved. The preservation of cytokine secretion at stimulation levels is important in order to maintain proper T cell function and the proper inflammatory environment. The cytokine profile is reinforced by cytokines binding to receptors that activate STAT molecules which in turn transcribe cytokine loci to further strengthen a subtype.

Calpain in known to act upon several of these STAT molecules so the proliferative and cytokine effects observed in these MBP specific T cells by inhibiting calpain activity may be related. The levels of STAT proteins in the whole cell lysate were examined following activation in the presence of several different concentrations of calpeptin. The effect of reduced cytokines (figure 22) observed at the highest concentration of calpeptin is repeated with the STAT protein levels at the highest concentration of calpeptin compared to the stimulated control (figure 25B). The level of STAT1 and STAT4 trend lower but the level of STAT6 is significantly lower compared to the stimulated control. This reduction in STAT protein levels at the highest concentration of calpeptin is probably due to non-specific cell cycle effects and the arrest of the cells in a phase in which the STAT proteins exist at a lower level. The two lower concentrations of calpeptin (10μM and 1μM) however result in STAT6 levels that trend higher than the stimulated control and are significantly higher than the

unstimulated control. This is in contrast to the highest calpeptin concentration. This result supports the hypothesis that calpain contributes to subtype bias through STAT6. This also correlates with the proliferation assay (figure 15) where proliferation of the E3 cells is increased at 10μM and 1μM calpeptin.

If calpain is acting to repress the Th2 subtype through cleavage of STAT6 and calpain inhibition results in increased proliferation of a Th2 subtype as seen in figure 15 and 17C as well as increased STAT6 protein levels (figure 25) it follows that the transcription factors downstream of STAT6 may be increased as well. This notion was explored by western blotting of the E3 MBP Ac1-11 specific T cells to examine the levels of transcription factors T-bet (Th1) and GATA3 (Th2) following stimulation in the presence of various concentrations of calpain inhibitors. There was a significant increase of T-bet and GATA3 protein levels in the groups exposed to the highest calpeptin concentration (100μM) compared to the unstimulated and stimulated controls (figure 26B). This trend is also observed in the *in vivo* studies in chapter 5 where the transcription factors tend to be present at higher levels in the groups that were treated with high levels of calpain inhibitor (figure 34). The reason why T-bet follows a somewhat similar trend to GATA3 can be ascribed to the idea that the protein levels may be controlled in some way by the activity of proteases in the cells. The activity and involvement of the protein to carry out its role of transcription and increasing certain cytokine concentrations may lie with a different signaling mechanism.

The expected trend is displayed in figure 27 which depicts levels of T-bet and GATA3 in activated $CD4^+$ Jurkat T cells in the context of calpain inhibitors.

The T-bet level in the activated groups is higher than the protein level in the activated plus calpeptin group. The GATA3 level in the activated groups is lower than the protein level in the activated plus calpeptin group. This trend is consistent with the hypothesis that calpain activity correlates with Th1 subtype and calpain inhibition correlates with Th2 subtype by influencing transcription factor levels. Unfortunately, the western blotting data from the E3 cells does not correspond to the Jurkat cell data. The whole cell protein levels do not reveal the entire story so the localization of the STAT proteins and transcription factors following stimulation and exposure to calpain inhibitors was also examined.

The subcellular localization of the STAT proteins and transcription factors was an effort to determine if the location in the cell of these proteins played a more important role than just the quantity of the protein themselves. Figure 28 reveals increased staining for both transcription factors in the groups that were incubated with calpeptin, especially in the case of GATA3 (figure 28B). The transcription factor staining also appears more perinuclear in the stimulated cell groups compared to the unstimulated control group. This fits with the mechanism that a transcription factor would move to the nucleus in order to accomplish its task when the cell becomes activated. The STAT proteins also display a more punctuate staining pattern in the stimulated groups compared to the unstimulated control which stains more diffuse through the cytoplasm. This pattern would correspond to increased concentrations of the protein at cytokine loci within the nucleus.

To summarize, the evidence and observations support the hypothesis that calpain is involved with Th cell bias as observed in 3 different cell systems as well as in some of the *in vivo* work that will be presented in chapter 5. Calpain inhibition increases the Th2 subtype by increasing proliferation but not by increasing cytokine secretion. The mechanism by which this occurs may be due in part to involvement of calpain in the STAT protein / transcription factor signaling pathway.

CHAPTER FIVE

COMBINED TREATMENT OF EAE WITH APL AND CALPAIN INHIBITOR

INTRODUCTION

The importance of T cells in the pathology of MS and EAE was identified in part by EAE studies in which the disease was caused by adoptively transferring these cells into naïve animals which subsequently develop clinical signs (Ben-Nun et al. 1981). T cells have therefore been the target of much study in the pursuit of the responsible subtypes. In order to prevent or lessen disease pathologies treatments to alter the cellular properties have been developed. Our lab, as well as others, have demonstrated the efficacy of calpain inhibitors in the reduction of clinical signs and pathologies of EAE (Guyton et al. 2006; Hassen et al. 2006). Likewise the efficacy of APLs of various myelin proteins has been demonstrated to reduce the occurrence and severity of EAE (Leadbetter et al. 1998; McCue et al. 2004; Smilek et al. 1991). APLs of MBP reached phase II trials as a treatment for MS (Bielekova et al. 2000; Kappos et al. 2000) when problems with the treatments overshadowed their efficacy. Calpain inhibitor treatment has not yet been published in a human trial but various improved versions have demonstrated somewhat impressive safety profiles (Inoue et al. 2003). There are shortcomings regarding solubility, cell permeability and specificity. Therefore, it is important to consider the feasibility of multidrug strategies in order to overcome the pitfalls and drawbacks of a single compound at higher doses, yet capitalize on the desirable properties still present at lower doses.

The calpain inhibitor used for these studies is ((1S)-((((1S)-1-benzyl-3-cyclopropylamino-2,3-di-oxopropyl)amino)-carbonyl)-3-methylbutyl)carbamic acid

5-methoxy-3-oxapentyl ester (SNJ1945) provided by Senju Pharmaceutical Co. (Hyogo, Japan). This compound is a peptidyl α-ketoamide with an amphiphile at the P3 site in order to increase water solubility, a known problem of calpain inhibitors (Shirasaki et al. 2005). Since this compound has never been used in an EAE model, a dosing study was designed assess the utility of SNJ1945 and attempt to identify an effective oral dose (Oka et al. 2006). This study was also used to further explore the effects of calpain inhibition on T cell bias and some of the mechanisms potentially involved with this bias.

In order to bring several of the topics from previous chapters together a study was designed to assess whether combination therapy with APL and calpain inhibitor treatment is more effective at preventing clinical disease signs than either treatment alone. The B10.PL mouse was chosen because the antigenic sequence against which this strain mounts an immune reaction is the same MBP sequence modified in chapter 2, MBP Ac1-8. Although it would be interesting to test an aza-APL, their effectiveness alone has not yet been established and would require extensive testing and optimization to select a candidate APL. Since these experiments are testing the combination of APL and calpain inhibitor treatment at low dose an APL that had already demonstrated the ability to reduce EAE signs was selected.

MATERIALS AND METHODS

Cell Culture

The cell types used for the cytokine studies and western blotting were B10.PL mouse leukocytes enriched from the spleen. The animals from the dose study were sacrificed 3 days following the onset of clinical signs and the spleen was dissected out and ground between fritted glass slides to make a single cell suspension. The cells were strained through a 70 μm cell filter (BD Biosciences) and enriched by gradient centrifugation over Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ). The cells were then washed twice and then plated onto petri-dishes coated with anti-CD3 (Santa Cruz Biotechnology) to provide stimulation to the T cells through the TCR. The cells are grown in RPMI 1640 (MediaTech, Inc., Herndon, VA) supplemented with 10% FCS (Hyclone, Logan, UT) at 37^0C in a 5% CO_2 atmosphere (Thermo Fisher Scientific, Waltham, MA). Cell free supernatant samples were isolated 48 hours later for cytokine analysis and the cells were harvested for western blotting. The cells and supernatant were stored at -80^0C until analysis.

EAE Animal Model

Male B10.PL-H2^{u}H2-T18a/(73NS)SnJ mice that were 3-5 weeks old were purchased from Jackson Laboratories (Bar Harbor, ME), provided water and food *ad libitum*, and maintained and used in the proposed experiments in accordance with the Laboratory Welfare Act and the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Mice were allowed to acclimate to the animal facility for at least 3 days before any experimental

manipulations were performed. EAE was induced when mice were about 7 weeks old or in the mass range of 18-20 grams. To induce EAE, mice were immunized with (1:1) CFA containing *Mycobacterium tuberculosis* H37Ra (10 mg/ml; Difco) and phosphate-buffered saline (PBS) containing guinea pig MBP (1 mg/ml). Each mouse received 4 s.c. injections, one over each haunch, totaling 400 µl of the emulsion which contains 400 µg of MBP. Control animals received PBS/CFA alone. At the time of the induction the mice also receive an i.p. injection of pertussis toxin (Sigma) (200 ng/mouse) and again 48 hours later. After induction of EAE, all mice were monitored daily for weight changes and analyzed for clinical signs. The clinical scores for severity of EAE signs were based on the following grades: 0-no change, 1-limp tail or waddling gait with tail tonicity, 2-partial hind-limb paralysis, 3-hind-limb paralysis, 4-hind-limb paralysis with front-limb weakness or partial paralysis, 5-quadriplegic or moribund. Half number scores were used if the animal was partway between clinical score criteria levels.

Drug Administration

The following dosing strategy for the SNJ1945 dosing study is based on doses utilized in a retinal degeneration model (Oka et al. 2006) and administered at a frequency to fit with the published oral pharmacokinetics (Shirasaki et al. 2005). SNJ1945 was suspended 4% (W/V) in distilled water containing 0.5% carboxymethyl cellulose (CMC). The SNJ1945 was then diluted 1:2 in CMC serially to obtain the various doses. Each mouse received 100 µl of the

respective dose by oral gavage two times daily beginning 7 days following EAE induction. The control and EAE animals received just CMC. These animals were monitored for mass changes and clinical signs of EAE. Animals were sacrificed three days after the onset of clinical signs in order to isolate the cells in an acute phase of the disease.

Alternatively, for the combined drug study, the SNJ1945 was dissolved at 0.1% in PBS for the 10 mg/kg dose and diluted ten fold to obtain the 1 mg/kg dose. The dose was given in 200 µl i.p. every other day starting on day 7 and continuing through day 21 for a total of 8 doses. The animals were monitored every day for mass changes and clinical signs through day 35 of the study when they were sacrificed. The SNJ1945 was given i.p. for this study so it could be injected together with the APL.

The MBP Ac1-8[4Y] (ASQYRPSQ) was synthesized as described in chapter two and was chosen because of known efficacy in the $H2^u$ EAE model system (Leadbetter et al. 1998). The APL was dissolved in PBS as a stock solution at 10 mM. This stock was used to prepare two concentrations either 1.25 mM or 0.5 mM which were used to dose the mice. The mice received 200 µl of the APL solution resulting in a dose of either 250 nmol or 100 nmol from the 1.25 mM or 0.5 mM solution respectively. The dose was given in 200 µl i.p. every other day starting on day 7 and continuing through day 21 for a total of 8 doses following the protocol established by a previous study (Leadbetter et al. 1998).

The combined drug groups received either a high combination (10 mg/ml of SNJ1945 + 250 nmol 4Y) or a low combination (1 mg/ml SNJ1945 + 100 nmol 4Y). The drugs were combined by diluting the stock APL into the SNJ1945 solution and again the dose was given in 200 µl i.p. every other day starting on day 7 and continuing through day 21 for a total of 8 doses.

Cytokine Analysis

Cytokines were measured on supernatant collected 48 hours following plating and stimulation on the isolated leukocytes. The cytokines were assessed using OptIEA Sandwich ELISA (BD Biosciences) and the method is described in further detail in chapter four.

Western Blotting

The methodology used for western blotting is described in chapter four.

Statistics Analysis

All statistical tests were chosen based on recommendations by Fleming, *et al.*, for analyzing data from EAE studies (Fleming et al. 2005) and performed using SAS® statistics software. Overall statistical significance for ordinal data including median clinical score, day of onset, and cumulative clinical score (total for all animals within each group) was performed using the Kruskal-Wallis test followed by the Mann-Whitney-U tests for pair-wise comparisons. The proportion of animals with an incidence of a clinical score ≥ 3 in each group was analyzed using the Fisher's exact test. The null hypothesis was rejected when $p < 0.05$.

RESULTS

EAE Clinical Scores were Reduced by Calpain Inhibitor and APL Treatment

Several clinical parameters are displayed in table 2 from the dose study in order to establish whether or not SNJ1945 given orally was at all effective in altering EAE clinical signs or disease pathologies. The incidence of disease is the number of animals that displayed any clinical signs during the time period of the experiment. The day of onset is the median of the first day that clinical signs were observed for animals that did display clinical signs. Animals that did not have clinical signs were not included in this parameter. The maximum clinical score is the highest clinical score observed for any animal in the group. The table is organized by the groups which received the various oral doses of SNJ1945 compared to control animals and EAE animals without treatment.

The incidence of disease was decreased in all of the treatment groups compared to the EAE-0 group that did not receive treatment. The day of onset of clinical signs was increased in the 6.25, 25, 50, and 200 mg/kg groups compared to EAE. The day of onset was similar to EAE-0 in the 12.5 and 100 mg/kg groups. The maximum disease severity in all of the treatment groups was less than the maximum disease severity in the EAE-0 group. The assessment of cumulative clinical score was not possible for this study because the animals were sacrificed three days after onset of disease signs so a complete disease profile was not obtained.

SNJ1945 Dose Study (Time to Disease)

Group	Incidence	Day of Onset	Maximum CS
Control	0/8	N/A	0
EAE-0	18/19 (95%)	16.4 ± 1.03	5
EAE- 6.25 mg/kg	2/3 (66%)	22.5 ± 0.41	2
EAE- 12.5 mg/kg	1/3 (33%)	16	3
EAE- 25 mg/kg	2/3 (66%)	20.5 ± 1.22	3
EAE- 50 mg/kg	2/3 (66%)	20 ± 3.27	4
EAE- 100 mg/kg	2/3 (66%)	15 ± 0.82	3
EAE- 200 mg/kg	2/3 (66%)	23 ± 0.82	4

Table 2: The summary disease scores for the oral dosing SNJ1945 EAE experiment. The groups are divided by the treatment dose. The animals were dosed orally twice daily from day 7 through sacrifice. Animals were sacrificed three days following the onset of clinical signs. The n value for each group is indicated in the incidence column.

Table 3 contains several descriptive disease parameters from the combined therapeutic study including incidence of disease, the day of onset of clinical signs, the maximum clinical score observed for the group and also the mean cumulative score for the group. The mean cumulative score was included in this experiment because the animals were all observed for the same set period of time. The clinical score from each day of the experiment was summed for each animal for the entire time. The mean was then taken of these scores for each group to compare to the other treatment groups.

The incidence of disease compared to the EAE untreated group was the same for the lower dose of SNJ1945 by itself and the highest dose of combined treatment. The incidence of disease compared to the EAE untreated group was decreased for the higher dose of SNJ1945, both of the APL groups, and for the low dose combined treatment. The day of onset for the low dose of SNJ1945 and the high dose of APL compared to the untreated EAE is not different. The day of onset for all of the other groups compared to the untreated EAE group was increased. The maximum clinical score for the SNJ1945 was decreased in the high dose group to a 2 compared to a 4 for the low dose group and a 5 for the untreated EAE group. The maximum clinical score was most markedly decreased in the groups that included an APL as the therapeutic. The mean cumulative score is a summation measure that assess overall disease burden and is only truly accurate in the time course studies where a complete data set for all animals is available. The 100 nmol 4Y group and the low dose combined treatment groups were significantly different than the EAE untreated group. The

other groups demonstrate a marked decrease in the mean cumulative score compared to the untreated EAE group but were not statistically significant.

Combined APL and SNJ1945 Study (Time Course Study)

Group	Incidence	Day of Onset	Maximum CS	Cumulative CS
Control	0/8	N/A	0	0
EAE	18/19 (95%)	16.4 ± 1.03	5	15.38 ± 4.50
EAE- SNJ1945 1 mg/kg	5/5 (100%)	17.6 ± 1.69	4	17.4 ± 9.99
EAE- SNJ1945 10 mg/kg	1/4 (25%)	21	2	4.37
EAE- 4Y 100 nmol	3/4 (75%)	20 ± 0.86	0.5	0.62 ± 0.31†
EAE- 4Y 250 nmol	2/4 (50%)	18 ± 0.71	1.5	5.12 ± 4.80
EAE- SNJ1945 1 mg/kg + 4Y 100 nmol	2/4 (50%)	25 ± 3.53	0.5	1.25 ± 1.09†
EAE- SNJ1945 10 mg/kg + 4Y 250 nmol	4/4 (100%)	20.25 ± 0.95	1.5	3.12 ± 0.94

Table 3: The summary disease scores for the combined therapy treatment. The groups are divided by the treatment dose. The animals were dosed i.p every other day from day 7 through day 21. The dosing groups are 1 mg/kg or 10 mg/kg SNJ1945 and 100 nmol or 250 nmol MBP Ac1-9 (4Y) or combination of these. Animals were sacrificed 35 days after induction of EAE. The n value for each group is indicated in the incidence column. †, $p < 0.05$ vs EAE.

As important as summary measures are in evaluating the disease levels to compare the control and experimental groups, the disease time curves are also important to assess the profile of the clinical disease. The disease time curve for the SNJ1945 dosing experiment does not reveal much information beyond the summary statistics presented in table 2 because the animals were sacrificed 3 days following the onset of signs so a complete disease profile curve is not possible. The combination therapy experiment was performed as a time course experiment so a complete data set exists for all of the days of the experiment which allows for the creation of figures 29 - 31. Figure 29 is the mean clinical score of the SNJ1945 doses alone compared to the EAE untreated and control groups. Figure 30 is the mean clinical scores of the 4Y APL doses alone compared to the EAE untreated and control groups. Figure 31 is the mean clinical score of the combination therapy groups compared to the EAE untreated and control groups.

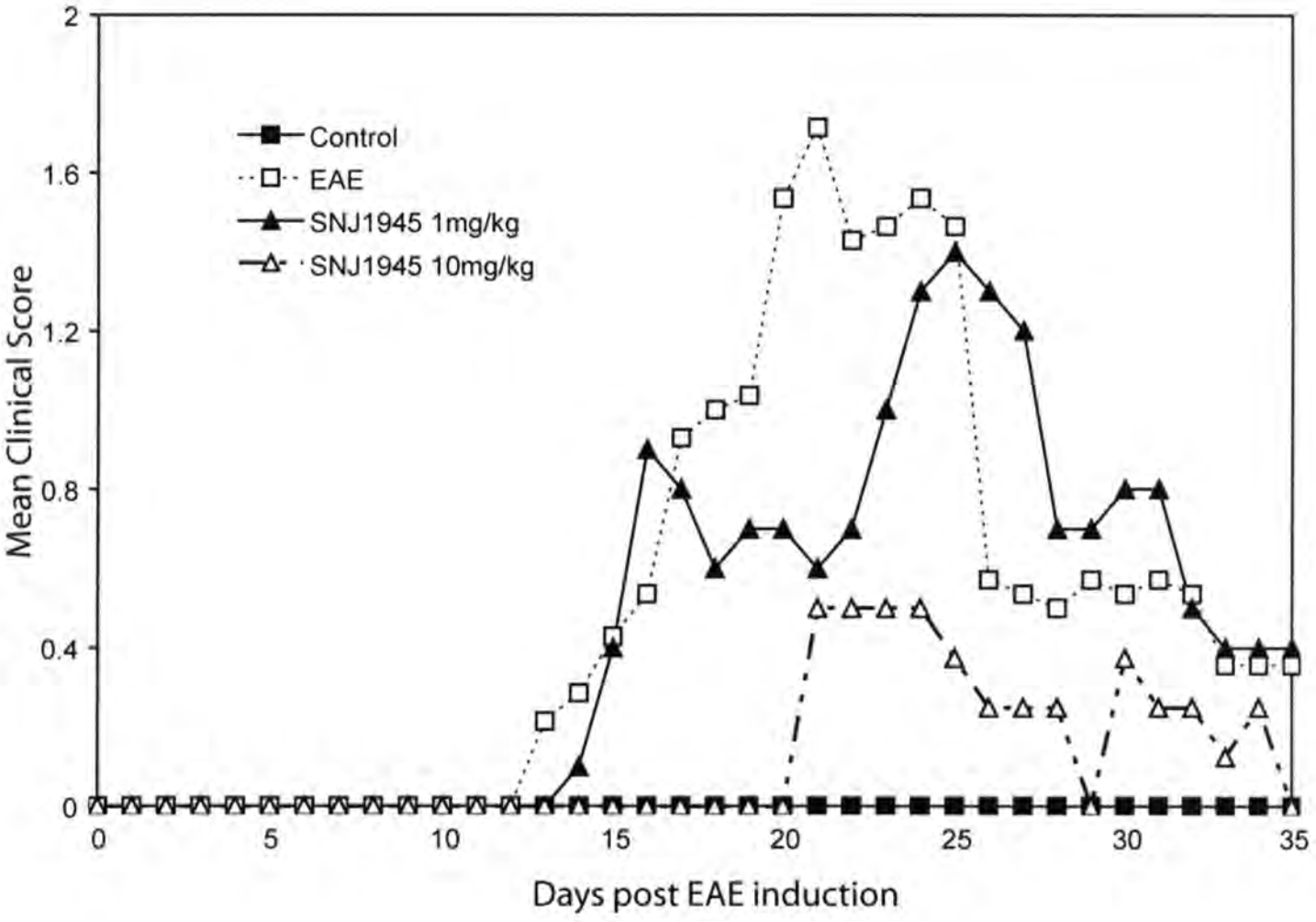


Figure 29: EAE Clinical Scores for the Calpain Inhibitor Treated Groups. Mean clinical score of the SNJ1945 groups compared to the control and EAE untreated groups for each day following EAE induction. The clinical score was assessed following the scale described in the methods section and averaged across the animals in the study group. Each time point represents the mean clinical score (n = 8 for control, n = 19 for EAE, n = 5 for each SNJ1945 group).

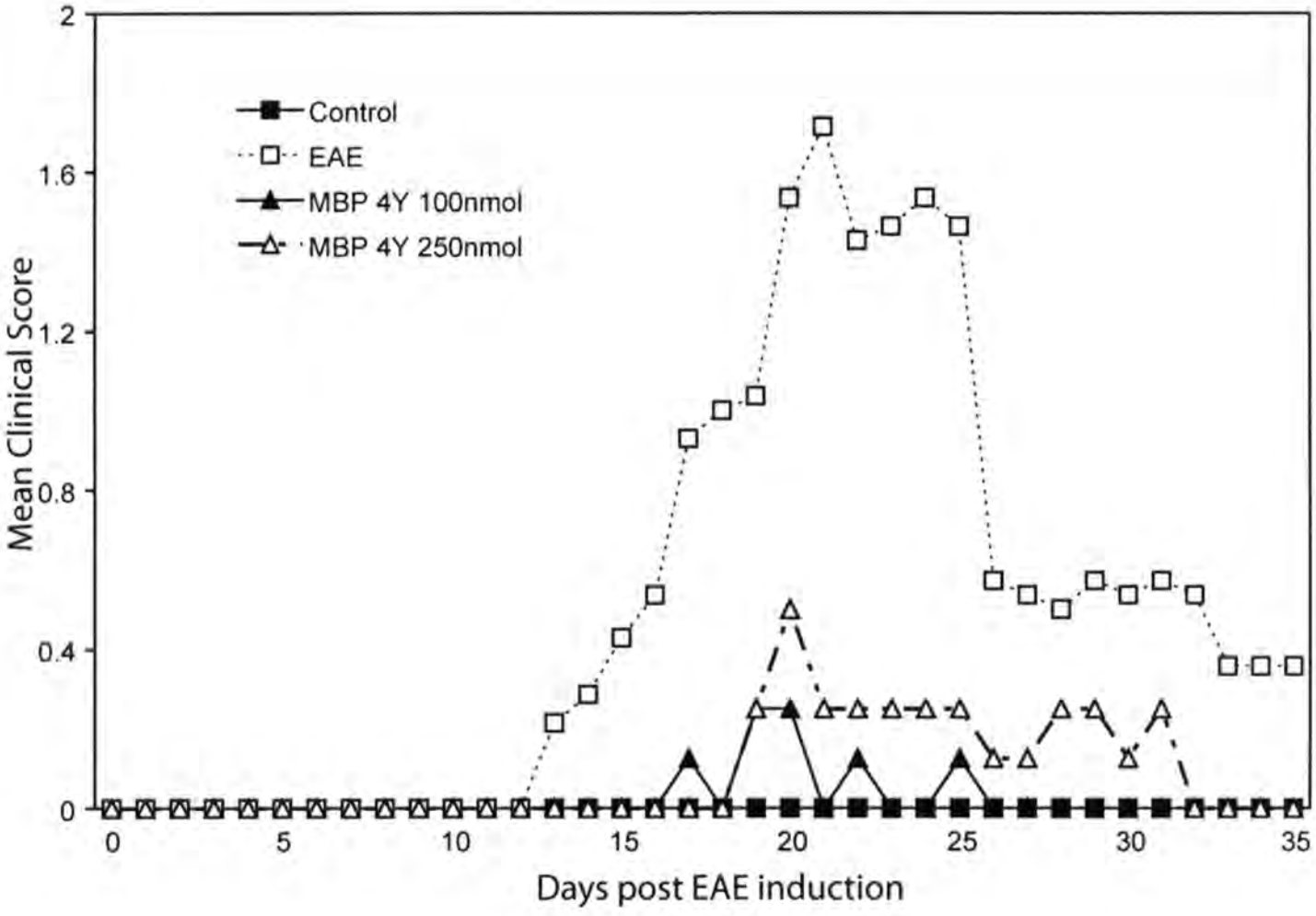


Figure 30: EAE Clinical Scores for the APL Groups. Mean clinical score of the MBP 4Y APL groups compared to the control and EAE untreated groups for each day following EAE induction. The clinical score was assessed following the scale described in the methods section and averaged across the animals in the study group. Each time point represents the mean clinical score (n = 8 for control, n = 19 for EAE, n = 4 for each of the MBP 4Y groups).

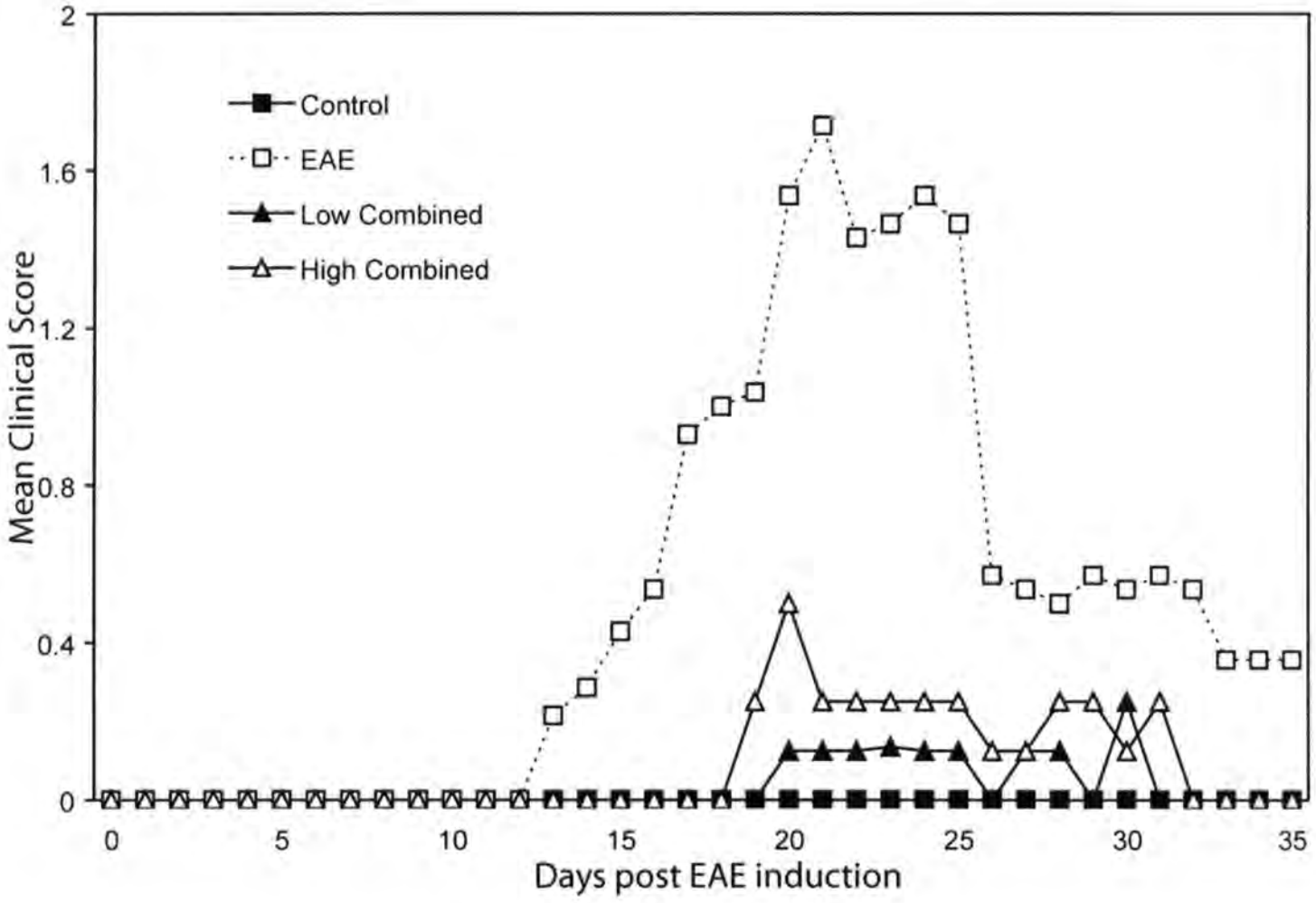


Figure 31: EAE Clinical Scores for the Combined Treatment Groups. Mean clinical score of the combined treatment groups compared to the control and EAE untreated groups for each day following EAE induction. The clinical score was assessed following the scale described in the methods section and averaged across the animals in the study group. The low combined treatment group received 1 mg/kg SNJ1945 and 100 nmol of MBP 4Y and the high combined treatment group received 10 mg/kg SNJ1945 and 250 nmol of MBP 4Y every other day i.p. starting on day 7 post induction and continuing until day 21 for a total of eight injections. Each time point represents the mean clinical score (n = 8 for control, n = 19 for EAE, n = 4 for each combined treatment groups).

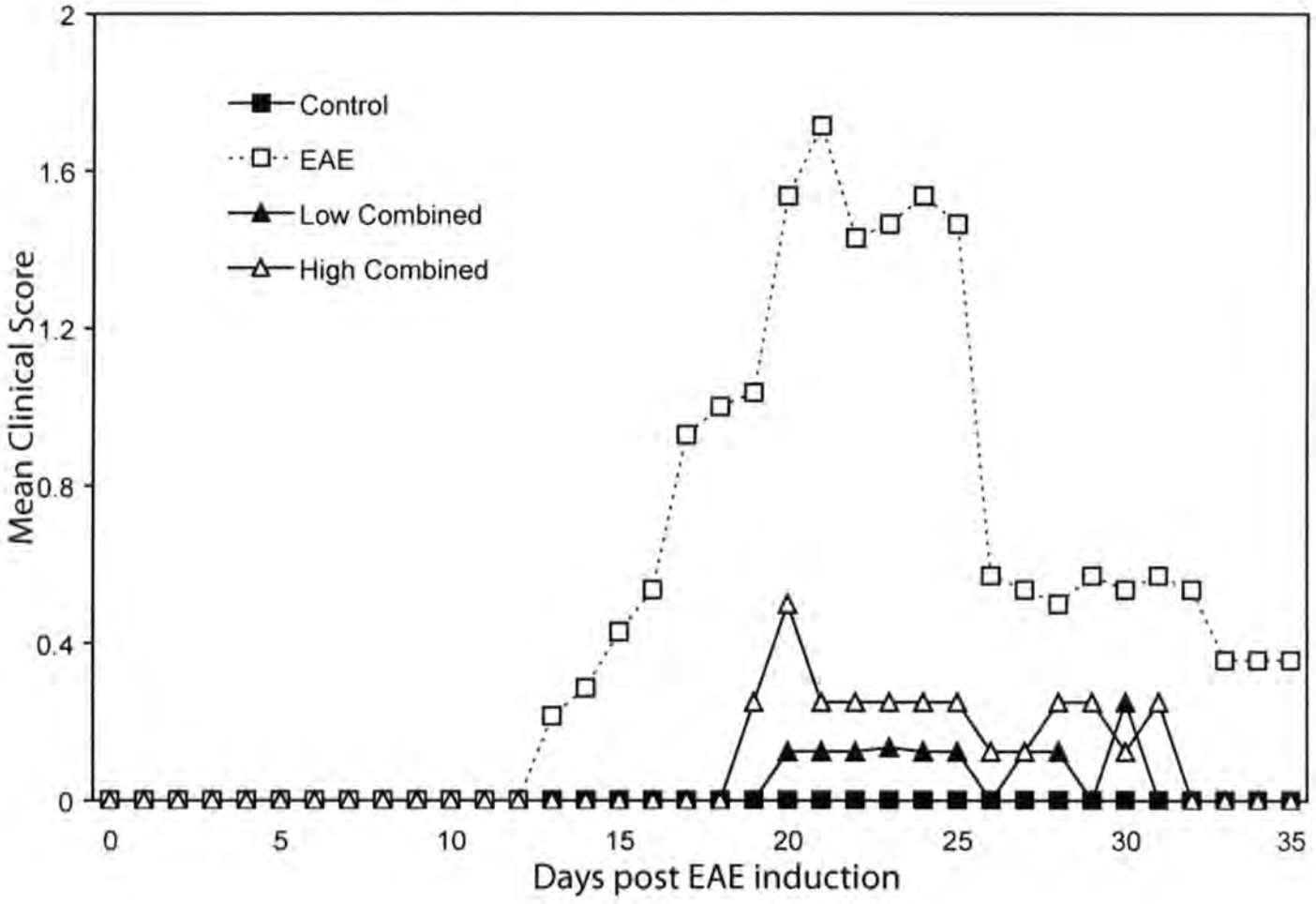


Figure 31: EAE Clinical Scores for the Combined Treatment Groups. Mean clinical score of the combined treatment groups compared to the control and EAE untreated groups for each day following EAE induction. The clinical score was assessed following the scale described in the methods section and averaged across the animals in the study group. The low combined treatment group received 1 mg/kg SNJ1945 and 100 nmol of MBP 4Y and the high combined treatment group received 10 mg/kg SNJ1945 and 250 nmol of MBP 4Y every other day i.p. starting on day 7 post induction and continuing until day 21 for a total of eight injections. Each time point represents the mean clinical score (n = 8 for control, n = 19 for EAE, n = 4 for each combined treatment groups).

Cytokine Analysis of Activated T cells

In an extension of the hypothesis from chapter 4 that calpain plays a role in the bias of T cells and that T cells treated with calpain inhibitor will display a bias toward Th2 cell subtype, the splenic T cells from the SNJ1945 treated animals were harvested, activated and assessed for cytokines. The secreted cytokines were measured to determine if calpain inhibitor treatment of an animal resulted in an altered T cell profile. These T cells were not treated with inhibitor *in vitro* like many of the previous studies but would have to alter their profile *in vivo* in the animal that was receiving the treatment. Figure 32 and 33 are the cytokines as measured by ELISA for the various treatment groups. The EAE untreated animals were divided into low to moderate disease (clinical score of 0-2) and moderate to severe (clinical score 3-5) groups due to the significant differences observed. Figure 32 shows the amounts of the cytokines IL-12p70, IFN-γ and IL-17A for each of the groups. Figure 33 shows the amounts of IL-4 and IL-10 cytokines for each of the groups. The cytokines for figures 32 and 33 were assessed for significance when compare to the control group, the mild EAE group or the severe EAE group. In figure 32 the IL-12p70 is significantly lower in the high grade EAE and all of the treatment groups compared to the control group. The IL-12p70 level is not significantly different in the EAE low grade group compared to the control group. Compared to the EAE low grade group none of the other groups are significantly different. Compared to the EAE high grade group the control group and the two lowest dose groups of SNJ1945, 6.25 and 12.5 mg/ml are significantly different. The levels of IFN-γ are significantly

higher in the low grade EAE and four highest doses of SNJ1945 compared to the control group. The IFN-γ levels are significantly lower in the high grade EAE group compared to control. The two lowest doses of SNJ1945 are not significantly different that control. The low grade EAE group is significantly different than all of the rest of groups either higher (control, EAE high, 6.25 mg/kg or 12.5 mg/kg) or lower (25 mg/kg, 50 mg/kg, 100 mg/kg, 200 mg/kg). The IFN-γ level of the high grade EAE group is significantly lower than all of the rest of the groups. The IL-17A level of the control group is significantly higher than the EAE (3-5) group and significantly lower than the 6.25 mg/kg, 25 mg/kg and 200 mg/kg groups. The IL-17A level of the EAE (0-2) group is also significantly higher than the EAE (3-5) group and significantly lower than the 6.25 mg/kg, 25 mg/kg and 200 mg/kg groups. The high grade EAE (3-5) group has an IL-17A level that is significantly less than all of the rest of the groups by quite a large amount.

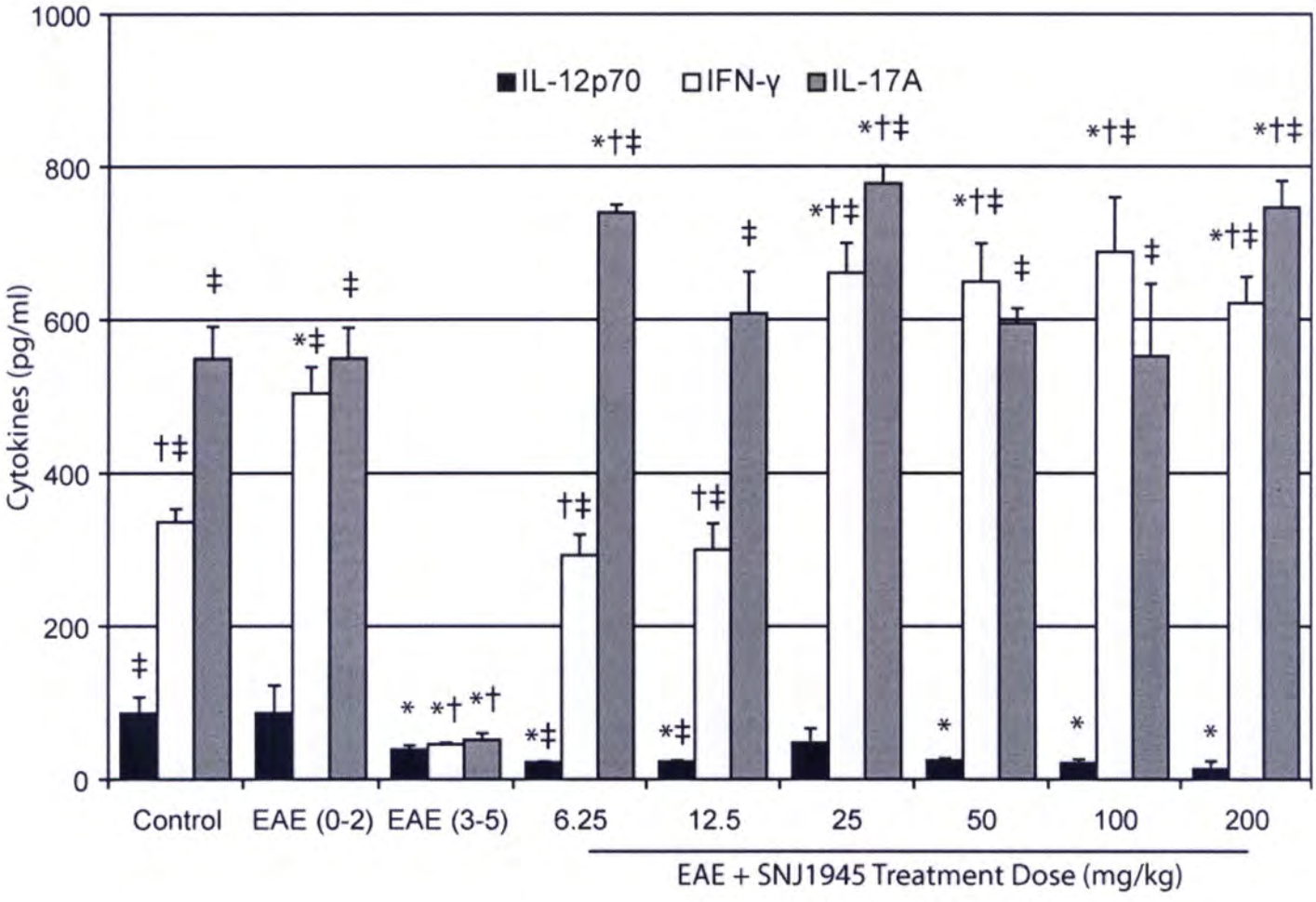


Figure 32: The Th1 and Th17 Subtype Cytokines for the Various Treatment Groups in the SNJ1945 Dose Study. The cytokines were measured in the supernatant from the leukocytes enriched from the spleen and activated with plate bound anti-CD3. The bars represent the mean ± SEM (n = 3 for control, n = 2 for each of the EAE groups and n = 3 for each the SNJ1945 treatment groups). *, $p < 0.05$ vs control. †, $p < 0.05$ vs EAE (0-2). ‡, $p < 0.05$ vs EAE (3-5).

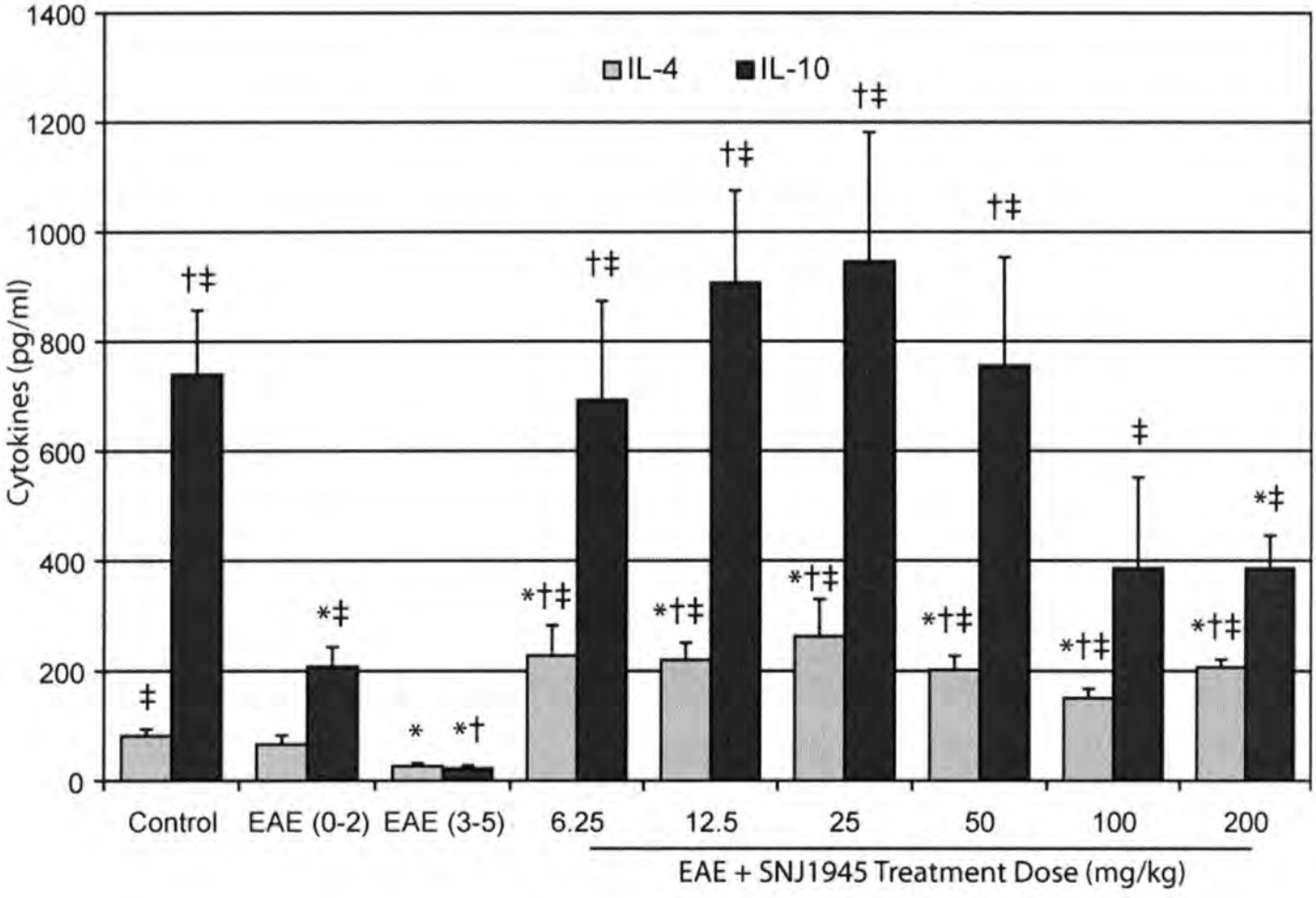


Figure 33: The Th2 and Treg Subtype Cytokines for the Various Treatment Groups in the SNJ1945 Dose Study. The cytokines were measured in the supernatant from the leukocytes enriched from the spleen and activated with plate bound anti-CD3. The bars represent the mean ± SEM (n = 3 for control, n = 2 for each of the EAE groups and n = 3 for each the SNJ1945 treatment groups). *, $p < 0.05$ vs control. †, $p < 0.05$ vs EAE (0-2). ‡, $p < 0.05$ vs EAE (3-5).

The cytokines displayed in figure 33 are most commonly associated with the Th2 subtype (IL-4) and Treg subtype (IL-10) even though Th2 will secrete some IL-10. The IL-4 levels are significantly lower in the high grade EAE group compared to the control group. The IL-4 levels in every SNJ1945 treatment group are significantly higher than the control, the low grade EAE and the high grade EAE groups. The IL-10 level of the two EAE groups and the 200mg/kg group are significantly lower compared to the control group. Compared to the EAE (0-2) group the IL-10 levels of the control and four lowest dose groups are significantly higher. The IL-10 levels of the EAE (3-5) group are significantly lower than the EAE (0-2) group. The IL-10 level of the EAE (3-5) group is significantly lower than the IL-10 levels of all of the rest of the groups measured.

Western Blotting of Activated T cells

In an attempt to further elucidate a possible mechanism by which calpain, or the inhibition of calpain, may be influencing the Th cell type bias the protein levels of several STAT proteins and transcription factors were assessed by western blotting. The samples used for these whole cell lysates are the leukocyte enriched cells from the spleen for which the cytokine profile was also assessed.

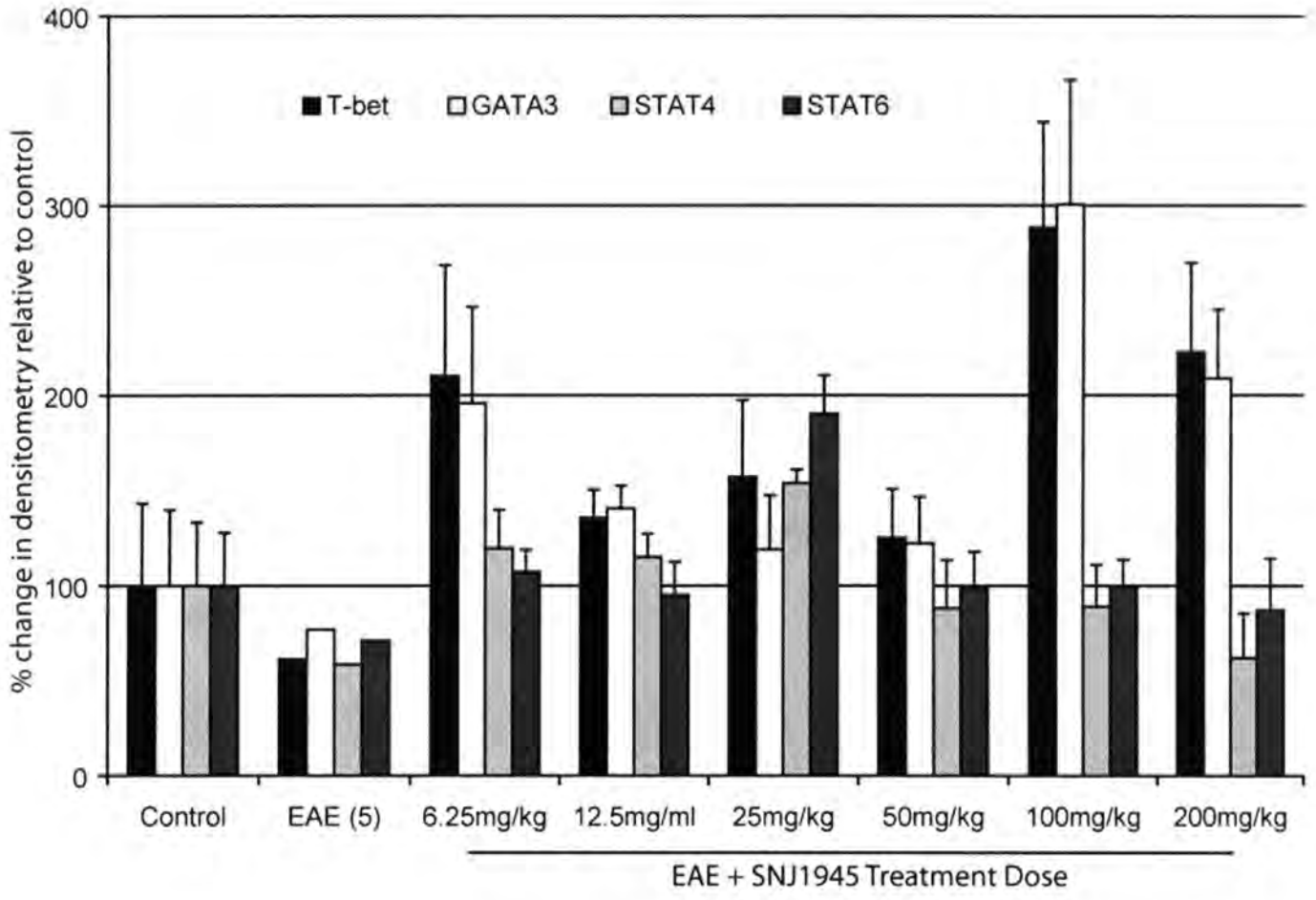


Figure 34: Transcription Factor Protein Levels from the SNJ1945 Dose Study. Western blotting for determination of 62 kD T-bet, 50kD GATA3, 89kD STAT4 and 110 kD STAT6 content in activated leukocyte enriched cultures from spleens of experimental animals following various treatments with SNJ1945. The control animal was induced without the MBP present in the CFA, the rest of the animals were induced as described in the methods and the treatments groups received SNJ1945 orally twice per day beginning on day 7 post induction. The densitometric analysis showing the mean ± SEM percentage change in OD of the various protein bands compared to the control group set at 100%. n = 3 for control, n = 2 for the EAE group and n = 3 for each the SNJ1945 treatment groups. None of the bars were significantly different than control.

Figure 34 is a representation of the mean densitometry of the protein bands for each of the various treatment groups compared to the control bands which were set to 100%. This data set only included one animal (grade 5) for the EAE untreated group. Unfortunately, while some of the data in figure 34 trends towards a significant difference compared to the control and EAE groups there is no real significance at these levels. This is somewhat similar to the experimental observations of the western blotting in chapter four performed on the whole cell lysate. The trend was to increase the protein levels of the transcription factors (T-bet and GATA3) at the higher doses of calpain inhibitors when compared to the untreated or activated groups.

DISCUSSION

Following the previous studies of potential modifications to autoimmunity therapeutics (Chapter 2) and mechanisms involved with EAE disease pathologies (Chapter 3 and 4) the application of a combination of these two approaches in an animal model is of great interest. All therapeutic compounds have side effects or drawbacks applicable to a risk/efficacy treatment paradigm. Combination therapy is a potential strategy to overcome some of the drawbacks that have in the past limited the application of various promising therapy candidates. This specific aim sought to explore the strategy of combining APL therapy with calpain inhibitor treatment in an EAE model to target several disease pathways simultaneously with lower doses of each compound. The lower doses together were hypothesized to reduce clinical signs more than either compound alone at a low dose. The dosing study in Table 2 does show that there is a delay in onset of disease for most groups and a reduction of disease maximum severity in the lower dosing groups more so than in the higher dosing groups. This is an interesting observation also observed in other experiments and EAE models that, at the higher doses of calpain inhibitor, there is either no benefit or an exacerbation of disease. This demonstrates that calpain activity is important for the proper regulation of the immune system and that high level inhibition is actually a problem. This demonstrates the difficulty of controlling the immune system. The observation is actually helpful because complete calpain inhibition is not required for the best clinical effect and the immune system may just need a little push in the correct direction to regulate itself. This increases the

relevance of low level dosing. The involvement of calpain in the neurodegenerative component of EAE (Hassen et al. 2008) makes the pairing of an inhibitor with some other compound that targets the inflammatory component more directly, such as APLs, an appealing strategy. Therefore, a study was performed using the calpain inhibitor, SNJ1945, and an APL of MBP to assess the effectiveness of combined therapy. Table 3 and figure 30 demonstrate that the APL appears more effective than the calpain inhibitor in this model system and at the doses tested but both compounds produced a reduction in clinical signs, as most thoroughly measured by cumulative clinical score. The combined treatment group that used the lower doses of SNJ1945 and the APL was the most efficacious at reducing clinical disease on all of the parameters measured. The lack of significant difference for more of the treatment groups compared to the untreated EAE animals is probably due to the low animal numbers. These studies were performed more as pilot studies and proof of concept so the trend is important. Significant differences are probably easily obtainable from these trends if the n were to be increased for these treatment groups. The combined treatment at low dose fits well with the idea that the immune system does not need to be completely suppressed. A benefit could be observed by only altering the overactive components with a calpain activity above the normal immune system function.

In order to tie these studies back to the previous chapters the cytokine profiles were examined from activated leukocytes enriched from the spleen of the animals in the SNJ1945 dosing experiment. The profile of the peripheral T cells

has an influence on how inflammation is going to affect the CNS (Bynoe et al. 2007) therefore assessing the peripheral T cell was an important measure of the disease model. The calpain inhibitor is thought to act at multiple points in the pathological pathways associated with EAE including, being associated with inflammatory type cells (Th1 cell types, microglia and macrophages) (Imam et al. 2007; Schaecher et al. 2002; Shields et al. 1998). Decreasing calpain activity may reduce inflammatory cells and delay disease or decrease disease burden. Several of the most important observations made from the cytokine profile measured from the dosing study (figures 32 and 33) were the extreme reduction in all cytokines for the severe disease animals. The cytokines for some of the treated groups also shifted from Th1 and Th17 to more Th2 and Treg types. The high grade EAE animals tend to lose cytokine secretion. Future studies may address whether this is due to a down regulation of inflammatory cells or due to suppressive Treg cytokines. This effect may also be due to cell death and massive organ failure in the high grade animals. Alternatively it would be determined if calpain inhibitor treatment is preserving cytokine function in high grade disease animals and if that has any bearing on recovery of those mice.

The total cell protein levels of STAT4, STAT6, T-bet and GATA3 are presented in figure 34 compared to the control level set at 100%. These results are somewhat disappointing but not really surprising. Their relation to calpain inhibition in the T cell subtype profile is not entirely clear though it does match the trend observed in chapter 4. The results trend toward increased transcription factors in the higher dosing groups which also agrees with the results observed

in chapter 4. Perhaps if the number of animals were increased, the differences may become significant but another mechanism may be at work such as phosphorylation in the case of STAT proteins or subcellular localization in the case of the transcription factors. The results from chapter 4 seem to lend evidence to subcellular localization and cell cycle regulation to achieve subtype bias.

The *in vivo* studies performed for this dissertation support the hypothesis that treatment of EAE with a combined therapy of APL and calpain inhibitor at low dose is more effective at reducing clinical disease than either treatment alone at that low dose. Importantly, the combined treatment is more efficacious at the low doses than either treatment alone at the higher doses. This represents an important strategy for exploring treatment options with therapeutics that may have been mildly effective yet not effective enough to rival conventional therapies and were therefore discarded. Addressing the multiple pathological pathways and mechanisms involved with this complex disease, or any complex disease of autoimmunity, is a strategy worth further exploration.

CHAPTER SIX

CONCLUSIONS AND FUTURE DIRECTIONS

SUMMARY

The studies in this dissertation were conducted to test several hypotheses addressing various therapeutics intended to treat EAE mediated by T cells. Chapter 2 includes experiments designed to test the feasibility of creating aza-amino acid substituted APLs that maintain bioactivity. The ultimate goal of testing these APLs was to demonstrate an increased protease resistance in order to address some of the shortcoming of current APL therapeutics. These experiments used aza-amino acid substitution to demonstrate the possibility of creating APLs with increased protease resistance yet maintain properties necessary to preserve bioactivity and specificity.

This dissertation also tested several mechanisms to explain the therapeutic benefit derived from treating EAE animals with calpain inhibitors, especially focusing on the effects on T cells. The experiments described in chapter 3 were designed to test the hypothesis that the Ca^{2+} activated protease calpain was involved with the cellular process of T cell chemotaxis. This was to further define the role that calpain inhibition was inhibiting the cells from entering the CNS; a process required for EAE pathology. The hypothesis was tested with intracellular Ca^{2+} analysis, migration assays, and live cell calpain activity assays. The major finding was that calpain does play a role in T cell migration and this role is restricted to chemotaxis.

The experiments described in chapter 4 were designed to test whether calpain plays a role in Th subtype bias and at what level. This hypothesis was tested with cellular proliferation assays, cytokine analysis and transcription factor

examination. The major findings in this chapter were that calpain does play a role in Th cell bias and seems to exert its greatest influence by negatively regulating the proliferation of the Th2 subtype. Calpain inhibition increased the proliferation of this subtype but the concentration of inhibitor needs carefully control because some calpain activity appears necessary for normal cellular processes. Inhibiting calpain does not appear to extend to increasing cytokine secretion but inhibiting activity at a high enough concentration also inhibits cytokines secretion. Calpain activity does appear to influence Th cell bias though the STAT signaling pathway and inhibition of activity can increase or decrease transcription factors depending on the concentration of inhibitor employed. The localization of transcription factors appears more important than total cellular protein levels with regards to calpain activity influencing Th cell bias.

The experiments performed in chapter 5 were designed to test the combination of several therapeutics in the EAE animal model of MS. Clinical score was monitored following several dosing protocols using the calpain inhibitor SNJ1945 and an APL of MBP independently and in combination. Enriched leukocytes from these animals were tested for cytokine secretion and transcription factor protein levels to determine the *in vivo* effect of calpain inhibition on T cells. The major finding of this chapter was that low dose combination therapy was more effective than either therapy alone.

INTERPRETING THE RESULTS

The involvement of T cells in the pathophysiology of MS and EAE are well established (Chitnis and Khoury 2003; Dittel 2008). The development of therapeutics to target these cells has been difficult and the currently approved therapies that target T cells have potential side effects or are only mildly successful for long term treatment. Improving upon promising therapeutics or illuminating the mechanism by which clinical benefit is derived is therefore important.

The most direct approach is to target the specific autoreactive T cells. An approach for targeting these cells is to modify the antigen to produce a desired immunomodulatory effect. This effect would either, deactivate the pathogenic T cells, or shift them to a more desirable subtype. APLs have produced promising results in many different EAE models but had problems in human trials. Chapter 2 contains experiments that attempted to improve on the hypothesized shortcomings of natural peptide APLs by employing a strategy of including aza-amino acids in order to increase protease resistance while still maintaining bioactivity. Figure 6 demonstrates that it is possible to make aza-amino acid substitutions and maintain binding to a MHC II molecule specific for another peptide sequence. This is an important property for bioactivity because the APLs need proper presentation to T cells. Figure 7 demonstrates the key property of aza-amino acid substitution; increased protease resistance compared to the native peptide sequence. This increase demonstrates that it is possible to alter the desirable drug characteristics of APLs and potentially address the large

dosage requirement for clinical benefit. The ability to activate T cells is an important characteristic to assess any antigen modification and figure 8 shows that T cell activation is still possible through the TCR when an aza-amino acid is included in the antigenic sequence. The sequences and cellular proteins used in this chapter relate to the EAE and MS autoimmune disease systems but this strategy is by no means restricted to MS. Inclusion of aza-amino acids may prove beneficial wherever APL therapy is utilized.

Calpain inhibitor treatment has been demonstrated to decrease the disease signs of EAE. The mechanisms for this observed effect are still being determined but ubiquitously expressed calpain is known to have many functions in cells. This dissertation focused on some of the possible disease modifying mechanisms involving calpain in T cell migration and Th helper cell bias development and proliferation. Chapter 3 demonstrated that calpain appears to play a role in T cell chemotaxis. The cellular calcium levels (figure 9) are favorable for calpain activity when exposed to T cell recruitment chemokines. Calpain inhibition reduced the migration of T cells toward CCL2 (figure 10D) and this effect appears restricted to chemotaxis (figure 10A-C) unlike some other immune cell types (Lokuta et al. 2003). This effect is also apparent in primary cells (figure 11). Calpain inhibitor treatment reducing EAE signs is potentially due to the reduced migration potential of pathological T cells entering the CNS which prevents the inflammatory pathology.

In addition to the role that calpain plays in migration, chapter 4 addresses the hypothesis that calpain plays a role in Th subtype bias. The $CD4^+$ Th cell

plays a large role in the pathology of EAE and the development of certain subtypes favor immunological conditions necessary for pathological sign development. If calpain plays a role in this process, then this might be a contributing mechanism by which administration of calpain inhibitors aids in clinical signs. Figures 14, 15 and 17C quite convincingly demonstrate an increase in Th2 subtype proliferation with calpain inhibition. Th2 cells are known to secrete cytokines that can counteract the effect of inflammatory T cells and reduce the inflammatory bias. This effect was observed in several MBP specific T cell lines as well as in primary MBP Ac1-8 responsive T cells. The proliferation of Th17 cells (figure 17D), one of the predominately pathological T cells in EAE, was conversely reduced at calpeptin concentration that caused a positive proliferation of Th2 cells. The bias toward Th2 subtype does not appear to translate into an increase in secreted cytokines (figures 19-24) but the cytokine levels are maintained at the calpain inhibitor concentrations that produce the increase in proliferation. This means that the cell type bias can be shifted away from an inflammatory subtype by favoring the proliferation of Th2 when calpain inhibitor is present. The caveat of this strategy is that the Th2 subtype tends to favor B cell assistance and may increase the possibility of antibody creation and allergic response. This effect would demand careful monitoring in any clinical trial but the dosing regiment required and the low concentrations necessary would possibly only act upon pathological cells with increased calpain activity, and not normal cellular processes. Several hypothesized mechanisms by which calpain exert its influence upon Th cell bias were tested. Figures 25-28 examine

the protein levels and cellular localization of transcription factors involved with the activation of cytokines that aid in the polarization of the subtypes. These pathways reinforce the dominant subtype which can influence the surrounding inflammatory environment. The protein level of T-bet and GATA3 in the *in vivo* results as well as the *in vitro* results (figures 26 and 34) were increased in the groups treated with the highest concentrations of calpain inhibitor. There was a decrease in the STAT protein levels (figures 25 and 34) in the same groups. While this high concentration of calpain inhibitor does not produce the desired effect of shifting away from an inflammatory cell bias, like the lower concentrations do, it does reveal that calpain activity plays a role related to the level of these proteins. The IHC images in figure 28 demonstrate that the number of cells predominately staining for T-bet and GATA3 are increased in the groups that contain calpain inhibitor. This could be due to localization of the transcription factor toward the nucleus in order to increase the cytokine production in the Th2 cell and increase cellular proliferation and bias. Another possible explanation for this observation is that calpain is involved with a cellular cycle mechanism and the cells become stuck in a phase where more transcription factor is present in the cell than STAT protein. This possibility would explain the western blotting protein levels (figures 25 and 26).

The results obtained in Chapter 5 confirm previous observations that calpain inhibitors can reduce clinical disease signs in EAE animals (Guyton et al. 2006; Hassen et al. 2006). The results also confirm that APLs based on the antigenic sequence of MBP are capable of reducing clinical signs of EAE. The

significant results of this chapter are the reproduction of observations from the *in vitro* studies on T cells with regards to cytokine profiles and transcription factor levels using an orally available calpain inhibitor. In addition these experiments also demonstrated the feasibility of the concept of lower concentration combined therapies to treat EAE. This strategy can produce effective clinical results better than either therapy alone. Targeting multiple pathways is an important strategy to continue pursuing in the future.

FUTURE DIRECTIONS

The concept of aza-amino acid incorporation into APLs can be taken to the next level by determining if increasing the number of aza-amino acids in an APL can further increase the protease resistance and still maintain the characteristics important for bioactivity such as MHC II binding and T cell activation. Furthermore, these APLs could be tested in an EAE model to determine if they possess the same ability to decrease clinical signs as the natural peptide APLs.

The examination of the involvement of calpain in T cell processes can go beyond the observations made in chapter 3 and 4. More mechanistic studies on cell cycle or Th cell bias development would be an interesting direction to pursue. Future studies can use transfected or knockout cells to determine more clearly where in these cellular processes calpain is exerting its effect. An expansion of the IHC studies in order to look at localization of the transcription factors in real time would also be an interesting direction to pursue. Live cell imaging with

transcription factors tagged with green fluorescent protein (GFP) and exposed to calpain activators and inhibitors could be performed.

The animal studies have the greatest range of possible future directions to explore even though they are the experiments that demand the most resources and time. An expansion on the current experiments performed in chapter 5 to determine if an altered cytokine profile returns to baseline after cessation of therapy would be of interest. Also manipulating the dosing concentration and frequency to determine the minimal effective amounts would be of value. The proliferation of Th2 subtype observed *in vitro* should be explored further *in vivo* before calpain inhibitors are used in any human studies for any disease as this has a potential side effect of increased allergic responses. These results may be minimized by dosing amounts, routes and frequency as well as application but they need testing. Calpain inhibitors are being explored in the context of neurodegeneration as well, so the integration of both of the effects, immunomodulatory and neurodegenerative (which is an active research component of the lab) should be performed.

CONCLUDING REMARKS

The data presented in this dissertation supports the hypotheses proposed in the introduction and points the direction toward future studies involving the effects of calpain inhibitor treatment and APL modification. APL treatment research for MS has lost quite a bit of momentum due to some unsuccessful clinical trials but the specificity of the concept is still sound. Future efforts made in field of APL therapeutics, either for MS or any other disease for which specific

peptide therapy may prove effective, would all benefit from strategies meant to increase the desirable clinical benefit while reducing the potential side effects. Aza-amino acid substitution, as demonstrated in chapter 2, is one such strategy that is worth further examination.

The current environment for drug approval by the Federal Drug Administration in the USA is quite stringent and requires knowledge of possible side reaction and deleterious clinical effects. The identification of the effects of a compound, such as calpain inhibitor, that acts upon a protease with a wide range of cellular processes is important if these molecules are ever to be applied in a clinical setting. Chapter 3 and 4 identify some of the effects of calpain inhibition on T cells. Taken in the context of MS, these effects may prove beneficial but they also hold value in the application of these compounds to treat other conditions and the immunomodulation that might occur with their application.

Chapter 5 demonstrated that the use of two compounds that act by different mechanisms is a viable strategy for treating disease. This chapter specifically showed that calpain inhibitor and APLs can work together but more important is the way in which the beneficial effect was achieved. Calpain inhibitors and APLs may have limited use by themselves in treating autoimmunity, due to broad action or past difficulties, but in combination, they demonstrate the important concept that mildly effective drugs may hold promise when logically combined.

The results presented in this dissertation encompass a large variety of scientific topics but demonstrate the importance of cross disciplinary research.

This approach allows for the application of concepts that may seem unrelated at first but prove useful when combined. This combination allows for rapid identification of medically necessary research topics as well as the application in the medical field of promising research results.

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