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ABBREVIATIONS USED IN THIS THESIS

β_2M	β_2-Microglobulin
APC	Antigen Presenting Cell
APL	Altered Peptide Ligand
BCR	B Cell Receptor
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
CLIP	Class II-Associated Invariant Chain Peptide
CTL	Cytotoxic Lymphocytes
DN	Double Negative
DP	Double Positive
FCS	Fetal Calf Serum
fM	Formylated Methionine
FTOC	Fetal Thymic Organ Culture
HSC	Hematopoietic Stem Cell
mAb	Monoclonal Antibody
MHC	Major Histocompatibility Complex
MMTV	Mouse Mammary Tumor Virus
Mta	Maternally Transmitted Antigen
OVA	Ovalbumin
PBL	Peripheral Blood Lymphocytes
PCR	Polymerase Chain Reaction
RAG	Recombination Activating Gene
SP	Single Positive
TAP	Transporter Associated with Antigen Processing
TCR	T Cell Receptor
TCR_{trans}^{+}	TCR Transgenic
TN	Triple Negative

List of Publications

Irion, S., Berg, E. R., and Staerz, U. D. (2000). A Physiological Ligand of Positive Selection is Seen with High Specificity. J Immunol *164*, 4601-06.

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ZUSAMMENFASSUNG

Junge T-Lymphozyten durchlaufen 2 zentrale Selektionsschritte, bevor sie in den Pool der immunkompetenten Zellen aufgenommen werden. Im Thymus wird sichergestellt, dass sie Fremdeiweiße nur in Verbindung mit körpereigenem MHC (Haupthistokompatibilitätskomplex) erkennen, und dass sie fremde von körpereigenen Eiweißbruchstücken unterscheiden lernen. Der erste Prozess wird als Positive Selektion bezeichnet, wohingegen man den zweiten Schritt als Negative Selektion kennt. Für diese beiden Prozesse werden Selbst-MHC Moleküle sowie Selbstpeptide benötigt. Mehrere Arbeitsgruppen haben versucht, die Charakteristika des selektierenden Peptidliganden zu beschreiben. Bisher konnte keine eindeutige Aussage bezüglich der Rolle dieser Proteinbruchstücke getroffen werden, da eine zu große Zahl von Peptiden an ein bestimmtes MHC binden kann. Mit einem neuen transgenen Mausmodell wurde kürzlich eine Möglichkeit geschaffen, die Rolle von Selbstpeptiden bei der Selektion von T-Lymphozyten zu studieren. Im C10.4 TCR_{trans}^{+} Modell wird der transgene TCR vom nicht-klassischen MHC Molekül der Klasse Ib, H2-M3, in Verbindung mit dem physiologisch vorkommenden Peptid ND1 selektiert. Das Peptid ND1 entstammt dem mitochondrialen Genom und codiert für die NADH-Dehydrogenase Untereinheit 1.

Unter Zuhilfenahme eines Fötalen Thymus Organ Kultur Systems (FTOC) war es uns möglich zu zeigen, dass ND1 Positive Selektion in einem definierten, jedoch schmalen Fenster von Peptidkonzentrationen induzieren kann. Ich konnte zudem zeigen, dass das ND1 Peptid von reifen C10.4 TCR_{trans}^{+} T-Zellen als schwacher Agonist erkannt wird. Das *Listeria monocytogenes* Peptid hingegen ist ein starker Agonist für reife C10.4 TCR_{trans}^{+} T-Zellen. Auch dieses Peptid konnte die Positive Selektion induzieren, wenn auch bei signifikant niedrigerer Peptidkonzentration. Bei gesteigerter Peptidkonzentration und folglich gesteigerter Epitopdichte konnten beide Peptide nur partiell funktionelle T-Zellen selektieren. Ich schloss, dass erfolgreiche Positive Selektion nur von einem schmalen Aviditätsfenster getrieben wird.

Im folgenden untersuchte ich die Spezifität der Positiven Selektion im beschriebenen Modellsystem. Bei geringer Peptidkonzentration beeinträchtigte das Kürzen des ND1 Selbstpeptides sowie das Mutieren von oberflächenexponierten Seitenketten die Positive Selektion. Ich folgerte, dass in einer physiologischen Situation Positive Selektion von C10.4 TCR_{trans}^{+} T-Zellen hochspezifisch ist und eine geringe Epitopdichte zur Vorraussetzung hat.

Die Definition eines natürlich vorkommenden Peptides, welches einen T-Zell - Klon selektieren kann, und das Wissen um die Peptidanforderungen für eine produktive TCR – MHC - Interaktion, erlaubt es uns vorzustellen, wie spezifische T-Zellen *in vitro* oder *in vivo* generiert werden könnten. Die Generation von spezifischen T-Zellen wäre eine nutzbare Strategie in der Kontrolle viraler oder bakterieller Infektionen. Das Humane Immunodefizienz - Virus beispielweise attackiert selektiv T-Lymphozyten. Spezifische T-Zellen könnten hier Schutz und die finale Klärung der infizierten Zellen bringen oder die vernichteten Zellen könnten dem Körper wieder zugeführt werden. Des weiteren könnten tumorspezifische T-Zellen entartete Zellen aufspüren, die entweder Selbstpeptide überexprimieren oder Mutanten von Selbstpeptiden präsentieren.

Mit der Sequenz eines Selbstpeptides in der einen und dem aktivierenden pathogenen Peptid in der anderen Hand rückt die Vorhersage eines für die Selektion von T-Zellen verantwortlichen Sequenzmotivs in greifbare Nähe.

CHAPTER I

Introduction

The immune system is comprised of two general effector systems: An innate immunity and an adoptive immunity. The innate immunity is able to provide a quick and non-antigen specific protection. It is represented by body tissues, such as skin and mucosa, by nonspecific phagocytotic cells such as macrophages and by the complement system. The second effector system provides the body with adoptive immunity that is acquired over time and is able to mount a specific and efficient primary immune response, that improves towards a possible secondary exposure. Two main cell types, namely B and T lymphocytes, are responsible for providing specific immunity. The specific immune system has evolved and adapted over time so that mammalian species are well equipped to respond to a vast number of foreign antigens. Yet the potential pathogens have evolved at least over the same period of time. Therefore the immune system must be able to adapt to novel infectious challenges unique to a given individual. The goal must be to maintain a well-balanced equilibrium between the evolving new immunological challenges and the education of new effector cells.

B cells originate and mature in the bone marrow. They possess B cell antigen receptors (BCR) that are able to recognize foreign antigens. Those antigens are either circulating through the blood stream, lymphatic system or challenge the body via other surface exposed tissues like lung mucosa. In most cases a supporting T cell signal is required for B cell activation. Upon activation B cells mature into antibody producing plasma cells. Those clonotypic antibodies produced by plasma cells can directly label foreign proteins and make them susceptible for phagocytosis. Or they can initiate the complement cascade, able to destroy pathogens directly.

T cells, on the other hand, are also bone marrow derived; however they mature in the thymus. T cells possess a similar receptor to B cells, termed the T cell antigen receptor (TCR). Although, T cells are only able to recognize foreign

antigen that is presented in the context of self-major histocompatibility (MHC) proteins on the cell surface. As T cells mature in the thymus, they must be educated only to recognize peptides in the conjunction with those MHC molecules expressed in the given individual. The process of shaping the T cell repertoire to recognize foreign antigens only in the context of self-MHC molecules is termed positive selection. Foreign antigens, which can be recognized by T cells, include proteins from viruses, bacteria, other pathogens, neo-antigens that are expressed by various tumor cells; as well as antigens found on allogenic tissue transplants. However, on occasion T cells specific for pathogens or tumors are either silenced, not present in sufficient numbers or directly attacked by viruses, such as the human immunodeficiency virus (HIV), resulting in a lack of sufficient immune response of the individual.

The introduction of this inherent bias towards recognition of self-MHC molecules during positive selection can lead to the development of T cells with strong reactivity towards self-antigens. Therefore, ridding the immune system of T cells dangerous to self-tissue must also be achieved as thymocytes mature in the thymus. This process is referred to as negative selection. Failure to delete or inactivate these self-reactive T cells can lead to autoimmune diseases, such as multiple sclerosis or diabetes, caused by T cells reactive to self-tissues.

Understanding the mechanisms of T cell development will provide insight views into how autoreactive T cells develop. Furthermore, being able to induce development of specific T cells might be beneficial to individuals with cancers or patients with immunodeficiencies.

This thesis examines the physiology of murine T cell development in the thymus. As a model system, the development of a subset of T cells able to respond to the intracellular bacterium *Listeria monocytogenes* is analyzed. The particular aspects of T cell development that are addressed in this study include the nature of the self-peptide recognized during positive selection as well as the specific recognition of this peptide antigen by the TCR.

Alpha/Beta T Cell Development in the Thymus

Hematopoietic stem cells (HSC) capable of differentiating into T cells arise from the bone marrow. These precursor cells then migrate to the thymus via the blood stream to begin their final maturation. There they undergo positive and negative selection. A general, but simplified, transition scheme of maturing thymocytes has been determined based on the expression of the CD4 and CD8 co-receptors. Maturing thymocytes proceed from being $CD4^-$ $CD8^-$ double negative (DN) to $CD4^+$ $CD8^+$ double positive (DP) and finally to a single positive (SP) stage expressing either CD4 or CD8, but not both (Fowlkes et al., 1985; Scollay et al., 1988). The progression of thymocytes through the thymus begins with DP cells entering the outer rim of the thymic cortex and then moving inward towards the medulla as they mature into SP cells (Sprent and Webb, 1987).

The earliest pre-T cells express neither CD4 nor CD8 nor αβTCR. Those so-called triple negative cells (TN) are mostly identified by their $CD44^+CD25^-$ phenotype. As these TN cells become $CD44^-CD25^+$, they also begin rearranging the β chain of the TCR locus (Fink and Bevan, 1995; Fehling and von Boehmer, 1997; Spits et al., 1998). Rearrangement links together a variable ('V') gene segment with one of many diversity ('D') gene segments and a joining ('J') gene segments to create the variable region of the TCR β chain. TCR α chain rearrangement links together a 'V' gene segment and a 'J' gene segment to create the variable region of the TCR α chain gene. There is a plethora of gene segments of each variety that may be used in this process. Thus, the rearrangement process creates an vast number of different α and β chains. This is the genetic basis for the ability of the specific immune system to raise T cells against virtually any known and unknown antigen. It is estimated that T cells can rearrange between 10^6 and 10^9 distinct TCRs capable to recognize peptide antigens. After the TCR β locus is rearranged, the TCR β gene is transcribed, translated and shuttled to the cell surface (Grawunder et al., 1998).

At this point the maturing thymocytes undergo a process known as β selection. β selection is mediated by the newly synthesized TCR β chain paired with the preTCRα (pTα) chain. The invariant pTα is able to pair with any

functional TCR β chain expressed on the cell surface. This preTCR complex delivers a signal that is thought to be independent of ligand binding. The signal generated by the preTCR complex results in survival of pre-T cells and allows pre T-cells to continue their path to create a functional TCR αβ heterodimer. In addition, β selection suppresses rearrangements at the second TCR β locus so that T cells only express one TCR β polypeptide (Fehling and von Boehmer, 1997; von Boehmer and Fehling, 1997). Expression of low levels of both the CD4 and CD8 co-receptors is initiated at this stage. The markers used to identify early thymocytes, CD44 and CD25, are no longer expressed during thymic differentiation (Fink and Bevan, 1995; Fehling and von Boehmer, 1997; Spits et al., 1998).

Upon completion of the β selection process, thymocytes begin rearranging one of their TCR α genes. Expression of the rearranged TCR α gene facilitates the assembly of the TCR αβ protein with the CD3 complex (γ, δ and ε chains). Subsequently, this larger complex is transported to the cell surface. Thymocytes that express both the CD4 and CD8 co-receptors and a low, but functional level of the TCR complex, undergo two independent selection processes. These processes are designed to ensure that T cells only recognize foreign antigens that are presented by self-MHC (positive selection) and to eliminate or silence auto-aggressive T cells (negative selection). (Bevan, 1977; Zinkernagel et al., 1978; Kappler et al., 1987)

Positive Selection of Thymocytes

Positive selection ensures that developing thymocytes are self-MHC restricted. Therefore, they are able to respond to foreign antigens presented by self-MHC molecules found on the peripheral cells. The phenomenon of positive selection was discovered in 1977 using bone-marrow chimeric mice. When donor bone marrow with mixed MHC (AxB) haplotype was used to reconstitute a host with a single MHC (A) haplotype, then most of the T cell responses were restricted to the MHC-A haplotype (Bevan, 1977; Fink and Bevan, 1978; Zinkernagel et al., 1978). Further evidence of positive selection was provided with αβ TCR transgenic mice in which T cells only matured in the presence of

the original restriction element (Kisielow et al., 1988b; Sha et al., 1988b; Berg et al., 1989). Since αβ TCR rearrangement is a random process, the vast majority of thymocytes are unable to recognize self-MHC. They fail positive selection and die in the thymus.

The requirement for self-peptides in positive selection was initially suggested using variant MHC molecules (Berg et al., 1990; Jacobs et al., 1990; Nikolic-Zugic and Bevan, 1990; Sha et al., 1990). Reconstitution of Class I-deficient Fetal Thymic Organ Culture (FTOC) systems with peptides or peptide extracts also indicated that self-peptides were required for positive selection (Ashton-Rickardt et al., 1993; Hogquist et al., 1993). However, the nature of the self-peptide that drives positive selection has remained controversial. Investigators have postulated numerous theories and models to define a mechanism for positive selection. An explanation of each theory, as well as evidence to support or refute it, follows.

Antagonist Theory

T cells respond to cognate (antigenic) peptides presented by self-MHC molecules in the periphery. These cognate peptides induce agonist responses characterized by full activation of T cells. Variants of the antigenic peptide normally seen by a T cell are referred to as altered peptide ligands (APL). These altered peptide ligands can either be non-, partial or full agonist peptides. They result in no, partial or full activation of the T cell, respectively. It also has been proposed that some APLs can inhibit the response of the T cell to the antigenic peptide (antagonistic peptides). These antagonistic peptides are able to stimulate some, but not all of the responses generated by the antigenic peptide (Evavold and Allen, 1991; De Magistris et al., 1992; Jameson et al., 1993; Sloan-Lancaster et al., 1993). The reason for the incomplete responses to antagonist peptides is attributed to incomplete signaling (Kersh and Allen, 1996; Madrenas and Germain, 1996; La Face et al., 1997), and to lowered binding affinities of the TCR for an individual antagonist / MHC complex compared to an agonist / MHC complex (Alam et al., 1996; Lyons et al., 1996; Alam et al., 1999).

The original proposition of an antigen model of positive selection stated that qualitatively different responses are mediated by the TCR during positive selection and negative selection (Janeway et al., 1989; Mannie, 1991). These signals required for positive selection could be generated following interaction of the TCR with an antagonist peptide. Therefore, the antagonist theory of positive selection states that self-peptides that are able to antagonize the response of the mature T cell are responsible for positive selection in the thymus (Jameson et al., 1995). Data to support this theory was provided by Bevan's group using a TCR transgenic mouse model able to recognize a peptide derived from ovalbumin (OVA) presented by the Class I MHC molecule, H-2K^b. By breeding their TCR transgenic mouse with a β2-Microglobulin (β_2M) deficient mouse expressing very low levels of MHC Class I (Koller et al., 1990; Zijlstra et al., 1990), they were able to examine the requirement of peptides for positive selection of a defined TCR. Using a FTOC system, it was possible to study T cell selection in the presence of a single peptide added to the culture. The addition of antagonist peptides led to the positive selection of CD8$^+$ T cells (Hogquist et al., 1994). The cognate OVA-derived peptide was also able to induce phenotypic, but not functional positive selection of CD8$^+$ T cells. The inability of T cells selected on the cognate peptide to mount responses to the cognate peptide was attributed to a low level of CD8 expression on the post-selected T cells (Jameson et al., 1994; Hogquist et al., 1995).

In contrast to the above experiments designed to analyze CD8$^+$ T cell selection, other reports have suggested that antagonist peptides can prevent the positive selection of CD4$^+$ T cells (Spain et al., 1994), or actually induce negative selection (Page et al., 1994) in Class II restricted T cell transgenic mouse models. Furthermore, antagonist-like signals can select CD8$^+$ T cells regardless of the original T cell specificity. Volkmann et al. demonstrated that antagonist peptides were not able to select T cells into the CD4$^+$ lineage using a FTOC system with a MHC Class II restricted TCR. Instead, these MHC Class II restricted T cells expressed the CD8 co-receptor (Volkmann et al., 1998). Induction of CD8$^+$ T cells from immature T cells destined to become CD4$^+$ T cells was demonstrated by Basson et al. using a modified monoclonal antibody

(mAb) that delivered antagonist-like signals (Basson et al., 1998). These observations could be reconciled by a difference in the signaling requirements for generation of $CD4^+$ and $CD8^+$ T cells (Jameson et al., 1995; Kisielow and von Boehmer, 1995). Although the 'antagonist' theory is attractive, no positively selecting self-peptide with antagonist characteristics has been identified.

Differential Avidity Theory

The quantitative model of positive selection introduced by Sprent and co-workers suggested that low affinity interactions between the TCR and peptide / MHC complexes would promote positive selection, while high affinity interactions would lead to negative selection (Lo et al., 1986; Sprent and Webb, 1987; Sprent et al., 1988). A more recent version of this model factors both TCR and peptide / MHC complex density into the equation. The differential avidity theory states that the avidity (i.e. strength of TCR signaling combined with the number of TCRs engaged) controls positive and negative selection (Ashton-Rickardt and Tonegawa, 1994). If this were true, then very low concentrations of cognate peptide would be sufficient to drive positive selection, while higher concentrations of the same cognate peptide could induce negative selection. Furthermore, high concentrations of a relatively weak agonist peptide could induce positive selection.

Data to support the differential avidity theory came from two independent groups who showed that a cognate peptide that functioned as a strong agonist could induce positive selection of a defined MHC Class I restricted TCR specific for lymphocytic choriomeningitis virus (LCMV) presented by $H\text{-}2D^b$ (Ashton-Rickardt et al., 1994; Sebzda et al., 1994). For these studies, Ashton-Rickardt et al. established FTOCs using TCR transgenic mice that had been bred onto a Transporter Associated with Antigen Processing 1 (TAP1) deficient background (Van Kaer et al., 1992). These mice expressed very low levels of MHC Class I and were unable to mediate positive selection of $CD8^+$ T cells in the absence of exogenous peptide. The addition of cognate peptides with strong agonist characteristics resulted in positive selection of $CD8^+$ T cells. Similar studies were performed with FTOCs from a TCR transgenic, β_2M knockout mouse

(Koller et al., 1990; Zijlstra et al., 1990). Although the peptide concentrations required for both positive and negative selection differed in the two systems, the authors came to the same conclusions concerning how these processes were mediated. They believed that very low expression levels of a strong agonist peptide / MHC combination would induce positive selection in the thymus. In keeping with the differential avidity theory, the same agonist peptide that mediated positive selection could also induce negative selection when added at higher concentrations.

One caveat of the above experiments is that effector functions of thymocytes generated by interactions with the cognate peptide were not analyzed. Subsequent experiments demonstrated that the T cells arising from the FTOCs of transgenic TCRs, TAP1 deficient mice in the presence of the cognate peptide did not respond to the peptide after positive selection (Girao et al., 1997). The conclusion of these studies was that the T cells that developed in the presence of a strong agonist peptide, at any concentration, down-regulated expression of CD8. This down-regulation of the CD8 co-receptor rendered these T cells unable to respond to restimulation with peptide (a similar result to that obtained by Jameson et al. (1994)). In contrast, another group found that although CD8 levels were down-regulated, functional T cells could be generated during FTOC using LCMV specific TCR transgenic mice cultured in the presence of the cognate peptide (Chidgey and Boyd, 1997). However, Chidgey et al. used an *in vitro* suspension culture system to induce positive selection whereas the original positive selection studies described above used FTOC systems (Jameson et al., 1994; Girao et al., 1997). These different culturing conditions could potentially explain the discrepancy in the results.

Using the 2C TCR transgenic system that is selected on H-2K^{b} and alloreactive for H-2L^{d}, data were presented showing that very low levels of H-2L^{d} were able to mediate positive selection instead of negative selection (Cook et al., 1997). This indicated that the same ligand responsible for peripheral activation could induce positive selection when cell surface densities were sufficiently lowered. Further support for the differential avidity theory was provided by data from a transgenic mouse with a MHC Class II restricted TCR.

In FTOCs from this mouse, there was an inverse relationship between antigenicity of a peptide for peripheral T cells and the concentration of peptide required for positive selection (Wang et al., 1998). The differential avidity model has received considerable support from a number of experiments and one could easily imagine low-level expression of a self-peptide possessing agonist-like qualities. These peptides could then provide the stimulation required to positively select T cells. However, a recent report showed that as few as three agonist peptide / MHC complexes were able to induce negative selection (Peterson et al., 1999). This observation challenges the theory that strong agonists expressed at low levels can mediate positive selection.

Gemisch Model

A third theory to explain positive selection of thymocytes states that individual T cells can be selected on a 'gemisch' of different peptides (Bevan et al., 1994; Jameson et al., 1995). Due to the large number of peptides that are able to bind a given MHC molecule (Hunt et al., 1992), it seems plausible that more than one of these peptides could be involved in the positive selection of a given TCR. Furthermore, although TCRs are known to be very specific, some TCRs have been shown to be promiscuous in their recognition of peptides (Tallquist et al., 1996; Loftus et al., 1997; Boesteanu et al., 1998). If the TCR exhibits peptide promiscuity, then numerous peptides in the thymus may contribute to the overall signal resulting in the positive selection of a particular T cell.

Data to support this theory has been obtained from FTOCs of the 2C TCR transgenic, β_2M knockout mouse thymi (Pawlowski et al., 1996). In this system, the addition of many unrelated H-2K^b binding peptides to the FTOC was able to induce the maturation of 2C T cells. Some of the peptides were naturally occurring peptides; however, none of them had the ability to activate peripheral 2C T cells. The ability of selecting peptides delivered *in vivo* to the thymus to select functional T cells has also been examined (Nakano et al., 1997). Delivery of either the antigenic peptide itself, peptides related to the

antigenic peptide, or even unrelated peptides, was capable of positively selecting I-E^k restricted, moth cytochrome C specific T cells.

Another group presented data indicating that at least eight different self-peptides were able to interact with a MHC Class I restricted TCR (Hogquist et al., 1997). One of these self-peptides was shown to induce positive selection of TCR transgenic T cells in a FTOC system, albeit at a very high concentration. Therefore, the authors argued, approximately 12% of all peptides could induce positive selection of a given TCR. Using a similar approach, Hu et al. demonstrated that of three naturally occurring self-peptides, one was able to select T cells from a defined TCR transgenic, while the other two self-peptides could promote selection of a different set of TCR transgenic T cells. They estimated that between 33% and 66% of self-peptides could function to select T cells bearing a particular TCR (Hu et al., 1997). Thus, it seemed that high frequencies of self-peptides could contribute to the selection of a defined TCR. Due to the high concentrations needed to reveal increased positive selection in these systems, productive interactions with numerous peptides may be required for positive selection.

Except for the *in vivo* peptide delivery system, all of the above data supporting the 'gemisch' or 'promiscuity' model were generated with $CD8^+$ T cells restricted to Class I MHC. To analyze the positive selection of $CD4^+$ T cells restricted to MHC Class II molecules, mice that were able to express only a limited number of peptides were engineered. The H2-M molecule is implicated in the loading of antigenic peptides onto Class II MHC molecules (Denzin and Cresswell, 1995; Sherman et al., 1995; Sloan et al., 1995; Sanderson et al., 1996). In H2-M knockout mice, the vast majority of MHC Class II molecules are associated with the Class II-associated invariant chain peptide (CLIP) (Fung-Leung et al., 1996; Martin et al., 1996; Miyazaki et al., 1996). These H2-M knockout mice possess fairly broad repertoires of $CD4^+$ T cells, presumably selected on the I-A^b/CLIP complexes. It was noted that a large percentage of these $CD4^+$ T cells were reactive to Antigen Presenting Cells (APC) from mice with an intact H2-M gene suggesting that although the I-A^b/CLIP complexes were capable of positively selecting a broad repertoire of T cells, they were not

efficient at mediating negative selection. These findings were further described by a recent report. Singh and van Kaer show that the T cell repertoire of H2-DM^+ and H2-DM^- mice was remarkably different, thereby explaining the those unexpected reactivities (Sing and van Kaer, 1999).

More recent reports using these mice revealed that not all of the I-A^b complexes were loaded with the CLIP peptide. Furthermore, of five TCR transgenes examined, none were selected by the H2-M knockout background (Grubin et al., 1997; Surh et al., 1997; Tourne et al., 1997). More extensive analysis has revealed that the repertoire of T cells selected by the I-A^b/CLIP complexes is different than that selected by the natural set of self-peptides bound to I-A^b. A similar study used mice with a transgene encoding a modified invariant chain that has the CLIP peptide replaced with a peptide from the Eα chain of MHC Class II, bred onto the invariant chain knockout background. Although it was expected that only the Eα peptide would be presented by I-A^b in these mice, approximately five percent of the I-A^b was occupied by diverse peptides. Furthermore, these diverse complexes were shown to be responsible for positive selection of $CD4^+$ T cells (Barton and Rudensky, 1999). Thus, the investigators suggested that in H2-M knockout mice and single peptide / MHC mice (see below), Class II MHC molecules present a low abundance of self-peptides and the semi-diverse repertoire of $CD4^+$ T cells does not arise only from degenerate recognition of these ‘single-peptide / MHC’ complexes.

The other approach used to create mice with a single peptide / MHC complex was to generate transgenic mice that expressed a peptide covalently linked to a MHC Class II molecule. This was initially achieved using a peptide from the Eα MHC Class II molecule tethered to the I-A^b Class II MHC molecule (Ignatowicz et al., 1996a). This mouse was bred onto a MHC Class II knockout background, as well as the invariant chain knockout background, to ensure that all the peptide / MHC complexes were of the transgenic nature. These mice were subsequently termed A^bEpIi- denoting the MHC molecule, the transgene and the invariant chain knockout background. As with the H2-M knockout mice, the A^bEpIi- mice were able to select a fairly broad repertoire of $CD4^+$ T cells. The A^bEpIi- mice also contained numerous T cells that were reactive to

syngeneic APCs presenting normal self-peptides. The inability of the selecting peptide to antagonize responses of the peripheral T cells was in disagreement with the postulated 'antagonism theory' (see above) (Ignatowicz et al., 1996b). Furthermore, it was shown using the A^bEpIi- mice that the activating peptides were not necessarily related to the selecting peptide (Ignatowicz et al., 1997).

Providing direct evidence against the 'gemisch' model is difficult due to the vast number of peptides able to bind MHC Class Ia and MHC Class II molecules.

Non-interference Model

The non-interference model of positive selection states that suitable ligands for selection merely function to stabilize MHC expression. These peptides do not participate directly in positive selection and should not interfere with T cells contacting MHC molecules in the thymus (Schumacher and Ploegh, 1994). Data to support this model has been scarce, and in most experimental systems positive selection requires recognition of the bound peptide. However, the 2C TCR can be selected in FTOCs with a poly serine octamer peptide that should be mostly buried in the MHC molecule and may not provide for much contact between the peptide and the TCR (Pawlowski et al., 1996). In TAP1 knockout mice, the presence of a transgene encoding β_2M resulted in increased numbers of $CD8^+$ T cells compared to TAP1 knockout or β_2M knockout mice. This increase in $CD8^+$ T cells was attributed to an increase in expression of Class I MHC molecules that are either unoccupied or occupied with non-TAP dependent peptides (Martien van Santen et al., 1995).

Altered Peptide Hypothesis

The altered peptide hypothesis, originally postulated by Marrack and Kappler, stated that peptides unique to thymic epithelial cells would be responsible for imprinting self-MHC reactivity on T cells (Marrack and Kappler, 1987). If this were true, then T cells could avoid autoreactivity because they would no longer see the same self-peptide / MHC combination once they exited the thymus. Although attractive, this hypothesis has received little support. One

early study was able to show that the thymic cortical epithelium presented epitopes in a unique manner compared to other cells (Marrack et al., 1989). The exact nature of the difference in the epitopes expressed by different cell types was not determined. A subsequent study which showed that spleen and thymus present nearly identical MHC Class II bound peptides strongly refuted the altered peptide hypothesis (Marrack et al., 1993). Furthermore, thymic epithelial cells have been shown to induce negative selection (Speiser et al., 1992; Bonomo and Matzinger, 1993), and cell types other than thymic epithelial cells can mediate positive selection (Bix and Raulet, 1992; Hugo et al., 1993; Pawlowski et al., 1993). All of these studies strongly argue that although thymic epithelium may induce positive selection, specialized self-peptides presented by thymic epithelial cells are probably not responsible for positive selection.

Cell Types Involved in Positive Selection

It was initially shown that thymic epithelial cells were responsible for positive selection (Lo and Sprent, 1986; Ron et al., 1986). However, subsequent studies revealed that bone marrow derived cells could also induce positive selection of Class I restricted T cells (Bix and Raulet, 1992). Injection of different cell types into mice demonstrated that thymic epithelial cells (Hugo et al., 1992; Vukmanovic et al., 1992) and fibroblasts were able to mediate positive selection (Hugo et al., 1993; Pawlowski et al., 1993). Another study showed that expression of a Class II transgene exclusively on thymic cortical epithelium resulted in efficient positive selection, but inefficient negative selection (Laufer et al., 1996). A more recent report shows similar results in a MHC Class I restricted system (Capone et al., 2001). Conversely, in another transgenic model in which MHC Class II molecules were expressed exclusively on dendritic cells, there was no positive selection of $CD4^+$ T cells (Brocker et al., 1997). Taken together the above results suggest that cortical thymic epithelial cells are primarily responsible for positive selection, but that other cell types may play a minor role in this process.

CD4 and CD8 Co-receptor Involvement in Positive Selection

The CD4 and CD8 co-receptors are expressed on T cells and contribute to the TCR interaction with MHC Class II and Class I, respectively. These co-receptors increase binding of the T cell to the APC and amplify TCR generated signals. The roles of CD4 and CD8 in positive selection have been analyzed with CD4 and CD8 knockout mice. Although CD4 was required for generation of Class II restricted T helper cells (Rahemtulla et al., 1991; Killeen et al., 1993), CD4 knockout mice did possess a very small number of T cells that acted like $CD4^+$ T cells with the ability to mediate effector functions normally attributed to $CD4^+$ T cells (Locksley et al., 1993). Using mutant MHC Class II molecules, one group was able to show that CD4 interaction with $I\text{-}A^k$ was necessary for the development of most $CD4^+$ T cells (Gilfillan et al., 1998). Conversely, in another study the interaction of CD4 with $I\text{-}E^k$ was not necessary for development of TCR transgenic mice restricted to $I\text{-}E^k$ (Yelon et al., 1999). This data suggests that some MHC Class II restricted TCRs bear high enough affinity to induce positive selection without the need of CD4 or utilize another co-receptor to aid in positive selection. Mice that are deficient in either CD8α (Fung-Leung et al., 1991), or CD8β (Crooks and Littman, 1994; Nakayama et al., 1994) do not possess Class I restricted CTLs. Furthermore, the TCR and CD8 molecules must interact with the same MHC molecule in order to mediate positive selection (Aldrich et al., 1991; Killeen et al., 1992).

Other Molecules Involved in Positive Selection

Numerous molecules are involved in T cell / APC interaction. However, the importance of most of these molecules in positive selection is not known. The CD28 / B7 costimulation pathway, while important for T cell activation, is not important in positive selection (Freeman et al., 1993; Shahinian et al., 1993). Analysis of CD45 knockout mice indicated that CD45 was required for the development of T cells (Kishihara et al., 1993). Antibody mediated cross linking of CD2, CD5, CD24, CD28, CD49d, CD81, or TSA-1 with the TCR was

shown to augment positive selection (Cibotti et al., 1997). It should be noted that in this system, only $CD4^+$ T cells were generated, and that cross-linking of CD45, a molecule known to be involved in positive selection, had no effect.

Negative Selection in the Thymus

Positive selection skews the repertoire of T cells to recognize self-MHC molecules. However, this self-MHC recognition can lead to autoreactive T cells that damage organ tissue because of their high affinity for self-peptide / self-MHC complexes. Therefore, the process of negative selection has evolved to eradicate this dangerous resource of thymocytes and potential self-reactive T cells. Negative selection is a form of tolerance induction that occurs in the thymus. It can be achieved either by deleting or anergizing maturing T cells. Negative selection is induced by the interaction of immature T cells at the $CD4^+CD8^+$ stage with self-peptides presented by self-MHC in the thymus. This interaction, which is thought to be one of high affinity, rids the body of T cells reactive to self-proteins.

Clonal Deletion

Evidence that clonal deletion was responsible for eliminating T cells with specificities for self proteins was first shown in mice where T cells bearing certain TCR Vβ chains that were specific for self-MHC molecules were eliminated in the thymus (Kappler et al., 1987; Kappler et al., 1988; MacDonald et al., 1988b). Elimination of the thymocytes occurred before they matured into $CD4^+TCR^{hi}$ or $CD8^+TCR^{hi}$ cells. Consequently, the particular Vβ TCR was expressed only on immature $CD4^+CD8^+$ DP hymocytes. This self-MHC reactivity was later shown to be due to presentation by MHC Class II molecules, of a determinant derived from an endogenous mouse mammary tumor virus (MMTV) (Woodland et al., 1990; Acha-Orbea et al., 1991; Choi et al., 1991; Woodland et al., 1991; Blackman et al., 1992). Furthermore, by injecting neonatal mice with infectious MMTVs the same deletion phenomenon was demonstrated (Marrack et al., 1991).

Another approach to analyze T cell development was provided by TCR transgenic mice. A transgenic αβ TCR specific for a self-antigen from male mice was expressed only on a very few peripheral T cells in male transgenic mice, but was expressed on most T cells in female transgenic mice (Kisielow et al., 1988a). The transgene of another αβ TCR transgenic mouse (2C) was generated from a T cell clone that was alloreactive to H-2^d. In H-2^d mice, no peripheral transgenic 2C T cells were found (Sha et al., 1988a), whereas 2C mice on the H-2^b background contained a high frequency of transgenic T cells in the periphery. The absence of mature peripheral T cells in the deleting backgrounds was due to the elimination of immature thymocytes at the $CD4^+CD8^+$ stage. Furthermore, injection of antigen into neonatal mice also resulted in deletion of mature thymocytes in mice transgenic for a TCR reacting with an ovalbumin-derived epitope presented by the MHC Class II molecule, I-A^d (Murphy et al., 1990). Taken together, these studies showed conclusively that clonal deletion in the thymus is very important in eliminating self-reactive T cells.

Cell Types Involved in Clonal Deletion

Early studies using either transplantation models (Ready et al., 1984; Von Boehmer and Schubiger, 1984), or chimeric mice (Longo and Schwartz, 1980) implicated bone-marrow derived antigen presenting cells (APCs) as the primary cell type involved in clonal deletion. However, subsequent studies both *in vivo* and *in vitro* have demonstrated that other cell types, including lymphocytes and thymic epithelium, are able to induce tolerance via a deletional mechanism in the thymus (Hoffmann et al., 1992; Iwabuchi et al., 1992; Speiser et al., 1992; Bonomo and Matzinger, 1993; Tanaka et al., 1993; Oukka et al., 1996). Demonstration that the same cell line can induce either positive or negative selection ruled out the possibility that these two processes involve presentation by different cell types (Hugo et al., 1994; Vukmanovic et al., 1994a). The expression of MHC Class II molecules solely on thymic cortical epithelium results in inefficient negative selection. This finding indicated that deletion involved other cell types (Laufer et al., 1996). A similar study showed

that expression of MHC Class II molecules on dendritic cells resulted in efficient negative, but not positive, selection (Brocker et al., 1997). Therefore, negative selection may occur on numerous cell types as long as the interaction between the TCR and peptide / MHC complex is of sufficient affinity.

Clonal Inactivation

A role for clonal inactivation in the establishment of thymic T cell tolerance has come from studies monitoring T cells expressing certain Vβ TCR chains normally deleted in the presence of defined MMTVs (see above). It was demonstrated that in some systems, T cells expressing these Vβ TCR subunits could be found in the periphery (Ramsdell et al., 1989; Blackman et al., 1990; Roberts et al., 1990; Speiser et al., 1990). It was also shown that very low levels of antigen expressed in the thymus induced a non-deletional mechanism of tolerance that resulted in down-regulation of both the CD8 co-receptor and the TCR (Husbands et al., 1992). A later study using a TCR transgenic mouse showed that these 'anergic' T cells actually had the capacity to respond to the foreign antigen for which they were specific. Even though they were specifically non-responsive to the MMTV, they could respond to a higher affinity cognate antigen (Kawai and Ohashi, 1995). The authors postulated that very high affinity interactions in the thymus resulted in deletion, whereas lower affinity interactions resulted in a state of non-responsiveness to restimulation by the self-antigen.

Using FTOC systems, several investigators have shown that Class I restricted TCR transgenic T cells can be rendered non-responsive to the peptide upon which they were selected (Jameson et al., 1994; Hogquist et al., 1995; Sebzda et al., 1996; Girao et al., 1997). Down regulation of the CD8 co-receptor was suggested as the reason for the specific non-responsiveness in some of the systems (Jameson et al., 1994; Girao et al., 1997).

Cell Types Involved in Clonal Inactivation

If clonal inactivation were indeed due to a lowered affinity / avidity interaction between the thymocyte and the tolerizing cell, then a likely candidate to induce this type of negative selection would be a cell type that does not possess strong co-stimulatory capabilities. In support of this, several groups reported that non-deletional self-tolerance is controlled by radioresistant elements within the thymic stroma (non bone-marrow derived APCs) (Ramsdell et al., 1989; Roberts et al., 1990; Speiser et al., 1990). In contrast, it has been shown that a cell line that does not exhibit co-stimulatory activity can induce thymic deletion (Vukmanovic et al., 1994a; Vukmanovic et al., 1994b). Other investigators suggest that epithelial cells in the thymic medulla mediate clonal inactivation (Hoffmann et al., 1992; Schonrich et al., 1992) as well as deletion (see above) of specific thymocytes.

CD4 and CD8 Co-receptor Involvement in Negative Selection

Evidence that the CD8 co-receptor is involved in negative selection comes from several groups who utilized different experimental approaches. Mutant MHC Class I molecules that were unable to bind CD8 are inefficient in the negative selection of Class I restricted T cells in TCR transgenic mice or in normal mice (Aldrich et al., 1991; Ingold et al., 1991; Killeen et al., 1992). Furthermore, TCR transgenic T cells that were normally positively selected could be deleted if they expressed higher levels of CD8 (Lee et al., 1992; Robey et al., 1992). Both sets of experiments presented strong evidence that CD8 is necessary for negative selection. However, some TCR transgenic lines may not be suited for this analysis because they are efficiently deleted in CD8 knockout mice (Fung-Leung et al., 1993). Thus, T cells bearing TCRs that bind self-peptide / MHC complexes with very high affinity may not require a CD8 / MHC Class I interaction for negative selection. Conversely, the deletion of T cells with lower affinity TCRs requires the CD8 / MHC Class I interaction.

A role for the CD4 co-receptor in negative selection was initially suggested by the observation that antibodies to CD4 inhibited the deletion of self-reactive T cells (Fowlkes et al., 1988; MacDonald et al., 1988a). In contrast, CD4 is not required for the deletion of MMTV reactive T cells (Wallace et al., 1992). Furthermore, in mice expressing a transgenic MHC Class II molecule that had been mutated so that it could not bind CD4, negative selection seemed to be intact (Gilfillan et al., 1998). These data would indicate that the role of the CD4 co-receptor is not as important as the role of the CD8 co-receptor in negative selection of MHC Class II restricted and MHC Class I restricted T cells, respectively.

Other Molecules Involved in Negative Selection

In order for peripheral T cells to be activated, a second signal must be provided. Generally, the CD28 receptor on T cells provides this signal. If deletion of immature thymocytes resembles activation in the periphery, then it would be reasonable to assume that a second signal was required for clonal deletion. Using an *in vitro* culture system, one group has provided data indicating that a second signal is needed, but that CD28 is not the molecule delivering this second signal (Page et al., 1993). Indeed, CD28 knockout mice do not show any perturbation in selection processes (Shahinian et al., 1993) and injection of a high affinity soluble CTLA4-Ig molecule (which binds B7 molecules and thus blocks CD28 signaling) does not inhibit negative selection (Jones et al., 1993). In contrast, another group found that the co-stimulatory signal provided by CD28 was required for negative selection (Punt et al., 1994). Furthermore, APCs transfected with B7-1 (a ligand for CD28) can augment negative selection (Kishimoto et al., 1996). The only real conclusion to be drawn from the above data is that different experimental systems have differing requirements for the induction of negative selection.

Other cell surface molecules implicated in negative selection include CD5 and CD43 (Kishimoto and Sprent, 1999), CD30 (Amakawa et al., 1996) and CD45 (Ong et al., 1994). There is also conflicting data as to whether ICAM

expression by APCs affects negative selection (Carlow et al., 1992; Kishimoto et al., 1996). A limited role for the Fas / FasL interaction in negative selection of small numbers of immature thymocytes has also been suggested (Castro et al., 1996; Kishimoto et al., 1998). It seems as though deciphering the exact role of cell surface molecules in deletion of a T cell that expresses a particular TCR is a very difficult task.

The Experimental System

The Non-Classical MHC Class Ib Molecule, H2-M3

The major histocompatibility gene complex (MHC) encodes two 'Classes' of MHC molecules. MHC Class II molecules are expressed primarily by professional APCs and are responsible for displaying peptides from soluble proteins (either self or foreign) found in the extracellular milieu. Murine MHC Class I molecules are expressed by almost all cells and present cytoplasmic peptides derived from self proteins or from foreign pathogens. MHC Class I molecules are further divided into the polymorphic Classical Class Ia molecules and the rather non-polymorphic, non-classical Class Ib molecules. The remainder of the introduction concerning MHC molecules will focus on Class I molecules and more specifically, on H2-M3, a MHC Class Ib molecule that functions as the restricting element of the C10.4 TCR_{trans}^{+} used in this study.

Discovery of H2-M3

H2-M3, like other MHC Class I proteins, is composed of a heavy chain containing a peptide binding groove and an associated light chain, β2-Microglobulin (β_2M). T cells specific for peptides (both self and foreign) presented by H2-M3 are $CD8^{+}$ and have cytolytic functions. The initial observation that eventually led to the discovery of H2-M3 occurred in 1980 when the maternally transmitted antigen of mice (Mta) was described as a target for CTLs that were not H-2 restricted (Fischer Lindahl et al., 1980). These CTLs were shown to be dependent on the maternally transmitted factor (Mtf) as well as a gene product termed HMT that was located distal of the MHC gene complex (Fischer Lindahl et al., 1983). The identification of the NADH dehydrogenase subunit 1 (ND1) gene as the molecule giving rise to Mtf was demonstrated by two independent groups in 1990 (Loveland et al., 1990; Shawar et al., 1990). A polymorphism in position six of the ND1 protein encoded the Mtf and was responsible for the CTL response that was not H-2 restricted. It was subsequently demonstrated that the restricting molecule was

encoded by the MHC Class Ib gene, H2-M3 (Wang et al., 1991). Within the majority of the common strains of mice that express H2-M3, there is very little polymorphism of this gene. Of particular interest appeared one strain of mice, *Mus musculus castaneus*, containing the *cas* allele of H2-M3. Immunologically, it behaves as a *null allele* that is unable to present peptides to any of the known H2-M3 restricted T cells. Several other alleles have been described, yet their experimental use was limited (Fischer Lindahl et al., 1997).

Requirement of Formyl Methionine for Peptide Binding to H2-M3

As discussed above, the ND1 gene was shown to give rise to the Mtf. The peptide from ND1 that sensitizes targets for lysis is unique in that its N-terminus contains a formyl-Methionine (fM). Using peptide competition assays, it was demonstrated that only synthetic peptides that contained fM could compete with Mtf for binding to H2-M3 (Shawar et al., 1990; Vyas et al., 1992). It was subsequently shown that the unformylated version of ND1 required approximately 10^4-fold more peptide in order to sensitize targets for lysis (Smith et al., 1994). However, a recent report indicated that the *in vitro* addition of a nonformylated viral epitope induced CTLs restricted to H2-M3 (Byers and Fischer Lindahl, 1998).

Presentation of Self-Peptides by H2-M3

There are four known forms of ND1. They are all immunogenic in mice when the proper strain combinations are used (Fischer Lindahl et al., 1997). The presentation of ND1-like peptides containing the different polymorphisms in the sixth residue results in a minor histocompatibility response restricted to H2-M3. Also a polymorphism in the COI gene of the mitochondria has been shown to induce a minor histocompatibility response in mice (Morse et al., 1996). An alternative role for self-peptides in H2-M3 restricted responses was suggested by the observation that alloreactive responses against H2-M3 could be

generated and that these CTLs had specific requirements for peptides that contained fM (Dabhi et al., 1998; Dabhi and Lindahl, 1998).

Presentation of Listeria Monocytogenes Antigens by H2-M3

Early studies showed that $CD8^+$ T cells from mice immunized with *L. monocytogenes* were able to confer MHC unrestricted immunity in recipient mice (Kaufmann et al., 1988; Lukacs and Kurlander, 1989). In 1992, two independent groups demonstrated that fM-peptides from *L. monocytogenes* were presented by H2-M3 for recognition by $CD8^+$ T cells (Kurlander et al., 1992; Pamer et al., 1992). Subsequent studies defined three precise amino acid sequences of hydrophobic fM containing peptides derived from *L. monocytogenes* that were presented by H2-M3 (Gulden et al., 1996; Lenz et al., 1996; Princiotta et al., 1998). The LemA peptide (fMIGWII) was shown to be derived from a transmembrane protein oriented with its N-terminus outside of the cell (Lenz et al., 1996). The N-terminal sequence of another fM-peptide (fMIVIL) from *L. monocytogenes* was determined using biochemical methods. Therefore the identity of the intact protein is not known (Gulden et al., 1996). The AttM-derived fM-peptide (fMIVTLFYSA) is encoded by a putative leader peptide that may function as a transcriptional attenuator (Princiotta et al., 1998).

Expression of H2-M3

The development of a monoclonal antibody specific for H2-M3 has enabled the analysis of the expression levels and patterns of H2-M3. The expression of H2-M3 on cells *in vitro* is very low due to the paucity of self-peptides containing fM (Chiu et al., 1999) and the low level of H2-M3 gene expression (Wang and Lindahl, 1993). Similarly, in the thymus, the basal levels of H2-M3 are undetectable with the monoclonal antibody. Only peripheral lymphoid organs show any basal surface expression of H2-M3. *In vitro*, H2-M3 expression can only be up regulated by fM containing peptides from *L. monocytogenes* or the two highest affinity self fM-peptides (ND1 and COI). The

authors in the above manuscript believe that H2-M3 is stored in an intracellular site in a peptide receptive state that can then traffic to the cell surface upon infection with bacteria such as *L. monocytogenes.*

Importance of H2-M3 Following Bacterial Infection

Upon infection with *L. monocytogenes*, MHC Class Ia knockout mouse (H2-$K^{b-/-}$ $D^{b-/-}$) are able to mount $CD8^+$ T cell responses restricted to two non-classical MHC Class Ib molecules, Qa-1^b and H2-M3 (Seaman et al., 1999). The antigenic epitopes recognized by these T cells include the three fM containing peptides from *L. monocytogenes* described above. Another study made use of an H2-M3 tetramer complexed with a known fM containing peptide from *L. monocytogenes* to examine the relative contribution of H2-M3 restricted T cell responses to *L. monocytogenes*. In normal mice infected with *L. monocytogenes,* H2-M3 restricted T cell responses are generated more rapidly than MHC Class Ia restricted T cell responses. However, upon re-challenge with *L. monocytogenes*, MHC Class Ia restricted responses are more prevalent than the H2-M3 restricted response (Kerksiek et al., 1999). The ability of MHC Class Ia knockout mice to clear *L. monocytogenes* infections and the rapid and strong H2-M3 restricted T cell response to *L. monocytogenes* demonstrate that Class Ib MHC molecules and T cell responses restricted to them are important for immunity to the bacterium.

The C10.4 TCR_{trans}^+ Mouse

The TCR transgenic mouse used in this thesis was established by Rance Berg, a graduate student in our lab. The C10.4 T cell is restricted to H2-M3 and is positively selected on a self-peptide derived from the NADH dehydrogenase subunit 1 (ND1). Addition of the 9 amino acid long version (ND1/9mer) to fetal thymic organ cultures FTOCs gives rise to functional $CD8^+$ T cells (Berg et al., 1999b). Further information is provided in chapters II and III.

Experimental Rationale

Earlier studies used cognate peptides or their variants to probe T cell development (Ashton-Rickardt et al., 1994; Hogquist et al., 1994; Sebzda et al., 1994), because physiological ligands of positive selection had not been defined. Different experimental systems lead to different hypotheses of positive selection. The ‘antagonism’ theory postulated that T cells were positively selected on peptides antagonistic to the cognate antigen (Hogquist et al., 1994; Jameson et al., 1994). The ‘differential avidity’ hypothesis suggested that positive selection depended on low avidity interactions between the TCR and the MHC / peptide complex (Ashton-Rickardt et al., 1994). If one assumes that TCR-MHC / peptides interactions by themselves were of low affinity, this theory could further explain how positive selection was promiscuous (Pawlowski et al., 1996), or even independent of the recognition of a specific peptide (Schumacher and Ploegh, 1994). The ‘differential avidity’ hypothesis also predicted that a low TCR affinity could be compensated for by large numbers of MHC / peptide epitopes. Thus, positive selection of immature T cells could be induced by a 'gemisch' of self-peptides rather than a specific antigen (Bevan et al., 1994; Jameson et al., 1995). However, recent experiments did not support this last conclusion. They suggested that positive selection of T cells was a specific interaction that occurred on low abundance self-peptides (Barton and Rudensky, 1999). Due to the nature of the used ligands it has been extremely difficult to answer the questions addressed in this thesis. We came to the conclusion that the question of specificity of positive selection can be best addressed in a model system in which the physiological ligand of positive selection is known.

Our lab recently produced a TCR transgenic (TCR_{trans}^{+}) mouse that has unique characteristics and the physiological ligand of positive selection has been defined (Berg et al., 1999b). Our lab had chosen this system because H2-M3 molecules preferentially bind peptides that carry a fM in the N-terminal position (Loveland et al., 1990; Shawar et al., 1990). In the mouse, only thirteen mitochondrial genes give rise to fM peptides (Lindahl et al., 1997). This limited

number of fM peptides enabled us to define a NADH dehydrogenase subunit 1 (ND1) derived peptide as the physiological ligand of positive selection for the C10.4 TCR_{trans}^{+} (Berg et al., 1999b). In this thesis, I first investigate the agonistic activity of the ND1/9mer self-peptide to then further study how alterations of the ND1/9mer self-peptide affect positive selection using a fetal thymic organ culture (FTOC) system. I shortened the ND1/9mer and mutated the exposed amino acid side-chains as determined by the crystal structure of the H2-M3/ND1 complex (Wang et al., 1995). My experiments demonstrate that a natural ligand of positive selection, such as the ND1/9mer self-peptide, acts as a weak agonist and is recognized with exquisite specificity when offered at physiological epitope densities.

CHAPTER II

Materials and Methods

Mice

C10.4 TCR_{trans}^{+} mice were produced by a graduate student in our laboratory. C57BL/6 mice were purchased from either The Jackson Laboratory (Bar Harbor, Maine) or Taconic Farms (Germantown, New York). B10.D2, B10.BR, and $TAP1^{-/-}$ mice were purchased from The Jackson Laboratory. $RAG2^{-/-}$ mice were provided by Fred Alt (Children's Hospital, Boston, Massachusetts). B6.R9 mice were provided by Kirsten Fischer Lindahl (University of Texas South Western, Dallas, Texas). All mice were housed and bred (if necessary) at the Biological Resource Center at National Jewish Medical and Research Center (Denver, Colorado). Mice were euthanized by CO_2 inhalation.

Cell Culture Medium

All cells were cultured at 37^{o} C in 7% CO_2 (unless otherwise indicated) in Iscove's Modified Dulbeccos's Medium (IMDM) (Sigma Chemical Co., St. Louis, Missouri) supplemented with 5 mM Hepes, 2 mM glutamine, 1 mM hydroxypyruvate, 50 mM 2-mercaptoethanol, nonessential amino acids, 100 IU/ml penicillin, 100 mg/ml streptomycin, 50 mg/ml gentamycin (all from Sigma), and 10% Fetal Calf Serum (FCS) (Hyclone, Logan, Utah). This medium is referred to as complete IMDM (cIMDM). The T cell clones had 4% Rat Concanavalin A Supernatant (RCAS) added to the complete IMDM to provide cytokines and growth factors. Some CTL assays were performed with IMDM containing supplements, but without FCS. Instead, 0.5% (w/v) bovine serum albumin (Sigma Chemical Co., St. Louis, Missouri) was substituted and this media is referred to as serum free IMDM (sfIMDM).

Rat Concanavalin A Supernatants

Rat Concanavalin A stimulated culture Supernatant (RCAS) was prepared from the spleens of juvenile male Lewis rats purchased from Harlan Sprague Dawley (Indianapolis, Indiana). Spleens were harvested aseptically and disrupted by passage through steel mesh. Erythrocytes were lysed with Tris-buffered ammonium chloride (Sigma), then spleen cells were washed 3 times in sterile phosphate buffered saline (PBS) (Sigma). Spleen cells were cultured for 48 h in complete IMDM containing 4 μg/ml concanavalin A (Sigma) at 37°C in 7% CO_2. Spleen cell cultures were harvested and cells were removed by centrifugation. RCAS was sterilized by filtration through 0.2 micron filters (Nalgene, Rochester, NY) and stored at -20°C until needed.

Cells and Cell Lines

EL4 is an H-2^b thymoma obtained from American Type Culture Collection (ATCC, Rockville, Maryland). RMA and its TAP-$2^{-/-}$ negative counterpart, RMA-S, are H-2^b derived T cell lymphomas obtained from ATCC. The 13S2 cell line is a fibroblast transfected with a chimeric H2-$M3^{wt}/L^d$ molecule (Vyas et al., 1994) that was provided by Robert Rich and John Rodgers (Baylor College of Medicine, Houston, Texas). C10.4 TCR_{trans}^+ CTLs were generated from the spleens of C10.4 TCR_{trans}^+ mice on a $RAG2^{-/-}$ background. C10.4 TCR_{trans}^+ CTLs were maintained by weekly restimulation with irradiated C57BL/6 spleen cells incubated with the cognate AttM/6mer peptide.

Peptides

All peptides were synthesized at the National Jewish Molecular Resource Center (Denver, Colorado) by Amy Marrs and Randal Anselment. Standard (non fM) peptides were synthesized using standard solid phase FMOC chemistry on an Applied Biosystems (Foster City, California) 433A peptide synthesizer. fM-peptides were synthesized manually using standard solid phase

FMOC chemistry. Protecting groups were removed from resin-bound amino acid residues using trifluoroacetic acid (Sigma). FMOC amino acids were purchased from AnaSpec, Incorporated (San Jose, California) or SynPep (Dublin, California) and N-formyl methionine was purchased from Bachem Bioscience, Incorporated (King of Prussia, Pennsylvania). Peptides were purified by reverse-phase high performance liquid chromatography (RP-HPLC) on a Luna 10μ C18(2) 250 X 2.12 mm column (Phenomenex, Torrance, California) or a Vydac C4 column (Hesperia, California) using a Rainin gradient HPLC system with model SD-200 pumps (Rainin, Inc., Walnut Creek, California). Integrity of the peptides was confirmed using a Voyager RP matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (PerSeptive Biosystems, Framingham, Massachusetts). Peptides were dissolved in 100% dimethylsulfoxide (DMSO) (Sigma), aliquoted and stored at -20°C.

Amino acid sequences of the naturally occurring peptides are as follows: AttM cognate peptides (5-mer, 6-mer, or 9-mer) from *L. monocytogenes* recognized by C10.4 TCR_{trans}^{+} T cells (fMIVTLFYSA); LemA peptide from *L. monocytogenes* able to bind H2-M3 but not recognized by C10.4 TCR_{trans}^{+} T cells (fMIGWII) (Princiotta et al., 1998; Lenz et al., 1996); OVA-derived peptide (SIINFEKL); ND1/9mer self-peptide (fMFFINILTL); COI self-peptide (fMFINRWLFS); ND2 self-peptide (fMNPITLAII); COII self-peptide (fMAYPFQLGL); ND4 self-peptide (fMLKIILPSL); ND3 self-peptide (fMNLYTVIFI); ND6 self-peptide (fMNNYIFVLS); ND5 self-peptide (fMNIFTTSIL); ATPase 6 self-peptide (fMNENLFASF); ND4L self-peptide (fMPSTFFNLT); ATPase 8 self-peptide (fMPQLDTSTW); COIII self-peptide (fMTHQTHAYH); and CytB self-peptide (fMTNMRKTHP).

Amino acid sequences for the variants are as follows, whereas x represents the original amino acid from the ND1/9mer and a '-' represents a deleted amino acid: ND1/8mer (fxxxxxxxx-); ND1/7mer (fxxxxxxx--); ND1/P2 (fxAxxxxxxx); ND1/P5 (fxxxxAxxxx); ND1/P6 (fxxxxxAxxx); ND1/P8 (fxxxxxxxAx); ND1/P9 (fxxxxxxxxA); ND1/P89 (fxxxxxxxAA) and ND1/P2589 (fxAxxAxxAA).

Cytotoxicity Assays

Target cells were labeled with [^{51}Cr]$NaCr_2O_4$ (ICN Biomedical Research Products, Costa Mesa, California) for 1 hour at 37^{o}C. The targets were then washed four times. The two final washes were done with sfIMDM if the targets were to be pulsed with peptides. sfIMDM was used because some fM-peptides are susceptible to degradation by peptidases in the FCS. Peptides were then added to the targets at the indicated concentration. After another 1 hour incubation at 37^{o}C, 10^4 targets (in 100 µl) were added per well to a 96-well round bottom plate (Becton Dickinson, Mountain View, California). Effectors (in 100 µl) were then added at the Effector to Target ratio (E:T) indicated. A brief, slow centrifugation was performed in order to cluster the effectors with the targets. The plates were then incubated at 37^{o}C. After four hours, the plates were centrifuged for six minutes at 500 x g and 100 µl per well of supernatant was harvested. ^{51}Cr release was measured on a γ-counter (Micromedic). Spontaneous release was determined from wells that contained targets, but did not contain effectors. Total release was determined by adding 100 µl of 2% Triton-X 100 (Sigma) per well without the addition of any effectors. % specific lysis was calculated as ([experimental release – spontaneous release]/[maximum release – spontaneous release]) X 100. Results are expressed as the mean of triplicate determinations.

Antibodies and Flow Cytometric Analyses

Monoclonal antibodies (mAbs) specific for CD4 (RM4-5) and CD8 (53-6.7) were purchased from Pharmingen (San Diego, California). Streptavidin Cy-Chrome (Sa-CyC) and Streptavidin Phycoerythrin (Sa-PE) for revealing biotinylated mAbs were also purchased from Pharmingen. The anti-Cβ mAb, H57-597 (Kubo et al., 1989), the anti-Vβ8.1-8.3 mAb, F23.1 (Staerz et al., 1985), the anti-α3 L^d mAb, 28-14-8S (Ozato et al., 1983), and the anti-CD24 mAb, J11d (Bruce et al., 1981) were purified from culture supernatants using Protein G Sepharose Beads (Pharmacia Biotech, Piscataway, New Jersey).

These mAbs were then biotinylated using Biotinamidocaproate N-hydroxysuccinimideester (Sigma) or directly conjugated to Fluorescein isothiocyanate (FITC) (Sigma), if necessary.

For staining, cell suspensions were made by pressing organs through either steel or nylon mesh (Bally Ribbon Mills, Bally, Pennsylvania). Between 10^4 and 10^6 cells were then added per well to a 96-well round bottom plate (Becton Dickinson) and washed with phosphate buffered saline containing 3% FCS (staining buffer) one time. Saturating concentrations of the indicated antibodies were then added in a volume of 50 µl per well. TCR staining was done at 37°C for 30 minutes. Other staining was performed at 4°C for 30 minutes. Between primary and secondary stains, the cells were washed with staining buffer two times. After the secondary stain, cells were washed with staining buffer three times and then brought up to a final volume between 200 and 500 µl per sample in staining buffer. Cells were analyzed on a FACScan or FACScalibur (Becton Dickinson) using the CellQuest Software (Becton Dickinson).

Staining of Peripheral Blood Lymphocytes (PBL) to Determine Phenotype

To screen mice for the presence of certain cell surface molecules and / or the presence of the C10.4 TCR_{trans}^{+}, PBLs were stained and analyzed by flow cytometry. Mice were bled from the tail vein, and 3-5 drops of blood were collected into complete IMDM containing 0.67 mg/ml heparin sulfate (Sigma). After centrifugation at 500 x g for five minutes, one milliliter of Tris buffered ammonium chloride was added to the cell pellet to lyse red blood cells (RBCs). The cells were incubated for ten minutes at room temperature and then washed three times with staining buffer. Cells were added to a 96-well round bottom plate. For the C10.4 TCR_{trans}^{+}, the lymphocytes were stained for CD4, CD8 and TCR expression levels using H57-597 and F23.1.

Screening of Tail DNA Using Microsatellite Mapping

Genomic DNA was prepared by digestion of tail fragments in 200 µl of 10 mM Tris, 50 mM KCL, 0.5% NP40 detergent, 0.5% Tween, and 0.2 mg/ml proteinase K (all reagents from Sigma) overnight at 56^{o}C. 150 µl of 6 Molar NaCl was then added to the microcentrifuge tube. After gentle mixing, the tubes were centrifuged at 12,500 x g and the supernatant was transferred to a new tube where 750 µl of cold 95% ethanol was added to precipitate the DNA. The DNA was then centrifuged at 12,500 x g and the precipitated DNA was washed once with 750 µl of 70% ethanol. After a quick drying period, the DNA was hydrated with 200 µl of double distilled H_2O.

A microsatellite procedure was used to distinguish between the H2-M3^{wt} allele expressed on most common strains of mice and the H2-M3^{cas} allele expressed on B6.R9 mice. It was previously shown that the D17Mit148 microsatellite marker could be used to distinguish between the H2-M3^{wt} and H2-M3^{cas} alleles (Xiao et al., 1997). D17Mit148 oligonucleotide pairs were purchased from Research Genetics, Inc. (Huntsville, Alabama) and used in a PCR reaction with genomic DNA as the template. PCR reagents contained 0.4 mM dNTP mix, 3 mM $MgCl_2$, 1 unit/ 50 µl Taq polymerase, and 1X PCR buffer (Reagents for PCR were all from Perkin-Elmer, Norwalk, Connecticut). Conditions used were an initial 95^{o}C (2 minute) cycle followed by thirty-five cycles of 94^{o}C (45 seconds), 57^{o}C (45 seconds), 72^{o}C (1 minute) followed by one cycle of a seven minute 72^{o}C elongation. Resulting PCR products were then separated on a 15% non-denaturing Acrylamide gel (Bio Rad Laboratories).

Fetal Thymic Organ Cultures

One male mouse was placed together with two female mice in the evening and the next morning vaginal plugs were checked. The day the mice were checked was considered day 1 of gestation. On day 16 of gestation, thymic lobes from C10.4$^{+/+}$TAP$^{-/-}$ fetuses were removed by dissection. The

thymic lobes were cultured in 6-well plates (Becton Dickinson) on 0.45 µM pore size nitrocellulose membranes (Gelman Sciences, Ann Arbor, Michigan) supported by gelatin sponge filters (Pharmacia and Upjohn, Kalamazoo, Michigan). The sponges were saturated with four milliliters of complete IMDM containing 1% Nutridoma SP (Boehringer Mannheim, Indianapolis, Indiana), potato carboxypeptidase inhibitor (10^{-4} nM final concentration), captopril (10^{-7} mM final concentration), E-64 (10^{-2} mM final concentration) (all from Sigma), 5 µg/ml human β_2M (as a source of exogenous β_2M) (Vital Products, St. Louis, Missouri) and the indicated HPLC purified peptides (if necessary). After incubating at 37^{o}C for 6 days, the resulting thymocytes were pressed through nylon mesh into a single cell suspension and stained for CD4, CD8, CD24, and TCR expression levels using the staining procedure and mAbs described above.

Expansion of FTOC Thymocytes and Their Use in a CTL Assay

After FTOCs were cultured for six days, a single cell suspension was made from the resulting thymocytes by pressing the lobes through nylon mesh. One third was used for FACS analysis, whereas the remainder was then added to 24-well plates (Becton Dickinson) that had been coated with 5 µg/ml of the H57-597 mAb. Media used was complete IMDM with 4% RCAS. After three days, the cells were harvested, counted and used as effectors at an E:T ratio of 10:1. Targets used were EL4 cells labeled with either the AttM/6mer peptide or the LemA 6-mer peptide. After four hours the supernatant was harvested and ^{51}Cr release was measured on an automatic γ-counter. % specific lysis was calculated as ([experimental release – spontaneous release]/[maximum release – spontaneous release]) X 100. Results are expressed as the mean of triplicate determinations.

H2-M3 Up-regulation Assay

To measure the relative binding affinities of fM-peptides, an up-regulation assay previously described was used (Vyas et al., 1994). The 13S2 cell line expressing the H2-M3^{wt}/L^{d} chimeric molecule was plated in 48-well plates (Becton Dickinson) at approximately 10^5 cells per well in complete IMDM containing potato carboxypeptidase inhibitor (10^{-4} nM final concentration), captopril (10^{-7} mM final concentration), E-64 (10^{-2} mM final concentration) (all from Sigma) and 100 units/ml of IFN-γ (Genzyme, Cambridge, Massachusetts). After incubation overnight at 37^{o}C, peptides were added to the cells at various concentrations and the cells were incubated overnight at 27^{o}C in 7% CO_2. The following day, the cells were harvested and stained for expression of the chimeric H2-M3^{wt}/L^{d} molecule using the 28-14-8S mAb conjugated to biotin followed by a secondary Streptavidin Phycoerythrin reagent from Pharmingen.

Naïve Activation Assay

Preparation of T cells:

C10.4$^{+/+}$ RAG2$^{-/-}$ lymph nodes (inguinal, paraaortic and intestinal) were harvested. The resulting cells were pressed through a nylon mesh. This T cell population had a purity greater than 99% CD8^{+}Vβ8^{+} as determined by flow cytometry. After washing in Hanks buffered salt solution (HBSS, Cellgro), to remove any extracellular proteins, cells were dyed for 30 minutes in the presence of 0.2 µM carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes Inc.) (Weston and Parish, 1990), then washed 4 times in HBSS.

Preparation of APCs:

BM derived enriched dendritic cells were generated as described earlier (Inaba et al., 1992). Shortly femur and tibia from TAP1$^{-/-}$ deficient mice were removed and the bone marrow was flushed out. After lysis of red blood cells the

cells were treated with an antibody cocktail (anti CD4 (GK 1.5, prepared from culture SN); anti CD8 (HO 2.2, prepared from culture SN); anti B cell (RA3-6B2, Pharmingen) and anti Class II (3F12, prepared from culture SN) in the presence of rabbit complement (Cedarlane). After washing with IMDM medium the cells were cultured in media containing recombinant murine GM-CSF (rmuGM-CSF) (Prepared as culture supernatant from chinese hamster ovary cells transfected with the murine GM-CSF gene). After 6 days in culture ~6,000 enriched DCs were transferred into 96 well plate flat bottom plates (Greiner) and incubated for 1h with the indicated peptides. Then ~80,000 enriched T cells were added. 6 days later the cells were harvested and analyzed by 4 color flow cytometry.

CHAPTER III

Characteristics of the C10.4 TCR_{trans}^{+} Mouse

Unraveling the role of self-peptides in positive selection seemed to be a very difficult task due to the vast number of peptides able to bind MHC Class Ia and MHC Class II molecules. In fact, most investigators had focused their efforts on analyzing selection of a given TCR using either cognate peptides or variants of cognate peptides. Alternatively, the selection of T cells by a single peptide / MHC complex was also examined. Although these approaches provided useful data concerning the nature of the interaction responsible for positive selection, the only unbiased way to analyze positive selection is to define a self-peptide, or peptides, responsible for positive selection of a subset of T cells expressing a defined TCR.

The non-classical MHC molecule, H2-M3, binds peptides carrying a formyl-Methionine (fM) on their N-terminus (Loveland et al., 1990; Shawar et al., 1990). Furthermore the only source of self fM-peptides in eukaryotes are thirteen gene products in the mitochondria (Fischer Lindahl et al., 1997). Additionally, several fM-peptides derived from bacteria have been shown to be specifically recognized by $CD8^{+}$ T cells restricted to H2-M3 (Gulden et al., 1996; Lenz et al., 1996; Princiotta et al., 1998). H2-M3 can also bind hydrophobic peptides without a formyl group on the N-terminus, but the binding affinities from non fM-peptides are at least 100-fold lower than the binding affinities for the formylated version of the same peptide (Smith et al., 1994; Byers and Fischer Lindahl, 1998; Princiotta et al., 1998). This large difference in binding abilities would probably result in self fM-peptides being responsible for positive selection of H2-M3 restricted T cells in the thymus.

To overcome the above mentioned difficulties and to take advantage of the unique characteristics of the H2-M3 MHC molecule, a graduate student in our lab established a new transgenic mouse. The TCR transgenes were derived from a previously described T cell clone (Princiotta et al., 1998). It expresses a

TCR that is restricted to the H2-M3 molecule and is positively selected on the ND1/9mer self-peptide.

The C10.4 TCR_{trans}^{+} phenotype is characterized as shown by flow cytometric analysis on peripheral blood lymphocytes (PBLs). The PBLs were stained for CD4, CD8 and TCR levels using the H57-597 mAb (specific for the constant region of the TCR β chain) or the F23.1 mAb (specific for Vβ8.1 - 3^{+} TCRs). As shown in Figure 1, PBLs from the C10.4 TCR_{trans}^{+} mouse exhibited a pronounced skewing towards the $CD8^{+}$ phenotype. Furthermore, the vast majority of the $CD8^{+}$ T cells stained positive with the F23.1 mAb, which detects $V\beta8^{+}$ T cells. In contrast, a non-transgenic (C57BL/6) mouse showed a skewing towards the $CD4^{+}$ compartment, as well as a much lower percentage of $CD8^{+}V\beta8^{+}$ T cells in the periphery. This data shows that the C10.4 TCR_{trans}^{+} was expressed on peripheral T cells.

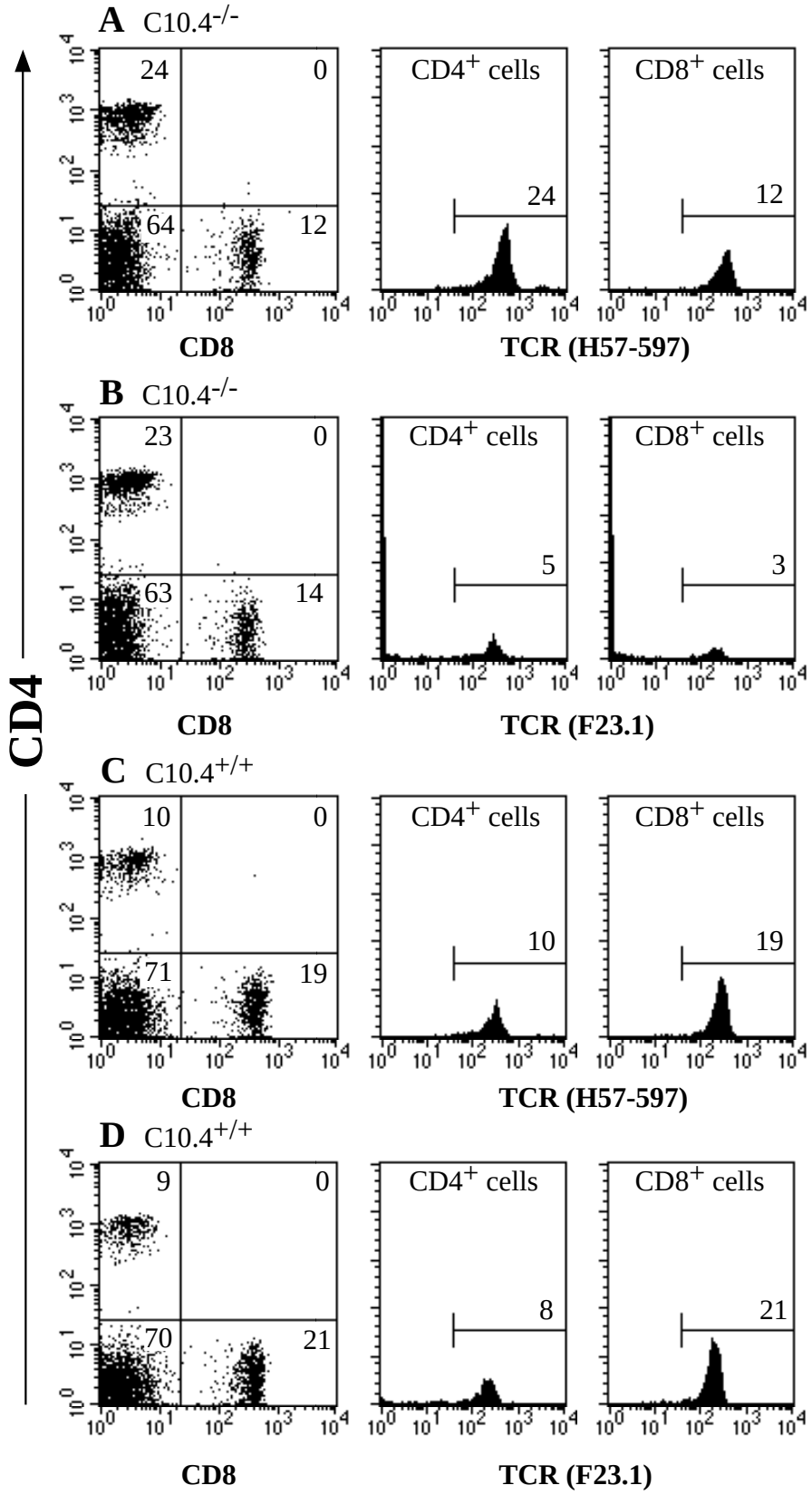
A C10.4-/-
24
0
64
12
CD4+ cells
24
CD8+ cells
12
CD8
TCR (H57-597)
B C10.4-/-
23
0
63
14
CD4+ cells
5
CD8+ cells
3
CD8
TCR (F23.1)
C C10.4+/+
10
0
71
19
CD4+ cells
10
CD8+ cells
19
CD8
TCR (H57-597)
D C10.4+/+
9
0
70
21
CD4+ cells
8
CD8+ cells
21
CD8
TCR (F23.1)
CD4

Figure 1

FIGURE 1: PERIPHERAL BLOOD STAIN OF THE C10.4 TCR $_{TRANS}^{+}$ MOUSE

This Figure shows a CD4 vs. CD8 dot plot in the first panel, followed by a histogram of the T-cell receptor levels on the CD4 single positive and CD8 single positive lymphocyte population. Quadrant labels represent percent of total cells as gated through the live cell population (determined by forward/side scatter, using flow cytometry). Marker label show percent of total live cells gated through the depicted quadrants. Panel A shows the PBL lymphocytes from a C57/Bl6 mouse. TCR is analyzed using the $\alpha C\beta$ mAb H57-957. Panel B shows the same mouse, but stained with the mAb F23.1, specific for the Vβ8.1-Vβ8.3 TCR region. Panel C shows the H57-957 stain on peripheral blood lymphocytes from a homozygotous C10.4 TCR $_{trans}^{+}$ mouse. Panel D shows the same mouse, yet TCR was stained using the F23.1 mAb. Similar results were obtained in independent analysis.

Staining and Specificity of T Cells Isolated from a C10.4 TCR_{trans}^{+} Mouse on a RAG2 Deficient Background

Mice deficient in the Recombination Activating Gene 2 ($RAG2^{-/-}$) do not contain T or B cells due to their inability to rearrange TCR or BCR genes (Shinkai et al., 1992). However, providing a rearranged TCR ensures that the only T cells present in the mouse are TCR transgenic in nature. In order to establish a pure population of C10.4 TCR_{trans}^{+} T cells, the C10.4 TCR_{trans}^{+} mouse was bred onto the $RAG2^{-/-}$ background. Indeed, as depicted in Figure 2, C10.4 TCR_{trans}^{+} $RAG2^{-/-}$ mice show a complete skewing towards $CD8^{+}$ T cells in peripheral blood. Furthermore, peripheral splenocytes from these mice give rise to CTLs that exhibited the specificity of the original C10.4 T cell (Figure 3, (Princiotta et al., 1998)). These mice provide a pure population of C10.4 TCR_{trans}^{+} T cells that do not express any endogenous TCR.

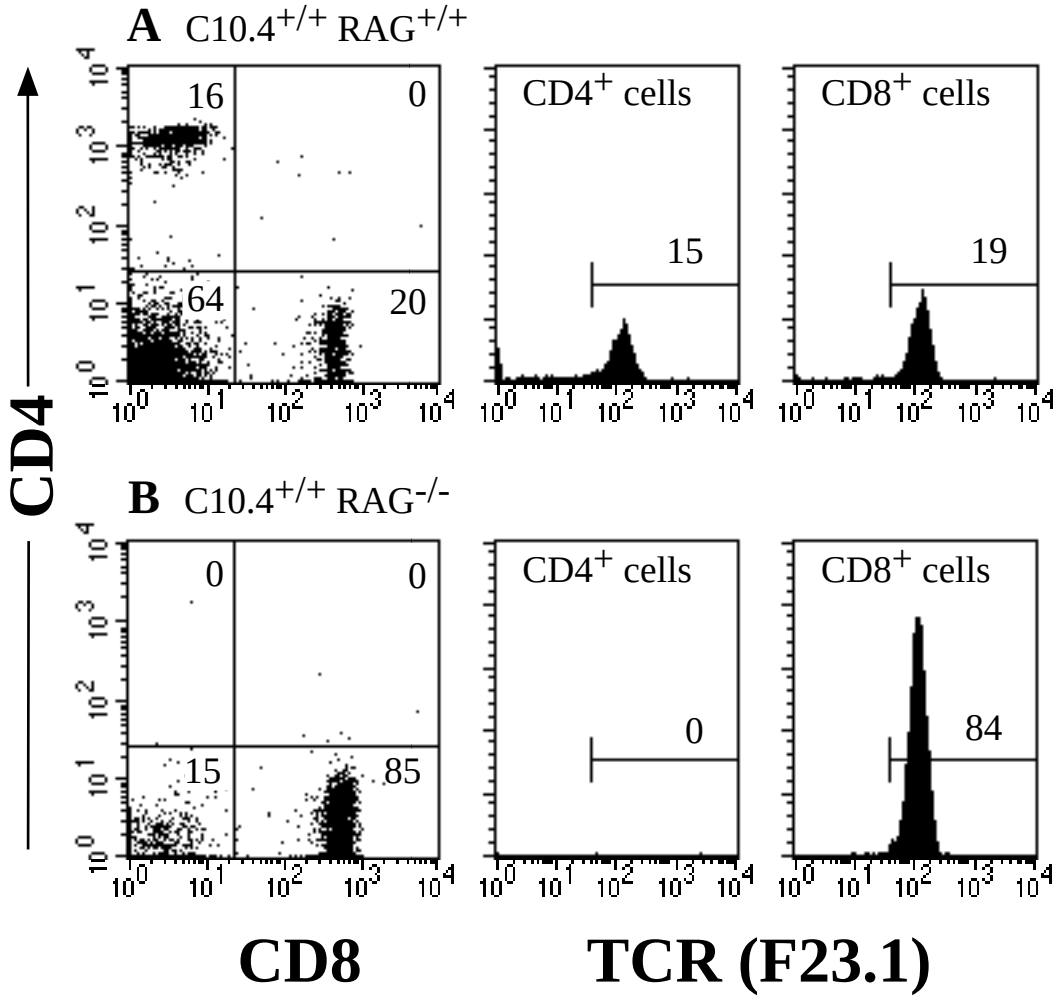

Figure 2

FIGURE 2: PHENOTYPE OF THE C10.4$^{+/+}$ RAG2$^{-/-}$ MOUSE

A dot plot of CD4 vs. CD8 was performed using flow cytometry on PBLs. The histograms show TCR levels on the depicted populations. Quadrant labels show percent of total live cells and marker label show percent of total live cells gated through the given quadrants. Panel A shows PBL from a C10.4$^{+/+}$ RAG2$^{+/+}$, Panel B shows a C10.4$^{+/+}$ RAG2$^{-/-}$ mouse. Data shows representative results from at least three independent experiments.

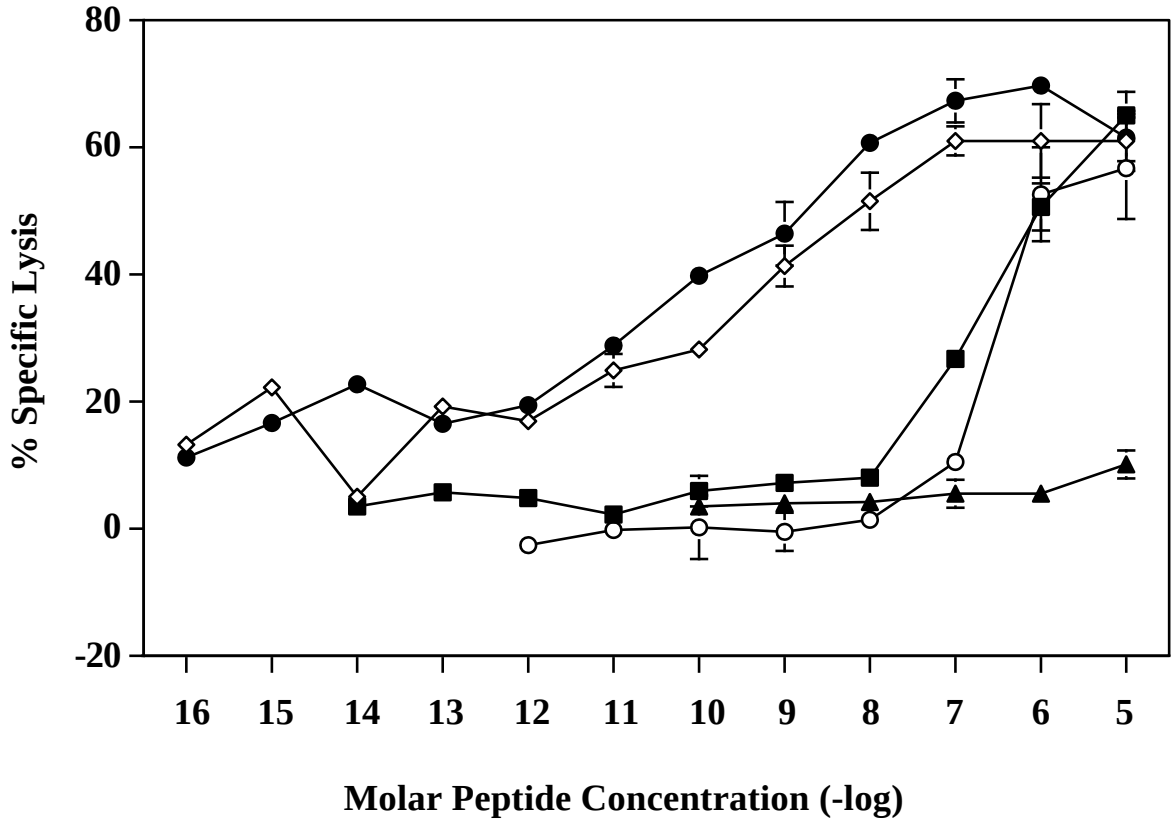

Figure 3

FIGURE 3: THE ND1/9MER IS A PARTIAL AGONIST TO PERIPHERAL C10.4 TCR_{TRANS}^{+} T CELLS

Effectors from a C10.4 TCR_{trans}^{+} T cell line generated from a C10.4$^{+/+}$RAG2$^{-/-}$ mouse were used in a standard CTL assay at an E:T ratio of 5:1. Targets used were EL4 cells incubated with either the ND1/9mer (filled squares), the AttM/9mer peptide (open diamonds), the AttM/6mer peptide (filled circles), the AttM/5mer peptide (open circles), or the LemA 6-mer peptide (filled triangles) at the indicated concentrations. Data is presented as percent specific lysis and is the mean of triplicate determinations for each data point.

Staining of T Cells Isolated from a C10.4 TCR_{trans}^{+} Mouse on a TAP1 Deficient Background

Mice deficient in the Transporter Associated with antigen Processing 1 ($TAP1^{-/-}$) cannot present peptides via MHC Class I to $CD8^{+}$ T cells. These mice cannot transport any processed peptides to the endoplasmatic reticulum (ER), where they are loaded onto empty MHC Class I molecules. Therefore only empty MHC Class I molecules traffic to the cell surface. These empty molecules are fairly unstable and only minimal amounts of empty molecules are present on the cell surface at a given time (York and Rock, 1996). Since $CD8^{+}$ T cells are restricted to MHC Class I molecules, mice deficient in TAP1 (or TAP2) cannot positively select $CD8^{+}$ cells.

Indeed, as depicted in Figure 4, C10.4 TCR_{trans}^{+} $TAP1^{-/-}$ mice lack the entire CD8 single positive (SP) population. There is a significant skewing towards the CD4 SP compartment probably due to TCR α chain rearrangement. As we bred the $C10.4^{+/+}$ $TAP1^{-/-}$ mouse onto the $RAG2^{-/-}$ background ($C10.4^{+/+}$ $TAP1^{-/-}$ $RAG2^{-/-}$) this developmental pathway was abolished (data not shown).

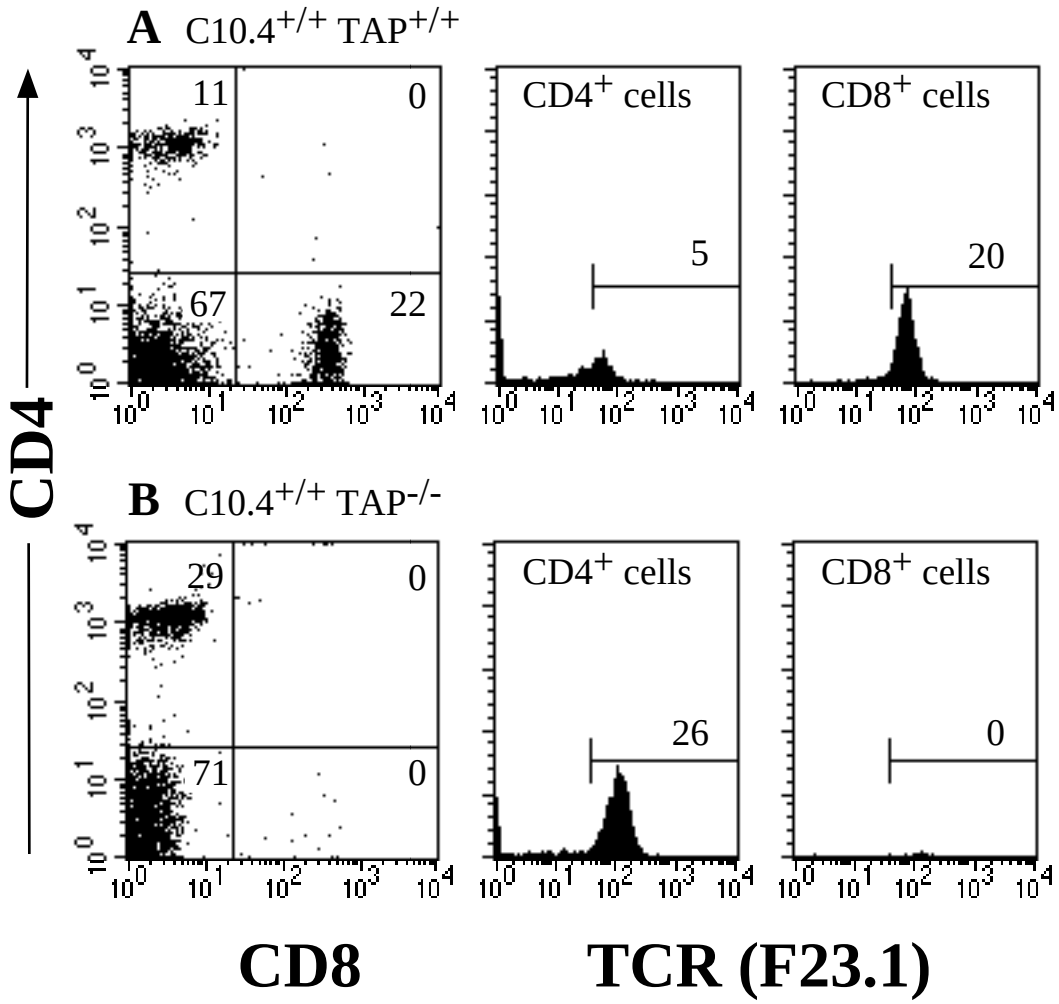

Figure 4

FIGURE 4: PHENOTYPE OF THE C10.4$^{+/+}$ TAP1$^{-/-}$ MOUSE

A dot plot of CD4 vs. CD8 was performed using flow cytometry. The histograms show TCR levels on the depicted populations. Quadrant labels show percent of total live cells and marker label show percent of total live cells gated through the given quadrants. Panel A shows PBL from a C10.4$^{+/+}$ TAP1$^{+/+}$, Panel B shows a C10.4$^{+/+}$ TAP1$^{-/-}$ mouse. Similar results were obtained in 3 consecutive experiments.

Positive Selection of C10.4 TCR_{trans}^{+} Thymocytes on Different MHC Backgrounds

To confirm that the C10.4 TCR_{trans}^{+} was selected on the non-polymorphic MHC molecule H2-M3, it was bred onto different MHC haplotypes that all carry the wild type allele of H2-M3 (H2-$M3^{wt/wt}$) (Fischer Lindahl et al., 1997). Figure 5 shows that C10.4 TCR_{trans}^{+} cytolytic T cells developed in mice of the different Classical MHC haplotypes, such as C57BL/6 (H-2^{b}), B10.D2 (H-2^{d}) and B10.BR (H-2^{k}) Splenocytes of those mice could be expanded *in vitro* in the presence of external cytokines and growth factors to become functional cytolytic T cells. CTLs generated from all different backgrounds specifically lysed target cells coated with the AttM peptide, but did not lyse target cells coated with the control H2-M3 binding LemA peptide (Figure 5).

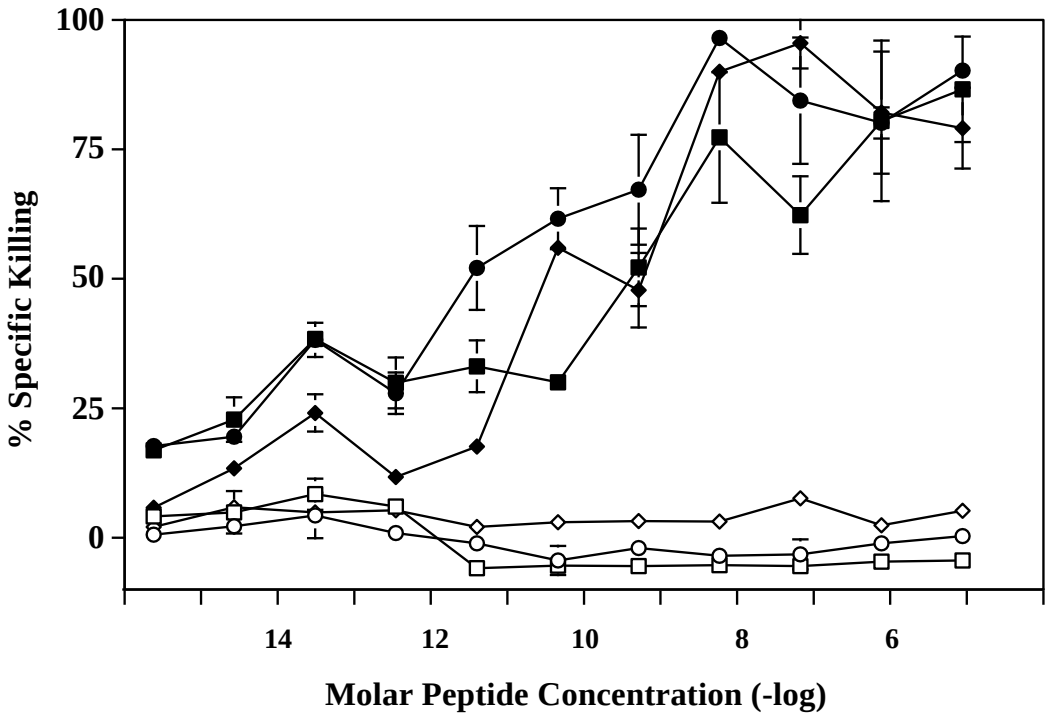

Figure 5

FIGURE 5: C10.4 TCR $_{TRANS}^{+}$ CYTOLYTIC CELLS FROM DIFFERENT H-2 BACKGROUNDS CAN LYSE TARGETS COATED WITH THE COGNATE PEPTIDE.

C10.4 TCR $_{trans}^{+}$ splenocytes have been expanded in vivo *on the cognate peptide in the presence of endogenous cytokines and growth factors. They can lyse EL4 target cells coated with the cognate Listeria monocytogenes AttM peptide impaired of their H-2 background in a standard chromium release assay. Circles show cells on an H-2^{b} background, boxes show cells from a C10.4$^{+/+}$ H-2^{d} mouse and diamonds are cells from a C10.4$^{+/+}$ H-2^{k} mouse. Filled symbols show lytic activity towards EL4 cells coated with the cognate AttM/6mer peptide, empty symbols show EL4 cells coated with the LemA/6mer peptide due to its ability to bind H2-M3 without sensitizing C10.4 T cells. E:T ratio was 5:1. Each data point is depicted as the mean of two data points.*

Requirement of H2-M3^{wt} for Positive Selection of C10.4 TCR$_{trans}^{+}$ Thymocytes

It had been shown that the castaneus allele of H2-M3 (H2-M3$^{cas/cas}$) could not present peptides to any of the known H2-M3^{wt} restricted T cells, including C10.4 (Princiotta et al., 1998). Therefore, the C10.4 TCR$_{trans}^{+}$ was bred onto mice that expressed H2-M3^{cas} on an H2^{b} background. These mice were then bred onto a RAG2$^{-/-}$ knockout background. The castaneus allele was derived from the B6.R9 mice, which utilize the H2-K through D region of C57BL/6 (H2^{b}) and the H2-Q through M region of *Mus musculus castaneus* (Fischer Lindahl et al., 1997). The H2-M3 genotype was determined by microsatellite mapping (Figure 6). As depicted in Figure 7, C10.4 TCR $_{trans}^{+}$ T cells were detectable in the PBL population of C10.4$^{+/+}$ RAG2$^{-/-}$ H2-M3$^{wt/wt}$, but not in C10.4$^{+/+}$ RAG2$^{-/-}$ H2-M3$^{cas/cas}$.

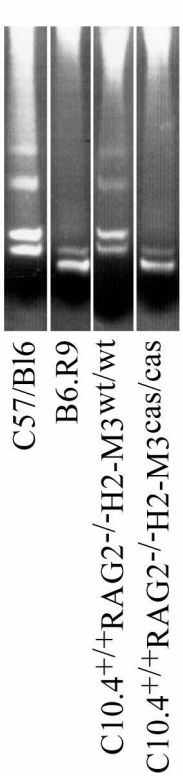

Figure 6

FIGURE 6: MICROSATELLITE MAPPING TO DETERMINE THE H2-M3 GENOTYPE

Genomic DNA from tail cells was extracted using standard protocols. The genotype was then determined by PCR using the MapPairs D17Mit148-F/R. Lane 1 shows the pattern from a C57/Bl6 encoding the H2-M3 wildtype. Lane 2 is from the B6.R9 mouse strain that encodes for the H2-M3 castaneus. Lane 3 and 4 show those genotypes on the depicted transgenes.

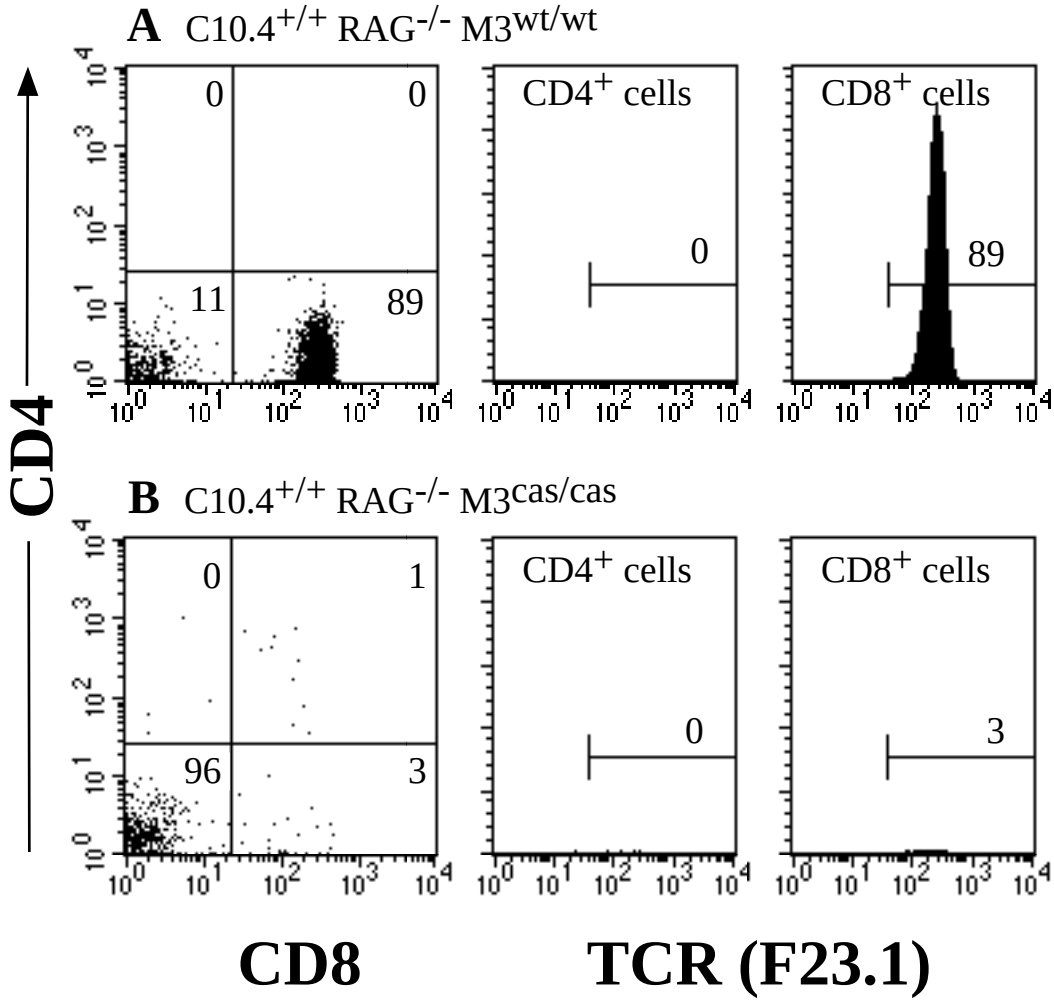

Figure 7

FIGURE 7: C10.4 TCR_{TRANS}^{+} T CELLS ARE SELECTED ON H2-M3

The C10.4 TCR_{trans}^{+} was bred onto the B6.R9 background, which carries a null-allele *of the H2-M3 gene (H2-$M3^{cas/cas}$) and the $RAG2^{-/-}$ knockout background. Therefore we could not detect a significant amount of $CD8^{+}/TCR^{high}$ T cells in peripheral blood by flow cytometry. mAbs used in this experiment were CD4-PE, CD8-FITC and F23.1-biotin. Streptavidin-CyC was used as a second step reagent (Panel B). Panel A shows PBL from a $C10.4^{+/+}$ $RAG2^{-/-}$ H2-$M3^{wt/wt}$ as a positive control.*

Development of a Fetal Thymic Organ Culture (FTOC) System to Analyze Positive Selection

FTOC systems using TCR_{trans}^{+} mice had previously been established to study the role of peptides in positive selection (Ashton-Rickardt et al., 1994; Hogquist et al., 1994; Sebzda et al., 1994). For these FTOC systems, the C10.4 TCR_{trans}^{+} was bred onto mice deficient for the TAP1 gene ($C10.4^{+/+}TAP^{-/-}$). Thymic lobes from day 16 fetuses were cultured for six days as described in Chapter II. T cells emerging from these cultures were analyzed for expression of CD4, CD8 and TCR. In FTOCs from C10.4 TCR_{trans}^{+} mice deficient in the TAP1 gene show very few mature $CD8^{+}V\beta8^{+}$ T cells (data not shown). Thus, this FTOC system could be used to analyze the impact of exogenous peptides on C10.4 TCR_{trans}^{+} T cell development.

Efficiency of the TAP knockout system

To test how efficiently exogenous fM peptides are presented to C10.4 T cells in $TAP^{-/-}$ mice, a standard CTL assay was performed. RMA cells, and their TAP2 deficient counterpart RMA-S, were exogenously pulsed with the AttM/9mer peptide. The peptide was titrated over 5 logs in 1:2 dilutions, to see even small differences. As shown in Figure 8 there is very little, if not no difference between the two cells. Therefore it can be concluded that exogenous loading of the fM peptides is a fair way to simulate the physiological situation, due to the comparable amount of presented peptide in the two cell lines.

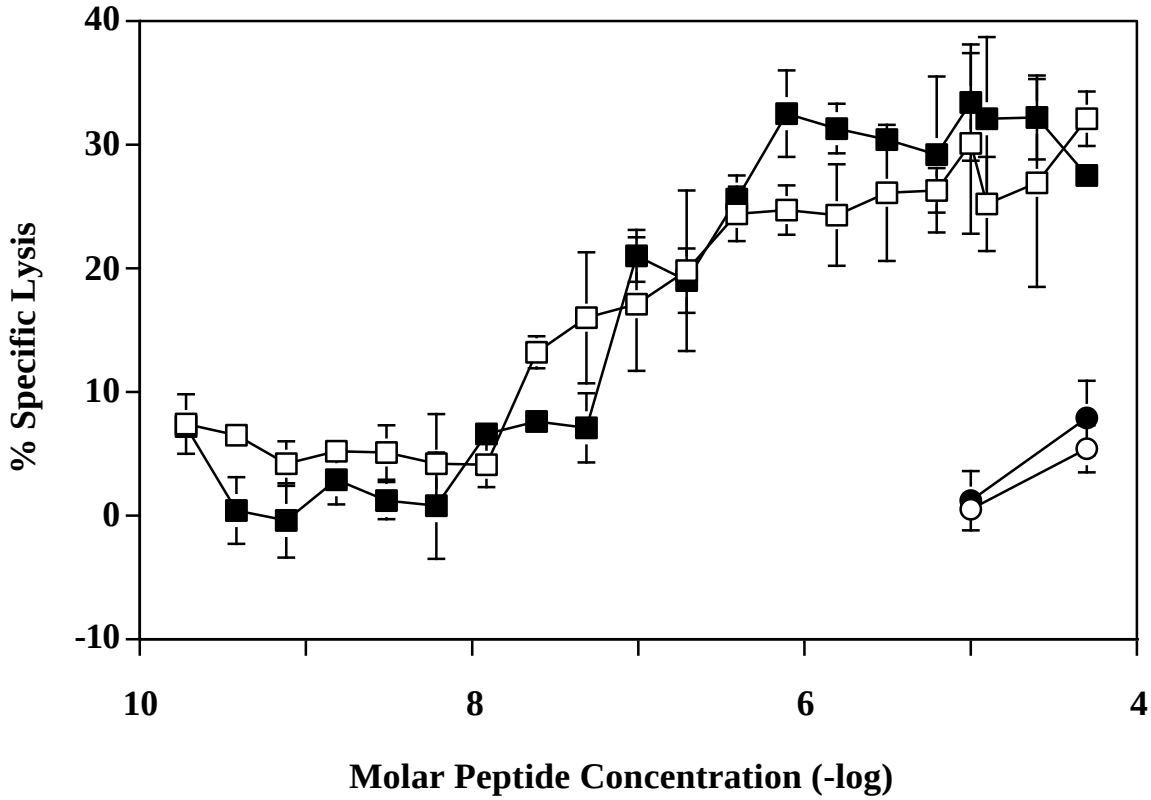

Figure 8

FIGURE 8: EFFICIENCY OF THE TAP KNOCKOUT SYSTEM

To investigate the efficiency of the endogenous peptide loading onto TAP deficient cells used in this study, we compared the ability of RMA vs. RMA-S cells to present the cognate AttM/9mer peptide to C10.4$^{+/+}$ RAG2$^{-/-}$ splenocytes. Empty symbols are RMA targets (parental cell line), whereas filled symbols are RMA-S targets, deficient of the TAP2 gene. Boxes show killing on the AttM/9mer, circles show the LemA/6mer peptide as a negative control. % specific lysis is expressed as the mean of triplicates.

ND1 is the Physiological Ligand of Positive Selection for C10.4 TCR_{trans}^{+} Thymocytes

Rance Berg focused on the 13 self-mitochondrial peptides and their role in positive selection of the C10.4 mouse. To determine the appropriate length we reviewed the given literature. The exact length of self fM-peptides presented by H2-M3 was unknown, but peptides from six to nine amino acids have been shown to be presented (Dabhi and Lindahl, 1998). The binding of peptides of nine amino acid lengths to $H2\text{-}M3^{wt}$ had been extensively studied (Wang et al., 1995; Fischer Lindahl et al., 1997; Dabhi et al., 1998). Furthermore, peptides extracted from MHC Class Ia molecules were generally eight to nine amino acids in length (Rammensee et al., 1993). Therefore, the thirteen corresponding N-terminal fM-peptide 9-mers were assayed for their abilities to induce positive selection of C10.4 TCR_{trans}^{+} thymocytes. These peptides were titrated into $C10.4^{+/+}TAP^{-/-}$ FTOCs starting at 10 μM. Control cultures that contained 0.1% DMSO (used as fM-peptide diluent) without peptide or with an $H\text{-}2K^{b}$ binding ovalbumin-derived peptide (SIINFEKL) failed to induce the development of $CD8^{+}V\beta8^{+}$ thymocytes (Berg et al., 1999b). When the N-terminal 9-mer fM-peptide derived from the NADH dehydrogenase subunit 1 (ND1) was added to FTOCs, concentrations down to the low nM range promoted the development of $CD8^{+}V\beta8^{+}$ thymocytes (Figure 9). In fact, a concentration of between 10 nM and 39 nM was able to induce the positive selection of levels of $CD8^{+}V\beta8^{+}$ T cells seen in the thymus of a normal $C10.4^{+/+}TAP^{+/+}$ mouse (Figure 4).

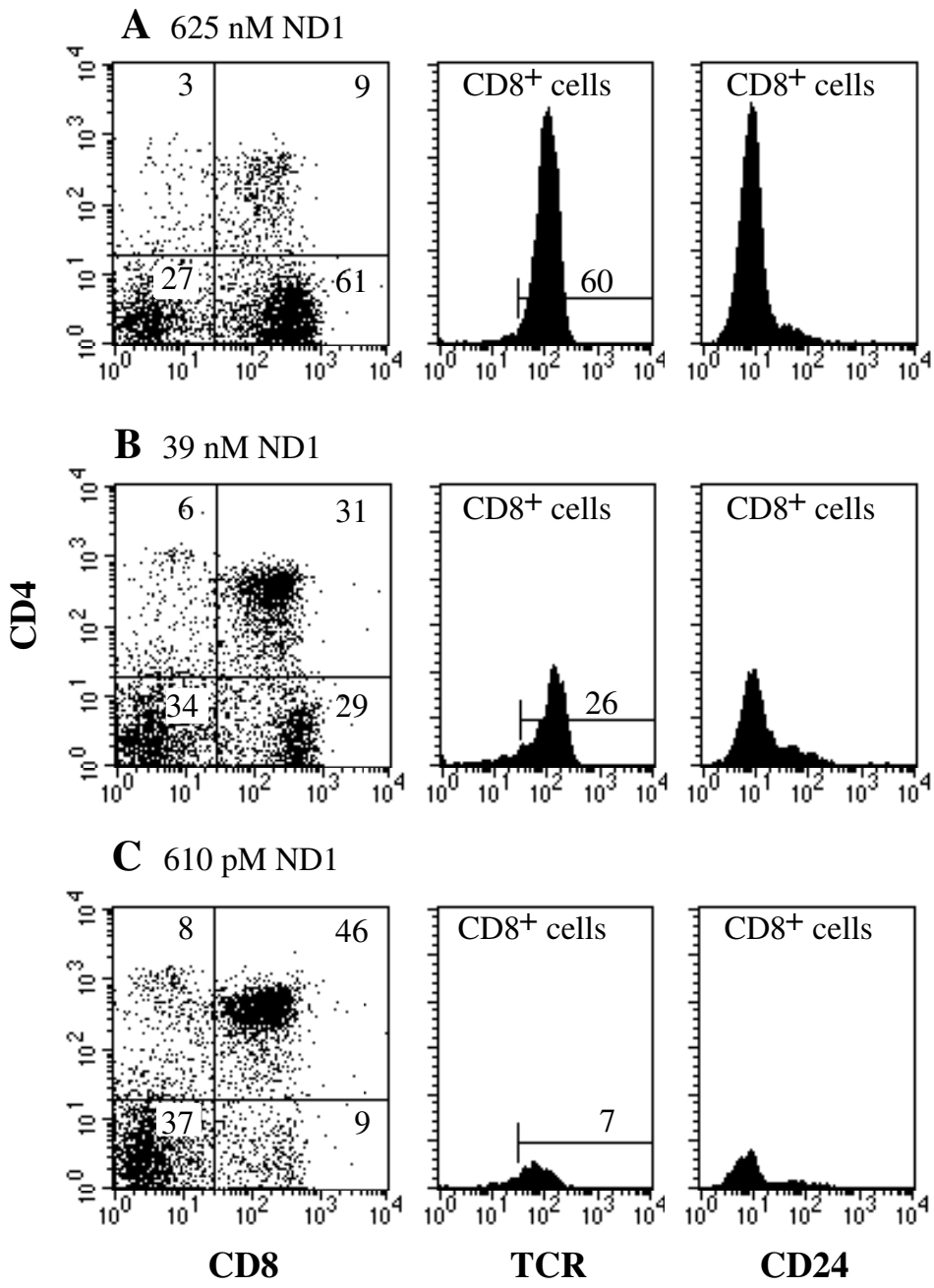
A 625 nM ND1
3
9
27
61
CD8+ cells
60
CD8+ cells
B 39 nM ND1
6
31
34
29
CD8+ cells
26
CD8+ cells
C 610 pM ND1
8
46
37
9
CD8+ cells
7
CD8+ cells
CD4
CD8
TCR
CD24

Figure 9

FIGURE 9: POSITIVE SELECTION ON THE NATURALLY OCCURING SELF-PEPTIDE ND1/9MER

Different concentrations of the ND1/9mer were added to the media of C10.4$^{+/+}$ TAP$^{-/-}$ FTOCs. After six days the thymocytes were stained for CD4, CD8, CD24, and TCR expression levels (using the F23.1 mAb for TCR analysis). The concentrations of peptide used for each experiment are depicted in the graph. Data is presented as CD4 versus CD8 staining for the dot plots, and TCR staining on the CD8 SP T cells in the first column of histograms. The second column of histograms depicts CD24 staining on CD8 SP T cells. The numbers in the dot plots indicate the percentage of cells within each quadrant out of all live-gated events. The numbers in the first column of histograms represent the percentage of CD8^{+}Vβ8^{+} T cells out of all live gated events. Data was obtained from at least 2 individual thymic lobes. Representative FACS analysis is shown.

The ND1/9mer Induces Positive Selection of Functionally Mature Thymocytes

To examine the functional development of C10.4 TCR_{trans}^{+} T cells generated from C10.4$^{+/+}$TAP$^{-/-}$ FTOCs cultured in the presence of the ND1/9mer, thymocytes were expanded for 3 days with the H57-597 mAb as described in chapter II and then tested for their ability to lyse target cells coated with the cognate AttM/6mer peptide. Specific CTLs were expanded from C10.4$^{+/+}$TAP$^{-/-}$ FTOCs that had been supplemented with the ND1 peptide (Figure 10, filled squares). In the absence of the ND1/9mer, specific CTLs were not expanded from the $CD8^{+}V\beta8^{+}$ thymocytes present in the control FTOC cultures. Thus, addition of the ND1/9mer induced a shift towards cells of the mature $CD8^{+}$ TCR_{trans}^{+} phenotype, and a development of functional thymocytes with the proper CTL specificity.

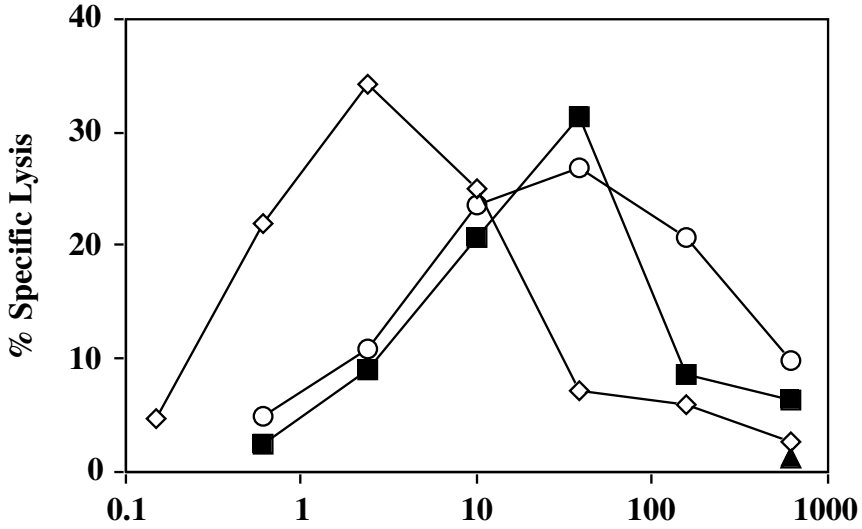

Figure 10

FIGURE 10: FUNCTIONAL CHARACTERISTICS OF C10.4$^{+/+}$TAP$^{-/-}$ FTOC DERIVED THYMOCYTES INCUBATED WITH THE ND1/9MER, THE ATTM/9MER PEPTIDE, OR THE ATTM/5MER PEPTIDE

Thymocytes resulting from C10.4$^{+/+}$TAP$^{-/-}$ FTOCs cultured with the indicated concentrations of peptide were expanded for three days on H57-597 coated plates. After expansion, the cells were used in a standard CTL assay. Targets used were EL4 cells pulsed with 10 µM AttM/6mer peptide or 10 µM LemA 6-mer peptide. This graph shows killing on the AttM peptide coated targets only. Specific lysis on the targets coated with the control LemA peptide was always less that five percent for all effectors. E:T ratio was 10:1. The peptides used in the C10.4$^{+/+}$TAP$^{-/-}$ FTOCs for deriving the thymocytes are as follows: the ND1/9mer (filled squares), the AttM/9mer peptide (open diamonds), the AttM/5mer peptide (open circles), or no peptide (filled triangle). Data is presented as percent specific lysis and is the mean of triplicate determinations.

Summary

The experiments performed in this chapter describe the phenotype of the C10.4 TCR_{trans}^{+} mice and the other knockout breeds used in this study. The different MHC backgrounds are further described to point out the unique characteristics of the H2-M3 Class I molecule. Furthermore the used FTOC system is described in detail. More information has been published earlier (Berg et al., 1999b).

CHAPTER IV

The ND1/9mer Functions as a Partial Agonist

Chapter III described the characteristics of the C10.4 TCR_{trans}^{+} mouse and the MHC and peptide requirements for positive selection of the transgenic T cells in this mouse. The ND1/9mer responsible for positive selection of C10.4 TCR_{trans}^{+} T cells shows little if any sequence homology with the cognate AttM peptide (fMFFINILTL vs. fMIVTLFYSA). Experiments in this section were designed to probe the recognition events during positive selection by using the ND1/9mer and the cognate AttM peptide to induce development of C10.4 TCR_{trans}^{+} T cells. The function of the ND1/9mer in the development of C10.4 TCR_{trans}^{+} T cells would be determined with this strategy. Furthermore, the potential role of the cognate peptide in positive selection of C10.4 TCR_{trans}^{+} T cells would be explored.

Some theories of positive selection have implicated weak agonists, antagonists, or low expression levels of strong agonists in positive selection (Ashton-Rickardt et al., 1994; Hogquist et al., 1994; Sebzda et al., 1994; Wang et al., 1998). However, all of these theories were generated using data derived from T cell development induced with cognate or variant cognate peptides. Now that the naturally occurring self-peptide responsible for selection of C10.4 TCR_{trans}^{+} T cells was defined, the validity of these theories could be directly measured using the physiological ligand of positive selection. The data in this chapter was produced in collaboration with Rance Berg (Berg et al., 2000).

The first question we asked was whether or not peripheral C10.4 TCR_{trans}^{+} T cells could recognize the ND1/9mer, hereby probing the agonist activity of the ND1 self-peptide. C10.4 TCR_{trans}^{+} CTLs were generated from $C10.4^{+/+}RAG2^{-/-}$ mice and used as effectors in a standard CTL assay. We examined the ability of the ND1/9mer and different lengths of the cognate AttM peptide to sensitize targets for lysis by these cytolytic T cells (Figure 3). A control H2-M3 binding LemA peptide was unable to sensitize targets at any concentration tested. Consistent with results from previous studies (Princiotta et

al., 1998), the AttM/9mer peptide and AttM/6mer peptide behaved as strong agonists under serum free assay conditions. Surprisingly, the ND1/9mer was scored as a weak agonist. However, it was significantly less potent than the AttM/9mer peptide or AttM/6mer peptide in sensitizing targets. The ND1/9mer did sensitize targets to a similar degree as the AttM/5mer peptide. The other fM self-peptides able to sensitize targets for lysis by peripheral C10.4 TCR_{trans}^{+} T cells (Figure 11) were also able to induce positive selection of C10.4 TCR_{trans}^{+} T cells although only at the highest concentration tested and to a lesser extent than the ND1/9mer (Berg et al., 1999b). These data show that in the C10.4 TCR_{trans}^{+} system, the physiological ligand of positive selection functions as a weak agonist for peripheral CTLs.

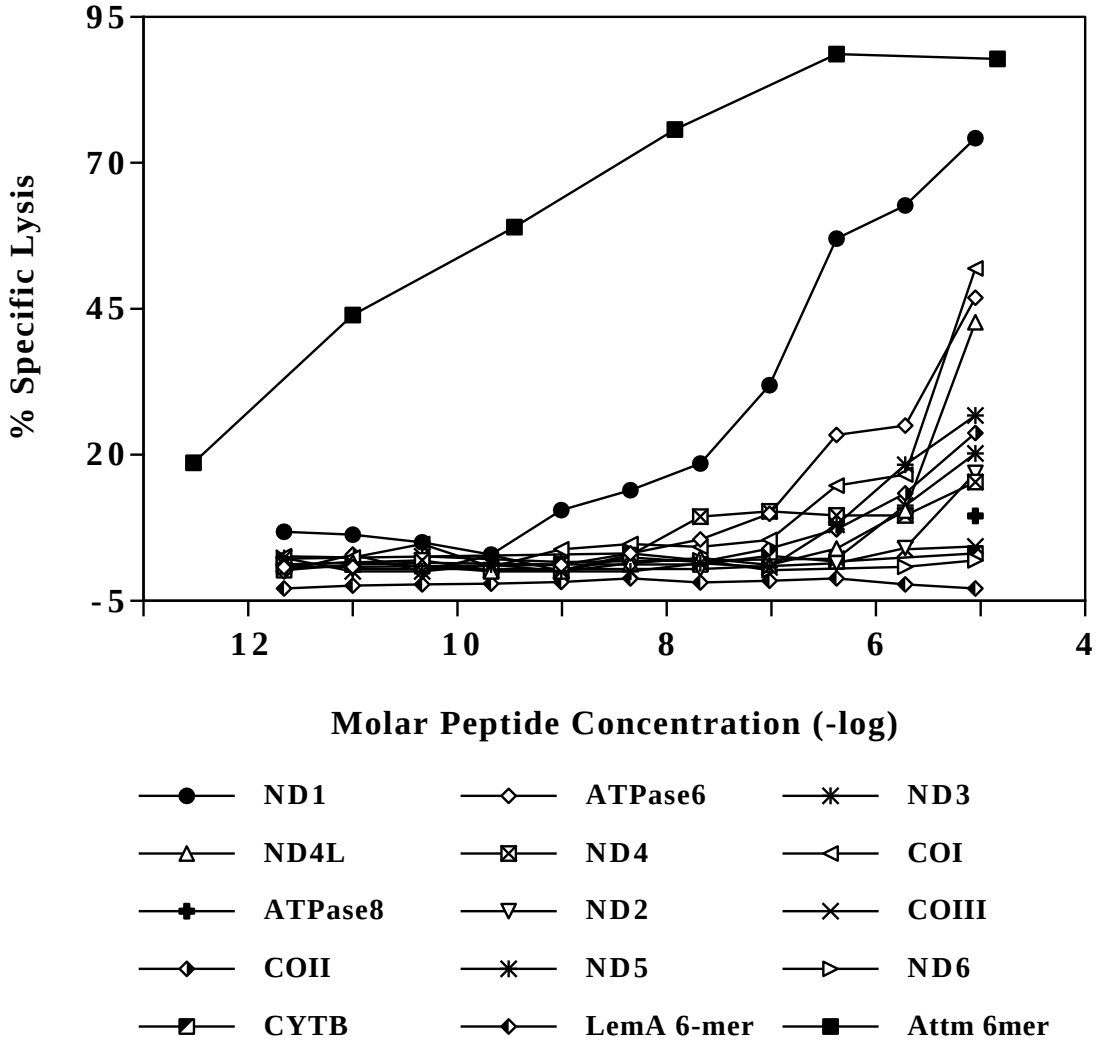
95
70
45
20
-5
% Specific Lysis
12
10
8
6
4
Molar Peptide Concentration (-log)
ND1
ATPase6
ND3
ND4L
ND4
COI
ATPase8
ND2
COIII
COII
ND5
ND6
CYTB
LemA 6-mer
Attm 6mer

Figure 11

FIGURE 11: AGONISTIC ACTIVITY OF THE MITOCHONDRIAL FM PEPTIDES

To test the agonistic activity of the 13 mitochondrial peptides were challenged in a standard CTL assay. Shortly, EL4 targets were labeled with ^{51}Cr and pulsed with the indicated peptide at the listed concentration. The cognate AttM/6mer functioned as a positive control, while the LemA/6mer was used as a negative control. Then C10.4$^{+/+}$ RAG2$^{-/-}$ CTLs were added at an E:T ratio of 5:1. After 4 hours supernatant was harvested and ^{51}Cr release was measured on an automatic gamma counter. Datapoints are means of duplicates.

Positive Selection Induced by the ND1/9mer and Cognate AttM Peptides

Using the C10.4$^{+/+}$TAP1$^{-/-}$ FTOC system described in Chapter II, the abilities of the ND1/9mer , the weak agonist AttM/5mer and the strong agonist AttM/9mer peptide to mediate positive selection were determined. The graph in Figure 12 depicts the percentages of CD8^{+}Vβ8^{+} T cells recovered from FTOCs incubated with different concentrations of the three peptides. The data clearly show that the ND1/9mer self-peptide and the AttM/5mer peptide, both weak agonists induce equivalent amounts of positive selection of C10.4 T cells, whereas the AttM/9mer, a full agonist, is about a 100 fold more potent.

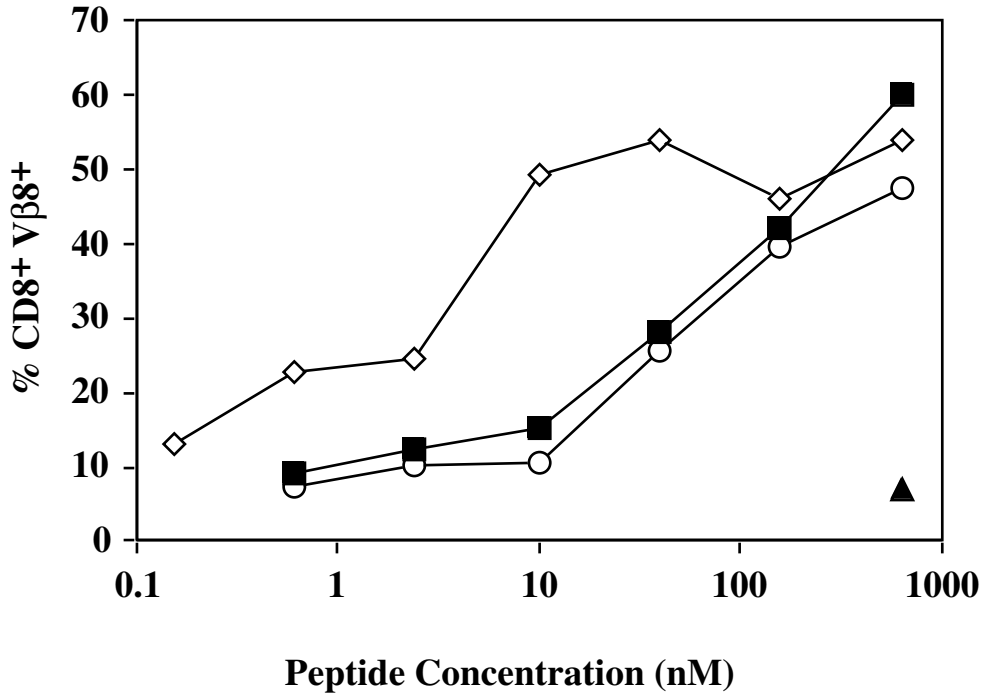

Figure 12

FIGURE 12: COMPARISON OF POSITIVE SELECTION ABILITIES OF THE ND1/9MER, THE ATTM/9MER PEPTIDE, AND THE ATTM/5MER PEPTIDE

A C10.4$^{+/+}$TAP$^{-/-}$ FTOC was performed as described in Chapter II. The ND1/9mer (filled squares), the AttM/9mer peptide (open diamonds), the AttM/5mer peptide (open circles), or no peptide control (filled triangle) were added to the FTOC media at the indicated concentrations. After six days in culture, the resulting cells were stained for CD4, CD8 and TCR levels using the F23.1 mAb. Data on the y-axes is presented as the percentage of CD8^{+} Vβ8^{+} T cells out of all live gated events. It shows the mean of two thymic lobes.

Prior to the described experiment we tested the potency of the AttM/6mer peptide in a FTOC system. We initially started with the 6mer version of the cognate peptide, since Mike Princiotta, a former graduate student in our lab, described the 6mer as the most potent peptide for his C10.4 clone. Figure 13 shows two curves, both depicting the percentages of $CD8^{+}V\beta8^{+}$ T cells recovered from FTOCs incubated with different concentrations of peptide. The filled circles shows the AttM/6mer peptide under FTOC conditions without the described peptidase inhibitors.

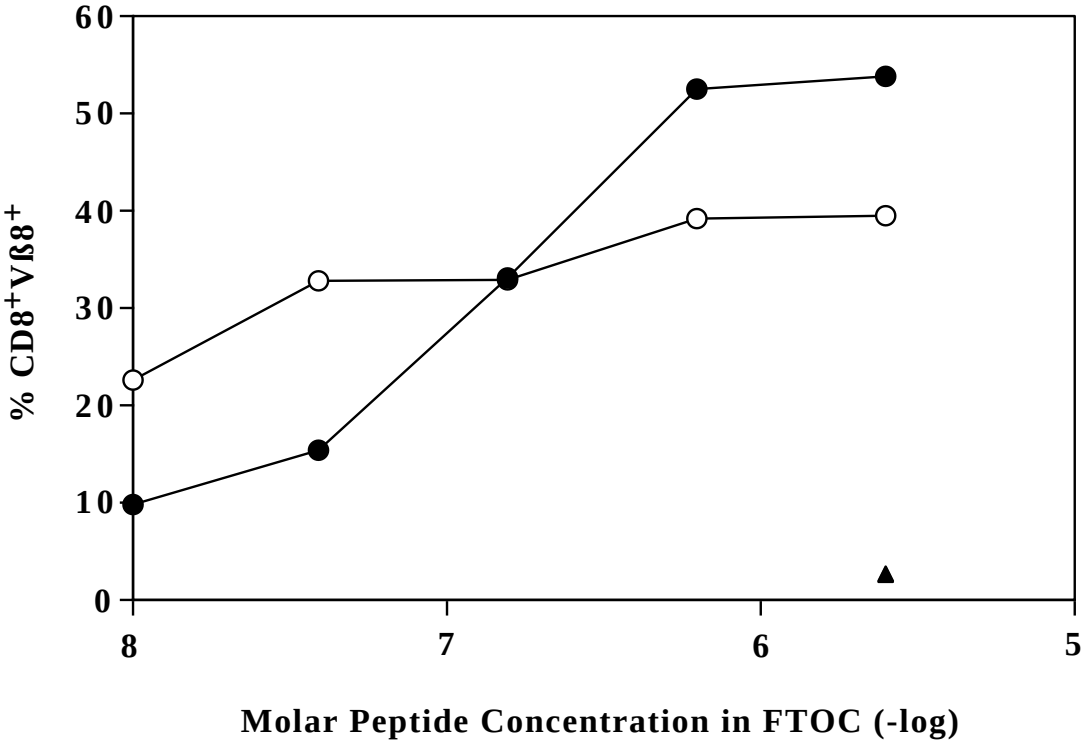

Figure 13

FIGURE 13: THE EFFECTS OF SERUM PEPTIDASES IN A FTOC WITH THE ATTM/6MER

Filled circles show percent CD8/Vβ8 emerging from FTOCs with the depicted peptide concentration of the AttM/6mer peptide in FTOC media without peptidase inhibitors. The numbers are clearly different from the numbers resulting from the same FTOC, yet with added peptidase inhibitor cocktail (empty circles). Background positive selection was determined using no peptide (filled triangle). All datapoints are expressed as a mean of two thymic lobes.

This result was very surprising to us, since the curve almost completely lines up with a similar curve from an FTOC exposed to the ND1/9mer, yet in the periphery there is a 100 to 1000 fold difference in recognition by mature, primed T cells if done in an *in vitro* assay using serum free medium. Since the only difference between the AttM/5mer and the AttM/6mer is the phenolalanine in position 6, we became curious to understand these functional differences. Previous work by other groups had demonstrated that fM-peptides are sensitive to degradation by peptidases in fetal calf serum (FCS), Dabhi and Lindahl, 1998; Princiotta et al., 1998). We therefore tested whether our peptide was similarly degraded by serum peptidases. We examined how a cocktail of peptidase inhibitors affected positive selection in the FTOC (Dabhi and Lindahl, 1998). This experiment is represented by the empty circles in Figure 13. Now the AttM/6mer induces a shift in the titration curve, we see more signs of negative selection at the highest peptide concentrations tested, and less peptide is required to induce positive selection. We therefore concluded that the AttM/6mer acts as a strong agonist under serum free conditions, yet in a six day FTOC, containing the necessary fetal calf serum, the peptide was either cut down to the AttM/5mer, or the amount of available AttM/6mer was reduced. To test this theory we performed a standard CTL assay. As shown in Figure 14, AttM/6mer exposed to FCS is less potent in sensitizing EL4 target cells for lysis by C10.4 clones. However if exogenous peptidase inhibitors are provided, the AttM/6mer keeps its strong agonist behavior.

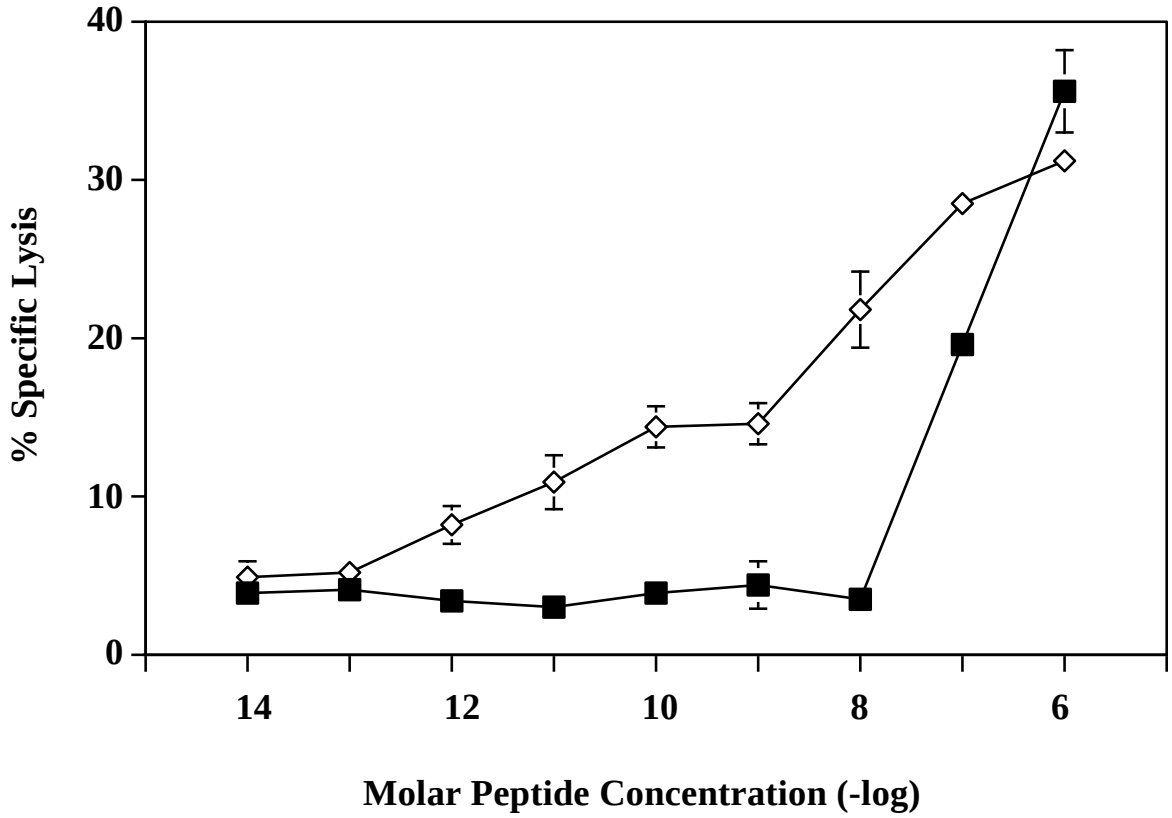

Figure 14

FIGURE 14: POTENCY OF THE USED PEPTIDASE INHIBITOR COCKTAIL

A standard CTL was performed to test the potency of the used peptidase inhibitors. EL4 target cells were labeled with ^{51}Cr and subsequently pulsed with the cognate AttM/6mer peptide, diluted either in complete IMDM (empty diamonds) or complete IMDM with a peptidase inhibitor cocktail (full boxes). Results are expressed as the mean of triplicates.

Degradation of Certain Cognate AttM Peptides in FTOC Media

We further examined the actual peptide integrity in the FTOC medium. The amino acid at position six of the AttM peptide is crucial for recognition by C10.4 T cells (Princiotta et al., 1998). Therefore degradation of the AttM/6mer at the C-terminus could convert it into a shorter peptide that would behave only as a weak agonist. FTOCs were established using either the ND1/9mer, the AttM/9mer peptide, or the AttM/6mer peptide. The media used in FTOCs without peptidase inhibitors was sampled at different time points. Since recognition of these three peptides can be monitored using a CTL assay with C10.4 TCR_{trans}^{+} T cells, the relative degree of peptide degradation in the FTOC media was determined. As a source of non-degraded peptides, media containing BSA (sfIMDM), instead of FCS, was used to dilute fresh peptides. The other sources of peptide were from FTOC media containing the peptides at known concentrations (either freshly prepared, after incubation in a FTOC for one day, or after incubation in a FTOC for six days). As depicted in Figure 15 the ND1/9mer self-peptide was not degraded in the FTOC media at any of the time points tested. All sources of the peptide sensitized targets equally well for killing by C10.4 TCR_{trans}^{+} CTLs. However, the AttM/6mer peptide was degraded in FTOC media containing FCS (Figure 17). Supernatants from FTOC cultures containing AttM/6mer peptide concentration of 100 nM did not sensitize target cells for lysis. In contrast, the AttM/6mer peptide diluted in serum free media sensitized targets down to the pM range. Even the AttM/6mer peptide that was freshly made in FTOC media containing FCS was degraded to a considerable extent. The AttM/9mer peptide (another strong agonist) was then tested for its stability in FTOC media (Figure 16). The addition of the extra three amino acids was sufficient to make the longer cognate peptide resistant to degradation in the FTOC media. Even after a six day culture in FTOC media, this peptide did not loose activity in sensitizing targets. Therefore we concluded, that the AttM/6mer lost its activity in the FTOC media, whereas the 9mer versions of either the cognate or the self-peptide remained stable.

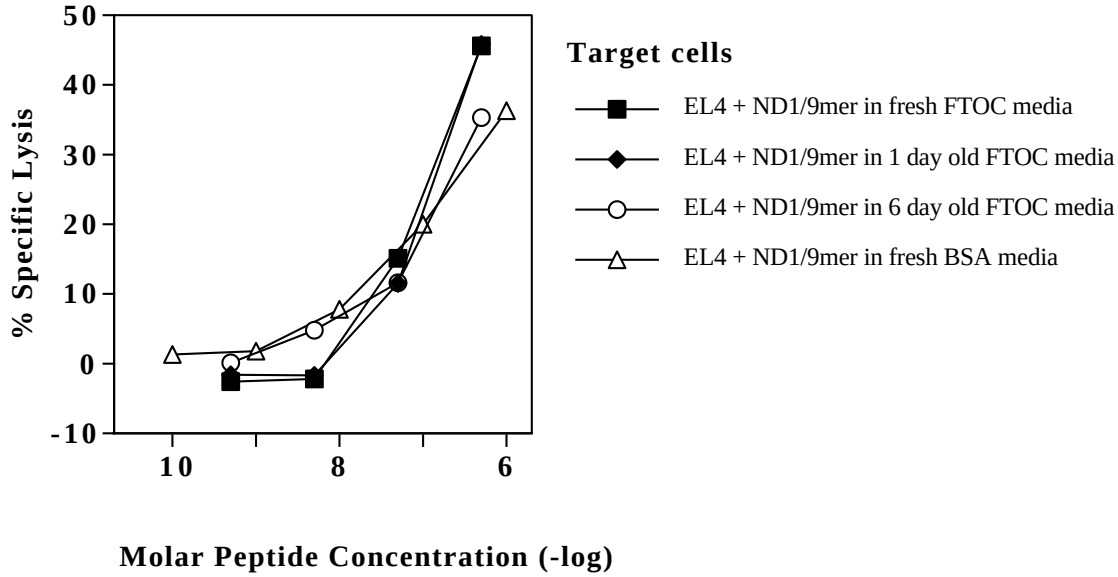

Figure 15

FIGURE 15: THE ND1/9MER IS STABLE IN FTOC MEDIA

Effectors from a C10.4 TCR_{trans}^{+} T cell line generated from a C10.4$^{+/+}$RAG2$^{-/-}$ mouse were used in a standard CTL assay at an E:T ratio of 5:1. The ND1/9mer was used to sensitize EL4 target cells. The source of the ND1/9mer was from either FTOC media (containing FCS) or from media containing BSA instead of FCS. The peptides were added to the EL4 target cells at the indicated concentrations. The filled squares are the ND1/9mer in fresh FTOC media. The filled triangles are the ND1/9mer in FTOC media that had been incubated with thymic lobes for one day. The open circles are the ND1/9mer in FTOC media that had been incubated with thymic lobes for six days. The open triangles are the ND1/9mer in fresh BSA media (no FCS). Data is presented as percent specific lysis and is the mean of triplicate determinations for each data point.

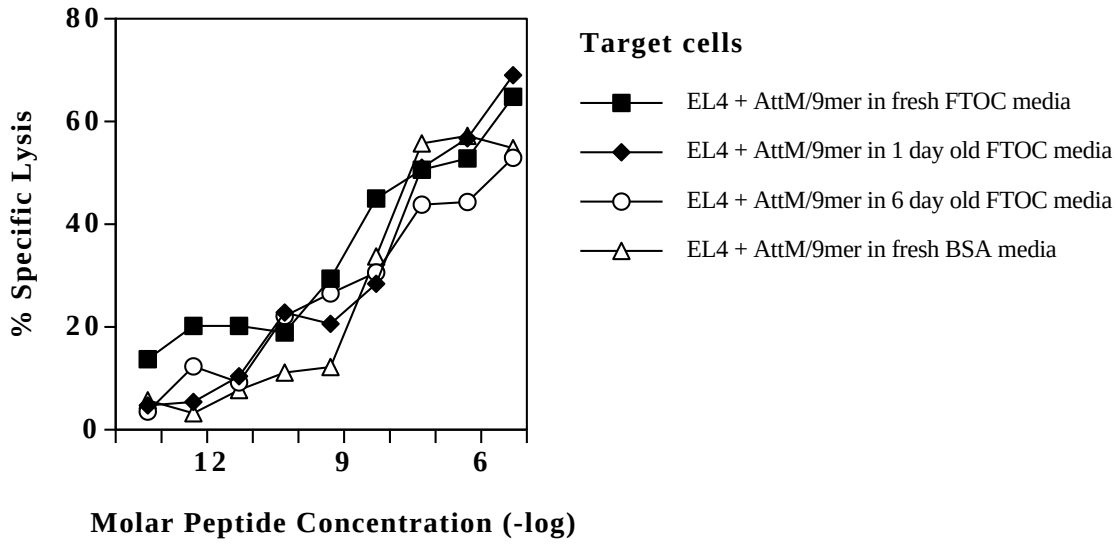

Figure 16

FIGURE 16: THE ATTM/9MER PEPTIDE IS STABLE IN FTOC MEDIA

Effectors from a C10.4 TCR_{trans}^{+} T cell line generated from a C10.4$^{+/+}$RAG2$^{-/-}$ mouse were used in a standard CTL assay at an E:T ratio of 5:1. The AttM/9mer peptide was used to sensitize EL4 target cells. The source of the AttM/9mer peptide was from either FTOC media (containing FCS) or from media containing BSA instead of FCS. The peptides were added to the EL4 target cells at the indicated concentrations. The filled squares are the AttM/9mer peptide in fresh FTOC media. The filled triangles are the AttM/9mer peptide in FTOC media that had been incubated with thymic lobes for one day. The open circles are the AttM/9mer peptide in FTOC media that had been incubated with thymic lobes for six days. The open triangles are the AttM/9mer peptide in fresh BSA media (no FCS). Data is presented as percent specific lysis and is the mean of triplicate determinations for each data point.

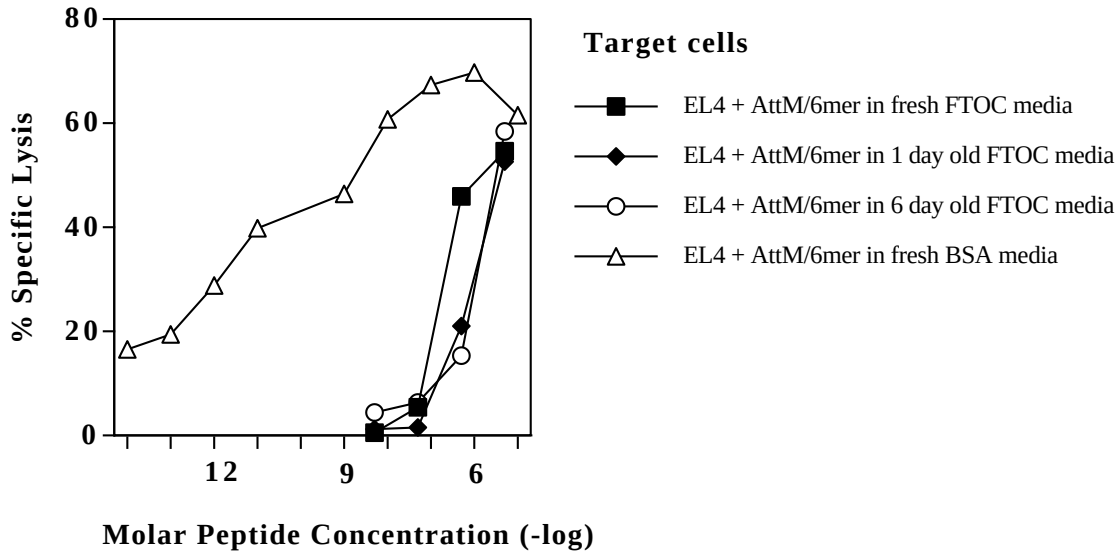

Figure 17

FIGURE 17: THE ATTM/6MER PEPTIDE IS DEGRADED IN FTOC MEDIA

Effectors from a C10.4 TCR_{trans}^{+} T cell line generated from a C10.4$^{+/+}$RAG2$^{-/-}$ mouse were used in a standard CTL assay at an E:T ratio of 5:1. The AttM/6mer peptide was used to sensitize EL4 target cells. The source of the AttM/6mer peptide was from either FTOC media (containing FCS) or from media containing BSA instead of FCS. The peptides were added to the EL4 target cells at the indicated concentrations. The filled squares are the AttM/6mer peptide in fresh FTOC media. The filled triangles are the AttM/6mer peptide in FTOC media that had been incubated with thymic lobes for one day. The open circles are the AttM/6mer peptide in FTOC media that had been incubated with thymic lobes for six days. The open triangles are the AttM/6mer peptide in fresh BSA media (no FCS). Data is presented as percent specific lysis and is the mean of triplicate determinations for each data point.

Positive Selection Induced by the ND1/9mer and Cognate AttM Peptides

In the C10.4 TCR_{trans}^{+} system, the ND1/9mer functions as the physiological ligand of positive selection and a *L. monocytogenes* derived AttM peptide is recognized as the cognate peptide by effector T cells. As mentioned earlier, these two peptides did not show any apparent sequence homology. Peripheral C10.4 TCR_{trans}^{+} T cells recognize the ND1/9mer as a weak agonist (Figure 3). The AttM/5mer peptide is recognized to a similar extent. The AttM/9mer peptide is recognized by peripheral C10.4 TCR_{trans}^{+} T cells approximately 100-fold better than either one of the two weak agonists.

In the next set of experiments, the abilities of the ND1/9mer, the AttM/9mer peptide, and the AttM/5mer peptide to induce T cell maturation in the $C10.4^{+/+}TAP^{-/-}$ FTOC system were compared. Each of the three peptides was titrated into the FTOCs from 625 nM to either 610 pM (for the ND1/9mer and the AttM/5mer peptide) or 152 pM (for the AttM/9mer peptide). After a six day incubation, thymocytes were harvested from the FTOCs and analyzed for surface expression of CD4, CD8, TCR and CD24. Figure 12 shows a graph giving a representative overview over the percent of $CD8^{+}V\beta8^{+}$ resulting from an FTOC induced with the indicated peptides. In Figure 9 and Figure 18 representative flow cytometric data are shown for FTOCs induced with the ND1/9mer and a negative control (containing 0.1% DMSO, used as the fM peptide diluent). The flow cytometry data depict staining for the highest concentration tested (625nM), the lowest concentration tested (610pM), and the concentration that resulted in the selection of the most efficient CTLs (39nM) for the ND1 peptide. At 625 nM, all three peptides induced strong phenotypic shifts, driving immature thymocytes to become $CD8^{+}V\beta8^{+}$ T cells.

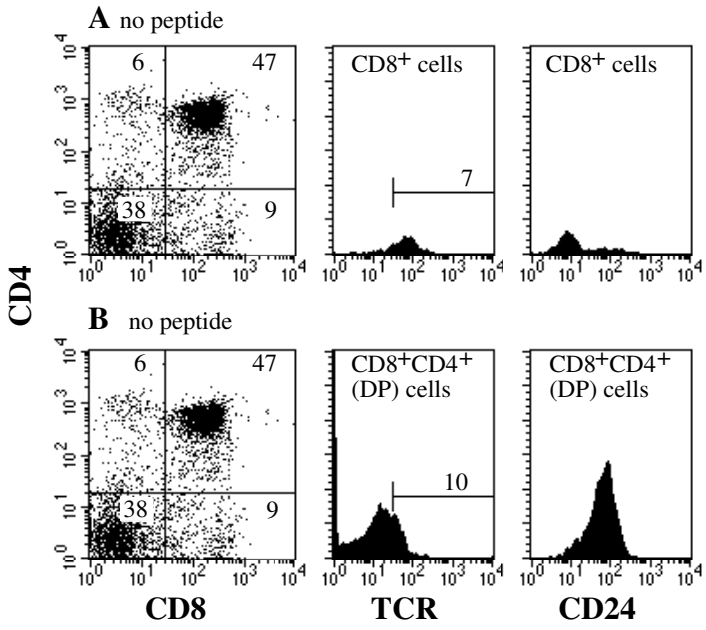

Figure 18

FIGURE 18: CONTROL C10.4$^{+/+}$TAP$^{-/-}$ FTOC STAINED FOR CD4, CD8, TCR, AND CD24

A control C10.4$^{+/+}$TAP$^{-/-}$ FTOC to which no peptide had been added was incubated for six days and the resulting thymocytes were stained for CD4, CD8, CD24, and TCR expression levels using the F23.1 mAb. Dot plots show CD4 versus CD8 staining and the numbers within the quadrants represent the percentage of all live gated events within the indicated quadrant. The first column of histograms shows F23.1 staining, while the second column depicts CD24 staining. The histograms in panel A show staining on CD8 SP T cells, while the histograms in panel B show staining on CD4/CD8 DP T cells. The numbers within the first column of histograms indicates either the percentage of CD8$^+$Vβ8$^+$ T cells out of all the live gated events (panel A), or the percentage of CD4$^+$CD8$^+$Vβ8$^+$ T cells out of all live gated events (panel B).

Another measure of thymocyte maturity was analyzed to ensure these $CD8^+V\beta8^+$ T cells had undergone positive selection. It had been suggested that positive selection results in the down-regulation of CD24, also known as the Heat Stable Antigen (HSA) (Crispe and Bevan, 1987; Lucas et al., 1994). Indeed, the newly appearing $CD8^+$ Single Positive (CD8 SP) T cells no longer expressed CD24 (Figure 9). In control FTOCs that had been incubated with 0.1% DMSO, the majority of cells consisted of immature $CD4^+CD8^+$ Double Positive (CD4/CD8 DP) cells with low levels of TCR and high levels of CD24 (Figure 18). This data suggested that the ND1/9mer could induce phenotypic positive selection of C10.4 TCR_{trans}^+ T cells.

In an unmanipulated $C10.4^{+/+}TAP^{+/+}$ mouse, approximately 20% of the thymocytes are $CD8^+V\beta8^+$ T cells, representing the physiological situation (Berg et al., 1999b). Peptide concentrations of approximately 39 nM of the ND1/9mer and the AttM/5mer peptide were required to induce this percentage of $CD8^+V\beta8^+$ T cells in FTOCs from $C10.4^{+/+}TAP^{-/-}$ mice (Figure 12). A lower concentration (approximately 2.4 nM), of the AttM/9mer peptide was required to induce these levels of positive selection. The AttM/9mer peptide lost activity at a concentration of 152 pM. Neither the ND1/9mer nor the AttM/5mer peptide showed any activity at 2.4 nM. Several conclusions can be drawn from the above set of experiments. A strong agonist, such as the AttM/9mer peptide, induced phenotypic shifts to $CD8^+V\beta8^+$ T cells at significantly lower concentrations than the physiological ligand of positive selection. The ND1/9mer and the partially agonistic AttM/5mer peptide behaved in a similar manner, even though they did not show any apparent sequence homologies and are of different length. Because both the ND1 and the AttM peptides are able to induce positive selection, C10.4 TCR_{trans}^+ thymocytes are not able to distinguish between self and foreign peptides during positive selection in the thymus. The only distinguishing feature of the peptides to the T cells was either an increased affinity of the TCR for the AttM/9mer peptide/MHC complex or an increased binding ability of this peptide for H2-M3, however the given peptide is seen with high specificity, as shown in chapter V.

Functionality of C10.4 TCR_{trans}^{+} T Cells after Selection with the ND1/9mer or the Cognate AttM Peptides

Although C10.4$^{+/+}$TAP$^{-/-}$ FTOCs incubated with self peptides or cognate AttM peptides had produced mature CD8^{+}Vβ8^{+} T cells, the possibility existed that these T cells were not functional. Indeed, previous reports had suggested that strong agonist peptides do not induce the positive selection of functional T cells (Hogquist et al., 1995; Girao et al., 1997). Therefore thymocytes resulting from C10.4$^{+/+}$TAP$^{-/-}$ FTOCs were expanded on H57-597 coated plates and used in a CTL assay to determine their lytic potential. When the functional capabilities of thymocytes developing from the C10.4$^{+/+}$TAP$^{-/-}$ FTOCs were examined, several observations were made. All cultures, even those established from control FTOCs to which peptide had not been added, underwent vigorous expansion (at least 7-fold, data not shown). When the different peptides were added, expansion of thymocytes increased up to approximately 15-fold (Berg et al., 1999a). Control C10.4$^{+/+}$TAP$^{-/-}$ FTOC thymocytes, which were selected in the presence of 0.1% DMSO did not contain thymocytes that gave rise to lytic C10.4 TCR_{trans}^{+} T cells (Figure 10). The ND1/9mer, the AttM/9mer peptide, and the AttM/5mer peptide all induced the maturation of lytic C10.4 TCR_{trans}^{+} T cells at specific peptide concentrations in the FTOCs. The ND1/9mer and the AttM/5mer peptide both efficiently selected CTLs at an optimal dose of 39 nM, while the AttM/9mer peptide was able to induce the most efficient CTL activity at 2.4 nM. None of the thymocytes tested were able to lyse targets coated with an H2-M3 binding control peptide; LemA (Berg et al., 1999a); indicating that specificity was maintained.

Although the appearance of AttM specific CTLs in the *in vitro* expansion cultures was dependent on the presence of peptide induced CD8^{+} C10.4 TCR_{trans}^{+} thymocytes, not all peptide-induced T cells were fully functional. Generation of fully functional CTLs was most efficient in FTOCs that contained levels of CD8^{+}Vβ8^{+} T cells (approximately 20%) seen under physiological conditions in the unmanipulated C10.4$^{+/+}$TAP$^{+/+}$ (Berg et al., 1999b). For the ND1/9mer and the weak agonist, AttM/5mer peptide, this percentage was

reached at approximately 39 nM, while for the strong agonist AttM/9mer peptide, less peptide (2.4 nM) was required. At lower than optimal peptide concentrations, the number of positively selected cells and thus the number of expanded CTLs decreased. Increasing the concentration of the relevant peptides above optimal concentrations for CTL development resulted in an even higher frequency of CD8 SP T cells, but did not boost the number of fully functional T cells. Instead, the lytic activity of the T cells decreased and eventually reached background levels. It seemed as though a mechanism similar to peripheral inactivation (tolerance) was occurring at the level of the thymus.

The Ability of the ND1/9mer and Cognate AttM Peptides to Bind H2-M3

Since the stability of a peptide/MHC complex is a crucial component of T cell recognition, the abilities of the ND1/9mer and the two cognate AttM peptides to bind H2-M3 were compared. H2-M3 preferentially binds fM-peptides (Loveland et al., 1990; Shawar et al., 1990). However, a peptide motif that predicts binding to H2-M3 has not yet been defined. Therefore, an H2-M3 upregulation assay to measure the relative abilities of the ND1/9mer, the AttM/9mer peptide, and the AttM/5mer peptide to bind to H2-M3 was utilized (Vyas et al., 1994). 13S2 fibroblasts that had been transfected with the chimeric H2-M3^{wt}/L^{d} molecule were incubated overnight with the ND1/9mer, the AttM/9mer peptide, or the AttM/5mer peptide. Surface expression of the chimeric H2-M3^{wt}/L^{d} molecule, as a measure of peptide/MHC stability and thus relative affinity, was determined using the anti-L^{d} specific mAb, 28-14-8S (Figure 19 and Figure 20). A conventional non-fM peptide derived from ovalbumin (OVA) was used as a negative control. This OVA peptide did not up-regulate H2-M3 expression over background staining levels. All three fM-peptides bound to H2-M3 and stabilized its expression in a manner similar to other high affinity fM-peptides (Dabhi et al., 1998; Chiu et al., 1999). The ND1/9mer bound H2-M3 slightly better than either the AttM/9mer peptide or the AttM/5mer peptide. Therefore, differences in the abilities of the peptides to positively select C10.4 TCR$_{trans}^{+}$ thymocytes, or to activate peripheral C10.4

TCR_{trans}^{+} T cells, were not attributed to different binding affinities of the peptides to H2-M3.

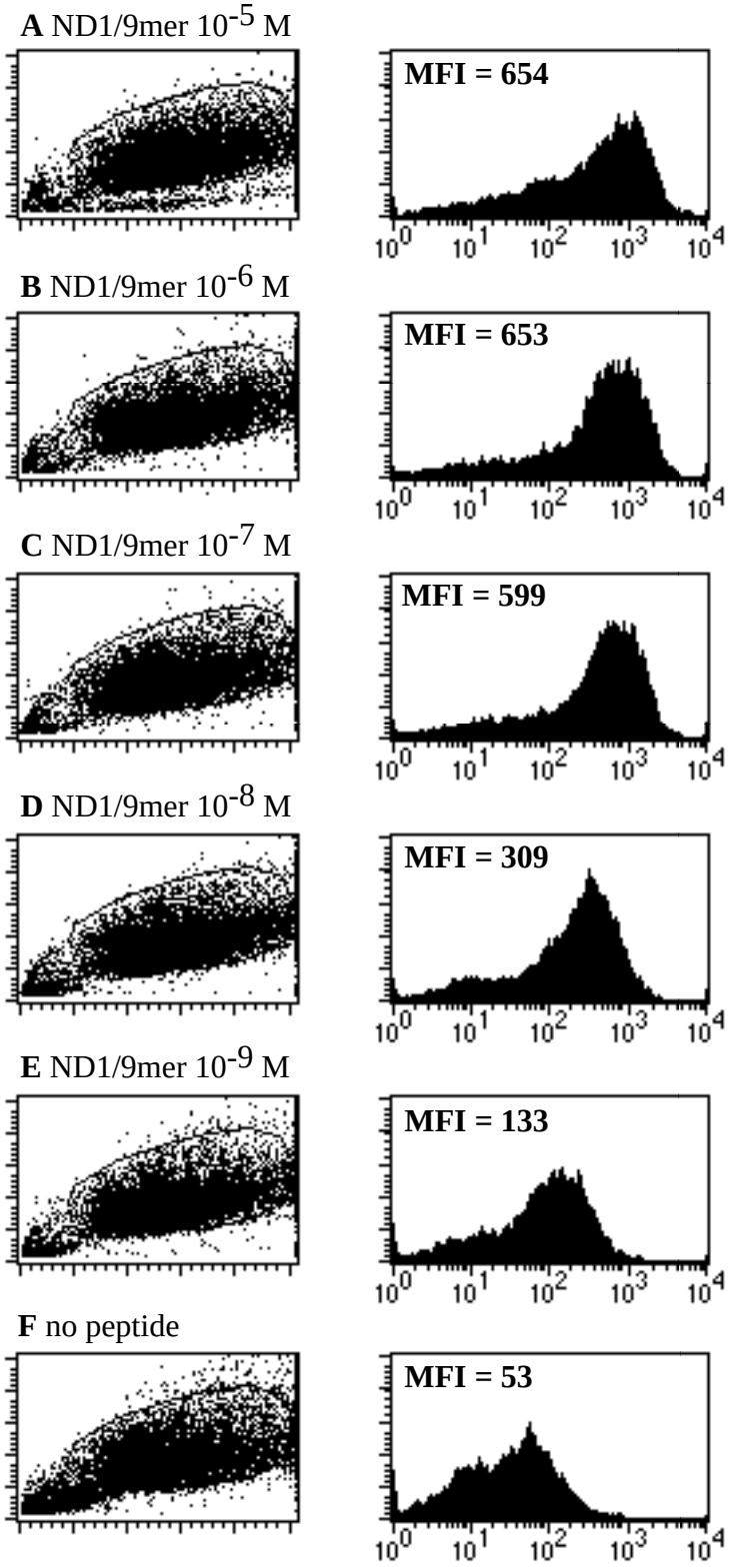
A ND1/9mer 10-5 M
MFI = 654
B ND1/9mer 10-6 M
MFI = 653
C ND1/9mer 10-7 M
MFI = 599
D ND1/9mer 10-8 M
MFI = 309
E ND1/9mer 10-9 M
MFI = 133
F no peptide
MFI = 53
10^0 10^1 10^2 10^3 10^4

Figure 19

FIGURE 19: PEPTIDE BINDING TO H2-M3

To determine how the different peptides of this study bind to H2-M3, We performed an H2-M3 upregulation assay. Shortly 13S2 fibroblasts (a B10.cas tail fibroblast cell line transfected with a chimeric Class I molecule, that uses the α1 and α2 domain of H2-M3 ^{wt} and the α3 domain of L^d) was incubated overnight at 27^0C with the indicated peptide concentrations. The next day H2-M3 surface levels were detected using the biotinylated 28-14-8S monoclonal antibody, specific for the α3 domain of L^d, and Streptavidin-PE as a second step reagent. The first column shows the forward/side scatter pattern as determined by flow cytometry. The second column shows the histogram plot for the 28-14-8S antibody and the resulting mean fluorescence intensity. Panel F shows a negative control where the cells have not been pulsed with peptide. Similar results were obtained in 2 consecutive experiments.

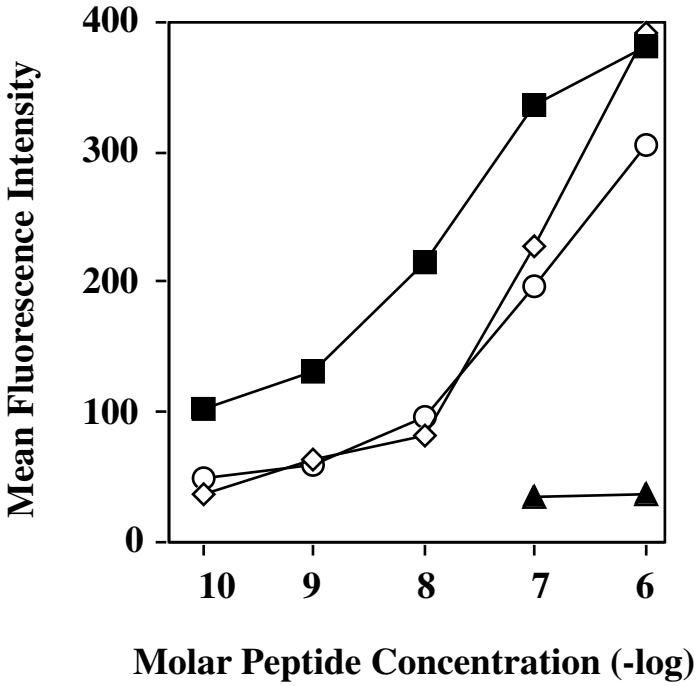

Figure 20

FIGURE 20: OVERVIEW OF THE BINDING CHARACTERISTICS OF THE ND1/9MER, THE ATTM/9MER AND THE ATTM/5MER

This graph shows the surface expression of the chimeric H2-M3^{wt}/L^{d} molecule over the titration range of the ND1/9mer peptide (filled boxes), the AttM/9mer peptide (empty diamonds) and the AttM/5mer (empty circles). Filled triangles show the negative control SIINFEKL.

Summary

The results presented in this chapter described the nature of a physiological ligand of positive selection, such as the ND1/9mer. Comparison of the ND1/9mer and the cognate AttM peptide revealed that the ND1/9mer functions as a weak agonist, both for C10.4 TCR_{trans}^{+} thymocytes and for peripheral C10.4 TCR_{trans}^{+} T cells. The data further describe selection of functional C10.4 TCR_{trans}^{+} T cells with the strong agonist AttM/9mer peptide. Other groups have been unable to induce functional T cells with agonist peptides.

Selection of $CD8^{+}V\beta8^{+}$ thymocytes that had the ability to proliferate, but not to mediate cytolytic functions, was established and these T cells were subsequently examined. Although the exact reason why these T cells did not possess lytic capabilities was not finally determined. Finally, by determining the relative concentrations of peptides needed for positive selection and peripheral activation, two mechanisms used to avoid autoimmunity can be discussed. The data presented suggests that positive selection occurs within a narrow window of avidity. Above this avidity, T cells are rendered anergic by an unknown mechanism. These T cells were shown to be unable to perform cytolytic functions. In addition, T cells become less sensitive to weak agonists after they survive the positive selection process. This should ensure that they cannot respond to the same peptide that they recognized in the thymus. However, this same peptide may be responsible for maintaining T cells in the periphery.

CHAPTER V

Peptide Specificity During Positive Selection

Design of ND1 Peptide Mutants

The exact lengths of naturally occurring peptides involved in positive selection have not been determined. Rance Berg had chosen the 9 amino acid length of the ND1/9mer for the original studies of positive selection, for reasons mentioned in chapter III.

Since we already knew that positive selection of C10.4 TCR_{trans}^{+} T cells depended on the N-terminal fM group, we looked for crucial residues at the C - terminus of the ND1/9mer by removing C-terminal amino acids (ND1/8mer and ND1/7mer). In the crystal structure of the H2-M3/ND1 complex, amino acid side chains at position P2, P5, P8 and P9 were surface exposed (Wang et al., 1995). In addition, functional studies demonstrated that some specific CTLs recognized amino acid side-chains in position P6 (Loveland et al., 1990; Princiotta et al., 1998), indicating that position 6 is seen by the TCR. Therefore, we synthesized ND1/9mer peptide variations in which the amino acids in position P2, P5, P6, P8 and P9 were exchanged for the amino acid alanine or the amino acids in positions P8 or P8 and P9 were removed (Table 1).

TABLE 1: NOMENCLATURE, SEQUENCE AND BINDING OF THE PEPTIDES USED IN THIS STUDY

This table shows the name and the sequence variations of the peptides compared to the ND1/9mer ('x' being the original amino acid, '-' indicating a removed amino acid). It also shows the peptide concentration required for half maximal H2-M3 binding.

Name	Sequence	Peptide concentration[1]
ND1/9mer	f-MFFINILTL	1.2×10^{-8}
ND1/8mer	f-xxxxxxxx-	2.8×10^{-8}
ND1/7mer	f-xxxxxxx--	77×10^{-8}
ND1/P2	f-xAxxxxxxx	44×10^{-8}
ND1/P5	f-xxxxAxxxx	29×10^{-8}
ND1/P6	f-xxxxxAxxx	7.4×10^{-8}
ND1/P8	f-xxxxxxxAx	33×10^{-8}
ND1/P9	f-xxxxxxxxA	10×10^{-8}
ND1/P89	f-xxxxxxxAA	19×10^{-8}
ND1/P2589	f-xAxxAxxAA	42×10^{-8}

[1] Peptide Concentration required for half maximal binding to the chimeric H2-M3^{wt}/L^{d} molecule as determined by MFI of the geometric mean of two independent experiments.

Binding of the Peptide Mutants to H2-M3

It would be impossible to judge the capabilities of the peptide mutants to induce positive selection without testing their binding potency to H2-M3. So we first studied how changes in the ND1/9mer peptide sequence affected binding to H2-M3. We measured the relative binding abilities of the different ND1 peptide mutants utilizing the H2-M3 upregulation assay described in chapter IV. The peptide concentration required for half maximum upregulation of the H2-$M3^{wt}/L^{d}$ molecule is shown in Table 1. Binding of a non-fM peptide, the ovalbumin-derived SIINFEKL, to H2-M3 was not detectable above background levels (Irion et al., 1999). Removing the amino acid in position P9 (ND1/8mer) did not alter affinity to H2-M3 (Table 1). The ND1/7mer peptide, in which the two C-terminal amino acids had been removed, required 60-fold higher peptide concentrations for half-maximal H2-M3 stabilization compared to the concentration of ND1/9mer required for this stabilization. Changing position 9 (ND1/P9) to an alanine did not decrease the stability of the MHC / peptide complex. However, individual changes at position 2 (ND1/P2), 5 (ND1/P5), 6 (ND1/P6), or 8 (ND1/P8), as well as multiple changes at positions 8 and 9 (ND1/P89) or 2, 5, 8, and 9 (ND1/P2589) reduced peptide binding by 6- to 36-fold.

Stability of a MHC / peptide complex is a crucial component of T cell recognition. Therefore, these binding studies would help us to discern to what extent changes in the function of the different peptide variants could be attributed to altered binding affinities to H2-M3 molecules.

Recognition of ND1 Mutant Peptides in an FTOC system

Positive selection induces shifts in the surface phenotype of immature thymocytes and leads to the functional maturation of T cells. We used the FTOC system described above to study how mutations within the ND1/9mer sequence affected this T cell differentiation process. $C10.4^{+/+}TAP1^{-/-}$ FTOCs were supplemented with decreasing concentrations of the different peptide variants. After 6 days of culture, the thymic lobes were harvested and the

developing thymocytes were analyzed for surface expression of CD4, CD8, CD24 (were indicated) and TCR (using the F23.1 and H57-597 mAbs). The ND1/9mer induced physiological levels of $CD8^{+}V\beta8^{+}$ SP thymocytes (20 to 30%) when added at 39nM (Figure 9 B; Berg et al., 1999). None of the peptide variants was as effective (Figure 21).

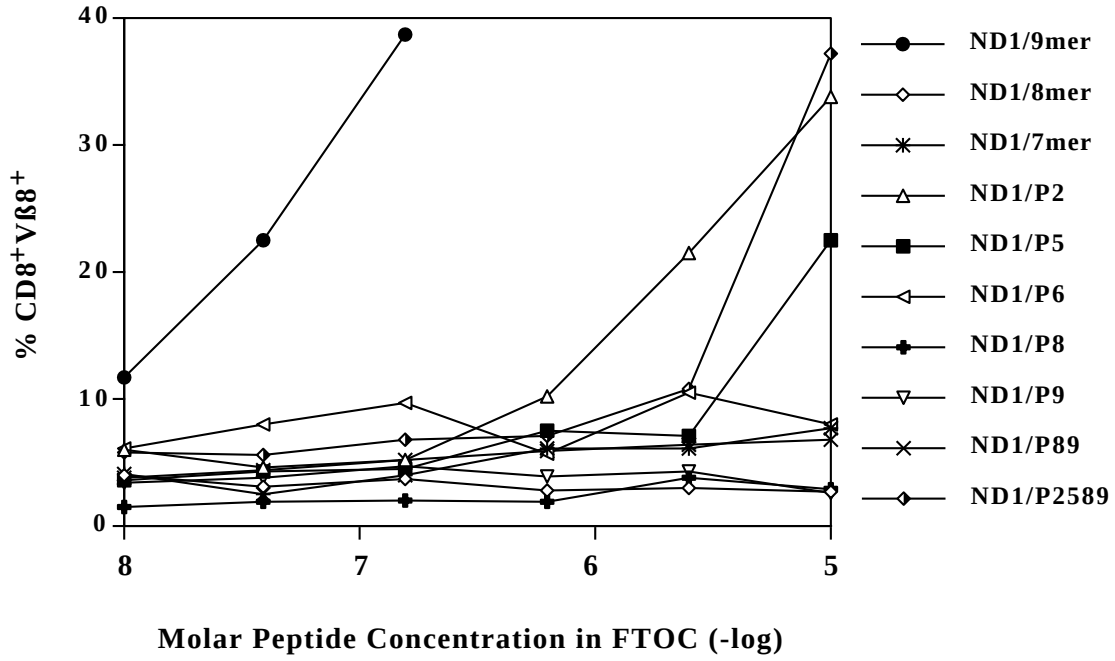

Figure 21

FIGURE 21: DEPENDENCE OF POSITIVE SELECTION ON THE CONCENTRATIONS OF THE PEPTIDE MUTANTS

An overview of the percentage of $CD8^+V\beta8^+$ positively selected T cells is provided covering the titration range of each peptide mutant used in FTOCs (as indicated in the Figure). Percentages are expressed as the mean of two thymic lobes.

Staining patterns for these peptides are presented for the highest concentration only due to the lack of selection in the lower peptide concentration range (Irion et al., 2000; Figure 22). Neither the ND1/8mer nor the ND1/7mer induced thymocyte maturation at the highest peptide concentrations of 10 µM even though the ND1/8mer bound to H2-M3 with high affinity. One of the peptide mutants, namely the ND1/P2, induced phenotypic shifts towards mature thymocytes at the two highest peptide concentrations. However, even the most potent peptide mutant (ND1/P2) was at least 100-fold less effective than the ND1/9mer in inducing positive selection in this assay. The ND1/P5 and the ND1/P2589 peptides showed activity only at the highest peptide concentration of 10 µM.

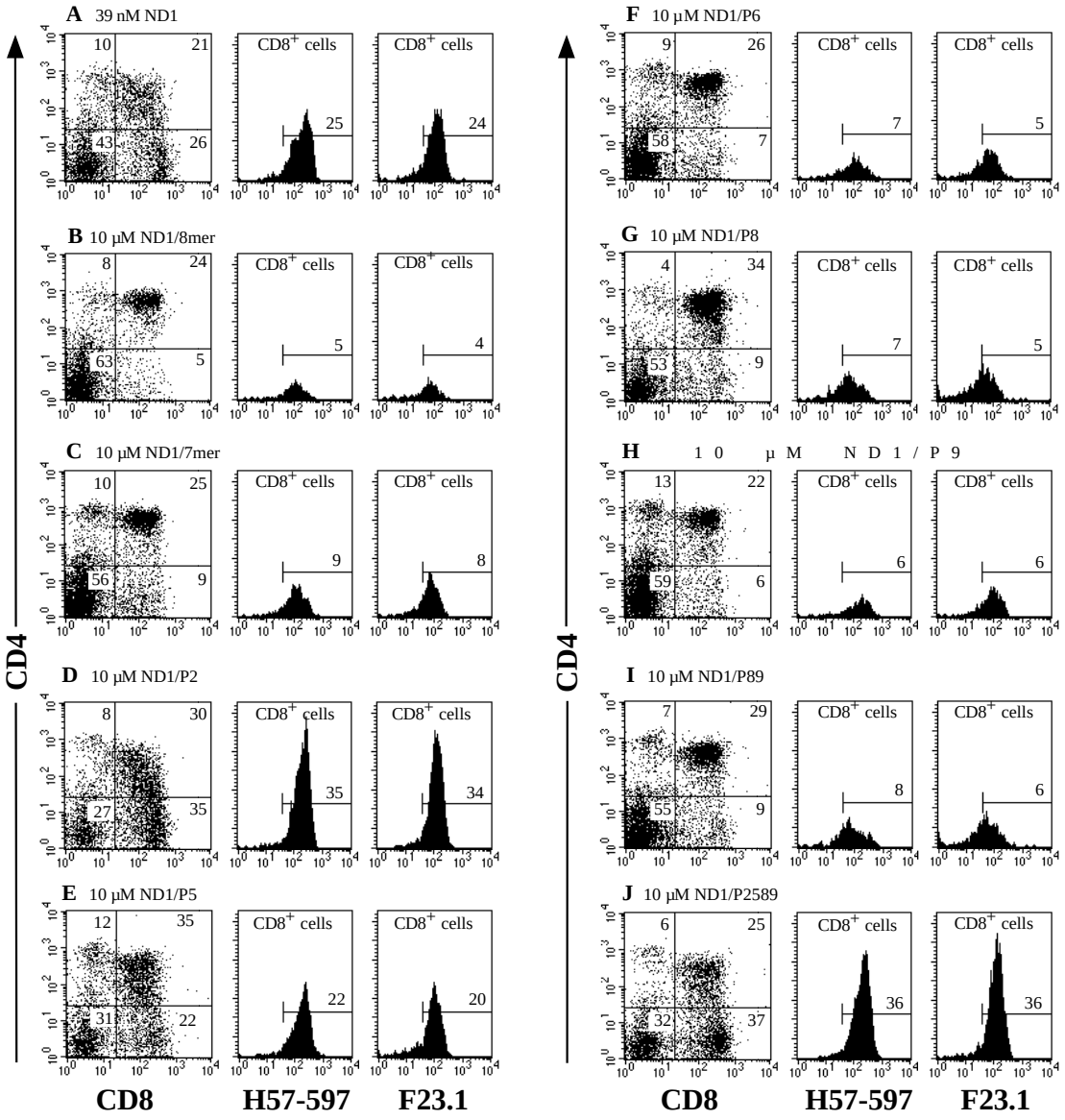
A 39 nM ND1
B 10 µM ND1/8mer
C 10 µM ND1/7mer
D 10 µM ND1/P2
E 10 µM ND1/P5
F 10 µM ND1/P6
G 10 µM ND1/P8
H 10 µM ND1/P9
I 10 µM ND1/P89
J 10 µM ND1/P2589
CD4
CD8
CD8+ cells
H57-597
F23.1

Figure 22

FIGURE 22: FTOCS USING DIFFERENT ND1 VARIATIONS

The peptide variants were added at the indicated concentration to C10.4$^{+/+}$ TAP1$^{-/-}$ FTOCs. After 6 days in the FTOC, thymocytes were brought into a single cell suspension and analyzed by flow cytometry using the mAbs shown. Panel (A) shows the staining patterns for cells selected on 39 nM ND1/9mer. Panels B through J show results for the different peptide mutants (all at 10 µM). Each quadrant label shows percentage of total live gated cells (as determined by forward / side scatter). The marker labels show percentage CD8^{+}Vβ8^{+} or CD8^{+}TCR^{+} T cells out of all live gated cells. Similar results were achieved in at least 2 consecutive experiments.

As mentioned earlier positive selection not only induces phenotypic T cell maturation, but also generates functional T cells. Indeed, we had demonstrated earlier that positively selected C10.4 TCR_{trans}^{+} thymocytes, when expanded *in vitro* by TCR cross-linkage, developed into specific CTLs (Berg et al., 1999b). In the thymus of normal C10.4 $TCR_{trans}^{+/+}$ $TAP1^{+/+}$ mice, between 20 to 30% of thymocytes are of the $CD8^{+}$ TCR^{hi} phenotype. This level of mature-type T cells was reached in FTOCs to which ND1/9mer (39 nM), ND1/P2 (2.5 µM), ND1/P5 (10 µM) or ND1/P2589 (10 µM) had been added. Thymocytes were harvested from all FTOCs, expanded and tested for specific CTL responses (Figure 23). $CD8^{high}$ TCR^{high} T cells proliferated from all FTOCs, including those that had not shown signs of positive selection (Irion et al., 1999). Only the ND1/9mer, the ND1/P2 and the ND1/P2589 peptides induced the development of specific CTLs. The ND1/P5 peptide that had been the weakest inducer of the phenotype shift showed only marginal CTL activity. T cells grown from any of the other cultures did not show any specific lytic activity.

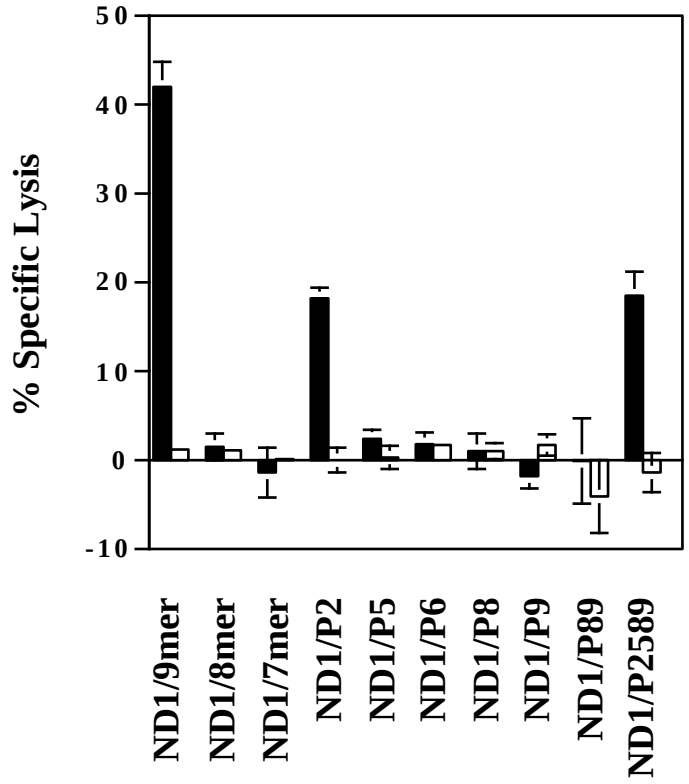

Figure 23

FIGURE 23: FUNCTIONALITY OF THE MATURED THYMOCYTES FROM FTOCS

After 6 days of FTOC with the different peptides, thymocytes were expanded for 3 days by TCR cross-linkage and tested for specific killing in CTL assays. EL4 target cells were labeled either with the cognate AttM fM peptide fMIVTLF (filled bars), or with the unrelated H2-M3 binding control peptide fMIGWII (empty bars). The E:T ratio was 10:1. Expanded thymocytes were derived from FTOCs supplemented with the ND1/9mer peptide at 39 nM, the ND1/P2 peptide at 2.5 µM and all other peptides at 10 µM.

We had determined in earlier experiments that naturally occurring fM peptides extracted from surface H2-M3 molecules, as well as the ND1/9mer peptide, reconstituted physiological levels of positive selection. They had to be added to FTOCs at low nM concentrations (Berg et al., 1999b). We assumed that these peptide concentrations had recreated natural conditions of positive selection and thus physiological numbers of H2-M3 / peptide complexes.

Others have shown that incubating cells with exogenous peptides at these concentrations did not result in detectable up-regulation of this MHC Class Ib molecule. H2-M3 surface expression indeed is extremely low (Chiu et al., 1999). Therefore, we concluded that under physiological conditions, positive selection of C10.4 TCR_{trans}^{+} T cells was driven by a low number of MHC / peptide complexes.

The ND1/9mer was active at low nM peptide concentrations. However, removing the lysine in P9 abolished the ability of the ND1/9mer (hence the ND1/8mer) peptide to induce positive selection at any peptide concentration. Therefore, at low epitope density, immature C10.4 TCR_{trans}^{+} thymocytes recognized the leucine in P9. Mutations of the surface side-chains profoundly affected the ability of any of the peptide variants to positively select. Thymocytes did not mature on either the ND1/P6, the ND1/P8, the ND1/P9 or the ND1/P89 peptides even though these peptides bound well to H2-M3. The ND1/P5 and the ND1/P2589 peptides induced CD8 SP thymocytes at the highest concentration tested. Yet, T cells expanded from ND1/P5 conditioned FTOCs did not give rise to functional CTLs. The best positive selector of the peptide mutants, the ND1/P2 peptide showed approximately 1/100 of the activity of the ND1/9mer peptide. This loss of activity could only be partially contributed to reduced binding to H2-M3. Therefore, these experiments demonstrated that recognition during positive selection is highly specific.

We knew from previous experiments that recognition of immature C10.4 TCR_{trans}^{+} T cells becomes promiscuous when the peptide concentration is raised to 10 µM (Berg et al., 1999b). Mitochondrial peptides with no apparent homology to the ND1 peptide induced positive selection. Thus, at those high concentration levels, recognition of amino acid side-chains became less

important. This fact would explain why the 'flat' ND1/P2589 peptide induced some positive selection at the highest peptide concentration tested. Its increased activity compared to the other mutants might be caused by slight conformational changes that it induced in the MHC structure as others have shown in different experimental systems (Chen et al., 1993; Rohren et al., 1994; Calbo et al., 1999).

To find further evidence that a normal cell does not present 10 µM of peptide, we looked at the peptide concentration required for activation of naïve C10.4 T cells *in vitro*. We cultured naïve, CFSE labeled C10.4$^{+/+}$ RAG2$^{-/-}$ LN cells with decreasing amounts of the ND1 self-peptide presented by TAP1$^{-/-}$ deficient bone marrow derived enriched dendritic cells, in the absence of any exogenous growth factors or cytokines. CFSE, a fluorescent dye that covalently couples with intracellular macromolecules splits up 50:50 on each resulting daughter cell, so it is ideal to study cell division. The C10.4$^{+/+}$ RAG2$^{-/-}$ LN cells had a purity of at least 99% (Figure 24).

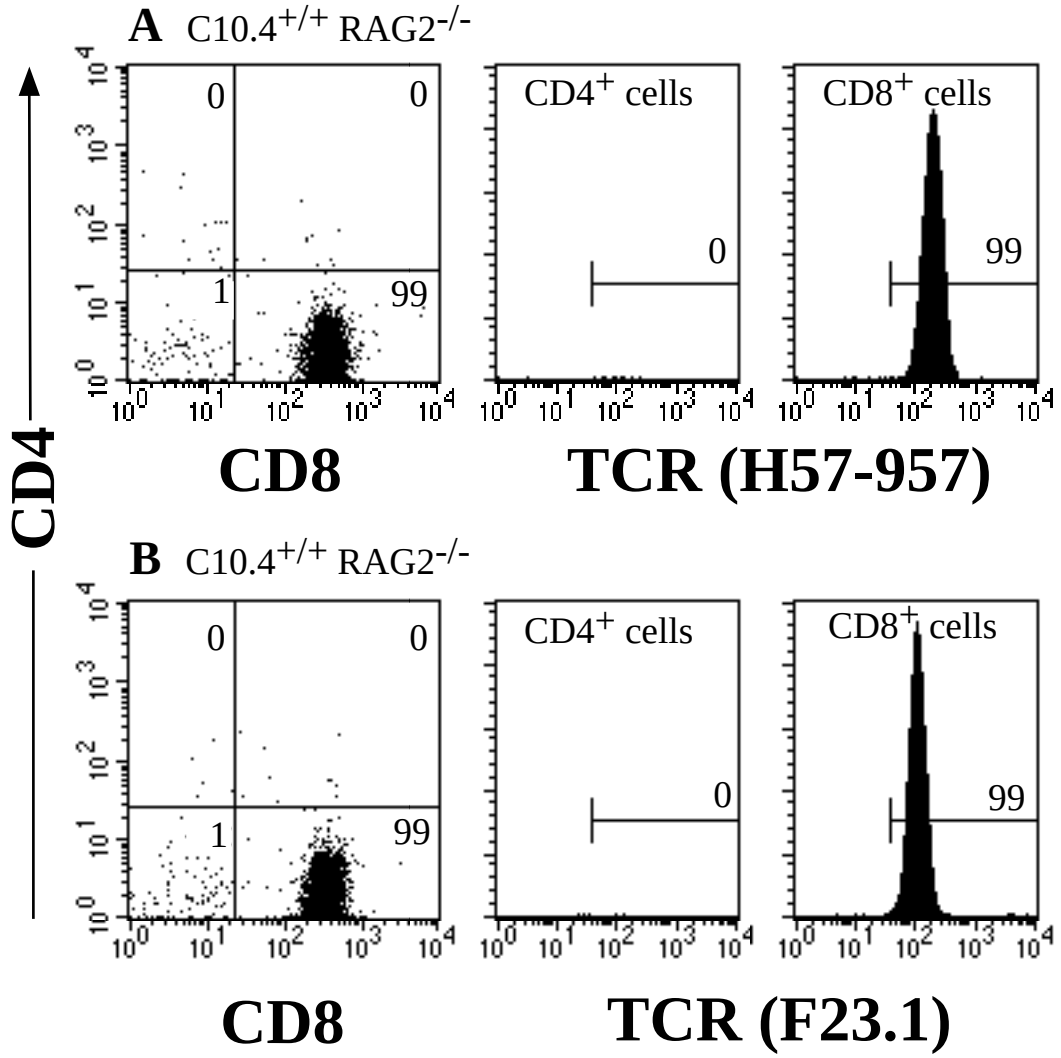

Figure 24

FIGURE 24: PURITY OF C10.4$^{+/+}$ RAG2$^{-/-}$ LYMPH NODE CELLS

Inguinal, intestinal and paraaortic lymph nodes were harvested and single cell suspensions were stained for their expression of CD4, CD8 and TCR. Panel A uses the H57-597 mAb. Panel B shows the same stain with F23.1 as a αTCR mAb. Quadrant labels show percent of total live gated cells. Marker labels are percent of total live gated cells gated through the depicted quadrant.

As shown in Figure 25 at high concentrations (10µM) of the ND1/9mer self-peptide only 5% of the T cells (determined by CD8 and TCR stain) are $CFSE^{high}$. With decreasing amounts of peptide the percentage of $CFSE^{high}$ T cells increases, denoting the T cells that had not received a proliferation stimulus. Background proliferation is shown by pulsing the APCs with ND2, a peptide known to bind H2-M3 without giving a stimulating signal to the C10.4 TCR_{trans}^{+}. In addition to that staining of naïve peripheral lymphocytes did not indicate activation of T cells as determined by forward / side scatter, CD25 and CD69 stain (Irion et al., 1999). They appeared non-blast like in size and were $CD25^{low}$ and $CD69^{low}$. Further, we could not observe signs of autoimmunity in our mouse colony, thus suggesting that the amount of peptide presented in a living animal is too low to activate naïve T cells, i.e. suggesting that the ND1 peptide is presented in the low nM range.

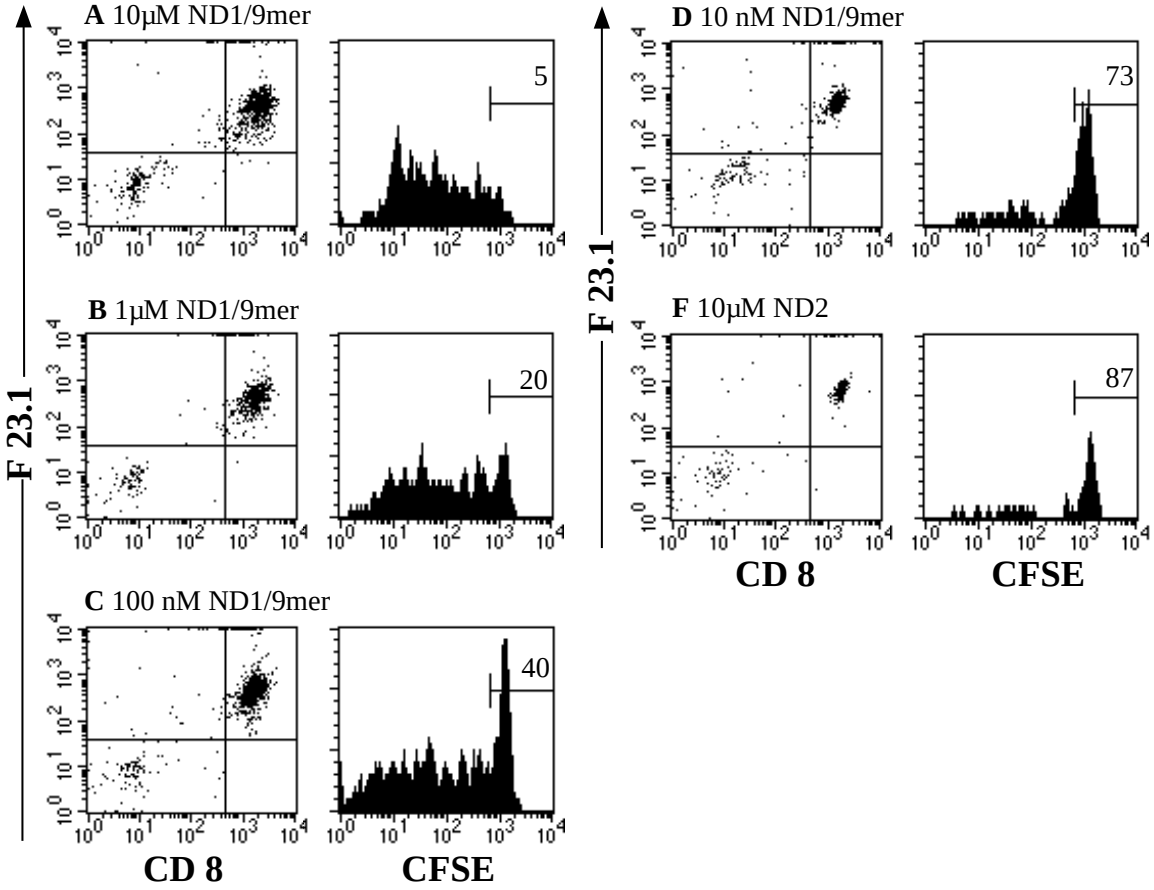

Figure 25

FIGURE 25: PHYSIOLOGICAL PEPTIDE CONCENTRATIONS PRESENTED BY H2-M3

Naïve C10.4$^{+/+}$ RAG$^{-/-}$ LN cells were labeled with 0.2 µM CFSE and then cocultured in the presence of enriched TAP1$^{-/-}$ deficient dendritic cells that had been pulsed with the indicated peptide prior to the experiment. 6 days later the cells were analyzed by 4 color flow cytometry using the mAbs αCD4-APC (data not shown), αCD8-PE (1 column) and αTCR (F23.1, y-axes). The mean CFSE fluorescence intensity is shown in the 2 column. Marker label show percent of total live gated cells.

As shown earlier, positive selection occurs on a weak agonist. Therefore we examined whether any of these peptides were seen by mature C10.4 TCR_{trans}^{+} CTLs (Figure 26). The ND1/9mer peptide sensitized targets at peptide concentrations that were about a 100 fold higher than those required for positive selection in FTOCs. When we incubated target cells with either the ND1/P2 or the ND1/P2589 peptide at concentrations at which they had induced T cell maturation, the targets were not lysed by C10.4 TCR_{trans}^{+} CTLs. In Table 1, we had determined that these peptides bound to H2-M3 molecules. Therefore, we concluded that the affinity of the C10.4 TCR_{trans}^{+} to either the H2-M3/ND1/P2 or the H2-M3/ND1/P2589 complex was too low to trigger the C10.4 TCR_{trans}^{+} CTLs. Similarly, this low interaction affinity should be responsible for the inefficiency of positive selection induced by these peptide variants.

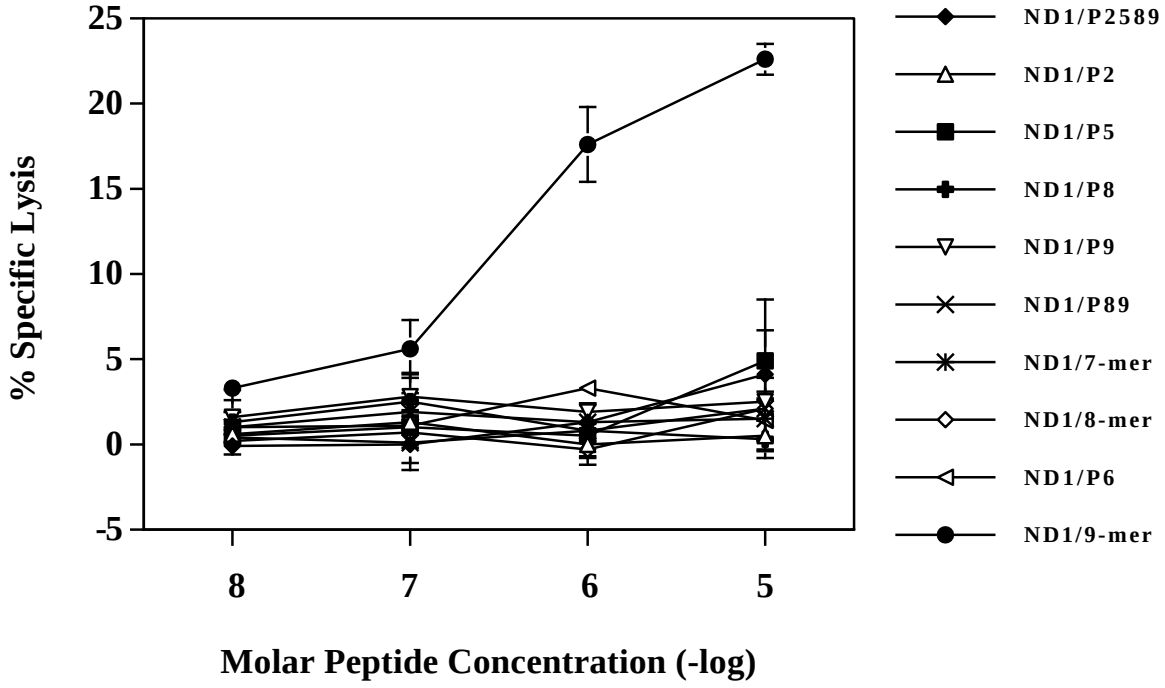

Figure 26

FIGURE 26: AGONISTIC ACTIVITY OF THE DIFFERENT PEPTIDE MUTANTS

EL4 target cells were incubated with decreasing concentrations of the different peptide mutants and exposed to C10.4 TCR_{trans}^{+} CTLs at an E:T ratio of 5:1. All data was collected in triplicates.

Summary

During positive selection the TCR engages with a self-peptide presented by MHC molecules. In our model system the TCR shows very little promiscuity and, at physiological peptide concentrations, only interacts with the physiologically presented ND1/9mer. All peptide variants used in this study failed to do so.

CHAPTER VI

Discussion

Positive selection is a process that permits the survival of T cells with the capacity to distinguish self peptides from foreign antigens in association with self-MHC molecules. The repertoire of T cells is thus prepared to engage self-MHC molecules bound with foreign peptides and mount a specific immune response in the individual. However, this selection process can lead to the development of T cells that recognize self-MHC molecules in conjunction with self-peptides with high affinity, resulting in potential autoimmunity. Therefore negative selection eradicates or anergizes potential autoreactive cells that bind self-MHC molecules with such high affinity. Since MHC molecules must bind peptides to be expressed and be maintained in a stable fashion on the cell surface, the role of self-peptides in both positive and negative selection has been intensely studied. Several theories have been proposed to explain recognition events of positive selection. They range from the non-interference model to a very precise recognition model.

The goal of this thesis was to address the nature of the self-peptide involved in positive selection and the specific recognition of this ligand by the C10.4 TCR_{trans}^{+}. In the C10.4 mouse the physiological ligand of positive selection was known, and positive and negative selection were carefully researched in parallel to my studies.

This Thesis in Context with Current Theories of Positive Selection

C 10.4 T cells are positively selected on concentrations of the ND1/9mer in the low nM range, however at high peptide concentrations, other mitochondrial fM-peptides as well as some of the peptide mutants were able to promote positive selection of C10.4 TCR_{trans}^{+} T cells. Yet, a correlation between the ability of a peptide to bind to H2-$M3^{wt}$ and to mediate positive selection was not detectable. Indeed, numerous peptide variants were able to stabilize surface expression of H2-$M3^{wt}$ at least as well as the ND1/9mer, nevertheless, the

ND1/9mer was the only one to induce positive selection at a very low peptide concentration. These observations suggest that peptide recognition during positive selection in the thymus is peptide specific and peptide dependent rather than highly promiscuous as proposed by the non-interference model (Schumacher and Ploegh, 1994). The authors in that paper argue that only MHC is required for positive selection, yet there is no peptide to be present in the binding groove. Thereby suggesting that the only function of a peptide during positive selection is limited to the stabilization of MHC on the cell surface, resulting in a high TCR / MHC avidity (where avidity is defined as the product of the number of MHC class I molecules carrying a peptide and the affinity of this complex for the TCR).

Experiments in this thesis demonstrated that at high peptide concentrations the specificity of positive selection relaxed. This broadening of positive selection at concentrations in the µM range may cause peptides to be scored as positive selectors even though they may in fact not be capable of positive selection *in vivo*, due to the low physiological peptide concentration. Indeed, in previous studies utilizing FTOCs, other naturally occurring peptides that were designated as 'positive selectors' were used at 20 µM or even higher concentrations (Pawlowski et al., 1996; Hogquist et al., 1997; Hu et al., 1997). It seems likely that these peptides were only able to induce positive selection due to the extremely high concentrations used in the FTOC system. This might also explain why some of the ND1 peptide mutants scored as positive selectors at high peptide concentrations. These excessive peptide concentrations would result in non-physiological, high epitope densities that offer a strong signal to the TCR, resulting in a loss of specificity.

The advantage of the system used in this study is, that such high MHC / peptide complexes are unlikely to be reached *in vitro* due to the limited surface expression of H2-M3, which is not only caused by the limited numbers of available H2-M3 binding f-Met peptides under physiological conditions, but also by the low levels of H2-M3 mRNA found in cells (Wang et al., 1991).

Other investigators have addressed the question of positive selection with mice that were designed to express high levels of a single peptide / MHC

complex. In H2-M knockout mice, the vast majority of the MHC Class II molecules contain the CLIP peptide from the invariant chain (or a variant). Here the CLIP fragment can not be removed during assembly of MHC class II in the endoplasmatic reticulum, and therefore is shuttled to the cell surface, without being removed and being replaced with self or foreign peptides (Fung-Leung et al., 1996; Martin et al., 1996; Miyazaki et al., 1996). In another experimental approach, mice were engineered to express a single peptide covalently bound via a linker region to all of the expressed MHC Class II molecules (Ignatowicz et al., 1996a; Fukui et al., 1997). In contrast to our system the cell surface in these systems was here covered with extremely high levels of a single MHC / peptide complex. In both cases, broad spectra of $CD4^+$ T cells were selected; however, the resulting T cells were different from those selected in a normal mouse. Those T cells contained a high frequency of cells that were reactive with self-peptide / self-MHC complexes. The non-specific positive selection of C10.4 TCR_{trans}^+ T cells in FTOCs containing high epitope densities suggests that in other experimental systems, such as single peptide / MHC mice, the broad repertoire of T cells is due to promiscuity of peptide recognition. In the thymus of a non-transgenic mouse, numerous unrelated peptides are probably required for complete positive selection of a diverse repertoire of T cells. Indeed, a recent report suggests that the majority of the repertoire selected in single-peptide / MHC mice is generated by interactions between TCRs and low-abundance diverse self-peptides (Barton and Rudensky, 1999). Support comes from another group, that was able to show that a single amino acid substitution in a Class II restricted system greatly reduced the amount of positively selected CD4 T cells (Fukui et al., 2000).

Positive selection of a particular T cells probably depends on the selecting MHC molecule, TCR / peptide / MHC affinity and expression levels of TCR and MHC molecules. However, recent experiments have suggested that a diverse collection of low-abundant peptides are required for the positive selection of $CD4^+$ T cells restricted to MHC Class II molecules (Barton and Rudensky, 1999). Therefore, the selection of all T cells may require an interaction with a specific peptide.

In Chapter IV, we demonstrated that the ND1/9mer, as well as a pentamer version of the cognate AttM peptide, functioned as weak agonists for peripheral C10.4 TCR_{trans}^{+} T cells. Both of these peptides also positively selected C10.4 TCR_{trans}^{+} thymocytes. Furthermore, the concentrations of peptide required for peripheral activation of C10.4 TCR_{trans}^{+} T cells or positive selection of C10.4 TCR_{trans}^{+} thymocytes was nearly identical for the ND1/9mer and the AttM/5mer peptide. The AttM/9mer peptide, a strong agonist for C10.4 TCR_{trans}^{+} T cells, was capable of inducing positive selection of functional $CD8^{+}V\beta8^{+}$ T cells at lower peptide concentrations than the weak agonist peptides (Figure 12). Yet, the ND1/9mer, the AttM/9mer peptide, and the AttM/5mer peptide all bound H2-M3 with comparable affinities (Figure 20). Successful positive selection of functional C10.4 TCR_{trans}^{+} T cells induced by the ND1/9mer, the AttM/9mer peptide or the AttM/5mer peptide only proceeded at a narrow range of avidities. Therefore, the data presented in this thesis fit best with the 'differential avidity' hypothesis of positive selection (Ashton-Rickardt et al., 1994; Ashton-Rickardt and Tonegawa, 1994; Sebzda et al., 1994). This hypothesis states that the outcome of thymic selection is determined by the affinity of the TCR for the peptide / MHC complex as well as the expression levels of TCR and peptide / MHC complexes. Very low-level expression of a self-peptide that is an agonist for a particular TCR could induce positive selection of T cells bearing this TCR. Furthermore, according to this theory, self-peptides that act as weak agonists for a particular TCR would have to be displayed at higher epitope densities to achieve the avidity required for positive selection.

The 'differential avidity' hypothesis was recently modified to state that peptides that are strong agonists for peripheral T cells only negatively select, whereas peptides that are weak agonists only positively select (Girao et al., 1997). These predictions were tested in the C10.4 TCR_{trans}^{+} model system. The AttM/9mer peptide, which functioned as a strong agonist, mediated positive selection of functionally mature C10.4 TCR_{trans}^{+} T cells. This data is inconsistent with the modified differential avidity hypothesis. Furthermore, the ND1/9mer that

functions as a weak agonist was able to induce negative selection (clonal inactivation) of C10.4 TCR_{trans}^{+} thymocytes.

Positive selection of functional C10.4 TCR_{trans}^{+} T cells depended on a certain avidity threshold. An increase in the avidity to a level greater than this threshold (by adding more peptide to the FTOC) resulted in non-lytic T cells ('boondoggled' T cells) that did not lyse target cells pulsed with the cognate peptide. A decrease in the avidity (by reducing the peptide concentration) results in a lack of positive selection. It has been shown that surface expression of H2-M3 is lower than that of classical MHC Class Ia molecules (Vyas et al., 1994; Chiu et al., 1999). Consequently, H2-M3 may mediate the development of T cells with relatively high affinities. These high affinity TCRs would be necessary to overcome the relatively low avidity due to the low H2-M3 surface expression. This consideration might explain why the ND1/9mer was a stronger agonist than other positively selecting peptides identified for T cells restricted to highly abundant MHC Class Ia molecules. In these model systems, peptides that were capable of positive selection were either very weak agonists/antagonists (Hogquist et al., 1994; Jameson et al., 1994; Hogquist et al., 1995), or were not recognized by mature T cells (Hogquist et al., 1997; Hu et al., 1997). Partial agonist peptides displayed on MHC Class Ia or MHC Class II molecules would most likely induce negative selection because of the increased avidity created by the relatively high level of expression. Therefore, as noted earlier, differences in the surface expression levels of MHC molecules may result in functional maturation of T cells bearing TCRs with different affinities for self-peptide / MHC complexes.

Mice that expressed a single peptide / MHC complex have been established (discussed above). In these animals, peripheral T cells express TCRs with exceedingly low affinities to these peptide / MHC complexes and do not react to these highly abundant epitopes (Fung-Leung et al., 1996; Ignatowicz et al., 1996a; Ignatowicz et al., 1996b; Martin et al., 1996; Miyazaki et al., 1996; Liu et al., 1998). T cells bearing TCRs with a modest affinity for the selecting peptide / MHC complexes would be deleted due to the increased avidity created by the high expression of peptide / MHC complexes. Therefore,

these systems are biased towards the positive selection of T cells with very low affinity for self-peptides.

Using a system that does not involve a high level of expression of the selecting epitope, the physiological ligand of positive selection for C10.4 TCR_{trans}^{+} T cells, the ND1/9mer, was also defined as a weak agonist. When present at a low abundance, ND1/9mer/H2-M3 complexes induced the positive selection of C10.4 TCR_{trans}^{+} T cells. This interaction was shown to be specific, and promiscuity was only demonstrated at non-physiological concentrations of peptide. Furthermore, the ND1/9mer did not function as an antagonist. Therefore, the data presented in this thesis support the original 'differential avidity' hypothesis of positive selection. However, as explained above, not all of the conclusions of the 'differential avidity' model are supported by data from the C10.4 TCR_{trans}^{+} mouse. In the C10.4 TCR_{trans}^{+} system, positive selection is induced by a weak agonist, but functional T cells only arise from productive TCR / MHC interactions occurring over a narrow range of peptide concentrations.

Our findings demonstrated that under physiological conditions positive selection of C10.4 TCR_{trans}^{+} T cells is highly specific and occurs as a low abundance interaction between the TCR and the MHC / peptide complexes. However, this exquisite specificity is lost at high epitope densities. Even a 'flat' peptide variant (ND1/P2589) induced positive selection at the highest peptide concentration (10 µM). In contrast to H2-M3, MHC Class Ia molecules are present on the cell surface at high density. The high number of MHC / peptide complexes could therefore compensate for the low affinity of a promiscuous TCR. Yet, others provided evidence that a repertoire of T cells restricted to MHC Class II molecules was selected on diverse peptides present at low levels (Barton and Rudensky, 1999), and was highly specific (Fukui et al., 2000). Therefore, we postulate that our observations of the positive selection of C10.4 TCR_{trans}^{+} T cells are representative for positive selection of all T cells. Thus, positive selection depends on a highly specific, low abundance TCR interaction.

Mechanisms of Positive Selection

The 'differential avidity' hypothesis suggests that positive selection occurs at lower triggering thresholds than peripheral activation. Data to support this has been derived from different experimental systems. For example, T cell hybridomas specific for a variant of the selecting peptide have been generated from single peptide / MHC mice (Liu et al., 1998). The TCRs of these hybridomas have at least a ten fold lower affinity for the selecting peptide / MHC complex than for the activating peptide / MHC complex. TCR_{trans}^{+} mice have been used to identify positively selecting peptides (Hogquist et al., 1994; Williams et al., 1999). Subsequent BIAcore studies suggested that the TCRs have such a low affinity for positively selecting peptide / MHC complexes they are not measurable (Williams et al., 1999) or, if measurable, the TCRs have a lower affinity for positively selecting peptide / MHC complexes than for peripheral activating (or thymic deleting) peptide / MHC complexes (Alam et al., 1996). To test these theories we expressed a soluble C10.4 TCR in a bacculovirus system and used a soluble H2-M3 purified from SC2 *drosophila* cell cultures (Wang et al., 1995). The resulting MHC was pulsed with either the ND1/9mer or the AttM/9mer. Resulting MHC / peptide complexes were then floated over a BIAcore chip that presented either the C10.4 TCR or the DO11.10 TCR (as a negative control, data not shown). The preliminary data available from these experiments suggest that positive selection is indeed a low affinity interaction. We could also demonstrate that the AttM/9mer bound to H2-M3 with a higher affinity than the ND1/9mer. This data is conclusive with the data shown in chapter IV. Additional studies to investigate this phenomenon are on their way.

Data obtained in this thesis indicate that positive selection by the ND1/9mer was induced at avidities 100- to 1000-fold lower than that required for the recognition of the same peptide by peripheral T cells. These numbers were determined by factoring in the inefficiency of presentation demonstrated in the FTOC system by Rance Berg in our lab (data not shown). Previous data suggested that thymocytes undergoing positive selection had a lowered

threshold for signaling and thus were easier to trigger than peripheral, mature T cells (Davey et al., 1998). Furthermore, T cells maintain sensitivity to strong agonists throughout development, even though the sensitivity to weak agonists is decreased after maturation (Lucas et al., 1999). In the C10.4 TCR_{trans}^{+} model system, the overall patterns of the relative efficiencies of both strong and weak agonists for positive selection and peripheral recognition were similar.

Outlook

Rance Berg has established the C10.4 TCR_{trans}^{+} mouse and its TCR was shown to be selected on a self-peptide, ND1, presented by H2-M3. Peripheral C10.4 TCR_{trans}^{+} T cells recognized this physiological ligand of positive selection as a weak agonist. Furthermore, the cognate AttM peptide was able to induce positive selection of functional C10.4 TCR_{trans}^{+} T cells. Assuming that the cognate AttM peptide was recognized with a higher affinity than the physiological ligand of positive selection, the success of positive selection in the induction of fully functional T cells depended on a fairly defined range of avidity. Increasing the epitope density beyond this point resulted in the selection of partially functional T cells with the ability to proliferate, but not to mediate cytolytic functions.

We could also show, that the C10.4 TCR recognizes the H2-M3 / peptide complex with exquisite specificity. A single amino acid change could lower or even abandon signs of positive selection. Yet at high enough peptide concentrations this phenomenon vanishes. In contrast to other studies our system is sensible enough to induce maturation of functional C10.4 T cells at very low epitope densities. We were therefore able to describe these phenomena within a physiological model system, circumventing high and therefore non-physiological epitope densities, as used in earlier studies.

Using this system I determined the specificity of positive selection in a very physiological manner. I came to the conclusion that positive selection is a highly specific interaction and is a low affinity / avidity interaction. It remains to be researched what the nature of these non-lytic cells from the ‘high-peptide-concentration’ FTOCs is. We were able to show, that these cells express lower levels of normal CD8 α/βheterodimers, yet these heterodimers are replaced by higher levels of CD8 β/β homodimers (Berg et al., 2000).

We further performed some preliminary *in vivo* studies. We therefore transferred C10.4 T cells that were selected on either their optimal peptide concentration, or C10.4 T cells that were selected to become anergetic, into $RAG2^{-/-}$ deficient mice. In those mice the functionally selected cells survived and

homed in the thymus whereas the anergized cells vanished (data not shown). It seems likely though that those cells are not able to survive, even in such a 'viable' environment. The expansion of the C10.4 TCR_{trans}^{+} thymocytes under physiological conditions instead of stimulation with the H57-597 mAb may be more appropriate for the analysis of their lytic potential. The analysis of key molecules such as FasL or perforin or analysis of signaling intermediates may provide an explanation as to why these T cells are non-lytic. Furthermore, the use of representational differential analysis (RDA) may be useful in dissecting the differences between the fully functional T cells and the partially functional T cells. Further studies to investigate this phenomenon are on their way.

The issue of T cell survival after thymic selection has received quite a bit of interest lately. Although MHC molecules are necessary for this survival, it is mostly unknown what role self-peptides play in this process. With the identification of the ND1/9mer as a physiological ligand of positive selection, the question of peripheral positive selection can be directly addressed. We therefore bred a $RAG^{-/-}$ and $TAP^{-/-}$ deficient mouse. By comparing the survival of C10.4 TCR_{trans}^{+} T cells in those mice compared to the survival in only $RAG^{-/-}$ deficient mice, the role of Class I MHC could be addressed. A more physiological way would be to leave the RAG gene intact, and to track the transferred T cells by expression of Thy 1.1 vs. Thy 1.2. Experiments to focus on this question are on their way. Another way to approach this matter would be to transfer C10.4 TCR_{trans}^{+} T cells into MHC Class I deficient mice. In these recipients, the C10.4 TCR_{trans}^{+} T cells should not survive. However, a source of peptide / MHC complexes could be added back to the system by coating APCs from Class I deficient mice with fM peptides and then transferring these cells along with the C10.4 TCR_{trans}^{+} T cells. It seems likely that the ND1/9mer will be responsible for survival of peripheral C10.4 TCR_{trans}^{+} T cells.

The role of the ligand involved in positive selection could be further studied by creating triple crystals with a H2-M3 / TCR / peptide trimer. It seems likely that the TCR can either engage with different parts of the ligand, or it has multiple binding sites that are by themselves strong enough to deliver a sufficient signal to the T cell. It was interesting that the AttM/5mer peptide could

select T cells in the same manner as the ND1/9mer. Since the ND1/9mer was seen with unique specificity, it was surprising that a 5mer peptide was able to induce positive selection, yet the ND1/8mer and the ND1/7mer failed to do so. It is therefore most likely that the TCR has highly specific, yet multiple binding sites.

Defining the nature of a naturally occurring self-peptide able to induce positive selection of a subset of T cells and the peptide requirements for an appropriate TCR – MHC interaction allows one to imagine the possibilities of inducing specific T cells either *in vitro* from T cell precursors or *in vivo*. The generation of specific T cells by inducing positive selection could be a useful strategy in controlling certain viral or bacterial infections. Diseases such as the Acquired Immuno Deficiency Syndrome (AIDS) render people immunocomprimised due to T cells being selectively attacked by the virus. T cell responses to cells infected by HIV could provide protection and ultimate clearance of the virus, or the vanishing T cells could be 'restocked'. Furthermore, generation of T cells specific for malignant cells, that might overexpress self peptides, or present mutants of self-peptides, could be induced *in vivo* or *in vitro*. With the sequence requirements of a self-peptide known to induce positive selection and the activating cognate peptide in hand a motif that allows for sequence determination of self-peptides with the ability to select certain T cells may possibly be identified.

A homologue of H2-M3 has not been identified in man. However, non-polymorphic MHC-like molecules such as CD1 may serve the same purpose as H2-M3 in the mouse. Therefore, in humans, the transfer of T cells restricted by CD1 to immunocomprimised individuals may overcome the transfer barrier normally encountered. The problem lies in the vast number of MHC molecules expressed in humans. In order to successfully transfer T cells, the restricting MHC molecule must be matched from the donor to the recipient. T cells restricted to H2-M3 or CD1 would function in any environment due to the non-polymorphic nature of these MHC molecules.

SUMMARY

In this thesis I demonstrate that positive selection of the C10.4 TCR_{trans}^{+} is highly specific and is a low affinity / avidity interaction. Further it is shown that C10.4 TCR_{trans}^{+} T cells are positively selected on the ND1/9mer as well as on the full agonistic cognate AttM/9mer and the weak agonistic cognate AttM/5mer peptide. One could imagine that the results from these studies would be useful in the *in vitro* generation of T cells to fight cancer and immune deficiencies.

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Acknowledgements

The Code Of the West

Write it in your heart. Stand by the code, and it will stand by you.
Ask no more and give no less than honesty, courage, loyalty, generosity, and fairness.

I guess this quote from 'A Cowboy's Guide To Life' best describes my experience in beautiful Denver, Colorado. People I met really followed that code, and it was a pleasure to live, work and enjoy the time in Denver.

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Curriculum Vitae

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