The Immunodominance of CD4 T Cell Epitopes is Peptide Intrinsic: Implications for Rational Vaccine Design

by

Jason Michael Weaver

Submitted in Partial Fulfillment
of the
Requirements for the Degree
Doctor of Philosophy

Supervised by
Professor Andrea J. Sant

Department of Microbiology and Immunology
School of Medicine and Dentistry
University of Rochester
Rochester, New York
2009
Dedication

To my children, who have filled my life with so much joy. May the World be your playground…

May the raindrops fall lightly on your brow.
May the soft winds freshen your spirit.
May the sunshine brighten your heart.
May the burdens of the day rest lightly upon you.
~Irish Proverb

And to Chrissy, “grá mo chroí”, the love of my life.

The rose of the valley may wither
The pleasures of youth pass away
But friendship will blossom forever
While all other flowers decay
~Mary Power ca. 1881
Curriculum Vitae

The author was born in New London, CT on February 9th, 1976. He attended The Rochester Institute of Technology from 1994 to 1998, and graduated with a Bachelor of Science degree in Biotechnology in 1998. He then attended The University of Rochester from 2001 to 2003, and graduated with a Master of Science degree in Microbiology and Immunology in 2003. He matriculated into the Doctoral Program at the University of Rochester in the summer of 2003 and began graduate studies in Immunology. He has since pursued his research in understanding the mechanisms of CD4 T cell immunodominance under the direction of Professor Andrea J. Sant.
Acknowledgements

I would like to thank the following:

First and foremost, to Andrea Sant, for her support and encouragement in all aspects of science and her incredible talents as a mentor and advisor. She has not only been instrumental in my professional life, but also has become a wonderful friend. Thank you.

My lab mates over the years; Scott Jenks, Christopher Lazarski, Francisco Chaves, Katie Richards, Scott Leddon, Jeni Nayak, Shabnam Alam, Fred Krafcik, Jackie Tung, and Kira Zebroski; for their discussions, assistance and most of all friendships.

Members of my thesis committee; Jim Miller, Deb Fowell, Craig Mullen and Paige Lawrence, you all have contributed greatly to my project and education.

The Department of Microbiology and Immunology and financial support through Dr. Edith Lord’s NIH Training Grant T32-AI0785.

The Center for Vaccine Biology and Immunology, especially Mariano Sanchez-Lockhart.

My family, extended family, and friends for all your love and support.
Foreword

The experimental work presented herein was performed by the author except for the following:

All of the dissociation assays to determine the kinetic stability of peptides in association with MHC class II molecules was performed by Francisco A. Chaves. This thesis could not have been possible without this immense amount of work!

Chapter 3, Table 3.1: analyses of proteins were performed in conjunction with Francisco A. Chaves.

Chapter 3, Figure 3.1: some of these experiments were performed by Christopher A. Lazarski.

Chapter 3, Figure 3.2: performed by Francisco A. Chaves.

Chapter 3, Figure 3.4: performed in conjunction with Katherine A. Richards.

Chapter 3, Figure 3.6D: some of these experiments were performed by Christopher A. Lazarski.
Thesis Abstract

Establishment of an immune response that is focused on a limited number of peptide determinants expressed by a complex antigen or pathogen is a phenomenon known as immunodominance. For CD4 T cells, many of the mechanisms used to explain this selectivity suggest that events related to antigen processing play a major role in determining a peptide’s ability to recruit CD4 T cells. However, more recent data have revealed that an intrinsic biochemical property of the peptide – the kinetic stability of the peptide:MHC class II complex – both predicts and controls CD4 T cell immunodominance. At the initiation of these studies, we hypothesized that peptide stability from MHC class II is the principle factor in determining the immunogenicity of a given peptide. Those peptides that have a very stable interaction with its presenting MHC class II molecule will not only have an advantage during the processing of antigen but also in the events after peptide presentation at the surface of the antigen-bearing cell. Thus selecting for CD4 T cells that recognize only very persistent peptide:class II complexes for the final specificity of the CD4 T cell repertoire after antigen challenge. The results herein show that the immunodominant heirarchy of peptides contained in complex antigens is independent of molecular context, competing peptides and epitope availability and is principally due to an intrinsic factor of the peptide, based upon the stability of that peptide for MHC class II molecules. Additionally, when antigen processing is bypassed, low stability peptide:class II complexes were capable of supporting the initial priming and
expansion of CD4 T cells but the expansion becomes strikingly aborted in the presence of competitive T cell responses to unrelated dominant peptides. These findings suggest that the immune system has adopted multiple independent mechanisms to select a CD4 T cell repertoire that is focused on long-lived peptide:class II complexes during a response to invading pathogens or vaccine-induced immune responses.
Table of Contents

Dedication............................................................................................................................ii

Curriculum Vitae..................................................................................................................iii

Acknowledgements...............................................................................................................iv

Foreword..............................................................................................................................v

Abstract................................................................................................................................vi

Table of Contents.................................................................................................................viii

List of Tables.........................................................................................................................xii

List of Figures.......................................................................................................................xiii

List of Abbreviations............................................................................................................xviii

Chapter 1  Thesis Introduction

1.1 Significance....................................................................................................................2
1.2 Overview of antigen processing and presentation..........................3
1.3 Dendritic cells orchestrate the immune response and prime naïve CD4 T cells.................................................................6
1.4 Classical view of immunodominance.........................................13
1.5 Competition.............................................................................17
1.6 The peptide-intrinsic model of immunodominance ......................20
1.7 Scope of thesis.........................................................................25

Chapter 2  Materials and Methods

2.1 Preparation of cross-linked amylose...........................................31
2.2 MalE protein purification..........................................................31
2.3 ANS binding assays..................................................................32
2.4 Mice........................................................................................33
2.5 Immunizations..........................................................................33
2.6 Cell purification.........................................................................34
2.7 Isolation of T cells from ear dermis..........................................35
2.8 ELISpot assays..........................................................................36
2.9 Bone marrow-derived dendritic cell preparation......................37
2.10 Construction of Flt3L-secreting cell line.................................38
2.11 Immunization with Flt3L-secreting cell line and isolation of DC-enriched spleen..............................................................39
2.12 Antibodies and peptides..........................................................39
Chapter 3  Immunodominance is independent of molecular context

3.1 Introduction .................................................................................. 43
3.2 Results ......................................................................................... 45
3.3 Discussion ................................................................................... 57

Chapter 4  Persistence of high stability peptide:class II complexes in vivo

4.1 Introduction .................................................................................. 73
4.2 Results ......................................................................................... 75
4.3 Discussion ................................................................................... 83

Chapter 5  Abortive activation of CD4 T cell responses during competitive priming

5.1 Introduction .................................................................................. 95
5.2 Results ......................................................................................... 99
5.3 Discussion ................................................................................... 111

Chapter 6  Development of a serum-free DC-based immunization system

6.1 Introduction .................................................................................. 128
6.2 Results ......................................................................................... 131
Chapter 7  Thesis Discussion

7.1 Summary.................................................................151
7.2 Mechanisms of immunodominance.................................152
7.3 Immunodominance and the fate and effector function of CD4 T cells ..161
7.4 Implications of selecting for persistent peptide:class II complexes......163
7.5 Application to rational vaccine design and immunotherapy..........166

Bibliography.............................................................................173

Appendix....................................................................................226
List of Tables

Table 3.1  Peptide accessibility does not differentiate the immunogenicity of several heterologous epitopes……………………………………………………………62

Table 5.1  Kinetic stability of wild-type and variant peptide epitopes in association with I-A^d………………………………………………………………………………..117
List of Figures

Figure 1.1 MHC class II-restricted antigen presentation ……………………29

Figure 3.1 Immunodominance is independent of protein context………………..63

Figure 3.2 Differences in acid-induced conformational stability of multiple proteins…………………………………………………………………………64

Figure 3.3 The efficiency of antigen presentation is not detectably altered by location of the peptide within a complex antigen…………………65

Figure 3.4 A peptide intrinsic factor rather than the site of localization in the antigen dictates immunogenicity……………………………………66

Figure 3.5 Efficient antigen presentation of a single peptide epitope is not detectably altered by location of the peptide within a complex antigen..67

Figure 3.6 The immunodominant phenotypes for two overlapping epitopes in a single peptide are distinct and correlate with kinetic stability………..68

Figure 3.7 Hypothesized enhanced cleavage of a cryptic peptide ………………..69
**Figure 3.8** Addition of a dibasic motif increases the liberation of a cryptic peptide *in vitro*………………………………………………………………………….70

**Figure 3.9** Increased processing of a cryptic peptide is not sufficient to confer immunodominance *in vivo*……………………………………………..71

**Figure 4.1** Persistence of high stability peptide:MHC class II complexes *in vivo*...88

**Figure 4.2** Phenotype of LPS activated BMDC………………………………………………..89

**Figure 4.3** Similar epitope densities between peptides of varying kinetic stabilities…………………………………………………………………………90

**Figure 4.4** High stability peptide:class II complexes persist *in vivo*……………..91

**Figure 4.5** Kinetic stability of the peptide:class II complex dictates T cell expansion…………………………………………………………………….92

**Figure 4.6** Differential rates of T cell division elicited by low or high stability peptide:class II complexes…………………………………………….93
**Figure 5.1** Expansion of T cells to low stability peptide:class II complexes is lost in the presence of ongoing responses to unrelated peptide antigens…118

**Figure 5.2** Similar patterns of T cell loss to low stability peptide:class II complexes when measuring IL-2 or IFNγ producers……………………………119

**Figure 5.3** The persistence of peptide:class II ligand controls the ability of CD4 T cells to expand during responses to unrelated peptide antigens……120

**Figure 5.4** Co-administration of a low stability peptide with a single dominant peptide is sufficient to induce a loss in CD4 T cell expansion to the low stability peptide………………………………………………………121

**Figure 5.5** Systemic suppression does not account for the failure of low stability complexes to sustain CD4 T cell expansion……………………………..122

**Figure 5.6** Competitive loss of responses to low stability complexes requires that the competitor and test peptides be introduced at the same immunization site……………………………………………………..123

**Figure 5.7** Loss in T cell responses to rapidly decaying peptides is not due to peptide competition for binding of class II molecules in vitro………124
Figure 5.8  Attenuation of the T cell responses is not due to competition for peptide binding to MHC class II molecules..............................125

Figure 5.9  Abortive CD4 T cell expansion to low stability peptide:class II complexes.........................................................126

Figure 6.1  Map of murine Flt3L inserted into pHβ Apr-1-neo.........................143

Figure 6.2  MHC expression on Line 1 tumor cells in vitro..............................144

Figure 6.3  Secretion of Flt3L from tumor cells ........................................145

Figure 6.4  Flt3L increases splenic-derived DC .......................................146

Figure 6.5  Flt3L generates a heterogeneous population of DC.........................147

Figure 6.6  Visualization of peptide loading onto in vivo expanded DC..............148

Figure 6.7  Flt3L expanded DC can prime CD4 T cells ...............................149
**Figure 7.1**  T cell expansion to dominant peptides is temporally linked to the reduction in responses to low stability peptides………………..……169

**Figure 7.2**  Persistence of peptide:class II complexes during the evolution of an immune response……………………………………………………..170

**Figure 7.3**  Modulation in chemokine expression may attenuate T cell responses to low stability peptide:class II complexes………………………………….171

**Figure 7.4**  Peptide persistence controls the evolution of a productive immune response at several levels…………………………………………… ..172

**Figure A.1**  Kinetics of CD4 T cell expansion to low and high stability peptide:class II complexes…………………………………………………………227
List of Abbreviations

APC   Antigen presenting cell
DC    Dendritic cell
MHC class II Major histocompatibility antigen class II
DM    H-2M
CLIP  Class II-associated invariant chain derived peptide
Ii    Invariant chain
CD4   CD4 positive
CD8   CD8 positive
FRC   Follicular reticular cells
FDC   Follicular dendritic cells
TcR   T cell Receptor
IL    Interleukin
Th1   T helper type 1
Th2   T helper type 2
Tfh   T follicular helper
Th17  T helper type 17
IFN-γ Interferon gamma
ELISpot Enzyme-linked immunosorbant assay
LACK  Leishmania homolog for activated C kinase
MalE  E subunit of E. coli maltose binding protein
HEL   Hen egg lysozyme
OVA   Ovalbumin
HA    Hemagglutinin
CFSE  carboxyfluorescein succinimidyl ester
CFA   Complete Freund’s Adjuvant
IFA   Incomplete Freund’s Adjuvant
BMDC  Bone marrow-derived dendritic cell
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>Flt3L</td>
<td>Fms-Like Tyrosine Kinase 3 Ligand</td>
</tr>
</tbody>
</table>
CHAPTER 1

Thesis Introduction

1.1 Significance
1.2 Overview of antigen processing and presentation
1.3 Dendritic cells orchestrate the immune response and prime naïve CD4 T cells
1.4 Classical view of immunodominance
1.5 Competition
1.6 The peptide-intrinsic model of immunodominance
1.7 Scope of thesis
1.1 Significance

The adaptive immune response by CD4 T cells is typically initiated when antigens or pathogens are introduced into a peripheral site, either through the skin or into organs, such as the gut or respiratory tract. At these sites, the pathogenic organism or antigen is typically taken up by resident professional antigen presenting cells (APC), either through endocytosis or receptor-mediated uptake. In endosomal compartments of APC, protein antigens are proteolyzed and the peptide fragments are loaded onto MHC class II molecules with the help of DM. The resulting peptide:class II complexes are then exported to the cell surface. During this time of antigen processing and presentation, the APC, typically a dendritic cell (DC) leaves its peripheral site and traffics to the draining lymph node where CD4 T cell recognition and expansion can be initiated. A highly selective CD4 T cell repertoire is usually primed under these conditions, leading to a distinct hierarchy of antigen reactive CD4 T cells known as immunodominance. Although the phenomenology of restricted CD4 T cell specificities to foreign antigens in vivo, known as immunodominance, is well established, the mechanism(s) that govern this process has been a focus of intense study and debate for many years.
1.2 Overview of antigen processing and presentation

Intracellular processing of a complex antigen into peptide fragments is an obligatory event for CD4 T cell recognition of cognate antigen on the surface of professional antigen presenting cells (Figure 1.1). Antigen processing by macrophage was first described by Ziegler (1), and was later shown to be a degradative process, based on the capacity of lysosomal (2), and protease inhibitors (3) to inhibit the display of peptide:MHC class II complexes following antigen internalization. It was further shown that this process could be bypassed in vitro by prior protease cleavage of antigen (4). This early data, and a large body of subsequent experiments (reviewed in (5-10)), has led to the current view that three molecules play an integral role in the presentation of antigenic peptides to CD4 T cells on the surface of antigen presenting cells; αβ MHC class II heterodimeric glycoproteins as antigenic receptors, and two accessory molecules, invariant chain (Ii) and DM (H-2M in mice and Human Leukocyte Antigen (HLA)-DM in humans). Ii, a non-polymorphic chaperone, has been shown to play a critical role in assisting in the folding of MHC class II in and transport out of the endoplasmic reticulum. During biosynthesis in the endoplasmic reticulum, with the help of calnexin, three αβ MHC class II heterodimers will bind sequentially with Ii to form a nanomer. The complex is subsequently targeted to the Golgi apparatus and actively sorted to endosomes by a sorting signal on the cytoplasmic domain of Ii (11). Ii prevents aggregation and maintains the integrity of the class II molecule at neutral pH (12), while also protecting the class II binding
groove from premature binding of peptides (13). Once the Ii:MHC class II complex reaches an acidic environment within the endosomes (ranging from pH 4.5-6.5, with optimal peptide exchange at pH 4.5-5.5), Ii is cleaved by cysteine and aspartic proteases (cysteine - B, F, H, L, S and Z, and AEP, and aspartic - D), leaving a set of Ii peptide fragments termed class II-associated Ii peptides (CLIP), represented by amino acid residues 81-104, forming an intermediate CLIP:class II complex (14-16). CLIP can then dissociate from, with a concurrent association of other self- or foreign-derived peptides to MHC class II molecules, and these complexes can then subsequently be expressed on the APC surface. Peptides bind the membrane-distal domain of the MHC class II molecule with both termini protruding from the binding groove. This binding involves interactions between peptide side chains and specificity pockets lining the binding groove (P-1, -4, -6, -9) (17, 18) with a lattice network of hydrogen bonds that stabilizes and orients the peptide into the binding groove of class II (19-23) resulting in the productive formation of peptide:class II complexes that are exported to the surface of the APC.

Another chaperone, DM, a non-classical MHC class II heterodimer without apparent peptide binding capability (24, 25), has a crucial role during antigenic peptide loading onto class II molecules. This was first observed when HLA-DMα and HLA-DMβ were identified as the genetic components necessary to restore presentation in mutant B cell lines (26-28), as these mutant cells had impaired antigen presentation, but were capable of presentation of exogenously added peptides. Furthermore, another DM
variant B cell line was found to present an abundance of CLIP:class II complexes on the surface (29), suggesting there may be a defect in the capability to process and present exogenous antigen adequately. This hypothesis was confirmed when cells transfected with the α and β chains of DM, a wild-type phenotype was observed, as they were now capable of presenting exogenous antigen (27, 28). DM has a targeting signal in its cytoplasmic domain that directs the transport and accumulation of it within endosomes. Within these highly acidic compartments, the pH dependent exchange of peptides is not only maximal (30), but also the activity of DM is maximal (31-33), where it can enhance the process of peptide exchange. DM facilitates the dissociation of CLIP and prevents the functional inactivation of empty class II molecules by maintaining class II in an “open” or peptide receptive conformation until a more stable binding peptide is bound in the binding groove of class II (34). The relative effect of DM on the dissociation of peptides, termed the J factor (31), has been shown to be directly proportional to the intrinsic ability of a peptide to stably bind MHC class II molecules. Hence, during this exchange process, DM alters the repertoire of non-CLIP peptides that bind MHC class II, shifting the determinant display of exogenous antigens (6, 31, 35-38) and alloantigens (39), thereby introducing a bias toward DM-resistant peptide:MHC class II complexes in a process known as DM editing.
1.3 Dendritic cells orchestrate the immune response and prime naïve CD4 T cells

Dendritic cells, the primary APC that is capable of priming naïve CD4 T cells, are sentinels spread throughout the body that are highly efficient in sensing the damage and destruction of tissues (danger signals) and uptake of antigens present in their immediate surroundings. They have the distinct ability to induce tolerance or immunity, and in doing so driving naïve lymphocytes to distinct T helper fates. There are at least 5 different types of dendritic cells in the mouse, characterized based upon cell surface markers and their capacity to induce tolerance or immunity when priming T cells (40). Upon antigen acquisition and sensing of maturation signals by recognition of pathogen-associated molecular patterns (PAMP) through pattern recognition receptors (PRR) (such as toll-like receptors) or other inflammatory mediators (e.g. Type I IFN or TNFα) the dendritic cell undergoes a phenotypic change termed ‘maturation’. Upon maturation, DC lose their ability to capture antigen and increase their expression of MHC class II molecules and costimulatory molecules such as CD80, CD86, and CD40 that aid in the efficient priming of naïve T cells. A comprehensive microarray analysis of DC shows they will modulate the expression of many genes upon maturation (41). Upon maturation the DC, which typically is not a very mobile cell, releases from collagen and efficiently traffics to secondary lymph nodes (reviewed in (42-44)). It is within these highly organized organs that priming of naïve T cells takes place. DC will cluster around high endothelial venules (HEV), where the CCR7 ligand CCL21 is highly expressed, to
efficiently interact with T cells entering the lymph node via the blood. T cells will use specific receptors (such as CD62L, CCR7, and lymphocyte function-associated antigen (LFA-1)) to recognize ligands on the high endothelial cells of venules in the lymph nodes, stick, and then pass between these cells to enter the central part of the node known as the paracortex (45). T cells can spend a few hours to days within the lymph node and if they do not recognize their cognate antigen return to the blood via the efferent lymph vessels and thoracic duct (46). Once in the blood, they will spend less than 30 minutes in the circulation before homing to another lymphoid organ (47).

Trafficking within the lymph node had been difficult to assess until more recently with the advent of the 2-photon microscopy. The deliberate and interactive behavior of T cells and the cellular components making up the lymphoid vessel network have been illuminated. T cells were first thought to have a seemingly random walk and stochastic interaction with antigen-bearing cells while “scanning” their environment (48, 49). This high motility of randomly migrating T cells, combined with the ability of highly motile dendrites on DC to spread out allows for highly efficient scanning of the lymph node. Published data showed that between 500 to 5,000 T cells may contact an individual DC per hour (50). In contrast to this view of ‘randomness’, there is now strong evidence showing T cell motility within the lymph node is not random and follows a complex cellular conduit system. T cells crawl along a lymphatic network consisting of a core of collagen fibers surrounded by extracellular matrix molecules, a basement membrane, and a sleeve of stromal cells; follicular
reticular cells (FRC; ERTR-7⁺) (51-54), that is highly abundant within the paracortex and not found within the B cell follicles (54). Interestingly, B cells crawl along a similar network of fibers consisting of follicular dendritic cells (FDC) (55) and the specific localization and exclusivity of FRC and FDC may play a role in the compartmentalization of the lymph node. Furthermore, T cells will ‘stop’ at bifurcations in the FRC, and then continue on, after seemingly receiving external cues to dictate their progression along the FRC. It is unknown if the T cells are being guided by chemokine gradients (guided directionality) or whether the FRC network simply enhances the possibility of interaction with antigen-bearing DC occupying small gaps between the FRC (52). But it is suggested that antigen-bearing DC spread out their dendrites along the FRC and produce chemoattractant factors to help guide naïve T cells along the reticular network toward them for scanning (56, 57). This may account for the T cell ‘stop’ and directional changes at bifurcations along the FRC toward mature, antigen-bearing DC as a means of increasing the efficiency of possible DC:T cell interactions.

Within the paracortex of the lymph node, conventional CD4 T cells recognize processed peptide fragments in the context of MHC class II molecules on APC by the cell surface expressed αβ T cell receptor (TcR). Naïve CD4 T cells must have the capacity to recognize self, as survival in the periphery is contingent on tonic interactions with self peptide:MHC class II complexes (58, 59), but also non-self determinants, in order to counteract pathogen invasion (60, 61). The typical
precursor frequency of T cells recognizing a foreign peptide antigen has recently been shown to be approximately 20-200 cells (62). When T cells encounter antigen expressed on a mature DC, they upregulate maturation markers (CD69, CD25, and CD44), secrete cytokines (IL-2), and down-regulate CD62L-selectin and CCR7. The expression of CD69 is an integral mechanism in retaining T cells within the lymph node. The increased expression of CD69 is thought to inhibit the expression of sphingosine 1-phosphate receptor 1 (S1P1) on the surface of the T cell through protein:protein interactions and maintain its internalization (63). After several divisions, CD4 T cells decrease the expression of CD69 allowing for the re-expression of S1P1 on the surface, which binds sphingosine 1-phosphate (S1P) on secondary lymphoid tissue for egress through the efferent lymph and thoracic duct to migrate to sites of tissue infection (63-66).

Acquisition of T cell effector function, the classical Th1 (IL-2 and IFNγ producing) or Th2 (IL-4, -5, -13 producing) phenotypes, as well as others (Tfh, IL-17 and -23 producing and Th17, IL-17 producing), have been hypothesized to be acquired by dendritic cells via several models. For example, DC may be precommitted to secrete either Th1 (IL-12) or Th2 (IL-4) promoting cytokines upon stimulus (DC1 or DC2), a temporal model in which DC receive a maturation stimulus and produce Th1 promoting cytokines, then further matures into a Th2 priming DC, or the flexible model in which a DC precursor is able to mature into either a Th1- or Th2-inducing DC (40, 67). Not only could the DC be directing the fate of the T cell through
cytokines, but also through the continued availability of cognate ligand for the TcR during productive interactions.

Most of our knowledge on the requirements of antigen in driving a T cell response derives from analyses of CD8 T cell responses. Using a *Listeria monocytogenes* model of infection, it was shown that CD8 T cells continued through several rounds of division and acquire effector function, despite the abolition of live bacteria with antibiotic treatment within 24 hours (68). Furthermore, *in vitro* studies showed that CD8 T cells were capable of continued expansion when removed from antigen or APC stimulation (69, 70). Similarly, when recently activated CD8 T cells were transferred into naïve hosts, they continued through several rounds of programmed cell division (71). More recently, it was shown that a brief period of antigen is sufficient to begin programmed cell division, but persistence of antigen, in concert with costimulation and cytokines, is required for the survival and effector function of an oligo-clonal population of CD8 T cells (72, 73). Collectively, these types of data reflect the view that CD8 T cells require only a brief encounter with their cognate antigen to induce a transcriptional program that drives T cell expansion and ultimately, their effector and memory functions. This process of programmed cell division has been termed “autopilot” (74).

Less clear in the literature is whether CD4 T cells follow the same “autopilot” programming upon antigen encounter. Early studies showed that the initial antigen
encounter supported subsequent cell divisions (75), but continued antigen encounters allowed for enhanced T cell expansion (76-78). Thus CD4 T cells were thought to be similar to CD8 T cells in their programming upon antigen challenge. This view was unchallenged until, when in an elegant model, Obst et al. demonstrated to the contrary. Using a transgenic mouse engineered to express a neo-self antigen (MCC inserted into Ii) under the control of tetracycline, these workers were able to vary the amount and time of antigen exposure (79). They showed that the response to antigenic stimulus was flexible for CD4 T cells as they were able to continuously adjust proliferation according to their environment. One of the caveats to this study was these DC expressing cognate antigen for presentation to transferred TcR transgenic T cells were not mature, and hence not supplying costimulatory signals to the T cells. Surprisingly in response to this caveat, the group further showed that the induction of persistent antigen is capable of mimicking aspects of a mature DC (the stabilization of peptide:class II complexes on the DC surface) and may be a mode of promoting immunity rather than tolerance induction (80). Moreover, it was shown that CD4 T cells may require persistent peptide: class II complexes to sustain optimal T cell division for effector function and the development of a memory population (79, 81, 82). In light of these more recent data, it seems CD4 T cells may be on a “manual” control while CD8 T cells are on “autopilot”.

Despite such a small number of potential responding T cells to a specific peptide antigen, there is a dramatic expansion of these cells, with a subsequent contraction
phase in which most of the responding T cell population die, returning to numbers slightly above that prior to infection. The remaining cohort of memory cells can be subdivided into two distinct phenotypes; central memory cells, continually recirculating through lymph nodes, and effector memory cells, which home to peripheral tissue sites, are critical for long-lived T cell immunity.

From the discussion above, it is clear that the lymph node is a central location in which the immune system can recruit and increase the numbers of antigen-dependent T cell responders that eventually acquire an effector function and leave the lymph node to combat the invading pathogen at a peripheral site. Although the migrating DC is an integral player in orchestrating the adaptive immune response, there are many other events that can influence the priming of CD4 T cells. The lymph nodes are not only receiving DC, but already contain resident DC as well as a steady flow of the interstitial fluid which may contain soluble antigens, intact microbes, microbial products, and locally produced extracellular signaling molecules like cytokines and chemokines, dependent on the pathogen or vaccination, which may play a crucial role in the priming process. Thus the process of priming naïve CD4 T cells in vivo is extremely complex and the influence of these factors on the outcome of the T cell response is still heavily researched.
1.4 Classical view of Immunodominance

When an individual is immunized with a complex antigen or a pathogen that contains multiple antigenic determinants, most of the responding T cells are specific for a few of the many potential peptide epitopes contained within the antigen or pathogen. These peptide determinants are termed immunodominant. Considering the vast number of potential epitopes in complex organisms and proteins, it is remarkable that T cells have the potential for a high degree of diversity in the specificity of their response and apparently react to a very limited number of peptides, while seemingly ignoring the other potential peptide epitopes. The earliest models to explain this unexpected restriction in T cell responses focused on two potential mechanisms (83, 84). First, it was speculated that the MHC molecules in the responding animal were able to bind only a limited subset of antigenic peptides that are released during antigen processing and those that did not bind would be invisible to the responding host. This was termed “determinant selection”. The other, non-mutually exclusive possibility was that in the immunized host, the TcR repertoire diversity was limited and only able to react with one or a few of the many potential peptide:class II complexes which led to the hypothesis termed “holes in the repertoire”. Subsequent studies showed that complex proteins have many peptides that could bind to the MHC molecule and elicit T cell responses when administered as a single peptide, suggesting “determinant selection” and “holes in the repertoire” were insufficient to explain the phenomenon of immunodominance (reviewed in (10)). These
determinants whose response was only detected after immunization with peptide were termed cryptic, because they remained in some way sequestered from the response after immunization with the intact complex immunogens.

Since these early models were insufficient in determining the mechanism(s) by which selective peptides were presented to T cells while others were sequestered during challenge to foreign antigens, further research focused on the intracellular events related to antigen processing as the determining factor. Using a number of experimental model antigens, (reviewed in (85-88)) significant effort has been put forth to understand the mechanisms that underlie immunodominance in CD4 T cell responses. Much of the data in the literature have suggested that events related to antigen processing, such as protease susceptibility of the antigen (typically within the residues that flank the peptide), tertiary structure of the antigen, and the site within the antigen might compete for binding to MHC class II molecules or elicitation of immune responses which may alter the immunodominance hierarchy (87, 89-98).

There is literature that lends support for all of these mechanisms. Studies on model protein antigens showing that treatment of APC with inhibitors of endosomal proteolysis could either enhance or inhibit antigen presentation, depending on the epitope (99). Mutation of a flanking sequence by introduction of a protease recognition sequence can enhance epitope display \textit{in vitro} (100). It has also been demonstrated that different APC may degrade antigens in selective compartments of
the endocytic pathway, suggesting differential liberation of peptides between APC may lead to an altered determinant display available for T cell recognition (101).

Similarly, knock-out mice were constructed to determine the singular role of each protease in the degradation of both Ii and newly acquired foreign antigen. This type of method had serious caveats for understanding the contribution of each protease, as there was redundancy in the usage, differential expression and localization within antigen presenting cells, as well as very broad and difficult to precisely define cleavage specificities (5). In addition to proteolysis by cathepsins, the reduction of disulfide bonds within antigenic proteins has been shown to be critical to the unfolding and efficient proteolysis of some antigens during processing. Disulfide bond cleavage by gamma-interferon-inducible lysosomal thiol reductase (GILT) may relax the antigen structure (ex. Hen egg lysozyme), allowing for protease susceptibility, and modulate the binding of newly exposed peptide epitopes to MHC class II molecules (102). Overall, these data suggest that proteolytic release may be limiting for some peptides.

Similarly, several studies have observed that immunodominant epitopes clustered in limited regions of the antigen under study, often within solvent exposed regions or adjacent to protease sensitive loops (103, 104). These types of data, which are largely correlative in nature, suggest that antigens that have a high degree of tertiary structure, such as RNAse (105), may be poorly immunogenic or peptides buried within protease resistant regions of an antigen may sequester peptides from
proteolytic release and subsequent association with class II molecules. In contrast, some studies have suggested that CD4 T cell epitopes are preferentially associated with structurally stable regions (106-109).

The concept that follows from this view is that differential release of some peptides over others can lead to variation in the yield of these peptides for MHC class II binding, resulting in a hierarchy of peptide:class II complexes at the cell surface of the priming APC. The peptides that are expressed at high epitope density on the APC surface would then recruit a correspondingly large number of CD4 T cells and these T cells would assume immunodominance in the immune response. Conversely, those peptides that are inefficiently released or degraded would be presented at limiting quantities with the MHC class II molecule and thus would be ineffective in recruiting CD4 T cells and would be “cryptic”. If this model is correct, one of the key implications is that it will not be possible to predict or control the immunodominance of a given target peptide because of the unpredictable nature of protein folding and degradation. Also, with respect to novel techniques to engineer new proteins in which to incorporate multiple antigenic peptides that target both CD4 and CD8 T cells, immunodominance patterns would change with every new protein into which a candidate peptide is incorporated, a consequence that would greatly complicate peptide-based vaccine design.
1.5 Competition

Another mechanism that restricts the final specificity of responding CD4 T cells, which can occur at multiple levels during an immune response to foreign antigens, is competition. Competition can theoretically occur during the initial intracellular binding of peptides to MHC class II, for binding to available MHC class II molecules on the surface of the priming APC (antigenic competition), or between T cell populations (for the same or disparate antigen) after presentation of peptide:class II complexes to the surface of the APC (T cell competition). Competition at the intracellular level could occur by the inhibition of peptides binding on the available MHC class II during the processing and presentation of a complex antigen.

Chemically related determinants were first shown to compete for association and subsequent presentation under limiting conditions in vitro by a reduction in the proliferation of antigen-specific T cells (110-112). In subsequent experiments, competition between various peptide antigens for MHC class II molecules was not only demonstrable in vitro (113-115), but also shown to be a relevant mechanism of T cell inhibition in vivo (91). These studies led to the concept that peptide determinants processed from a complex antigen may interfere with the binding of other peptides and thus alter the specificity of the responding T cell population. This could occur by differences in the association and / or dissociation rates between different peptides, and also through a mechanism of steric hinderance where peptides have the ability to “push-off” other less favorable peptides from the class II molecules (116-119).
Although much of the literature dealing with mechanisms underlying immunodominance has pointed to events related to antigen processing, there are also studies that suggest preferences in T cell priming may relate, in part, to competition among the responding T cell population. Competition can occur between T cells of the same antigen specificity (intra-peptide competition) and between T cells of disparate antigen specificity (cross-peptide competition). Analogous to homeostasis which is thought to regulate the population of the naïve and memory T cell populations (120, 121), T cells to exogenous antigen could conceivably compete for resources within a local niche thus potentially narrowing the repertoire of responding T cells. The mechanisms driving both intra-peptide and cross-peptide competition have been under investigation in recent years and have provided some insight for CD8 T cells, although little for CD4 T cells.

Early work with CD8 T cells, of which the majority of studies relating to competitive T cell activation \textit{in vivo} have been performed, dealt with CD8 T cell responses across minor histocompatibility antigen differences (122-124). Responses to dominant antigens caused an inhibition of responses to subdominant epitopes, and importantly, removal of one epitope allowed responses to others to emerge. Moreover, immunodominance was found to be abrogated when the immunizing antigens were presented by separate dendritic cells (122, 125, 126), implicating competition at the level of the APC:T cell interaction. Several reports, using TcR transgenic T cells,
show that intra-peptide competition (125, 127) and cross-peptide competition (123, 126, 128) may be dependent on the ratio of DC:T cells interacting within the draining lymph node during the immune response. Furthermore, those T cell clones that most successfully compete may express a high affinity TcR and cause decreased expression of cognate but not bystander peptide:MHC class I complexes on the antigen presenting cell surface (129). In an attempt to identify the kinetics of competition, a recent study showed that the loss in T cell priming occurs within the first few hours after antigen exposure (130). This is consistent with strong evidence that CD8 T cells do not require persistent antigen, but rather only 12 hours of stimulation to reach a threshold for programmed cell division and differentiation (68-71, 74).

In contrast to these studies with CD8 T cell responses, there are very few reports in the literature on whether CD4 T cells influence or ‘compete’ with each other during an immune response. Vacchio et al. performed an early study suggesting that immunodominance could be affected by competitive events (131). These workers evaluated the immunodominance hierarchy to S. nuclease and found that eliminating the binding of the dominant peptide by mutation of a key MHC class II anchor residue allowed responses to other subdominant peptides to emerge. Although the results were striking, it was unclear if the competition occurred at the level of peptide binding during processing and presentation or among the responding population of T cells. An additional body of evidence supporting the notion of CD4 T cell
competition derives from analyses of the TcR repertoire changes that occur early in the primary immune response to the peptide pigeon cytochrome C (132-136).

Although the exact mechanism by which TcR affinity maturation occurs is not known, it is clear that at low antigen doses there is a shift in the representation between days 3-6 toward higher affinity T cells. Those T cells bearing the requisite threshold of affinity (136) or duration of TcR binding (137) to cognate antigen are apparently more ‘fit’ and selectively proliferate during subsequent antigen encounters. Efforts to explicitly evaluate competition among CD4 T cells have largely used analyses of TcR transgenic T cells which have shown evidence of intra-peptide competition (82, 138-140) and altered T cell differentiation (140-142), but observed modest, if any effects of cross-peptide competition (138, 143) and, in some cases, even cooperation (144) for CD4 T cells concurrently responding to different cognate antigens.

1.6 The peptide-intrinsic model of immunodominance

A different perspective from the aforementioned studies that suggest location and hence proteolytic release determine the immunodominance hierarchies, our laboratory has accumulated substantial evidence that a biochemical property of the peptide:class II complex - its spontaneous kinetic stability - is a critical parameter that determines the immunodominance of that peptide:class II epitope in the developing immune response (10, 145, 146).
The peptide-intrinsic model of immunodominance states that the potency of a peptide’s immunogenicity is principally a function of its primary sequence, not its position in the larger protein or its flanking residues. Therefore, during antigen processing of a complex antigen, irrespective of the protein structure, location, and possibly overall yield in antigenic peptides, there will be preferential loading of very stable binding peptides onto class II by DM, leading to an altered determinant display on the surface of the APC toward peptide:class II complexes that are kinetically stable. These persistent, high stability peptide:class II complexes will in turn be capable of recruiting a correspondingly large number of CD4 T cells that would dominate the immune response. Furthermore, peptides with rapid off-rates from MHC class II would be underrepresented, as they are efficiently removed from class II during peptide loading, and remain inaccessible from the immune response.

Studies were initiated to determine if it was possible to accurately predict the immunogenicity of a given peptide using a set of known immunodominant and cryptic peptides. The peptide epitopes utilized all come from different proteins, each with unique structures and processing characteristics, providing an opportunity to identify a potential biochemical attribute that distinguishes peptide immunogenicity. A comparison of both peptide competition and peptide dissociation assays were used to identify a biochemical parameter between these peptide epitopes of disparate origin. Both of these assays have been previously used to determine the relative
strength of peptide:MHC class II interactions (147-149). The competition assay determines the ability of a peptide to inhibit formation of complexes between a standardized, labeled peptide with purified class II molecules. Conversely, dissociation assays measure the kinetic stability of interaction between a test peptide and soluble MHC class II molecules after their formation. The kinetic stability or “off-rates” of these peptides are an accurate measure of the “affinity” for a peptide for its class II molecule because it has been previously shown that the differences among on-rates between different peptides to be negligible, while the off-rates can vary up to 7-fold (149). When these two assays were compared, the immunodominant and cryptic epitopes only segregated when analyzed using the dissociation assay. This observation was paramount to the initiation of these studies because it provided evidence for a biochemical feature to accurately predict the immunogenicity of a given peptide based upon its primary sequence.

Furthermore when these peptides of known dominance or crypticity were taken from the native protein and inserted into a new protein context to determine the immunogenicity of these peptide epitopes within the same location within a larger protein, competing peptides and proteolytic accessibility, they were shown to maintain their respective immunodominance. Hence, the off-rate of a peptide from the class II molecule was the most accurate measure of affinity or stability of the peptide:MHC class II complex and the only measure of affinity that has been identified that correlates with immunodominance (150). Moreover, the construction
of altered peptide ligands with substitutions in the key anchor residues with optimal (stabilizing) and sub-optimal (destabilizing) anchor interactions identified from murine I-A<sup>k</sup> and I-A<sup>d</sup> molecules (151-154) demonstrated that the immunogenicity of those peptides could be rationally modulated by altering the primary sequence and hence associated stability with MHC class II molecules. The preceding results indicated that the kinetic stability of a given peptide:class II complex and its immunodominance in vivo were causally linked and most importantly, could be rationally modulated.

Early studies by various groups on antigen presentation by MHC class II molecules suggested that DM functions as a peptide exchange factor and can act as either a positive or negative regulator of expression of peptide:class II complexes (85, 155-158). These studies suggested that DM expression within APC can have opposing effects on peptide:class II complexes, depending on the peptide and can either enhance or antagonize peptide presentation by MHC class II molecules. This led to the speculation that cryptic peptides may fail to elicit T cell responses when introduced as part of an intact antigen because these peptides are removed from the MHC class II molecule through the catalytic activity of DM within endosomal compartments of APC. This possibility was investigated when T cells specific for cryptic and immunodominant peptide epitopes were collected for analyses and studied using class II positive APC that differed only in their expression of DM (36). These studies revealed a complete concordance between DM effects and
immunodominance. All immunodominant peptides behaved similarly, and were enhanced by DM editing while consistently antagonizing the cryptic epitopes. This commonality in patterns occurs despite the fact that these peptides each come from unique molecular contexts.

This relationship between DM editing and immunodominance provided a novel mechanism to explain immunodominance. Because the priming APC in vivo is a DM-positive DC, DM editing is likely to antagonize or completely eliminate the presentation of cryptic peptides by class II molecules. These low stability peptide:class II complexes will therefore be unavailable to recruit CD4 T cells. Consequently, DM editing accounts for the sequestration of the cryptic peptides from the emerging immune response. Additionally, the finding that DM-negative APC are active at presentation of the cryptic peptides shows that these determinants are neither over-proteolyzed or inadequately released during endosomal processing. This finding argues against differential proteolysis of cryptic and immunodominant peptides. Collectively, these findings assert that immunodominance is due to an intrinsic property of the peptide:class II complex.

Essential to the peptide-intrinsic view is that a peptide’s immunogenicity can be rationally manipulated by altering the amino acids that interact with specificity pockets on the MHC class II molecule. The demonstration that it is possible to predict and rationally promote any peptide’s immunogenicity in vivo has immense
repercussions not only on the fundamental understanding of how the selectivity in the CD4 T cell response develops, but also with respect to vaccine design. Such modified peptides could be incorporated into vaccines where the peptides will maintain their immunogenicity and expand host CD4 T cells of the desired specificity, while retaining recognition of the native sequence.

1.7 Scope of thesis

The discovery that DM editing has such a profound impact on the repertoire of peptides available for CD4 T cell recognition and final specificity in the T cells elicited was a key observation that has led us to consider if there are other levels of control that the affinity of a peptide for its class II molecules may have a consequence on the immune outcome. There may be several checkpoints throughout the immune response that may lead to selective presentation of the highly persistent peptide:class II complexes, preferentially recruiting and expanding T cells to which their cognate ligand is continuously available. This thesis investigates the impact of peptide:class II stability on the final specificity of the CD4 T cell population at several stages during an immune response \textit{in vivo}.

During intracellular processing of antigen, we know that DM alters the repertoire of peptides presented on the surface of antigen presenting cells. What remains to be established is how frequently DM-independent factors, such as the molecular context
of a foreign peptide within an antigen accounts for immunodominance hierarchies. We asked how commonly such factors as location within an antigen, competing peptides within the antigen and / or differences in sensitivity of the peptide to the proteases that liberate it from the complex antigen are the deciding factor in establishing immunodominance hierarchies? The data presented in Chapter 3 suggest that immunodominance hierarchies are largely independent of the molecular context and are intrinsic to the peptide:class II complex that elicits the CD4 T cells.

It also remains to be determined if the selective editing of peptides by DM during antigen presentation is the only stage at which the kinetic stability of a peptide is most critical or if peptide:class II stability can have an influence on the potential responding T cell populations during subsequent events, after peptide:class II presentation on the surface of the antigen-bearing cell. One would imagine there to be persistence of very stable peptide:class II complexes and a rapid decay of unstable peptide:class II complexes during the transit to and priming within the lymph node. Moreover, if there is differential persistence of these peptides after expression on the surface of the APC that lives for several days, then there may be an advantage in the recruitment and expansion of T cells to more stable complexes that ultimately plays a role in shaping the CD4 T cell repertoire to a foreign antigen. We asked if DM editing is bypassed and the initial epitope densities of low and high kinetic stability peptides loaded onto the surface of antigen-bearing cells are equivalent, is there a functional consequence of the affinity of the peptide for its presenting class II
molecule in the stages after intracellular processing? To address this question, peptides of differing off-rates from class II were evaluated for functional persistence under biological conditions *in vivo* (Chapter 4). Furthermore, we sought to comprehensively evaluate the impact of simultaneous T cell responses to peptides that differ in their kinetic stabilities with MHC class II on the final specificity and magnitude of the primary CD4 T cell response (Chapter 5). The results presented in Chapters 4 and 5 provide evidence that the off-rates measured *in vitro* are biologically significant and stable binding peptides to MHC class II molecules on the surface of APC have an advantage in eliciting and maintaining the activation of cognate CD4 T cells in the absence of DM editing.

In addition, the results in Chapter 5 has led to the development of a serum-free DC-based immunization scheme to address the molecular mechanisms driving the selective loss of T cells elicited by low stability peptides during competitive activation *in vivo* (Chapter 6). This system can also be used to broaden our understanding of the role the APC plays in directing what signals modulate the determinant display available for T cell priming and how the APC may direct the fate of the ensuing CD4 T cell response.

The studies presented within this thesis provide strong evidence that the immunodominance of a peptide in a developing immune response is largely a property intrinsic to the peptide itself. The data not only provide novel insight into
the events that regulate CD4 T cell priming \textit{in vivo}, but also provide a new conceptual framework to understand the parameters that select the final specificity of the T cell repertoire during pathogen or vaccine-induced immune responses.
Figure 1.1 MHC class II-restricted antigen presentation

Depicted are the stages of MHC class II-restricted antigen presentation to foreign or self antigens. Stage 1 is the uptake of the pathogen or self-antigen by the antigen presenting cell. Stage 2 is the proteolysis of antigen into peptide fragments in endocytic vesicles. These vesicles then fuse with vesicles containing CLIP:MHC class II complexes, and with the help of DM, CLIP is replaced with more stable binding peptides with MHC class II (Stage 3). The vesicular microenvironment becomes more neutral and there is sorting of the peptide:MHC class II complex to the cell surface (Stage 4). Once on the surface of the antigen presenting cell, there can be recognition of the peptide:class II complexes by CD4 T cells.
CHAPTER 2

Materials and Methods

2.1 Preparation of cross-linked amylose
2.2 MalE protein purification
2.3 ANS binding assays
2.4 Mice
2.5 Immunizations
2.6 Cell purification
2.7 Isolation of T cells from ear dermis
2.8 ELISpot assays
2.9 Bone marrow-derived dendritic cell preparation
2.10 Construction of Flt3L-secreting cell line
2.11 Immunization with Flt3L-secreting cell line and isolation of DC-enriched spleen
2.12 Antibodies and peptides
2.13 Antigen presentation assays
2.14 CFSE staining – cell tracking
2.1 Preparation of cross-linked amylose

Amylose (Sigma-Aldrich, St. Louis, MO) (5 g) was dissolved in 20 ml of water, 30 ml 5 N NaOH, and warmed to 50 °C in a water bath. Once fully dissolved, 15 ml of epichlorohydrin was mixed in, and the solution was allowed to solidify at 50 °C. The resulting mass was allowed to cool to room temperature (~1 hr), and pulse-blended with 200 ml of water in a Waring single speed blender for approximately 10 sec. The subsequent suspension was transferred to a 1000 ml glass cylinder and allowed to settle. The remaining supernatant and fine particles that had not settled were decanted. The amylose slurry was washed 3 times with 200 ml 50 mM glycine-HCl, 0.5 M NaCl (pH 2.0), removing the supernatant and fine particles still in suspension each time. The slurry was further refined 3 times with 200 ml water, and 3 times with 200 ml 10 mM Tris-HCl buffer (pH 7.2). The gel was resuspended in 10 mM Tris-HCl (pH 7.2) to approximately 150 ml and stored at 4 °C in a glass bottle.

2.2 MalE protein purification

MalE constructs were designed and purified as previously described with some modifications (159). Briefly PAGE-purified synthetic oligonucleotides encoding the desired peptide were ligated into BamH1-digested MalE133, Xho1-digested MalE206, or BamH1-digested MalE303 vector. Sequenced clones were transformed into MalE (-/-) ER2507 E. coli. 40 ml of overnight bacterial cultures expressing the MalE construct were added to 4 L of LB:ampicillin with 0.2% maltose and grown for 6-7.5 hr at 37 °C. Bacteria were pelleted by centrifugation at 7000xg for 10 min at 4
°C and resuspended in 800 ml of pH 8.0 30 mM Tris:Cl/20% sucrose/1 mM EDTA. After shaking for 10 min, bacteria were pelleted by centrifugation at 8000xg for 10 min at 4 °C and resuspended in 400 ml of ice cold 5 mM MgSO₄ and shaken for 10 min on ice. After centrifugation at 8000xg for 10 min at 4 °C, the supernatant was collected and 8 ml of 1 M Tris:HCl, pH 7.4 was added. The osmotic shock fluid was filtered over a 0.45 µM membrane and applied to an amylose column of 15 ml bed volume with a flow rate of ~ 1 ml/min (160). The column was washed with 250 ml column buffer (20 mM Tris:HCl, pH 7.4; 0.2 M NaCl, and 1 mM EDTA) and protein was eluted in 50 ml of column buffer/10 mM maltose. Collected fractions positive for protein (Bradford analysis, BioRad) were pooled, dialyzed against PBS, and concentrated with a Centricon µm-10 KDa cutoff filter to ~ 1 ml final volume. Concentrated protein in PBS was sterile filtered through a 0.2 µM syringe filter, quantified by Bradford assay and SDS-PAGE, and stored at -20°C.

2.3 ANS binding assays

The proteins LACK (a gift from Dr. Deborah Fowell, University of Rochester), MalE, Ovalbumin (Sigma-Aldrich, St. Louis, MO), or HEL (Sigma-Aldrich, St. Louis, MO) were diluted and transferred to 0.2 M phosphate/0.1M citrate McIlvaine buffers at a pH range between pH 4.0 and 7.0, at a final concentration of 2 µM and incubated with 60 µM 8-anilino-1-naphthalenesulfonic acid (ANS) (Sigma-Aldrich, St. Louis, MO) for 10 min at room temperature. Measurement of bound fluorescent ANS was performed in a FLUOROMAX (Horiba Jobin Yvon, Edison, NJ); exciting the probe
at 350 nm and the fluorescence intensity was measured between 490 nm to 600 nm. Where indicated, the proteins were reduced in the presence of 1 μM DL-dithiothreitol (DTT) (Sigma-Aldrich, St. Louis, MO) for 10 min prior to incubation with ANS probe.

2.4 Mice
BALB/c mice were purchased from (NCI, Rockville, MD), DO11.10 TcR transgenic and control BALB/c mice were purchased from (Jackson Laboratories, Bar Harbor, ME). HNT TcR transgenic mice (BALB/c Thy1.1) were a gift from Susan Swain, Trudeau Institute. Mice were maintained in the pathogen-free animal care facility at the University of Rochester Medical Center. All animals were handled according to the regulations set by the University Committee on Animal Care at the University of Rochester, Rochester, NY.

2.5 Immunizations
Mice (NCI, Rockville, MD) were immunized in the hind footpad with 50 μl of 0.2 mg/ml of protein antigens emulsified in Complete Freund’s Adjuvant (Sigma-Aldrich, St. Louis, MO) or in the hind footpad with 50 μl (unless otherwise indicated) of an Incomplete Freund’s Adjuvant (IFA)/PBS emulsion containing synthetic peptides (25nmol or 5nmol/mouse) and 0.6 μg/ml lipopolysaccharide (LPS) (Sigma-Aldrich, St. Louis, MO). For some experiments, mice were immunized in the ear pinna with 10 μl of an IFA/PBS emulsion containing synthetic peptides (25 nmol or 5
nmol/mouse) and 0.6 µg/ml LPS or 10 µl of protein antigens of the indicated concentration in PBS administered using a 29G ½`` tuberculin syringe (modified from Itano et al. (161)). Where indicated, ears were removed at either 6 hr or 2 days post immunization. Unless otherwise indicated, ten days post-immunization, the mice were sacrificed and draining lymph nodes were harvested and used as the source of CD4 T cells for ELISpot analyses.

2.6 Cell purification

A single cell suspension from lymph node of individual immunized mice was prepared and depleted of non-CD4 T cells by incubation at 2x10^7 cells/ml with monoclonal antibody supernatants from the cell lines MKD6 (anti-I-A^d^) and M5/114 (anti-I-A^d^, anti-I-E^d^) for class II^+^ cells, with 3.155 for CD8 T cells, and RA3-3A1/6.1 (anti-B220) for B cells at a final dilution of 1:4. Cells were then washed and resuspended at 2x10^7 cells/ml in Rabbit Low Tox-M complement (Cedarlane, NC) diluted 1:10 in media at 37°C for 30 min. Dead cells were removed by density gradient centrifugation with Lympholyte-M (Cedarlane, NC). CD4 enriched, viable cells were recovered, washed, and resuspended at 3x10^6 cells/ml. CD4 T cell purity was assessed before and after complement treatment by staining for CD4 (RM4-4), typically indicating 90-95% purity. During kinetic experiments, a single cell suspension of lymph node from an individual mouse was enriched for CD4 T cells by negative selection per the manufacturer recommendations (Miltenyi Biotech). Again CD4 T cell purity was assessed before and after negative selection by staining for
CD4 (RM4-4), typically indicating 90-95% purity. A single cell suspension of splenocytes from naïve mice were depleted of red blood cells by treatment with ACK lysis buffer (0.15 M NH₄Cl, 1 mM KHCO₃, and 0.1 mM Na₂-EDTA in H₂O, pH 7.2; Sigma-Aldrich, St. Louis, MO) (5ml/spleen) for 5 min at room temperature. After washing, the lymphoid cells were depleted of T cells by incubation with the monoclonal antibody supernatant from the cell line J1j.10 (anti-Thy1.2) at 2x10⁷ cells/ml that was diluted 1:2 in media. Viable cells were recovered as described above, and then resuspended at 1x10⁷ cells/ml for use as stimulators in ELISpot assays.

2.7 Isolation of T cells from ear dermis

The ear pinnae from a group of 5 mice were removed, cut into pieces, and then digested with 1 mg/ml collagenase/dispase (Roche, New Jersey) for 1 hr at 37°C. The tissue was mashed through a metal strainer, releasing a single cell suspension, which was washed two times with media. Staining for CD4 (RM4-4) typically yielded 0.5-1.8 % CD4 T cells in the cell suspension. Cells were resuspended in media at 0.5 ear equivalents/well (approximately 5x10⁶ cells/well) and were stimulated with freshly isolated, T cell depleted, syngeneic splenic antigen presenting cells in the presence or absence of peptide. Cytokine production was measured by a 6 hr ELISpot assay. The mean number of spots for each condition was determined from triplicate wells.
2.8 ELISpot assays

96-well filter plates (Millipore) were coated with 50 µl of rat anti-mouse capture cytokine at 2 µg/ml in PBS at room temperature for 2 hr and then washed with media to remove any unbound antibody. CD4 T cells (3x10^5 cells/well) were cocultured with syngeneic T cell depleted splenic antigen presenting cells (5x10^5 cells/well) with the indicated peptides at a total volume of 0.2 ml for 6 hr or 18 hr at 37°C and 5% CO2. Cells were removed from the plates and the plates were washed with PBS/0.1% Tween 20 wash buffer. 50 µl of biotin rat anti-mouse secondary cytokine was prepared in wash buffer with 10% fetal bovine serum was added to the plates and incubated for 30 min at room temperature. The plates were washed again, and 50 µl of alkaline phosphatase-streptavidin (Jackson ImmunoResearch, PA) was added at a dilution of 1:1000 in wash buffer/10% Fetal Bovine Serum (FBS). The plates were incubated for an additional 30 min at room temperature, then washed, drained, and developed using Vector blue substrate kit III (Vector Laboratories, CA) prepared in 100 mM Tris, pH 8.2 per manufacturer instructions. The quantification of spots was performed with an Immunospot Reader Series 2A, using Immunospot Software V.2.0. The mean number of spots for each condition was determined from triplicate wells. In some instances, the total number of responders / pooled LN in a mouse were calculated as follows:

\[
\frac{(\text{Total # LN cells})(\% \text{CD4 in LN})}{100} = \text{Total CD4 in LN} \quad \frac{(\text{Total CD4 in LN})(\text{Spot #})}{3 \times 10^5 \text{ cells/well}} = \text{Total # Responders}
\]
2.9 Bone marrow-derived dendritic cell preparation

Bone marrow cells were obtained by first removing the femurs from mice and stripping all the tissue from the surface of the bone. Using scissors, cut both ends off of the femur. With a 25G ½`` syringe, insert the tip into one open end of the femur, and expel 5 ml media, removing all of the bone marrow cells, into a petri dish containing 10 ml media. The cell suspension was vigorously pipetted to form a single cell suspension, filtered through a 45 µM sterile filter, washed, and resuspended to 4x10^5 cells / ml. 10 ml of the suspension was plated onto Petri dishes in the presence of 20 ng/ml Granulocyte-macrophage colony stimulating factor (GMCSF). At day 3 an additional 10 ml of media containing 20 ng/mL GMCSF was added to each plate. At day 6 10 ml of media was aspirated and spun down to pellet any cells. These cells were resuspended with fresh media and added back to the original plate. On day 7, 10 ng/ml LPS (Sigma-Aldrich, St. Louis, MO) was added to each plate to mature the dendritic cells. The next day, the plates were gently washed and pooled, collecting only the floating cells. Enriched bone marrow-derived denritic cells (BMDC) were resuspended at 5x10^5 / ml in media, and pulsed with peptide at the indicated dose.

Verification of enrichment for BMDC was performed by staining with class II-FITC (39-10-8) and CD11c-PE (HL3) (BD Pharmingen, San Jose, CA) and analyzed on a BD FACS Caliber.
2.10 Construction of Flt3L-secreting cell line

The DNA encoding Flt3L was excised from Flt3L-sfHAV-EO (American Type Culture Collection, Rockville, MD) using SalI. The Flt3L fragment was purified by phenol / chloroform extraction and gel purification and then inserted into pHβ Apr-1-neo (a gift from Dr. John Frelinger) (fragment:vector ratios 5:1, 1:1, and 1:5) with the vector at a constant concentration of 50ng. The resulting ligations were transformed into DH5α, plating the cell suspension at 10% and 90% onto agar plates containing ampicillin. Single colonies were chosen from the plates with a 5:1 ratio that gave an 8-10-fold induction of colonies over linearized SAP-treated vector incubated with ligase and the absence of insert. Single colonies were tested for insert and orientation by restriction enzyme digestion using the restriction enzymes SacI, BamHI, HindIII, NdeI, and double digestions of SacI / BamHI, SacI / HindIII, and SacI / NdeI for 90 mins. at 37˚C. Visualization of fragments was performed using a 0.8% agarose gel run at 50V. Clone 4 was chosen and used for subsequent expression experiments.

Line 1 (a gift from Dr. Edith Lord) was transfected with clone 4 using Lipofectamine 2000. 20,000 or 50,000 cells / well in a 6-well plate were transfected with either 0.8 µg or 20 µg of DNA and Lipofectamine (DNA:lipofectamine ratios 1:2 and 1:0.5) under selection with 1 mg/ml G418. Selected clones from the 20,000 cells / well at 1:2 ratios were initially screened for secretion of Flt3L cytokine by ELISA and then a single clone (4.23) was subcloned to make stable cell lines. The different subclones
from (4.23-IE5.1, .2, and .3) were plated at low density and 2 days later the supernatants were harvested and tested for the secretion of Flt3L by ELISA.

2.11 Immunization with Flt3L-secreting cell line and isolation of DC-enriched spleen

BALB/c mice (NCI, Rockville, MD) were immunized in the left flank intradermally with 200 µl of 0.5-1x10^6 Flt3L-secreting cells in PBS using a 29G ½ `` tuberculin syringe. When the tumor was 2-3 cm in diameter or the animal was in distress, the spleen was excised and placed into a Petri dish. The spleen was injected with 1ml of 2 mg/ml collagenase D in HBSS containing Mg and incubated at 37˚C for 20 mins. A single cell suspension was created by crushing with the blunt end of a 3ml syringe and filtered through a 45 µM filter (Millipore). The collagenase D (Roche, New Jersey) was diluted out by washing the cells 3 times with HBSS. The single cell suspension was then subjected to enrichment by positive selection with CD11c^+ MACS beads per the manufacturers recommendations except that the MACS purification buffer was substituted with HBSS (Miltenyi Biotech). One spleen / column was used to purify the CD11c^+ cells, resulting in a typical yield >90% pure population as measured by flow cytometry with the CD11c clone HL-3 (BD Pharmingen, San Jose, CA).

2.12 Antibodies and peptides
Purified rat anti-mouse IL-2 (JES6-1A12), biotinylated rat anti-mouse IL-2 (JES6-5H4), purified rat anti-mouse IFNγ (AN-18), biotinylated rat anti-mouse IFNγ (XMG1.2), purified rat anti-mouse Flt3L, biotinylated rat anti-mouse Flt3L, and anti-CD4-FITC (RM4-4) antibodies were obtained from BD Pharmingen, San Jose, CA. The monoclonal antibody producing hybridomas MKD6 (162), M5/114 (163), 3.155 (164), RA3-3A1/6.1 (165), and J1j.10 (166) were acquired from American Type Culture Collection, Rockville, MD. All synthetic peptides (BioPeptides, San Diego, CA or Invitrogen Corporation, Carlsbad, CA) were reconstituted at either 10 mM or 1 mM stocks with or without 1 mM dithiothreitol (for cysteine-containing peptides).

2.13 Antigen presentation assays

Cell lines were maintained at 37°C and 5% CO₂ in DMEM containing 5% FBS supplemented with 5 mM HEPES, 2 mM glutamine, 1 mM nonessential amino acids, and 5x10⁻⁵ M 2-ME. All medium and supplements were purchased from GIBCO (Carlsbad, CA) unless otherwise noted. T cell hybridomas were maintained as previously described (167). For antigen presentation assays, 5 x 10⁴ of the I-A<sup>d</sup>-restricted T cell hybridomas specific for the HA [126-138]; TS2 (168), HEL [20-35] (169), MalE [269-284]; M1 (170), and MalE [69-84]; M3 (170) were co-cultured with 5x10⁴ A20 cells, 1.5x10⁴ BMDC, or 1.5x10⁴ CD11c<sup>+</sup> purified dendritic cells from BALB/c spleen in 0.2 ml in a 96 well flat bottom plate with increasing concentrations of antigen or peptide in duplicate. In some groups, 1 µM of MalE [69-84], MalE [102-115], and MalE [269-284] were added to the cell culture as competing peptides.
After overnight culture, 50 µl of supernatant was removed, frozen, thawed, and added to 3x10^4 CTLL cells. After 16-20 hr, CTLLs were incubated with 0.4 mg/ml MTT (Sigma-Aldrich, St. Louis, MO) for 6 hr, followed by incubation overnight with 100 µl 10% SDS/0.01 N HCl. Results appear as a mean Optical Density in the MTT assay from duplicate wells read at 570-650 nm on a Vmax plate reader.

2.14 CFSE staining – cell tracking

Obtain a single cell suspension of peripheral LN and splenocytes from DO11.10 TcR or HNT TcR transgenic mice and wash cells with PBS 2 times. Take an aliquot of cells to ascertain the percent of KJ(1-26)^+CD4^+ or Vβ8.3^+CD4^+ expressing cells by FACS analysis. Taking the total number of cells, resuspend cells at 3x10^6 cells / ml in PBS in a Falcon tube and add CFSE at 1:5000 dilution (0.2 µM). Invert three or four times to mix in the CFSE dye and incubate 5 min at 37°C in the waterbath. Immediately wash the suspension with Media three times, and then PBS five times. Cells were then resuspended in PBS at the indicated concentration of KJ(1-26)^+CD4^+ or Vβ8.3^+CD4^+ T cells for the individual experiments and 200 µl was transferred via retro-orbital injection using a 29G ½`` tuberculin syringe.
CHAPTER 3
-------------------------------

Immunodominance is independent of molecular context

-------------------------

3.1 Introduction

3.2 Results

The immunodominance of peptides track with their primary sequence rather than the protein in which they are contained

The immunogenicity of a given peptide is not detectably altered by its location in a complex protein antigen

The immunodominant phenotypes for two overlapping epitopes in a single peptide are distinct and correlate with kinetic stability

Addition of a dibasic motif increases the liberation of a cryptic peptide in vitro but does not convert it to an immunodominant peptide in vivo
3.3 Discussion

3.1 Introduction

Historically, immunodominance has been attributed to the events related to antigen processing, while more recent data from our laboratory has shown it to be an intrinsic biochemical property of the peptide itself. In light of these opposing views, it is critical to understand the molecular events that control immunodominance to complex antigens and how significantly the molecular context of a peptide determines its fate in a developing immune response.

It seems intuitive that peptide release from an intact antigen during proteolysis within the endosomal compartments will depend on both the flanking sequences and the location of the peptide within the antigen. This issue has been difficult to experimentally address due to a lack of a usable in vitro system that successfully recapitulates endosomal antigen processing. The proteases involved in endosomal antigen processing are poorly defined, display cell lineage variability and are subject to heterogeneity in endosomal localization. Also, the cleavage specificities of most endosomal proteases are very broad and difficult to precisely define. Finally, at present, there are no direct ways to quantify and sequence free antigen-derived peptides from endosomal extracts. Collectively, these factors make predictions
regarding protein unfolding, intermediate steps in processing and final cleavage events very difficult. This has limited our ability to demonstrate any causal relationships between the context of a peptide, its proteolytic release from the antigen, and ultimately the immunodominance of that peptide in the immune response.

What remains to be established in studies on immunodominance is how frequently the context of a foreign peptide within an antigen accounts for immunodominance hierarchies. We began these studies by asking how commonly such factors as location within an antigen, competing peptides within the antigen and / or differences in sensitivity of the peptide to the proteases that liberate it from the complex antigen are the deciding factor in establishing immunodominance hierarchies? We have used genetic engineering to change the molecular context of several independent peptide antigens; hence changing the overall secondary structure in which it is contained and the competing peptides. We also have altered the efficiency of proteolytic release and thus the T cell determinant display on the surface of antigen presenting cells. In each case we evaluated whether the immunodominance hierarchies track with the molecular context of a peptide or with the peptide itself. The data presented here suggests that immunodominance hierarchies are largely independent of the molecular context and are intrinsic to the peptide: class II complex that elicits the CD4 T cells.
3.2 Results

The immunodominance of peptides track with their primary sequence rather than the protein in which they are contained

In the first series of experiments, we evaluated the role of a peptide’s molecular context by comparing its immunogenicity \textit{in vivo} when contained within its native protein versus that displayed when it is encoded within a heterologous protein. As previously described (171), the MalE gene encodes a subunit of the \textit{E. coli} maltose binding protein and has several permissive sites that accept peptide inserts of 10-40 amino acids without changing its overall conformation. We used amino acid 133 as the site of insertion for the test peptides, which is known to be solvent exposed and to readily accommodate heterologous sequences (Table 3.1). MalE is an attractive protein vector because it allows insertion of antigenic peptides in a new context and enables concurrent analyses of responses to dominant MalE peptides. In our experiments, peptides that were inserted within MalE contained several flanking residues of the native sequence on the amino- and carboxy-terminus to preserve potential TcR contact residues (172, 173). An independent set of peptides with previously characterized immunodominance hierarchies were compared in these experiments, including the immunodominant peptides Myo [102-117] (174), LACK
(156-173) (175), the subdominant OVA epitope [273-288] (4, 150), the cryptic peptides HEL [20-35], HEL [11-25] (96, 176), OVA [327-339] (4, 150, 177) and a modified cryptic form of the LACK [156-173] peptide with a substitution at the P4 pocket (166 I>A) that reduces its kinetic stability with I-A^d and correspondingly its immunodominance (150) (Table 3.1). BALB/c mice were immunized with these antigens either in their native context or encoded in MalE and CD4 T cells were isolated from the draining lymph nodes 9-10 days after priming. CD4 T cells were analyzed for the number of IL-2 spots elicited in response to synthetic peptides or intact antigen in order to evaluate the number of CD4 T cells recruited into the response.

Figure 3.1 shows a comparison of CD4 T cell responses to peptides contained in their native protein context or inserted within MalE at amino acid 133. The data for each peptide are presented as the percent of spots elicited by peptide relative to the total number of IL-2 producing cells that were elicited in response to the intact antigen. Strikingly, each of the peptides maintained their relative immunodominance observed in their native protein (Figure 3.1 top panel) or when they were moved into the heterologous bacterial protein MalE (Figure 3.1, bottom panel). The peptide MYO [102-117] and WT LACK [156-173] each binding very stably to class II (t_{1/2}>150 hrs) remain immunodominant in MalE, recruiting approximately 50% of the CD4 T cells that the intact protein antigen does. The OVA [273-288] peptide is subdominant, eliciting 15-25% of the total IL-2 response, both in its native ovalbumin context and
within MalE. In a similar fashion, the cryptic peptides OVA [327-339], HEL [11-25], HEL [20-35] and the low stability LACK peptide variant consistently displayed an inability to prime CD4 T cell responses, independently of their molecular context, eliciting only 2-14% of the total IL-2 spots. These peptides have been shown to exhibit a half-life in association with I-A\(^d\) of less than 10 hrs at endosomal pH (150).

To determine if there are differences in the conformational stability of the antigenic proteins, we assessed their sensitivity to acid-induced unfolding at the range expected to be encountered in endosomal compartments of APC. In Figure 3.2, the fluorescent probe 8-anilino-1-naphthalenesulfonic acid (ANS), a fluorescent probe that binds to exposed hydrophobic patches (178-181), was used to detect pH-dependent conformational changes. If there were differences in the unfolding of the proteins an increase in the fluorescence intensity between 440 and 480nm would be observed. Each of the proteins studied, LACK, MalE, OVA, and HEL, displayed distinct sensitivities to low pH, as indicated by the variable ANS enhancement at each pH tested. For example, the LACK protein from Leishmania (Figure 3.2A, upper left) exhibited enhancement of ANS fluorescence at a modestly acidic condition of pH 5.5, MalE (Figure 3.2A, upper right) showed pH-dependent conformational changes only at pH 4.5, while strikingly, HEL (Figure 3.2A, lower right) displayed almost no sensitivity to acidic pH but did show evidence of unfolding in the presence of reducing agents (Figure 3.2B). This data suggests that during antigen processing
within an APC, each antigen may be distinct in its progressive unfolding and
differentially susceptible to proteolytic attack.

Collectively, these results, using unrelated foreign antigenic peptides, provide strong
evidence that immunodominance is independent of the molecular context and that
conversely, the affinity for MHC class II is an accurate predictor of immunogenicity,
irrespective of the protein context in which the peptide epitope is delivered.

**The immunogenicity of a given peptide is not detectably altered by its location in a complex protein antigen**

To further explore if molecular context can influence the hierarchy of
immunodominance, we asked if there were differences in the immunodominance of
antigenic peptides when they are located at different sites within a complex protein.
We inserted a well-defined cryptic peptide from influenza hemagglutinin (HA) [126-138] or a variant HA [126-138] peptide, with a substitution at the residue that anchors the peptide into the P1 pocket of I-A<sup>d</sup>, into several distinct sites of MalE. A T128>V substitution at the P1 residue in the HA peptide increases its affinity to I-A<sup>d</sup> by 3-fold (from 26 hrs t<sub>1/2</sub> dissociation for the WT peptide to 85 hrs for the variant) (154) and when inserted at amino acid 133 of MalE, causes a corresponding enhancement in its immunodominance in the CD4 response in BALB/c mice (150). Insertion of peptides into amino acids 133, 206, and 303 within MalE has been previously described by
LeClerc and colleagues (182), who found that insertion of peptides at these sites does not affect the binding affinity for maltose, arguing that conformational integrity is largely intact, in agreement with crystallographic data (183). Table 3.1 describes the location of the peptides within the complex antigens, when known, from crystallographic studies. The structure of MalE shows that the segments around residues 133 and 206 are solvent-exposed, whereas the region around amino acid 303 is buried.

One of the challenges in comparing individual responses to independent MalE constructs is that each molecular form is independently produced and purified in the laboratory. This could potentially introduce quantitative errors in estimation of concentration or purity of the constructs used for immunologic studies. To deal with this concern, prior to assessing the in vivo immunodominance of these test constructs, an in vitro antigen presentation assay was utilized to verify the functional concentrations of each antigen and to assess the release of the test HA peptides from the MalE constructs. When dose response curves using T cell hybridomas specific for internal MalE peptides; M1, reactive to MalE [268-285] and M3, reactive to MalE [69-84], respectively were compared, similar dose response curves between each set of constructs was observed (Figure 3.3A top and middle panels; respectively) indicating that the protein antigens were at similar functional concentrations. To assess whether release of the test HA peptides was similar in the different HA:MalE constructs, each construct was assessed in antigen dose response assays using a T cell
hybridoma specific for the HA peptide: I-A\textsuperscript{d} complex. HA:MalE (Figure 3.3B, left) and HA-T128V:MalE (Figure 3.3B right); 133 (squares), 206 (triangles), and 303 (circles) constructs displayed no differences in class II-restricted presentation of the HA peptide. These results suggest that the epitope density resulting from processing and presenting of the HA peptide encoded in the three different sites was similar. This in itself is informative because it suggests that the effective yield of peptide:class II at the cell surface of APC was not significantly impacted by the localization of the HA peptide within the different MalE protein constructs.

Having confidence that the three different constructs for WT HA and the three different constructs for the higher stability variant of HA were at the same effective concentration after their production and purification \textit{in vitro}, it was then possible to compare the immunogenicity of the HA peptides within the constructs \textit{in vivo}. The CD4 response to internal MalE peptides were used to verify the quality of the immunization of different groups of mice and thus to provide internal controls for the immunogenicity of the HA peptide. Figure 3.4 shows that for each of the six constructs tested, the MalE [69-84] peptide was the most immunogenic, followed by MalE [102-115], while the MalE [268-285] peptide was subdominant. Quantification of HA-specific CD4 T cells from the mice revealed that the responses elicited by the constructs bearing the HA peptide at the three different sites in MalE were very similar. The wild type HA [126-138] peptide was poorly immunogenic in each of the constructs, eliciting well below half of the number of responding CD4 T cells than the
subdominant MalE [268-285] peptide, independent of the insertion site within MalE (Figure 3.4, left). In contrast, when the responses to the higher stability HA T128V variant were tested, the responses were increased and now were approximately equal to the responses elicited by the MalE [268-285] peptide (Figure 3.4, right). Again, the responses to the peptide variant inserted into the three sites were similar.

Overall, these data involving MalE constructs with peptides at different sites shows first, that the effective epitope density achieved on an APC in vitro of a given peptide is maintained when the peptide is located within different sites of a complex protein and secondly, that the CD4 immunodominance patterns in vivo toward peptides are largely maintained when they are processed and released from different sites within the same protein context. Importantly, the only difference between these test (WT and T128V variant) HA peptides that recruited differing numbers of CD4 T cells in vivo in each site is their affinity for MHC class II molecules, a finding that supports the concept that an intrinsic factor of the peptide- its kinetic stability with its presenting MHC class II molecule - dictates its relative immunogenicity in vivo.

**The immunodominant phenotypes for two overlapping epitopes in a single peptide are distinct and correlate with kinetic stability**

In the next series of experiments we made side-by-side comparison of the immunodominance patterns of three different peptide epitopes in ovalbumin (OVA).
Each of these peptides are presented by I-A<sup>d</sup> and were originally described by Grey and colleagues (184) as present in the proteolytic fragments of OVA recognized by T cell hybridomas, 3DO54.8, 3DO11.10, 8DO51.15 and 3DO18.3. The 3DO18.3 T cell recognizes a cyanogen bromide fragment [273-288], while 54.8, 51.15 and 11.10 all recognize peptides within a tryptic fragment [323-339]. Both of these peptides are buried within the native ovalbumin protein (Table 3.1), although the segments flanking the [323-339] peptide are solvent exposed (185). More precise mapping of this segment of OVA (186) suggested that independent registers of the [323-339] peptide were stimulating the different T cell clones (Figure 3.5A). Peptide dissociation studies using I-A<sup>d</sup> (177) indicate that binding of [323-339] to I-A<sup>d</sup> displays complex kinetics, with the most amino terminal fragment [323-335] having the highest stability (t<sub>1/2</sub> > 200 hrs), likely corresponding to the register crystallized with I-A<sup>d</sup> (187). These studies in conjunction with our own (unpublished F.R. Krafcik and A.J. Sant) show that the amino-terminal fragment is recognized by 3DO51.15 and 3DO54.8 while the most carboxyterminal fragment, [327-339] contains the register recognized by 3DO11.10 and the derived TcR transgenic mouse 11.10. Dissociation studies with purified I-A<sup>d</sup> indicate that this peptide binds with very low stability (t<sub>1/2</sub> < 5 hrs) (150).

The immunodominance of these different peptides in BALB/c mice using native OVA as the immunizing antigen are shown in Figure 3.6B. The [323-339] fragment, encompassing all the different registers stimulates the highest number of CD4 T cells,
and virtually all the responses seem attributable to the amino-terminal [323-336] fragment, while the carboxy-terminal [327-339] fragment recruits very few CD4 T cells above background (<5%). Also shown in panel 3.6B is the immunodominance of the [273-288] peptide from OVA, which elicits significant but somewhat lower numbers of CD4 T cells than does the [323-339] peptide. Therefore, in OVA, the [323-336] peptide is immunodominant, the [273-288] peptide is subdominant and the [327-339] peptide is cryptic.

The OVA [323-339] peptide was then inserted into MalE, at amino acids 133, a site that is solvent exposed in MalE or at amino acid 303, a site that is buried in MalE. Protein constructs bearing these peptides were produced and then tested in vivo for their immunodominance hierarchies. Again, the functional concentration was similar between the different MalE constructs (Figure 3.5). As can be seen in Figure 3.6C, the OVA [327-339] peptide remains cryptic in both sites of MalE, while, in contrast, the OVA [323-336] peptide maintains its dominant phenotype, recruiting similar numbers of cells as does the subdominant MalE [102-115] peptide. This finding indicates that distinct but overlapping peptide epitopes can display quite discreet immunodominance patterns that correlate with their binding affinity to MHC class II molecules rather than with their site within the native antigen. Finally, OVA [273-288] inserted at amino acids 133 and 303 in MalE maintained its subdominant phenotype observed with the native antigen (Figure 3.6D). Overall, the
immunogenicity of the OVA peptides tracks with their primary sequence rather than with the protein context in which they are introduced into the immune system.

**Addition of a dibasic motif increases the liberation of a cryptic peptide *in vitro* but does not convert it to an immunodominant peptide *in vivo***

As a final strategy to addressing the impact of context in immunodominance, we sought to increase the efficiency of proteolytic release of a cryptic peptide and evaluate any changes in immunodominance. Dibasic motifs are recognized by a wide family of endoproteases (188-193) and several reports have indicated that if these motifs flank antigenic peptides, peptide:class II presentation efficiency can be enhanced (100) or immunodominance can be conferred (194). For example, when the native HEL protein was altered to contain a dibasic endosomal cleavage motif (189, 191, 192, 195) carboxy-terminal to the cryptic peptide HEL [20-35], class II-restricted presentation of the peptide by APC *in vitro* was enhanced (100) but this study did not examine whether this change was associated with any change in immunogenicity *in vivo*. Conversely, another report found that introduction of dibasic motifs flanking other epitopes increased immunogenicity *in vivo*, but did not examine antigen presentation efficiency (194). To address this issue more comprehensively, we asked if the addition of an endopeptidase cleavage site (F34>R)
flanking the cryptic peptide HEL [20-35] inserted in MalE (Figure 3.7) leads to increased peptide liberation, and if so, using the same construct, whether this affects the immunogenicity in vivo. Examination of peptide-specific responses using an HEL [20-35] specific T cell hybridoma indicated the amino acid substitution did not interfere with MHC class II or T cell receptor binding (Figure 3.8A), consistent with a modified residue being outside the core peptide binding register [23-32] as defined previously (100). To examine antigen presentation of this modified peptide, the wild type and F34R variant sequences were encoded into the MalE shuttle protein at amino acid 133. The MalE proteins bearing the WT or modified peptide were produced and dose response curves were performed. As before, presentation of the endogenous MalE [268-285] and MalE [69-84] epitopes were used to evaluate the functional protein concentrations of the independently prepared antigens.

When dose response curves using the intact MalE constructs bearing WT HEL [20-35] or the dibasic motif variant were performed, we observed a 4.5 fold increase in the presentation of the F34R peptide by A20 cells (Figure 3.8B, left) and a 6-fold increase in the presentation of the F34R peptide by freshly isolated splenocytes (Figure 3.8B, right). The observed differences were not due to protein concentration differences (Figure 3.8C and 3.8D), as there were no differences in any of the dose response curves obtained to the internal MalE epitopes. We next asked whether the enhanced presentation was sufficient to alter the hierarchy of immunodominance in vivo. Figure 3.9 shows that the HEL [20-35] peptide is approximately 2% of the total
immune response, in agreement with previous studies demonstrating this peptide to be cryptic (96, 176, 196). When the responses to the dibasic motif variant F34R were similarly analyzed, it was found to maintain its crypticity in vivo, despite the increase in its antigen presentation by APC in vitro. The internal MalE control peptides from both constructs each elicited significant responses, and at similar levels among the immunized animals, confirming the mice were immunized equivalently. These data demonstrate that increased liberation of a cryptic peptide from a complex protein, at least at the levels possible through the introduction of a dibasic cleavage sequence, is not sufficient to confer immunodominance in vivo.
3.3 Discussion

The question we sought to address is whether the potency of a peptide in recruiting CD4 T cells *in vivo*, its immunodominance, is seriously impacted by the protein context in which the peptide is contained or whether the immunodominance patterns are intrinsic to the primary sequence of the peptide. We have used a comprehensive but empirical approach to assess this issue of the role of “context” in immunodominance by changing the sites of expression of a series of unrelated peptides in the protein vector MalE and by comparing the immunogenicity of peptides that differ only in their affinity for class II within the same site of MalE. Comparing responses to peptides in their native context versus the same peptides inserted into MalE, we were able to change the competing peptides in the response and the secondary and tertiary structure of the antigen. By changing the localization of a peptide within a given protein or across different proteins, we anticipated that accessibility to proteases would vary, although we have not experimentally assessed this. Finally, in this study, we analyzed the potential of peptide liberation from the intact antigen to have a contribution to immunodominance through insertion of a dibasic motif adjacent to the cryptic peptide HEL [20-35]. Collectively, these
experiments revealed no differences in the immunogenicity of peptides when they are introduced in different molecular contexts and find the immunodominance of a peptide tracks with the peptide itself, rather than the site in a given protein or the protein in which it is contained.

The results of our study raise the question of why a peptides “context” apparently plays such a minor role in immunodominance, as we have measured it experimentally. The first consideration is whether and by how much the yield of antigenic peptides varies among potential immunogenic peptides contained in the antigen and how these differences compare to the total quantity of competing peptides in endosomal class II peptide loading compartments. Experimentally, microgram quantities of antigen are typically introduced subcutaneously and it can be expected that only a fraction of this will be taken up by APC. These antigen-derived peptides must compete for binding to class II molecules with peptides derived from a highly diverse and abundant pool of endogenously synthesized peptides (reviewed in (60)). It is quite possible that under these conditions, even dramatic differences in yield of one peptide over another, perhaps as much as 10-20 fold within the antigen due to a favorable location and flanking sequences, will lead to only minor differences in the yield of cell surface expressed peptide:MHC class II complexes because of competition with the tremendous excess of endogenously supplied peptides. These differences in ultimate yield may not offer a significant advantage of the more highly expressed complexes during CD4 T cell priming.
Secondly, DM editing may dramatically override the effects of differences in the initial yield of peptides from the intact antigen. Existing data on the function of DM (reviewed in (6, 10)) indicate that a subset of antigenic peptides are removed from the class II binding pocket by DM, as CLIP is, prior to their export to the cell surface (31, 32, 37-39, 157, 158, 197). Biochemical studies with purified class II molecules (31, 32, 35, 37, 38) and antigen presentation studies (36, 37, 158) suggest that DM preferentially removes peptides that contain suboptimal side chains at their anchor positions and that DM-antagonized peptides have lower stability interactions with the presenting class II molecule. Our laboratory has found that DM editing in APC can cause up to a 1000-fold change in initial epitope density, depending on the kinetic stability of the peptide:class II complex (36). This dramatic variability in the yield of peptide:class II complexes for different peptides due to differential DM editing is likely to profoundly diminish the impact of variation in initial proteolytic release of a peptide on its initial epitope density and thus its ability to successfully recruit CD4 T cells.

In the last set of experiments, we modified a cryptic epitope to have a protease sensitive site carboxy-terminal to the peptide epitope, and shown there was an increase in the liberation of this epitope in vitro. This result was very striking, and again showed how complex the processing of antigen within APC truly is. Despite being able to enhance the epitope release in vitro, there was no increase in the
immunogenicity of that peptide. This may be multi-factorial, with DM removing the newly released epitope and as the APC is migrating to the draining lymph node to prime naive CD4 T cells, the half-life in association with its class II molecule is so fast, that even a 6-fold increase in epitope density is not sufficient to alter its immunogenicity. Thus, there may never be enough of this peptide remaining on the surface of the APC for a long enough period of time for any enhancement in the recruitment of CD4 T cells simply because the stability of the peptide:class II complex is so weak. There have been several reports showing that T cells required approximately 40-200 complexes (198-200) and more recently at least 10 complexes to initiate a CD4 T cell response (201). These antigenic complexes will make up only about 0.1-0.4% of the total peptide:class II complexes on the surface of the APC (60, 202). To more comprehensively address this question, further experiments using a known subdominant epitope, with a modest half-life with MHC class II should be used to determine if there is a difference in 1) epitope yield and 2) immunogenicity in vivo. One would imagine that a subdominant peptide would have an enhanced epitope display, and possibly less susceptibility to DM editing effects, that may remain at a sufficient level and persist long enough in complex with class II for APC trafficking to and adequate T cell priming within the draining lymph node. This is an important question to address further because it will aid in determining if the molecular context of a peptide can be utilized in conjunction with peptide affinity for MHC class II molecules to have an impact on CD4 T cell selectivity, and if so, how this alters the design of new protein vaccines carrying novel peptide epitopes.
One can imagine that under some conditions of *in vivo* priming, molecular context may play a more dominant role. One of these conditions is when antigen is taken into APC by receptor-mediated uptake. Dendritic cells are known to take up antigen selectively by a number of cell surface receptors, as do B cells with their antigen specific receptors. *In vitro* evidence suggests that the facilitated uptake of antigen can amount between 100 to 1000-fold enhancement in antigen presentation (203-208). Also, some intracellular pathogens replicate rapidly within dendritic cells and the abundance of their associated proteins may be quite high. Under these types of conditions, and when endogenously synthesized antigens are the target of CD4 T cells (97, 146), differences in yield among the peptides released from the target antigens may lead to biologically relevant differences at the cell surface that are determined by the initial yield of the peptide from proteolytic processing events.

Although our laboratory has not yet established model systems to systematically examine immunodominance hierarchies under these types of conditions. There are potential models to specifically target antigens to both DC by DEC-205, DC-SIGN, Fc receptors, or the mannose receptor or B cells by antigen-specific targets to the B cell Receptor (BcR) using transgenic B cells. For example, by altering MalE, we could target it to the DEC-205 endocytic receptor on DC by conjugation with monoclonal antibodies (209) and assess the immunodominance hierarchy of peptide epitopes generated from facilitated uptake *in vivo*. 
Table I. Peptide accessibility does not differentiate the immunogenicity of several heterologous epitopes

<table>
<thead>
<tr>
<th>Protein</th>
<th>Regio</th>
<th>ID</th>
<th>Solvent Accessibility</th>
<th>Competing Peptides a</th>
<th>PDB #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm Whale Myoglobin</td>
<td>[102-117]</td>
<td>Dominant</td>
<td>Exposed</td>
<td>None</td>
<td>1VXG</td>
</tr>
<tr>
<td>MaIE</td>
<td>[69-84]</td>
<td>Dominant</td>
<td>Exposed</td>
<td>[102-115] [268-285]</td>
<td>1OMP</td>
</tr>
<tr>
<td>Insertion site</td>
<td>[102-]</td>
<td>Dominant</td>
<td>Buried b</td>
<td>[69-84] [268-285]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>[268-]</td>
<td>Dominant</td>
<td>Exposed</td>
<td>[69-84] [102-115]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>133</td>
<td></td>
<td>Exposed</td>
<td>[69-84] [102-115]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>206</td>
<td></td>
<td>Exposed</td>
<td>[69-84] [102-115]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>303</td>
<td></td>
<td>Buried</td>
<td>[69-84] [102-115]</td>
<td></td>
</tr>
<tr>
<td>Hen Egg Lysozyme</td>
<td>[11-25]</td>
<td>Cryptic</td>
<td>Exposed</td>
<td>[107-]</td>
<td>1LYS</td>
</tr>
<tr>
<td></td>
<td>[20-35]</td>
<td>Cryptic</td>
<td>Exposed c</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>[107-]</td>
<td>Dominant</td>
<td>Exposed</td>
<td>[107-]</td>
<td></td>
</tr>
<tr>
<td>Chicken Egg Ovalbumin</td>
<td>[323-]</td>
<td>Dominant</td>
<td>Buried b</td>
<td>[273-288] [323-339]</td>
<td>1UHG</td>
</tr>
<tr>
<td></td>
<td>[327-]</td>
<td>Cryptic</td>
<td>Buried d</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>[273-]</td>
<td>Dominant</td>
<td>Buried</td>
<td>[323-]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>[273-]</td>
<td>Cryptic</td>
<td>Buried b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LACK</td>
<td>[156-]</td>
<td>Dominant</td>
<td>ND</td>
<td>None</td>
<td>ND</td>
</tr>
</tbody>
</table>

Peptides from several proteins were analyzed for solvent exposure using Protein Explorer 2.45 based upon known crystal structures submitted to the Protein Data Base as referenced by the PDB numbers. 

a Potential competing peptides as defined in H-2<sup>d</sup> mice.

b Peptides that were buried except for the amino- and carboxy-termini that were solvent exposed.
Accessibility was determined in monomeric form.

Peptide that was buried except for the carboxy-terminus that was solvent exposed.

Amino-terminus only determined because structure may alter depending on the inserting sequence.

ND Not Determined.

---

**Figure 3.1 Immunodominance is independent of protein context**

The dominant epitopes MYO [102-117] and LACK [156-173], the subdominant OVA [273-288], and cryptic epitopes HEL [11-25], HEL [20-35], LACK[156-173]I166A, and OVA [327-339] were assessed for their immunogenicity at the peak of an immune response in the native context (top) or genetically encoded within the protein
shuttle MalE at amino acid 133 (bottom). Groups of two BALB/c mice were immunized in the footpad with 0.2mg/ml of the indicated protein emulsified in CFA. IL-2 ELISpot assays were performed and total spot counts of triplicate wells were normalized to the total IL-2 producers from the intact antigen and shown as a percent of the immunizing antigen. Data represent the mean percent of the immunizing antigen of at least three independent experiments ± SD.

Figure 3.2 Differences in acid-induced conformational stability of multiple proteins

The proteins LACK, OVA, HEL, and MalE were assessed for their ability to maintain conformational stability under acid-induced unfolding conditions. Each protein was incubated at a final concentration of 2μM with 60μM ANS for 10 min at room
temperature. Measurement of bound fluorescent ANS was performed at a pH range between pH 4.0 and 7.0, exciting the probe at 350nm and measuring the fluorescence intensity between 490nm and 600nm (A). In Figure 2B, native HEL was either not reduced (B, left) or reduced with DTT (B, right) prior to incubation with ANS. Results are presented as the Fluorescence Intensity at each pH from duplicate readings and is representative of two independent experiments.

Figure 3.3 The efficiency of antigen presentation is not detectably altered by location of the peptide within a complex antigen

The peptide epitopes HA [126-138] and higher stability variant HA [126-138] T128V were encoded within MalE at amino acids 133 (squares), 206 (triangles), and 303 (circles). The constructs were assessed for functional equivalence in protein concentration by testing the release of the internal MalE peptide epitopes using T cell
hybridomas M1 and M3, reactive to MalE [268-285] (A top) and MalE [69-84] (A bottom), respectively. TS2 reactive to HA [126-138] was used to assess the peptide release from the different insertion sites within MalE (B). Results are presented as the Mean Optical Density ±SD from duplicate wells with background (no antigen) subtracted and is representative of two independent experiments.

Figure 3.4 A peptide intrinsic factor rather than the site of localization in the antigen dictates immunogenicity

HA [126-138]:MalE and HA [126-138] T128V:MalE 133, 206, and 303 constructs were emulsified 1:1 with Ag-PBS:CFA at 0.2mg/ml. Groups of two BALB/c mice were immunized in the footpad with 50μl of the mixture. At day 10, draining popliteal lymph nodes were harvested and CD4 T cells purified as described in the
Materials and Methods and fresh splenocytes as restimulating APC were Thy1.2-depleted. CD4 T cells were plated in 1:2 dilutions titrating from 300,000 cells/well and restimulated with 10µg immunizing protein and 5µM of test peptides or MalE internal control peptides in an IL-2 ELISpot assay. The results are the average ±SEM of at least three independent experiments from triplicate wells and are presented as a percent of the immunizing antigen.

Figure 3.5 Efficient antigen presentation of a single peptide epitope is not detectably altered by location of the peptide within a complex antigen

The peptide epitope OVA [323-339] was encoded within MalE at amino acids 133 (squares) and 303 (circles). The constructs were assessed for functional equivalence in protein concentration by testing the release of the internal MalE peptide epitopes using T cell hybridomas M1 and M3, reactive to MalE [268-285] (A top) and MalE
23.12 T cell hybridomas reactive to OVA [323-339] was used to assess the peptide release from the different insertion sites within MalE (B). Results are presented as the Mean Optical Density ±SD from duplicate wells with background (no antigen) subtracted and is representative of two independent experiments.

Figure 3.6 The immunodominant phenotypes for two overlapping epitopes in a single peptide are distinct and correlate with kinetic stability

The putative recognition sites of the T cell hybridomas 3DO11.10, 8DO51.15, and 3DO54.8 for the peptide OVA [323-339] are shown in panel A. Groups of two BALB/c mice were immunized as described previously with native OVA (B), OVA
[323-339]: MalE encoded at 133 or 303 (C), and OVA [273-288]: MalE encoded at 133 or 303 (D) and assessed in an IL-2 ELISpot assay. Shown is the number of IL-2 spots elicited in response to the test peptides or internal MalE control peptides. The results are presented as IL-2 spots (background subtracted) / 300,000 cells and are representative of two or more independent experiments from triplicate wells ± SD.

Figure 3.7 Hypothesized enhanced cleavage of a cryptic peptide

An amino acid substitution at residue 34 from F>R was introduced into the minimal peptide epitope HEL[20-35] to create a well-characterized recognition sequence of two basic amino acids (KR) that is recognized by a broad family of proprotein convertases. It is hypothesized that the peptide will be cleaved from the whole protein (HEL[20-35] inserted into MalE at amino acid 133) carboxy terminally to the
two basic amino acids. Subsequent to the endopeptidic cleavage event, there is frequently a carboxypeptidase-mediated removal of both amino acids. Leaving the cleavage product of HEL[20-32], which has been shown to be the core determinant.

**Figure 3.8 Addition of a dibasic motif increases the liberation of a cryptic peptide in vitro**

HEL:MalE133 (open triangles) or HEL(F34R):MalE133 (closed squares) were tested for presentation on A20 cells (panel A) or on freshly isolated splenocytes (panel B) for T cell hybridomas reactive to HEL [19-37] with titrating doses of antigen. Each peptide, HEL [19-37] (open circles) and HEL [19-37] F34R (closed circles) was tested with titrating doses on A20 cells to show there was no intrinsic difference in
their presentation (inset panel A). M1 and M3 hybridomas reactive to the peptides MalE[268-285] (panel C) or MalE[69-84] (panel D) respectively, were used as internal protein concentration controls when each antigen was presented on A20 cells. Triplicate wells from each condition were frozen, thawed, and assayed for IL-2 by the IL-2 dependent cell line CTLL. Data are presented as the Mean Optical Density and are representative of two independent experiments ±SD.

Figure 3.9 Increased processing of a cryptic peptide is not sufficient to confer immunodominance in vivo

HEL:MalE133 (white bars) or HEL(F34R):MalE133 (black bars) were emulsified 1:1 with Ag-PBS:CFA at 0.2mg/ml. Groups of two BALB/c mice were immunized in the footpad with 50μl of the mixture. At day 10, draining popliteal lymph nodes were harvested and CD4 T cells purified as described in the Materials and Methods. CD4 T cells were plated in 1:2 dilutions titrating from 500,000 cells/well and restimulated
with 10µg immunizing protein, 5µM of test peptide or MalE internal control peptides in an IL-2 ELISpot assay. The results are representative of two independent experiments from triplicate wells and are presented as a percent of the immunizing antigen. * denotes no spots were detected above background.

CHAPTER 4
---------------------------------

Persistence of high stability peptide:class II complexes in vivo

------------------------

4.1 Introduction

4.2 Results

Kinetic stability of the peptide:class II complex correlates with antigen persistence

Hierarchy of peptide persistence in the absence of DM editing

Persistent peptide:MHC class II complexes contribute to the expansion of CD4 T cells in vivo

4.3 Discussion
4.1 Introduction

The interaction between CD4 T cells and antigen-bearing DC initiates a process that generally leads to immunity, but can also result in deletion, anergy, or conversion to a regulatory phenotype. Upon stimulation, signals through the TcR (signal 1) and costimulatory molecules (signal 2) result in the proliferation of T cells. These activated T cells will receive environmental cues (cytokines / chemokines – signal 3) that cumulatively result in the progression toward effector cells that combat the invading pathogen and memory cells which protect against future encounters to the same antigen (reviewed in (60, 210, 211)). It is clear that CD4 T cells are capable of dividing with an initial encounter of antigen, but it is unknown if clonal expansion or progression to effector or memory T cells is dependent on continuous reengagement with the antigen-bearing cell. The contribution of antigen has been difficult to assess due to the environmental cues that are integrated by the T cell in conjunction with TcR ligation by its cognate antigen. There have been a few reports trying to address this question using in vivo model systems (79, 81, 82), but much debate still remains whether daughter cells require their cognate antigen to continue through the full activation programming.
In the present study we began to evaluate the requirement of antigen on the fate of the responding population of CD4 T cells. We have utilized peptides of varying off-rates from MHC class II molecules to determine the functional stability of these peptides \textit{in vivo} by the proliferation of adoptively transferred TcR transgenic T cells. Collectively, we show that peptide dissociation rates from class II molecules \textit{in vitro} do accurately reflect the persistence of these complexes \textit{in vivo}. Furthermore there is a hierarchy of expansion of the T cells elicited that correlates with the kinetic stability of the peptide:class II complexes; the more persistent peptide:class II complexes were capable of expanding a larger cohort of CD4 T cells than rapidly decaying complexes. These studies provide a more comprehensive approach to address the requirement of antigen on the programming of naïve CD4 T cells and the fate of those T cells responding to cryptic or dominant peptides.
4.2 Results

**Kinetic stability of the peptide:class II complex correlates with antigen persistence**

As previously described, our studies indicate that immunodominant and cryptic peptides segregate dependent upon the singular biochemical property of either stable or unstable, respectively, interactions with the presenting MHC class II molecules (36, 150, 156). These studies determined the kinetic stability of the peptide:class II complex by measuring the off-rate of peptide from purified MHC class II molecules in acidic and basic conditions. However, it is unknown whether the kinetic stability of these peptide:class II complexes is correlative to their persistence *in vivo* and if so, whether there is any physiological relevance or functional consequences of persistent peptide:class II complexes on the fate of the responding T cells. Potentially, if the antigen density between high and low stability peptide:class II complexes decay differentially and T cells require continuous signaling through the TcR, then there may be differences in the quantity and quality of the T cells elicited. More stable complexes may be capable of recruiting more T cells over time, concomitant with a kinetic advantage due to faster expansion, and establish more ‘fit’ effector and
memory populations. Not only would this drive selectivity in the oligo-clonal population responding to a single antigen (132-137), there may be consequential effects to other T cells responding to their own cognate antigen being presented by the same dendritic cells (addressed in Chapter 5).

To assess if peptides of varying kinetic stability with MHC class II molecules differentially persist we used a model antigen to assess the availability of peptide:class II complexes on APC in vivo. As described in Chapter 3, our laboratory utilizes an antigen, MalE, to assess immunodominance hierarchies of heterologous test peptides in the context of the same protein. MalE is a bacterial protein that is expressed at high levels, is easily purified, strongly immunogenic, and permits the addition of 20-30 amino acids into several sites without altering the protein structure (159).

BALB/c mice were immunized in the ear pinnae with limiting doses of the low stability OVA:MalE133 or a higher stability variant A332I-I334V:MalE133 emulsified 1:1 in Complete Freund’s Adjuvant (CFA). A 10-fold advantage in antigen dose was given to the low stability OVA [327-339] in order to compensate for intracellular processing and the effects of DM editing. On days 1, 3, 7, and 10 post immunization 2x10^6 carboxyfluorescein succinimidyl ester (CFSE) -labeled, 11.10 TcR transgenic CD4 T cells were adoptively transferred into a group of the immunized mice to determine if complexes remained. Thirty-six hours after the
adoptive transfer, T cells were visualized for dilution of the CFSE indicating CD4 T cell proliferation and the presence of cognate ligand. Also, it is known that immunization with CFA causes a local depot of antigen that persists for very long periods of time (212). One could imagine that there may be continually migrating DC from the site of immunization carrying antigen, and if so whether they contribute to the expansion and recruitment of T cells. Hence persistence was assessed when the cohort of DC were limited by removal of the immunization site as well.

In Figure 4.1 the persistence of the low stability OVA peptide and higher stability variant A332I-I334V peptide:class II complexes are shown over time when encoded within MalE at amino acid 133. For the intact antigen site, on day 1, we observed robust proliferation profiles of T cells elicited from both the high and low stability antigens. However, a drastic decrease in complexes was observed for the low stability OVA peptide, as determined by the inability of the newly transferred T cells to proliferate after day 7. Conversely, the high stability A332I-I334V variant peptide persisted at a sufficient density to drive newly transferred T cells into cell division for over 10 days. Similarly, when the site of immunization was removed to limit the numbers of antigen presenting cells migrating to the draining lymph node (213), the high stability complexes persisted for over 7 days, while the low stability peptide:class II complexes were essentially undetectable after day 3. Unexpectedly, when we compared the intact to removed site for either protein, there was not a drastic difference between the CFSE profiles for each of the days. This in itself is
interesting because it would argue that there may not be continual trafficking of antigen-bearing DC from the site of immunization into the lymph node, at least at the level of our detection. If there were continual trafficking of DC, I would have expected the first cohort of DC and any subsequent cohort of DC to be capable of presenting antigen to the newly transferred T cells if the antigen persists long enough. Overall, when the test peptides were introduced in the context of a protein, the kinetic stability of the peptide:class II complex can predict the functional persistence of a peptide antigen in vivo.

**Hierarchy of peptide persistence in the absence of DM editing**

One of the difficulties in assessing the persistence of peptide:class II complexes with protein immunizations is that DM editing will selectively skew the determinants displayed to be represented mostly by stable binding peptides to class II molecules and drastically limiting the availability of T cell recognition to low affinity peptides (31, 32, 35-38, 158). To bypass DM editing and load equivalent numbers of complexes on the surface of the APC, synthetic peptides of varying kinetic stabilities in association with MHC class II were used to assess the dissociation kinetics in vivo. OVA [327-339] and peptide variants of higher binding stability, A339S, A329M-A339S, A332I-I334V, have been shown to exhibit a half time of dissociation on I-A\textsuperscript{d} of 1.3, 4, 10.4, and >250 hrs at pH=7.4 (unpublished, F.A. Chaves and A.J. Sant). Bone marrow-derived dendritic cells (BMDC) were used as the immunizing vector
over peptide / CFA immunizations for several reasons. One can obtain large numbers of mature DC in vitro (~$10 \times 10^6$/mouse) with a purity of 80-85% I-A<sup>d</sup>CD11c<sup>+</sup> with a high expression of costimulatory molecules (Figure 4.2), determine the relative initial epitope density, and the ability to control for DC numbers available for CD4 T cell priming. BMDC were peptide-pulsed for 1 hour and either used in vitro to determine relative complex numbers or to immunize mice to determine the functional persistence of peptide:class II complexes of varying stabilities in vivo.

Initially, peptide-pulsed BMDC were mixed with T cell hybridomas reactive to the OVA [323-339] peptide in a 1:3 ratio in the presence of diluting concentrations of an anti-MHC class II antibody and assayed for IL-2 production by ELISA. Figure 4.3 shows an example of the anti-MHC class II blocking assay to assess the loading of peptide onto the immunizing BMDC. Each of the OVA peptides loaded similar numbers of peptide:MHC class II complexes as shown by the inflection points in the IL-2 curves at ~1:100 – 1:1000 of anti-MHC class II. Thus, at the concentration of peptides used, we have confidence that they were initially similar in their ability to stimulate T cells.

Subsequently, the peptide-pulsed BMDC were used to immunize BALB/c mice and on days 1-4 post immunization, $2 \times 10^6$ CFSE-labeled, 11.10 TcR transgenic CD4 T cells were adoptively transferred into a new group of the immunized mice each day. Draining lymph nodes were harvested 48 hours later and assayed for the presence of
antigenic complexes as indicated by the dilution of CFSE. The data in Figure 4.4 are represented as the percent of the total cells in the parental peak, generations 2-5, and generations 3-5. Again we observed a similar trend as our initial studies with protein, in that at day 1, there are sufficient complexes of all test peptides to drive proliferation of the transferred T cells. Furthermore, these results argue that the initial loading of the peptides observed in Figure 4.4 were sufficiently equivalent to promote comparable levels of T cell proliferation. Over time, the kinetic stability of the peptide:MHC class II complexes dictates the duration those peptides persist in complex with class II as visualized by the ability or inability of newly transferred T cells to recognize their cognate ligand and proliferate. Surprisingly, only the most stable binding peptide variant of OVA persisted in sufficient numbers of complexes to prime the newly transferred T cells at day 4. Thus a hierarchy of peptide persistence was observed as predicted based upon the kinetic stability of that peptide in association with its presenting class II molecule.

**Persistent peptide:MHC class II complexes contribute to the expansion of CD4 T cells in vivo**

The previous result demonstrated that peptides displaying a range in kinetic stabilities on purified class II in vitro display a similar hierarchy of persistence in vivo. This observation led us to begin investigating if there are functional consequences of persistent peptide:MHC class II complexes in vivo. Upon maturation with LPS or
TNFα, human DC have been shown to increase the half-life of surface class II from 10 hours to >100 hours, increase the expression of class II ~50-fold, with steadily decreased levels of class II recycling (202) and their life-span to have a half-life from 1.5-3 days (214). Thus, DC presenting very stable peptide:class II complexes may be capable of stimulating CD4 T cells for several days, as long as the DC did not perish (215) or lose the capability to stimulate adequately (216-219), recruiting and expanding a cohort of T cells that would assume immunodominance; while rapidly decaying peptides would be incapable of long-term T cell stimulation. This provocative scenario would lead to another level of control where peptide stability on its presenting class II molecule could alter the repertoire of T cells ultimately elicited. Those T cells that do recognize less stable peptide:class II complexes may be driven to anergy, death, a regulatory phenotype, or if persistence is not necessary, to an effector cell. In order to address this, we asked if long-lived peptide:class II complexes have an advantage over low stability peptide:class II complexes by contributing to the recruitment and expansion of CD4 T cells. In other words, do T cells need to continuously recognize their cognate ligand to progress through the full activation program?

Here, we used a panel of OVA peptides that vary in their kinetic stabilities with the presenting class II molecule, as described earlier, and the 11.10 TcR transgenic CD4 T cells for an increased T cell precursor frequency to evaluate the functional consequence(s) of peptide stability during an immune response. Mice were
adoptively transferred with 2,000 transgenic CD4 T cells and 24 hours later
immunized in the ear pinnae with 25 nmol of each OVA peptide emulsified (1:1) in
CFA. Two days later the site of immunization was removed to have an adequate but
limited number of DC presenting antigen within the draining lymph node. Ten days
post immunization, draining lymph nodes and spleen were removed and purified CD4
T cells were rechallenged with the immunizing test peptide in a 4 hour IL-2 ELISpot
assay to determine the frequency of responding TcR transgenic T cells.

Figure 4.5 shows the frequency of responding T cells within the lymph node (solid
bars) and spleen (transparent bars). Interestingly, there was an increase in the
expansion of T cells that corresponded to the stability of the test peptide.
Furthermore, a similar trend was observed when assessing the T cells that have
trafficked out of the lymph node and are in the spleen. Although it is clear high
stability peptides are capable of increasing the frequency of responding T cells during
an immune response, we have not determine if this is a selective increase in
recruitment, expansion, or both. In summary, we observed a hierarchy in the recall
response to the immunizing antigen, translating to preferential CD4 T cell expansion
based upon the kinetic stability of the peptide:class II complexes.
4.3 Discussion

The main question we began to address in this chapter is if there is a requirement of continual TcR engagement by naive CD4 T cells in order to drive programmed cell division of the daughter progeny. We have taken a very systematic approach to address this important issue. We have immunized mice with a well-defined, heterologous protein inserted with peptides of differing kinetic stabilities in association with MHC class II, as well as peptides loaded onto antigen-bearing cells and followed the fate of those complexes over time with adoptively transferred TcR transgenic T cells. First, we show that the differences in off-rates of peptides from MHC class II molecules measured in vitro are functionally relevant in vivo. Additionally, when those T cells responding to peptides of various kinetic stabilities were assessed for their capacity to expand over time, a hierarchy of T cell expansion was observed that correlates with the kinetic stability of the peptide:class II complexes. Most importantly, these latter observations are made when antigen processing and DM editing are bypassed, deeming these processes as a critical checkpoint, but not the only stage at which peptide stability on MHC class II molecules plays a role in altering the repertoire of T cells elicited.
Although the aforementioned results are encouraging, there are questions to be addressed still. Future experiments will need to separate the contribution of recruitment into the lymph node and the requirement of antigen, cytokines, and tonic interactions with MHC class II or costimulatory molecules for the progression of daughter cells into further divisions.

To begin to separate these potentially non-mutually exclusive mechanisms in maintaining division and focus on the requirement of antigen for this process in an in vivo environment, we can use the TC / APC adoptive transfer system established in this Chapter. One could transfer low or high stability peptide-pulsed APC, allow for the migration to the lymph node, and subsequently transfer naïve CD4 T cells. Then block newly immigrating naïve CD4 T cells into the lymph node by blocking CD62L-selectin, a molecule required for T cells to enter the lymph node (see Chapter 1), shortly after T cell transfer. This allows only a single cohort of T cells to respond to the antigen that is being presented by the transferred APC and the ability to address if there is a difference in the early events in cellular division between low and high stability peptide:class II complexes. Pilot studies performed thus far show there is evidence of differential rates of T cell division elicited by low or high stability peptide:class II complexes when a single cohort of transferred T cells is followed over the first few days of an immune response (Figure 4.6), in agreement with published data from Henrickson et al. (220).
Second, we can then address the role of continuous TcR engagement in driving daughter cell progeny by utilizing mice that express the diptheria toxin receptor under the control of the CD11c promoter to ablate those transferred antigen-bearing DC at different time points during the response (221). One major caveat to this experiment is if there is cross presentation of foreign antigen from the diptheria killed DC on the endogenous APC. Although the original report did not detect any antigen being transferred (221), harvesting the endogenous APC and performing reverse ELISpots could easily test this caveat. If there is a truncation in the proliferation of the daughter cells, then we know that antigen is required for the full progression of expansion and other factors, such as cytokines or MHC molecules may not play a major role. Conversely, there may be no differences observed and the contribution of cytokines (like IL-2) and cell surface associated molecules, such as MHC class II and costimulatory molecules, may need to be segregated from that of antigen.

We can separate the role of IL-2 in driving division by transferring TcR transgenic T cells that are IL-2Rα-/- . This would allow for the continued interactions with costimulatory and MHC class II molecules, in the absence of antigen and stimulation by IL-2. A caveat to this experiment may be that the IL-2Rα-/- T cells may not divide as efficiently as wild-type Tg cells, even in the presence of antigen. Furthermore, blocking antibodies, such as 7D4, could be used, but may be inadequate because the antibody is of low affinity and may not be capable of blocking all of the IL-2 from binding.
Lastly, since MHC class II and costimulatory interactions could occur in trans, we could also perform our transfer system in class II-/- mice, with or without DC ablation, and assess the progression of T cell division at select time points. Although this may be informative, it has been shown that tonic interactions with self:MHC class II is not only required for survival in the periphery (58, 59), but a loss in this interaction leads to hyporesponsiveness in vitro (59). Although these types of experiments each come with their own caveats, they could aid in determining the contribution of antigen from other factors on selecting the final specificity of the responding T cell repertoire in vivo.

One alternative reason for the differential persistence in peptide:class II complexes observed in this Chapter could be explained by the fixed affinity of the TcR utilized. The 11.10 TcR transgenic is of low affinity (222) and may be capable of only sensing antigen to a certain threshold of complex numbers. Even if there is a drastic reduction in low stability peptide:class II complexes in comparison to higher stability peptides over time, there still may be a sufficient number of peptide:class II complexes to prime CD4 T cells expressing a higher affinity receptor. The reactivity of these T cells to antigen could lead to the elimination of the differences observed in the recruitment and expansion. To test this, these experiments could be reiterated with a TcR transgenic of a higher affinity or with a more heterogeneous population of
CD4 T cells with a higher precursor frequency, such as a β-chain transgenic, to determine the presence of antigenic complexes over time.

Essential to interpreting the results presented here is that the differential persistence of high and low kinetic stability peptides measured *in vivo* is independent of DM editing. Exogenous peptides have been hypothesized to be loaded onto newly synthesized class II molecules in MHC class II containing compartments (MIIC) (223) or on recycling class II molecules in peripheral or early endosomes (202, 224-226). Reports have shown DM to be localized to the MIIC compartment (227, 228) and not found within the recycling pathway (33, 229). Although, there is one report demonstrating the detection of DM on the surface of B cells and immature DC (230) suggesting that DM may be active at the cell surface or have access to the recycling pathway, but not abundant enough to detect, and if the environment is sufficiently acidic may alter the determinant display upon recycling. If this report is correct, the role of DM should be considered when utilizing immunization schemes with peptide. Because the endogenous T cell repertoire in DM-/- mice is selectively skewed (170, 231), experiments would have to use splenic DC or BMDC from DM-/- mice to assess the persistence of fluorescently labeled peptide *in vitro* or by detection of antigenic complexes with adoptively transferred TcR transgenic T cells *in vivo*. 
Figure 4.1 Persistence of high stability peptide:MHC class II complexes \textit{in vivo}

Mice were immunized S.C. in the ear pinnae with 0.2mg/mL OVA \{323-339\}:MalE133 or 0.02mg/mL variant OVA \{323-339\} A332I-I334V:MalE133 proteins emulsified 1:1 in CFA. In the indicated groups, the ear pinnae were removed 6 hours after immunization. CFSE-labeled DO11.10 transgenic T cells were adoptively transferred intravenously at days 1, 3, 7, and 10, and subsequently harvested 36 hours later. T cells were analyzed by flow cytometry for the dilution of
Figure 4.2 Phenotype of LPS activated BMDC

Femurs from BALB/c mice were harvested and cleaned of all muscle tissue in media. Bone marrow was extracted by pushing media through the femur using a 27G needle. Bone marrow cells were washed, counted and plated at 4x10^6 cells/10cm petri dish in the presence of 7% fetal calf serum and 20ng/mL GMCSF in 10mL of media. At day 3 an additional 10mL of media containing 20ng/mL GMCSF was added to each plate. At day 6 10mL of media was aspirated and spun down to pellet any cells. These cells were resuspended with fresh media and added back to the original plate.
On day 7 10ng/mL LPS was added to each plate for DC maturation. The next day cells were harvested, counted and stained for cell surface markers I-A<sup>d</sup>-PE in combination with CD11c-FITC, CD80-FITC, CD86-FITC, B220-FITC, or CD40-FITC.

Figure 4.3  Similar epitope densities between peptides of varying kinetic stabilities

LPS matured BMDC were peptide-pulsed with PBS, OVA [327-339], and the variants A337S, A329M-A337S, A332I-I334V for 1 hour, washed, and added to an <i>in vitro</i> anti-MHC class II blocking assay. 15,000 peptide-pulsed BMDC were
cocultured with 50,000 T cell hybridomas (5G5) reactive to OVA [323-339] overnight at 37°C. In some wells, anti-MHC class II antibodies were titrated starting at 1:1 to 1:1000000. Supernatants from triplicate wells from each condition were frozen, thawed, and assayed for IL-2 by ELISA. Data are presented as ng / ml IL-2 secreted and are representative of at least two independent experiments ±SD.

**Figure 4.4** High stability peptide:class II complexes persist *in vivo*

LPS matured BMDC were peptide-pulsed with PBS, OVA [327-339], and the variants A337S, A329M-A337S, A332I-I334V for 1 hour, washed and used to immunize BALB/c mice. Shown here, mice were immunized with 200,000 peptide-pulsed BMDC per ear pinnae. On days 1-4 separate animals were adoptively
transferred intravenously with CFSE-labeled DO11.10 TcR transgenic total spleen and peripheral lymph node cells that were normalized to 2x10^6 CD4^+KJ^+ T cells via flow cytometry. 48 hours later draining lymph nodes were separately harvested and analyzed by flow cytometry for dilution of CFSE. CFSE profiles were analyzed and are represented as the percent of the total number of cells and are an average of 2 independent experiments, n=2 per experiment.

Figure 4.5 Kinetic stability of the peptide:class II complex dictates T cell expansion

BALB/c mice were adoptively transferred intravenously with DO11.10 TcR transgenic total spleen and peripheral lymph node cells that were normalized to 2,000 CD4^+KJ^+ T cells via flow cytometry. T cells were allowed to park for 24 hours, then mice were immunized s.c. in the ear pinnae with 25 nmol peptide emulsified in CFA.
The immunization groups were PBS, OVA [327-339] [black], and the variants A337S [green], A329M-A337S [blue], A332I-I334V [red]. The pinna of the ear was removed at 48 hours post immunization. Draining lymph nodes and spleen were harvested at day 10, and bulk lymphocytes were recalled to the immunizing peptide in a 4-hour IL-2 ELISpot assay. Lymph node spots were normalized to 500,000 cells (solid bars) and splenocytes were normalized to 1x10⁶ cells (transparent bars). * denotes no spots above background.

Figure 4.6 Differential rates of T cell division elicited by low or high stability peptide:class II complexes

LPS matured BMDC were peptide-pulsed with either 10 μM and 50 μM OVA [327-339] or 2 μM and 10 μM of the higher stability variant A332I-I334V for 1 hour, washed and used to immunize BALB/c mice. Mice were immunized with 200,000 peptide-pulsed BMDC per ear pinnae. 24 hours later mice were adoptively
transferred intravenously with $0.5 \times 10^6$-sorted CD4$^+$CD44$^{lo}$ CFSE-labeled DO11.10 TcR transgenic T cells from peripheral lymph node (excluding mesenteric lymph nodes). 6 hours later, mice were injected with $100 \mu g \alpha$CD62L-selectin (MEL-14) intraperitoneal. 48 hours later draining lymph nodes were separately harvested and analyzed by flow cytometry for dilution of CFSE. CFSE profiles were analyzed and are represented as the percent of the total number of cells.

CHAPTER 5

---

Abortive activation of CD4 T cell responses during competitive priming

5.1 Introduction

5.2 Results

Peptides with fast off-rates from class II elicit diminished T cell responses only when immunized with dominant peptides

Kinetic stability of the peptide:class II complexes predicts resistance to competitive T cell activation

Competitive T cell activation is a local event that is limited to the draining lymph node

Peptide competition for binding of available class II does not account for diminished T cell responses
Abortive T cell activation by cryptic peptides occurs during the expansion phase of the primary immune response.

5.3 Discussion

5.1 Introduction

Most of the efforts to reveal the major mechanisms driving the selectivity in the primary CD4 T cell response to complex antigens, including our own, have focused on the intracellular events associated with antigen processing (reviewed in (10, 85, 87, 146, 232, 233)). In bypassing intracellular processing events, including DM editing, we will be able to elucidate other non-processing factors, such as antigen persistence, that may influence the immunodominance hierarchy established during an immune response. To date, there have been no studies to evaluate the magnitude and final T cell specificity elicited by cryptic or rapidly decaying peptides from class II when in the presence of other immunogenic peptides after arrival at the cell surface.

Recent findings describing the dynamic interactions of antigen-bearing dendritic cells and T cells suggests that peptide off-rates from class II may impact the immune outcome at several levels: T cell signaling, expansion, effector function, and memory formation. The quality of signals received through the TcR by peptides of different affinity for MHC class II has recently been suggested to regulate the fate of those
cells, inhibiting the induction of autoimmunity (234, 235). Similarly, altered peptide ligands of the TcR can send a qualitatively different signal, as strong agonist peptides were capable of activating while low-potency peptide:class II complexes caused anergy of a monoclonal population of T cells (236). Despite being able to visualize the different phases of DC:T cell interactions to antigenic stimulus occurring within the lymph node (81, 237-243), the factors that contribute to efficient T cell activation and how the kinetic features of those interactions governs the fate of the responding T cells is still unknown. The decision for a T cell to ‘stop’ and form long-lived contacts with a DC when scanning for cognate antigen may depend on multiple parameters, but antigen density (242) and peptide affinity for class II (220) have been shown to augment the frequency of prolonged DC-T cell contacts, increasing the efficiency of the T cell ‘stop’ signal. In conjunction with these reports, are several studies that have suggested the requirement of stable DC:T cell interactions for the induction of immunity, rather than tolerance (240, 244-246).

Interestingly, as described in detail in the Thesis Introduction and Chapter 4, there is mounting evidence that CD4 T cells are flexible in their response to antigenic stimulus, as they divide according to antigen load, and require persistent peptide:class II complexes to sustain optimal T cell division for effector function and the development of a memory population (79, 81, 82). This is in sharp contrast with evidence that CD8 T cells do not require persistent antigen, but rather only 12 hours of stimulation to reach a threshold for programmed cell division and differentiation
Thus, if the antigen density between high and low stability peptide:MHCI complexes decays differentially and T cells do require continuous signaling through the TcR, we speculate that the peptide off-rates from MHC class II might lead to differences in the quantity and quality of the final repertoire of CD4 T cells that ultimately expand and differentiate during an immune response.

In the present study we sought to comprehensively evaluate the impact of simultaneous T cell responses to peptides that differ in their kinetic stabilities with MHC class II on the final specificity and magnitude of the primary CD4 T cell response. Previous studies using the transgenic system have broadened our understanding of the cellular immune dynamics, particularly at the early phases of a primary immune response. Although informative, there are several caveats to these systems such as high precursor frequencies and a fixed TcR that are inadequate for probing the intercellular dynamics involved in the full evolution of an immune response. Thus, we have devised an immunization strategy to prime T cells of disparate antigen specificities using the natural endogenous TcR repertoire with its corresponding low precursor frequencies as the responding population. Collectively, our studies revealed that during competitive CD4 T cell responses, peptides with low stability interactions with class II molecules lose the ability to sustain T cell responses. Furthermore, we demonstrate that CD4 T cells that are specific for rapidly decaying peptides from class II molecules are initially recruited into an immune response, but fail to progress further in the activation program by an apparent stall in
proliferation when in the presence of other ongoing T cell responses to dominant peptides. These studies are important to understanding the mechanisms driving competitive T cell activation *in vivo* both to aid in the design of vaccine strategies and also to allow a more comprehensive and sophisticated understanding of the cellular interactions that play a vital role in imparting protection after antigen exposure.
5.2 Results

**Peptides with fast off-rates from class II elicit diminished T cell responses only when immunized with dominant peptides**

Cryptic peptide determinants are defined by their failure to recruit CD4 T cells when introduced in the context of a protein antigen, but have the ability to elicit T cell responses when administered as a single synthetic peptide (reviewed in (87)). These results are most often interpreted as evidence for events associated with antigen processing as the major force that dictates immunodominance. However, an additional difference between protein and peptide immunizations is the presence of multiple, different epitopes, and thus ongoing T cell responses to different peptides. To explore this issue, we evaluated whether CD4 T cell responses to cryptic peptides are sustained in the face of ongoing CD4 responses to unrelated foreign peptides. In contrast to previous studies that have utilized adoptively transferred TcR transgenic CD4 T cells, we have chosen to study this issue in the context of the endogenous CD4 T cell repertoire, with its naturally low precursor frequency and heterogeneity in TcR affinity. Because previous work has shown that immunodominance is controlled by the off-rate or kinetic stability of a peptide in association with MHC class II, in these
studies we have used both wild type and variant peptide epitopes that differ in their off-rates from the class II molecules. These peptide variants offer the advantage of allowing side-by-side comparisons of T cell responses to related peptides that differ only in their affinity for MHC class II. The strategy in all the studies described here, unless otherwise stated, is to immunize BALB/c mice in the hind footpad with wild type or variant peptides (Table 5.1) alone or with a mixture of defined dominant peptides. Immunodominance hierarchies were evaluated by assessing the total number of responding T cells at the peak of the immune response via IL-2 ELISPOT assays. All peptides were emulsified in Incomplete Freund’s Adjuvant (IFA) containing (lipopolysaccharide) LPS instead of CFA as an adjuvant because the mycobacterium contains its own immunodominant peptides and would hinder our analyses. LPS was used to ligate TLR4 for the maturation and mobilization of APC at the site of immunization.

Initial studies utilized the well-defined, classic cryptic peptide HEL [11-25] (176) from the model antigen hen egg lysozyme (HEL). Our laboratory has found this peptide to have a half time of dissociation from I-A^d of 12 hrs. At the peak of the immune response, the HEL peptide elicited a robust CD4 T cell response (Figure 5.1A). We then analyzed the response to HEL [11-25] when it was co-administered with several high stability dominant peptides, OVA [327-339] A332I-I334V, HA [126-138] T128V, and MalE [69-84]. In an attempt to give the HEL peptide an advantage in epitope density, the dominant peptides used as competitor peptides were

Unexpectedly, when the cryptic HEL peptide was administered with three high
stability peptides, there was a loss in the total number of HEL [11-25]-specific T cells
recalled at the peak of the immune response (Figure 5.1A).

We wanted to verify this important finding by evaluating the ability of another low
stability peptide to recruit CD4 T cells with or without co-administration of
competitor peptides. Therefore, we next utilized a cryptic peptide from ovalbumin, of
which we have identified several peptide variants of altered kinetic stabilities with
class II. OVA [323-339] has three known registers in which the peptide binds to I-A\textsuperscript{d}
(186). The carboxy-terminal register from the peptide [327-339] (4) known to
activate the 11.10 TcR transgenic mouse, was shown by our laboratory to be cryptic
(150) when assayed using the normal endogenous repertoire of CD4 T cells as
responders. As expected, a high dose of this OVA peptide (25 nmol/mouse) was able
to elicit a robust CD4 T cell response when administered as a single peptide, despite
having a very fast off-rate from I-A\textsuperscript{d} (t\textsubscript{1/2} < 2 hrs)(Figure 5.1B, left). In accordance
with the HEL peptide results, when the OVA peptide was co-administered with three
defined dominant peptides (LACK [156-173], HA [126-138] T128V, and MalE [69-
84]), the OVA specific response became almost undetectable (over a 75% reduction)
and the majority of the responding T cells were specific for the competing dominant
peptides (Figure 5.1B, right). This result was encouraging and led us to pursue other
peptide epitopes of OVA to determine if this was a unique property of cryptic
peptides or if there was a relationship between the kinetic stability of a peptide and its ability to maintain T cell responses during other simultaneous CD4 T cell responses. When the slightly higher kinetic stability variants A337S, A329M-A337S, and the much more stable binding A332I-I334V OVA peptides (Table 1) were introduced alone, each elicited robust immune responses that increased in magnitude as the kinetic stability of the peptide epitope increased (Figure 5.1C-E, left). Again, there was an attenuation in T cell responses when the low stability peptide variants were introduced with three known dominant peptides, being suppressed between 25-60% in comparison to peptide alone (Figure 5.1 C and D, left, respectively). Interestingly, sustained T cell responses were consistently observed only with the high stability variant A332I-I334V (Figure 5.1 E, left). This result indicates that extended persistence of peptide:class II complexes may be needed to maintain the ability to recruit T cells during concurrent T cell responses. Independent of the test OVA peptide used, the three heterologous peptides, LACK [156-173], HA [126-138] T128V, and MalE [69-84], recruited similar numbers of IL-2 secreting T cells at the peak of the immune response (Figure 5.1B-E, right). Similar patterns of responses were observed when IFNγ-producing, rather than IL-2-producing, CD4 T cells were quantified as an indication of T cell priming (Figures 5.2 and 5.9), while there was no detection of Th2-like cytokines, such as IL-4, IL-5, and IL-10 (data not shown). As the only difference between the native OVA [327-339] and kinetic stability variants is the off-rate from class II, our results suggest a direct correlation between the kinetic
stability of the peptide: class II complex and the ability to recruit T cells in the face of competitor responses.

**Kinetic stability of the peptide: class II complexes predicts resistance to competitive T cell activation**

We next wanted to extend our initial observations and determine if the susceptibility to decreased T cell priming during competitive T cell responses was a general phenomenon of low stability or cryptic peptides. To evaluate this, an independent set of peptides with previously characterized immunodominance patterns were co-administered with competing dominant peptides (Table 5.1). There are advantages in using a set of independent peptides of known immunodominance patterns with the endogenous CD4 T cell repertoire, in that there will be a range in TcR affinities and pre-immune precursor frequencies, with the common feature of the known peptide off-rates in association with class II molecules. As described in the Materials and Methods, the data for each peptide is presented as the percent response gained or lost when co-administered with competing peptides. Figure 5.3 shows that strikingly, there was a segregation of the peptide epitopes in that the immunodominant peptides (black) elicited similar or enhanced T cell responses while in the presence of other dominant peptides, while cryptic peptides (white) consistently showed diminished T cell responses. The selective loss of T cell responses to cryptic, but not dominant peptides while in competition directly correlated with the kinetic stability of these
peptides in association with I-A\(^d\) (Spearman \(r^2=0.7818, \ p<0.0064, \) Figure 5.3B). The
dominant peptides OVA [327-339] A332I-I334V, LACK [156-173], the high stability
recruited similar or enhanced T cell responses over the numbers elicited by peptide
alone. These dominant peptides exhibit half-lives in association with I-A\(^d\) of >250,
70, 62, 85, and >200 hrs at neutral pH, respectively. The cryptic peptides OVA [327-
339], and kinetic stability variants A337S, A329M-A337S (\(t_{1/2} 1.3, 4, 14\) hrs,
respectively), the low stability variant LACK I166A (\(t_{1/2} 1.0\) hr), HEL [11-25] (\(t_{1/2} 12\)
hrs), and MalE [69-84] Q72T-L75A (\(t_{1/2} 2.6\) hrs), consistently displayed an inability to
prime similar numbers of antigen specific CD4 T cells when immunized with a
mixture of dominant peptides. Interestingly, we found that as few as one
immunodominant peptide is sufficient to induce the observed loss in T cell responses
(Figure 5.4). Thus, in general, peptides with rapid off-rates from class II that
ordinarily fail to elicit responses when they are contained in a complex antigen also
selectively lose the ability, while dominant peptides maintain the ability, to recruit
CD4 T cells in the presence of other dominant CD4 T cell responses when in the form
of peptides.

**Competitive T cell activation is a local event that is limited to the draining lymph node**
To begin evaluating the potential factors that contribute to the attenuation of T cell responses to cryptic peptides in the face of ongoing responses to other peptides, we needed to determine if the negative effect occurred systemically or locally. Evidence in the literature argues that when CD4 T cells are responding to a foreign antigen, pro-inhibitory cytokines, such as IL-10, can be systemically produced inhibiting the activation of T cells (247-249). If some cytokines can suppress the immune response, we speculate that there may be a selective loss in T cells elicited by cryptic determinants because they are phenotypically different or more susceptible to the inhibitory effects.

To address this, mice were immunized with test and competitor peptides in two peripheral sites that drained to separate lymph nodes. We reasoned that if there were a systemic inhibition in the recruitment of T cells by cryptic peptides, we would observe the loss when immunizations were introduced in separate footpads. Conversely, if the selective loss develops only when both responses are occurring within the same local environment, we would observe the loss when the immunizations were introduced in the same site. Upon administration of OVA [327-339] or the higher stability variant as a single peptide immunogen, robust T cell responses were elicited (Figure 5.5A, black bars). As previously observed, when introduced with a mixture of dominant peptides in the same footpad there was about a 90% loss in the total number of antigen specific responders to WT OVA while there were similar numbers of T cells recruited by the variant A332I-I334V (Figure 5.5A,
white bars). Importantly, when mice were immunized with WT OVA and the mixture of dominant peptides into separate footpads (Figure 5.5A, grey bars), the loss in responses to the cryptic peptide was not observed. These results indicate that for the loss in T cell priming to be observed, the T cell responses must occur within the same local lymph node environment.

Since the priming of T cells occurs within the lymph node, the preceding results led us to consider factors at the level of the APC: T cell interactions that may account for the localized attenuation in T cell priming to cryptic peptides. Unfortunately, the dynamics of APC:T cell interactions with the normal T cell precursor frequency for antigen, recently shown to be in the range of 20-200 T cells for any given peptide (62), is extremely difficult to assess using current techniques in vivo. However, we do have the ability to manipulate the initial antigen-bearing cell by altering our immunization scheme. The strategy chosen was to introduce peptides at separate sites that drain to the same lymph node. Two separate cohorts of APC bearing the test and competing peptides would migrate and present antigen to T cells within the same draining lymph node. We reasoned that if an abrogation in the loss in T cell responsiveness to cryptic peptides were observed only when competitor peptides were administered at the same site, it would imply a key role for the antigen-bearing cell in competition. This would be limiting the likely mechanism(s) to the niche of localized cytokine milieu, access to cell surface associated molecules or the APC itself.
The experimental design was as follows. BALB/c mice were immunized in the footpad with 10 µl of emulsion using three different conditions i) a low or high stability peptide alone on one side of the footpad, with IFA / LPS on the opposite side; ii) a low or high stability peptide in a mixture of dominant peptides on one side while the opposite side was immunized with IFA / LPS; or iii) a low or high stability peptide alone on one side and the mixture of dominant peptides on the other side of the same footpad. IFA / LPS sham injections were used to control for the cellular influx from the site of immunization and possible increased stimulatory capacity of mature APC entering the lymph node. The results of this experiment are shown in Figure 5.6. The low stability MalE [69-84] variant and WT dominant MalE [69-84] both elicited robust antigen specific T cell responses when introduced alone (Figure 5.6A top and bottom black bars, respectively). As expected, there was a decrease in the number of antigen specific responders (50%) when the low stability variant (Figure 5.6A top, white bar) was immunized with the mixture of dominant peptides in the same emulsion (Figure 5.6B), while those T cells recruited to WT MalE [69-84] (Figure 5.6A bottom, white bar) were unaffected by the presence of other unrelated peptides. Remarkably, when the test peptides and competing peptides were administered in separate emulsions at two different sites, loading separate cohorts of APC that migrate and present antigen within the same lymph node, both cryptic and dominant peptides primed similar numbers of T cells as the peptide alone group (Figure 5.6A top and bottom, grey bars, respectively). We concluded from these results that the antigen must be delivered at the same site, and must be presented by
the same-antigen bearing cell in order for the loss in T cell responsiveness to occur. This result also points out that the majority of the peptide presentation is limited to the migrating APC from the site of antigen introduction and not as free peptide draining through the lymphatic system. Collectively, these data show that for the competitive loss in T cell priming to occur, it must be within the same draining lymph node microenvironment and the same antigen-bearing cell must present the competing peptides.

**Peptide competition for binding of available class II does not account for diminished T cell responses**

One of the simplest explanations for the failure of low stability complexes to recruit CD4 T cells under competitive conditions is that the presence of the competitor peptides diminishes presentation of the low affinity peptides on the priming APC. To address whether there is competition for peptide binding to class II molecules on the surface of the APC, several approaches were taken. First, purified CD11c+ dendritic cells were incubated with HA [126-138] alone or in a mixture of three dominant peptides *in vitro*. IL-2 production by T cell hybridomas was used to compare the efficiency of peptide:class II presentation. Figure 5.7A shows there was no difference in the dose response curves of HA alone, HA with the addition of three dominant peptides, or when a kinetic advantage was given to the competitor peptides by pre-pulsing the APC with the three dominant peptides. Because it could be argued that
the local APC have a more limited MHC class II density than the APC used *in vitro*, we next tested the impact of competition on the initial epitope density on the priming APC using CFSE-labeled TcR transgenic T cells. There was no difference in the initial expansion of the transferred T cells when in the presence or absence of competitor peptides (Figure 5.7B and C). In order to verify this important issue using an alternative method, the effect of competitor peptides that are restricted by a different class II molecule with the endogenous pool of T cells was employed. Mice were immunized with a low stability I-A<sup>d</sup> restricted peptide alone or with either I-A<sup>d</sup> or I-E<sup>d</sup> restricted dominant peptides. Published data on both HA [107-120] (113) and HEL [103-117] (250) in addition to our own unpublished results (F.A. Chaves and A.J. Sant) indicate neither peptide detectably binds to I-A<sup>d</sup>. If there is competition for available class II, then the I-E<sup>d</sup> restricted peptides should not inhibit the T cells responding to the I-A<sup>d</sup> peptide. The results of this experiment show that both the MHC matched (I-A<sup>d</sup>) and unmatched (I-E<sup>d</sup>) competitor peptides elicited robust responses (Figure 5.8B) and were equally potent in attenuating the expansion of I-A<sup>d</sup> restricted T cells (Figure 5.8A closed circles and closed squares). Collectively, these results argue that peptide competition for class II does not account for the dramatic loss of T cells responding to low stability peptides.

**Abortive T cell activation by cryptic peptides occurs during the expansion phase of the primary immune response**
Recently, the kinetics of competition during the priming of CD8 T cell responses was shown to occur within the first few hours after antigen exposure (130). In order to determine when during an evolving immune response the CD4 T cell recruitment to unstable peptides became attenuated, we performed kinetic analyses. Mice were immunized in the ear pinnae, and at different days, the draining lymph nodes were harvested and antigen-specific CD4 responses were assessed for the production of IL-2 and IFNγ by ELISpot assays. The CD4 T cells accumulating at the site of immunization after priming in the lymph node were also examined for number and phenotype at days 3, 4, 5, 7 and 10. The results shown in Figure 5.9 indicate that late in the response, after day 7, the disparity in T cell expansion from mice immunized with peptide alone vs. peptide in competition was striking (p<0.0005). However, responses to unstable peptides measured at early time points, days 3, 4, and 5, were indistinguishable with or without additional peptides in the emulsion. Initial priming and expansion was apparent when either IL-2 (Figure 5.9A) or IFNγ (data not shown) was used to quantify T cells above IFA/LPS alone (Figure 5.9B). The later loss in responses to low stability complexes in the context of competitive responses to other peptides was not only detected by CD4 T cells in the draining lymph node (Figure 5.9A and 5.9C left), but also by CD4 T cells that migrate back to the peripheral site of immunization (Figure 5.9C right and data not shown). This striking result indicates that when an unstable peptide is introduced concurrently with dominant peptides, the complexes initially recruits and primes antigen-specific CD4 T cells, but this response fails to progress, where other responses to dominant peptides continue to expand.
5.3 Discussion

In the current study, we sought to evaluate the role of peptide persistence on MHC class II molecules for CD4 T cell priming and expansion *in vivo*. Our results show that peptides with rapid decay from class II molecules are able to initially expand CD4 T cells, but fail to sustain T cell activation throughout the full course of an evolving T cell response. Importantly, this failure is only manifested when the unstable peptides are co-administered with other dominant peptides. Peptides that naturally possess stable interactions with class II molecules or those that are engineered to have this property were able to maintain, and in some cases demonstrated an enhanced ability to recruit their respective T cells when they were introduced with other immunodominant peptides. Collectively, these results argue that during an immune response, even when antigen processing and DM editing is bypassed, there are other potent regulatory events that drive the selectivity in the T cell repertoire to a restricted set of peptides that possess highly stable interactions with the presenting MHC class II molecules.

In evaluating the potential mechanism(s) that could account for the loss in T cell expansion *in vivo*, we must first consider the complexities of the cell-cell associations
needed for optimal T cell activation and continued cell division (see Figure 7.4). First, upon administration of peptides and adjuvant, peripheral APC will mature as a consequence of ligation of toll-like receptors, and peptides within the emulsion will be loaded onto MHC class II molecules at the cell surface. Once peptide is acquired, it takes approximately 18 hours for the antigen-bearing dendritic cells to leave the peripheral site, migrate and enter the lymph nodes (161). Furthermore, antigen-reactive CD4 T cells in the host require approximately 24 hours to recirculate to the draining lymph node where antigen is localized (251, 252). Here, CD4 T cells sample the microenvironment, and if cognate antigen is present, the CD4 T cells form many serial engagements with antigen-bearing dendritic cells (phase 1). Within 1-3 hours, the CD4 T cells apparently reach a threshold of activation, stop and make stable (10-20hr) cell interactions with the antigen-bearing DC (phase 2) (81, 238-243). After the initial signaling events that drive antigen-specific CD4 T cells into cell division have occurred, it is thought that T cells may require only transient interactions with peptide:MHC class II complexes to continue expanding for several generations (phase 3) until they are capable of exiting the lymph node as effectors (251).

Heterologous peptides co-introduced in vivo with single peptide immunogens and the concomitant immune responses that these competitor peptides induce might down modulate or arrest responses at any of these stages. In the first stage, at the peptide loading step, if MHC class II molecules were limiting and the competitor peptides
were present in sufficiently high quantities, there could be diminished recruitment of antigen-reactive CD4 T cells to the test peptide simply by competitive binding to the presenting class II molecule. Evidence presented in this chapter, both in vitro with cultured dendritic cells, and in vivo with competitor peptides that bind to alternate class II molecules other than the test peptide, argues that the competitors are not acting at this level. Also arguing against this early effect of the competitor peptides is that the initial activation and priming of T cells to low stability peptides is unaffected by the presence of other dominant peptides in the emulsion. If the competitor peptides are not acting here, then the subsequent events in recruitment and expansion must be considered. As the CD4 T cell response matures through expansion and affinity maturation of antigen-specific T cells (132-137) there is thought to be a coincident loss in cognate antigen on the APC through trogocytosis or “membrane–stealing” that can transfer MHC:peptide, costimulatory molecules, or other plasma membrane proteins from the APC to the antigen-specific T cells after a productive contact is established (216-219). The loss in available peptide:MHC class II molecules over time due to trogocytosis will be compounded for unstable peptides through the additional factor of peptide:class II dissociation. The dominant high stability peptides typically persist on class II molecules for more than 100 hrs (4-5 days) while the low stability peptides have half-lives of association with class II molecules for less than 20 hrs (150). This decay in available peptide:MHC class II will be kinetically linked to the increase in the number of T cells, driven initially by antigen-dependent expansion, and into successive generations by continued contact
with their MHC:peptide ligand. Recent data suggests that the continued expansion of
the responding CD4 population, unlike CD8 T cells, relies on the continued
opportunities to interact with antigen-bearing APC (79, 82). Thus, it is possible that
competition among CD4 T cells for available peptide:MHC on APC surfaces may
explain the failure of CD4 T cells reactive to low stability complexes to continue to
expand, because the selective decay of these peptides may abbreviate their ability to
stimulate CD4 T cells through their full expansion program. This simple kinetic
model is consistent with our data showing little effect of competitor peptides at the
early time points, and an apparent failure of T cells to progress at later time points.

In addition to this relatively simple model, is the possibility that CD4 T cells
responding to stable and unstable peptides may differ with respect to the number of
serial engagements with the APC that they need to reach a similar threshold of
activation. There is some evidence that very stable peptide:class II complexes may
drive T cells into more rapid cell cycles, leading to a more rapid progression of these
T cells through their proliferative program relative to T cells specific for low stability
cryptic peptides (unpublished, J.M. Weaver and A.J. Sant, Chapter 4 Figure 4.6 and
(220)). Therefore, T cells recruited by very stable peptide:class II complexes may
have already reached a level of activation where only transient interactions with
antigen, class II molecules, or other activation molecules of the APC are adequate to
sustain expansion of these T cells.
It is also possible that the diminished CD4 T cell responses in the presence of competitors might be accounted for, at least in part, by a deviation from the classical T helper subsets. We have begun to address this issue and have found no IL-4, IL-5, or IL-10-producing T cell responses (data not shown). An alternative possibility from deviation of the CD4 T cells to produce a different cytokine from the type we have analyzed is that under competitive conditions, T cells specific for the low stability peptide proliferate and continue to expand in number but cease to produce cytokines altogether. The only method to examine this and to quantify antigen-reactive cells that are not secreting cytokines would be to use MHC class II:peptide tetramers. Just as with ELISpot analyses, there may be caveats to using tetramers that may perform well in the detection of antigen-specific CD4 T cells. The main concern is that they may react with only subsets of antigen specific cells, e.g. CD4 T cells with receptors of a certain threshold of affinity (253, 254), and again the total enumeration of elicited CD4 T cells would be underestimated.

Moreover, the possibility that the T cells elicited by low stability peptides may be inherently more sensitive to the normal regulatory mechanisms involved in the contraction of the immune response. For example, in light of recent literature on the mechanisms by which regulatory T cells inhibit T cell responses \textit{in vivo}, the role of Tregs in CD4 T cell competition should be considered here. It is clear that regulatory T cells are capable of suppressing the activation or proliferation of other T cells and by doing so, controlling homeostasis, autoimmunity, and the response to foreign
antigens (reviewed in (255, 256)). It is conceivable that Tregs may inhibit the responses to T cells of differing antigen specificity, by either directly suppressing actively proliferating cells, or by competing for access to the antigen-bearing cell surface, cytokines or other molecules around the local niche of the APC. Additionally, Tregs may down-modulate all T cells indirectly by interacting with the antigen-bearing cell (245, 246) and altering their phenotypes (257-261). Suppression may be most selective for T cells elicited by low stability cryptic determinants because of the diminished peptide:class II complexes density available to these T cells later in the immune response. Also, if high stability peptide:class II complexes deliver a qualitatively different signal to the responding T cell (220), then T cells elicited by low stability peptides may be selectively sensitive to negative regulatory mechanisms.

Although the primary focus of this Chapter has been to determine if the response to cryptic peptides is selectively lost during CD4 T cell responses to unrelated peptides, the persistence or sometimes enhancement of responses elicited by dominant peptides in the presence of bystander peptides is equally interesting. The mechanism(s) by which this occurs may be distinct from the loss in responses imparted to T cells elicited by cryptic determinants. There is evidence that CD4 T cells can increase the priming of other CD4 T cells. An effect thought to be mediated via the APC through upregulation of costimulatory molecules (262) or by paracrine cytokine production (144), both of which require their cognate antigen to be presented by the same APC.
Table 5.1 Kinetic stability of wild-type and variant peptide epitopes in association with I-A$^d$

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Sequence (Putative Register)</th>
<th>Dominant / Cryptic</th>
<th>$t_{1/2}$ (hr)</th>
<th>Anchor Changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>MalE [69-84] WT</td>
<td>GY AQSGLLAEITPDKA</td>
<td>Dom</td>
<td>62</td>
<td>P1,P4</td>
</tr>
<tr>
<td>Q72T-L75A</td>
<td>GY A TSGALAEITPDKA</td>
<td>Crypt</td>
<td>3</td>
<td>P1,P4</td>
</tr>
<tr>
<td>[102-115] WT</td>
<td>KLIAYPIAVEALSL</td>
<td>Dom</td>
<td>&gt;200</td>
<td></td>
</tr>
<tr>
<td>HEL [11-25] WT</td>
<td>AMKRHDNYRGYSL</td>
<td>Crypt</td>
<td>12</td>
<td>P1,P6,P9</td>
</tr>
<tr>
<td>R14Q-N19A-G22S</td>
<td>AMKQHGLDAYRSLYS</td>
<td>Dom</td>
<td>62</td>
<td>P1,P6,P9</td>
</tr>
<tr>
<td>OVA [327-339] WT</td>
<td>KHAHAEINEAGR</td>
<td>Crypt</td>
<td>1</td>
<td>P9</td>
</tr>
<tr>
<td>A339S</td>
<td>KHAHAEINESGR</td>
<td>Crypt</td>
<td>4</td>
<td>P9</td>
</tr>
<tr>
<td>A329M-A339S</td>
<td>KMAHAEINESGR</td>
<td>Crypt</td>
<td>14</td>
<td>P1,P9</td>
</tr>
<tr>
<td>A332I-I334V</td>
<td>KHAHIEVENAEGR</td>
<td>Dom</td>
<td>&gt;250</td>
<td>P4,P6</td>
</tr>
<tr>
<td>LACK [156-173] WT</td>
<td>SFGPSEHPIVVGSWD</td>
<td>Dom</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>I166A</td>
<td>SFGPSLEHPAVVGSWD</td>
<td>Crypt</td>
<td>1</td>
<td>P4</td>
</tr>
<tr>
<td>HA [126-138] WT</td>
<td>HNTNGVTAASSHHE</td>
<td>Crypt</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>T128V</td>
<td>HNVNGVTAASSHHE</td>
<td>Dom</td>
<td>160</td>
<td>P1</td>
</tr>
<tr>
<td>MYO[102-118] WT</td>
<td>KYLEFISEAAIHVLHSR</td>
<td>Dom</td>
<td>139</td>
<td></td>
</tr>
</tbody>
</table>

Half-lives of peptide: I-A$^d$ complexes were calculated from the time required to dissociate 50% of the FITC-labeled peptide initially bound to soluble I-A$^d$ at pH 7.4. The immunodominance of different peptide epitopes were determined from protein
immunizations and denoted as Dom or Cryp; respectively. Putative pocket residues for peptides are indicated in bold, and the mutations at pocket residues that modulate kinetic stability are indicated in bold italics. Lys in OVA[327-339] was substituted for Val to eliminate binding of an alternate register.

Figure 5.1 Expansion of T cells to low stability peptide:class II complexes is lost in the presence of ongoing responses to unrelated peptide antigens

Mice were immunized in the hind footpad with 25nmol of HEL[11-25] in an emulsion containing IFA/PBS and 0.6µg/mL LPS either alone (Panel A, open circles) or in a mixture containing 5nmol of each of the dominant peptides LACK[156-173], HA[126-138]T128V, and MalE[69-84] (Panel A, closed circles). In Panels B-E, mice were immunized with 25nmol of OVA[327-339], or 5nmol of higher stability
variants, OVA A339S, OVA A329M-A339S, and OVA A332I-I334V alone (open circles) or in a mixture containing dominant peptides (closed circles). Draining lymph nodes from individual mice were pooled, n=2 mice per group. Shown is the total number of IL-2 secreting antigen-specific cells at day 10 after restimulation with increasing doses of the test peptides or 5µM of competing dominant peptides (Panels A-E, right). The results are representative of between 2-7 independent experiments.

Figure 5.2 Similar patterns of T cell loss to low stability peptide:class II complexes when measuring IL-2 or IFNγ producers

Mice were immunized with 25nmol of OVA [327-339] (Panel A, top and bottom) or 5nmol of OVA A332I-I334V (Panel B, top and bottom) in an emulsion containing IFA/PBS and 0.6 µg/mL LPS alone (black) or as a mixture with 5nmol of the dominant peptides MalE [69-84], HA[126-138]T128V and MYO [102-118] (white).
Shown is the total number of CD4 T cells producing IL-2 (top) or IFNγ (bottom) when recalled to titrating doses of the test peptides or with 5µM of each competing dominant peptide. The results are representative of at least two independent experiments.

Figure 5.3  The persistence of peptide:class II ligand controls the ability of CD4 T cells to expand during responses to unrelated peptide antigens

Panel A, mice were immunized with the low stability peptides OVA [327-339], OVA A339S, OVA A329M-A339S, LACK [156-173]I166A, HEL[11-25], MalE [69-84]Q72T-L75A (white bars) or high stability peptides OVA A332I-I334V, LACK[156-173], HEL[11-25]R14Q-N19A-G22S, MalE[69-84], and MalE[102-115] (black bars) to assess the ability of these peptides to elicit T cell responses when immunized with a mixture of dominant peptides. The results are presented as the percent of the response gained or lost with competitor peptides in the immunization, relative to the peptide alone responses and are an average, from triplicate wells of IL-2 ELISpots of the indicated number of experiments ±SEM. Panel B, the percent loss or gain of T cell activation of the indicated peptides was quantified across experiments by comparing the total number of antigen-specific IL-2 responders from
test peptide immunized alone to test peptide immunized with competitors at the peak of the immune response. The percent of the response with competitors was then plotted against the $t_{1/2}$ in association with I-A<sub>d</sub> of the peptide: class II complex under study. The data points were subjected to Spearman’s non-parametric test, resulting in an $r^2$ value = 0.7818, p <0.0064 (significant correlation).

Figure 5.4  Co-administration of a low stability peptide with a single dominant peptide is sufficient to induce a los s in CD4 T cell expansion to the low stability peptide

BALB/c mice were immunized with 25nmol of MalE[69-84]Q72T-L75A in an emulsion containing IFA/PBS and 0.6 µg/mL LPS either alone (left, open circles) or included in a mixture containing 5nmol of the dominant peptide MalE [102-115] (left, closed squares), MalE [102-115] and MYO [102-118] (left, closed triangle), or MalE [102-115], MYO [102-118] and OVA[327-339]A332I-I334V (left, closed circles) in the hind footpad. The total number of IL-2 secreting antigen-specific cells at day 10 was determined using an IL-2 ELISpot assay and restimulated with increasing doses
of the test peptide or 5μM of competing dominant peptides (right). Draining lymph nodes from individual mice were pooled and there were 2 mice per group. The results are presented as the total number of responding T cells and are representative of three independent experiments ±SEM.

Figure 5.5  Systemic suppression does not account for the failure of low stability complexes to sustain CD4 T cell expansion

In Panel A, mice were immunized with 25nmol of OVA [327-339] (top) or 5nmol of OVA A332I-I334V (bottom) in an emulsion containing IFA/PBS and 0.6 μg/mL LPS alone (black) or as a mixture with 5nmol of the dominant peptides MalE [69-84], HA[126-138]T128V and MYO [102-118] (white). Alternatively, the test peptide was introduced in an emulsion alone in one footpad and the dominant peptides in the contralateral hind footpad (grey) and recalled to 5μM of the test peptide. Shown in
Panel B is the total number of CD4 T cells producing IL-2 with 5µM of each competing dominant peptide. The results are representative of four independent experiments ±SEM.

Figure 5.6 Competitive loss of responses to low stability complexes requires that the competitor and test peptides be introduced at the same immunization site

Shown in Panel A are the total number of IL-2 producing cells at the peak of the immune response after restimulation with 20µM of the test peptide when mice were immunized with 10µl of 25nmol of the low stability variant MalE[69-84]Q72T-L75A (top) or 5nmol of MalE[69-84] (bottom) in an emulsion containing IFA/PBS and 0.6µg/mL LPS alone (black), as a mixture with 5nmol of the dominant peptides MalE[102-115], OVA[327-339]A332I-I334V and MYO[102-118] (white), or the test peptide alone in one side of the footpad and the dominant peptides in the other side of the same hind footpad (grey). Panel B are the total number of IL-2-producing cells after restimulation with 5µM of competing dominant peptides. Draining lymph nodes from individual mice were pooled, n=2 mice per group. The results are an average of two independent experiments ±SEM. Three other independent experiments using
alternate test peptides also show similar results (data not shown). Results were analyzed with the one-way Students t-test, p-values are ** = p<0.005.

Figure 5.7 Loss in T cell responses to rapidly decaying peptides is not due to peptide competition for binding of class II molecules in vitro

In Panel A, HA [126-138] was tested for its ability to stimulate TS2 hybridoma cells using CD11c+ enriched splenocytes in the presence or absence of competing peptides. IL-2 production by TS2 alone (open circles), in the presence of 1 µM MalE [69-84], MalE [102-115], and MalE [269-284] (closed circles), HA [126-138] preincubated with APC for 30 min prior to the addition of dominant peptides (closed squares), or the dominant peptides preincubated with APC for 30 min prior to the addition of HA [126-138] (closed triangles) is shown as described in the Material and Methods. Results are presented as the Mean Optical Density from the secondary CTLL co-culture and are representative of two independent experiments ±SEM. In Panels B and C groups of mice were immunized with 5nmol HA[126-138] in the absence or presence of competitor peptides. In B, 24 hours later, some of the mice were injected IV with 5x10⁶ CFSE-labeled HNT TcR transgenic T cells. Forty eight hours later, the draining lymph nodes were harvested and proliferation determined by FACS (Panel B, left). The profiles were subjected to Modfit 3.0 analyses for the % of cells divided (Panel B, right). With the remaining mice, the total number of IL-2 secreting endogenous T cells at day 10 was determined using an ELISpot assay and restimulated with increasing doses of the test peptide (Panel C, left) or 5µM of
competing dominant peptides (Panel C, right). Draining lymph nodes from individual mice were pooled and there were 2 mice per group. The results are presented as the total number of responding T cells and are representative of two independent experiments ±SEM.

Figure 5.8 Attenuation of the T cell responses is not due to competition for peptide binding to MHC class II molecules

To assess competition for peptide binding in vivo, BALB/c mice were immunized with 25nmol of the low stability variant MalE[69-84]Q72T-L75A in an emulsion containing IFA/PBS and 0.6µg/mL LPS alone (A, open circles) or as a mixture with 5nmol of the dominant peptides that bind I-A^d HEL[11-25]R14Q-N19A-G22S and MalE[102-115] (A, closed circles) or as a mixture with 5nmol of the dominant peptides that bind I-E^d HA[107-120] and HEL[103-117] (A, closed squares) in the hind footpad with increasing doses of the test peptides. Shown in the Panel B are the CD4 T cell responses after restimulation with 20µM of competing dominant peptides. Draining lymph nodes from individual mice were pooled, n=2 mice per group. The results are presented as the total number of responding T cells and are an average of
two independent experiments ±SEM. Results were analyzed with the one-way Students t-test, p-values are *** = p<0.0005.

Figure 5.9 Abortive CD4 T cell expansion to low stability peptide:class II complexes

Mice were immunized in the ear with 10µl of 25nmol of MalE[69-84]Q72T-L75A in an emulsion containing IFA/PBS and 0.6µg/mL LPS alone or in a mixture with 5nmol of the dominant peptides MalE[102-115], OVA[327-339]A332I-I334V and MYO[102-118]. At days 3, 4, 5, 7, and 10, CD4 T cells purified by negative selection from deep cervical lymph nodes (Panels A and C) or unpurified ear pinnae tissue extract (Panel C and data not shown) were restimulated with 20µM of the test peptide in the presence of T cell depleted splenocytes in IL-2 (Panels A-C, squares) and IFNγ (Panel C, circles and data not shown) ELISpot assays. There was no antigen-specific T cell response detected at the peripheral site of immunization prior to day 7 (data not shown). Panel B shows the response of CD4 T cells purified from LN of mice immunized with IFA/LPS at the early time points for production of IL-2 when restimulated with 20µM of the test peptide. CD4 T cells enriched from the LN were plated at 300,000 cells/well and unpurified cell suspensions from the ear were plated at 0.5 ear equivalents/well. Draining lymph nodes from individual mice were pooled and there were 5 mice per group. The results are presented as the number of cytokine spots/300,000 T cells per pooled lymph node of individual mice from triplicate wells or as the number of cytokine spots/0.5 ear equivalents from triplicate
wells at each time point, with the mean number of spots indicated. Results were analyzed with the one-way Students t-test, p-values are * = p<0.05 and *** = p<0.0005.

CHAPTER 6
-------------------------

Development of a serum-free DC-based immunization system

6.1 Introduction

6.2 Results

Construction of a Flt3L-secreting cell line
Phenotype of in vivo expanded dendritic cells
Semi-quantification of peptide loading onto MHC class II molecules on the surface of DC using fluorescently conjugated peptides
Priming of naïve CD4 T cells by in vivo expanded dendritic cells

6.3 Discussion
6.1 Introduction

Cumulatively, our studies on immunodominance, particularly those involving peptide-based immunization regimens, have raised the issue of the role of the antigen-bearing cell in modulating the selectivity and magnitude of the endogenous T cell response to foreign antigens. In order to address the role of the APC in shaping immunodominance hierarchies *in vivo*, it would be tremendously advantageous to develop a dendritic cell (DC)-based immunization strategy.

Early attempts to generate DC used bone marrow-derived dendritic cells (BMDC) or splenic-derived dendritic cells that were cultured for extended periods of time in the presence of fetal calf serum (FCS), as had been previously published (263-269). The components of FCS contain known antigens of CD4 T cells and would therefore, seriously impact immune responses, similar to that observed during competitive T cell responses in Chapter 5. Consequently, the utilization of APC grown in the presence of FCS would not be useful in assessing the role of the antigen-bearing cell in immunodominance. In order to circumvent the use of FCS, we initially generated DC in serum-free conditions *in vitro*, in the presence of GM-CSF, IL-4, or both. The results of these experiments, and experiments purifying DC directly from spleen,
have led to a very low yield of CD11c+ cells that would not be adequate for the planned studies (J.M. Weaver and A.J. Sant, unpublished).

An alternative approach to produce a large number of DC, which has become more prevalent in recent years, is to preferentially expand DC in vivo. This model uses Fms-Like Tyrosine Kinase 3 Ligand (Flt3L) to expand hematopoietic precursors of DC in their natural environment (270-274). Previous studies have demonstrated that the total cellular composition of the spleen after treatment with Flt3L increases 3-4 fold in total cell numbers, with a selective increase in DC populations (~30%) without altering other committed cellular subsets, such as T cells, B cells, NK cells and macrophage (274, 275). This suggests that Flt3L is selectively expanding hematopoietic precursors of DC and not subverting or enhancing precursors to other committed lineages.

We adopted this experimental strategy to develop a DC-based immunization system that implemented these in vivo expanded DC that can be highly enriched, pulsed with peptide, and then reintroduced into mice, free of FCS. The gene for murine Flt3L was obtained and inserted into a new plasmid under the control of the human β-actin promoter. The Flt3L construct was then introduced into a tumor cell line, and the Flt3L-secreting tumor cells were selected for high secretion of Flt3L after subcloning by limiting dilution. When Flt3L-secreting tumor cells were injected into mice, there was a selective expansion of splenic-derived DC over time. We also demonstrate that
the *in vivo* expanded DC can be highly enriched, yielding large numbers, have peptides loaded onto class II molecules for display on the surface of the DC, and then introduced into mice to prime the endogenous pool of naïve CD4 T cells. All of this was achieved in the absence of FCS, as FCS responsive T cells were not detected. Collectively, we show that a serum-free DC-based immunization strategy can be used to assess the role of the antigen-bearing APC in focusing the T cell response to foreign antigens *in vivo*. 
6.2 Results

Construction of a Flt3L-secreting cell line

In order to determine the role that the APC is playing in competitive T cell responses to multiple peptides, a system to expand, purify and characterize dendritic cells for a DC-based priming regimen of CD4 T cells was generated. Previous reports have injected purified recombinant Flt3L to expand large cohorts of splenic-derived DC in vivo (276-279). Bone marrow (272) and tumor cell lines (270, 280) expressing Flt3L have also been used to obtain large numbers of DC. Presently, purified Flt3L is extremely costly, making it cost prohibitive for long-term use in the generation of DC in mice. The tumor line that has been constructed to secrete Flt3L that is currently utilized in vivo originates from B16-F10 melanoma cells that are syngeneic to C57Bl/6 mice (H-2\textsuperscript{b} MHC haplotype) (270). The majority of our studies on immunodominance of peptide epitopes in vivo have focused on utilizing BALB/c mice of the H-2\textsuperscript{d} MHC haplotype and peptide epitopes presented by the I-A\textsuperscript{d} class II molecule. Despite the MHC mismatch and potential for rejection, we did try to inject a large number of B16 melanoma cells into BALB/c mice to determine if the tumor could survive for sufficiently long periods of time to expand DC in vivo, as observed
by others (personal communication, Alexander Chervonsky, University of Chicago). Those efforts resulted in the rejection of the tumors in the MHC mismatched mice without any substantial expansion of DC (data not shown, J.M. Weaver and A.J. Sant). Therefore, the generation of a Flt3L-secreting tumor cell line capable of implantation into mice of the H-2^d MHC haplotype was paramount to the initiation of these studies.

The Flt3L-secreting tumor cells were constructed as described in detail in the Materials and Methods section. Briefly, the DNA encoding murine Flt3L was excised from the vector Flt3L-sfHAV-EO and inserted into pHβ Apr-1-neo (Figure 6.1) so that Flt3L could be expressed under the control of the human β-actin promoter. The resulting constructs were introduced into E. coli and the transformants were selected on agar plates containing ampicillin. Single colonies were chosen and tested for the presence and orientation of the insert by restriction enzyme digestion. Clone 4 was chosen and used for subsequent expression experiments.

We next transformed a weakly immunogenic tumor cell, Line 1, a murine lung carcinoma initially isolated from BALB/c mice (281), with the Flt3L-pHβ Apr-1-neo construct using Lipofectamine 2000. Line 1 has been previously used to over-express many cytokines, including granulocyte-macrophage colony stimulating factor (GM-CSF) in BALB/c mice (282). Our studies indicate that these cells only express H-2D^d, in agreement with previous findings (282), and do not express class II molecules
under *in vitro* conditions (Figure 6.2). The Line 1 cells, transfected with Flt3L-pHβ Apr-1-neo, were cloned by limiting dilution and initially screened for secretion of Flt3L cytokine by an ELISA assay. The highest expressing clone (clone 4.23) was further subcloned by limiting dilution to make a stable clone and again screened for Flt3L secretion. To test the stability of the subclones, several of the highest expressing subclones were passed in culture for 3-4 weeks. The subclones were then plated at 50,000 cells / 6-well plate and 2 days later the supernatants were harvested and tested for the secretion of Flt3L by ELISA. Shown are three different stable subclones, each expressing approximately 75 ng/ml Flt3L (Figure 6.3). Collectively, these results show that we were able to construct a tumor cell line that secretes the cytokine Flt3L, for the expansion of splenic-derived DC in mice of an H-2d MHC haplotype.

**Phenotype of *in vivo* expanded dendritic cells**

In order to determine if the Flt3L-secreting cells can expand splenic-derived DC *in vivo*, BALB/c mice were injected intradermally with 0.5-1x10⁶ Flt3L-secreting tumor cells in PBS into the left flank above the anatomical location of the spleen. The tumors were allowed to grow for 2.5-3 weeks, usually a size of 2-3 cm or until the animal was exhibiting discomfort. Both treated and untreated mice were sacrificed, the spleens excised, and dispersed into single cell suspensions through the use of collagenase D according to the manufacturers recommendations. Single cell
suspensions were depleted of red blood cells, and subjected to flow cytometry for analysis of cell surface associated phenotypic markers and other markers corresponding to populations of T cells, B cells, macrophage, and DC. Figure 6.4A shows there was a 3-4 fold increase in total cellularity of the spleen, a 10-15-fold increase in the percent of DC within the spleen (Figure 6.4B) and approximately a 40-fold increase in the total yield of DC after enrichment by positive selection using MACS beads when used according to the manufacturers recommendations, except for the substitution of MACS buffer with HBSS (Figure 6.4C). Substituting the MACS buffer with HBSS allowed for the purification and isolation of DC in the absence of FCS. The expanded DC were of a mixed phenotype, heterogeneous in expression of MHC class II, B220, CD11b, and CD8α (Figure 6.5A and data not shown, J. Tung and A.J. Sant, Rotation Report-Winter 2009, unpublished) and were capable of upregulating activation markers upon stimulation with LPS (Figure 6.5B). Further analyses of the overall cellular composition of the spleen, including CD4 and CD8 T cells, B cells, and macrophage indicated that the spleen cells were not different from the untreated spleen, as there were similar percentages and numbers of each population (data not shown, J. Tung and A.J. Sant, Rotation Report-Winter 2009, unpublished). These results demonstrate that the Flt3L-secreting tumor cells can stimulate DC expansion in vivo and these DC can be isolated at a high purity and number ex vivo.
Semi-quantification of peptide loading onto MHC class II molecules on the surface of DC using fluorescently conjugated peptides

One of the advantages in obtaining a highly purified population of DC for the priming of T cells is that it allows the opportunity to evaluate if the initial epitope densities of antigenic peptides (between low and high kinetic stability peptide:class II complexes) are equivalent prior to immunization. Our previous experiments to determine the initial epitope density on the priming APC measured IL-2 production after the activation of T cell hybridomas. In those studies, BMDC were pulsed with variable doses of either high or low kinetic stability peptides for less than 2 hours, washed and subsequently added to a 96-well plate containing T cell hybridomas reactive to OVA[323-339], in the absence or presence of titrating concentrations of αMHC class II antibodies in an in vitro assay (Chapter 4 Figure 4.3). The culture was incubated overnight and the presence of complexes was assessed by the production of cytokine. Because cytokine secretion is a downstream event of TcR stimulation, a more direct method to quantify peptide:MHC class II complexes on the surface of DC would be to exploit fluorescently conjugated peptides. Fluorescently conjugated peptides would allow for the direct assessment of peptide binding onto MHC class II
molecules immediately after pulsing a cohort of DC ex vivo, resulting in the quantification of the initial epitope density of the priming APC prior to immunization.

In this set of experiments, splenic-derived DC from Flt3L-treated mice were enriched for CD11c\(^+\) cells by positive selection using MACS beads as previously described. CD11c\(^+\) enriched DC were pulsed with increasing concentrations of low (used at 10-100\(\mu\)M) or high (used at 2-20\(\mu\)M) kinetic stability peptides in the presence of 0.6 \(\mu\)g/ml LPS for 1 hour. The results of these experiments reveal a dose dependent shift in the MFI of both high and low stability peptides when pulsed onto DC directly ex vivo (Figure 6.6). In addition, we used directly conjugated peptides to address whether the loading of a cryptic peptide was affected by the presence of known dominant peptides, MalE[102-115], MYO[102-117], and OVA[327-339]A332I-I334V, as competitors. Using this technique, we corroborated previous conclusions suggesting that the addition of dominant peptides does not alter the initial loading of cryptic or dominant peptides onto MHC class II molecules on the surface of the antigen-bearing APC (Chapter 5 Figures 5.7 and 5.9). Altogether, we can detect the loading of labeled peptides onto MHC class II molecules on the surface of an enriched population of DC ex vivo and the density of epitope displayed is not influenced by the presence of competitor peptides.

**Priming of naïve CD4 T cells by in vivo expanded dendritic cells**
We next asked if the peptide-pulsed DC were capable of priming naïve CD4 T cells upon transfer in vivo. Splenic-derived DC from Flt3L-treated mice were enriched for CD11c+ cells by positive selection using MACS beads, and then pulsed with 5μM of the high stability peptides MalE[69-84], MalE[102-115], MYO[102-117], and OVA[327-339]A332I-I334V in the presence of 0.6 μg/ml LPS for 1 hour. These dominant peptides were chosen because they have been previously used as competitor peptides in the experiments described in Chapter 5 and the magnitude and selectivity of the T cell response to these peptides is well characterized, allowing us to compare responses elicited by peptides delivered in an emulsion with those elicited via the peptide loaded DC. After a 1 hour pulse, all unbound peptide was removed by washing two times in HBSS. BALB/c mice were injected with 300,000 peptide-pulsed DC / 50μl in the hind footpad. Seven days later, the lymphocytes harvested from the draining lymph nodes were assessed for their ability to be restimulated to the different immunodominant peptides in an IL-2 ELISpot assay. Figure 6.7 demonstrates that multiple peptides can be loaded onto the enriched population of DC and can stimulate naïve CD4 T cells when they are introduced subcutaneously. There was also no T cell response detected upon restimulation with FCS (data not shown). In comparison, the response elicited by a single injection of peptide-pulsed DC was less efficient than that achieved with immunization with peptide emulsified in adjuvant / LPS, but was readily detectable. Recently, when others in the laboratory, under my guidance, have used this general approach to optimize the conditions of the DC-based immunization scheme, more efficient priming was observed (data not
shown, J. Tung and A.J. Sant, Rotation Report-Winter 2009, unpublished). We can conclude from these experiments that \textit{in vivo} expanded DC are capable of priming an endogenous pool of naïve CD4 T cells.

\section*{6.3 Discussion}

The primary goal of these studies was to develop a DC-based immunization system to prime CD4 T cells to a defined set of peptide antigens. This goal also required avoidance of methods that involve culturing DC with media containing heterologous serum. The results presented here indicate that a tumor cell line engineered to secrete Flt3L induces the selective expansion of DC \textit{in vivo}. Furthermore, the enriched DC population can be manipulated \textit{ex vivo} to present peptides at a known antigen density, a parameter that has been previously difficult to control. Lastly, the enriched DC can be peptide-pulsed and reintroduced into syngeneic mice to prime CD4 T cells. Taken together, these studies demonstrate that a tumor cell line engineered to secrete Flt3L can stimulate the \textit{in vivo} expansion of DC that are capable of priming naïve CD4 T cells upon subsequent transfer into syngeneic mice.

The studies presented in Chapters 4 and 5 have shown that the persistence of peptide:class II complexes on the surface of antigen-bearing cells is a major factor in determining the magnitude and selectivity of the T cells elicited when intracellular processing is bypassed. Peptides with rapid off-rates from MHC class II molecules were found to initially expand CD4 T cells, but failed to sustain T cell activation
throughout the full course of an immune response. In evaluating the possible mechanism(s) that could account for the selective loss in T cell expansion to low stability peptide:class II complexes, the events surrounding the antigen-bearing cells need to be considered. In order to begin addressing the possible mechanism(s) by which the APC may be playing a role in the apparent stall or aborted proliferation program by T cells recognizing unstable peptides, it would be very helpful to recapitulate the results obtained when using peptide emulsified in adjuvant.

In contrast to immunizing mice with a peptide emulsified in adjuvant, the inefficient priming of antigen-specific T cells obtained upon immunizing with a single cohort of DC loaded \textit{ex vivo} may have resulted from several factors in this protocol. First, the number of antigen-bearing DC may be suboptimal when DC immunization is used compared to peptide emulsified in IFA/LPS, where there may be multiple cohorts of DC migrating from the site of antigen introduction. We also do not know the efficiency of DC migration to the draining lymph node after subcutaneous introduction. Another factor that may lead to a lower number of antigen-specific responding T cells may be differences in the life span of the transferred DC compared to endogenously derived DC after a subcutaneous antigen/emulsion-based priming regime. In order to optimize the efficiency of the T cell response, a single injection of various numbers of DC, pulsed with various concentrations of peptide could be introduced and the kinetics of the CD4 T cell responses followed.
If an immunization regime can be optimized to reproduce our previous findings using peptide emulsified in adjuvant, some basic questions regarding the mechanism(s) by which T cell competition occurs can then be asked. First, is to formally address if the peptide immunogens are required to be presented by the same antigen-bearing cell for the loss in T cell responses to unstable peptides to be observed, as our previous findings suggest (Chapter 5 Figure 5.6). Mice could be immunized with low stability and dominant, competitor peptides pulsed onto the same or separate cohorts of DC, to assess if there is an aborted expansion of T cells responding to low stability peptides. Furthermore, if there is a stall in the T cell response to unstable peptides when both high and low stability peptides are loaded onto the same antigen-bearing cell, an additional cohort of DC pulsed with the low stability peptide alone could be introduced during the expansion phase to determine if those T cells can be reengaged and continue through the proliferation program. However, if competitive T cell responses are observed when immunizing mice with the low or high stability peptides onto separate DC, then the number of DC carrying a sufficient density of antigen may be limiting in conjunction with a T cell intrinsic mechanism. For example, the antigen-bearing cells and/or all of the T cells may be non-selectively suppressed by the antigen-specific expansion of Tregs to stable peptides.

Another important question to address is how the phenotype or the functionality of the APC may be changing during the evolution of the immune response to multiple peptide antigens. One method is to utilize a mouse model on the H-2d background
where CD11c+ cells, expressed by DC, can be tracked and selectively ablated. Mice, which are insensitive to diphtheria toxin (DT)(283), were engineered to express the diphtheria toxin receptor fused with eGFP under the control of CD11c (DTR-CD11c) (221). Upon administration of DT, the CD11c+ DC population is transiently depleted for 24 hours. Another advantage to using the DT-CD11c mice is that all CD11c+ cells will express GFP and can be easily identified from the endogenous DC after transfer. In future work, we will expand a cohort of DTR-CD11c DC with Flt3L that could be used to introduce antigen into mice and at different time points during the immune response all of the cells could be retrieved from the draining lymph nodes. Using GFP to identify only the DTR-CD11c DC, they can be assessed for the expression of MHC, costimulatory, or adhesion molecules and total DC numbers by flow cytometry. If there is a change in immunomodulatory molecules or the DC have been phenotypically altered and secrete suppressive cytokines, the DTR-CD11c DC can be functionally assessed for the range of cytokines secreted and the ability to prime naïve T cell hybridomas or TcR transgenic T cells in vitro in a reverse ELISpot.

A significant observation made in Chapter 5 was that when a low stability peptide was immunized alone or in the presence of three dominant peptides, there was no detectable difference in the expansion of T cells to the unstable peptide for the first 5 days of the immune response (Chapter 5 Figure 5.9). This observation in the kinetics of CD4 T cell responses provides strong evidence that DM editing is not detectably
altering the repertoire of peptides presented on the surface of the APC. However, we still need to formally address whether DM editing is playing a role in selecting the repertoire of peptides presented to T cells when peptides are loaded onto class II molecules on the surface of APC. To date, we have not been able to directly address this question using DM-/- mice because they have been suggested to have a difference in the repertoire of peripheral T cells in comparison to wild-type mice (170, 231). We can now get around the differences in the T cell repertoires between wild-type and DM-/- mice by using the DC priming regimen to formally address this issue. First, wild-type and DM-/- splenic-derived DC can be pulsed with fluorescently labeled low or high kinetic stability peptide alone or in the presence of competitor peptides. The kinetics of peptide dissociation from MHC class II molecules on the surface of the APC can then be performed \textit{in vitro} to determine if there is a change in the mean fluorescence intensity of the test peptides when in the presence of competitor peptides in comparison to peptide alone. Second, we can analyze the kinetics of the T cell responses can also be followed upon transfer of peptide-pulsed wild-type or DM-/- splenic-derived DC \textit{in vivo}.
Figure 6.1 Map of murine Flt3L inserted into pHβ Apr-1-neo

The purified fragment encoding the murine Flt3L was ligated into pHβ Apr-1-neo at the SalI site. This plasmid has ampicillin resistance (Amp R), G418 resistance (SV2-neo), and has the Human β-actin promoter to express the Flt3L.
Figure 6.2  MHC expression on Line 1 tumor cells *in vitro*

Line 1 tumor cells were pipetted into a single cell suspension and 500,000 cells / well were stained with MHC class I H-2D<sup>d</sup>-FITC (top, blue), and MHC class II, I-A<sup>d</sup>-FITC (bottom, blue) and I-A<sup>b</sup>-FITC (bottom, green) for 20 minutes at 4°C in the dark. Cells were washed three times and resuspended in 200µl FACS buffer. The labeled cells were analyzed by flow cytometry for the expression of each MHC molecule. Shown are the fluorescence intensities of each marker in comparison to no stain controls (red).
Figure 6.3  Secretion of Flt3L from tumor cells

Subclones from the parental clone 4.23, IE5.1, IE5.2, and IE5.3 were plated at 50,000 cells / well in a 6-well dish in media at 37°C for 48 hours. The supernatants were collected, frozen, thawed, and subjected to a Flt3L ELISA assay. Shown is the quantity of Flt3L in ng/ml secreted from each subclone.
Figure 6.4 Flt3L increases splenic-derived DC

Splenocytes were harvested 2-3 weeks after live Flt3L-secreting cells were injected. Shown are 4 representative spleen preparations. The total cell numbers from each spleen (A) and the percent of cells within that population expressing CD11c (B) prior to purification are shown. (C) After enrichment for CD11c+ cells using MACS purification, the typical purity was >90% (left) and the overall yield was approximately 40-fold more DC.
Figure 6.5 Flt3L generates a heterogeneous population of DC

Splenocytes were harvested 2-3 weeks after injection of live Flt3L-secreting tumor cells, enriched for CD11c<sup>+</sup>, and stained with I-A<sub>d</sub>, CD11b, B220, and CD8α (A). After culture with 0.6µg/ml LPS for 1 hour, the enriched DC were stained with CD40, CD80, and CD86 for the upregulation of maturation markers (B). Shown are histograms of each marker (red) in comparison to no stain control (blue).
Figure 6.6 Visualization of peptide loading onto in vivo expanded DC

CD11c⁺ enriched splenic dendritic cells from Flt3L treated mice were peptide-pulsed with increasing doses of the low stability variant MalE[69-84]Q72T-L75A-FITC or high stability peptide MalE[69-84]-FITC alone or in the presence of 5μM of MalE[102-115], MYO[102-117], and OVA[327-339] A332I-I334V for 1 hour at 37°C in HBSS. Unbound fluoresceinated peptide was removed by washing 2 times with HBSS. Peptide loading was analyzed by flow cytometry. Shown are the fluorescence intensities of FITC labeled peptide (blue) over the no stain control.
(green) alone (left column for each peptide) or in competition (right column for each peptide).

Figure 6.7 Flt3L expanded DC can prime CD4 T cells

CD11c+ enriched splenic dendritic cells from Flt3L treated mice were peptide-pulsed with 5µM of MalE[69-84], MalE[102-115], MYO[102-117], and OVA[327-339] A332I-I334V for 1 hour at 37°C in HBSS. Unbound peptide was removed by washing 2 times with HBSS. Mice were immunized in the hind footpad with 300,000 splenic DC and draining lymph nodes from 2 mice were pooled. Shown is the total number of IL-2 secreting antigen-specific cells at day 7 after restimulation with 20µM of the test peptides.
CHAPTER 7
-------------------------------

Thesis Discussion

7.1 Summary
7.2 Mechanisms of immunodominance
7.3 Immunodominance and the fate and effector function of CD4 T cells
7.4 Implications of selecting for persistent peptide:class II complexes
7.5 Application to rational vaccine design and immunotherapy
7.1 Summary

Prior to the initiation of this thesis, work within our laboratory demonstrated that immunodominance hierarchies in the peptide specificity of CD4 T cells could be predicted and controlled by peptide off-rates from MHC class II molecules. Further studies indicated that DM acts as a “peptide editor” to selectively remove unstable peptides from the class II molecule, thus enriching for presentation of peptides with stable interactions with class II at the cell surface. In the studies presented here, we have explored whether the immunodominance of CD4 T cell epitopes was absolutely “peptide intrinsic” or whether the molecular context plays an additional role in dictating immunodominance. We also evaluated whether peptide persistence on MHC class II molecules on the surface of antigen presenting cells after the initial epitope density is established is an important contributor in developing the final specificity of the CD4 T cell response.

Within this thesis we have progressively determined at what stages during an immune response very stable peptide:class II complexes have an advantage in elicitation of CD4 T cells over those of rapid off-rates from class II. At the level of intracellular processing, we found no detectable impact of the molecular context in which a
peptide is expressed on immunodominance hierarchies. When the events after antigen processing were assessed, our experiments revealed that the off-rates of peptides measured \textit{in vitro} are biologically significant and peptides that persist on class II have an advantage in eliciting and maintaining the activation of cognate CD4 T cells. Furthermore, our studies revealed that T cell expansion to low stability, but not high stability determinants, become aborted when introduced with other dominant peptides. The mechanism(s) by which the attenuated responses occur required at least one dominant peptide to be presented to T cells by the same antigen-bearing cell and manifests itself during the expansion phase of an immune response. Collectively, our findings suggest that the immune system has adopted multiple independent mechanisms to select a CD4 T cell repertoire that is focused on long-lived peptide: class II complexes during a response to invading pathogens.

7.2 Mechanisms of immunodominance

\textit{Classical view of immunodominance}

An important question is why the molecular context of a given peptide plays such a minor role in immunodominance as we have determined it experimentally. We have comprehensively evaluated the role of molecular context by moving peptides from their native protein into a new molecular context, with the same protease accessibility and competing peptides and have found no difference in their immunogenicity. In addition, increasing the availability of a cryptic peptide for presentation to CD4 T
cells on the surface of the APC by the addition of a dibasic motif flanking the cryptic peptide was insufficient to alter the immunodominance observed. Collectively, all of the experiments we have performed to directly address the role of molecular context have indicated that for exogenous foreign antigens, the site of localization of the peptide in the antigen, the sequences flanking the peptide and the actual protein carrier itself has minimal influences on the ability of that peptide to elicit CD4 T cells and promote expansion in vivo, but rather can be attributable to the biochemical properties of the peptide itself.

The peptide-intrinsic view of immunodominance

One key clue to the mechanism of competitive T cell responses was provided when the kinetics of T cell responses to a defined set of peptides was evaluated in peptide priming strategies. Peptides with a rapid decay from MHC class II molecules were able to initially expand CD4 T cells, but displayed abbreviated expansion during the evolving immune response. The initial activation and priming of T cells to unstable peptides was apparently unaffected by the presence of other heterologous peptides. We have not yet addressed if there is a functional consequence of the competitor peptides during the early kinetics that might eventually alter the fate of the T cells. On the contrary, the experiments performed thus far have identified the expansion of T cells to stable peptides to be temporally but not causally linked to the abbreviated activation program of T cells to unstable peptides (Figure 7.1). Therefore, in considering the potential mechanism(s) by which the failure in expansion of T cells
with specificity to low stability peptides occurs, the expansion phase of the T cell response will be the main focus of our discussion as this is the time point where the apparent stall in the activation program takes place.

One possibility to explain our results is related to the potential that there are time dependent changes in the APC that occur in the presence of competitor peptides. Since the antigen-bearing cell is a central component in initiating and maintaining the immune response, it may become limiting by losing some stimulatory capacity when multiple peptide determinants are eliciting robust T cell responses (Figure 7.2). As T cells proliferate in response to antigenic stimulus, there is a shift in the representation of effector T cells toward T cells of a higher affinity for peptide:class II complexes (132-137). Coincident with the ‘maturation’ of the T cell response, it is possible that T cells expressing high affinity receptors induce a loss in potency of the APC through trogocytosis (216-219), a process that occurs upon close contact of the APC and T cell, that can transfer peptide:class II complexes, costimulatory and adhesion molecules, or other plasma membrane proteins from the APC to the antigen-specific T cells. If such “membrane stealing” occurs, the loss in available peptide:MHC class II molecules over time will be compounded for low affinity peptides through the additional factor of peptide:class II dissociation. If the loss in peptide:class II complexes drops below a requisite density, it would create an unfavorable environment for T cells responding to unstable peptides because they may need to reengage their cognate ligand for optimal expansion (79, 80, 82).
Additionally, there may be differences in the number of serial engagements required to reach a threshold of activation between T cells with specificities for stable and unstable peptides. Recent data describes T cells interacting with cognate antigen on DC will pass through three distinct phases of activation (238, 243). The first phase occurs during the initial T cell encounter with cognate antigen on the surface of the DC and can last for 1-3 hours where brief interactions of 11-12 minutes are made (phase 1). Brief serial engagements between the T cell and the APC occur until a threshold of activation is reached, where T cells will then stop, and form long-lived 10-20 hour interactions over the next 3-16 hours (phase 2). Subsequently, T cells will receive sufficient signaling to promote the initial antigen-specific cell division and it is thought that only short-lived 10-20 minute interactions with antigen-bearing DC are required to continue to expand for several generations (phase 3). Therefore, the differential T cell expansion due to peptide dissociation and trogocytosis could be exacerbated as T cells responding to high stability peptides progress through phase 1 to phase 2 faster than low stability peptides (220), resulting in more rapid expansion and thus a larger number of effector cells. This hypothesis is supported by preliminary data using a limited number of peptides, the high stability peptides MalE[102-115], MalE[69-84], LACK[156-173] and low stability variants MalE[69-84]Q72T-L75A and LACK[156-173]I166A, showing that CD4 T cells elicited by dominant peptides are either recruited or expanded faster than T cells to cryptic peptides (Appendix Figure A.1).
Coincident with this kinetic model, there are several mechanisms by which regulatory T cells could be down-modulating T cells to unstable peptides during the expansion phase of an immune response to multiple peptide epitopes. It has been shown that regulatory T cells proliferate within the draining lymph node and accumulate at the site of infection at a rate similar to effector T cells (284). This could lead to the active suppression of all effector T cells either by directly suppressing T cells or by acting indirectly through the modulation of the APC. If Tregs are acting by modulating T cell responsiveness through cytokines or direct contact, both of which have been shown to inhibit IL-2 gene transcription in responding T cells (reviewed in (255)), it may lead to an apparent selective inhibition of T cells to unstable peptides. T cells responding to unstable peptides may not be as far into the activation program (220) or there may be a “sensitivity clock” where a threshold of activation may need to be reached in order to be resistant to the suppressive effects of Tregs (285). Additionally, the slower progression through the different phases of activation could contribute to the lag in expansion of effector T cells responding to unstable peptides, a consequence that would augment suppression.

In conjunction with this potential mechanism, we must consider the data showing that the strength of TcR signaling (286) and costimulation (287, 288) can modify the susceptibility of effector T cells to Treg-mediated suppression. If antigen is decaying rapidly from the APC, and the antigen-bearing cell is undergoing trogocytosis and
becoming less potent over time, then those effector T cells requiring additional antigen or cell surface-associated molecules on the APC late in the expansion phase would be at a disadvantage in being more susceptible to mechanisms of suppression.

Moreover, Treg-mediated suppression may be occurring through the modulation of the APC via several independent mechanisms (255, 256, 289). Potent immunosuppressive effects can occur when Tregs interact with costimulatory molecules (CD80 or CD86) and MHC class II molecules with CTLA-4 and LAG3, respectively, or by the secretion of immunosuppressive cytokines (IL-10 or TGF-β). Another possible intracellular change in the APC is through the induction of indoleamine 2,3-dioxygenase (IDO) in APC when CD80 or CD86 is ligated by CTLA-4 expressed by Tregs. These types of mechanisms are aimed at modulating the potency of or killing the APC, both of which render the immune response incapable of further progression. If the APC is modulated during the expansion phase of T cells to multiple peptide epitopes, then it may lead to an apparent selective inhibition of T cells to unstable peptides. T cells responding to unstable peptides may not be as far into the activation program and their slower progression through the different phases of activation could contribute to the lag in expansion of effector T cells elicited, resulting in a detectable aborted T cell response. Conversely, the large cohort of expanded T cells specific to stable peptides would appear unaffected by the decrease in APC capacity to prime.
The maturation signal received by the DC can play a role in secreting cytokines during the evolving immune response that may confer protection on the responding T cells. Cytokines produced by DC after maturation via toll-like receptors, such as LPS and CpG, may be secreted during the acute phase of the response and provide protection to the effector T cells primed during the first few days (290, 291). Evidence in the literature shows that APC transiently produce IL-6 and there is a concomitant down-regulation of the IL-6Rα chain on the surface of CD4 T cells during the same time period (292). This type of data suggests that protection from regulatory T cell-mediated suppression may be selectively conferred on T cells recruited into the immune response early, while those antigen-specific T cells expanding later, in the absence of IL-6, may be more vulnerable to suppression.

Another mechanism by which T cell expansion is modulated during the course of an immune response is by chemokine expression that can regulate both the cellular influx into and the cellular interactions within the draining lymph node. As described earlier, both migrating antigen-bearing DC and circulating CD4 T cells express CCR7 to interact primarily with CCL21 on HEV and lymphatic endothelium for entry into the secondary lymph nodes (42-44). Furthermore, initial encounters between T cells and DC may be promoted by the common attraction of the cells to CCL21-producing follicular reticular cells within the lymph node (293, 294). Interestingly, there can be a transient down-regulation of CCL21 in response to several pathogens, reducing the number of lymphocytes trafficking through the draining lymph nodes (295) and
possibly decreasing the interactions of antigen-bearing DC and T cells within the lymph nodes. Moreover, the expression of IFNγ was required for chemokine down-regulation, suggesting that activated macrophage, NK cells or T cells could initiate the modulation in CCL21 expression.

Our results indicate the T cells that are elicited by peptides emulsified in IFA containing LPS are Th1-like. Accordingly, the secretion of IFNγ from the effectors may initiate the modulation in the homeostatic expression of CCL21. Therefore, one can speculate the down-regulation of CCL21 might “close” the lymph node and inhibit potentially antigen-reactive T cells and migrating antigen-bearing DC from entering the draining lymph nodes (Figure 7.3). The “closing off” of the lymph node might be selectively detrimental to the response to low stability peptides for two reasons i) there is a dramatic loss in peptide:class II complexes on the APC within the lymph node due to the half life in association with class II as well as trogocytosis and ii) the potential for any newly immigrating antigen-bearing APC to migrate into / within the draining lymph node to provide antigen would decrease, stalling or aborting the progression of T cells through the activation program. Additionally, T cells to stable peptides would be unperturbed by this mechanism because their cognate peptide:class II complexes would persist, allowing for continued expansion of the T cells. Also, within the lymph node, if there is a concomitant down-regulation of CCL21 by FRC, then T cells scanning the lymph node microenvironment will be
less efficient in finding their cognate antigen and forming productive interactions with antigen-bearing APC.

An alternative explanation that may contribute to the apparent loss of antigen-specific T cell responses to unstable peptides in the presence of multiple determinants is that some of the antigen-reactive T cells are not being detected. It is possible that there may be a deviation from the classical T helper subsets, and that other T helper subsets (Tfh, Th17, or Treg) and their associated cytokines need to be considered and quantified. Other possibilities from a deviation of the CD4 T cells to produce an alternate cytokine other than the types we have analyzed is that under competitive priming conditions T cells specific for unstable peptides proliferate and continue to expand in number but cease to produce cytokines altogether, become anergic or die. A more comprehensive analysis of the final repertoire of T cells elicited under competitive conditions, that could be used in parallel with ELISpot analyses, would be to enumerate the total number of antigen-reactive T cells by peptide:MHC class II tetramers.

It is possible that any or all of these mechanism(s) may be interconnected or additive in causing a reduction in expansion of T cells responding to unstable peptides late in the evolving immune response. Although the causality between the aborted activation of T cells to unstable peptides with the expansion of other T cells may be
experimentally difficult to assess, these mechanisms we have discussed above can be experimentally addressed and should be the focus of future studies.

7.3 Immunodominance and the fate and effector function of CD4 T cells

A key observation from the studies presented herein is that CD4 T cells that are specific for rapidly decaying peptides from class II molecules are initially recruited into an immune response, but fail to progress further in the activation program by an apparent stall in proliferation when in the presence of other ongoing T cell responses to dominant peptides. Despite the fact that there is attenuation in the proliferation program, one relevant question remaining is to determine what is the fate of the T cells that are initially elicited by low stability peptides under conditions of competitive T cell priming. This is an important question to address not only in understanding how the immunodominance of CD4 T cell peptide epitopes is established but also because of the current use of polyepitope peptide vaccines. Accordingly, with the use of polyepitope peptide vaccines, many candidate peptides may fail to promote the full expansion and efficient effector function of CD4 T cells and it is unknown if the resultant CD4 T cells to those peptides helps or hinders future adaptive immune responses to invading pathogens. Therefore, identifying the fate of T cells responding to unstable peptides will enhance our understanding of the adaptive immune response at multiple levels.
One major difficulty in following the fate of T cells in vivo is being able to identify the antigen-specific CD4 T cells during the course of an immune response. If we utilized a model antigen system, such as cytochrome c, we could more easily track the fate of a population of T cells responding to several peptide variants of differing off-rates from class II molecules (149, 296). Additionally, this system offers the advantage in that the ‘maturation’ of the T cell response is well documented (132, 133, 135, 136), allowing for the direct assessment of the phenotype of the T cells over time by tracking distinct Vα and Vβ expressing TcR. Both of these factors would enable us to more efficiently track the evolution of the immune response to either a low or high stability peptide while in the presence of other peptides and gain insight into understanding the mechanism(s) controlling the magnitude, selectivity, and ultimately the fate of the responding population of CD4 T cells.

Moreover, to assess the generation of long-term adaptive immunity, studies to determine if T cells responding to unstable peptides are capable of establishing a memory population under competitive conditions are critical. While there is a decreased frequency of T cells elicited by low stability peptides when in the presence of competitor peptides after the primary response, it is possible that an efficient pool of memory cells is generated. However, if these cells remain in the peripheral pool of memory cells at homeostasis but are unable to mediate protection upon encounter with a pathogen, they may be detrimental to other cellular immune responses by
limiting the peripheral pool of functional memory cells by “taking up space”.
Understanding the capacity of these cells to facilitate memory T cell responses will
enable the implementation of better vaccination strategies aimed at eliciting the
desired immune response.

For example, with respect to vaccine development, the issue of how the magnitude
and selectivity of the primary CD4 T cell response reflects the immune response to
subsequent vaccination or pathogen encounters is important. If the number of
memory CD4 T cells is determined by the burst size of the primary T cell response to
antigen, then the immunodominance hierarchy established during the initial
expansion may dictate subsequent T cell responses. Thus, those very stable binding
peptides to MHC class II molecules eliciting the majority of T cells during the
primary response would also be highly represented and dominate the immune
response in successive antigen encounters. Furthermore, if the contraction rates are
the same for all T cells that are responding to either a high or low stability peptide
under competitive conditions, yielding disparate numbers at the end of the contraction
phase, then one would imagine the immunodominant hierarchy of CD4 T cells would
become progressively more pronounced upon successive antigen encounters.
However, if the contraction phase yielded equal numbers of memory cells
independent of the number of cells at the peak of the response, immunodominance
may still be observed because of the persistence of peptide:class II complexes during
antigen-driven expansion. T cells responding to high stability peptides progress
through phase 1 to phase 2 faster than low stability peptides (220), which would result in more rapid expansion and thus a larger number of effector T cells. Therefore, if the ability to form protective immunity is predicted by the initial burst size or quantity of antigen-reactive T cells during the peak of the primary response, then the results of these experiments conducted here would have strong implications on the design of both protein and peptide-based vaccinations. The stability of a peptide in association with MHC class II molecules would thus be considered to be the key parameter that dictates and controls the magnitude and selectivity of the primary CD4 T cell response.

However, memory formation may also be a function of the quality of the primary CD4 T cell response and not solely predicted by the magnitude of the effector T cells expanded (reviewed in (297)). During a primary T cell response to low stability peptides, effector T cells are generated, even under competitive conditions. The elicitation of effector T cells by low stability peptide:class II complexes may be sufficient to be above a threshold required to convert to and be maintained as a memory population. In this case, pre-primed T cells specific for low stability peptides would be effective in providing protective immunity.

Lastly, an essential feature of the peptide-intrinsic view of immunodominance is the capability to either stabilize or destabilize the association of a peptide with MHC class II molecules through modification of key amino acids in the peptide. Our
laboratory has a significant amount of data showing that by modulating the key amino acids of a peptide, we are able to enhance the stability of that peptide for class II and subsequently elicit predictable primary T cell responses (36, 150, 169). Future studies should be aimed at determining if the generation of a strong primary response from T cells responsive to a peptide of modified stability in its binding to MHC class II molecules can generate an effective memory population that would provide a memory response that will be protective in subsequent antigen encounters of the native pathogen. Studies such as these would be extremely helpful with respect to vaccine design, as conserved epitopes across different strains of a pathogen, such as Influenza, that are not normally recognized by T cells, could be engineered to be immunogenic and possibly confer heterosubtypic immunity.

7.4 Implications of selecting for persistent peptides: class II complexes

Multiple levels of controlling the T cell response to exogenous antigens have recently been described in the literature as well as in this study, where highly persistent peptide: class II complexes preferentially drive the selectivity in an adaptive immune response. In considering the events that might be influenced by the ability of a peptide to persist on MHC class II molecules, it is useful to review what is known about the steps involved in CD4 T cell responses initiated by peripheral introduction of antigen (Figure 7.4). At the site of infection or immunization, dendritic cells sampling the environment are triggered to mature in the presence of foreign antigens
and inflammatory signals, such as those contained in pathogens or adjuvants. Coincidentally, DC detach from the tissue site of antigen introduction and begin migrating to the lymph node. During intracellular processing, DM editing plays a critical role as the first level of control in acting as a “peptide editor”; differentially skewing the repertoire of expressed peptide:class II complexes to kinetically stable ones. It has been shown that DM editing is capable of skewing the epitope display over 100 to 1,000-fold in favor of high affinity peptides for its presenting MHC class II molecule (36). An additional level of control identified in this thesis is that peptides that bind stably to class II have an advantage over less stable peptides because they persist longer on the surface of the migrating APC than less stable peptides during transit; broadening the difference in epitope density initially created by DM editing. Once the APC has migrated to the draining lymph node, there is evidence suggesting the dynamics of CD4 T cell priming to stable binding peptides will have faster kinetics in the transition through phase 1 to phase 2 upon recognition of cognate antigen on an antigen-bearing APC (220), thus leading to a kinetic advantage in the expansion of T cells elicited by high stability peptide:class II complexes. Persistent peptide:class II complexes may then subsequently determine the fate of the responding CD4 T cells. Our studies show that those peptides that persist on the antigen-bearing APC expand a larger cohort of effector T cells than unstable peptides, and are the predominant effector cells within the lymph node and at the site of antigen introduction during the primary response. Delivery of effector function during pathogen-specific responses may be more efficient for CD4 T cells if
their TcR ligand on the target cells persists at high epitope density after pathogen protein synthesis has diminished, allowing for complete removal of pathogen-bearing cells. Also, engagement of CD4 T cells, particularly follicular helper T cells, with antigen-specific B cells in the germinal center may be most efficient for long-lived stable peptide:class II complexes. Recent studies suggest that follicular helper cells are sequestered with antigen-bearing APC for extended periods of time in the germinal center of lymph nodes during a T cell-dependent antibody response (298). These prolonged interactions between CD4 T cells with antigen-bearing B cells may be essential for the formation of a productive germinal center reaction (299).

However, we have not yet addressed if CD4 T cells specific for unstable peptide:class II complexes are useful for providing this help for immunoglobulin isotype switching and affinity maturation, as we have not yet examined all subsets of T cells generated. Another possibility is that persistence of a peptide on the class II molecule provides an advantage for stable CD4 memory generation and maintenance. Although it is known that antigen persistence is not required to generate memory cells (300-303), it is possible that continued opportunities to engage the CD4 T cells agonist ligand provides some advantage in the quantity or quality of memory T cells (251, 304-307).

Any or all of these activities of CD4 T cells may be most efficiently delivered if the ligand for the TcR persists for extended periods of time on the antigen-bearing APC. If so, then vaccination strategies should be targeting T cell responses to persistent peptides, as it may be counterproductive to initially prime and enrich for CD4 T cells
that are specific for low stability peptide:class II complexes because these CD4 T
cells may ultimately be less capable of providing effector function activity or
providing long term memory responses to pathogens.

7.5 Application to rational vaccine design and immunotherapy

The results of this study also have major implications from the practical standpoint of
vaccine design and immunotherapy. By understanding the forces that drive the
adaptive immune response, one can design better vaccines that will promote the
desired immune response. There have been significant recent advances in epitope
discovery that have allowed the identification of CD4 and CD8 peptide epitopes from
tumors, viruses, bacteria and other pathogenic organisms (308-315). Consequently,
there is increasing interest and promise in such strategies as multiepitope vaccines
(309, 312, 316-321) where candidate tumor-derived or pathogen-derived peptides of
interest are linked together to make recombinant multiepitope proteins. Such
strategies, frequently intended to target both CD4 and CD8 T cells, have been
developed to enhance responses to tumors and pathogenic organisms. These
advances in epitope discovery and vaccine design can now be coupled with our
increased understanding of peptide:MHC class II interactions (153, 309, 312, 322-
329) to modulate the type of immune response generated.
Also, there are major advances in the mode of peptide delivery (330-333), by identifying some of the critical proteins that mediate uptake of antigens in DC, such as DEC-205 and DC-SIGN. These advances have led to new efforts to target antigenic peptides to the DC by incorporating the peptides into antibodies that engage these cell surface proteins (331, 334-337). In targeting specific receptors of antigen acquisition on the surface of the DC, one can design better vaccines that would focus the responding population of T cells to those of the desired specificity and possibly effector phenotype. It was previously unclear whether polyepitope peptide vaccines would elicit predictable and immunogenic CD4 T cell responses. The results presented in this thesis suggest that many candidate peptide immunogens may fail to promote T cell priming if administered with other peptides. However, as we have shown here and in other studies (36, 150, 155, 156, 169), it may be possible to predict and rationally promote any peptide’s immunogenicity by optimizing the anchor residues that bind to the MHC class II pockets, thus allowing it to persist for extended periods of time on the priming APC. Encouraging results from our laboratory using human class II molecules in mice have shown the major epitopes elicited by vaccines were the same as those elicited by live influenza infection, consistent with our previous conclusion that immunodominance is a property intrinsic to the peptide itself (unpublished, K.A. Richards and A.J. Sant). Furthermore, preliminary analyses on the biochemical properties of the dominant influenza epitopes discovered suggest that these peptide epitopes, like those identified in our studies using experimental antigens, formed very long-lived complexes with the presenting human class II
molecule (unpublished, F.A. Chaves and A.J. Sant). Thus the natural epitopes that are the focus of the primary CD4 response to influenza virus fit the paradigm established through the use of experimental protein antigens. Therefore, such modified peptides can be incorporated into vaccines where the peptides will maintain their immunogenicity and expand host CD4 T cells of the desired specificity, while retaining recognition of the native sequence.
Figure 7.1  T cell expansion to dominant peptides is temporally linked to the reduction in responses to low stability peptides
Schematically shown is the expansion of T cells in response to high (blue) or low (green) stability peptide:class II complexes over time. T cells elicited by low stability peptides are truncated in their activation program (grey) during the expansion phase (red box) of the immune response under competitive conditions.

**Figure 7.2 Persistence of peptide:class II complexes during the evolution of an immune response**

Schematically shown are the number of peptide:class II complexes remaining on the surface of the antigen-bearing APC during the evolution of an immune response when
introduced as a single peptide immunogen in the presence of multiple peptides (blue or red) or when introduced as a single peptide immunogen in the presence of multiple peptides and being removed by trogocytosis (light blue or orange). Also indicated is the hypothesized requisite density of complexes necessary to prime CD4 T cells (dashed black).
Figure 7.3 Modulation in chemokine expression may attenuate T cell responses to low stability peptide: class II complexes

Early in the immune response the expression of CCL21 allows the entry of lymphocytes into and interaction within the draining lymph node (left). The modulation of the expression of CCL21 by the production of IFN\(\gamma\) may “close off” the lymph node for any further access by lymphocytes and decrease the efficiency of DC:T cell interactions within the lymph node (right).

Figure 7.4 Peptide persistence controls the evolution of a productive immune response at several levels

Multiple levels of control where persistent peptide:class II complexes preferentially drive the selectivity in an adaptive immune response. At the site of infection or immunization (1), DC sampling the environment are triggered to mature, detach from the tissue and begin migrating to the LN for presentation of peptides to CD4 T cells.
During intracellular processing, DM editing plays a critical role in acting as a “peptide editor”. Stable binding peptides have an advantage in being more readily able to prime cognate CD4 T cells once in the lymph node because they are more abundant, and persist on the surface of the migrating DC during transit. The dynamics of priming for stable binding peptides will have faster kinetics in the transition through phase 1 to phase 2. Persistent peptide:class II complexes determine the fate of the responding CD4 T cells, playing a role in a productive germinal center formation, to be the primary effectors migrating to the peripheral tissue site of antigen introduction and the formation of memory T cells.


and chaperoning: regulation of MHC class II peptide binding and expression.  


*Nat Immunol* 8:1041-1048.


different epitopes and predicts repertoire diversity and response magnitude.


84. Schaeffer, E. B., A. Sette, D. L. Johnson, M. C. Bekoff, J. A. Smith, H. M.
Grey, and S. Buus. 1989. Relative contribution of "determinant selection" and
"holes in the T-cell repertoire" to T-cell responses. *Proceedings of the

selection of immunodominant epitopes. *Critical Reviews in Immunology*
17:411-417.

86. Fairchild, P. J. 1999. Reversal of immunodominance among autoantigenic T-

87. Sercarz, E. E., P. V. Lehmann, A. Ametani, G. Benichou, A. Miller, and K.
*Annual Review of Immunology* 11:729-766.

processing in determinant selection by class II MHC and its relationship to

Immunodominance: a single amino acid substitution within an antigenic site
alters intramolecular selection of T cell determinants. *Journal of Immunology*
151:1852-1858.

90. Nikcevich, K. M., D. Kopielski, and A. Finnegan. 1994. Interference with the
binding of a naturally processed peptide to class II alters the


131. Vacchio, M. S., J. A. Berzofsky, U. Krzych, J. A. Smith, R. J. Hodes, and A. Finnegan. 1989. Sequences outside a minimal immunodominant site exert
negative effects on recognition by staphylococcal nuclease-specific T cell clones. *The Journal of Immunology* 143:2814-2819.


172. Arnold, P. Y., N. L. La Gruta, T. Miller, K. M. Vignali, P. S. Adams, D. L. Woodland, and D. A. Vignali. 2002. The majority of immunogenic epitopes generate CD4+ T cells that are dependent on MHC class II-bound peptide-

*Journal of Immunology* 169:739-749.


class II I-A(d) protein using two functionally distinct registers. *Biochemistry* 38:16663-16670.


of CD8(+) T cells by exogenous cell-associated antigens. *Immunity* 17:211-220.


271. Karsunky, H., M. Merad, A. Cozzio, I. L. Weissman, and M. G. Manz. 2003. Flt3 ligand regulates dendritic cell development from Flt3+ lymphoid and


Appendix

A.1 Kinetics of CD4 T cell expansion to low and high stability peptide:class II complexes
A.1 Kinetics of CD4 T cell expansion to low and high stability peptide:class II complexes

Faster kinetics of CD4 T cell expansion to long-lived peptide:class II complexes. (A) BALB/c mice were immunized in the ear pinnae with 25nmol of MalE[69-84]Q72T-L75A in an emulsion containing IFA/PBS and 0.6µg/mL LPS in a mixture containing 5nmol of each of the dominant peptides MalE[102-115], MYO[102-117], and OVA[327-339]A332I-I334V. Shown are the responses to Q72T-L75A and MalE[102-115] when recalled to 20µM peptide. In Panel B, mice were immunized with 25nmol of MalE[69-84]Q72T-L75A or 5nmol of MalE[69-84] alone (left) or 25nmol of LACK[156-173]I166A or 5nmol of higher stability LACK[156-173] alone (right). Draining lymph nodes from individual mice were pooled, n=2 mice per group. Shown is the total number of IL-2 secreting antigen-specific cells at each day after restimulation with 20µM of the test peptides.