The roles of ERK pathway localization and the regulation of β-catenin/Bcl-XL kinetic in thymic selection.

A THESIS SUBMITTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF MISSOURI BY:

William Joseph Olson

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE MASTER OF SCIENCE

Dr. Mark A. Daniels, Advisor.

December, 2011

The undersigned appointed by the dean of the Graduate School, have examined the thesis entitled

The roles of ERK pathwaylocalization and the regulation of β-catenin/Bcl-XL kinetic in thymic selection

Presented by William J. Olson,

a candidate for the degree Master of Science

and hereby certify that, in their opinion, it is worthy of acceptance.

Dr. Mark Daniels

Dr. Emma Teixeiro

Dr. David Lee

Dr. Charles Brown

Acknowledgements:

Dr. Mark A. Daniels: Advisor
Dr. Emma Teixeiro: Committee Member
Dr. Charlie Brown: Committee Member
Dr. David Lee: Committee Member

Dr. Bumsuk Hahm: Former Committee Member
Dr. Susan McKarns: Former Committee Member
Members of the Daniels Lab
Members of the Teixeiro Lab
Jana Clark: MMI Administrator
MMI Administrators
Wes and Julaine Olson: Parents
Morgan Olson: Sister

Abstract:

Thymocytes undergo a process of development that serves to favor the differentiation of T-cells bearing self-MHC (major histo-compatibility complex) restricted T-cell receptors (TCR). Signaling through the TCR is crucial for the development from double positive ($CD4^+CD8^+$, referred to as DP) thymocytes into mature T-cells. Strong or no affinity interaction between MHC and the TCR leads to apoptosis and is considered as negative selection or death by neglect respectively. A weak to moderate affinity signal initiates differentiation of DP cells into single positive cells (positive for either CD4 or CD8, SP) termed positive selection. The importance of several pathways for either negative or positive selection has been described. However, the molecular events that allow the differentiation of a positive versus negative selecting signal are unclear because, engagement of the TCR leads to activation of all pathways known to play a role in selection. It has been suggested that differential localization of the extra cellular regulated kinase 1/2 (ERK) pathway plays a key role in selection. Here we examine the localization of ERK activation in thymic selection by the manipulating calcium levels leading alterations in RasGRP location and through a Raf construct targeted to either the plasma membrane or the golgi. We also examine differences in β-catenin expression and its role in the expression of Bcl-XL, a pro-survival molecule critical for survival at the DP stage.

We find that activation of the ERK pathway from the plasma membrane converts positive selection to negative selection, while the activation of ERK from the golgi upon stimulation by a negative selector causes a deviation of these cells from apoptosis into the $CD8\alpha\alpha^+$ lineage. In addition we show that the kinetics of β-catenin and Bcl-XL are initially consistent with a role in mediating survival. However, later in selection β-catenin may be more important in preventing cells that have been negatively signaled from maturing further, while Bcl-XL levels remain low, leading to their eventual death.

Table of Contents:

List of Figures

Chapter 1

Chapter 2

Introduction:

T-cells are the primary effectors of cell mediated immunity and are thus responsible for the clearance of intracellular pathogens. Each T-cell expresses multiple copies of a single TCR, through which the cells mediate cellular immunity by interacting with peptide antigen presented by self-MHC. Upon, infection antigen presenting cells (APCs) become activated, take up and process the invading pathogen and enter the lymph node where they interact with naive T-cells, activating those that recognize the foreign antigen presented. These T-cells then move to the peripheral sites of infection and clear the pathogen.

The TCR is rearranged in a random fashion from multiple genes and put together by a process that alters the composition of the joints between variable regions. Rearrangement can lead to greater than 10^{15} different possible TCRs (1) (2). However, only 3×10^{11} naive T-cells are present in an individual, mouse at any given time (2). Because the TCR must interact with MHC and only a few MHC alleles are present in an individual the majority of rearranged TCRs will be useless. A small number will even be potentially dangerous due to high affinity for self. Therefore, only receptors with a low to moderate affinity for MHC will be useful. The problem for the organism is how to retain T-cells that express useful receptors while deleting those that express useless and harmful ones and still maintain as much TCR diversity as possible. This is accomplished through the process of thymic selection.

Thymocytes arise from a seeding population of bone marrow precursor cells. These cells arrive in the thymus via blood vessels near the junction of two distinct anatomical regions of the thymus, the cortex and the medulla. Once in the thymus these cells develop through several stages, defined first by the DN1-4 (double negative for CD4 and CD8) stages by expression of CD44 and CD25 (3). Upon, successful rearrangement of the TCRβ chain, at the DN3 stage, and surface expression of the receptor, with a pre-TCRα chain, the nascent receptor initiates a signaling event that allows the cells to proceed to

the DP stage (Juliet Mahtani-Patching (4), (5). It is at the DP stage that thymocytes express a fully rearranged TCRαβ receptor and positive or negative selection can occur.

Pre-selection DP thymocytes are unique among lymphocytes in that they are unresponsive to pro-survival cytokines such as IL-7 (6) due to low receptor expression, and the expression of negative regulators of cytokine signaling, such as the protein, suppressor of cytokine signaling (SOCS)(6, 7). Therefore, the survival signal the cells need to continue in development comes from a low- to moderate affinity TCR interaction with self-peptide bound MHC expressed on cortical thymic epithelial cells (cTECs). Thymocytes expressing receptors that do not interact with self-MHC die by "neglect". Cells that interact too strongly undergo apoptosis and are removed from the repertoire. They may also undergo receptor editing or anergy. The result of this process is a T-cell repertoire that is self-restricted but self-tolerant. Upon positive selection, DP cells become sensitive to CCR7 signaling via CCR7L and migrate to the medulla of the thymus (8, 9). Here, DP cells also up regulate IL-7R, cease SOCS production and are instructed to become either CD8 or CD4 SP cells, depending on TCR signal duration (10). In the medulla, thymocytes are able to scan a larger pool of self-antigen, including some peripheral gene products that would not normally be expressed in the thymus, due to the expression of the autoimmune regulator AIRE (11, 12). Strong interactions between TCR and MHC in the thymic medulla lead primarily to apoptosis. But there are alternatives to death upon TCR engagement in the medulla; including diversion into regulatory T-cell, CD8αα, and natural killer T-cell lineages (13). In the event the receptor does not interact strongly with self peptide displayed by MHC the cell will eventually leave the thymus and take up residence in the periphery as a naive T-cell.

The signaling threshold for positive selection has been well defined (14-16) and a number of signaling mediators involved in selection have been characterized. Extra-cellular regulated kinase 5, c-Jun N-terminal kinase 1/2 (JNK1/2) and p38 all play roles in negative selection (17-19). The mitogen activated protein kinase (MAPK), ERK is crucial for survival and differentiation of DP thymocytes (20, 21). Nuclear ERK kinase activity is known to be important in positive selection, including activation of

the transcription factor, SRF accessory protein 1 (SAP-1). Activation of SAP-1 leads to expression of the transcription factor early growth response protein 1 (EGR-1), and EGR-1 activity is critical for positive selection (22). Targets in the cytoplasm have been characterized as well and include, JNK1/2 and the pro-apoptotic protein Bcl-2 like 11 (Bim); both play important roles in negative selection (18, 23).

Together these studies suggest that we know the signaling mechanism that establishes the selection decision. However, upon TCR-pMHC interaction in the cortex, the pathways involved in both positive and negative selection become activated. Thus, it is unclear how thymocytes distinguish positive from negative selection. One possibility is the differential localization of the ERK-MAPK pathway(14).Other groups have shown that different ERK-MAPK signaling locations can alter signaling duration and the outcome of signaling (24, 25). Indeed, differential localization of ERK, as well as upstream mediators of the pathway, has been described in thymocytes (14). Members of the ERK pathway, and ERK, were observed via confocal microscopy to be sequestered exclusively at the plasma membrane when cells were stimulated with a negative selector. The pattern of ERK was more diffuse, indicating both nuclear and cytoplasmic localization, upon positive selector stimulation. These observations suggest two possibilities: First, sequestering ERK at the plasma membrane may prevent it from interacting with downstream targets. Second, ERK activation at the plasma membrane may lead to an altered signal that ultimately leads to negative selection. The former possibility is tempting given that JNK activity alone is sufficient to induce apoptosis.

Bim is a Bcl-2-Homology-Domain 3 (BH3) only, pro-apoptotic protein that plays an important role in apoptosis. Deficiency can lead to altered negative selection and has been shown to play a role in some models of autoimmunity, including the non-obese diabetic (NOD) mouse model of type 1 diabetes (26). Bim mediates apoptosis by binding pro-survival Bcl-2 family members, Bcl-XL, Bcl-2 and Mcl-1. In a cell with survival cues these molecules sequester BAX and BAK, by binding via the BH3 domain. It is interesting to note that ERK and JNK differentially phosphorylate Bim, phosphorylation by ERK leads to degradation, while JNK activity stabilizes Bim. Increasing levels of Bim and other pro-apoptotic

mediators such as PUMA and BAD relative to pro-survival proteins, determines whether or not a cell will undergo apoptosis. Bim and other pro-apoptotic molecules bind to pro-survival proteins through the BH3 domain with greater affinity than BAX or BAK thus releasing them. BAX and BAK are then able to form pores in the outer mitochondrial membrane, resulting in release of cytochrome-c that initiates a caspase cascade eventually resulting in apoptosis.

Bcl-XL is the most abundant pro-survival protein in DP thymocytes. It is considered to be responsible for cell survival during the DP stage (27-30). However, the signaling involved in the regulation of Bcl-XL is unclear Huimin et. al. correlated the level of Bcl-XL to β-catenin level. In this study, artificially increased β-catenin levels correlated with an increase in Bcl-XL, while a decrease in β-catenin correlated with decreased Bcl-XL. This was linked to the activation of the transcription factor TCF-1by β-catenin (30, 31). Interestingly, signaling pathways that have been shown to stabilize β-catenin are also important in the survival and positive selection of thymocytes. ERK, PI_3K and WNT signaling pathways can positively regulate β-catenin levels.

Here, we examine two roles for signaling in the selection of thymocytes. In Chapter 1 we investigate the role of ERK localization in selection. Different affinity ligands have the ability to alter the localization of ERK (14). Strong (negative selectors) activates ERK at the plasma membrane, weak (positive selectors) activate the ERK pathway on endomembranes and leads to diffuse pERK (active ERK). Differential localization of the ERK pathway has been reported in other cell and receptor types. Localization of an upstream member of the pathway, Ras, can lead to different signaling outputs and different biological outcomes (24, 25). In Chapter 1 we test the role of ERK localization by altering the localization of members of the ERK pathway during selection and examine changes in the outcome of selection.

Chapter 2 focuses on the role that signaling plays in regulating the pro-survival molecule Bcl-XL in thymocytes. Because apoptosis is the primary mechanism of negative selection, its regulation must be tightly controlled. Because, Bcl-XL plays an important role in the survival of DP thymocytes, it may be

regulated based on the TCR signals that determine selection. Several reports suggest that the regulation of Bcl-XL is dependent on β-catenin levels (27, 30, 31). Several pathways are known to positively regulate β-catenin level including PI_3K, ERK and WNT. In Chapter 2, we investigate the TCR component of these pathways and examine the role of PI_3K and AKT signaling in the regulation of β-catenin and Bcl-XL in early T-cell selection.

References:

1. Patten P, T., Yokota, J. Rothbard, Y. Chien, K. Arai, and M.M. Davis. 1984. Structure, expression and divergence of T-cell receptor beta-chain variable regions. *Nature* 312: 40-6
2. Marc K. Jenkins HHC, James B. McLachlan, and James J. Moon. 2008. On the Composition of the Preimmune Repertoire of T Cells Specific for Peptide–Major Histocompatibility Complex Ligands. *Annu. Rev. Immunology* 28: 275-94
3. Dale I. Codfrey JK, Takashi Suda, and Albert Zlotnik. 1993. A Developmental Pathway Involving Four Phenotypically and Functionally Distinct Subsets of CD3-CD4-CD8- Triple-Negative Adult Mouse Thymocytes Defined by CD44 and CD25 Expression. *J. Immunol* 150: 4244-52
4. Juliet Mahtani-Patching JFN, Dick John Pang, Kostadin V.Stoenchev, Ana M. Aguirre-Blanco, Bruno Silva-Santos and Daniel J. Pennington. 2011. PreTCR and TCR{gamma}{delta} Signal Initiation in Thymocyte Progenitors Does Not Require Domains Implicated in Receptor Oligomerization. *Science Signaling* 4
5. Bryan A. Irving FWA, Nigel Killeen. 1998. Thymocyte Development in the Absence of Pre-T Cell Receptor Extracellular Immunoglobulin Domains. *Science* 280: 905-8
6. Qing Yu J-HP, Loretta L. Doan, Batu Erman, Lionel Feigenbaum, and Alfred Singer. 2006. Cytokine signal transduction is suppressed in preselection double-positive thymocytes and restored by positive selection. *Journal of Experimental Medicine* 1: 165-75
7. Mark M.W Chong ALC, Rima Darwiche, Edouard G Stanley, Jared F Purton, Dale I Godfrey, Douglas J Hilton, Robyn Starr, Warren S Alexander,Thomas W.H Kay. 2003. Suppressor of Cytokine Signaling-1 Is a Critical Regulator of Interleukin-7-Dependent CD8+ T Cell Differentiation. *Immunity* 18: 475-87
8. Hirotsugu Kurobe CL, Tomoo Ueno, Fumi Saito, Izumi Ohigashi, Natalie Seach, Rieko Arakaki, Yoshio Hayashi, Tetsuya Kitagawa, Martin Lipp, Richard L. Boyd, Yousuke Takahama. 2006. CCR7-Dependent Cortex-to-Medulla Migration of Positively Selected Thymocytes Is Essential for Establishing Central Tolerance. *Immunity* 24: 165-77
9. Tomoo Ueno FS, Daniel H.D. Gray, Sachiyo Kuse, Kunio Hieshima, Hideki Nakano, Terutaka Kakiuchi, Martin Lipp, Richard L. Boyd, and Yousuke Takahama. 2004. CCR7 Signals Are Essential for Cortex–Medulla Migration of Developing Thymocytes. *J. Exp. Med.* 200: 493-505
10. Alfred Singer SA, Jung-Hyun Park. 2008. Lineage Fate and Intense Debate: myths, models, and mechanisms, of CD4-, versus CD8- lineage choice. *Nat. Rev. Immunol.* 8: 788-801
11. Mark S. Anderson, Emily S. Venanzi, Ludger Klein, Zhibin Chen, Stuart P. Berzins, Shannon J. Turley, Harald von Boehmer, Roderick Bronson, Andrée Dierich, Christophe Benoist, Mathis aD. 2002. Projection of an Immunological Self Shadow Within the Thymus by the Aire Protein *Science* 298

12. Mathis JVCBD. 2005. AIRE and APECED: molecular insights into an autoimmune disease. *Immunological Reviews* 204: 156-64
13. Troy A. Baldwin KAHaSCJ. 2004. The Fourth Way? Harnessing Aggressive Tendencies in the Thymus. *J. Immunol* 173: 6515-20
14. Mark A. Daniels ET, Jason Gill, Barbara Hausmann, Dominique Roubaty, Kaisa Holmberg, Guy Werlen, Georg A. Holla¨nder, Nicholas R. J. Gascoigne & Ed Palmer. 2006. Thymic selection threshold defined by compartmentalization of Ras/MAPK signalling. *Nature* 444: 724-9
15. S. Munir Alam PJT, Jay L. Wung, Wade Nasholds, Stella Redpath, Stephen C. Jameson & Nicholas R. J. Gascoigne. 1996. T-cell-receptor affinity and thymocyte positive selection. *Nature* 381: 616-20
16. S.Munir Alam GMD, Christina M. Lin, Tomasz Zal, Wade Nasholds, Stephen C. Jameson, Kristin A. Hogquist, Nicholas R.J. Gascoigne, Paul J. Travers. 1999. Qualitative and Quantitative Differences in T Cell Receptor Binding of Agonist and Antagonist Ligands. *Immunity* 10: 227-37
17. Sue J Sohn GMLaAW. 2008. Non-redundant function of the MEK5–ERK5 pathway in thymocyte apoptosis *EMBO J* 27: 1896 - 906
18. Mercedes Rincón AW, Derek D. Yang, Linda Weiss, Benoit Dérijard, Prash Jayaraj, Roger J. Davis, and Richard A. Flavell. 1998. The JNK Pathway Regulates the In Vivo Deletion of Immature CD4+CD8+ Thymocytes *J. Exp. Med.* 188: 1817-30
19. Takehiko Sugawara TM, Eisuke Nishida, Yousuke Takahama. 1998. Differential Roles of ERK and p38 MAP Kinase Pathways in Positive and Negative Selection of T Lymphocytes. *Immunity* 9: 565-74
20. Jose Alberola-Ila KAH, Kathryn A. Swan, Michael J. Bevan, and Roger M. Perlmutter. 1996. Positive and Negative Selection Invoke Distinct Signaling Pathways. *J. Exp. Med.* 184: 9-18
21. Jose Alberola-lla KAF, Rony Seger, Edwin G. Krebs & Roger M. Perlmutter. 1995. Selective requirement for MAP kinase activation in thymocyte differentiation. *Nature* 373: 620-3
22. Patrick S Costello RHN, Yasuyuki Watanabe, Ian Rosewell & Richard Treisman. 2004. Ternary complex factor SAP-1 is required for Erk-mediated thymocyte positive selection. *Nature Immunology* 5: 289-98
23. Andreas Strasser HP, Lorraine A O'Reilly and Philippe Bouillet. 2008. What do we know about the mechanisms of elimination of autoreactive T and B cells and what challenges remain. *Immunology and Cell Biology* 86: 57-66
24. Adam Mor MRP. 2006. Compartmentalized Ras/MAPK Signaling. *Annu. Rev. Immunol.* 24: 771–800
25. Chiu VK BT, Hach A, Sajous JB, Silletti J, Wiener H, Johnson RL, Cox AD, Philips MR. 2002. Ras signalling on the endoplasmic reticulum and the Golgi. *Nat. Cell Biol.*: 343-50
26. Takuma Hayashi DLF. 2003. Role of Defective Apoptosis in Type 1 Diabetes and Other Autoimmune Diseases *Recent Prog Horm Res* 58: 131-53
27. Vassilios Ioannidis FB, Hans Clevers & Werner Held. 2001. The beta-catenin−TCF-1 pathway ensures CD4+CD8+ thymocyte survival. *Nature Immunology* 2: 691-7
28. D A Grillot RM, G Núñez. 1995. Bcl-XL displays restricted distribution during T cell development and inhibits multiple forms of apoptosis but not clonal deletion in transgenic mice. . *J. Exp. Med.* 182: 1973-83
29. Korsmeyer DTCaSJ. 1998. BCL-2 FAMILY: Regulators of Cell Death. *Annu. Rev. Immunology* 16
30. Damian Kovalovsky YY, Marei Dose, Anastasia Emmanouilidou,Tassos Konstantinou,Kristine Germar, Katayoun Aghajani, Zhuyan Guo, Malay Mandal Fotini Gounari. 2009. Beta-Catenin/Tcf Determines the Outcome of Thymic Selection in Response to Alpha/Beta TCR Signaling. *Journal of Immunology* 183: 3873-84

31. Huimin Xie ZH, Maureen S. Sadim, and Zuoming Sun. 2005. Stabilized -Catenin Extends Thymocyte Survival by Up-Regulating Bcl-xL. *J. Immunol* 175: 7981-8

Chapter 1:

The role of ERK localization in thymic selection.

Summary:

The molecular events that allow a DP thymocyte to properly interpret the TCR signals involved in selection are unclear. It has been suggested that a pathway of critical importance in positive selection, ERK, is differentially localized depending on the selection signal received by the thymocyte. Here we directly test the role of ERK localization in selection by chemically altering the levels of intracellular calcium thereby affecting RasGRP localization, and by targeting Raf to different cellular compartments. We find evidence in favor of ERK localization determining the outcome of selection.

Introduction:

The process of thymic selection is crucial for the prevention of autoimmunity (1, 2). Selection is necessary due to the random production of TCRs most of which have no affinity for self MHC and the small percent that have an affinity for MHC that is too high. TCR affinity for self MHC determines the outcome of selection. This model of selection is often referred to as the "Goldilocks" model; only TCRs, with intermediate affinity will be positively selected. Strong interactions will induce apoptosis, and no interaction will lead to "death by neglect" while a weak to moderate affinity interaction will cause thymocytes to undergo positive selection. Yet the signaling events that mediate this process are not clear. Several signaling pathways have been demonstrated to be important for either positive or negative selection. Extra cellular regulated kinase 5 (ERK5), p38, c-Jun-NH_3 terminal kinase 1/2 (JNK 1/2) are important mediators of negative selection (3-5). The MAP kinase, ERK 1/2 (referred to here as ERK), is essential for the positive selection of thymocytes (4, 6). Interestingly, TCR engagement, regardless of affinity, activates all of these pathways. Thymocytes must interpret all of these signals to decide cell fate.

The small GTPase Ras, an upstream member of the ERK pathway, may become active in several cellular compartments including endosomes, the ER, and the Golgi (7). Ras activation within different compartments leads to distinct signaling outputs as well as different biological outcomes (7). Work by Chiu *et. al*. suggests that Ras activation at the Golgi stimulates JNK activity, while active Ras at the ER and plasma membrane induce PI_3K and ERK activation (8). More recent studies indicate that activation of Ras on endomembranes is dependent on the guanine exchange factor (GEF) RasGRP (9-11). In thymocytes, it has been shown that TCR affinity affects the levels of calcium and diacylglycerol (DAG), both secondary messengers important for the localization and activation of RasGRP(9). Ras activation on the plasma membrane is probably due to a combination of son-of-sevenloss (SOS) associated with GRB2 and RasGRP. One possibility is that differential localization of the ERK pathway via RasGRP and Ras accounts for thymocytes ability to distinguish positive and negative selecting signals.

Indeed, differential localization of ERK pathway members, RasGRP, Ras, Raf, and pERK (active ERK) has been observed in thymocytes. These molecules are exclusively localized to the plasma membrane upon stimulus with a negative selector. The same molecules exhibited a pattern consistent with Golgi staining when stimulated by a positive selecting peptide, with the exception of pERK, which appeared diffuse(12).

Given the data from Daniels *et. al*., it is tempting to postulate that activation of Ras/ERK on the plasma membrane sequesters ERK from the intracellular targets ERK must act on to induce positive selection and mediate cell survival. Consistent with this JNK, ERK and AKT compete for the regulation of members of the apoptotic pathway, specifically Bim. Bim is an important pro-apoptotic protein that is differentially phosphorylated by JNK and ERK. Phosphorylation by JNK leads to stabilization, whereas ERK activity leads to its degradation. The previous work done by Daniels *et. al.* only explores differences in signal localization in vitro (not a selecting environment) and at early time points (selection takes about three days).

To investigate if ERK localization does indeed determine the outcome of selection, we artificially altered ERK localization. We also manipulated cellular calcium levels to alter RasGRP localization and introduced a Raf construct into thymocytes in a selecting thymic environment to prime different cellular compartments for ERK signaling.

Results:

Calcium manipulation alters RasGRP and pERK localization.

To test if differential localization of ERK is the mechanism that thymocytes use to distinguish between a positive or negative selecting signal, we manipulated calcium levels in thymocytes. It is known that calcium level influences the localization and activation of RasGRP, the guanine exchange factor responsible for the activation of Ras an upstream member of the ERK pathway. Previous work suggests that RasGRP is the "fork" in ERK signaling, as it is the most upstream member of the ERK pathway to exhibit differences in localization during selection (12). In keeping with this idea, it is known that calcium levels differ based on the strength of TCR signal: a strong signal induces a large calcium flux into the cytoplasm from both the intracellular endoplasmic reticulum (ER) stores as well as extracellular calcium via CRAC channels; while a weak signal induces a small calcium flux predominately from the ER.

To test if calcium levels influence RasGRP localization and activation in thymocytes, we isolated OT-I DP thymocytes from a non-selecting background ($Rag^{-/-}$, $\beta_2m^{-/-}$, OT-I) and stimulated them with either OVA (negative selector) or Q4H7 (positive selector) tetramers *in vitro*.

As expected, stimulation of thymocytes with Q4H7 localized RasGRP to the Golgi, and active ERK exhibited a diffuse staining pattern upon Q4H7 stimulation. Cells stimulated with OVA localized both ERK and RasGRP to the plasma membrane (Fig 1A). Increasing of calcium levels with the CRAC channel activator thapsigargan (THAPS) led to the localization of RasGRP and pERK at the plasma membrane. Localization was independent of the stimulus (Fig 1B). Reducing the calcium flux by using

EGTA localized RasGRP to the Golgi and distributed pERK evenly throughout the cell, even with OVA stimulation (Fig 1C). Thus, THAPS and EGTA successfully compartmentalized RasGRP and ERK.

Figure 1

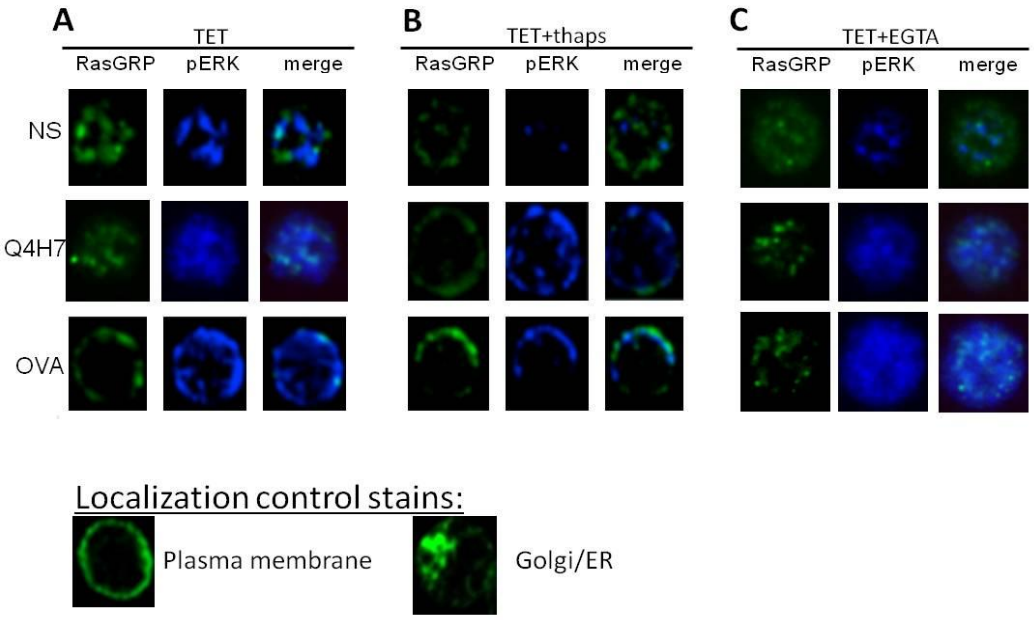

Figure 1: Altering calcium levels alters RasGRP/ERK localization. Thymocytes were isolated from a non-selecting thymus and stimulated by tetramers. A.) DP Thymocytes stimulated with Q4H7, OVA tetramer or left unstimulated (NS) were stained for localization of RasGRP and ERK. Confocal microscopy was performed to obtain the images. B.) Cells were treated as in (A) except stimulation was done in the presence of the CRAC channel activator thapsigargan. (20nM) C.) Thymocytes were treated as in (A) and (B) with the exception that the calcium chelator EGTA (4μM) was added to the media during stimulation..

Chemical manipulation of RasGRP localization alters Bim levels.

Since, altering calcium flux in thymocytes leads to differential localization of the ERK pathway (Fig 1), we wanted to examine if localization also has effects on selection. Signals that induce positive selection must also lead to cell survival. Negative selection signals lead to apoptosis. We know signaling pathways involved in selection compete for regulation of members of the Bcl-2 family, specifically, regulation of the pro-apoptotic molecule Bim. ERK phosphorylation of Bim leads to its degradation and

protection from apoptosis. Phosphorylation of Bim by JNK stabilizes Bim and can induce apoptosis. Bim, and other pro-apoptotic protein levels relative to the levels of pro-survival proteins determines cell survival. Other proteins are known to be important for survival. Bcl-XL a pro-survival protein is thought to be important for survival of $CD4^+$- $CD8^+$ double positive cells (DP) (13, 14). Another protein of interest is Bcl-2, a protein important for survival of cells post selection (15, 16).

Isolated DP thymocytes were stimulated with tetramers (OVA or Q4H7) in media alone or media containing either THAPS or EGTA. Protein levels of Bim, Bcl-2 and Bcl-XL were analyzed by flow cytometry. Consistent with the role of Bcl-2 late in selection, Bcl-2 levels were unchanged under all conditions (Fig 2A). The levels of Bcl-XL are important during the DP stage of T-cell development for survival. We would expect more Bcl-XL when cells are stimulated with Q4H7 than with OVA stimulation. However, under *in vitro* conditions, Bcl-XL levels remained unchanged (Fig 2B). If localization of ERK at the plasma membrane prevents the phosphorylation of ERK targets such as Bim, we would have expected to see increased levels of Bim when ERK was at the plasma membrane. As expected, ERK activity at the plasma membrane, using either OVA alone or Q4H7 in the presence of THAPS (by altering RasGRP localization), Bim levels were increased. When RasGRP activity was localized to the Golgi, Bim levels decreased (Fig 2C). Consistent with a role for the location of ERK in selection its localization correlates with differences in Bim levels.

Altering localization of Raf alters selection.

Because thymocytes cannot undergo selection *in vitro* we wanted to alter signaling in a selecting environment. In order to accomplish this, signaling in cells was manipulated in fetal thymic organ culture (FTOC), an *ex vivo* system. Use of this method allows for the introduction of gene constructs while the cells are in a selecting thymic environment.

Raf gene constructs were used to redirect ERK signaling. With the Raf-CTK construct Raf, is fused with the C-terminal portion of K-Ras, leading to plasma membrane localization and activation of ERK (Fig. 3A). Raf-181S is attached to a mutated version of the C-terminal portion of H-Ras (Fig 3A).

Figure 2

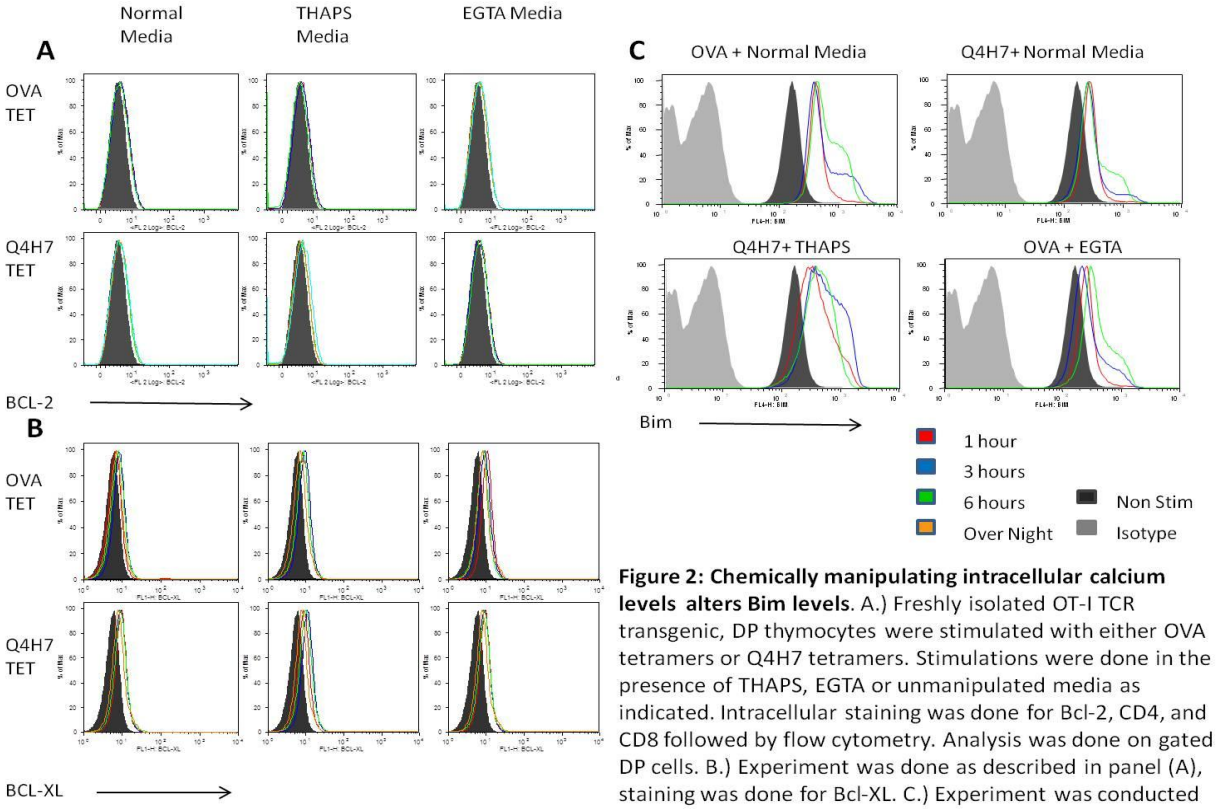

Figure 2: Chemically manipulating intracellular calcium levels alters Bim levels. A.) Freshly isolated OT-I TCR transgenic, DP thymocytes were stimulated with either OVA tetramers or Q4H7 tetramers. Stimulations were done in the presence of THAPS, EGTA or unmanipulated media as indicated. Intracellular staining was done for Bcl-2, CD4, and CD8 followed by flow cytometry. Analysis was done on gated DP cells. B.) Experiment was done as described in panel (A), staining was done for Bcl-XL. C.) Experiment was conducted as in (A) with the addition of an isotype control and staining for Bim .

Both constructs were fused with the enhanced green fluorescent protein (eGFP) gene. By localizing the Raf constructs to these cellular compartments, we are priming them for ERK signaling.

Thymi from β_2m -/-, Rag -/-, OT-I TCR transgenic mice were removed on day 15 of embryonic development and incubated for one day in the presence of a viral vector containing our Raf construct

(either Raf-CTK or Raf-181S) or media alone as a control. Thymocytes that populate the thymus at day 15 of development are in the double negative (DN) stages. Cells transitioning from the DN4 stage to the double positive (DP) stage undergo a brief proliferative burst thereby increasing the chances of infection with the retroviral vector. By embryonic day 16, DP cells have started to develop from the seeding population of DN cells. On day 16, β_2m and peptide (OVA or Q4H7) were added to reconstitute MHC allowing selection to proceed.

As expected, stimulation with OVA led to the accumulation of DN cells, consistent with negative selection. Stimulation with Q4H7 lead to positive selection as shown by the accumulation of CD8 SP cells (Fig.3A). When cells treated with the viral vector containing Raf-CTK were analyzed based on GFP expression, GFP negative cells developed normally (Fig. 3B). Cells that were positive for GFP and thus contained Raf-CTK had normal negative selection (Fig. 3B). However, positive selection was disrupted in these cells and they accumulated a significant DN population consistent with negative selection (Fig. 3B). When cells were treated with Raf-181S, again the GFP negative cells proceeded through selection normally (Fig, 3C). In infected cells, where Raf was targeted to the Golgi, the positive selector lead to the accumulation of CD8 SP cells indicative of positive selection (Fig. 3C). However, when the thymocytes were treated with a negative selector while Raf was targeted to the Golgi, conventional positive selection did not occur (Fig. 3C). Interestingly, a population of CD8αα cells, a regulatory T-cell population found in the gut, accumulated (Fig. 3C). Thus, the location of ERK pathway activation appears to determine the outcome of selection.

Figure 3

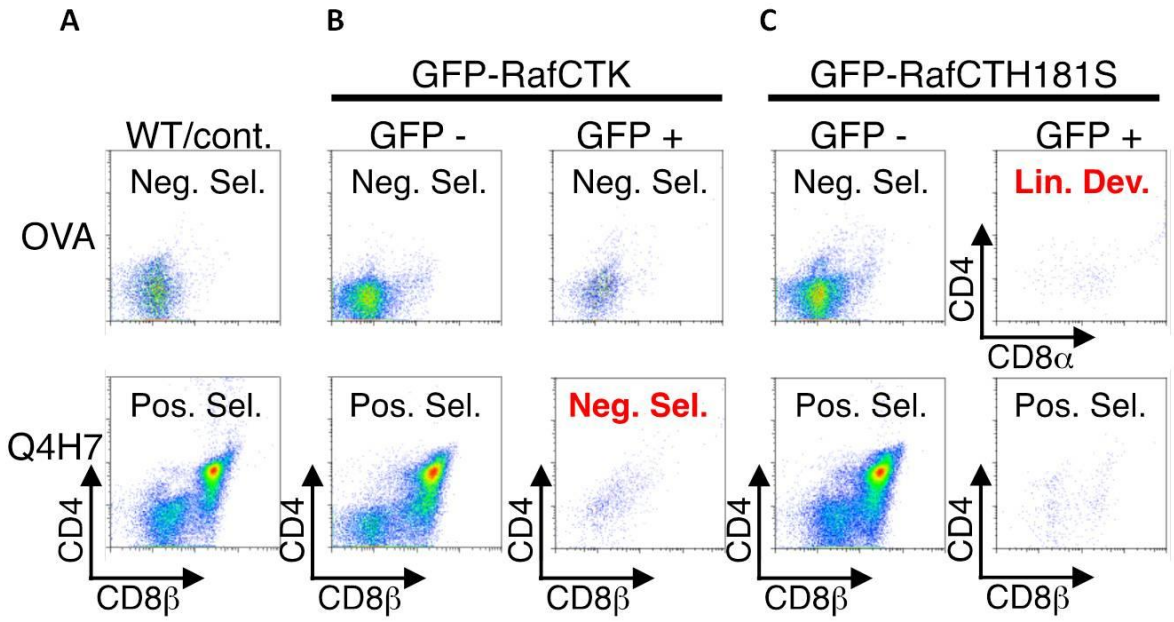

Figure 3: Altering Raf localization alters selection outcome. A.) FTOC cultures were allowed to progress for four days in the presence of OVA or Q4H7 and β2M. Thymocytes were isolated and stained for CD4, CD8β and CD8α. The cells were then analyzed by flow cytometry. B.) FTOC cultures were done in the presence of lentiviral vectors containing GFP tagged Raf-CTK constructs (added at E15, the day before stimulation was initiated) otherwise cultures were treated as in (A). Cells were analyzed based on GFP expression as indicated. C.) Cultures were treated as in (B) with viral vectors containing GFP tagged Raf-181S.

Discussion:

We were able to test the role of subcellular ERK localization in thymic selection by manipulating calcium levels and through manipulation of Raf localization. Both methods effectively localized the ERK pathway independently of the selection stimulus. Altering ERK localization via calcium altered the levels of the pro-apoptotic protein Bim. Manipulating ERK using a Raf construct altered thymic selection. Our data suggests that ERK localization determines the outcome of thymic selection.

From previous studies it is clear that variations in affinity of the TCR for pMHC, as well as interaction and duration, determine the outcome of selection (17-19). Because the pathways important for negative selection JNK, ERK5, and p38, as well as the pathway necessary for positive selection, ERK1/2,

are activated during TCR engagement, it is not clear how thymocytes integrate these signals (4, 5, 20, 21). It has been demonstrated that ERK activation kinetics differ based on selection signal. Negative selectors induce rapid and brief ERK activation, while positive selectors cause a slower sustained activation of ERK. Sustained activation of ERK is necessary for positive selection (6).This difference in ERK activation kinetic is at least partially due to the kinetics of co-receptor engagement (22) as well as negative regulators of Ras activity, which primarily reside on the plasma membrane(7).

How is ERK prevented from inducing survival during negative selection? The answer may lie in differential localization of ERK during selection. Work from our lab indicates that members of the ERK pathway, including pERK (active ERK), localize exclusively to the plasma membrane during negative selection. But, during positive selection the pathway localizes to what appears to be the Golgi, with pERK distributed diffusely throughout the cell (12). ERK signaling from different cellular compartments has been well established, especially with G-protein coupled receptors (GPCRs). Interestingly, Ras activation on endomembranes leads to a sustained ERK signal, while activation of Ras on the plasma membrane by both SOS-GRB2 and RasGRP leads to robust yet transient ERK activation(7). Activation of Ras in different compartments also results in different signal outputs and thus different biological outcomes (7, 8).These observations are consistent with known kinetics of ERK activation in thymocytes and our localization data (Fig 1). It is also of interest to note that Chiu *et. al.* observed JNK activation by Ras at the Golgi, while we have observed ERK pathway activation on this organelle. The discrepancy may lay in the cells used (COS-1 cells by Chiu,) in the method used to target Ras to the Golgi, or the Ras construct that was used.

It isn't clear if compartmentalized signaling is the mechanism thymocytes use to differentiate selection signals or if it is a side effect of negative versus positive selection. To test this, we demonstrated that by altering calcium levels we altered the location of RasGRP and thus where Ras was activated in the cell; we also change the levels of the pro-apoptotic molecule Bim (Fig. 2). Work by Barrett et. al. suggests PKC (possibly PKCα) activated by calcium and diacylglycerol (DAG) is critical for induction of

Bim and thymocyte apoptosis(23). This data, along with our localization data, strongly suggests that calcium plays a pivotal role in negative selection by increasing the expression of the pro-apoptotic molecule Bim, and by altering the cellular compartment upon which ERK is activated. Both of these events are necessary for the negative selection of thymocytes.

It was also of interest to determine what effect early localization of ERK had on later events, i.e. selection outcome. Previous work on ERK localization only looked at the first signaling events (12). Our data was consistent with a role of ERK localization affecting selection outcome (Fig. 3). Alteration, of Raf location to the plasma membrane induced apoptosis (Fig. 3B), while Raf located at the Golgi prevented apoptosis but did not lead to conventional positive selection (Fig. 3C). Intriguingly, Raf localized to the Golgi lead to the accumulation of CD8αα$^+$ cells, a regulatory T-cell which resides in the gut, when the cells were stimulated with the negative selector OVA. One, of the caveats of our experiment is that endogenous Ras and Raf may become active elsewhere in the cell. It is therefore possible that what led to an accumulation of CD8αα$^+$ thymocytes was signaling from both the Golgi by the Raf construct and signaling by endogenous Ras and Raf at the plasma membrane. Selection of CD8αα intraepithelial lymphocytes (IELs) has been the subject of intense debate. Previous work on IELs has suggested that they do not pass through a DP stage, and whether or not the thymus is the location of selection has been under debate (24). CD8αα$^+$ IELs can have variations in TCR expression; they may be TCR αβ$^+$ or γδ$^+$. These cells likely arise at different developmental stages of the host, with γδ$^+$ cells developing in the fetal stage and αβ$^+$ cells from the neonatal stage onward. Variations in the phenotype of these cells are at least partially responsible for the difficulty in defining the selection requirements for them. Work by several groups on athymic mice has concluded that selection of CD8αα IELs occurs in the crypto patches (CP) of the gut wall as CD8αα cells are still found in the gut of these mice. TCRγδ$^+$ CD8αα$^+$ cells were at equal numbers when compared to normal mice, but TCRαβ$^+$ CD8αα$^+$ cells were reduced (25-28). Other groups have concluded, by thymic grafting into athymic mice or through TCR transgenic models that the thymus is required for at least the initial development of CD8αα$^+$ cells (29-31).

Interestingly, many of the IELs in the gut in the TCR transgenic model were DN cells, which suggests these cells do not pass through a DP stage (24). These studies have been cited as data in favor of CD8αα IELs initially developing in the thymus, but leaving at the DN stage and finishing development in the CP. Our work would suggest that these cells develop in the thymus rather than extra-thymically (Fig. 3). The CD8αα cells in our system likely passed through a DP stage, as they were positive for GFP and thus virally infected. Thymocytes are normally very resistant to viral infection but are more receptive if they are undergoing proliferation. Cells going from the DN3 stage to the DP stage undergo a proliferative burst which would suggest that our cells were likely DP. It is also possible that the fetal stage is the developmental stage where CD8αα IELs are produced. At later stages in life, these IELs may develop differently. More work will be needed to determine the role of ERK localization in CD8αα selection.

Our work certainly doesn't rule out the possibility of a negative selecting signal emanating from the ERK pathway active on the plasma membrane during negative selection. As discussed above, endogenous Ras and Raf are not under the control of our constructs they are free to signal from other compartments in the cell. It is interesting to note that thymocytes with Raf construct targeted to the plasma membrane underwent apoptosis efficiently, which may be evidence of a dominant, negative signal from the plasma membrane. However, it may also be due to the Raf construct interacting with most of the endogenous MEK and ERK and preventing a strong enough signal from the Golgi. More work is needed to determine the actual mechanism ERK localizations plays in selection.

Materials and Methods:

Mice and Cells

OT-I TCR transgenic, Rag $^{-/-}$, $\beta_2M^{-/-}$ mice were generated in our lab and maintained under pathogen free conditions. Thymi from six-week old to 11 month old mice were removed and smashed between two

nylon filters (Sefar Nitex). Cells were then washed off of the filters with RPMI media containing 10% FCS. Thymocytes were then resuspended in 2ml of the RPMI media per thymus. Most of the thymic epithelial cells remain trapped in the nylon filter and since the mice are non-selecting at least 85% of the cells are DP thymocytes, the rest are DN and either not expressing the receptor or are dying. No further purification was done.

Thymocyte Stimulation

Stimulation of thymocytes *in vitro* was done using synthetic tetramers made in the lab. Two million cells were stimulated in 100µL of RPMI (Gibco) media containing 10% FCS on ice, a 1:100 dilution tetramer was used for stimulation. After the tetramer was added the cells were incubated at 37°C. The thymocytes were then centrifuged at full speed (13,300 RPM) for 10 seconds and the supernatant aspirated.

Fetal Thymic Organ Culture

FTOC was done as described(32). Six well culture dishes containing RPMI (Gibco) with 10% FCS, a square of sterile surgical foam (Ferrosan) and a nitrocellulose filter (Millipore) was used to hold the thymi. Stimulation was done using (25µM) β_2M (Invitrogen) and 20µM OVA (SIINFEKL) or 400nM Q4H7 (SIIQFEHL), peptides were made in house. Viral vectors were courtesy of Dr. Marc Johnson (University of Missouri-Columbia). Vectors were added at a ratio of 5:1 with the thymocytes. Media containing peptide and vectors from each well were used to "baste" or cover the thymi of that well at least twice per day. Thymocytes were isolated by smashing between two nylon filters as described above.

Flow Cytometry and Antibodies

Isolated thymocytes were stained for CD4 (BD), CD8α (BD) and CD8β (BD) for 30 minutes on ice. The cells were then washed once with FACS buffer containing 1%FCS. The samples were then fixed for 10 min on ice with 1% Formaldehyde to destroy any excess vector. Cells were than run on a FACScalibur and the data analyzed by FlowJo .

References:

1. Takuma Hayashi DLF. 2003. Role of Defective Apoptosis in Type 1 Diabetes and Other Autoimmune Diseases *Recent Prog Horm Res* 58: 131-53
2. Andreas Strasser HP, Lorraine A O'Reilly and Philippe Bouillet. 2008. What do we know about the mechanisms of elimination of autoreactive T and B cells and what challenges remain. *Immunology and Cell Biology* 86: 57-66
3. Mercedes Rincón AW, Derek D. Yang, Linda Weiss, Benoit Dérijard, Prash Jayaraj, Roger J. Davis, and Richard A. Flavell. 1998. The JNK Pathway Regulates the In Vivo Deletion of Immature CD4+CD8+ Thymocytes *J. Exp. Med.* 188: 1817-30
4. Takehiko Sugawara TM, Eisuke Nishida, Yousuke Takahama. 1998. Differential Roles of ERK and p38 MAP Kinase Pathways in Positive and Negative Selection of T Lymphocytes. *Immunity* 9: 565-74
5. Sue J Sohn GMLaAW. 2008. Non-redundant function of the MEK5–ERK5 pathway in thymocyte apoptosis *EMBO J* 27: 1896 - 906
6. Lisa K. McNeil TKS, Kristin A. Hogquist. 2005. A requirement for sustained ERK signaling during thymocyte positive selection in vivo. *PNAS* 102: 13574-9
7. Adam Mor MRP. 2006. Compartmentalized Ras/MAPK Signaling. *Annu. Rev. Immunol.* 24: 771–800
8. Chiu VK BT, Hach A, Sajous JB, Silletti J, Wiener H, Johnson RL, Cox AD, Philips MR. 2002. Ras signalling on the endoplasmic reticulum and the Golgi. *Nat. Cell Biol.*: 343-50
9. Carrasco S MI. 2004. Diacylglycerol-dependent binding recruits PKCtheta and RasGRP1 C1 domains to specific subcellular localizations in living T lymphocytes. *Mol. Biol. Cell*: 2932–42
10. P´erez de Castro I BT, Philips M, Pellicer A. 2004. Ras activation in Jurkat T cells following low-grade stimulation of the T-cell receptor is specific to N-Ras and occurs only on the Golgi. *Mol. Cell. Biol.*: 3485–96
11. Caloca MJ ZJ, Bustelo XR. 2003. Exchange factors of the RasGRP family mediate Ras activation in the Golgi. *J. Biol. Chem.*: 33465–73
12. Mark A. Daniels ET, Jason Gill, Barbara Hausmann, Dominique Roubaty, Kaisa Holmberg, Guy Werlen, Georg A. Holla¨nder, Nicholas R. J. Gascoigne & Ed Palmer. 2006. Thymic selection threshold defined by compartmentalization of Ras/MAPK signalling. *Nature* 444: 724-9
13. D A Grillot RM, G Núñez. 1995. Bcl-XL displays restricted distribution during T cell development and inhibits multiple forms of apoptosis but not clonal deletion in transgenic mice. . *J. Exp. Med.* 182: 1973-83
14. A Ma JCP, B Chang, E Margosian, L Davidson, F W Alt, C B Thompson. 1995. Bclx regulates the survival of double-positive thymocytes. *PNAS* 92: 4763-7
15. J Gratiot-Deans LD, LA Turka, G Nunez. 1993. bcl-2 proto-oncogene expression during human T cell development. Evidence for biphasic regulation. *J. Immunol* 151: 83-91
16. DJ Veis CS, EA Bach, SJ Korsmeyer. 1993. Expression of the Bcl-2 protein in murine and human thymocytes and in peripheral T lymphocytes. *J. Immunol* 151: 2546-54
17. McKeithan TW. 1995. Kinetic proofreading in T-cell receptor signal transduction. *PNAS* 92: 5042-6
18. S. Munir Alam PJT, Jay L. Wung, Wade Nasholds, Stella Redpath, Stephen C. Jameson & Nicholas R. J. Gascoigne. 1996. T-cell-receptor affinity and thymocyte positive selection. *Nature* 381: 616-20

19. Calvin B. Williams DLE, Gilbert J. Kersh, J. Michael White, Paul M. Allen. 1999. A Kinetic Threshold between Negative and Positive Selection Based on the Longevity of the T Cell Receptor–Ligand Complex. *J. Exp. Med.* 189: 1531-44
20. Jose Alberola-lla KAF, Rony Seger, Edwin G. Krebs & Roger M. Perlmutter. 1995. Selective requirement for MAP kinase activation in thymocyte differentiation. *Nature* 373: 620-3
21. Jose Alberola-Ila KAH, Kathryn A. Swan, Michael J. Bevan, and Roger M. Perlmutter. 1996. Positive and Negative Selection Invoke Distinct Signaling Pathways. *J. Exp. Med.* 184: 9-18
22. Pia P. Yachi JA, Tomasz Zal, Nicholas R.J. Gascoigne. 2006. Altered Peptide Ligands Induce Delayed CD8-T Cell Receptor Interaction—a Role for CD8 in Distinguishing Antigen Quality. *Immunity* 25: 203-11
23. Kirsten Canté-Barrett EMG, Monte M. Winslow, Gerald R. Crabtree. 2006. Thymocyte Negative Selection Is Mediated by Protein Kinase C- and Ca2+-Dependent Transcriptional Induction of Bim. *J. Immunol* 176: 2299-306
24. Vassalli DG-GaP. 2002. Gut intraepithelial lymphocyte development. *Current Opinion in Immunology* 14: 255-9
25. B Rocha PV, and D Guy-Grand. 1994. Thymic and extrathymic origins of gut intraepithelial lymphocyte populations in mice. *Journal of Experimental Medicine* 180: 681-6
26. S R Guehler JAB, and T A Barrett. 1996. Immune deviation of 2C transgenic intraepithelial lymphocytes in antigen-bearing hosts. . *Journal of Experimental Medicine* 184: 493-503
27. Daniel Cruz BCS, Kristine Hetzel, Gian Yakoub, Mitchell Kronenberg, and Hilde Cheroutre. 1998. An Opposite Pattern of Selection of a Single T Cell Antigen Receptor in the Thymus and among Intraepithelial Lymphocytes *Journal of Experimental Medicine* 188: 255-65
28. Christiaan N. Levelt YPdJ, Emiko Mizoguchi, Cliona O'Farrelly, Atul K. Bhan, Susumu Tonegawa, Cox Terhorst, and Stephen J. Simpson. 1999. High- and low-affinity single-peptide/MHC ligands have distinct effects on the development of mucosal CD8αα and CD8αβ T lymphocytes. *PNAS* 96: 5628-33
29. Tesu Lin GM, Hiroyuki Kenai, Takao Nakamura and Kikuo Nomoto. 1993. Thymus influences the development of extrathymically derived intestinal intraepithelial lymphocytes. *European Journal of Immunology* 23: 1968-74
30. Olson LLaS. 1994. A novel pathway of thymus-directed T lymphocyte maturation. *Journal of Immunology* 153: 987-95
31. Delphine Guy-Grand NP, Sylvie Darche, Olivier Lantz, Philippe Kourilsky and Pierre Vassalli. 2001. Contribution of double-negative thymic precursors to CD8α α + intraepithelial lymphocytes of the gut in mice bearing TCR transgenes *European Journal of Immunology* 31: 2593–602
32. Hogquist KA. 2001. Assays of Thymic Selection. *Methods in Molecular Biology* 156: 219-32

Chapter 2:

The Regulation of Bcl-XL and β-catenin in early thymic selection.

Summary:

The pro-survival molecule Bcl-XL has been shown to play an essential role in the survival of DP thymocytes. However, the role of Bcl-XL during the selection process is unclear. We looked at the regulation by the known Bcl-XL regulator, β-catenin, and determined that Bcl-XL functions initially to protect positively signaled DP cells from apoptosis, but is later down regulated (along with the pro-apoptotic molecule Bim). For negative selection, we saw reduced Bcl-XL expression, while Bim levels were initially higher than that seen during the course of positive selection. Interestingly, we also found evidence during negative selection that suggests β-catenin may prevent further differentiation.

Introduction:

Thymic selection is critical for the development of a healthy immune system. Roughly 95% of thymocytes will die during development, due to unsuccessful rearrangement of TCR chains, lack of affinity for self MHC or high affinity for MHC. Only a small percentage of thymocytes will display the proper affinity for self-pMHC. Apoptosis is the major mechanism for the disposal of non-reactive or auto-reactive TCR-bearing thymocytes. Thus, the regulation of apoptosis is critical for the production of a healthy T-cell repertoire. Indeed, defects in apoptosis have been shown to at least partially account for the phenotype of non-obese diabetic mice (NOD), as well as other auto immune disease models in mice (1, 2).

The Bcl-2 family of proteins regulates apoptosis. This family can be divided into two groups: the pro-survival members, and the pro-apoptotic members. Pro-survival members of the Bcl-2 family include Bcl-XL, Mcl-1, Bcl-2, A1 and Bcl-W. These proteins contain all four of the Bcl-2-Homolgy-Domains (BH1-4), with the exception Mcl-1, which contains only BH1-3. They prevent apoptosis by binding

through BH3 domains to the proteins BAX and BAK, sequestering and preventing them from oligomerizing at the outer mitochondrial membrane, an event that initiates apoptosis. Pro-apoptotic molecules contain only the BH3 domain (they are also called BH3-only proteins); these proteins are able to displace BAX and BAK from the pro-survival molecules via BH3 interaction. Free BAX and BAK are able to initiate apoptosis by mitochondrial outer membrane permeabilization (MOMP) and the subsequent release of cytochrome-c. It is the balance between pro-survival and pro-apoptotic proteins that determines if the cell will undergo apoptosis(3).

Several of the pro-survival proteins are known to be important for the survival of thymocytes at different stages of development. Bcl-2 for example, is the predominant pro-survival molecule in DN and SP thymocytes. Bcl-XL is critical for survival during the DP stage(4-7). The role of Bcl-XL at the DP stage makes it particularly relevant to early selection events. Yet, how Bcl-XL is regulated during this process is not clear. Work by Huimin and colleagues correlated the levels of β-catenin with levels of Bcl-XL(8). Consistent with signaling pathways known to be important for thymocyte positive selection and survival, pathways that positively regulate β-catenin are ERK, PI_3K and WNT. Of particular interest are ERK and PI_3K, which are both downstream of TCR signaling. ERK is necessary for positive selection and survival of these cells(9, 10). PI_3K has been shown to positively regulate the positive selection of thymocytes possibly through the regulation of Itk and calcium flux(11, 12). WNT signaling in thymocytes should be a constant and should not differ between positive and negative selection.

We tested the role of PI_3K and ERK signaling in the regulation of Bcl-XL during thymic selection and showed that signaling through ERK had no effect on the levels of either β-catenin or Bcl-XL. However, blocking AKT activity lowered the overall levels of Bcl-XL and β-catenin. Effects of the AKT inhibitor were not dependent on TCR signaling as there was no difference observed with a positive selecting or negative selecting signal, indicating AKT regulates the steady state levels of these molecules. These experiments also elucidate the kinetics of β-catenin during selection at the DP stage.

Results:

Differential signaling and β-catenin level during *in vitro* stimulation.

To test the role of signaling in Bcl-XL regulation, $\beta_2m^{-/-}$, $Rag^{-/-}$ DP thymocytes transgenic for the MHC K^b-restricted OT-I TCR, were stimulated *in vitro* with OVA (negative selector) or Q4H7 (positive selector) tetramers. If the regulation of β-catenin is important for thymocyte survival during selection, then the activating phosphorylation of AKT at threonine 308 and the inactivating phosphorylation of GSK3β at serine 9 should be increased during positive selection in contrast to negative selection (Fig.1). This differential phosphorylation should in turn lead to an increase in β-catenin and Bcl-XL with a positive selector (Fig.1).

Figure 1

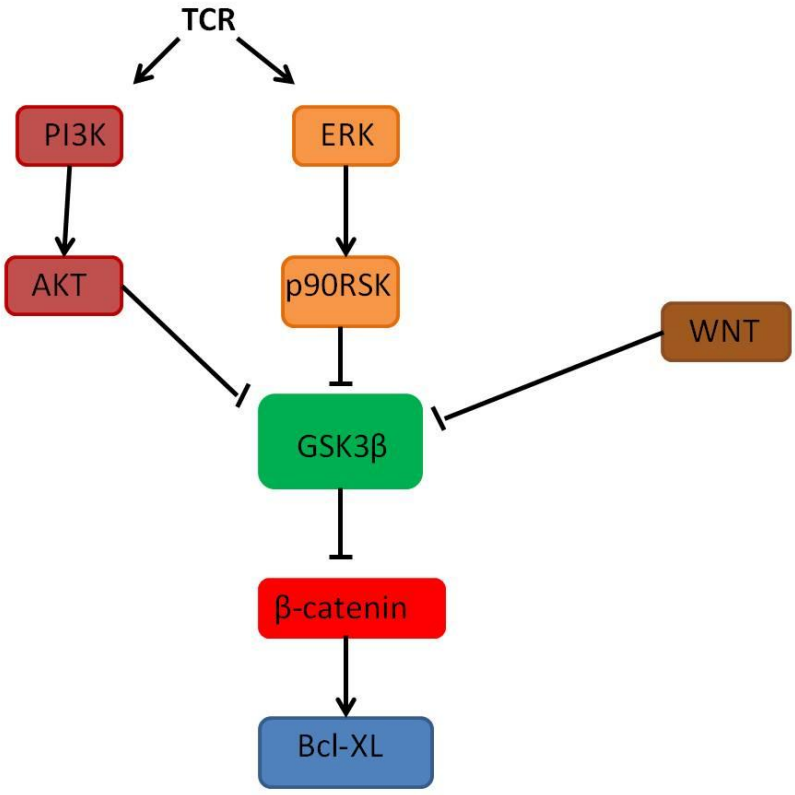

Figure1: Known signaling pathways involved in the stabilization of β-catenin. Three pathways converge on the constituitively active kinase GSK3β. PI3K phosphorylation of AKT allows AKT to phosphorylate and inactivate GSK3β. ERK phosphorylation of p90RSK allows p90RSK to inactivate GSK3β. WNT signaling also leads to inactivation of GSK3β. Signaling by these pathways leads to stable β-catenin. Of particular interest are the PI3K and ERK pathways due to activation via the TCR.

Levels of AKT and GSK3β phosphorylation were measured by Western blot. β-catenin and Bcl-XL protein levels were also measured by Western blot. Protein and phosphorylation levels were normalized to unstimulated samples. Stimulation by both OVA and Q4H7 tetramers led to a decrease in total levels of AKT and GSK3β phosphorylation. However, loss of phosphorylation was less pronounced with Q4H7 stimulation (Fig. 2A and 2B). These data are consistent with the role of these molecules in stabilizing β-catenin and maintaining thymocyte survival during positive selection. Indeed, β-catenin and Bcl-XL levels were increased with the positive selector over the negative selector (Fig. 2C and 2D). Although Bcl-XL levels were not significantly different with OVA or Q4H7 stimulation, this was most likely due to the short time frame for these experiments, as *in vitro* stimulation for longer than six hours results in apoptosis of thymocytes regardless of the strength of stimulus.

Figure 2

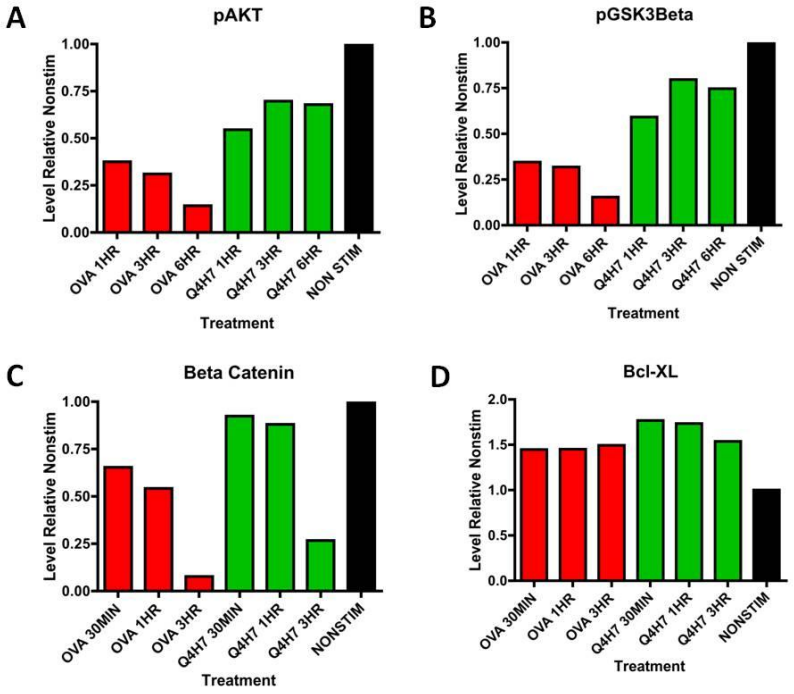

Figure 2: In vitro levels of β-catenin and upstream signaling. A.) DP thymocytes were stimulated with either OVA or Q4H7 tetramers for up to six hours. Protein levels were measured by western blot. Levels were set relative to an unstimulated control (Nonstim). B.) Western blot for inactive phosphorylated GSK3β, stimulation performed as in (A). Level is relative to the unstimulated control C.) and D.) Western blots for β-catenin and Bcl-XL protein levels, relative to unstimulated cells. Stimulations performed as in (A).

Differential signaling and Bcl-XL levels in selecting thymi.

Because the *in vitro* environment is not a selecting environment and due to the short lifespan of thymocytes in media alone, we needed to examine signaling in a more physiologically relevant setting. To do this, we utilized the FTOC system which allowed us to measure signaling and Bcl-XL levels in the context of the fetal thymus. FTOC experiments were set up as described in (13). Briefly, embryonic day 15 (E15) fetuses were removed from β2m $^{-/-}$, Rag$^{-/-}$, OT-I TCR transgenic mice. Thymi from these mice were removed and placed in FTOC culture for one day. This allowed the development of DP cells, as most of the cells at day 15 are DN. The following day fetal thymi were treated with either positive selecting Q4H7 peptide or negatively selecting OVA peptide as well as β_2m to reconstitute class I MHC.

Selection was allowed to proceed for one, two or three days; the thymi were then harvested, and protein levels were measured by Western blot.

At 24 hours, the phosphorylation level of AKT was increased in the presence of Q4H7 as was the phosphorylation of GSK3β (Fig. 3A). This suggests that very early signals involved in positive selection favor the inhibition of GSK3β by AKT. In agreement with these data, the levels of both Bcl-XL and β-catenin were alsoincreased with the positive selector over the negative selector at 24 hours (Fig. 3A). This data is consistent with β-catenin being stabilized early in positive selection to prevent apoptosis by maintaining Bcl-XL. Loss of β-catenin corresponded with a decrease in the level of Bcl-XL. Stimulation of thymocytes with OVA also led to an increase in Bim expression. Taken together, early negative selection signaling appears to lead to a loss of Bcl-XL and β-catenin, and a concomitant increase in Bim, thus making the cells susceptible to apoptosis. Indeed even OVA stimulation after only 24 hours led to an increase in caspase 3 cleavage when compared to Q4H7 stimulation (data not shown). Interestingly, at later time points, 48 or 72 hours, thymocytes displayed the opposite pattern of GSK3β phosphorylation and β-catenin levels with OVA stimulation eliciting more phosphorylation and higher β-catenin levels (Fig. 3B and 3C). However, Bcl-XL levels remained similar to the 24 hour time point with lower levels under negative selecting conditions. Bim levels at 48 hours were similar between positive and negative selection. However, at 72 hours the levels of Bim decreased under negative selecting conditions (Fig. 3B and 3C). Caspase 3 cleavage remained higher in the presence of OVA at 48 hours, but by 72 hours it had decreased to levels below the positive selector (Data not shown).

Figure 3

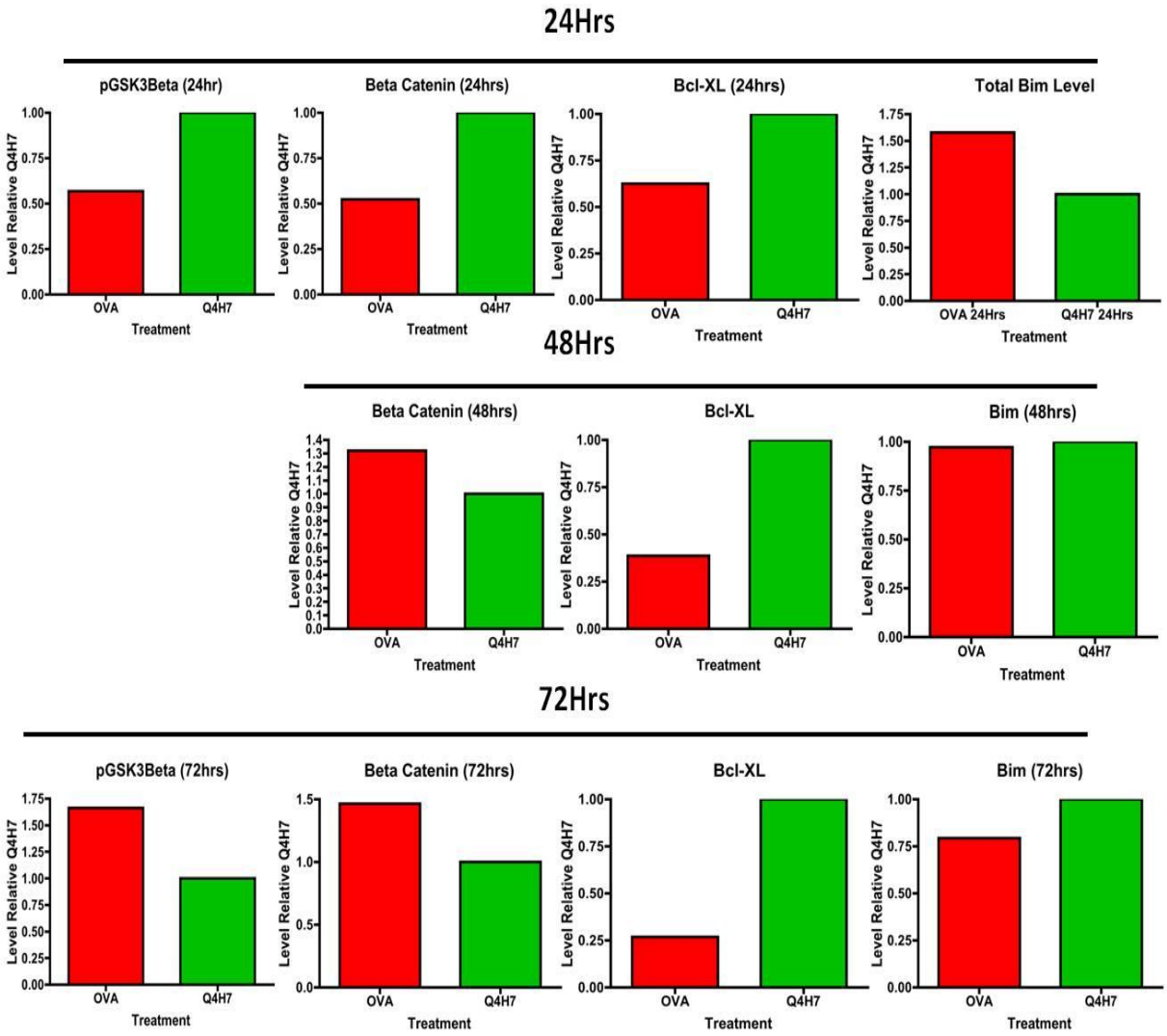

Figure 3: Kinetics of β-catenin and Bcl-XL during early thymic selection. A, B and C FTOCs were stimulated for 24 hours, following stimulation thymocytes were isolated and whole cell lysates prepared. Western blots were performed to determine protein and protein phosphorylation levels. Results are depicted relative to the positive selector Q4H7. Data is representative of three experiments.

Inhibition of AKT but not ERK alters β-catenin/Bcl-XL levels *in vitro*.

To determine the signaling pathways important for β-catenin and Bcl-XL regulation during selection, we stimulated DP thymocytes with tetramers in the presence of either an ERK inhibitor or an

AKT inhibitor. Both the AKT and ERK pathways are of interest because they are downstream of the TCR and both pathways have been shown to regulate β-catenin levels. Inhibition of the ERK pathway with the MEK1/2 inhibitor UO129 did not alter the levels of pGSK3β, β-catenin or Bcl-XL from samples that were not treated with the inhibitor (Fig.4A and 4B). Thus, ERK signaling does not appear to be the dominant signal in the early regulation of β-catenin.

Figure 4

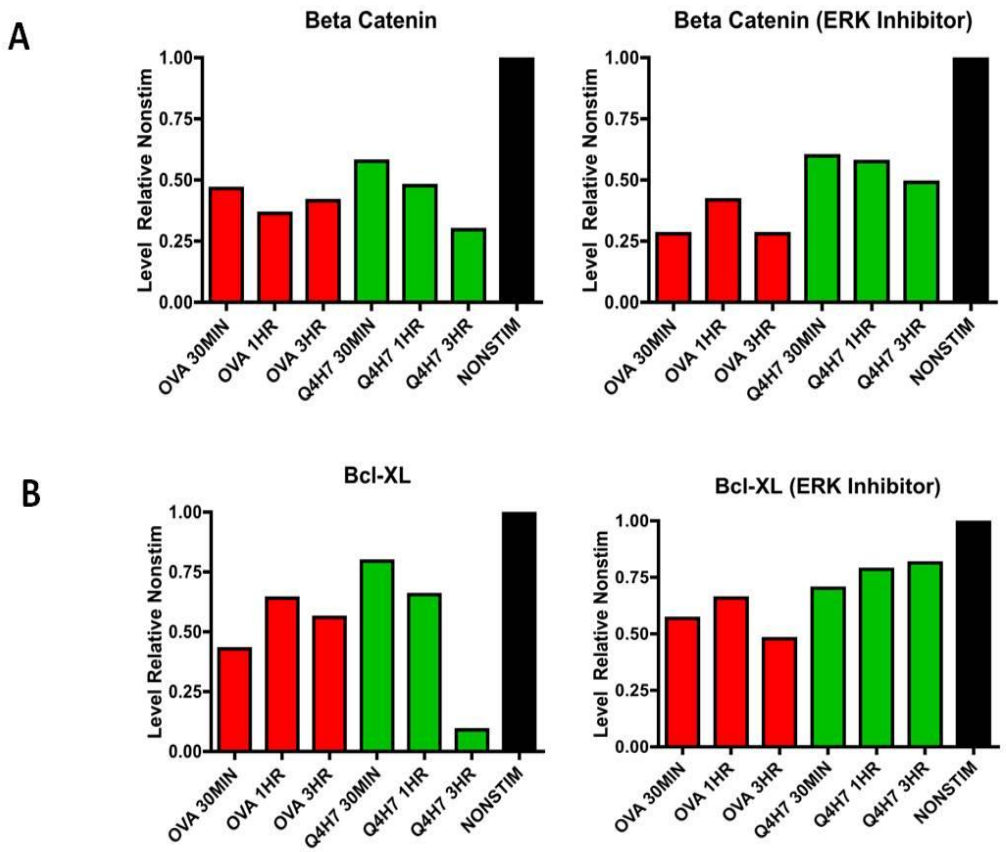

Figure 4: ERK inhibition has no effect on the levels of β-catenin or Bcl-XL. (A) and (B) DP thymocytes were stimulated *in vitro* with OVA or Q4H7 tetramers with or without the ERK inhibitor U0129 (10μM) for the indicated time. Whole cell lysates were prepared and analyzed by Western blot.

AKT has been shown to play multiple roles in the development of thymocytes, including survival, differentiation, and proliferation. The role of AKT in survival has been linked to its ability to upregulate Bcl-XL through phosphorylation and subsequent inhibition of GSK3β activity, thus preventing β-catenin degradation. ((14) (Figure 1)). However, the role of AKT in the regulation of Bcl-XL during selection of DP thymocytes is not clear. To examine this, we stimulated DP thymocytes in the presence of an AKT inhibitor and measured the level of GSK3β phosphorylation as well as the protein levels of β-catenin and Bcl-XL. Inhibition of AKT did not alter the levels of GSK3β phosphorylation or the protein levels of β-catenin and Bcl-XL between positive and negative selecting signals (Fig. 5A and 5B), indicating that the regulation of these proteins is not predominantly AKT dependant. Interestingly, there were differences in the steady state levels of these proteins and their phosphorylation (Compare Fig.5A and 5B). Taken together these data suggest that AKT is important for the maintenance of steady state levels of Bcl-XL and β-catenin but not changes during selection.

Figure 5

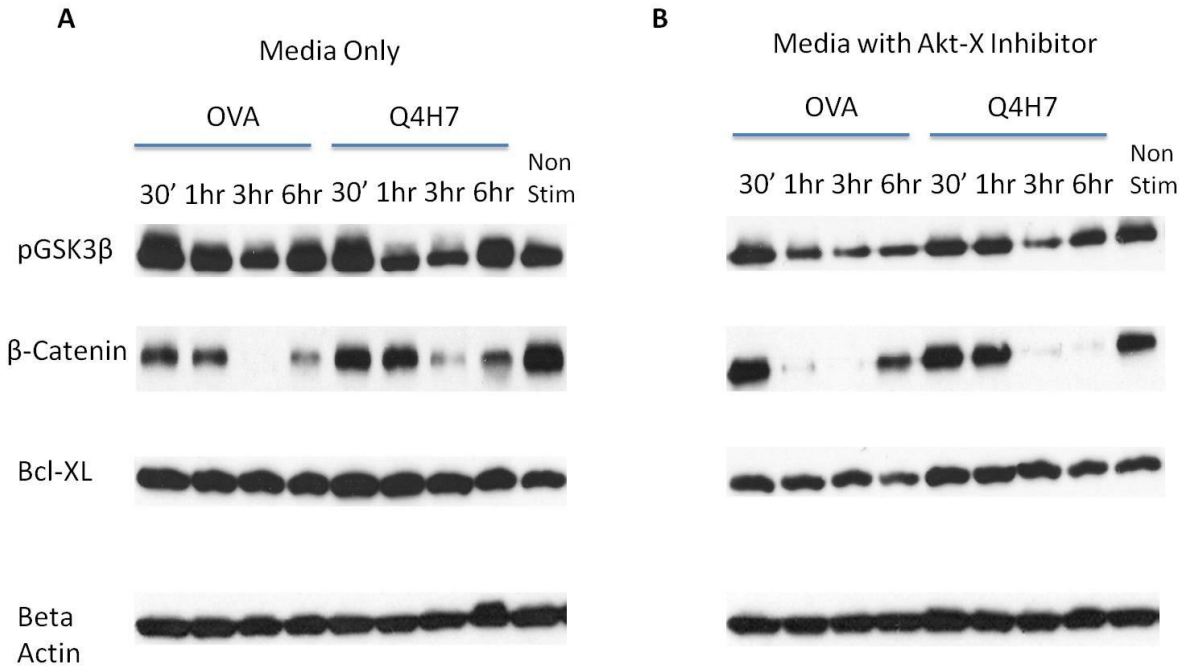

Figure 5: AKT mediates the steady state levels of β-catenin. A.) DP thymocytes were stimulated in vitro for the indicated time. Whole cell lysates were prepared and analyzed by western blot. Beta actin was used as the loading control. B.) DP cells were stimulated as in (A) in the presence of the AKT inhibitor AKT-X (5μM). Western blotting was performed to determine protein and phosphorylation levels. Beta actin was used as a loading control.

Discussion:

Through the use of the OT-I TCR transgenic system, FTOC and inhibitors of ERK and AKT, we were able to demonstrate the role of AKT but not ERK in maintaining the steady state levels of β-catenin and Bcl-XL. We were also able to demonstrate the kinetic of β-catenin through early selection. Our data coupled with data from other groups suggest a pro-survival role for β-catenin very early in selection, but a role later in negative selection characterized by the prevention of differentiation in negatively signaled DP thymocytes(15, 16).

β-catenin has been linked to the survival of thymocytes in the DN and DP stages and in naive peripheral T-cells through TCF-1 and its role in Bcl-XL expression(7, 17, 18). However, the role of β-catenin during DP selection has not been resolved. Several groups have concluded that β-catenin is pro-apoptotic. Gounari *et. al.* 2001 used mice expressing a β-catenin mutant lacking exon 3, under the control of the proximal *lck* promoter this mutant is not phosphorylated and therefore not degraded. Cells expressing the mutant β-catenin were able to pass through the DN3 stage without rearranging a TCR. They also observed an increase in apoptosis that was likely due to the lack of TCR expression in these cells. Another group, Kovalovsky *et. al.* 2009 attempted to circumvent problems with stabilized β-catenin and β-selection by expressing mutant β-catenin under the control of the CD4 promoter. In this study, increased β-catenin was still associated with a decrease in TCR expression, and again likely accounted for most of the apoptosis observed. In addition, an unusual stimulation method makes the results from this study difficult to interpret. Other studies have concluded that β-catenin plays a pro-survival role in DP thymocytes by upregulating Bcl-XL. Huimin *et. al.* 2005 expressed a stabilized β-catenin under the CD4 promoter. Stabilization of β-catenin led to increased Bcl-XL expression and resistance to apoptosis. Another group used various domain knockouts of TCF-1 to demonstrate the requirement of TCF-1, β-catenin interaction in DP thymocyte survival (7).

Because the PI_3K/AKT and ERK pathways have been linked to β-catenin stabilization and because they are downstream of the TCR, they are attractive candidates for the regulation of β-catenin during selection in the DP stage where the TCR mediates survival. Apoptosis is the primary means of negative selection. Therefore, the ability of β-catenin to upregulate Bcl-XL would suggest β-catenin is a crucial regulator of apoptosis during the selection process. Since the role of β-catenin in DP selection is not clear, we tested the ability of the ERK and AKT pathways to alter β-catenin and Bcl-XL during selection. Our data is consistent with a role for AKT in maintaining cell survival via β-catenin and Bcl-XL in pre-selection DP thymocytes, but not later in selection. Interestingly, β-catenin appears to mediate survival through Bcl-XL expression early in selection. OVA stimulated cells lose both β-catenin and Bcl-

XL after 24 hours, whereas expression of these molecules with the positive selector Q4H7 is not affected, when compared with a null peptide (data not shown and Fig. 3). However, by days two and three β-catenin levels are much higher under negative selecting conditions, yet Bcl-XL remains low.

These observations suggest two interesting possibilities. Given the ability of artificially stabilized β-catenin to allow cells into the DP stage without first passing through the β-selection checkpoint, it is possible that the levels of β-catenin helps determine the developmental stage of thymocytes (15, 16). Here low levels of β-catenin are associated with survival and proliferation in the DN stage. Upon successful signaling through the pre-TCR, β-catenin levels increase and facilitate the transition to the DP stage. One of the known targets of β-catenin/TCF-1 is CD4, providing some direct evidence for β-catenin mediating the transition to the DP stage (19). Our data indicate that early after stimulation with a negative selector, β-catenin is lost, but returns to higher level at about 48 hours. The loss of β-catenin explains the early loss of Bcl-XL in these cells as well as the increased apoptosis. The resurgence of expression may play a role in keeping these cells in the DP stage and may prevent any further differentiation to the SP stage. Consistent with this RoRγt as well as TCF-1, must be down regulated for further differentiation of DP thymocytes and the concomitant increase in TCR expression (20, 21). β-catenin levels may also help to facilitate secondary rounds of TCRα rearrangement in an attempt to rearrange a useful TCR, a process termed "receptor editing"(22). Further work will be needed to fully elucidate the role of β-catenin in thymocyte selection.

Our work also suggests a possible role for post-translational modifications to Bcl-XL that lead to its degradation, but little is known about post-translational modifications to Bcl-XL, however other pro-survival members of the Bcl-2 family have been extensively studied, including Mcl-1 and Bcl-2. Modification of Mcl-1 by different pathways leads to distinct outcomes. JNK phosphorylation of Mcl-1 leads to its degradation and prevents its binding of Bim, both of which result in susceptibility to apoptosis. Phosphorylation of Mcl-1by ERK leads to its stabilization of Mcl-1 and protection from

apoptosis(23). Bcl-2 is known to be phosphorylated by both JNK 1 and the ERK1/2 pathway, yet the physiological relevance of these modifications is unclear (24-26).

The transcription factor c-Myb has been linked to the expression of Bcl-XL in thymocytes as well as their proliferation and differentiation. Of note, loss of c-Myb results in a phenotype similar to the loss of the β-catenin linked transcription factor, TCF-1. Both c-Myb and β-catenin appear to play important roles in the transition from DN3 to the DP stage as well as survival during the DP stage(27, 28). Interestingly, loss of c-Myb appears to be required for the differentiation of thymocytes, as suggested above with β-catenin (29). It is not immediately clear why one molecule cannot compensate for the loss of the other. It has been demonstrated that β-catenin/TCF-1 mediated survival of DP thymocytes is dependent on RoRγt, suggesting that the loss of TCF-1 may be compensated by regulation of RoRγt through other mechanisms (30). Potential cross talk between these molecules warrants further study.

Materials and Methods:

Mice and Cells

OT-I TCR transgenic, Rag $^{-/-}$, $\beta_2M^{-/-}$ mice were generated in our lab and maintained under pathogen free conditions. Thymi from six-week old to 11 month old mice were removed and smashed between two nylon filters (Sefar Nitex). Cells were then washed off of the filters with RPMI media containing 10% FCS. Thymocytes were then resuspended in 2ml of the RPMI media per thymus. Most of the thymic epithelial cells remain trapped in the nylon filter and since the mice are non-selecting at least 85% of the cells are DP thymocytes, the rest are DN and either not expressing the receptor or are dying. No further purification was done.

Thymocyte Stimulation

Stimulation of thymocytes *in vitro* was done using synthetic tetramers made in the lab. Two million cells were stimulated in 100μL of RPMI (Gibco) media containing 10% FCS on ice, a 1:100 dilution tetramer

was used for stimulation. After the tetramer was added the cells were incubated at 37°C. The thymocytes were then centrifuged at full speed (13,300 RPM) for 10 seconds and the supernatant aspirated. AKT-X inhibitor (EMD), 5μM, or U0129 (EMD) 10μM were added fresh to media containing thymocytes to be stimulated; cells were placed with the inhibitor at 37°C for 30 minutes before stimulating.

Fetal Thymic Organ Culture

FTOC was done as described(13). Six well culture dishes containing RPMI (Gibco) with 10% FCS, a square of sterile surgical foam (Ferrosan) and a nitrocellulose filter (Millipore) was used to hold the thymi. Stimulation was done using (25μM) β_2M (Invitrogen) and 20μM OVA (SIINFEKL) or 400nM Q4H7 (SIIQFEHL) peptides were made in house. Viral vectors were courtesy of Dr. Marc Johnson (University of Missouri-Columbia). Vectors were added at a ratio of 5:1 with the thymocytes. Media containing peptide and vectors from each well was used to "baste" or cover the thymi of that well at least twice per day. Thymocytes were isolated by smashing between two nylon filters.

Western Blotting

After the thymocytes were spun down and the supernatant removed the cells were lysed. The lysis buffer used 10mM Tris (Sigma), 5mM EDTA (Sigma), 250mM Glucose (Sigma), 4% Triton-X100 (Invitrogen), 2% Na Deoxycholate (Sigma), 0.4% SDS (Sigma), 1 mM DTT. The following were were added fresh 1mM Na_3VO_4 (Sigma), 1mM NaF (Sigma), 100μM PMSF (Invitrogen), 1μg/ml Leupeptine (Sigma) and 1μg/ml Aprotinin (Sigma). The lysis step was performed on ice for 30 minutes. Following lysis the samples were centrifuged for 60 minutes at full speed (13,300 RPM, 4°C). Supernatant was removed and placed in a fresh 1.5ml eppendorf tube. Lysates were run on 12.5% acrylimide gel (Bio Rad) and proteins were transferred to a nitrocellulose membrane (Bio Rad). Membranes were blocked with 5% milk for 60 min prior to staining. Primary antibodies in 5% milk with 0.1% sodium azide (Sigma) were left on membranes overnight at 4°C with agitation. Membranes were washed four times for seven minutes with TBS buffer containing 0.1% Tween 20 with agitation. Secondary antibodies were incubated at room

temperature in 5% milk. Washes were repeated as described above. Films (Invitrogen) were developed using chemi-luminescent substrate (Bio Rad) following manufacturer recommendations.

References:

1. Takuma Hayashi DLF. 2003. Role of Defective Apoptosis in Type 1 Diabetes and Other Autoimmune Diseases *Recent Prog Horm Res* 58: 131-53
2. Adrian Liston SL, Daniel H.D. Gray, Lorraine A. O'Reilly, Andreas Strasser, Aude M. Fahrer, Richard L. Boyd, Judith Wilson, Alan G. Baxter, Elena M. Gallo, Gerald R. Crabtree, Kaiman Peng, Susan R. Wilson, and Christopher C. Goodnow 2004. Generalized Resistance to Thymic Deletion in the NOD Mouse: A Polygenic Trait Characterized by Defective Induction of Bim. *Immunity* 21: 817-30
3. Andreas Strasser HP, Lorraine A O'Reilly and Philippe Bouillet. 2008. What do we know about the mechanisms of elimination of autoreactive T and B cells and what challenges remain. *Immunology and Cell Biology* 86: 57-66
4. D A Grillot RM, G Núñez. 1995. Bcl-XL displays restricted distribution during T cell development and inhibits multiple forms of apoptosis but not clonal deletion in transgenic mice. . *J. Exp. Med.* 182: 1973-83
5. Korsmeyer DTCaSJ. 1998. BCL-2 FAMILY: Regulators of Cell Death. *Annu. Rev. Immunology* 16
6. Damian Kovalovsky YY, Marei Dose, Anastasia Emmanouilidou,Tassos Konstantinou,Kristine Germar, Katayoun Aghajani, Zhuyan Guo, Malay Mandal Fotini Gounari. 2009. Beta-Catenin/Tcf Determines the Outcome of Thymic Selection in Response to Alpha/Beta TCR Signaling. *Journal of Immunology* 183: 3873-84
7. Vassilios Ioannidis FB, Hans Clevers & Werner Held. 2001. The beta-catenin−TCF-1 pathway ensures CD4+CD8+ thymocyte survival. *Nature Immunology* 2: 691-7
8. Huimin Xie ZH, Maureen S. Sadim, and Zuoming Sun. 2005. Stabilized -Catenin Extends Thymocyte Survival by Up-Regulating Bcl-xL. *J. Immunol* 175: 7981-8
9. Lisa K. McNeil TKS, Kristin A. Hogquist. 2005. A requirement for sustained ERK signaling during thymocyte positive selection in vivo. *PNAS* 102: 13574-9
10. Jose Alberola-lla KAF, Rony Seger, Edwin G. Krebs & Roger M. Perlmutter. 1995. Selective requirement for MAP kinase activation in thymocyte differentiation. *Nature* 373: 620-3
11. Alberola-Ila SDBaJ. 2006. Phosphatidylinositol 3-kinase improves the efficiency of positive selection. *International Immunology* 18: 921-30
12. Elisabeth Fayard GM, Brian A. Hemmings and Georg A. Holländer. 2010. Phosphatidylinositol 3-Kinase Signaling in Thymocytes: The Need for Stringent Control. *Science Signaling* 3: re5
13. Hogquist KA. 2001. Assays of Thymic Selection. *Methods in Molecular Biology* 156: 219-32
14. Marisa M. Juntilla GAK. 2008. Critical roles of the PI3K/Akt signaling pathway in T cell development. *Immunology Letters* 116: 104–10
15. Fotini Gounari IA, Khashayarsha Khazaie, Sonja Hoeflinger, Naomoto Harada, Makoto M. Taketo & Harald von Boehmer. 2001. Somatic activation of beta-catenin bypasses pre-TCR signaling and TCR selection in thymocyte development. *Nature Immunology* 2: 863-9

16. Zhuyan Guo MD, Damian Kovalovsky, Rui Chang, Jennifer O'Neil, A. Thomas Look, Harald von Boehmer, Khashayarsha Khazaie and Fotini Gounari. 2007. Beta-catenin stabilization stalls the transition from double-positive to single-positive stage and predisposes thymocytes to malignant transformation. *Blood* 109: 5463-72
17. Matthew Lovatt M-JB. 2010. Stabilisation of Beta-Catenin Downstream of T Cell Receptor Signalling. *PLOS One* 5: e12794
18. Huimin Xie ZH, Maureen S. Sadim, and Zuoming Sun. 2005. Stabilized -Catenin Extends Thymocyte Survival by Up-Regulating Bcl-xL. *J. Immunol* 175: 7981-8
19. Zhaofeng Huang HX, Vassilio Ioannidis, Werner Held, Hans Clevers, Maureen S. Sadim, Zuoming Sun. 2006. Transcriptional Regulation of CD4 Gene Expression by T Cell Factor-1/ Beta-Catenin Pathway. *Journal of Immunology* 176: 4880-7
20. You-Wen He CB, Michael L. Deftos, Ethan W. Ojala, Katherine A. Forbush and Michael J. Bevan. 2000. Down-Regulation of the Orphan Nuclear Receptor RORγt Is Essential for T Lymphocyte Maturation. *Journal of Immunology* 164: 5668-74
21. Qing Yu BE, Jung-Hyun Park, Lionel Feigenbaum, and Alfred Singer. 2004. IL-7 Receptor Signals Inhibit Expression of Transcription Factors TCF-1, LEF-1, and RORgammat: Impact on Thymocyte Development *Journal of Experimental Medicine* 200: 797-803
22. Maureen A. McGargill JMDKAH. 2000. Receptor editing in developing T cells. *Nature Immunology* 1: 336-41
23. Luke W. Thomas CL, Steven W. Edwards. 2010. Mcl-1; the molecular regulation of protein function. *FEBS Letters* 584: 2981-9
24. Wagner SWaSD. 2010. Post-transcriptional and post-translational regulation of Bcl2. *Biochemical Society Transactions* 38: 1571–5
25. Xingming Deng FG, Tammy Flagg, W. Stratford May, Jr. 2004. Mono- and multisite phosphorylation enhances Bcl2's antiapoptotic function and inhibition of cell cycle entry functions. *PNAS* 101: 153-8
26. Kazuhito Yamamoto HI, Stanley J. Korsmeyer. 1999. BCL-2 Is Phosphorylated and Inactivated by an ASK1/Jun N-Terminal Protein Kinase Pathway Normally Activated at G2/M. *Mol. Cell. Biol.* 19: 8469-78
27. Timothy P Bender CSK, Manfred Kraus, Thorsten Buch & Klaus Rajewsky. 2004. Critical functions for c-Myb at three checkpoints during thymocyte development. *Nature Immunology* 5: 721-9
28. Kylie T. Greiga SC, Stephen L. Nutt 2008. Critical roles for c-Myb in hematopoietic progenitor cells. *Seminars in Immunology* 20: 247–56
29. Idoya Lahortiga KDK, Pieter Van Vlierberghe, Carlos Graux, Barbara Cauwelier, Frederic Lambert, Nicole Mentens, H Berna Beverloo, Rob Pieters, Frank Speleman, Maria D Odero, Marijke Bauters, Guy Froyen, Peter Marynen, Peter Vandenberghe, Iwona Wlodarska, Jules P P Meijerink & Jan Cools. 2007. Duplication of the MYB oncogene in T cell acute lymphoblastic leukemia. *Nature Genetics* 39: 593-5
30. Ruiqing Wang HX, Zhaofeng Huang, Jian Ma, Xianfeng Fang, Yan Ding, Zuoming Sun. 2011. T Cell Factor 1 Regulates Thymocyte Survival via a RORγt-Dependent Pathway. *Journal of Immunology* 187: 5964-73

Conclusions:

We have looked at several roles for signaling in the selection decision of DP thymocytes, specifically the role of ERK pathway localization, and the signaling required for the regulation of the survival molecule Bcl-XL through β-catenin.

In Chapter 1, we demonstrate a role for ERK localization in the thymic selection decision. ERK localization is critical for the selection decision, as alterations in ERK localization lead to outcomes that are inconsistent with the selecting stimulus provided the cells. ERK artificially targeted to the plasma membrane by a Raf construct induced negative selection, regardless of the selector stimulus. Localizing ERK to the Golgi and stimulating the cells with a negative selector induced the differentiation of cells into an alternate T-cell fate, the CD8αα lineage. Given the necessity of sustained ERK signaling for positive selection (1) and the known kinetics of ERK signaling on endomembranes versus the plasma membrane (2), it is entirely consistent that ERK localization plays an essential role in the thymic selection decision.

The coactivator β-catenin has been shown to play a critical role in the development of thymocytes, through both an increase in the pro-survival protein Bcl-XL and its ability to regulate RoRγt and TCF-1 activity (3-6). Our data suggest a unique role for β-catenin in negative selection. Initially, a negative selecting signal leads to both lower levels of β-catenin and Bcl-XL. Continued expression or induction of Bim sensitizes the cells to apoptosis. However, the cells that survive the initial negative selection signal increase the expression of β-catenin while keeping Bcl-XL levels low, a mechanism that may prevent these DP thymocytes from differentiating further and most likely leads to their death. This block in development has been suggested previously (7) in a study where a mutant stabilized form of β-catenin was expressed under the control of either an *lck* or *CD4* promoter. However, other work by this group demonstrated a loss in TCR expression after β-catenin stabilization. Therefore, it is not clear if loss of TCR is preventing further differentiation of DP cells or if the stable β-catenin is responsible for observed defects in differentiation with this study (8). We were able to observe the β-catenin kinetics

without manipulation of thymocytes and provide some preliminary evidence in favor of the conclusions reached by Guo and colleagues.

TCR signaling mediates the fate of DP thymocytes, either a strong or lack of signal leads to death by apoptosis and is considered either negative selection or death by neglect (respectively), while a weak to moderate strength signal facilitates the differentiation of DP thymocytes into SP cells, known as positive selection. The molecular mechanism that determines positive or negative selection is not clear. It has been shown that the calcium flux downstream of a negative selecting signal is higher than that seen during positive selection (9). Negative selectors are known to induce transient activation of ERK at the plasma membrane, while positive selectors lead to RasGRP activation on the Golgi, diffuse pERK (active), and sustained ERK activity. High calcium, levels as well as its activation of PKC (likely PKCα), has also been linked with an increase in the pro-apoptotic molecule Bim and is crucial for negative selection (10). Calcium and DAG production are also pivotal in the localization of ERK via RasGRP activation and localization (2). We demonstrate in Chapter 1, a role for calcium in the differential localization of ERK during thymocyte selection. By increasing intracellular levels of calcium we were able to activate ERK primarily at the plasma membrane. Decreasing intracellular calcium targeted RasGRP to the Golgi and allowed production of diffuse ERK, thus mimicking positive selection(9). ERK localization was also crucial for the final outcome of selection, as artificial targeting of ERK altered selection of DP thymocytes (Chapter 1). This data is also consistent with previous reports describing negative selection in the cortex. In our study thymocytes underwent negative selection but were only allowed to develop for four days, roughly the time it has been reported that thymocytes remain in the cortex(11, 12).

Targeting Raf to the Golgi, coupled with a negative selecting stimulus, induced the differentiation of CD8αα^{+} T-cells (Chapter 1). Previous work done on the development of this cell type came to one of two conclusions. Development was either independent of the thymus and took place entirely in CP (13-16); or the thymus was needed initially but later differentiation took place in the gut wall(17-19). The

property of the gut as the primary organ for the development of immune cells in lower organisms was given as evidence for the possibility of extra thymic development of CD8αα T-cells in either case (20). However, it is hard to reconcile this with the fact that the thymus appears to be the only location for all other T-cell development in higher animals. Contrary to the previous work done on these cells (20), our data suggests that these cells pass through a DP stage. GFP expression in the pre-selected controls and CD8αα population suggests these were infected during their proliferative burst, as they transition from DN to DP. Previous studies suggested that cells did not pass through the DP stage, but developed directly into CD8αα^{+} T-cells from the DN stages (20). It would appear from our data, that CD8αα cells develop based on a unique localization pattern of ERK where the ERK pathway localizes activation to the plasma membrane and the Golgi. Our experiments were done with endogenous Raf still present in the cells. Therefore, it is plausible that the negative selecting stimulus localized endogenous Raf to the plasma membrane, while our targeted Raf signaled from the golgi and that this combination of signals leads to lineage deviation. This does not rule out the possibility of weak signaling from the Golgi alone as the mechanism for CD8αα development, as it is still unclear if ERK at the plasma membrane propagates a signal. If there is no ERK signal from its apparent sequestration at the plasma membrane, our Raf construct may have served to dilute the endogenous ERK reducing the signal from the Golgi. This type of signaling may occur when the strength TCR signal is at or near the threshold between negative and positive selection, and the ERK pathway localizes to multiple compartments. Future work on the signaling output of this unique localization pattern, as well as the signal that leads to this pattern and its relation to CD8αα development, is needed to fully corroborate these conclusions. It cannot be ruled out that active ERK at the plasma membrane activates a unique signal leading to negative selection, or when coupled with Golgi signaling, lineage deviation. It is also possible that the developmental state of the fetal thymi or thymocytes plays a role in the unique cellular output observed, as has been described with other cell types (21, 22).

As described above, high calcium levels are necessary for both the increase in Bim by PKC and the localization of ERK to the plasma membrane. Both of these events are necessary for the proper negative selection of thymocytes (Chapter 1). However, calcium levels may also play a role in the loss of β-catenin and Bcl-XL levels that are seen early in negative selection (Chapter 2). Destabilization of β-catenin may initially be due to PKCα activation as has been demonstrated (23), as well as a decrease in the levels of inactive GSK3β (Chapter 2). A role for PKCα in β-catenin degradation during negative selection is also consistent given that its activation is mediated by calcium and DAG, two secondary messengers that are highly induced during negative selection. However, later time points in selection are inconsistent with a role in survival for β-catenin under negatively selecting conditions as the levels of β-catenin eventually increase, while Bcl-XL levels remain low. Given the ability of β-catenin to mediate the transition of DN cells into DP cells (7, 8), it is plausible that β-catenin prevents the further differentiation of these cells by keeping them in the DP stage. Consistent with this, it has been demonstrated that RoRγt and TCF-1 must be down regulated to allow further differentiation of DP thymocytes (6, 24). By keeping negatively stimulated cells in the DP stage, increased β-catenin may help to facilitate further TCRα chain rearrangements (25).

It is not clear how β-catenin is regulated later in selection. Our data suggest, as mentioned above, that PKCα coupled with decreased phosphorylation of GSK3β is the cause of the initial loss of β-catenin. In our system, the cells should be continuously receiving negatively selecting signals. It is not clear how PKCα and GSK3β are regulated differently under these conditions. Later time points and the increase in β-catenin are correlated with increases in pGSK3β which would implicate ERK, PI_3K or WNT signaling. An intriguing possibility is that the cells found later in selection are a distinct subset of thymocytes that are "pre-programmed" to become agonist selected T-cells such as T_{regs} as has been suggested by several groups(21, 22). This would explain the unique response to a negative selecting stimulus, but would suggest that negative selection of traditional thymocytes happens within the first 48 hours of selection.

This is consistent with the idea that deletion can occur in the cortex and similar to studies that suggest it takes three days for selection to be completed in this compartment (11, 12).

The phenotype of c-Myb knockouts are similar to that of TCF-1 knockouts, and are characterized by a block in T-cell development at the DN3 stage, and decreased survival in the DP stage due to the loss of Bcl-XL expression, c-Myb must be considered in future studies (26, 27). Work in the FTOC system will be needed to sort out the signaling involved at days two and three of thymic negative selection, specifically, experiments where inhibition of PI_3K and ERK are done to determine the pathways necessary for the observed β-catenin kinetic. Other experiments will need to be done to determine the role of PKC in β-catenin down regulation and the role of c-Myb and RoRγt in development.

Our data also suggest possible post-translational modifications to Bcl-XL (Chapter 2), yet, little is known about post-translational modifications to Bcl-XL. It is not clear how Bcl-XL is regulated after two days of negative stimulation.β-catenin levels are high at this time, but Bcl-XL levels remain low. One possibility is phosphorylation leading to degradation which has been observed in another pro-survival Bcl-2 family member, Mcl-1. Phosphorylation of Mcl-1 by JNK leads to its proteasome dependent degradation(28). Modifications to Bcl-2 by ERK1/2 and JNK 1 have also been observed, but the relevance of these phosphorylations is unclear (29-31). Another possibility is an increase in a negative regulator of transcription. Further study is required to determine which of these possibilities results in the loss of Bcl-XL.

This study has helped to elucidate the molecular mechanisms of conventional thymocyte development and may have revealed part of the mechanisms involved in the development of unconventional $CD8\alpha\alpha^+$ T-cells. We have also highlighted some of the molecular changes that differentiates positive selection and negative selection of DP thymocytes.

References:

1. Lisa K. McNeil TKS, Kristin A. Hogquist. 2005. A requirement for sustained ERK signaling during thymocyte positive selection in vivo. *PNAS* 102: 13574-9
2. Adam Mor MRP. 2006. Compartmentalized Ras/MAPK Signaling. *Annu. Rev. Immunol.* 24: 771–800
3. Vassilios Ioannidis FB, Hans Clevers & Werner Held. 2001. The beta-catenin−TCF-1 pathway ensures CD4+CD8+ thymocyte survival. *Nature Immunology* 2: 691-7
4. Huimin Xie ZH, Maureen S. Sadim, and Zuoming Sun. 2005. Stabilized -Catenin Extends Thymocyte Survival by Up-Regulating Bcl-xL. *J. Immunol* 175: 7981-8
5. Zhaofeng Huang HX, Vassilio Ioannidis, Werner Held, Hans Clevers, Maureen S. Sadim, Zuoming Sun. 2006. Transcriptional Regulation of CD4 Gene Expression by T Cell Factor-1/ Beta-Catenin Pathway. *Journal of Immunology* 176: 4880-7
6. You-Wen He CB, Michael L. Deftos, Ethan W. Ojala, Katherine A. Forbush and Michael J. Bevan. 2000. Down-Regulation of the Orphan Nuclear Receptor RORγt Is Essential for T Lymphocyte Maturation. *Journal of Immunology* 164: 5668-74
7. Zhuyan Guo MD, Damian Kovalovsky, Rui Chang, Jennifer O'Neil, A. Thomas Look, Harald von Boehmer, Khashayarsha Khazaie and Fotini Gounari. 2007. Beta-catenin stabilization stalls the transition from double-positive to single-positive stage and predisposes thymocytes to malignant transformation. *Blood* 109: 5463-72
8. Damian Kovalovsky YY, Marei Dose, Anastasia Emmanouilidou,Tassos Konstantinou,Kristine Germar, Katayoun Aghajani, Zhuyan Guo, Malay Mandal Fotini Gounari. 2009. Beta-Catenin/Tcf Determines the Outcome of Thymic Selection in Response to Alpha/Beta TCR Signaling. *Journal of Immunology* 183: 3873-84
9. Mark A. Daniels ET, Jason Gill, Barbara Hausmann, Dominique Roubaty, Kaisa Holmberg, Guy Werlen, Georg A. Holla¨nder, Nicholas R. J. Gascoigne & Ed Palmer. 2006. Thymic selection threshold defined by compartmentalization of Ras/MAPK signalling. *Nature* 444: 724-9
10. Kirsten Canté-Barrett EMG, Monte M. Winslow, Gerald R. Crabtree. 2006. Thymocyte Negative Selection Is Mediated by Protein Kinase C- and Ca2+-Dependent Transcriptional Induction of Bim. *J. Immunol* 176: 2299-306
11. McCaughtry TM BT, Wilken MS, Hogquist KA. 2008. Clonal deletion of thymocytes can occur in the cortex with no involvement of the medulla. *Journal of Experimental Medicine* 11: 2575-84
12. Zuniga-Pflucker HTPaJC. 2007. Zoned out: functional mapping of stromal signaling microenvironments in the thymus. *Annu. Rev. Immunol.* 25: 649-79
13. B Rocha PV, and D Guy-Grand. 1994. Thymic and extrathymic origins of gut intraepithelial lymphocyte populations in mice. *Journal of Experimental Medicine* 180: 681-6
14. S R Guehler JAB, and T A Barrett. 1996. Immune deviation of 2C transgenic intraepithelial lymphocytes in antigen-bearing hosts. . *Journal of Experimental Medicine* 184: 493-503
15. Daniel Cruz BCS, Kristine Hetzel, Gian Yakoub, Mitchell Kronenberg, and Hilde Cheroutre. 1998. An Opposite Pattern of Selection of a Single T Cell Antigen Receptor in the Thymus and among Intraepithelial Lymphocytes *Journal of Experimental Medicine* 188: 255-65
16. Christiaan N. Levelt YPdJ, Emiko Mizoguchi, Cliona O'Farrelly, Atul K. Bhan, Susumu Tonegawa, Cox Terhorst, and Stephen J. Simpson. 1999. High- and low-affinity single-peptide/MHC ligands have distinct effects on the development of mucosal CD8αα and CD8αβ T lymphocytes. *PNAS* 96: 5628-33
17. Tesu Lin GM, Hiroyuki Kenai, Takao Nakamura and Kikuo Nomoto. 1993. Thymus influences the development of extrathymically derived intestinal intraepithelial lymphocytes. *European Journal of Immunology* 23: 1968-74

18. Olson LLaS. 1994. A novel pathway of thymus-directed T lymphocyte maturation. *Journal of Immunology* 153: 987-95
19. Delphine Guy-Grand NP, Sylvie Darche, Olivier Lantz, Philippe Kourilsky and Pierre Vassalli. 2001. Contribution of double-negative thymic precursors to CD8α α + intraepithelial lymphocytes of the gut in mice bearing TCR transgenes *European Journal of Immunology* 31: 2593–602
20. Vassalli DG-GaP. 2002. Gut intraepithelial lymphocyte development. *Current Opinion in Immunology* 14: 255-9
21. Yan Jin MX, Christina M. Saylor, Kavitha Narayan, Joonsoo Kang, David L. Wiest, Yanming Wang, and Na Xiong. 2010. Cutting Edge: Intrinsic Programming of Thymic gdT Cells for Specific Peripheral Tissue Localization. *Journal of Immunology* 185: 7156–60
22. Jeff E. Mold SV, Trevor D. Burt, Jakob Michaëlsson, Jose M. Rivera, Sofiya A. Galkina, Kenneth Weinberg, Cheryl A. Stoddart, and Joseph M. McCune. 2010. Fetal and Adult Hematopoietic Stem Cells Give Rise to Distinct T Cell Lineages in Humans *Science* 330: 1695-9
23. Jungsug Gwak MC, Soo-Jung Gong, Jaejoon Won, Dong-Eun Kim, Eun-Young Kim, Sang Sup Lee, Mina Kim,Tae Kook Kim, Jae-Gook Shin andSangtaek Oh. 2006. Protein-kinase-C-mediated beta-catenin phosphorylation negatively regulates the Wnt/beta-catenin pathway. *J Cell Sci* 119: 4702-9
24. Qing Yu BE, Jung-Hyun Park, Lionel Feigenbaum, and Alfred Singer. 2004. IL-7 Receptor Signals Inhibit Expression of Transcription Factors TCF-1, LEF-1, and RORgammat: Impact on Thymocyte Development *Journal of Experimental Medicine* 200: 797-803
25. Maureen A. McGargill JMDKAH. 2000. Receptor editing in developing T cells. *Nature Immunology* 1: 336-41
26. Kylie T. Greiga SC, Stephen L. Nutt 2008. Critical roles for c-Myb in hematopoietic progenitor cells. *Seminars in Immunology* 20: 247–56
27. Timothy P Bender CSK, Manfred Kraus, Thorsten Buch & Klaus Rajewsky. 2004. Critical functions for c-Myb at three checkpoints during thymocyte development. *Nature Immunology* 5: 721-9
28. Luke W. Thomas CL, Steven W. Edwards. 2010. Mcl-1; the molecular regulation of protein function. *FEBS Letters* 584: 2981-9
29. Kazuhito Yamamoto HI, Stanley J. Korsmeyer. 1999. BCL-2 Is Phosphorylated and Inactivated by an ASK1/Jun N-Terminal Protein Kinase Pathway Normally Activated at G2/M. *Mol. Cell. Biol.* 19: 8469-78
30. Xingming Deng FG, Tammy Flagg, W. Stratford May, Jr. 2004. Mono- and multisite phosphorylation enhances Bcl2's antiapoptotic function and inhibition of cell cycle entry functions. *PNAS* 101: 153-8
31. Wagner SWaSD. 2010. Post-transcriptional and post-translational regulation of Bcl2. *Biochemical Society Transactions* 38: 1571–5