TCR-mediated signaling in thymocyte selection

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Immunology
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Ph.D. 2001

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ABSTRACT

TCR-mediated signals have been shown to induce both positive and negative selection of thymocytes. How the same TCR can induce both forms of selection introduces a conceptual paradox. A generally accepted model of thymocyte selection is the “strength of signal” or “affinity/avidity” hypothesis. This model predicts that the absence of any signal from the TCR results in death by neglect, a “strong” signal results in negative selection and a “weak” signal results in positive selection. My thesis examines how these TCR-peptide/MHC interactions are translated into intracellular signals that discriminate between positive and negative selection.

The thesis investigates the relationship between the extent of TCR internalization in response to various peptide/MHC stimuli and thymocyte selection outcomes. Our results demonstrate a direct correlation: peptides that induce strong TCR down-regulation are most efficient at mediating negative selection, whereas ligand interactions that induce suboptimal TCR internalization are more efficient at triggering positive selection. These data suggest that T cell selection is mediated by differing intensities of the same TCR-mediated signal, rather than by distinct signals.
My thesis also assesses the role of the mitogen-activated protein kinase (MAPK) signaling cascade, specifically the extracellular signal-regulated kinase (ERK) module in regulating both positive and negative selection. We find that negatively selecting conditions induce stronger ERK activation in thymocytes than do positively selecting conditions. We further demonstrate that attenuating the ERK activity by pharmacological inhibition can shift the activation threshold for negative selection to that for positive selection. Moreover, in order to study the kinetics of ERK activation upon stimulation with positive and negative selection stimuli, we devised an in vitro assay system for thymocyte selection. Data from these studies suggest that weak, sustained ERK activation is characteristic of positive selection, whereas strong but transient ERK activation leads to positive selection. Therefore, both timing and extent of ERK activation appear to be important for thymocyte selection. Since positively selecting stimuli do not induce efficient TCR internalization, the large proportion of TCR complexes that remain on the cell surface may relay continuous signals required for survival and differentiation. Together, these results add a novel viewpoint to thymocyte selection.
Publications (1997-2001)

Refereed Papers:


Reviews:


Acknowledgments

Cogito. ergo sum
I think, therefore I am
Descartes

Sentio, ergo sum
I sense, therefore I am
T cell

In order to understand a T cell, sometimes one needs to 'think' and 'sense' like a T cell. I believe that my supervisor, Dr. Pam Ohashi, embodies this notion. I have observed, admired and learned many things from her, but what stands out the most is her acute 'sixth sense' to understand the intricacies of immunology. Pam, thank you for inviting me to play and learn in this thymic kindergarten (the Ohashi lab). Special thanks for also bearing with my negative bearings and rescuing me from many of my self-induced negative selection (hari-kiri) attempts. I feel truly lucky to have worked with you.

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To

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Chapter I

Introduction

Overview of T cell development

Thymus

The first definitive experiments demonstrating the crucial role of the thymus in the development of the immune system were carried out in the early 1960's by Jacques Miller and colleagues (Miller and Osoba, 1967). Miller was investigating the role of the thymus in leukogenesis when he performed experiments which showed that if mice are thymectomized within 1 or 2 days of birth, they have a marked deficiency of lymphocytes, fail to reject foreign grafts and are susceptible to infections.

Within the thymus, the thymic stromal cells provide both direct cell contact and soluble molecules for the developing thymocytes (reviewed in (Anderson et al., 1996b)). The major stromal cell types that make up the thymic microenvironment are epithelium, macrophages, dendritic cells and fibroblasts. The thymic epithelial cells are derived from the Pax-1+ endoderm of the third and/or fourth pharyngeal pouch and the ectoderm of the third branchial cleft and the neural cleft. These domains fuse together to provide an epithelial thymic premordium which, between day 11 and day 12 of gestation, is populated with lymphoid progenitor cells. Thymic stromal cells of hematopoietic origin include macrophages, which appear around day 14 of gestation, and dendritic cells, which appear late in ontogeny.
Histologically, the thymus is composed of two major regions, the cortex and the medulla, and is encapsulated by connective tissue (Boyd et al., 1993). T cell precursors are thought to enter the thymus via the subcapsular region during fetal life or the cortico-medullary junction of the adult thymus. Following entry, the precursor cells proceed to the cortical subcapsular region and then migrate from the cortex to the medulla as they differentiate into mature cells. Thus, the thymic cortex largely contains immature DN and DP thymocytes whereas the medulla is the site for mature SP thymocytes.

**Ontogeny of Thymocyte Differentiation**

T lymphocytes are derived from pluripotent hematopoietic stem cells (HSCs) present in the yolk sack or fetal liver and migrate to the fetal thymus preferentially between E12-14. In adults, they migrate to the thymus from the bone marrow (reviewed in (Shortman and Wu. 1996; Akashi et al., 2000)). During T cell development, precursor cells are confronted with distinct cell fate specification events. First, a common lymphoid progenitor cell (CLP) which is capable of giving rise to T, B and NK cells, but which has lost its ability to differentiate along erythroid and myeloid lineage, must adopt a T cell fate. CLPs express CD117 (ckit receptor) and CD127 (IL-7Rα). Once the T cell lineage is specified, a pro-T (or pre-T) cell in the thymus must choose between the αβ and γδ T cell lineage. Finally, thymocytes that are committed to the αβ T cell lineage must undergo positive selection (and survive negative selection) to differentiate along either CD4+ or CD8+ mature T cells. These cell fate choices are outlined in Fig 1-1.
Figure 1-1: Different stages of T cell differentiation. Some of the key molecules that have been shown to be involved during these differentiation events are shown. Molecular determinants that influence DP to SP transition are covered in Fig 1-5.
CLP to T cell lineage commitment

Murine T cell precursors (CLPs) possess a rapid and potent reconstitution potential limited to T, B and NK cells (Kondo et al., 1997). Whether the commitment of CLPs to the T cell lineage occurs in the bone marrow (BM) or after they migrate to the thymus is currently unknown. As far as the T/B lineage choice is concerned, two independent types of evidence indicate that Notch-1 (a transmembrane receptor involved in multiple cell fate decisions in invertebrates) plays an essential role. Thus overexpression of Notch-1 in HSCs leads to ectopic development of immature T cells in the BM and concomitant inhibition of B cell development at an early stage (Pui et al., 1999). In reciprocal experiments, inactivation of Notch-1 in BM stem cells led to an early block in T cell development in the thymus that was accompanied by ectopic development of thymic B cells (Radtke et al., 1999). These results suggest that CLPs migrate to the thymus where they receive an instructive signal through Notch-1 to adopt a T cell fate. A role for Notch in thymic development can be inferred from the expression of a variety of Notch receptors (Notch 1-3) and their ligands (Jagged 1 and 2) in thymocytes and thymic stromal cells (reviewed in (Robey, 1999)).

Another potential fate that can be adopted by CLPs is NK cells. Studies have indicated that CD117+NK1.1+CD44+CD25- fetal thymocytes exhibit bipotential T/NK precursor activity (Carlyle et al., 1998). Recent studies have confirmed at the clonal level these T/NK precursors from E14 fetal thymi could give rise to both T and NK cells (Ikawa et al., 1999; Michie et al., 2000). As no comparable progenitor cell has yet to be isolated in
Figure 1-2: Sequence of TCR gene rearrangements and the resulting phenotypic changes in thymocytes.
adult thymi, these precursors represent a development stage that seems to be restricted to fetal development.

**αβ versus γδ T cell lineage determination**

A second stage of early T cell development involves the commitment to either the αβ or the γδ T cell lineage (reviewed in (Shortman and Wu, 1996)). Following definitive commitment to the T cell lineage, intrathymic precursors (CD44+CD25-CD117+) express CD25 (α chain of the IL-2 receptor). Based on CD44 and CD25 expression these CD4+CD8- double negative (DN) thymocytes can be further divided into four stages: DN1 (CD44+CD25- thymic lymphoid precursors); DN2 (CD44+CD25+ Pro-T cells); DN3 (CD44-CD25+ Pre-T cells); and DN4 (CD44+CD25-post-pre-T cells) (Fig 1-1).

As DN2 pro-T cells transit to DN3 pre-T cells, they begin to rearrange and express their TCR β,γ and δ genes (Fig 1-2). During this process the coding regions for the variable domains of the antigen receptors are created by joining of exons that are randomly selected from arrays of tandemly repeated V,D (sometimes) and J segments by the process of V(D)J recombination (Tonegawa, 1983).

Pro T cells that successfully rearranged TCRγ and TCRδ will express γδ TCR and be eligible to develop further as γδ T cells. By contrast productive TCRβ rearrangement will lead to expression of pre-TCR (consisting of a TCRβ chain associated with an invariant pTα chain), which is compatible with further development as αβ T cells. The extent to which γδ TCR and pre-TCR play an instructive (as opposed to a selective) role
in the αβ versus γδ lineage commitment process remains controversial (reviewed in (Fehling et al., 1999)).

DN to DP transition of αβ T cell lineage thymocytes.

During early T cell development, only cells that generate a functional TCRβ chain mature from the DN3 to DN4 stage and are selected to develop into αβ T cells (reviewed in (von Boehmer et al., 1999)). This process has been termed 'β selection' and is controlled by the pre-TCR. The pre-TCR is a multi-subunit receptor complex comprising the TCRβ chain, a pre-TCα chain, and several non-covalently associated polypeptides, referred to as CD3 protein complex (Fig. 1-3). The signaling networks initiated by the pre-TCR complex serve many functions:

1) DN cells lacking a pre-TCR have a survival defect, implicating the pre-TCR in providing crucial anti-apoptotic signals.

2) Transition from the DN to DP stage is accompanied by prolific expansion, and signaling through the pre-TCR is important of this burst of proliferation.

3) Once functional rearrangements of one TCRβ allele have occurred and the pre-TCR is formed, pre-TCR signals terminate rearrangements at the other TCRβ allele, thus ensuring allelic exclusion.

4) Pre-TCR signals control further maturation to the DP stage, regulating numerous genes, including those required for induction CD4, CD8 expression, downmodulation of pTCα gene expression, and those involved in transactivation of the TCRα locus prior to its rearrangement.
Figure 1-3. Subunit composition of pre-TCR and the TCR. The precise arrangement of TCR polypeptides and the CD3 molecules in a given cluster is unknown. There may be even two TCR heterodimers in each cluster. CD3δ is difficult to detect and is not required for pre-TCR function, but is required for abTCR assembly and function. The "YLYL" motifs are immunoreceptor tyrosine-based activation motifs (ITAMs) that are critical for signaling. S-S represent covalent di-sulphide bonds. V and C represent 'variable' and 'constant' regions, respectively.
How the signaling specificity is achieved by the pre-TCR to control many of these functions is an intensely researched field (reviewed in (Kruisbeck et al., 2000)). In fact the precise mechanism by which pre-TCR signaling is initiated in still unknown. Although most lymphocyte surface receptor complexes are activated by ligand engagement, data suggests that engagement by a specific ligand in not necessary for pre-TCR signaling in vivo. Along these lines it has been shown that the potential ligand binding exodomains of pTα and TCRβ are dispensable for the pre-TCR function (Irving et al., 1998). However, it is likely that the pre-TCR needs to be transported to the cell surface of immature thymocytes, presumably to meet some crucial signaling components. Recent data suggests that pre-TCR might confer its signaling ability by directly targeting pre-TCR complexes into lipid micro-domains (rafts, GEMS), which are enriched in glycolipids and signaling molecules involved in T cell activation (Saint-Ruf et al., 2000). Some of the proteins involved in pre-TCR signaling include members of the pre-TCR complex – pTα, TCRβ, CD3γ, CDε, CD3ξ, as well as signaling molecules such as SLP-76. LAT, Lck/Fyn, Ras, ERK, Zap-70/Syk, Rho, Vav, CD45, FADD etc. The interrelationship of these molecules in orchestrating different cell-fate decisions is beginning to be worked out.

Following completion of TCRα gene rearrangements (induced by the pre-TCR signaling) and the production of the clonotypic TCRαβ-CD3 complex, CD4+CD8+ (DP) thymocytes are subjected to a rigorous positive and negative selection process.
Positive and negative selection at DP stage

Positive selection

The next important step in intrathymic T cell maturation, after expression of $\alpha \beta$TCR, CD4, and CD8 molecules, is the selection of cells that will make up the repertoire of mature T cells in the periphery. The concept of positive selection originated from the necessity of explaining self-MHC restriction of T cell function (Zinkernagel and Doherty, 1974). Therefore, positive selection is the process in which thymocytes, whose TCRs have sufficient affinity for self-MHC molecules complexed with self peptides, are permitted to survive and the process in which all those thymocytes whose TCRs have no reactivity for self-MHC die. Bone marrow chimeras were used to first demonstrate that the MHC genes of the host animal in which T cells develop determine the MHC restriction pattern of the mature T cells (Bevan, 1977; Zinkernagel et al., 1978). Bone marrow from an (AxB) F1 heterozygous mouse (A and B representing different sets of MHC genes) was used to rescue the lethally irradiated homozygous A parent. T cells from such chimeras preferentially responded to antigen in the context of host MHC (type A) rather than the MHC type of the unshared parent (type B), indicating that self-MHC present on radiation-resistant thymic stromal cells selected the T cell repertoire.

Definitive evidence for positive selection was provided for by the construction of TCR transgenic mice. In such a mouse model, large numbers of lymphocytes expressing the receptor derived from a T cell clone of known antigen specificity and MHC restriction are generated, and their behavior as a function of their environment is readily tracked. In one of the early TCR transgenic models, mice were created that express $\alpha \beta$TCR
transgenes specific for the male H-Y antigen presented in association with MHC molecule H-2D^b (Teh et al., 1988). T cell development is arrested at the DP stage, unless the restricting H-2D^b molecule is expressed in the thymus (Teh et al., 1988; Kisielow et al., 1988; Scott et al., 1989). The anti H-Y TCR is predominantly expressed on DP and CD8+ thymocytes and mature T cells, but not on CD4+ thymocytes in H-2b female mice. Results from the anti-H-Y TCR transgenic model have also been extended to other MHC class I-specific TCRs (Sha et al., 1988; Sha et al., 1988; Pircher et al., 1989) as well as to MHC class II-specific TCRs (Kaye et al., 1989; Berg et al., 1989b). Accordingly, there is a bias towards the generation of CD4+ peripheral T cells in MHC class II-specific TCR transgenic models. In summary, the results indicate that the MHC specificity of the TCR determines whether DP thymocytes develop into CD4 or the CD8 lineage.

The nature of the cells that display appropriate self-MHC molecules to the differentiating thymocytes during positive selection has been an important issue. Multiple studies suggest that the thymic cortex is the primary site of positive selection (Lo and Sprent, 1986; Cosgrove et al., 1992). In the cortex, positive selection is thought to be primarily mediated by radioresistant thymic epithelial cells based upon studies of both bone marrow and thymic chimeras. However, several reports have provided evidence that stromal cells' requirements for positive selection may not be restricted to thymic epithelial cells, and that fibroblasts (Pawlowski et al., 1993; Hugo et al., 1993b) and bone-marrow derived cells can mediate this process (Bix and Raulet, 1992). It is possible that most APCs could provide the initial signals for positive selection via a TCR-
peptide/MHC dependent pathway but would require in addition the thymic epithelium for further differentiation and survival (Yasutomo et al., 2000b).

Negative selection

As in positive selection TCR-MHC interactions also direct thymocyte negative selection. Negative selection in the thymus is the primary checkpoint for establishing self-tolerance in the T lymphocyte repertoire. An effective way to counteract autoreactivity is to eliminate potentially harmful thymocytes by clonal deletion. Evidence for such deletion initially came from studies on tolerance to superantigens (SAgs) and from analysis of self-reactive TCR transgenic mice.

A seminal study was the analysis of the frequency of CD4+ T cells expressing Vβ17 region, which imparts reactivity to endogenous mtv SAgs presented by the class II E molecule, and can be detected with a Vβ17-specific monoclonal antibody (mAb). Significantly fewer Vβ17+ cells were found in I-E' than in I-E' mice (Kappler et al., 1987). This deletion was found in peripheral T cells and mature TCRhi thymocyte pool but not to DP's, indicating that immature Vβ17+ T cells did exist in I-E' mice, yet somehow were eliminated before maturity. The second clear proof for negative selection came from experiments also from TCR transgenic mice with specificity for the male H-Y antigen. Deletion of the TCR-expressing thymocytes was evident in male mice displaying the relevant MHC molecule (Kisielow et al., 1988). Similar observations were made in a variety of TCR transgenic mice, with many forms of antigens and different
restricting elements (Pircher et al., 1989; Murphy et al., 1990; Berg et al., 1989a; Zal et al., 1994).

The precise location and the mode of negative selection have also been controversial. Initial studies suggested that hematopoietic thymic APCs, especially dendritic cells, play a crucial role in tolerance induction by clonal deletion (Marrack et al., 1988). As thymic dendritic cells are concentrated at the cortico-medullary junction, this process predominantly occurs at the transition between the cortical DP and SP stage of thymocyte differentiation. However, several independent studies have indicated that tolerance induction could occur in the cortex and the medulla following contact with epithelial cells (Houssaint and Flajnik, 1990; Bonomo and Matzinger, 1993; Hoffmann et al., 1992). In most TCR transgenic mice directed towards self minor (H-Y), viral (LCMV) or allo-MHC antigens, evidence for clonal deletion could be found in vivo in the early cortical CD4^+^8^+^ stage of maturation prior to the phenotypically positively selected T cells (Kisielow et al., 1988; Hengartner et al., 1988; Sha et al., 1988). However, the nature of the deleting antigen could also play a role in the timing of negative selection. In the LCMV-glycoprotein specific TCR transgenic model, the TCRs expressing the Vβ8.1 (TCRα chain) are also reactive to the Mls^3^—superantigens, in addition to the cognate LCMV-derived peptide. When Mls^3^ Sags expressed by hematopoietic cells were chosen as deleting self-antigens, positively selected antigen specific thymocytes were found in the thymic cortex but deletion of these thymocytes occurred later where apoptotic Mls^3^-reactive cells were found in the medulla (Ohashi et al., 1990). However deletion to LCMV-derived peptides could occur earlier in the cortex. These studies suggested that,
at least in TCR transgenic models, negative selection could occur at different stages of thymocyte maturation depending on the cortical or the medullary location or the dose of the deleting antigen (Speiser et al., 1992). These studies also suggested that any stromal type, being either hematopoietic or epithelial, could mediate negative selection.

The role of peptides in T cell selection

It is now well established that the maturation of DP thymocytes into CD8+ and CD4+ T cells is dependent upon interaction with complexes of peptides and MHC Class I and Class II molecules expressed on thymic epithelium. Studies of MHC mutant mice, where mutations in MHC class I molecules affected the residues involved in peptide binding without altering residues that contact the TCR, showed a role for peptides in thymocyte selection (Nikolic-Zugic and Bevan, 1990; Sha et al., 1990). These MHC mutant mice demonstrated impaired development of CD8+ cells bearing a class I specific TCR transgene, suggesting a role for peptides in positive selection. However, whether or not peptides play a direct role in positive selection or merely present to stabilize the surface expression of MHC molecules is unclear (Schumacher and Ploegh, 1994). To investigate this, fetal thymic organ cultures (FTOCs) which were established from mice deficient in MHC class I expression in TAP-1 (Ashton-Rickardt et al., 1993) and β2m knockout mice have been used (Hogquist et al., 1993). The stable expression of Class I molecules is dependent on the non-covalent association of β2m with MHC class I molecules as well as cytosolic peptides, which are translocated into the endoplasmic reticulum by a peptide pump derived from the products of the peptide transported associated with antigen processing-1 and -2 (TAP-1 and TAP-2) genes. Since mice lacking either TAP-1 or β2m
express unfolded MHC class I heavy chains, expression of Class I molecules can be restored by the addition of exogenous β2m and peptides to FTOCs (Ashton-Rickardt et al., 1993; Hogquist et al., 1993). While several peptides were identified which restored MHC class I expression in these systems, only a few of these class I stabilizing peptides were capable of rescuing positive selection of CD8+ cells (Ashton-Rickardt et al., 1993). Thus MHC bound peptides do not solely function to stabilize surface expression of Class I molecules, but appear to make a contribution to the specificity of positive selection. Subsequent studies used FTOCs from MHC class I-restricted, MHC class I-mutant mice to address directly the role of peptide specificity in thymocyte positive and negative selection (Ashton-Rickardt et al., 1994; Sebzda et al., 1994; Hogquist et al., 1994). These will be discussed in detail in the subsequent section.

**Models to explain paradox of thymocyte positive and negative selection.**

Thymic selection has a built-in paradox: both positive and negative selection involve interactions between the TCR and the peptide-MHC complexes on thymic epithelial cells and other thymic APCs. Any coherent view of thymic education must integrate the two processes.

**Altered Ligand Model**

Various models have been put forward to address the paradigm of how positive and negative selection could result from the peptide/MHC interactions through identical TCRs. An early microenvironment-based model suggests that positive and negative
selection are mediated by ligands presented by thymic epithelial cells and hematopoietic cells, respectively (Sprent et al., 1988) (Fig. 1-4A). By assuming that positive and negative selection are induced by mutually exclusive cell populations at defined T cell developmental stages, the altered ligand model explains how the same TCR can be involved in both types of selection. However, as discussed in the previous sections, data from various studies challenge this model. Thus, positive and negative selection are not strictly mediated by two distinct subsets of thymic stromal cells.

Other models have focused on the nature of peptides during selection. Do distinct peptides induce positive versus negative selection? Do these peptides trigger the generation of unique TCR-mediated signals that induce either positive or negative selection? Alternatively, positively and negatively selecting peptides may not have to be exclusive. Rather, the influential factor that dictates positive versus negative selection may be an integrated intracellular signal mediated by TCR triggering that meets the quantitative threshold required for positive versus negative selection. In this case, the nature of TCR interactions mediated by various peptide-MHC complexes influences the selection outcome: weak, sub-optimal interactions induce positive selection, whereas stronger interaction induce negative selection.

The Efficacy Model (qualitative/peptide model)

This model proposes that mutually exclusive qualitatively different peptides promote the distinct selection outcomes (Fig. 1-4B). This model was primarily supported by studies demonstrating that non-stimulatory antagonist peptides could promote positive selection.
while stimulatory agonist peptides induce negative selection. Hogquist et al, using 
FTOCs from β2m deficient mice crossed to the chicken ovalbumin (c-OVA) specific 
TCR transgenic mouse (OT-1), failed to demonstrate positive selection in the presence of 
agonist peptide, but observed positive selection in the presence of antagonist peptides 
(Hogquist et al., 1994; Jameson et al., 1994; Hogquist et al., 1995). In addition, agonist 
peptides were shown to induce efficient clonal deletion. Other studies using an antibody-
mediated model of thymocyte selection also implied that antagonist-like interactions 
promoted the maturation of CD8+ T cells (Basson et al., 1998).

According to this model, efficacy was defined as the ability of a ligand to catalyze 
receptor mediated biological functions (Mannie, 1991). Non-stimulatory antagonist 
peptides were originally shown to inhibit the subsequent activation of mature T cells 
using stimulatory agonist peptides (De Magistris et al., 1992). The stimulatory agonist 
peptides (hence efficacious) would induce negative selection. On the contrary, antagonist 
peptides that lack efficacy were expected to promote positive selection. This model was 
further supported by studies showing that interactions with altered peptide ligands 
transmit distinct intracellular signals, leading to altered ζ-chain phosphorylation and 
ZAP-70 activation (Sloan-Lancaster et al., 1994; Madrenas et al., 1995; Sousa et al., 
1996).

However, thymocytes have been shown to be more sensitive to T cell stimulation than 
mature T cells (Pircher et al., 1991; Yagi and Janeway, 1990; Davey et al., 1998). 
Therefore, antagonists peptides may be perceived as weak agonist-like interactions by
immature thymocytes. Therefore, it is misleading to study parameters that govern mature T cell activation (agonists, antagonists, etc) and apply them to T cell selection.

**Affinity/avidity (quantitative model)**

There is increasing evidence to favor a quantitative/avidity model, which proposes that thymocyte response is determined by the relative overall avidity of the interaction between thymocytes and APCs (Fig 1-4C). This is influenced by the affinity and the expression levels of peptide/MHC ligands to the TCR. In support of this model, when TAP-1 or β2m deficient mice were crossed with mice bearing transgenic TCR for LCMV (P14 TCR transgenic line), both positive and negative selection were observed in FTOCs, depending on the concentration of peptide used (Ashton-Rickardt et al., 1994; Sebzda et al., 1994). Thus, lower concentration of agonist peptide induced positive selection, while higher concentration of the same agonist peptide induced negative selection. Conversely, lowering the surface expression of particular MHC ligands in vivo also results in the shift in development of TCR transgenic thymocytes from negative to positive selection (Sha et al., 1990; Cook et al., 1997). MHC interactions with TCRs as well as coreceptors are important in determining the overall avidity, as shown by the fact that increased coreceptor expression results in a shift from positive to negative selection (Robey et al., 1992; Lee et al., 1992). Together, these studies suggest that the overall avidity of the TCR/MHC-peptide interaction determines whether the outcome is either positive or negative selection.
Intracellular signals involved in thymocyte selection

The quantitative/avidity model explains cell fate determination on the basis of the nature of TCR-peptide/MHC interactions. However, we do not understand how these interactions are translated to intracellular signals that distinguish between positive and negative selection. Although triggering through the same TCR mediates both positive and negative selection, the question remains whether similar signaling molecules influence both events (Fig. 1-5). TCR mediated intracellular signaling includes a multitude of molecules and pathways. At some point, the signals for positive and negative selection must diverge. Where does this occur? In addition, other molecules have been suggested to modify and contribute to the signals that are received by the thymocyte (Tarakhovsky et al., 1995; Plas et al., 1996; Pani et al., 1996; Foy et al., 1996; Punt et al., 1997; Kishimoto et al., 1998). How does this become incorporated into the 'signals' received by the immature T cells? Based on existing experimental data, a couple of models have emerged to explain how downstream signaling molecules (cytoplasmic) influence thymocyte selection.

Unique signaling model

The unique signaling model proposes that the distinction between positive and negative selection occurs by initiating independent signals at the level of the TCR/CD3 complex. These initial events then influence the propagation of distinct downstream signaling cascades and the activation of unique transcription factors and genes, leading to positive or negative selection.
Figure 1-5: TCR-mediated intracellular signals that influence thymocyte selection. Molecules that are involved specifically in positive selection (green), negative selection (red) and both positive and negative selection (yellow) are shown. These are based on studies from various transgenic systems.
Several lines of evidence suggest that mutations affecting either the TCR or the associated CD3 components influence thymocyte selection. By deleting the TCR α-chain connecting peptide domain (α-CPM), Backstrom et al were able to inhibit positive selection, while negative selection was unaltered using a defined-TCR transgenic mouse model (Bäckström et al., 1998). In addition, immuno-precipitation studies using these thymocytes showed that the mutant TCRs were not associated with the CD3δ chain. They concluded that ligands with different affinities may associate with different elements of the TCR/CD3 complex, such that low affinity ligands may initiate positive selection through α-CPM and CD3δ. High affinity ligands, on the other hand, may induce negative selection through additional elements of the TCR/CD3 complex.

MAP-Kinase signaling in thymocyte positive and negative selection

After engagement of the TCR by APCs, multiple T cell signaling systems come into play. The extracellular-regulated kinase (ERK) pathway, which includes Ras/Raf-1/MEK1,2/ERK1,2, is one such signaling module of the MAPK signaling pathways. MAP-kinases are activated by dual phosphorylation of threonine and tyrosine residues by upstream kinases, collectively known as MAP-kinase kinases (MKKs). The ERK MAP-kinases are phosphorylated on the Thr-Glu-Tyr amino acid motif by MEK1 and MEK2. Transgenes overexpressing the dominant negative forms of Ras, Raf, MEK-1 or both Ras and MEK-1 have inhibited positive selection, yet left negative selection intact (Swan et al., 1995; Alberola-Illa et al., 1996a; Alberola-Illa et al., 1996b; O'Shea et al., 1996). Furthermore, expression of activated MEK-1 using retroviral gene transfer into TCRα-/- thymocytes resulted in the generation of SP cells, while the selective inhibition of MEK-1
with PD98059 impeded positive selection but not negative selection (Sugawara et al., 1998). Mice lacking p44 MAP kinase (ERK1) also show moderately impaired positive selection and apparently normal negative selection (data not shown), further providing support for the role of ERK signaling in positive selection (Pagès et al., 1999). These studies support the role of ERK signal transduction in positive but not in negative selection. In addition, ERK has also been shown to play a role in thymocyte lineage commitment, such that overexpression of a hypersensitive form of ERK2 skews differentiation towards CD4+ T cell lineage (Sharp et al., 1997) and conversely, pharmacological inhibition of ERK modifies signals that would normally induce CD4+ differentiation to promote CD8+ lineage commitment instead (Sharp et al., 1997; Bommhardt et al., 1999; Sharp and Hedrick, 1999).

Ras-Grp is a Ras activator with Calcium binding EF hands and a diacyleglycerol (DAG)-binding domain, that has been shown to enhance ERK activation and IL-2 secretion when over-expressed in Jurkat T cells. Ras-Grp-deficient mice show that there is a block in T cell development at the DP stage and that these DP thymocytes are unable to activate Ras or downstream ERK after TCR cross-linking (Dower et al., 2000). However, its role in negative selection was not investigated in this study.

The JNK/SAPK group MAPKs consists of three different members - JNK1, JNK2 and JNK3. Targeted disruption of individual genes did not alter normal T cell development or CD4/CD8 ratios (Dong et al., 1998; Yang et al., 1998; Yang et al., 1997). However, expression of a dominant negative JNK-1 in transgenic mice inhibited the SAPK pathway
and led to an increased resistance to anti-CD3 mediated thymocyte death (Rincón et al., 1998b). A similar defect in anti-CD3 mediated thymocyte deletion was also obtained in an another independent JNK2-deficient transgenic model (Sabapathy et al., 1999).

P38 kinase is another member of the MAPK family of proteins that has been reported to be highly activated in response to intrathymic signals in vivo (Sen et al., 1996). By either blocking this pathway by using a specific pharmacological inhibitor SB203580 or by retroviral gene transduction of MKK6 (which activates p38), Sugawara et al showed that this pathway is critically involved in negative selection, but not positive selection (Sugawara et al., 1998). In addition, no significant alterations in the distribution of thymic subpopulations were reported in dominant-negative p38 and constitutively active MKK6 transgenic mice (Rincón et al., 1998a). However, experiments that specifically address the efficiency of negative selection were not tested in the latter study. While both studies concur that p38 is not essential for positive selection, Sugawara et al suggest that it may also be involved in negative selection.

Grb2 is an adapter protein that binds to Sos guanine nucleotide exchange factor (GEF) and potentiates Ras activation in T cells. Recently, a report by Gong et al (Gong et al., 2001) reported that mice heterozygous for Grb2 gene have impaired activation of the JNK and p38 MAP kinases but surprisingly normal activation of ERK. Interestingly, studies in these mice showed impaired deletion in various negative selection models and positive selection remained normal. This may lend further support to the model that positive and negative selection invoke different MAPK pathways.
Taken together, these results showing differential involvement of ERK in positive selection and JNK and p38 in negative selection, suggesting that activation of differential MAPK pathways lead to opposite fates for immature thymocytes. Indeed studies have suggested that cells either survive (and possibly proliferate) or undergo apoptosis, depending on the strength and the balance of the various MAPK signals (Xia et al., 1995). These observations lend support to the unique signaling model.

**Integrated signaling model**

Alternatively an integrated signaling model predicts that productive thymocyte/stromal interactions generate TCR/CD3-mediated signals cascades with different intensities. The strength of these signals from several pathways (but possibly not all pathways) are “integrated” by the cell. These cumulative signals in response to positively selecting stimuli or in response to negatively selecting stimuli then orchestrate a spectrum of downstream events, that lead to positive or negative selection. In support of the integrated signaling model many studies have shown that a particular signaling molecule can influence both positive and negative selection. In this case, the extent of activation of these molecules may play a role in affecting the ultimate activational threshold needed for different outcomes.

The induction of two TCR-proximal kinases, Lck and Fyn, is one of the earliest signaling events following TCR stimulation (Qian and Weiss, 1997). Once activated, these Src kinases phosphorylate ITAMs in the CD3 complex and, subsequently, the ZAP-70
tyrosine kinase. The specific role of Lck in T cell selection was addressed by over-expressing a catalytically inactive form of Lck, which inhibited positive selection (Hashimoto et al., 1996). Although deletion proceeded normally in H-Y transgenic thymocytes, superantigen mediated negative selection was impaired in these mice. On the other hand, initial studies using thymocytes from Fyn -/- mice showed normal positive selection (Stein et al., 1992; Appleby et al., 1992). The absence of Fyn in T cells also resulted in limited clonal deletion to Mls-sa, but not to SEA (Stein et al., 1992). Further detailed analysis using TCR transgenic mice that do not express Fyn showed impaired maturation of selected thymocytes and a slight reduction in negative selection (Utting et al., 1998). Therefore, although Lck appears to play a prominent role, both Lck and Fyn contribute to positive and negative selection.

The activity of both Lck and Fyn is positively regulated by CD45, a transmembrane tyrosine phosphatase that is essential for positive selection (Kishihara et al., 1993; Byth et al., 1996). Although superantigen-mediated deletion proceeds normally, anti-CD3-mediated negative selection is also impaired in the absence of CD45. However, a recent study using a novel CD45-deficient mice expressing transgenic TCR show that there is a large increase in the thresholds of TCR stimulation required for both positive and negative selection in the absence of CD45 (Mee et al., 1999). In this model a role for CD45 in mediating efficient superantigen-mediated deletion was revealed in response to both Mls superantigens and exogenously administered SEB.
On the other hand, Csk functions as a repressor of Lck and Fyn activity and deletion of Csk activity causes thymocytes to mature exclusively into CD4\(^+\) cells, even in the complete absence of TCR signaling (Schmedt et al., 1998). However, TCR-mediated negative selection in Csk-deficient TCR transgenic thymocytes is normal (Schmedt and Tarakhovsky, 2001). Since the absence of Csk enhances TCR activation thresholds it would be difficult to see increased thymocyte deletion in the H-Y TCR transgenic model, where deletion of thymocytes in male mice is generally quite efficient. Other negative selection models in which the efficiency of negative selection could be experimentally varied would be a better suited for these studies.

Mutational analysis of the ζ-chain family also influences both positive and negative selection (Shores and Love, 1997a). The cytoplasmic domains of CD3 chains contain immunoreceptor tyrosine-based activation motifs (ITAMs) which become phosphorylated upon antigen stimulation. Each of the CD3 molecules γ, δ, and ε contains one ITAM, while the associated ζ chain contains three intracellular motifs. Although the ζ chain-associated ITAMs have been shown to be dispensable for thymocyte selection (Ardouin et al., 1999), studies by Shores et al have shown that multiple ITAMs could modulate TCR-mediated signal intensities and influence thymocyte selection (Shores et al., 1997b). Breeding transgenic ζ chains with one to three ITAMs to a CD3ζ-/- background served to amplify the signals generated by TCR engagement. A direct relationship was observed between the number of ITAMs and the efficiency of both positive and negative selection (reviewed in (Love and Shores, 2000)). In addition, Yamanakazi et al. observed a shift from negative to positive selection of H-Y specific T
cells in male mice deficient for the ζ-chain (Yamazaki et al., 1997). Together, these studies demonstrate the integral role of the CD3 components in influencing both selection events.

Phosphorylated ITAM motifs can recruit further signaling molecules including the Syk family PTKs such as Zap-70 and Syk. Activated ZAP-70 phosphorylates a number of downstream substrates, including LAT, PLCγ1, Cbl, Vav, and SLP-76 (Qian and Weiss, 1997; Alberola-Illa et al., 1997; Peterson et al., 1998). The essential role of ZAP-70 in T cell development has been established by studies in both humans and mice. In humans, a rare immunodeficiency syndrome results from a mutation in ZAP-70 that inhibits the development of CD8+ T cells and exhibits non-functional CD4+ T cells (Arpaia et al., 1994; Chan et al., 1994; Elder et al., 1994). Thymocytes from ZAP-70 deficient mice show a severe block in positive selection of both CD4+ and CD8+ T cell lineages as well as negative selection of DP thymocytes (Negishi et al., 1995). Ablation of ZAP-70 homologue Syk does not affect the development of αβ T cells (Turner et al., 1995; Cheng et al., 1995), although it may compensate for some ZAP-70 functions (Cheng et al., 1997; Gong et al., 1997).

Cbl, SLP-76, LAT and Gads are adapter molecules that lack intrinsic enzymatic activity, but which mediate critical protein-protein interactions with other signaling molecules, influencing T cell activation (Peterson et al., 1998). Recent data indicates that tyrosine-phosphorylated Cbl negatively regulates TCR signaling (Boussiotis et al., 1997; Murphy et al., 1998). In Cbl-deficient mice, positive selection of thymocytes expressing a MHC
class II restricted transgenic TCR was significantly enhanced (Naramura et al., 1998).

The generation of null mutations in mice in either SLP-76 or LAT exhibited a more profound block in thymocyte development with an absence of DP thymocytes and peripheral T cells (Clements et al., 1998; Pivniouk et al., 1998; Zhang et al., 1999). Curiously, gene deficiency of Gads, an adapter molecule that associates SLP-76 to LAT showed maturation of DN thymocytes to the DP stage; however, Gads -/- thymocytes demonstrated impaired responses to CD3 cross-linking in vivo and impaired positive and negative selection in TCR transgenic models (Yoder et al., 2001).

Vav, a Rho-family guanine-nucleotide exchange factor, is required for efficient TCR signaling and thymopoiesis in mice (Fischer et al., 1998a). Studies from Vav-deficient mice have shown inefficient thymocyte positive and negative selection (Fischer et al., 1995; Turner et al., 1997; Kong et al., 1998). This may be due in part to compromised TCR-mediated signaling, such as reduced calcium flux that may be associated with impaired actin polymerization and TCR capping in Vav-deficient thymocytes (Fischer et al., 1995; Turner et al., 1997; Fischer et al., 1998b; Holsinger et al., 1998; Kong et al., 1998). In addition to defective intracellular Ca2+ influx, some studies have also shown impaired activation of ERK and NF-κB in Vav-/- T cells (Costello et al., 1999).

Differential effects on positive and negative selection have also been reported for the transmembrane molecule CD5. DP thymocytes increase their CD5 expression during selection events (Fowlkes and Pardoll, 1989), and the increase in CD5 expression seems to directly correlate to the extent of TCR triggering (Azzam et al., 1998). Tarakhovsky et
al (Tarakhovsky et al., 1995) have examined positive and negative selection in CD5-deficient mice. Their data showed that the absence of CD5 renders the thymocytes hyper-responsive and accordingly alters thymocyte selection in different MHC class I-specific TCR transgenic mice. The lack of CD5 improved positive selection in F5 TCR transgenic mice (influenza peptide-specific), improved negative selection (at the expense of positive selection) in P14 TCR transgenic mice and enhanced positive and negative selection in H-Y TCR transgenic female and male mice, respectively. These data suggest that CD5 modulates TCR signaling during selection, but the outcome may depend on the overall avidity of TCR-peptide/MHC interactions.

One potential membrane proximal binding partner for CD5 is SHP-1, an intracellular tyrosine phosphatase and a negative regulator of T cell signaling (Pani et al., 1996). In this regard it is interesting that SHP-1 deficient mice showed similar defects in thymocyte selection compared to CD5-deficient mice (Plas et al., 1999; Zhang et al., 1999; Carter et al., 1999). A consistent observation of these studies is that SHP-1 and SHP-1 regulated molecules could influence the strength of TCR-mediated signals and, in turn, help set the threshold for thymocyte selection.

TCR stimulation also leads to the recruitment and activation of PLCγ1 at the cell membrane (Alberola-Illa et al., 1997). PLCγ1 stimulates the catabolism of membrane phospholipids and the release of phosphoinositide second messengers: inositol 1,4,5 triphosphate (IP3) and diacylglycerol (DAG). One of the roles of Itk, a Tec family
tyrosine kinase that is activated upon TCR ligation, includes phosphorylation of PLCγ1. Accordingly, T cells from Itk-deficient mice have shown defective TCR-induced IP3 generation and calcium mobilization (Liu et al., 1998). In addition, mutant mice exhibited decreased numbers of mature T lymphocytes and an appreciable block in development of thymocytes bearing defined transgenic TCRs (Liao and Littman, 1995). However, deletion of male-specific H-Y TCR transgenic T cells was not affected by the absence of Itk. Recently, mice lacking both Tec family genes, Rlk and Itk were generated (Schaeffer et al., 2000). Interestingly, CD8+ T cells were generated in the normally negatively selecting HY TCR-transgenic male environment. Again, a threshold model for thymocyte selection seems to explain these mutations: impaired TCR signal strength results in a shift from negative to positive selection and from positive selection to neglect.

The elevation of intracellular calcium in response to T cell activation induces the calcium/calmodulin-dependent serine phosphatase calcineurin (Alberola-Ila et al., 1997). Studies using the calcineurin inhibitors cyclosporin A and FK506 have shown that the calcium/calcineurin pathway is important for both positive and negative selection (Jenkins et al., 1988; Gao et al., 1988; Shi et al., 1989). In addition, thymocytes undergoing negative selection have been shown to induce elevated intracellular calcium (Nakayama et al., 1992; Mariathasan et al., 1998). However, other studies using the same inhibitors have suggested that this pathway does not play a role in thymic deletion (Vasquez et al., 1994; Anderson et al., 1995; Wang et al., 1995; Hayden-Martinez et al., 2000). Though these studies may suggest that the role of calcineurin in thymocyte
selection is controversial, they may reflect the differential sensitivity to inhibition of calcineurin to the relative strengths of deleting stimuli. In support of this, studies by Kane et al have shown that, although deletion can occur in the absence of calcium in response to a strong stimulus, a role for calcium influx was revealed under conditions of limiting stimulation (Kane and Hedrick, 1996). These results suggest it may be easier to block positive selection and weak negative selection but not strong deletion events, when using pharmacological agents that inhibit the Ca2+/calcineurin pathway. Together, these studies support the integrated signaling model by suggesting that the intensity of the early signals derived from TCR/peptide-MHC interactions determine thymocyte fate.

The two models (unique signaling model vs integrated signaling model) appear to be mutually exclusive but overlapping models are also plausible. Such complexity reflects the nature of the intracellular circuitry downstream from the TCR/CD3 complex. The above models provide a simplified framework to accommodate existing data concerning signaling molecules that affect T cell selection. Although these models take into consideration the nature and the intensity of TCR-mediated signals as key factors for influencing thymocyte selection, other parameters should also be considered. For instance, positive selection is a multi-step process, requiring continuous signaling between the stroma and T cells in order to ensure differentiation of thymocytes (Kisielow and Miazek, 1995; Wilkinson et al., 1995). This suggests that kinetics of T cell signals may influence thymocyte fate. The manner in which TCR-mediated signaling pathways are “connected” in different ways provides yet another dimension for regulating thymocyte fate.
LCMV-specific TCR transgenic mice

The generation of TCR transgenic mice has greatly enhanced our understanding of thymocyte positive and negative selection. By creating a mouse expressing a specific TCR, thymocyte selection can be easily analyzed at a population level. The strain of TCR transgenic mice used in this thesis was derived from TCR α and β chains extracted from the CTL clone, P14, which recognizes lymphocytic choriomeningitis virus glycoprotein (LCMV-gp) in the context of MHC class I molecule, H-2D^b (Fig 7A). The full length TCRα (Va2-JαTA31-Cα gene segments) and TCRβ (Vβ8.1-Dβ-Jβ2.4-Cβ2 gene segments) cDNA were subcloned into an expression vector (Pircher et al., 1989; Pircher et al., 1989). The cDNA was set under the control of the MHC class I promoter, H-2K^b, and a genomic fragment of human β globin gene was joined 3' to introduce RNA splice sites and poly(A) signal. Additionally, an immunoglobulin heavy chain (IgH) enhancer element was inserted downstream within this expression cassette. The use of productively rearranged TCR transgenes has a profound effect on the TCR repertoire since these transgenes inhibit endogenous TCR rearrangement. In P14 TCR transgenic mice, the vast majority of H-2D^b-restricted T cells express Va2 and Vβ8.1 TCR chains. Since H-2D^b is an MHC class I molecule, most mature transgenic T cells express CD8 coreceptors. Thymocytes expressing this transgenic TCR cannot be positively selected using MHC II molecules; therefore class II-restricted T cells must rearrange and express endogenous α chain if they are to survive. Since the transgenic TCR expressed on immature thymocytes confers recognition of self-MHC class I as an
Figure 1-6: The P14 TCR Transgenic model (A). Thymocyte profiles of positively selecting (H-2D$^{b\beta}$) and non-selecting (H-2D$^{d\alpha}$) MHC backgrounds, (B) and (D) respectively.
appropriate selecting ligand, CD8+ thymocyte development is favored (Fig. 1-6B). This results in the skewing of the post-selection thymocyte population towards the CD8 lineage, and consequently, a reduced proportion of mature CD4 T cells. Using antibodies specific for the TCR chains and co-receptors, it is possible to study transgenic thymocyte development in positively selecting and non-selecting backgrounds (Fig. 1-6B, C). As previously discussed in earlier sections, the P14 TCR transgenic mice have contributed towards elucidating many aspects of thymocyte selection (Pircher et al., 1989; Ohashi et al., 1990; Sebzda et al., 1994; Ashton-Rickardt et al., 1994).

Thesis

Although much work had been directed towards defining peptides or stimulation conditions that induce thymocyte positive and/or negative selection, the precise biochemical events that discriminate these processes are still unknown. The foundation for the affinity/avidity model for T cell selection was based on studies that focused on the extracellular interactions between the TCR and the peptide-MHC complexes: weak interactions favor positive selection, while strong interactions trigger clonal deletion.

The manner by which these extracellular interactions influence TCR triggering and activation of downstream intracellular signaling pathways is the focus of my thesis. To begin with, we asked whether positively and negatively selecting stimuli influence the dynamics of TCR triggering, thereby studying one of the earliest signaling events associated with thymocyte selection. Past studies have suggested that there is good
correlation between the ability of the ligand/MHC complexes to induce TCR down-modulation and its relative strength as an agonist for TCR triggering (Bachmann et al., 1997b; Preckel et al., 1997). During activation, mature T cells seem to keep count of the number of internalized TCRs and elicit various biological responses corresponding to different activating thresholds (Viola and Lanzavecchia, 1996). Since there is also a quantitative aspect to T cell selection, we then asked whether the extent of peptide-mediated TCR down-modulation could identify TCR-mediated events that discriminate between positive and negative selection. We examined the influence of a set of defined peptide variants of LCMV-gp peptide (p33) on T cell development using our P14 TCR-transgenic model. The results demonstrated that peptides mediating sub-optimal TCR internalization are effective at triggering positive selection, whereas peptides mediating efficient TCR internalization are proficient at triggering negative selection. We also showed that the magnitude of Ca2+ flux set by these peptides also reflects the hierarchy of TCR internalization and correlates with positive and negative selection of thymocytes. This is in accordance with the affinity/avidity model for selection. This work, entitled “Degree of TCR internalization and Ca2+ flux correlates with thymocyte selection” was published in the Journal of Immunology (1998) 161:6030-6037.

During T cell activation, the Ras-Raf-MEK-ERK module plays an important role in linking extracellular membrane proximal signals to the cell interior (Alberola-Ila et al., 1997). Activated ERKs translocate to the nucleus and activate transcriptional regulatory proteins. Studies addressing the role of the Ras pathway in thymic development indicate that it plays a selective role in thymocyte positive selection and not negative selection.
(Swan et al., 1995; Alberola-Ila et al., 1996a; Alberola-Ila et al., 1996b). Primarily these studies were conducted in dominant-negative transgenic mice expressing the Ras, MEK-1 or both Ras and MEK-1. However, it is not clear if the Ras dependent signaling pathway was completely blocked in these dominant negative systems. Negatively selecting stimuli deliver substantially stronger signals than do positively selecting stimuli. However, partial inhibition of the MAPK cascade might attenuate only the weakest signaling events, i.e., positive selection. We used PD98059, a selective inhibitor of MEK1 and MEK2 as an alternative approach to attenuate ERK signals (Alessi et al., 1995). We examined the role of ERK in negative selection, by adding PD98059 to P14 TCR β2m-/ FTOCs in the presence of negatively selecting stimuli. Analysis of these cultures showed impaired clonal deletion and a concomitant increase in positive selection of functionally mature transgenic T cells. This demonstrates that altering ERK activity switched negative selection to positive selection. In addition, we showed that positive selection could also be abrogated by the addition of PD98059 to cultures that have been positively selected by weak endogenous ligands. Collectively our data suggests that the ERK-MAP Kinase cascade is involved in both positive and negative selection. These results were reported in the European Journal of Immunology (2000) 20:1060-1068.

An unequivocal role for ERK signal transduction in guiding positive selection has been shown in thymocytes. In addition our results indicated that ERK signaling is also involved in negative selection. How positively and negatively selecting ligands induce apparently similar signals and yet are able to evoke very different outcomes is of great interest. Differences in the “strength” of signals could be one criterion. Thymocyte
selection (DP to SP transition) is a differentiation process and the "duration" of ERK signaling has also been shown to influence differentiation processes in other biological systems (Marshall, 1995).

In order to ascertain the kinetics of ERK activation in thymocytes receiving either positively or negatively selecting stimulus, we devised a novel in vitro culture system (Fig. 1-7). The system involved an initial signaling culture (~16 hours) and a follow-up FTOC culture (~60 hours) in which cells from the primary signaling culture were placed for further differentiation (Fig. 1-8). The primary culture consisted of a monolayer of peritoneal macrophages as APCs presenting either positively or negatively selecting ligands. Naive TCR transgenic DP thymocytes from P14/RAG2-/- H-2\textsuperscript{d}d mice were co-cultured with these APCs and at various time points thymocytes were harvested, subjected to flow cytometry and biochemical analysis. Data demonstrated that positively and negatively selecting stimuli induced sustained versus transient ERK activation, respectively. Blocking the transient ERK activity in thymocytes also prevented the apoptosis induced by negatively selecting stimuli. (The cultured thymocytes were also placed in the secondary FTOC cultures to demonstrate that the primary monolayer culture provided the necessary signals for thymocyte selection.) The manuscript comprising this data is under review for publication.

As opposed to what has been previously proposed in the literature, these studies suggest that both positive and negative selection, are influenced by the ERK MAP Kinase module. However, the extent and timing of ERK activation could differentiate the two
opposing biological processes. Finally, our data suggests that there is a link between the extent of TCR down-modulation and activation of downstream signaling molecules.
Pre-incubate macrophages APCs (H-2b) with peptides

Coculture thymocytes with APCs

Harvest thymocytes

Discard residual APCs with Dynal beads complexed to anti F4/80 Ab (macrophage marker)

P14 RAG2-/- d/d (thymocyte profile)

Purified thymocytes

-- Whole cell lysates
-- Western Blot analysis
-- Activation specific antibodies
(e.g.: phospho-p42/44 ERKs)

FCM analysis with Anti-CD4, CD8, Vα2, CD5, CD69, Annexin/PI

Figure 1-7: Monolayer culture system in which naïve P14RAG2-/- d/d DP thymocytes would receive the appropriate initial signals for positive and negative selection (Signaling culture-1).
P14 RAG2-/ d/d
Thymocytes

“signaling culture” (-16 hours)

Harvest thymocytes

Transfer to ‘empty’ thymic lobes

E.day 14/15 (C57BL6)
Harvest thymic lobes

Treat FTOCs with deoxyguanosine for 5 days

Culture reconstituted FTOC lobes for ~ 60 hrs

Flow cytometric analysis

Figure 1-8: Fetal thymic organ culture system to induce further maturation (DP to SP) of signaled thymocytes.
Chapter II
Chapter II

Materials and Methods

Mice.

P14 TCR transgenic mice were previously generated using α and β chains isolated from CTL clone P14, which recognized the LCMV glycoprotein (peptide p33-41), presented by H-2D\(^b\) (32). P14 RAG2\(^{-/-}\) and P14 β2m\(^{-/-}\) were previously described (Kawai and Ohashi, 1995; Sebzda et al., 1996). P14 RAG2\(^{-/-}\) H-2\(^{\text{dld}}\) mice were generated by crossing P14 RAG2\(^{-/-}\) (H-2\(^b\)) with BALB/c (H-2\(^{\text{dld}}\)) (Taconic Farms). RAG2\(^{-/-}\) deficiency was screened by staining peripheral blood for the lack of B cells with PE-conjugated anti-mouse CD45R/B220 (RA3-6B2) mAb. H-2\(^d\) and H-2\(^b\) haplotypes were screened by using PE-conjugated anti-mouse H-2K\(^b\) and H-2K\(^d\) mAbs (PharMingen). The presence of P14 (Vα2) TCR transgene was determined by PCR. (PCR primers: Vα2 - CTG ACC TGC AGT TAT GAG GAC AGC AC and Cα - CGA GGA TCC TTT AAC TGG TAC ACA GCA GG). All mice were kept at the Ontario Cancer Institute in accordance with institutional guide-lines.

MEK1 inhibitor: PD98059 was purchased from Calbiochem and was resuspended in DMSO (50mM stock solutions). Aliquots were frozen at -20°C.

Peptides

The peptides p33 (KAVYNFATM), S7A (KAVYNFSTM), Y6F (KAVVNYATM), A4Y (KAVANFATM), L6F (KAVYNLATM), W4Y (KAVWNFATM), adenovirus peptide
AV (SGPSNTPPEI), and LCMV nucleoprotein 116-127 (RPQASGVYMG) were synthesized by a solid-phase method using the F-moc/tBu-based protocol. Chain assembly was conducted at the Amgen Institute (Thousand Oaks, CA) as previously described (Sebzda et al., 1996).

**Peptide binding assay (Chapter 3)**

In peptide-pulsing experiments, 10⁶ RMA-S cells which were previously cultured overnight at 29°C in RMPI plus 10% FCS, were incubated with various concentrations of peptide at 29°C for 30 min. These cells were then transferred to a 37°C incubator for 3 h, after which the cells were washed and stained with anti-H-2Dᵇ mAb from tissue culture supernatant (B22.249) (Hammerling et al., 1979; Allen et al., 1986) and then FITC-conjugated rat anti-mouse Ig (Sigma, St. Louis, MO). RMA-S cells were incubated with LCMV nucleoprotein 118-127 (H-2ᵈ restricted) (von Herrath et al., 1994) to determine background H-2Dᵇ expression.

**Induction of TCR down-regulation (Chapter 3)**

Spleen cells from TCR transgenic mice (10⁵/well) were mixed with peptide-pulsed macrophages (2 X 10⁵/well), centrifuged, and incubated at 37°C (5% CO₂) in IMDM supplemented with 10% FCS in round-bottom 96-well plates. Five hours later, cells were harvested and stained for CD8 (PE; PharMingen, San Diego, CA) and Vα2 (FITC; PharMingen) and were analyzed by FCM. Median Vα2 expression is shown for CD8+ T cells.
Mature T cell Proliferation assays (Chapter 3)

Spleen cells (10^3/well) from TCR-transgenic or TCR-transgenic recombination-activating gene-2^{+} mice were incubated in triplicate in 96-well flat-bottom plates with 10^5/well irradiated C57BL/6J (H-2^{b}) splenocytes that had been prepulsed with various concentrations of peptide for 1 h at 37°C. After 48 h of cocultivation, the cells were pulsed with 1 μCi of \textsuperscript{3}H-thymidine (Amersham, Arlington Heights, IL) for 16 h. Cells were harvested and counted on a direct beta counter (Matrix96, Canberra Packard Canada, Mississauga, Canada).

Thymocyte Proliferation Assay (Chapter 4).

P14 CD8\(^+\) thymocytes were harvested from P14 RAG2^{−/−} mice by magnetic sorting with anti-mouse anti-CD4 MACS® mAbs (negative selection) and anti-mouse anti-CD8 MACS® mAbs (positive selection) as per the manufacturer's protocol (Miltenyi Biotech). When applicable, these thymocytes were preincubated with various concentrations of PD98059 for 1 hr at 37\(^\circ\) C prior to stimulation. 5 x 10\(^4\) thymocytes were incubated in triplicate in 96-well flat-bottom plates with 10^5 irradiated spleen cells (H-2^{b}) that had been prepulsed with various concentrations of peptide p33. After 24 hr cocultivation in IMDM supplemented with 10\% FCS, 2mM glutamine, 5 x 10^{-5} M 2-βME, penicillin and streptomycin, the cells were pulsed with 1 μCi of \textsuperscript{3}H − thymidine (Amersham) for 16 hr. Cells were harvested and counted on a direct beta counter (Matrix96; Canberra Packard Canada Ltd).
**FTOC proliferation assay (Chapter 3)**

Cultured thymic lobes were teased apart and stained at 4°C in PBS containing 2% FCS with FITC-conjugated anti-CD8 (Cedarlane) and PE-conjugated anti-CD4 (Cedarlane). These cells were then sorted using a FACStar Plus (Becton Dickinson) to collect CD8+ thymocytes. Irradiated spleen cells from a C57BL/6J mouse were prepulsed with 10^-7 M peptides for 1 h at 37°C, washed, and distributed in triplicate on a flat-bottom 96-well plate at a concentration of 10^5 cells/well. CD8+ thymocytes (5 X 10^4/well) resuspended in IMDM, 10% FCS, penicillin, streptomycin, and 5 x 10^-5 M 2-ME were then added to these wells. The cells were cultures at 37°C for 1 day, pulsed with 1 μCi of ³H-thymidine for 16 h, and harvested as described.

**FTOC proliferation assay (Chapter 4).**

Thymic lobes that were cocultured with PD98059 and peptide (as described above) were rested with media alone for an additional 36 hr. CD8+ thymocytes from these lobes were harvest by MACS® sorting as also described above. 5 x 10^4 thymocytes were incubated in triplicate in 96-well flat-bottom plates with 10^5 irradiated spleen cells (H-2b) that had been prepulsed individually with various peptides (p33, AV). After 24 hr cocultivation in IMDM supplemented with 10% FCS, 2mM glutamine, 5 x 10^-5 M-2-βme, penicillin and streptomycin, the cells were pulsed with 1 μCi of ³H – thymidine (Amersham) for 16 hr. Cells were harvested and counted on a direct beta counter (Matrix96; Canberra Packard Canada Ltd).
Flow cytometry

Thymocytes were stained with PE-conjugated anti-CD4, FITC-conjugated anti-CD8 and biotin-conjugated anti-CD69, HSA, anti-CD5, anti-Vα2, H-2D^b. All antibodies were purchased from Pharmingen. Biotinylated Abs were detected with streptavidin red 670 (Life Technologies, Gaithersburg, MD). Cell viability was determined by enumerating trypan blue negative cells or by flow cytometry, staining thymocytes with FITC-conjugated Annexin-V and propidium iodide (Biosource International). All flow cytometric analysis was performed on a FACScan instrument (Becton Dickinson, Mountain View, CA). Samples were gated for live cells based on forward and side scatter parameters (10,000 events/sample) and were analyzed using LYSIS II software (Becton Dickinson).

Fetal thymic organ cultures (FTOC) (Chapter 3)

For positive selection assays, timed breedings were established between TCR β_2m^{+/-}H-2^b males and β_2m^{-/-}H-2^b females. To test for negative selection, TCR β_2m^{+/-} males were bred with C57BL/6J females. On day 16 of gestation, females were sacrificed, and thymic lobes were removed from the fetuses. The fetal thymic lobes were placed on 0.8μm polycarbonate filters (Costar, Cambridge, MA), which floated on 1ml of IMDM, 1X Nutridoma-SP (Boehringer Mannheim, Indianapolis, IN), 5 X 10^{-5} M 2-ME, penicillin, streptomycin, 2mM glutamine, 2.5μg human β_2m (Sigma), and designated peptides. These lobes were then cultured for 6 days at 37°C, during which time the medium and peptides were added daily. After this incubation period, the thymic lobes were teased apart and stained with mAbs at 4°C in PBS containing 2% FCS and 0.2% NaN_3. Three-color analysis was performed with rat anti-mouse PE-conjugated anti-CD4,
FITC-conjugated anti-CD8, and biotinylated anti-Vα2 (B20.1) (PharMingen) or biotinylated anti-heat stable antigen (HSA; M1/69; PharMingen).

Addition of PD98059 to FTOCs (Chapter 4)

FTOC lobes were always preincubated with 25 μM PD98059 for 1 hr prior to the addition of p33 peptide. The lobes were cultured for 6 days at 37°C, during which time the media, PD98059 and peptide were changed daily.

Ca²⁺ flux (Chapters 3 and 5)

TCR-transgenic thymocytes were loaded with indo-1 (10 μM) for 1 h at 37°C in IMDM supplemented with 2% FCS. Indo-1⁺ cells exhibiting a large forward scatter corresponding to thymocyte–APC duplexes (Valitutti et al., 1995) were analyzed with FACS Vantage (Becton Dickinson) and CellQuest software, using an ion laser (Innova Enterprise: Coherent, Santa Clara, CA) optimized for UV argon ions, set for 355-nm excitation at a power setting of 50 mV. For stimulation of thymocytes, peritoneal macrophages were pulsed with various peptides (p33, S7A, Y6F, A4Y, W4Y, L6F, and AV) for 1 h. Macrophages (4 x 10⁶/ml) were mixed with thymocytes (1 x 10⁷ cells/ml) at 4°C, centrifuged, and warmed to 37°C for 3 min. Cells were gently resuspended and immediately analyzed. The basal level of Ca²⁺ flux observed in thymocytes in the presence of unpulsed APCs was calibrated at 200, as an arbitrary value. The Ca²⁺ flux induced by the nonstimulatory AV peptide was superimposable on this basal Ca²⁺ flux and hence should be read as the baseline response.
Western Blot Analysis (Chapters 4 and 5)

Thymocytes were harvested from P14 RAG2\(^{-}\) H-2\(^{dd}\) mice and kept on ice for 3 hours as single cell suspension in IMDM supplemented with 10% FCS, 2 mM glutamine, 5 \(\times\) 10\(^{-5}\) M 2-ME, penicillin and streptomycin. An adherent monolayer of peptide-pulsed 2A9 (H-2\(^{b}\)) thymic epithelial cell line was used as APCs (Hugo et al., 1993a). Whenever PD98059 was used in the assay, thymocytes were preincubated with the inhibitor for 1 hr at 37\(^{\circ}\) C prior to stimulation. For peptide stimulation, these thymocytes were then complexed with the peptide-pulsed 2A9 cells by quick-spinning the cells at 1300 RPM for 30 seconds. The cells were placed in the 37\(^{\circ}\) C incubator for 1 hour. Thymocytes were then harvested by disrupting them from the APCs by gentle pipetting. These were washed once in PBS and pelleted. Whole cell lysates were prepared by lysing cells with lysis buffer containing 50mM Tris pH 7.5, 20mM EDTA, and 1% Triton x-100 supplemented with 10 mg/ml Aprotinin, 10 mg/ml Leupeptin, 0.2mM PMSF on ice for 20 minutes. The lysates were cleared by centrifugation. Protein concentration were determined using a commercially available kit (Bio-Rad). Cell lysates with equivalent protein content were electrophoresed in a 12% SDS-polyacrylamide gel, transferred to a nylon membrane and detected for 'activated' ERK1/2 by using PhosphoPlus\(^{\circledR}\) p44/42 MAP Kinase (Thr202/Tyr204) antibody kit (NEB Inc). Signals were detected by ECL system (Amersham).

In vitro culture of thymocytes – Culture I (Chapter 5)

C57BL/6 mice or \(\beta_2m\)-deficient mice were used for harvesting day 4 thioglycollate activated peritoneal macrophages as APCs. Macrophages were plated at 3x10\(^{5}\) cells/well
in 24-well plates in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FCS, 2 mM glutamine, 5 x 10^{-5} M 2-ME, penicillin and streptomycin. After overnight incubation non-adherent cells were gently washed away. The APCs were then pulsed with appropriate concentration of peptides for two hours. Thymocytes were harvested from P14 RAG2^{-/-} H-2^{dd} mice and kept on ice for 2 hours as a single cell suspension. For peptide/APC stimulation thymocytes (2 x 10^6 cells/well) were then complexed with the peptide-pulsed macrophages by quick-spinning the cells at 1300 RPM for 30 seconds. The cells were placed in the 37°C incubator for appropriate periods of time. Thymocytes were then harvested by gentle pipetting. Usually thymocytes from 2-3 wells were pooled to obtain sufficient number of cells to perform biochemistry. Residual macrophages were removed by incubating these thymocytes with anti-F4/80 antibodies conjugated with anti-Rat-IgG Dynal magnetic beads for 30 minutes at 4°C and subjecting them to a magnetic field. For studies using PD98059, thymocytes were preincubated with the inhibitor for 1 hour at 37°C prior to stimulation.

*In vitro* culture II – FTOC culture (Chapter 5)

Day 15 fetal thymic lobes from C57BL/6 mice were cultured for 5 days in 1.35 mM deoxyguanosine (Sigma, St. Louis, MO). The lobes were washed three times in media. 2x10^5 thymocytes that had been stimulated for 16 hours in the monolayer signaling cultures were placed in Terasaki wells along with deoxyguanosine treated lobes. These were cultured overnight as hanging drops in a humidified chamber in the 37°C incubator. Reconstituted lobes were then placed on 0.8 μm polycarbonate filters (Costar Corp), and further incubated for 40 hours in media containing 12.5% FCS. After this incubation, the
thymic lobes were teased apart, the cells were enumerated by trypan blue exclusion and stained for flow cytometric analysis with anti-CD4-PE, CD8-FITC and H-2K\(^d\)-biotin.
Chapter III
Chapter III

Degree of TCR Internalization and Ca\(^{2+}\) Flux Correlates with Thymocyte Selection

Sanjeev Mariathasan, Martin F. Bachmann, Denis Bouchard, Toshiaki Ohteki, and Pamela S. Ohashi.


The consequences of TCR-peptide/MHC interactions have been extensively studied for mature T cells. For instance, it has shown that TCR down-regulation reflects the number of TCRs that have encountered the antigenic peptide/MHC ligands (Valitutti et al., 1996). During activation, T cells count the number of internalized TCRs and respond by various biologic stimuli when an activation threshold is reached (Viola and Lanzavecchia, 1996; Valitutti et al., 1996). Further studies also indicate a good correlation between the ability of a ligand/MHC to induce TCR down-regulation and its strength as an agonist (Bachmann et al., 1997b; Preckel et al., 1997; Cai et al., 1997). Strong antigenic peptides are believed to maintain relatively stable TCR-peptide/MHC complexes. Such interactions may augment the natural oligomerization of TCRs during Ag recognition and subsequent internalization (Reich et al., 1997; Bachmann et al., 1998). This leads to the production of second messengers that mediate the release of intracellular calcium ([Ca\(^{2+}\)]\(_{i}\)) (Sloan-Lancaster et al., 1996; Wülfing et al., 1997). Accordingly, weak agonists have a lower capacity to down-regulate TCRs, which correlates with diminished calcium signaling (Bachmann et al., 1997a).
In the present study we asked whether the extent of peptide-mediated TCR down-regulation could identify TCR-mediated events that discriminate between positive and negative selection. We examined the influence of a set of defined peptide variants on T cell development using the P14 TCR-transgenic mouse model. Our results demonstrate that peptides that mediate suboptimal TCR down-regulation are effective at mediating positive selection, whereas peptides that mediate efficient TCR down-regulation are efficient at triggering negative selection. Furthermore, our results indicate that the magnitude of Ca$^{2+}$ signaling induced by these peptides may be important in the determination of survival versus cell death of developing thymocytes. Based on our data, we propose that thymocyte fate is induced by varying intensities of the same TCR-mediated signal.

Contributions

Dr. Martin Bachmann was instrumental in teaching me various cellular-immunology techniques which included assays for TCR proliferation and TCR-downregulation. Dr. Toshiaki Ohteki taught me how to harvest thymii from mouse embryos for fetal thymic organ cultures. Dr M. Bachmann and Dr T. Ohteki were former post-doctoral fellows in the Ohashi lab. However, data presented in this chapter were generated by myself. Denis Bouchard operated the FACS machine for the Ca$^{2+}$ analysis.
Results

Differential TCR down-regulation by altered peptide ligands

Using LCMV-specific H-2D\textsuperscript{b}-restricted transgenic mice we have identified peptides that were efficient at inducing either positive or negative selection (Sebzda et al., 1994; Sebzda et al., 1996). The peptide p33 (KAVYNFATM) readily induced negative selection at high concentrations (10^{-8}-10^{-9} M) and only induced detectable positive selection within a narrow concentration range (10^{-11}-10^{-12} M). An altered peptide ligand, A4Y (KAVANFATM), promoted efficient positive selection over a wide range of concentrations (10^{-4} – 10^{-9} M) in the absence of detectable clonal deletion in FTOC. To identify unique properties associated with these peptides, we tested their ability to induce TCR down-regulation on mature T cells. In this assay, macrophages were pulsed with p33, A4Y, or control H-2D\textsuperscript{b}-binding adenovirus (AV) peptide and were incubated with TCR transgenic spleen cells. Five hours later, T cells were harvested and analyzed for TCR expression. The strong agonist peptide, p33, induced strong down-modulation of the TCRs. The control peptide, AV, failed to alter TCR expression, similar to that in an unstimulated T cell population (Fig. 3-1A). This raised the possibility that positively selecting ligands stimulate the TCR but do not lead to maximal TCR triggering, while negatively selecting ligands trigger efficient TCR internalization.

To identify other variant peptides that would also induce differential TCR down-regulation, macrophages were pulsed with graded doses of peptides and incubated with TCR transgenic splenocytes for 5 h. By analyzing TCR expression on CD8\textsuperscript{+} T cells, peptides were divided into categories defined by TCR down-regulation profiles. Peptide
variants S7A (KAVYNFSTM) and Y6F (KAVYNYATM) induced strong TCR internalization similar to p33, whereas W4Y (KAVWNFATM) and L6F (KAVYNLATM) showed weak down-regulation profiles approximating that of A4Y (Fig. 3-1, B and C). Even at the highest concentration of peptide tested (10^-4 M), peptide variants A4Y, L6F, and W4Y could not reach the magnitude for TCR down-regulation that was achieve using similar concentrations of p33, S7A, or Y6F.

**TCR down-regulation correlates with the efficiency of T cell proliferation**

To evaluate the biologic significance of differential TCR internalization induced by the variant peptides, we determined the proliferative capacity of naïve transgenic T cells. Proliferation assays were performed by cocultivating transgenic spleen cells with irradiated nontransgenic splenic APCs pulsed with various concentrations of different peptides (Fig. 3-2). The highest peptide-specific proliferation was observed in the presence of p33 and S7A, which induced strong TCR down-regulation. Y6F, the weakest of the three strongly down-regulating peptides, showed an intermediate proliferative response. Therefore, with this set of peptides, a direct correlation was observed between the abilities to induce TCR down-regulation and proliferation.

We also examined the effectiveness of these peptides to bind H-2D^b to exclude the possibility that suboptimal peptide presentation was responsible for the diminished proliferative response induced by weak agonist peptides. Although the amino acid substitutions in these peptide variants did not occur at the MHC contact residues (Fig. 3-1B), we tested all peptides for H-2D^b binding using the murine lymphoma cell line RMA-
Figure 3-1: TCR internalization by peptide variants. 

A. TCR transgenic spleen cells were incubated with peritoneal macrophages coated with 10^6 M p33, A4Y, or AV peptides. After 5 h, TCR (Vα2) expression was analyzed for CD8+ T cells. The control histogram (black) is stained with an isotype-matched control Ab. 

B. The sequences of the wild-type peptide p33 and altered peptides are listed. The main TCR contact residues are indicated with an upward arrow; the main MHC contact residues are indicated with a downward arrow. Only the residues that deviate from the wild-type peptide sequence are shown. 

C. TCR transgenic splenocytes were incubated with peritoneal macrophages that were prepulsed with varying concentration of different peptides. After 5 h, TCR (Vα2) expression was analyzed for CD8+ T cells. Results are expressed as the media Vα2 intensity. One representative experiment of three is shown.
Figure 3-2. Degree of TCR down-regulation correlates with degree of T cell proliferation. Mature spleen cells from TCR transgenic mice were stimulated with H-2b spleen cells pulsed with various concentrations of peptides, and proliferation was measured. This experiment was repeated three times with similar results.
Figure 3-3. Peptide variants bind to H-2D<sup>b</sup>. The ability of these peptides to rescue H-2D<sup>b</sup> surface expression on RMA-S cells was characterized by the percent increase in mean fluorescence relative to that of the control peptide, LCMV nuclear protein 118-127 (H-2<sup>d</sup> restricted).
S (Fig. 3-3). We observed that some weak agonists (A4Y and L6F) and the nonstimulatory control peptide (AV) bind as well as, if not better than the strong agonist peptides. These results demonstrate that the amino acid modifications did not adversely affect the binding of peptides to the H-2D<sup>b</sup> groove.

Peptides that moderately down-regulate mature TCRs mediate efficient positive selection.

Since A4Y efficiently induced positive selection (Sebzda et al., 1996), it was possible that peptides inducing similar TCR down-modulation should also mediate positive selection of transgenic thymocytes. Therefore, fetal thymic lobes from TCR β<sup>−/−</sup> mice were cultured with A4Y, L6F, and W4Y in the presence of exogenous β2m to examine their influence on positive selection. A4Y, L6F, and W4Y (10<sup>−7</sup> M) efficiently induced the development of CD8<sup>+</sup> T cells in the FTOC (Fig. 3-4). In lobes where control peptide AV was added, the percentage of CD8<sup>+</sup> T cells was 11.5±1.9%. These cells expressed reduced levels of the transgenic TCR. However, upon incubation with A4Y, L6F, and W4Y the percentage of CD8<sup>+</sup> cells increased to 30.1±7.9% (p<0.0003), 30.6±8.4% (p<0.003), and 29.0±2.9% (p<0.0001), respectively. These cells had high levels of the transgenic TCR and low expression of HSA, characteristic of mature thymocytes. As shown in Table I, the positively selecting peptides that trigger moderate TCR internalization are efficient at positively selecting transgenic thymocytes.

We observed a modest down-regulation of CD8α expression on thymocytes maturing in the presence of A4Y, L6F, and W4Y (Fig. 3-4). Using the P14 transgenic system, other
Figure 3-4. Peptides that induce suboptimal TCR down-regulation mediate efficient positive selection of thymocytes. Three-color analysis of TCR β2m+ thymic lobes cultured for 7 days with 10⁻⁷ M A4Y, L6F, W4Y, or AV (a control H-2Db-restricted adenovirus peptide) were stained with Abs specific for CD4, CD8, and Va2 or HSA. CD8+ or CD4⁺CD8+ cells were gated, and the profiles of Va2 and HSA are shown. These data are representative of five experiments. The mean CD8α expressions on thymocyte selected on AV, A4Y, L6F, and W4Y are 195, 154, 168, and 153, respectively (arbitrary units).
researchers have also shown that A4Y selects T cells with down-regulated CD8β expression (Chidgey and Boyd, 1998). Given the similarity of A4Y to W4Y and L6F, we would predict that W4Y and L6F would induce a similar CD8β down-regulation. T cells being selected on moderate agonist peptides could down-modulate their coreceptors to decrease the avidity to their cognate ligands to escape negative selection.

A4Y-, W4Y-, and L6F-selected thymocytes are responsive to p33

To further examine the functional maturity of the thymocytes that were selected in the presence of the positively selecting peptides, proliferation assays were performed. Purified CD8+ cells from TCR β2m+ thymic lobes as well as TCR β2m− thymic lobes incubated with A4Y, L6F, and W4Y were cocultured with irradiated splenocytes that were pulsed with p33, AV, or the corresponding positively selecting peptide. Although the positively selected TCR β2m− thymocytes did not respond to the selecting peptide, they mounted a proliferative response to the strong agonist peptide p33 (Fig. 3-5). In contrast, TCR β2m+ thymocytes selected by natural endogenous ligands proliferated not only in response to p33, but also in response to the positively selecting peptides. These results demonstrate that the positively selected TCR β2m− thymocytes are functionally mature, since they proliferated against p33 and are self tolerant.

Peptides that strongly down-regulate TCRs on mature T cells induce negative selection of thymocytes

The ligand p33, which induces strong TCR down-regulation, has been shown to negatively select transgenic thymocytes in FTOCs (Sebzda et al., 1994; Ashton-Rickardt
et al., 1994). Based on the correlation between the activity of p33 in the TCR down-regulation assay and that in the FTOCs, it was possible that the variant peptides S7A and Y6F, which induce maximal TCR down-regulation, might also effectively delete CD8+ T cells. TCR β2m− thymic lobes were cultured with p33, S7A, or Y6F at 10^−6 M to investigate their roles in negative selection. We consistently observed a four-to fivefold decrease in total cell recovery from cultures treated with these peptides compared with that from control cultures that were incubated with AV peptide (Table 3-1). Notably, the residual CD8+ cells that were found in these cultures expressed reduced levels of transgenic Vα2 TCRs and intermediate to high amounts of HSA, indicating their lack of maturity (Fig. 3-6A). In contrast, fetal thymic lobes cultured with A4Y, L6F, or W4Y did not show a decrease in total cell recovery compared with that in cultures treated with AV, suggesting that these peptides were not efficient in mediating deletion. The CD8+ thymocytes were Vα2^highHSA^low, demonstrating that A4Y, L6F, and W4Y did not trigger detectable negative selection of transgenic thymocytes (Fig. 3-6B). These data demonstrate that peptides that induce strong TCR down-regulation also induce negative selection, whereas peptides that moderately down-regulate TCRs promote positive selection.

Positively and negatively selecting peptides induce distinct calcium signals

Differential calcium signaling has been implicated in the regulation of T cells effector functions in response to altered peptide ligands (Sloan-Lancaster et al., 1996; Wülfing et al., 1997; Bachmann et al., 1997a). Therefore, we tested positively and negatively selecting ligands for their ability to induce increases in intracellular calcium [Ca^{2+}]_i in
Figure 3-5. TCR thymocytes selected in the presence of A4Y, L6F, or W4Y are tolerant to the selecting peptide, but proliferate in response to the strong agonist p33. TCR^+^CD8^+^ thymocytes from TCR β_2_ m^−^ thymic lobes cultured with A4Y (A), L6F (B), or W4Y (C; shaded bars) or TCR β_2_ m^+^ thymic lobes cultured in medium alone (solid bars) were cocultured with peptide-coated APC. Proliferation was measure 24 h later. These results are representative of three experiments.
Figure 3-6. Peptides that strongly down-regulate TCRs are most efficient at inducing negative selection of thymocytes. TCR β2m+ thymic lobes were cultured daily for 7 days with $10^{-6}$ M peptides. Thymocytes were then analyzed with Abs specific for CD4, CD8, and Vα2 or HSA. Vα2 and HSA profiles for gated CD8+ cells are shown. These data are representative of five experiments.
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<th>Peptide</th>
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<th>CD8^+ Cell Number</th>
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</tbody>
</table>

<sup>α</sup> 10<sup>−7</sup> M peptides.

<sup>β</sup> 10<sup>−7</sup> M peptides.

Table 3-1: Total and CD8<sup>+</sup> thymocyte numbers from TCR β₂m<sup>−/−</sup> and TCR β₂m<sup>−−</sup> FTOCs treated with various peptides.
TCR transgenic thymocytes. To examine the changes in intracellular calcium following stimulation, thymocytes were loaded with indo-1 mixed with peptide-pulsed macrophages. \([Ca^{2+}]_i\) was determined immediately for indo-1\(^+\) thymocytes that had formed complexes with APCs, as judged by a large forward scatter. In this assay, negatively selecting peptides p33, S7A, and Y6F induced a strong calcium flux (Fig. 3-7A). However, positively selecting peptides A4Y, L6F, and W4Y incubated under the same activating conditions induced a quantitatively different \([Ca^{2+}]_i\) profile. Although the initial increase in \([Ca^{2+}]_i\) was high, it declined rapidly. However, the response did not result in a convergence at a minimum. The AV peptide, which induced neither positive nor negative selection, did not induce any calcium flux over the basal level observed in thymocytes in the presence of unpulsed APCs.

These experiments were performed on H-2D\(^b\) thymocytes, in which positive selection is an on-going process. Therefore, the observed calcium flux in response to the strong and moderate agonists is in addition to the selection signals recently received by the thymocytes in vivo. To measure the calcium flux in cells that have not yet undergone selection, we used thymocytes from TCR-transgenic non-selecting H-2\(^d\) mice. As shown in Fig. 3-7B, we observed similar results with these mice. Furthermore, we observed that the calcium flux induced by these peptides tightly correlated with their TCR internalization profiles. Thus, the magnitude of calcium signaling induced by positively and negatively selecting ligands suggests that there is a quantitative role for calcium and/or calcium-dependent pathways during thymic selection.
Figure 3-7. Positively and negatively selecting ligands induce different calcium signals. A, TCR transgenic H-2D\textsuperscript{b} preloaded with indo-1 were centrifuged and incubated at 37°C with various macrophages pulsed with 10\textsuperscript{-5} M p33 or variant peptides. Three minutes later, free intracellular Ca\textsuperscript{2+} was measured (by the indo-1 405/485 ratio) for the indicated time span. One representative experiment of three is shown. B, TCR transgenic thymocytes (H-2D\textsuperscript{b}) were compared with TCR transgenic mice from H-2D\textsuperscript{d} mice. Representative calcium dot plots over time are shown for thymocytes responding to the nominal peptide, p33; a strong agonist, S7A; a moderate agonist, L6F; and the nonstimulatory peptide, AV.
Discussion

Extent of TCR triggering correlates with positive and negative selection.

Using a defined set of peptide variants of the LCMV-gp, we have shown that these peptides induce different degrees of transgenic TCR internalization (Fig. 3-1C). Using mature T cells, we have previously shown that the extent of TCR down-regulation correlates with the strength of TCR-mediated signals and the induction of a variety of effector functions (Bachmann et al., 1997b). Accordingly, peptides that strongly down-regulated TCRs (p33, S7A, and Y6F) induced a greater proliferative response than peptides that induced intermediate TCR internalization (A4Y, L6F, and W4Y; Fig. 3-2). We show here that all variant peptides used in this study bind to H-2D\(^b\) efficiently (Fig. 3-3). Therefore, the relative agonist strength of the peptides in these assays (p33, S7A > Y6F > A4Y, L6F, W4Y > AV) reflected the presumed affinities of these peptides for the LCMV transgenic TCR and paralleled the TCR down-regulation profiles. By testing these peptide in FTOCs, we demonstrated that weak agonist peptides that moderately triggered the TCRs were most efficient at mediating positive selection (Fig. 3-4). However, these peptides were inefficient at inducing clonal deletion (Fig. 3-6 and Table 3-1). Conversely, peptides that induced strong TCR-mediated signals were most efficient at triggering negative selection at similar peptide concentrations. It is worth noting that maximal TCR down-regulation seen with weak agonists never reached the levels induced by strong, agonist peptides.
Several studies have shown that partial agonist and antagonist peptides transmit distinct TCR-mediated intracellular signals from agonist ligands (Madrenas et al., 1995; Sloan-Lancaster et al., 1994; Sousa et al., 1996). Our data demonstrate that positive or negative selection may be induced by varying intensities of the same TCR-mediated events. We have shown that the level of TCR internalization induced by each peptide is dependent on peptide concentration (Fig. 3-1C). This suggests that different outcomes of TCR-mediated interactions could be induced by different intensities of the same signal in a peptide-specific, concentration-dependent manner. Accordingly, previous studies have shown that the same peptide could mediate positive selection at low concentrations and clonal deletion at high concentrations (Sebzda et al., 1994; Ashton-Rickardt et al., 1994). Together, these findings are consistent with the affinity/avidity model for thymocyte selection.

**TCR dimerization, internalization, and thymic selection**

Several experiments have suggested that oligomerization of TCRs is important for TCR internalization and subsequent T cell activation (Reich et al., 1997; Bachmann et al., 1998). Consistent with this idea, prolonged TCR occupancy has to occur to achieve efficient multimerization (Germain et al., 1995; McKeithan, 1995; Rabinowitz et al., 1996). Numerous studies have shown that low affinity APLs have a faster dissociation rate than the high affinity, agonist ligands (Alam et al., 1996; Lyons et al., 1996; Matsui et al., 1994). The decreased occupancy time associated with weak agonists would affect the degree of oligomerization and subsequent internalization, as observed by the
positively selecting peptides. Thymocytes may require such weak triggering for survival, but may die if significant numbers of TCRs are engaged within a certain time frame.

Reactivity of mature T cells altered during positive selection

Functional analysis of TCR transgenic CD8+ thymocytes maturing in the presence of defined, positively selecting ligands showed that they responded strongly to the agonist ligand p33. However, they reacted poorly to selecting peptides. However, proliferation assays performed on purified CD8+ T cells from β2m+/+ FTOCs indicate that TCR transgenic T cells developing in the presence of endogenous peptides could proliferate in response to A4Y, W4Y, and L6F (Fig. 3-5). Therefore, maturation of thymocytes in the continued presence of A4Y, W4Y, and L6F (rather than endogenous peptides) has resulted in functional tolerance to the selecting ligand. This ability to adjust the resting threshold and alter the ligands capable of inducing a T cell response has been previously reported in this model (Sebzda et al., 1996; Kawai and Ohashi, 1995). These positively selected thymocytes expressed high levels of transgenic TCRs, comparable to the TCR-transgenic β2m+/+ thymocytes that matured in the presence of endogenous peptides, arguing against the possibility that reduced TCR expression leads to unresponsiveness. However, coreceptor (Jameson et al., 1994; Chidgey and Boyd, 1997), adhesion, and signaling molecules could play a role in altering the reactivity profile of thymocytes. Thus, T cells may be “tuned” during positive and negative selection by interaction with peptide/MHC complexes on thymic stromal cells, thereby limiting the spectrum of activating ligands (Ohashi, 1996; Grossman and Singer, 1996).
It remains controversial whether functional thymocytes may be selected in the presence of agonist ligands. Some reports have suggested that T cells selected on agonist peptides are not bonafide, functional T cells (Hogquist et al., 1995; Girao et al., 1997). However, we have shown here and in previous studies that T cells selected by agonist ligands are functional, since they can fully respond to the stronger agonist peptides, but are not unresponsive to their selection peptide or other peptides that have similar or lower reactivity. Others have also shown that positive selection in the presence of agonist ligands lead to functional mature T cell (Nakano et al., 1997; Chidgey and Boyd, 1997; Wang R et al., 1998; Williams et al., 1998). One possible interpretation is that thymocytes are selected so that they do not respond to their positively selecting ligand (Hogquist et al., 1994; Sebzda et al., 1994; Liu et al., 1998). As long as the positively selecting ligand persists and continue to "contact" selected T cells, these T cells will have adjusted their basal resting threshold so that they do not respond to the selecting ligand. Only stronger stimulation will lead to activation of effector function. If the selection peptide is not continually present to tune or alter the reactivity of the selected thymocyte, the resting threshold may be lowered so that the selecting peptide can now induce T cell activation.

Role of Ca\(^{2+}\)/calcineurin in thymocyte selection

The importance of calcium signaling pathways has been implicated in T cells undergoing thymocyte selection. Studies using calcineurin inhibitors such as FK506 and cyclosporin A have shown that Ca\(^{2+}\)/calcineurin pathways are essential for positive and negative selection (Jenkins et al., 1988; Gao et al., 1988; Shi et al., 1989). A role for elevated
[Ca\(^{2+}\)]_i in cell death induction has also been previously reported (Nakayama et al., 1992; Kane and Hedrick, 1996). However, these studies do not directly correlate differential calcium signaling with peptide-induced positive and negative selection. In this study we clearly demonstrate that peptides that efficiently trigger positive selection induce an intermediate calcium flux in immature transgenic thymocytes, whereas the peptides that efficiently trigger negative selection induce a strong calcium flux. (Fig. 3-7). Difference in the magnitude of calcium mobilization may lead to the activation of different subsets of calcium-dependent enzymes and, hence, elicit different functional results. There are precedents for this in several different systems. In B cells, the amplitude and the duration of calcium signals control different sets of transcriptional regulators that have different sensitivities to intracellular calcium (Dolmetsch et al., 1997; Healy et al., 1997). Therefore, the intensity of TCR-mediated [Ca\(^{2+}\)]_i flux in thymocytes may play a role in defining the outcome of T cell maturation.

**Importance of continuous signaling through the TCRs for positive selection**

Several studies have indicated that survival of positively selected thymocytes involves sustained interactions with the thymic microenvironment, transduced via their TCRs (Wilkinson et al., 1995; Kisielow and Miazek, 1995; Groves et al., 1995) It also includes an obligatory role for TCRs in cooperating with other surface molecules to drive developing thymocytes through DP to SP transition (Kearse et al., 1995; Cibotti et al., 1997). Therefore, where negatively selecting peptides induce maximal TCR internalization, the thymocytes may no longer be receptive to these signals. However, thymocytes, in which positively selecting peptides do not fully internalize TCRs, can
continue to perceive signals from the microenvironment, suggesting that continual TCR engagements are required to induce the full spectrum of differentiation events associated with positive selection.

The findings described in this report address the relationships among TCR internalization, intracellular calcium levels, and T cell selection events induced by altered peptide ligands. We demonstrated that peptides that induce strong TCR down-regulation accompanied by elevated Ca^{2+} levels are most efficient at mediating negative selection, whereas peptides that induce suboptimal TCR internalization and weaker Ca^{2+} elevation are more efficient at triggering positive selection. We do not propose that all selecting ligands will necessarily have a measurable ability to mediate TCR internalization. Instead, our observation with this group of peptides suggest that the quantitative differences, rather than the quality of TCR-derived signal, determine the fate of the thymocyte. This is in accordance with the affinity/avidity model for thymocyte selection. Furthermore, our data explain how positively selecting ligands, due to their inability to fully internalize TCRs, may provide a way in which thymocytes receive the constant signals necessary for differentiation and survival.
Chapter IV
Chapter IV

Degree of ERK Activation Influences Both Positive and Negative Thymocyte Selection

Sanjeev Mariathasan, Shirley S.N. Ho, Arsen Zakarian, Pamela S. Ohashi


Introduction

Considerable evidence suggests that the ERK pathway is required for positive but not negative thymocyte selection. In this study we asked whether the TCR-peptide/MHC induced Ras pathway could also influence thymocyte negative selection. Using the P14 TCR transgenic model, we first examined the extent of ERK activation in thymocytes in response to positively and negatively selecting peptides. High levels of ERK activation were observed when thymocytes were stimulated with negatively selecting conditions, suggesting that the strength of ERK signaling may influence negative selection. We therefore wanted to determine whether modulating ERK activation would alter the outcome of thymocyte selection. It is not clear from the prior studies whether Ras-dependent signaling was effectively blocked in mice transgenic for dominant-negative molecules for this pathway (Swan et al., 1995; Alberola-Ila et al., 1996a; Alberola-Ila et al., 1996b; O'Shea et al., 1996). Therefore we adopted an alternate approach to block ERK signaling by using a pharmacological reagent PD98059, a potent and specific
inhibitor of MEK1/2 (Alessi et al., 1995; Dudley et al., 1995). Inhibition of the ERK pathway in the presence of negatively selecting stimuli altered thymocyte fate from deletion to survival. Accordingly, ERK activation remained undetectable in thymocytes from a non-selecting background. As a result this study suggests that the magnitude of ERK activation contributes to the threshold that distinguishes between positive and negative selection.

Contributions

Shirley Ho was a wonderful summer student who worked under my supervision. She helped me with set up fetal thymic organ cultures. Arsen Zakarian bred the P14 TCR RAG2−/− transgenic line into the H-2^d^d MHC-haplotype.

Results

Intensity of ERK activation correlates with thymocyte fate.

Although several studies have suggested that T cell selection is influenced by differing intensities of the same TCR-mediated signal, the degree of ERK activation in response to positively and negatively selecting stimuli in immature CD4^+^CD8^+^ (DP) thymocytes has not yet been characterized. We therefore examined the extent of TCR-mediated ERK activation in thymocytes from P14 TCR transgenic RAG2−/− deficient H-2^d^d (P14 RAG2−/− H-2^d^d) (non-selecting background) in response to various ligands. P14 RAG2−/− H-2^d^d thymocytes were incubated with adherent, 2A9 thymic epithelial (H-2^bb^) cells that were prepulsed with a determined peptide concentration that could induce either efficient positive or negative selection.
Figure 4-1. Degree of ERK activation correlated with thymocyte selection.

P14 RAG2\(^{-/-}\) H-2\(^{d/d}\) thymocytes were incubated with adherent 2A9 thymic epithelial cell lines (H-2\(^{b}\)) pre-pulsed with either the negatively selecting peptide p33 (10\(^{-6}\)M), the positively selecting peptide A4Y (10\(^{-6}\)M) or the non-selecting peptide AV (10\(^{-6}\)M) for 1 hour. Western blots were performed on whole-cell lysates from these thymocytes, and probed with antibodies specific for the activated forms of p42/44 ERKs (phospho-specific). p42 MAP kinase anti-sera that detects total p42 MAP kinase levels (phosphorylation-state independent) was used as control for sample variations. The data shown are representative of four independent experiments.
(Mariathasan et al., 1998; Sebzda et al., 1996; Ashton-Rickardt et al., 1994; Sebzda et al., 1994). These included a negatively selecting strong agonist peptide - p33 (10^{-6} M), a positively selecting weak agonist peptide - A4Y (10^{-6} M), or a non-stimulatory adenovirus-derived peptide - AV (10^{-6} M). Western blots were performed on whole cell lysates from the stimulated thymocytes and the level of ERK activation was determined using antibodies specific for the “activated” phospho-specific forms of p42/44 ERKs. As seen in Fig. 4-1, the strong agonist peptide p33 that triggers clonal deletion at high concentrations induces a strong ERK phosphorylation compared to the positively selecting weak agonist peptide A4Y. These data demonstrate that the degree of ERK activation strongly correlates with thymocyte fate.

**PD98059 modulates p33-induced activation of ERKs in P14 thymocytes**

If the degree of ERK activation does influence thymocyte fate, we reasoned that modulating the endogenous ERK activity in response to antigenic stimulation would have an effect on thymocyte selection. To investigate this possibility we took advantage of PD98059, a highly selective compound that has been shown to inhibit the phosphorylation of MEK1/2 by upstream activators of the MAP kinase cascade (Alessi et al., 1995; Dudley et al., 1995). Inhibition of MEK1/2 by PD98059 abolishes the activation of downstream effectors ERK1/2. To examine the effectiveness of PD98059, P14 RAG2^{+/−} H-2^{b/d} thymocytes were incubated with 25μM PD98059 for 1 hr and then cocultured with 2A9 thymic epithelial cells (H-2^{b}) prepulsed with different concentrations of p33. Fig. 4-2A shows a decrease in the level of ERK activation in 10^{-6} M p33-induced thymocytes as compared to 10^{-7} M p33-induced thymocytes, suggesting
Figure 4-2. PD98059 inhibits p33-induced activation of ERKs in P14 thymocytes.

(A) P14 RAG2<sup>−/−</sup> H-2<sup>dd</sup> thymocytes that were pre-incubated with or without 25μM PD98059 were cultured with adherent 2A9 thymic epithelial cell line pre-pulsed with either 10<sup>−7</sup> or 10<sup>−8</sup> M negatively selecting peptide p33 for 1 hour. Western blots were performed as described in Figure 1. The data shown are representative of three independent experiments. (B) Proliferative response of purified CD8<sup>+</sup> P14 RAG<sup>−/−</sup> H-2<sup>b[b]</sup> thymocytes were preincubated with various concentrations of PD98059 and cocultured with 10<sup>8</sup> M p33-pulsed irradiated spleen cells. (C) Purified CD8<sup>+</sup> P14 RAG<sup>−/−</sup> H-2<sup>b[b]</sup> thymocytes were preincubated with 0, 10 or 25μM PD98059 and cocultured with various concentrations of p33-pulsed irradiated spleen cells.
that the degree of TCR-mediated ERK activation is ligand concentration dependent. The data also shows that 25μM PD98059 markedly diminishes ERK activity in p33-induced thymocytes in a peptide concentration dependent manner. This demonstrates that the efficiency of ERK inhibition by PD98059 is dependent on the level of TCR-mediated triggering.

To further evaluate the effectiveness of PD98059 in blocking ERK activation, we examined thymocyte proliferation in response to p33 stimulation. Purified CD8+ P14 RAG2−/− thymocytes were preincubated with various amounts of PD98059 and were cocultured with p33-pulsed irradiated spleen cells. As shown in Fig. 4-2B, PD98059 inhibited the proliferative response to about 55% and 30% of the maximum at a concentration of 12.5μM and 25μM, respectively. However, consistent with the above biochemical data we also observed a marked reduction in the efficiency of PD98059 to inhibit proliferation of thymocytes incubated with higher concentrations of p33 peptide (Fig. 4-2C). Together, these data demonstrate that PD98059 can modulate TCR-mediated ERK activation in a dose dependent and peptide dependent fashion and accordingly alter thymocyte responses.

**PD98059 blocks positive selection of P14 CD8+ thymocytes**

The ERK MAP kinase pathway has been shown to be important in thymocyte positive selection (Swan et al., 1995; Alberola-Ila et al., 1996a; Alberola-Ila et al., 1996b; O'Shea et al., 1996; Sugawara et al., 1998). To examine whether PD98059 could inhibit positive selection in FTOCs, embryonic day 17.5, P14 Class I+ thymic lobes were cultured with or
P14 $\beta_{2}m^{+/+}$ thymic lobes (embryonic day 17.5) were cultured with or without 25$\mu$M PD98059 for 6 days. Three color analysis of thymocytes were done using antibodies specific for CD4, CD8 and Va2. Numbers indicate the percentage of cells within each quadrant. Va2 proliferates gated on CD8$^+$ cells are also shown. Total number of viable cells (trypan blue exclusion) in PD98059 treated lobes: 36 ± 4.2 x 10$^4$ cells/lobe ($n$=4). Total number of viable cells in control lobes: 37.0 ± 2.6 x 10$^4$ cells/lobe ($n$=4).
without 25μM PD98059. A reduction in the percentage and number of mature TCR transgenic CD8+ T cells, as well as CD4+ T cells was seen in the presence of PD98059 compared to controls (Fig 4-3). Based on the current literature, it would be predicted that attenuating ERK activation (downstream of signals derived from weak TCR-mediated interactions with endogenous ligands) would impede positive selection of both single-positive (SP) subsets. Concurrent with the decrease in SP cells, there was an increase in DP cells indicating a block in the DP to SP transition. Moreover, the total number of viable thymocytes remained constant for either lobes treated with or without PD98059, indicating a lack of inhibitor-mediated toxicity. These findings confirm the role of ERK in positive selection and demonstrate that PD98059 can effectively alter positive selection in FTOCs.

**ERK activation is detected in thymocytes undergoing positive selection**

These and other studies (Swan et al., 1995; Alberola-Illa et al., 1996a; Alberola-Illa et al., 1996b; O'Shea et al., 1996; Sugawara et al., 1998) suggest that ERK stimulation is necessary for thymocytes to escape from ‘death by neglect’ and undergo positive selection. To examine this possibility directly, we compared the relative intensity of ERK activation from thymocytes derived from P14 RAG2+H-2b mice and P14 RAG2+H-2d mice. Since the P14 TCR is H-2D^b restricted, positive selection is blocked in H-2d mice as expected (Fig. 4-4A). Interestingly, P14 RAG2+H-2b thymocytes showed elevated levels of ERK activation compared to thymocytes from the non-selecting environment (Fig 4-4B). This data clearly substantiates the role of ERK in influencing thymocyte selection processes.
Figure 4-4. Reduced ERK activation in 'non-selected' thymocytes.

(A) Two-color flow cytometric analysis using antibodies specific for CD4 and CD8 was performed on thymocytes harvested from P14 RAG2\textsuperscript{--}/-H-2\textsuperscript{bb} and P14 RAG2\textsuperscript{--}/-H-2\textsuperscript{dd} mice. (B) Western blots were performed to determine the relative level of endogenous ERK activation in these thymocytes derived from P14 RAG2\textsuperscript{--}/-H-2\textsuperscript{bb} or P14 RAG2\textsuperscript{--}/-H-2\textsuperscript{dd} mice.
Inhibition of ERK activation switches negative to positive selection

Our studies examining the activation of ERK levels in thymocytes in response to positively and negatively selecting peptides also demonstrate a hierarchy in ERK activation (Fig. 4-1). This suggests that the signaling threshold that defines positive and negative selection might also be influenced by ERK activation. To investigate this possibility, we examined the effect of PD98059 in p33-mediated negative selection in FTOCs. In this experiment, we wanted to ensure that selecting events were mediated by the exogenous peptide p33, rather than endogenous ligands. Therefore, thymic lobes from β2-microglobulin deficient (β2m−/−) mice were used. When embryonic day 17.5, P14 β2m−/− fetal thymic lobes were cultured with peptide p33 (10−8M), a substantial deletion of DP cells was seen compared to lobes that were cultured with the non-stimulatory AV peptide (Fig. 4-5A). The remaining CD8+ T cells that were found in these cultures expressed reduced levels of transgenic Vα2 TCRs and intermediate levels of HSA. In contrast, DP cells were protected from deletion in the lobes that were cultured with p33 and 25μM PD98059 (Fig 4-5A and Table 4-1). Interestingly, an increase in the total number of CD8+ Vα2hiHSAlo T cells, characteristic of mature thymocytes, was also seen. This demonstrates that the addition of PD898059 was able to block p33-mediated negative selection, and concomitantly led to p33-mediated positive selection of P14 thymocytes. These results demonstrate that lowering the ERK activation following strong TCR triggering results in a shift from negative to positive selection.
Figure 4-5. Inhibition of ERK activation switches negative selection to positive selection.

(A) P14 β₂m⁺⁺ thymic lobes (embryonic day 17.5) were cultured with or without 25 μM PD98059 for 6 days in the presence of 10⁻⁸M negatively selecting p33 peptide. Control lobes were cultured with 10⁻⁷ M non-stimulatory AV peptide. Three-color analysis was performed on thymocytes using specific antibodies for CD4, CD8 and Va2. Va2 and HSA profiles gated on CD8⁺ cells as well as HSA profiles on gated DP cells are also shown.
Table 4-1:
Inhibition of MEK rescues thymocytes from peptide-induced negative selection.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total (x 10⁴)</th>
<th>DN (x 10⁴)</th>
<th>DP (x 10⁴)</th>
<th>CD4 (x 10⁴)</th>
<th>CD8 (x 10⁴)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AV (10⁻⁷ M)</td>
<td>58.1 ± 7.1</td>
<td>7.6 ± 0.2</td>
<td>34.5 ± 7.7</td>
<td>8.7 ± 0.2</td>
<td>6.9 ± 1.6</td>
</tr>
<tr>
<td>P33 (10⁻⁸ M)</td>
<td>21.0 ± 7.5</td>
<td>6.0 ± 3.0</td>
<td>4.0 ± 1.0</td>
<td>6.9 ± 2.8</td>
<td>3.7 ± 1.3</td>
</tr>
<tr>
<td>P33 (10⁻⁸ M) + 25 μM PD98059</td>
<td>56.0 ± 12.5</td>
<td>9.5 ± 1.7</td>
<td>25.4 ± 11.0</td>
<td>8.0 ± 2.2</td>
<td>12.5 ± 3.1</td>
</tr>
</tbody>
</table>

(a) Total and absolute number of thymocytes from DN, DP, CD4⁺ and CD8⁺ are given as means for triplicate lobes ± SD. Similar results were obtained from at least 5 different experiments.
CD8+ thymocytes selected in the presence of p33 and PD98059 are functional

To further characterize the functional maturity of the P14 β2m+/+ thymocytes that were selected in the presence of p33 and PD98059, proliferation assays were performed. These cultures were treated with media alone for an extra 36 hours to terminate the effect of MEK inhibition prior to the proliferation assay. Purified CD8+ thymocytes were incubated with irradiated spleen cells (H-2b) pulsed with 10^-8 M p33 or the non-stimulatory peptide AV (10^-7 M). Thymocytes from P14 β2m+/+ thymic lobes cultured in media alone were used as controls, where positive selection was mediated on "endogenous" ligands. CD8+ thymocytes selected in the presence of 10^-8 M p33 and PD98059 were able to mount a proliferative response against p33, albeit at slightly reduced levels compared to control thymocytes (Fig 4-6). The response was specific, since no proliferation was observed with the AV peptide. Together, these data show that the positively selected thymocytes were functional.

Discussion

The present study directly examines the role of ERK activation during thymocyte selection. Activated phospho-ERK is detected in thymocytes from a positively selecting background but not from the non-selecting background (Fig. 4-4). In addition, we demonstrate that ERK activation is increased in thymocytes after stimulation with negatively selecting stimuli compared to positively selecting ligands (Fig. 4-1). Decreasing the level of ERK activation by the addition of the MEK inhibitor PD98059 in FTOCs greatly compromised the efficiency of the antigenic peptide to induce negative selection and notably led to positive selection of functional antigen specific CD8+ T cells.
Figure 4-6. CD8+ T cells selected in the presence of p33 and PD98059 are functional.

P14 β2m− thymic lobes were cultured with 25μM PD98059 for 6 days in the presence of 10^{-8}\text{M} p33 peptide and rested for an additional day with media alone. Purified CD8+ T cells harvested from these lobes (shaded bars) or CD8+ T cells from P14 β2m+ thymic lobes cultured in media alone (solid bars) were cocultured with 10^{-8}\text{M} p33 pulsed APC and proliferation was measured. These results are representative of four experiments.
(Figs. 4-5, 4-6). These studies suggest that the extent of TCR-mediated ERK activation is involved in setting the thresholds for both positive and negative selection.

Previous studies have shown that different concentrations of the same peptide can mediate both positive and negative selection (Ashton-Rickardt et al., 1994; Sebzda et al., 1994). Using the P14 FTOC system, it has been demonstrated that the nominal peptide p33 induced negative selection at high concentrations ($10^{-6}$ - $10^{-9}$M) and induced detectable positive selection with a low concentration range ($10^{-11}$ - $10^{-12}$M) (Sebzda et al., 1994). Our current study shows that different doses of peptide activate different levels of ERK (Fig. 2A). This suggests that low levels of ERK activation favor positive selection. This could be achieved by triggering thymocytes with 'weak' peptides such as A4Y (Fig. 4-1) or decreasing the dose of the strong antigenic peptide -- p33 (Fig. 4-2A).

Alternatively, the same effect was shown in these studies by incubating thymocytes with PD98059 in the presence of deleting concentrations of p33. Therefore, modulating the degree of ERK activation either by altering the peptide concentration or by the addition of PD98059 modified thymocyte positive and negative selection. In addition, the strength of ERK stimulation also influences activation threshold between 'null-selection' and positive selection. Together these support the contention that null-selection -> positive selection -> negative selection are influenced by varying intensities of ERK activation.

Our finding that ERK activation is involved in defining thresholds for thymocytes negative selection is in direct conflict with other previous studies. Expression of
dominant-negative forms of Ras, MEK-1 and both Ras and MEK-1 in thymocytes have been shown to interfere with positive selection while leaving negative selection unaffected (Swan et al., 1995; Alberola-Ila et al., 1996a; Alberola-Ila et al., 1996b). However, it was not clear whether the Ras-dependent signalling pathway was completely or partially blocked in these dominant negative systems. Studies have shown that Ras and MEK-1 are important for the DN to DP transition (Crompton et al., 1996; Swat et al., 1996), and this impaired transition between these stages was not reported in the other studies using dominant negative transgenes. Therefore, inefficient blockade of the Ras pathway in the dominant-negative transgenic models may still deliver signals necessary for negative selection when triggered by strong antigenic ligands.

Another approach to examine the role of the ERK pathway in thymocytes selection is to utilize pharmacological inhibitors such as PD98059 (Alessi et al., 1995; Dudley et al., 1995; Sharp et al., 1997; Sugawara et al., 1998; Bommhardt et al., 1999). Sugawara et al. have recently shown that ERK signal transduction was crucial for positive selection, and not essential for negative selection using PD98059 in FTOCs (Sugawara et al., 1998). Their results showed that clonal deletion of H-Y specific DP thymocytes in male fetuses as well as anti-CD3-induced negative selection in non-TCR transgenic mice proceeded normally in FTOCs treated with 10μM PD98059. Several points could be made to address the disparity observed between our system and the model presented by Sugawara et al. Our studies have shown that the degree of ERK inactivation by PD98059 is highly dependent on concentration of the inhibitor as well as the peptide concentration (Fig. 4-2A,B,C). We also observed that the concentration of PD98059 required to inhibit
thymocyte proliferation titrated with the amount of inhibitor that was used in the culture system (Fig. 4-2C). Therefore, 10μM PD98059 might not overcome clonal deletion with strong antigenic stimuli such as anti-CD3 antibodies or the abundant deleting self-peptides (H-Y antigen) because it does not efficiently inhibit ERK activity (Sugawara et al., 1998). However, by titrating the deleting peptide (p33) in the P14 transgenic system to a level where 25μM PD98059 could reduce ERK activation (Fig. 4-2A), we were able to demonstrate the role of ERK signalling in thymocyte negative selection. These results strongly suggest that thymocyte selection is influenced by ERK activity reflecting differing intensities of TCR-mediated signals.

Our results indicating an important role for ERK in the clonal deletion process may appear paradoxical given the more classical role for Ras in promoting cell survival, proliferation, differentiation, or some combination of the three. Nevertheless, many reports also associate Ras with enhanced apoptosis (Downward, 1998). In fibroblasts, it has been found that Ras is an effective promoter of apoptosis, through the Raf pathway (Kauffmann-Zeh et al., 1997). In B lymphocytes (WEHI 231), ERK2 has been implicated in playing an active role in mediating anti-IgM-induced apoptosis (Lee and Koretzky, 1998). The murine WEHI 231 immature B lymphoma cell line has been used widely as an in vitro model system to study negative selection during B cell development (Scott, 1993). During T cell development, in addition to TCR-mediated signals, other stimuli provided by TNF receptor families (such as Fas, CD30, CD40 and TNFR) have also been suggested to sensitize DP thymocytes to clonal deletion (Sebzda et al., 1999; Penninger and Kroemer, 1998). Interestingly, the Ras signalling pathway has been
implicated in TNF and Fas-induced apoptosis (Gulbins et al., 1995). Furthermore, TCR-CD3 ligation, which leads to Ras activation in mature T cells (Downward et al., 1990), induces apoptosis in immature thymocytes and T cell hybridomas (Green and Scott, 1994). Together, these data also suggest a possible role for ERK in the induction of apoptosis.

Current evidence suggests that positive and negative selection arise from the activation of distinct MAPK cascades (Swan et al., 1995; Alberola-Ila et al., 1996a; Alberola-Ila et al., 1996b; O'Shea et al., 1996; Sugawara et al., 1998; Sabapathy et al., 1999; Rincón et al., 1998b). Our data implies that the discrimination between thymocyte selection events may not arise solely by signaling via independent pathways. We have shown that thymocyte fate can be switched from negative to positive selection by altering ERK activation. Consistent with this finding, several other studies have shown that a single component of the TCR-mediated signaling cascades can shift negative to positive selection. Altering the number of ITAMs on the TCR-associated ζ chain has been shown to influence the efficiency of both selection processes (Shores et al., 1997b). Yamakazi et al. have observed a shift from negative to positive selection of HY-specific T cells in male mice deficient in ζ chain (Yamazaki et al., 1997). Although the role of the Ca²⁺/calcineurin pathway in negative selection is controversial, there is evidence that blocking calcineurin activity can shift negative to positive selection under certain circumstances (Urdahl et al., 1994). Together, these studies support an “integrated signaling model” for thymocyte selection. This model suggests that 1) positively and negatively selecting stimuli induce different intensities of many TCR-mediated signals.
and that 2) the 'integrated' strength of the signals from several pathways (but possibly not all pathways) influence thymocyte selection. A weak integrated signal induces positive selection, whereas a strong cumulative signal triggers clonal deletion. A prediction from this model is that any alteration in the cumulative signal intensity would alter the final selection outcome. Consistent with this notion, our data suggests that the ERK pathway influences both cell differentiation (positive selection) and cell death (negative selection), in part by altering the 'strength' of ERK activation. Similar mechanisms whereby quantitative differences in signal strength can be turned into large qualitative differences in gene expression and subsequent cell fate decision is a familiar paradigm in developmental biology (Hill and Treisman, 1995). Further experiments will clarify how the interplay between signaling cascades influences gene transcription and thymocyte fate.
Chapter V
Chapter V

Duration of T cell receptor signaling dictates positive versus negative thymocyte selection

Sanjeev Mariathasan, Arsen Zakarian, Denis Bouchard, Pamela S. Ohashi

Introduction

Several studies have suggested that positive selection requires multiple TCR-mediated interactions with the thymic stromal cells to promote efficient differentiation (Kisielow and Miazek, 1995; Wilkinson et al., 1995). Our previous studies (Chapter III) have shown that positively selecting stimuli induce suboptimal TCR internalization. As a consequence of inefficient TCR down-regulation, the proportion of the TCR complexes that remain on the cell surface may be able to relay continuous signals required for survival and differentiation. We have also shown that activation of ERK is sensitive to the extent of TCR triggering. Studies from other biological systems have suggested that sustained ERK activation is conducive for differentiation processes (Marshall, 1995). Therefore, we asked the question if positively and negatively selecting ligands induce ERK activation with differential kinetics.
In order to gain further insights into TCR-mediated signals that during thymocyte development, we developed an in vitro culture system in which a population of naive DP thymocytes were triggered with a defined set of ligands that induced either positive or negative selection. Biochemical analysis revealed that while positively selecting ligands triggered sustained low level ERK activation, negatively selecting ligands induced strong but transient ERK activation. Furthermore, the transient ERK activation induced by negatively selecting ligands triggered important downstream events, since inhibition of such initial ERK activation abrogated clonal deletion. Together, our data suggests that the extent and duration of ERK activation could influence both positive and negative thymocyte selection.

Contributions

Arsen Zakarian bred the P14 TCR RAG2/- transgenic line into the H-2^d^d MHC-haplotype. Denis Bouchard operated the FACS machine for the Ca2+ analysis.

Results

An in vitro culture system

One of the limitations in directly examining various signaling molecules during thymocyte development is that approximately 2x10^6 thymocytes are required for Western blot analysis. Another limitation is that thymocytes should synchronously receive a defined TCR signal that will induce positive or negative selection. In order to follow the induction of the ERK pathway during thymocyte selection, we established an in vitro system for thymocyte selection. P14 transgenic mice that express an H-2^b^-restricted
LCMV-gp-specific TCR was bred onto a non-selecting H-2^{dd} RAG2 deficient background (referred to as P14 RAG2^{+/−} H-2^{dd}). Since thymocytes from these mice are arrested at the double positive cell stage, they could be cultured in conditions that promoted either positive or negative selection. For most experiments, macrophages were used as antigen presenting cells (APCs). Previous studies using different models have shown that other cell types including macrophages, could promote positive selection (Hugo et al., 1992; Pawlowski et al., 1993; Hugo et al., 1993b; Yasutomo et al., 2000a).

To define conditions that promoted negative selection, P14 RAG2^{+/−} H-2^{dd} thymocytes were cultured on H-2^{b} macrophages prepulsed with either the nominal peptide p33 (KAVYNFATM, 10^{-6}M) or a strong agonist variant S7A (KAVYNFSTM, 10^{-6}M). These peptides were previously shown to induce effective negative selection in fetal thymic organ cultures (FTOCs) (Ashton-Rickardt et al., 1994; Sebzda et al., 1994; Mariathanasan et al., 1998). After various times, thymocytes were harvested and stained with antibodies specific for CD4, CD8, CD69, CD5 and Vα2. In addition, cell viability was assessed using propidium iodide and annexin V. When thymocytes were cultured with p33 or S7A on H-2^{b} APCs for 20 hours CD4 and CD8 co-receptor down-regulation was observed together with an increase in apoptotic cells (Fig. 5-1A, B). These events have been correlated with negative selection (Swat et al., 1991). Under these conditions, thymocytes also rapidly upregulated the activation markers CD69 and CD5 (Fig. 5-1C, D) reflecting the intensity of TCR triggering.
Figure 5-1 A, B. In vitro culture of thymocytes induces early aspects of negative and positive selection.

Naïve CD4+8+ thymocytes from P14 TCR RAG2+/− H-2<sup>dd</sup> mice were cultured for the indicated time periods with either MHC class I+ or β<sub>2</sub>m-deficient macrophages prepulsed with various peptides. Thymocytes were stained for the following cell surface markers and examined by flow cytometry. A. Expression levels of co-receptors CD4, CD8. B. cell-death markers annexin-V and propidium-iodide. The absolute number of recovered viable thymocytes is given on each contour plot on panel (A). These data are representative of 6 experiments.
Figure 5-1 C.D.E: In vitro culture of thymocytes induces early aspects of negative and positive selection.

Naïve CD4+8+ thymocytes from P14 TCR RAG2−/− H-2dΔ mice were cultured for the indicated time periods with either MHC class I+ or β2m-deficient macrophages prepulsed with various peptides. Thymocytes were stained for the following cell surface markers and examined by flow cytometry; (C) activation markers CD69, (D) CD5 and, (E) P14 TCR (anti-Vα2 in bold) and isotype-matched control antibody are shown. These data are representative of 6 experiments.
Positive selection was induced by culturing thymocytes on β₂m-deficient H-2b macrophages prepulsed with either the nominal peptide p33 (10⁻⁷M) or the weak agonist peptide variant L6F (KAVYNLATM, 10⁻⁸M). We postulated that decreasing the level of available peptide/MHC complexes using β₂m-deficient macrophages would favor positive selection by reducing the avidity of the thymocyte/APC interactions. Both p33 and L6F have been shown to mediate positive selection of functional thymocytes in FTOC (Mariathasan et al., 1998; Mariathasan et al., 2000). A non-stimulatory adenovirus peptide, AV (10⁻⁸M), was used as a control peptide. Upon culture of thymocytes with p33 or L6F on β₂m⁻/⁻ APCs, initial events associated with positive selection were detected, such as upregulation of CD69 (Swat et al., 1993) and CD5 (Punt et al., 1996) (Fig. 5-1C, D). Moreover, interactions that favor positive selection did not dramatically affect cell viability (Fig. 5-1A, B) and TCR levels remained high (Fig. 5-1E). This analysis demonstrates that these culture conditions are able to induce early events associated with either positive or negative selection.

We next examined whether the thymocytes that have received positively selecting stimuli could further differentiate into CD8⁺ SP T cells. P14 RAG2⁻/⁻H-2⁻⁻⁻ thymocytes that have received different signals in the monolayer culture system for 16 hours were transferred to culture wells containing deoxyguanosine treated C57BL/6 (H-2ᵇᵇᵇ) fetal thymic lobes. After 60 hours of culture, flow cytometric analysis was performed on thymocytes that repopulated the fetal thymic lobes. Thymocytes that received positively selecting stimuli in the initial in vitro culture system (10⁻⁷M p33/β₂m⁻/⁻ or 10⁻⁸M L6F/β₂m⁻/⁻) were able to mature into CD8⁺ T cells, compared to thymocytes that received 'null stimulation' with
Figure 5-2. Further differentiation of signaled thymocytes.

DP thymocytes from P14 TCR RAG2<sup>−/−</sup> H-2<sup>d</sup>d mice that were stimulated in the monolayer culture system were transferred to deoxyguanosine treated C57BL/6 (H-2<sup>b</sup>b) thymic lobes. After 60 hours of culture the lobes were teased apart, thymocytes were enumerated by trypan blue exclusion and stained with anti-CD4, CD8 and H-2K<sup>d</sup> for flow cytometric analysis. CD4, CD8 profiles are shown for H-2K<sup>d</sup> positive cells. The percentage of CD8+ T cells are shown in the lower right quadrant along with total cellularity at the top of each plot. These are representative of three individual experiments.
AV peptide (Fig. 5-2). However, thymocytes that initially received strong negatively selecting stimuli (10^{-6} \text{M} \text{p33/} \beta_2m^{+/+} \text{ or } 10^{-6} \text{M} \text{S7A/} \beta_2m^{+/+}) were unable to survive and differentiate in the fetal thymic lobes. This was demonstrated by the 8-10 fold reduction in thymic cellularity and the presence of only the double negative thymocyte subset. This analysis demonstrates that this in vitro system is appropriate for examining early selection events that promotes positive and negative selection.

Positive and negative selection trigger sustained versus transient ERK activation

In order to investigate the activation of the Ras/Raf/MEK/ERK pathway during selection events, Western blot analysis was done using an antibody specific for the phosphorylated activated form of ERK. As shown in Fig. 5-3A, P14 RAG2^{-/-} H-2^{dd} thymocytes cocultured in positively selecting conditions induced sustained ERK activation. During positive selection with either p33 or L6F, activated ERK was still detectable after 16 hours compared to ‘null selecting’ AV peptide (Fig. 5-3A, B). Notably, positive selection with low avidity interactions (10^{-7} \text{M} \text{p33/} \beta_2m^{-/-} \text{ APCs}) or low affinity peptides (10^{-6} \text{M} \text{L6F/} \beta_2m^{-/-} \text{ APCs}) were similar, consistent with the affinity/avidity model.

The duration of ERK activation was examined in thymocyte cultures that induced negative selection. P14 RAG2^{-/-} H-2^{dd} thymocytes were cultured with p33 (10^{-6} \text{ M}) and S7A (10^{-6} \text{ M}) on \beta_2m^{+/+} \text{ APCs}. At various time points cell lysates were prepared and Western blot analysis was done using the antibody specific for the phosphorylated forms of ERK. The activation of ERK reached maximum at 3-4 hours and then declined 5-6 hours after stimulation (Fig. 5-4A). A similar transient kinetics was observed for
Figure 5-3. Positively selecting ligands induce sustained ERK activation.

Western blots were performed on whole cell lysates from thymocytes that were incubated with various ligands as in Fig 5-1, and probed with antibodies specific for the activated forms of p42/44 ERK (phosphospecific). p42/44 ERK antiserum that detects total p42/44 ERK levels (phosphorylation state independent) was used as a control for sample variations. (A) Kinetics of ERK activation in response to positively selecting (p33/β2m-/-) interaction. (B) Extent of ERK activation in thymocytes at 3 hours and 16 hours of positively selecting conditions. Peptides and APCs that were used are indicated.
Figure 5-4. Negatively selecting ligands induce stronger but transient ERK activation.

Western blots were performed as in Figure 5-3. A, Kinetics of ERK activation in response negatively selecting (p33/β2m+/+) interaction. B, Extent of ERK activation at 3 hours and 10 hours during negative selection. Peptides and APCs that were used are indicated. C, Comparison of ERK activation in thymocytes receiving negatively and positively selecting stimuli.
negative selection that was induced by the strong agonist ligand S7A (Fig. 5-4B). Therefore, negative selection is associated with a strong transient activation of ERK, while conditions that induce positive selection generate sustained ERK activation.

The degree of initial ERK activation was also compared between positively and negatively selecting conditions. Again, P14 RAG2⁻/⁻ H-2^{d/d} thymocytes were cocultured with β₂m⁻/⁻ and β₂m⁺/+ APCs that were prepulsed with positive (10⁻⁷ M p33/β₂m⁻/⁻ or 10⁻⁶ M L6F/β₂m⁻/⁻) or negative (10⁻⁶ M p33/β₂m⁺/+ or 10⁻⁶ M S7A/β₂m⁺/+ ) selecting ligands. Cultures were prepared in parallel and harvested after 3 hours. Fig. 5-4C shows that negatively selecting conditions induced strong ERK phosphorylation in contrast to weak phosphorylation triggered by the positively selecting interactions.

**Negative selection leads to attenuation of TCR signals**

In these culture conditions, negative selection has been correlated with peptides that were able to induce rapid TCR modulation (Fig. 5-1E) (Mariathasan et al., 1998). In order to examine if any remaining or re-expressed TCR were able to trigger other downstream pathways, we examined the ability of the thymocytes to mobilize calcium. P14 RAG2⁻/⁻ H-2^{d/d} thymocytes were complexed with negatively or positively selecting ligand/APCs. After 3 minutes, calcium mobilization was monitored. Initially, negatively selecting interactions induced a strong Ca²⁺ flux, whereas positively selecting interactions induced a weaker Ca²⁺ flux (Fig. 5-5A). After 5 hours of coculture, thymocytes were transferred to plates with fresh APCs pulsed with high concentration of p33 (10⁻⁶ M). Thymocytes that were cocultured with negatively selecting ligands for 5 hours were refractive to
Figure 5-5. Thymocytes undergoing negative selection are unable to generate a Ca2+ flux to further TCR stimulation.

A. P14 TCR RAG2+/− H-2Kd thymocytes that were preloaded with indo-1 were centrifuged and incubated with various peptide/APCs. Three minutes later, free intracellular calcium was measured (by the indo-1 405/485 ratio) for the indicated time span. B. P14 TCR RAG−/− H-2Kd thymocytes that have been previously cultured with various peptide/APCs as in panel A for 5 hours, were loaded in indo-1 and incubated with classI+ APCs pulsed with the strong antigenic peptide, p33 (10−6 M). Intracellular calcium was measured as in panel A.
further Ca2+ flux on re-exposure to class I+ APC pulsed with the strong agonist p33 (Fig. 5-5B). On the contrary, thymocytes that were incubated with peptide/MHC ligands that induced positive selection (10^{-7} \text{ M } \text{p33/}\beta2m/-\text{ or } 10^{-6} \text{ M } \text{L6F/}\beta2m/-\text{) were able to generate a Ca2+ flux in response to a strong antigenic stimulus (Fig. 5-5B). This data provides direct evidence that negatively selecting TCR-peptide/MHC interactions decrease TCR expression to the extent where the remaining TCRs are unable to trigger other downstream signal transduction pathways.

Attenuating MEK/ERK signals inhibits clonal deletion

The next question we wanted to address was whether the transient ERK activation transmits downstream signals leading to negative selection. In order to address this issue, experiments were done to block the activation of ERK using the pharmacological inhibitor PD98059. P14 RAG2+/H-2^d thymocytes were preincubated with 25\mu M PD98059, a selective compound that has been shown to inhibit the activation of MEK1/2 by upstream activators of the MAP kinase cascade (Alessi et al., 1995; Dudley et al., 1995). These thymocytes were cultured for 1 hour with \beta2m+/+ APCs that have been prepulsed with 10^{-6} \text{ M } \text{p33. The data show that 25 \mu M PD98059 markedly diminishes ERK activity in p33-induced thymocytes (Fig. 5-6A).}

We have previously shown that the addition of PD98059 in fetal thymic organ cultures in the presence of negatively selecting stimuli abrogates negative selection (chapter IV). However, the role of MEK in thymic stromal cell physiology and antigen presentation is unknown. In this case it is difficult to definitely show that the inhibition on MEK in
Figure 5-6. See following page for text.
Figure 5-6. Inhibition of MEK-mediated ERK activation abrogates negative selection.

A. DP thymocytes from P14 TCR RAG2+/− H-2<sup>bd</sup> mice were stimulated in the monolayer culture system with either the non-stimulatory AV peptide or the agonistic p33 peptide in the presence or absence of MEK inhibitor, PD98059 (25 µM). All stimulation was carried out with β<sub>2</sub>m<sup>+</sup>/+ APCs. Thymocytes were harvest at three hours and Western blot was performed on whole cell lysates. Activation of ERKs were determined by phospho-specific antibodies. 

(B-E), Flow cytometric analysis of thymocytes incubated for 16 hours. (B), CD4, CD8 profiles indicating percentage of DP thymocytes in upper right quadrant. C, Cell viability was determined by annexin/PI staining. Percentage of live cells is given in lower left quadrant. D, Extent of CD69 expression. Percentage of CD69 high cells are shown. E, Vα2 staining on gated DP thymocytes.
thymocytes is the cause for impaired negative selection. However, this novel in vitro selection system provides a way to selectively inhibit ERK activation in thymocytes.

To evaluate the consequence of blocking ERK activation in clonal deletion, P14 RAG2^{−/−} H-2^{d} thymocytes that were treated with media alone (control) or pre-treated with PD98059 for an hour and then washed, were cultured with β_{2}m^{+/-} APCs pulsed with either the non-stimulatory AV (10^{-6} M) peptide or the antigenic p33 (10^{-6} M) peptide. After 16 hours of culture a substantial CD4, CD8 down regulation of DP thymocytes and apoptosis were observed under conditions that promote negative selection (Fig. 5B, C). In contrast, PD98059 was able to inhibit clonal deletion by negatively selecting stimuli. Consistent with the block in ERK activation, flow cytometric analysis (FCM) analysis showed that up-regulation of Ras-dependent CD69 expression was also abrogated (Fig. 5-6D). Examining the P14 transgenic TCR levels confirmed that the thymocytes received the p33 peptide/MHC signal and internalized the P14 TCR (Fig. 5-6E). Together these data demonstrate that PD98059 could inhibit p33 induced clonal deletion. These data suggest that MEK/ERK pathway does generate downstream signaling events necessary to induce negative selection.
Discussion

In the present study, we have developed a novel \textit{in vitro} culture system that is suitable for evaluating early biochemical events associated with positive and negative selection of naive DP thymocytes (Fig. 5-1). We have shown that the early signals received during the initial 16 hour culture were sufficient to lead to death or promote CD8+ positive selection after further maturation in deoxyguanosine treated thymic lobes (Fig. 5-2). Although several attempts were made to promote maturation \textit{in vitro}, successful maturation was only achieved in the thymic microenvironment. This is consistent with previous studies, suggesting that this environment is essential for the maturation of thymocytes (Anderson et al., 1996a; Yasutomo et al., 2000a).

**Positive selection requires sustained TCR signals**

Although it is possible that maturation of CD8+ T cells may need extended signals from the class I molecules encountered in the fetal thymic lobes, the initial culture conditions provided the appropriate signals to direct positive selection of these cells. We observed that the non-stimulatory peptide AV induced minimal maturation of CD8+ T cells (6%), whereas the weak agonist ligand (10^{-6} M L6F/\beta_{2m/-}) or low concentrations of strong agonist ligand (10^{-7} M p33/\beta_{2m/-}) triggered maturation of a significant proportion of CD8+ T cells (27% and 30% respectively). It possible that 'neutrally stimulated' (AV) thymocytes would eventually give rise to mature CD8+ T cells in H-2^{b} thymic lobes, since it is a selective environment for the LCMV TCR transgenic system. However, the kinetics of positive selection may be slower in neutrally stimulated cells compared with thymocytes that received positively selecting stimuli early during culture. Unfortunately,
we were unable to recover thymic lobes with sufficient cellularity to assess the extent of thymocytes selection past 60 hours. Finally, the ability of weak agonists or a low concentration of strong agonist to promote positive selection is fully consistent with an affinity/avidity model for selection.

How do low affinity/avidity ligand interactions induce 'sustained' ERK activation? Our data suggest that positively selecting, low affinity/avidity ligands may achieve sustained signaling by their inability to induce maximal TCR internalization (Fig. 5-1E) and thereby continue to trigger TCR-mediated signaling cascades. This may be one mechanism by which less-stable TCR-peptide/MHC complexes achieved a greater stable accumulation of signaling intermediates for efficient differentiation/survival. This is consistent with observations from other models that show that multiple interactions are required to promote thymocyte differentiation (Kisielow and Miazek, 1995; Wilkinson et al., 1995). In addition to ERK MAP kinases, these interactions could trigger many other intracellular signaling pathways, which ultimately activate transcription factors. These could include the NF-kB family (Hettmann and Leiden, 2000), IFN-regulating factor (Penninger et al., 1997), as well as calcium dependent signaling cascades (Glimcher and Singh, 2000; Hayden-Martinez et al., 2000).

Negative selection

This study demonstrates that relatively short exposure to either strong agonist peptide, p33 or S7A, is sufficient to induce intracellular events that trigger negative selection (Fig. 5-1). The interaction with relatively higher concentrations of strong agonist ligands lead
to rapid down-regulation of surface TCR (Fig. 5-1E), to a degree where remaining TCRs were unable to transmit further signals, as measured by the mobilization of intracellular calcium (Fig. 5-5). Evidence of apoptosis was detected after 20 hours in the initial culture, and further culture in the thymic microenvironment demonstrated that no detectable double positive or single positive cells repopulated the thymic lobes (Fig 5-2). Therefore the induction of negative selection is a relatively rapid event that is associated with the loss of TCR at the surface.

Models for negative selection

These data suggest two possible models for the induction of clonal deletion of thymocytes. The first model predicts that strong agonist ligands would induce maximal TCR internalization and the lack of further TCR-mediated signals would result in clonal deletion. This mechanism of death may be similar to death by neglect. By inference, the transient TCR triggering/ERK activation would be irrelevant. An alternative model predicts that this transient but strong ERK activity contributes to the induction of apoptosis during negative selection.

The first model is supported by findings from studies of transgenic mice expressing dominant negative molecules of Ras and MEK kinases that show no impairment in negative selection. These reports suggest that ERK does not play a role during clonal deletion (Swan et al., 1995; Alberola-Illa et al., 1996a; Alberola-Illa et al., 1996b; O'Shea et al., 1996). However, the role of ERK in negative selection in these studies is not definitive, since the endogenous molecules may induce a signal sufficient for negative
selection. Similarly, intact negative selection in Erk1-knockout mice could be explained by the presence of Erk2, an isoform that is more abundantly expressed in thymocytes (Pagès et al., 1999).

Several reports support the second model that ERK is involved in negative selection. Studies have shown that reducing the intensity of ERK signals may shift negative to positive selection (Bommhardt et al., 2000; Mariathasan et al., 2000). In addition, several studies have now shown that negative selection is correlated with a transient ERK signal (Shao et al., 1999; Werlen et al., 2000). However, the explicit biological significance of this ERK signaling was not addressed in these studies. In this report we investigate the requirement of ERK activation during negative selection. Our studies demonstrate that abrogating the ERK activity in thymocytes effectively blocked clonal deletion in response to negatively selecting stimuli (Fig 5-6). This data supports the second model, where the activation of ERK transmits necessary signals to execute clonal deletion of thymocytes.

Previously, using the P14 TCR β2m-/- transgenic system in FTOC, we have shown that peptide-mediated negatively selecting stimuli could be converted into positively selecting signals by diminishing MEK-mediated ERK activity with the use of PD98059 (Mariathasan et al., 2000). In the FTOC model the MEK inhibitor can reduce, but not completely block, ERK activation. PD98059 may not effectively reduce ERK activity and block negative selection in FTOC for a variety of reasons. In FTOC, the lobes are not completely submersed in media. Therefore the ability of the drug to permeabilize
each cell at the same concentration is very unlikely. It is likely that a gradient is achieved
where the level of active ERK is reduced and not completely eliminated. Also in FTOC,
it is unlikely that all thymocytes are synchronously receiving the negative selection
stimuli. As the activity of PD98059 declines in culture, some thymocytes may receive a
slightly reduced ERK signal and become positively selected. Nonetheless, these studies
clearly show a role for ERK during negative selection, and support a model where
negative selection signals are stronger than positive selection signals.
Chapter VI
Chapter VI

Summary and Perspectives

The main objective of my thesis was to understand the nature of TCR mediated signals that discriminate positive and negative thymocyte selection. Specifically, I examined the TCR down-modulation, Ca\(^{2+}\) influx and ERK signaling in response to various peptide/MHC ligands. As a whole, the data presented in this thesis demonstrates that the decision to survive and differentiate (positive selection) or to undergo clonal deletion (negative selection) is influenced by the 'intensity' and 'duration' of the above-mentioned responses. That negatively and positively selecting conditions initially induce 'strong' versus 'weak' signaling responses, respectively, is in accordance with the 'affinity/avidity' model for thymocyte selection.

Affinities of TCR-peptide/MHC interactions

T cell signaling is a series of complex events which begins with the localization of the TCR to the areas of contact between T cells and peptide/MHC ligands on APCs (Bromley et al., 2001). This localization or clustering results in a series of signal transduction events that is dependent on the TCR-peptide/MHC interactions. The mechanism by which these interactions are translated to different biological outcomes has been explained by the kinetic proofreading model (McKeithan, 1995). This model postulates that there is a time lag between ligand binding and the molecular events that characterize the full complement of receptor binding activities. By binding to the receptor for a time
less than the time lag, weak ligands such as partial agonists and antagonists fail to evoke one or more downstream signaling events typically evoked by full agonists. Various methods have been used, including direct analysis by surface plasmon resonance (using BIAcore biosensors) of the binding and dissociation of soluble αβTCR and soluble peptide-associated class I or class II molecules. Most data show a relationship between T cell activation and the disassociation rate and/or affinity of the TCR to its ligand (Sykulev et al., 1994; Lyons et al., 1996). These data implicate the relative off-rates of the ligands (determined in part by 'half-life' of the TCR-MHC complex) as the principal determining factor in discriminating optimal and suboptimal ligands (McKeithan, 1995; Rabinowitz et al., 1996). Accordingly, plasmon resonance data for both MHC class I and class II restricted receptors have supported the notion that positive selection ligands have faster dissociation rates from the TCR (hence weak ligation) compared to negatively selecting ligands (Alam et al., 1996; Williams et al., 1999).

However, there are limitations in these plasmon resonance studies. In studies using soluble αβTCR, we do not know if the removal of the TCR from the membrane environment affects its binding to the ligand. Along these veins there is normally significant contact between the αβTCR and regions of the CD3 components; the extent to which this influences how the αβTCR interacts with ligands is unknown. Some studies have even suggested conformational changes in TCR being required for signal transduction (reviewed by (Janeway, 1995)). Since most of these BIAcore biosensor studies were performed at room temperature (25°C) rather than at 37°C, relating these values to the actual rates of disassociation during physiological conditions is not possible.
In order to address the latter issues, Alam and colleagues have performed further studies at 37°C. Using the cOVA-specific OT-1 TCRs, they demonstrated a striking difference in the kinetics of interaction between strong negatively selecting agonist ligands and the TCR at physiological temperatures compared to lower temperatures (Alam et al., 1999). This difference is not seen with weak agonist or antagonist ligands. The kinetics at 37°C are compatible with a model in which a single TCR molecule binds to the MHC-peptide complex, after which a second TCR molecule binds to the first. This could likely represent the first stage TCR-ligand multimerization that occurs on the cell surface on recognition of antigens. In addition, these studies have now been extended to LCMV-gp–specific P14 TCRs (S.M. Alam and N. Gascoigne, personal communication). These data are summarized in Table 6-1. Overall, it is satisfying that the observed half-lives and affinities observed in these studies correlate well with our in vitro data on thymocyte selection and T cell activation (Fig 6-1). These studies provide further evidence for the affinity/avidity model for thymocyte selection.

**TCR dimerization, internalization and intracellular signaling**

The data from plasmon resonance studies presented by Alam et al (Alam et al., 1999) suggest that TCR discrimination between agonist and suboptimal ligands includes both a kinetic and an allosteric component, with TCR dimerizing after binding to an agonist ligand. TCRs that have productively engaged by peptide-MHC complexes are readily internalized (Valitutti et al., 1995). Interestingly, previous data from our laboratory by Bachmann et al (Bachmann et al., 1998) have shown that TCRs oligomerize as dimers
Table 6-1: P14 TCR binding kinetics constants for different H-2D\textsuperscript{b} binding peptides measured by BIAcore biosensor. The binding constants, $k_{on}$ and $k_{off}$ were determined by non-linear fitting of curves derived from a titration of P14 TCRs in immobilized D\textsuperscript{b}-peptide surfaces maintained at 37°C, as described for the OT-1 TCR transgenic system (Alam et al., 1999). Apparent $K_d$ were calculated as $k_{off} / k_{on}$. For strong agonist ligands (p33, S7A and A3V) both the fast and slow binding kinetics are given. This is based on the dimerization model (Step 1, A + B = AB; Step 2, A + AB = AAB). See Alam et al for further details (Alam et al., 1999). Peptide A3V has been previously characterized as strong stimulatory peptide for the P14 TCR transgenic model (Sebzda et al., 1997). This unpublished data was kindly provided by S.M.Alam and N.R.J.Gascoigne.

<table>
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<th>Peptide-H-2D\textsuperscript{b} Complex</th>
<th>$k_{on}$ M\textsuperscript{-1}s\textsuperscript{-1}</th>
<th>$k_{off}$ s\textsuperscript{-1}</th>
<th>$K_d$ μM</th>
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<tr>
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<td>0.028</td>
<td>7.2</td>
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Figure 6-1: Correlation between half-life of TCR-peptide/MHC binding (Table 6-1) and biological activity. Here, biological activity is defined as the concentration of peptide ($10^{-4}$M) that is required to induce 50% maximal TCR down-regulation. TCR internalization assays were performed on mature P14 TCR transgenic T cells incubated with APCs (macrophages) complexed with various altered peptide ligands.
before internalization during peptide specific T cell activation. This explains why negatively selecting stimuli (10^6 M p33/ β2m+ APC; 10^6 M S7A/ β2m+ APC) were efficient at inducing maximal TCR internalization in mature T cells (fig. 3-1) and immature thymocytes (fig. 5-1). Since maximal TCR internalization has been correlated with robust effector functions in mature T cells (Valitutti et al., 1996; Bachmann et al., 1997b), it is likely that such intense signals deliver negatively selecting signals to developing thymocytes.

On the other hand, positively selecting weak agonist peptides induce suboptimal TCR internalization (Figs 3-1, 5-1). Curiously, the agonist peptide p33 in low concentrations also induces inefficient TCR down-modulation (Fig 3-1). These studies suggest that in addition to intrinsic affinities of the TCR-peptide/MHC complexes, ligand concentration also influences the extent of TCR internalization. Since thymocytes are more readily activated by low avidity interactions than mature T cells (Lucas et al., 1999; Davey et al., 1998), measurable TCR internalization may not be obligatory for signal propagation. Indeed, our data demonstrate that such weak interactions were able to induce Ca^{2+} influx (Figs 5-5) and ERK signaling (Fig. 4-1, 5-3, 5-4). TCR cross-linking versus TCR ligation without aggregation (induced by engineered anti-TCR antibodies) has also been shown to induce negative or positive selection, respectively (Takahama et al., 1994). Therefore, it is tempting to speculate that monomeric TCR:peptide/MHC interactions are sufficient for weak signals associated with positive selection, whereas dimerization is required for potent intracellular signals during negative selection.
Data on affinity studies from BIAcore analysis also clearly correlates with the efficacy of TCR internalization in mature T cells, demonstrating a relationship between TCR affinities for peptide-ligands, TCR multimerization, TCR internalization, and the ability of such peptide-ligands to induce positive and negative selection in thymocytes (Fig 6-1). Since BIAcore analysis measures specific affinity between a TCR-peptide/MHC interaction and not the overall avidity between all TCRs and peptide/ligand interactions on living cells, the data from these studies should not be merely interpreted that only high affinity ligands induce negative selection and low affinity ligands trigger positive selection. In vivo, these ligands could be readily titrated and hence 'avidity' would play a major role in determining the appropriate biological outcome. In this regard, we and others have shown that high affinity peptide-ligands, can induce positive selection if present in appropriately low concentrations (Sebzda et al., 1994; Ashton-Rickardt et al., 1994; Mariathasan et al., 2000; Nakano et al., 1997).

Several studies from the OT-1 TCR transgenic model challenged the affinity/avidity model and proposed that positively selecting ligands are inherently different from negatively selecting ligands (Hogquist et al., 1995). Based on properties of mature T cell activation, agonistic and antagonistic peptides were categorized as negatively and positively selecting peptides, respectfully. These studies were further supported by the fact that in mature T cells, agonistic ligands were able to phosphorylate the full complement of ITAM motifs on the CD3ζ chain, whereas antagonistic ligands were only able to phosphorylate selective ITAM motifs (Sloan-Lancaster et al., 1994; Madrenas et al., 1995). Differential phosphorylation patterns were thought to recruit unique signaling
effectors that would result in either positive or negative selection. However, recent studies on immature thymocytes from several thymocyte development models demonstrated that similar phosphorylation events occur during positive selection by low affinity ligands compared to that elicited by negatively selecting ligands, albeit at lower levels (Smyth et al., 1998; Lucas et al., 1999). Positively selecting antagonist peptides from the OT-1 model were also shown to induce CD69 upregulation and Ca\(^{2+}\) influx in immature thymocytes (Davey et al., 1998). Similarly our data demonstrate that positively and negatively selecting interactions induce different intensities of Ca\(^{2+}\) flux and ERK activation. An affinity/-avidity model does not distinguish between selecting ligands; instead thymocyte selection is governed by the strength of TCR-peptide/MHC interactions. Therefore, whether a peptide is an agonist or an antagonist distinction is a moot point when applied to thymocyte selection.

**Transient versus sustained signaling**

There is evidence to suggest that phosphorylation of CD3\(\gamma\) chain leads to internalization of functionally triggered TCR-CD3 complex and mark it for degradation in the lysosomes (Letourneur and Klausner, 1992). Hence internalization of rapidly triggered TCRs would help terminate signals transmitted through the TCR (Valitutti and Lanzavecchia, 1997). In mature T cells, such a mechanism would confirm TCR-ligand specificity by increasing the 'signal/noise' ratio and appropriate effector functions would ensue. However, in immature thymocytes such a scheme would not only deliver a rapid and strong signal, but also abrogate sustained signaling known to be essential for survival and differentiation. Consistent with this notion, our data demonstrates that TCRs get rapidly
internalized when subjected to negatively selecting stimuli. Consequently ERK and Ca$^{2+}$ signaling are transient (Figs 5-4, 5-5). A closer look at the data present in the literature also supports the notion that strong TCR down-regulation is correlated with negative selection. For instance, in a class II restricted TCR transgenic model, the concomitant transgenic expression of the cognate peptide, or the expression of altered peptide ligands that also maintain longer TCR-ligand half-lives, showed a good correlation between the ability of these peptides to induce efficient TCR internalization and negative selection (Williams et al., 1999).

In addition to TCR internalization and consequent TCR-mediated signal cessation, other biochemical mechanisms may also operate to quench TCR mediated signaling. For instance, activation of tyrosine phosphatases SHP-1 and HePTP may negatively regulate ERK activation. Antibody-mediated signaling through the TCR of mature thymocytes from mutant mice for SHP-1 (motheaten) has revealed prolonged activation of ERK (Pani et al., 1996). HePTP is another tyrosine phosphatase that is expressed in T and B cells and has been shown to dephosphorylate ERK1/2. Analysis of HePTP +/- mice shows enhanced positive selection and negative selection, perhaps due to enhanced ERK activation (Sebzda, E., Ohashi, P.S., unpublished data).

Conversely, our data demonstrate that positively selecting stimuli induce suboptimal TCR internalization and sustained intracellular TCR signaling. Studies have suggested that continual engagement of the TCR with appropriate MHC environment to be essential for thymocyte survival and differentiation. (Kisielow and Miazek, 1995; Yasutomo et al.,
This underscores the importance of maintaining elevated TCR expression during positive selection. Although our monolayer signaling culture was able to initiate necessary signaling for thymocyte positive selection - such as, minor downregulation of CD4 and CD8 and simultaneous induction of CD69, it was unable to complete the maturation of these CD4loCD8loCD69+ cells to mature CD8+ T cells (Fig 5-1). Further culture in the FTOCs was required for a successful differentiation process (Fig 5-2). Lucas et al (Lucas and Germain, 1996) have shown evidence that these CD4loCD8loCD69+ cells represent a transitional stage in T cell maturation and that sustained interactions of the TCR and CD8 molecules with class I MHC would permit the maturation of these cells to the CD8+ SP T cell lineage. In addition to providing the necessary MHC interactions, FTOC cultures also may provide the essential 3D architecture, as well as the critical survival factors for development. Consistent with our observations, several recent reports have also shown that either intrathymic injections or stromal reaggregate cultures that mimic FTOCs were necessary to complete the lineage commitment of in vitro signaled CD4loCD8loCD69+ thymocytes (Kisielow and Miazek, 1995; Yasutomo et al., 2000a; Yasutomo et al., 2000b). Thus, both survival and maturation of DP thymocytes into mature T cells are dependent on sustained TCR engagements with thymic stromal cells.

Survival signals

In addition to TCR mediated events, distinct mechanisms are important for regulating thymocyte survival. Cytokines produced by the thymic stroma, such as Interleukin-7 (IL-7) have a critical role in thymocyte selection. IL-7 is a potent survival factor that
upregulates Bcl-2 expression in thymocytes (Akashi et al., 1997). Many studies have addressed the role of Bcl2 family members, such as Bcl2 and Bcl-xL, in thymocyte selection. Bcl2 is expressed in immature DN and mature SP thymocytes and T cells, whereas Bcl-xL is reciprocally expressed mainly in DP thymocytes (Chao and Korsmeyer, 1998). These expression profiles suggest that Bcl2 family members play a role in thymocyte selection. Overexpression of Bcl2 and of Bcl-xL has been shown to protect thymocytes from various apoptotic stimuli (Chao and Korsmeyer, 1998). However, a direct role for these proteins in blocking apoptosis in thymocytes still remains somewhat controversial since different models have given contradictory results (Williams and Brady, 2001).

Several molecules have been shown to regulate Bcl-xL expression in DP thymocytes. Reduced Bcl-xL expression with a concordant increase in apoptosis of DP thymocytes is seen in mice deficient in orphan nuclear hormone receptor, RORγ (Sun et al., 2000; Kurebayashi et al., 2000). Conversely, mice constitutively expressing protein-kinase B (PKB) in thymocytes show enhanced cell survival and increase in Bcl-xL expression without any apparent change in repertoire selection (Jones et al., 2000). Another molecule that has been implicated in thymocyte survival is PI3K (γ-isofrom) and studies from mutant mice deficient in this PI3Kγ show impaired thymocyte survival (Sasaki et al., 2000). Interestingly, PI3Kγ has been also shown to regulate PKB in neutrophils (Sasaki et al., 2000); however, if such a link exists in thymocytes is unclear.
Sustained versus transient ERK activation in other biological models: proliferation versus differentiation

In PC12 cells, the cellular responses such as proliferation and differentiation have been shown to be determined by the duration of ERK activation (Marshall, 1995). EGF induces proliferation in PC12 cells, whereas NGF induces differentiation of these cells into neurites. Signal transduction studies show that EGF stimulation produces a short-lived rise in RasGTP whereas NGF produces a persistent elevation in RasGTP (Muroya et al., 1992). The similar patterns of transient versus sustained signaling have also been shown for the downstream molecule ERK (Heasley and Johnson, 1992; Traverse et al., 1992; Nguyen et al., 1993).

Interestingly, the kinetics of ERK activation in part seem to be modulated by receptor internalization: EGF receptors are more readily down-regulated than NGF receptors by receptor internalization and phosphorylation (Countaway et al., 1992). At first glance, these events seem to mirror thymocyte development. Negative selecting stimuli induce rapid TCR internalization and transient ERK activation. In mature T cells, such stimuli induce efficient T cell activation (Fig. 3-2).

On the other hand, positively selecting stimuli induce suboptimal TCR internalization and sustained ERK activation. During positive selection, DP thymocytes undergo a differentiation program to become mature SP T cells. Interestingly, if PC12 cells are engineered to overexpress EGF receptors, there is prolonged ERK and as a result, cells differentiate but not proliferate in response to EGF stimulation (Traverse et al., 1994).
Furthermore, the process of receptor editing has been proposed in thymocytes (under certain circumstances), whereby novel expression of TCRα can rescue cells that would have be expected to undergo apoptosis in response to strong antigenic stimulation (McGargill et al., 2000). Sustained ERK activation also promotes differentiation in other models. Sustained ERK activation mediated by PKC has also been shown to induce megakaryocyte differentiation in K562 cells. In this system, TPA and bryostatin are known to activate PKC but paradoxically have opposing effects on megakaryocyte differentiation. TPC, a differentiation inducer, causes sustained ERK activation (>24 hours), whereas bryostatin, a differentiation blocker, only transiently activates ERK (~6 hours) and attenuates the activation of ERK by TPA (Racke et al., 1997; Whalen et al., 1997).

Proliferation and apoptosis: a possible link?

Ras has been implicated in T cell activation and proliferation (Downward et al., 1990). Inactivation of the Ras pathway has also been associated with anergy induction in mature T cells (Li et al., 1996; Fields et al., 1996; Boussiotis et al., 1997). Recent studies on ERK1 -/- and RasGRP -/- mice have indicated clearly that activation of ERK is essential for mature T cell proliferation (Dower et al., 2000). Similarly, our studies of the attenuation of ERK signaling by PD98059 addition also have demonstrated that downstream effectors of the ERK pathway influence T cell activation and proliferation (Fig. 4-2). However, such a ‘proliferative’ signal may be detrimental to immature thymocytes.
DP are mostly non-cycling cells which are sensitive to various apoptotic stimuli (Penninger and Kroemer, 1998). However, it is possible that negatively selecting stimuli could confer a 'proliferative' signal to these DP thymocytes at an inappropriate stage during development. Indeed, aberrant activation of cell cycle has been proposed to play a role in apoptosis (mitotic catastrophe) (Raff, 1992; Evan et al., 1995). Cdk2 is a key kinase that is involved in cell-cycle (G1 to S progression) and mitosis (Morgan, 1997). Its activity has been shown to increase in thymocytes during apoptosis induced by various stimuli (Hakem et al., 1999). Interestingly, inhibition of Cdk2 activity has been associated with the protection of apoptosis induced by negatively selecting ligands (Hakem et al., 1999). Activation of Cdk2 during thymocyte selection also parallels the degradation of Cdk2 inhibitor p27kip (Gil-Gomez et al., 1998) and as such, it is not surprising Cdk2 activity is elevated about 10-fold in p27kip−/− thymocytes (Nakayama et al., 1996). Accordingly, the deficiency of p27kip in mice resulted in an enlarged thymus, implying a possible role for p27kip as a pro-apoptotic molecule in thymocytes (Nakayama et al., 1996). However, additional studies need to be performed in negative selection models to validate this hypothesis. The connection between Ras signaling pathways and cell cycle regulators in regulating thymocyte negative selection is provocative and warrants further examination.

**Putative downstream targets of ERK that influence thymocyte selection.**

Collectively, studies have established that ERK is essential for thymocyte selection, the identification of downstream elements of the MAP kinase cascade that regulate these
Figure 6-2: An ERK-centric view of thymocyte selection. DAG activates both RasGRP and PKC, both of which may contribute to the activation of ERKs. Not detailed are the Ras-Raf-MEK intermediates of the ERK module. TCR mediated signaling via ERKs have been shown to activate some nuclear proteins such as, Nur77, Egr1, and Id3 proteins. Id proteins can bind to E Proteins (E2A, HEB etc) and inhibit E proteins' DNA binding activity. Studies on gene-targeting of E proteins suggest that E proteins inhibit TCR-mediated signaling, whereas upregulation of Id proteins enhance TCR-mediated signaling. The activity of ERKs may also be negatively modulated by protein tyrosine phosphatases: SHP-1 and HePTP.
processes becomes imperative. Towards this end, several nuclear proteins whose activity seems to be influenced by the ERK module have been reported in the literature (Fig. 6-2).

One of these transcription factors that are required for proper thymocyte selection is E2A. The E2A protein belongs to a class of basic helix-loop-helix (bHLH) transcription factors, known as E proteins (Massari and Murre, 2000). Members of this family include HEB and E2A gene products E12 and E47 (alternative splice variants) (Murre et al., 1989). E proteins form heterodimers and function transcription factors at conserved E box motifs (Murre et al., 1989). αβT cell development in mice lacking E2A shows partial block at the DN stage prior to the onset of TCRα rearrangement (Bain et al., 1997). In addition, E2A-deficient mice show impaired thymocyte selection, since there is a significant reduction in DP cells and a corresponding increase in CD4 and CD8 SP cells (Bain et al., 1997). Similar results were also seen in E47/- mice bred to class I and class II restricted T cell transgenic models (Bain et al., 1999). Furthermore, thymocytes derived from both E2A- and E47-deficient thymocytes had 3 to 4 fold more thymocytes expressing TCR-mediated activation marker CD69, suggesting enhanced selection in these mice (Bain et al., 1999). Collectively, these data indicate that activation of E proteins negatively regulates TCR-mediated signals and that the lack of E-protein activity lowers the threshold of avidity required for positive selection.

In contrast with E proteins, Id gene products lack DNA binding domains, but exert their transcriptional influence by dimerizing with E protein and thereby, disrupting E protein
activity (Benezra et al., 1992) (Fig 6-2). Therefore, Id gene products may enhance TCR-mediated responses by attenuating E protein activity. In accordance with this model, Id3-deficient mice show impaired thymocyte positive and negative selection (Rivera et al., 2000). In addition, thymocyte subpopulations are restored to normal levels in E2A-/Id3-/- mice, suggesting that these two gene products play a major role in influencing thymocyte selection by regulating one another (Rivera et al., 2000).

Recent data by Bain et al (Bain et al., 2001) have positioned ERK MAPK signaling and the regulation of E proteins in a common pathway. According to this in vitro data, the TCR-mediated lck-Ras-MEK-ERK module induces Id3 expression. However, E protein DNA binding activity but not induction of its expression is influenced by TCR-mediated signals. The DNA binding activity is most likely regulated by the extent of Id3 expression. Interestingly, the extent of Id3 induction is responsive to the dose of PD98059 expression. These data clearly suggest that Id3 mRNA can differentially regulate ERK MAPK signaling and previous evidence shows that Id3 deficiency can perturb both positive and negative selection. This clearly correlates with our studies where we have shown that inhibition of ERK activity by PD98059 both, (a) shifts negative selection to positive selection in FTOCs (Fig 4-5) and (b) impairs negative selection in in vitro signaling cultures (Fig. 5-6). The latter study suggests that this phenotype is not due to possible perturbations in APC function, since only the thymocytes were pre-incubated with the MEK-inhibitor.
The ERK-MAPK cascade has also been implicated in regulating other nuclear factors that are known have an impact on thymocyte selection. The above mentioned studies by Bain et al have also determined that the activated ERK MAPK module up-regulates the expression of early immediate-early gene Egr1, which has the ability to activate (either directly or indirectly) Id3 expression (Bain et al., 2001). Previous studies have shown that Egr-1 is induced during positive selection in the thymus and overexpression of Egr1 shows disrupted thymocyte selection (Shao et al., 1997; Miyazaki and Lemonnier, 1998).

In addition to Egr1, up-regulation of Nur77 has also been shown to be sensitive to PD98059-mediated ERK inhibition (Shao et al., 1999). Nur77 is an orphan member of the steroid nuclear superfamily and is rapidly induced by TCR-mediated signaling in immature thymocytes (Woronicz et al., 1994; Liu et al., 1994). Transgenic mice that express dominant-negative forms of Nur77 show protection from thymocyte apoptosis (Calnan et al., 1995; Zhou et al., 1996), whereas over-expression of wild-type Nur77 facilitated thymocyte apoptosis (Calnan et al., 1995; Weih et al., 1996). However, the precise role of Nur77 in thymocyte selection is controversial, since Nur77/- mice do not show impaired negative selection (Lee et al., 1995). Given the phenotype of dominant negative Nur77 transgenic mice, redundancy of Nur77 related molecules in influencing negative selection cannot be ruled out (Cheng et al., 1997).

Collectively, these studies suggest that the ERK-MAPK module regulates genes that are involved in thymocyte positive and/or negative selection. Again, delineation for either
of these processes may be determined by the extent and the timing of activation of ERK-sensitive nuclear genes.

Models for T cell selection: revisited

Unique signal model

Evidence for this model has come from several studies where unique signaling pathways were shown to regulate either positive or negative selection. Early studies with dominant negative forms of Ras and MEK showed that this MAPK module is essential for thymocyte positive selection and not negative selection (Swan et al., 1995; Alberola-Ila et al., 1996a; Alberola-Ila et al., 1996b). However, there is a possibility that the dominant negative molecules in these transgenic systems were not strong enough to inhibit negative selection. Since negative selection induces a stronger TCR-mediated signaling response, it is conceivable that it would be harder to block negative selection compared with positive selection. Several lines of evidence suggest that these transgenic models do not effectively block ERK signaling. For instance, ERK MAPK-module has also been implicated in DN to DP (Swat et al., 1996; Crompton et al., 1996; Michie et al., 1999); however, Ras and MEK dominant-negative transgenic expression under the influence of the proximal-lick promoter have shown no obvious defects in this transition (Swan et al., 1995; Alberola-Ila et al., 1996a; Alberola-Ila et al., 1996b). Although, MEK dominant-negative transgenes were expressed in mature LN T cells, TCR-mediated proliferative responses were normal (Alberola-Ila et al., 1996a). However, inhibition of MEK activity with PD98059 (Fig. 4-2), as well as specific attenuation of ERK activity by deletion of ERK1, showed impaired T cell proliferative responses (Pagès et al., 1999). These
observations lend support to the notion that these dominant-negative transgenic systems may be inefficient in blocking negative selection signaling.

Studies from the TCR transgenic mice with a deletion in the TCRα connecting peptide motif (αCPM) also supported the view that unique signals may influence thymocyte selection (Bäckström et al., 1998). Deletion of αCPM abrogated positive selection, but negative selection appeared normal. As an extension of these studies, a recent manuscript by Werlen et al (Werlen et al., 2000) showed that sustained ERK activation induced by positively selecting ligands is impaired in TCR transgenic models with the αCPM deletion. An interesting finding was that transient ERK activation in response to negatively selecting ligands was unaffected. These data correlated well with our studies showing that positively and negatively selecting ligands induce qualitatively different ERK signals.

The αCPM deletion mutation also affected the recruitment of CD36 to the TCR complex (Bäckström et al., 1998). An early interpretation of these results was that the αCPM motif by its virtue of association with CD36 would transmit unique signals for positive selection (Bäckström et al., 1998). Indeed, positive selection is impaired in CD36-deficient mice (Dave et al., 1997) and a recent study from the same group also reported impairment of ERK activation in CD36-deficient thymocytes (Delgado et al., 2000). Interestingly, negative selection is also impaired in CD36-deficient H-Y TCR transgenic mice (Dave et al., 1997), a relevant fact that seems to be ignored in many discussions
(Werlen et al., 2000; Delgado et al., 2000). In conclusion, these studies don’t rule out the possibility that ERK is also involved in negative selection.

Another recent report that is relevant for this discussion is that of Grb2-haploid insufficiency in thymocytes (Gong et al., 2001). Grb2 is an adapter protein that promotes Ras activation in association with Sos guanine nucleotide exchange factor (GEF). Unexpectedly, mice heterozygous for Grb2 null-mutation showed impaired JNK and p38 MAPK activities but not impaired ERK activation. Grb2 +/- mice also showed reduced negative selection in several selection models but positive selection remained normal. At first glance this study further supports the model that positive and negative selection invoke different MAPK pathways. However, quantitative analysis of the three MAP kinases showed a lower threshold for ERK activation compared with JNK and p38. This led the authors to propose a modified model for thymocyte selection where weak interactions that induce positive selection would selectively activate ERK, whereas stronger TCR-mediated interactions that induce negative selection would activate all ERK, JNK and p38. However, it is uncertain why positive selection is not affected in this model in which Grb2-deficiency should first impair weaker signals induced by positively selecting ligands.

Integrated signal model

As discussed in the introduction (Chapter I), inactivation of many TCR-triggered signaling molecules such as CD45, Lck, Fyn, CD3ε-associated ITAM motifs, Zap-70, Gads, Vav, and Itk / Rlk have shown diminished signaling abilities and decreased
positive and negative selection. One possible explanation is that these signaling molecules contribute to the overall signaling threshold that is required for different biological outcomes. Different levels of nuclear accumulation of signaling molecules and transcription factors could influence different biological outcomes. Our study shows that levels of ERK activity also contributes to this initial signaling threshold and thereby alter both positive and negative selection.

A more unified model: an attempt...

Based on analysis of our own study and as well as observations from the current literature, we propose the following model. Primarily, TCR-peptide/MHC interactions govern thymocyte selection by inducing differential TCR internalization. Indeed there is a reasonable correlation between the TCR-peptide/MHC affinities and the ability of the peptide/MHC ligands to induce TCR down-regulation. The extent of TCR internalization also corresponds to the level of TCR triggering and different biological outcomes ensue. Maximal TCR internalization is observed to occur with negatively selecting stimuli. The consequence of maximal TCR internalization is two-fold. First, maximal TCR internalization is a good indication that a strong stimulus is initiated for negative selection. Secondly, such a signal is transient due to the rapid clearance of TCRs from the cell surface. Continuous TCR-peptide/MHC interactions have been shown to be necessary for thymocyte survival. Therefore negative selection could be induced by strong TCR-mediated signals and/or the lack of continuous signals for survival (akin to death by neglect). Conversely, positively selecting stimuli would induce suboptimal
Figure 6-3: A model for thymocyte selection based on TCR-mediated signals. ERK activity is influenced by extent of TCR triggering. Strong but transient ERK activation induces negative selection, whereas weak, sustained ERK activation induces positive selection. Thymocytes show constitutive p38 activity. Absence of survival/differentiation signals via ERK activation may sensitize thymocytes to p38-mediated apoptosis. The direct role of SAPK/JNKs in thymocyte negative selection is contentious.
TCR internalization, providing constant tickling of the TCRs for sustained weak signaling.

It is rather evident now that there exists a 'kinetic' component to thymocyte selection. In this study, we focused on ERK signaling and showed that positively and negatively selecting stimuli invoke sustained and transient ERK activation, respectively. Similar scenarios in other signaling cascades may also influence thymocyte selection. For instance, it is very conceivable that the extent and duration of Ca^{2+} induced responses may also regulate both selection processes.

In addition, activation of the other two MAPK's, namely JNK/SAPK and p38, also adds another dimension to the scheme of thymocyte selection. The activation of these kinases has been implicated in thymocyte negative selection (Rincón et al., 1998b; Sabapathy et al., 1999; Sugawara et al., 1998; Werlen et al., 2000; Gong et al., 2001). The role of JNK/SAPK in negative selection remains controversial, since deletion of all major forms of JNKs in thymocytes seems to have no deleterious effect on thymocyte development (Dong et al., 2000). Attenuation of p38 activation impairs negative selection (Sugawara et al., 1998). However, in most studies the activity of p38 in thymocytes in response to both positively and negatively selecting peptides seems to be similar (Werlen et al., 2000). Therefore, a speculative new viewpoint would be that during both positive and negative selection p38 is constitutively active as a default (built-in) death pathway. During positive selection, survival/differentiation signals induced by sustained ERK activation would protect thymocytes from p38-mediated apoptosis; in contrast, premature
termination of ERK activity during negative selection would not afford this protection (Fig. 6-3).

Finally our studies demonstrate, that mechanisms for differentiation and apoptosis have common features among various biological systems. In this regard, the extent of receptor internalization and the duration of ERK activation for optimal differentiation, and finally the balance between ERK versus JNK/SAPK and p38 for life as opposed to death (Xia et al., 1995), seem to have striking similarities between thymocyte development and PC12 biology. Whether the mechanisms proposed here apply to most neuronal and non-neuronal cells in general has yet to be defined. Furthermore, it should be noted that ERK and p38 activation patterns during thymocyte selection are correlative at this juncture and hence, more experiments are needed to confirm if such a model is the key to different selection outcomes.
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Reference List


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