Regulation of T cell development by RasGRPs 1 and 3

by

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ABSTRACT

T cell development is a highly dynamic process driven by interactions between developing hematopoietic progenitor cells and numerous cell types that nurture their development. Ultimately, signals detected by developing progenitors lead to the activation of cellular signaling pathways that instruct the development of these cells into mature T cells. One signaling pathway that has proven to be an important regulator of T cell development is the highly conserved Ras pathway. Signals transduced through the Ras pathway regulate numerous cellular processes such as differentiation, proliferation, migration and survival, all of which are important during T cell development. The activation of Ras in T cells has been shown to regulated by members of the Ras guanine nucleotide releasing protein (RasGRP) family, which includes RasGRPs 1, 2, 3 and 4. RasGRP1 has proven to be the most potent regulator of T cell development, however the roles of the remaining family members remain unclear. Another RasGRP family member, RasGRP3, closely resembles RasGRP1 but has not been examined during T cell development. Furthermore, the contribution of RasGRP1 to many of the hallmark events of T cell development has not been examined in detail. Therefore, we sought to explore the roles of RasGRP1 and RasGRP3 throughout T cell development. To determine the contributions of these molecules during T cell development we examined gene knock-out mice deficient in RasGRP1, RasGRP3 and RasGRPs 1 and 3. Global analysis of T cell development was performed, uncovering numerous novel roles of RasGRPs 1 and 3 during T cell development.

Immature T cell progenitors originate in the bone marrow (BM) and populate the thymus following migration out of the BM via circulation. Early thymic progenitors (ETPs) are the most immature progenitors found within the thymus and are derived from incoming BM progenitors. Mice deficient in RasGRPs 1 and 3 showed impaired development of ETPs, suggesting that RasGRPs 1 and 3 regulate the earliest stages of T cell development. Downstream of ETPs, early development of $CD4^-CD8^-$ 'double negative' (DN) thymocytes culminates with development through the β-selection checkpoint. We determined that RasGRP1, but not RasGRP3, was critical for efficient passage through thymocyte β-selection. The product of β-selection is the development of the $CD4^+CD8^+$ 'double positive' (DP) thymocyte population. DP thymocytes expressing a mature αβ T cell receptor (TCR) experience a number of different fates depending on the affinity with which their clonal TCRs bind self-peptides presented on major histocompatibility complex (MHC) molecules. Generally, cells weakly recognizing self-peptide undergo positive selection and develop into mature T cells, while cells strongly recognizing self-peptides undergo negative selection and are eliminated from the T cell repertoire to prevent autoimmunity. However, some cells strongly recognizing self-peptides undergo an alternative positive selection process termed agonist selection. Numerous lineages of cells are derived from agonist selection, including a unique subset of intestinal T cells known as $TCR\alpha\beta^+CD8\alpha\alpha$ intraepithelial lymphocytes (IELs). Our data indicate that RasGRP1 is critical for the development of $TCR\alpha\beta^+CD8\alpha\alpha$ IELs through controlling thymocyte agonist selection. Collectively, the examination of RasGRP1 and RasGRP3 activity during T cell development has shed light onto the varied roles of these molecules throughout this complex developmental program.

PREFACE

This thesis is composed of original work by Dominic Golec. Throughout, it contains content co-authored by collaborators and republished with permission:

- Golec, D.P., Caviedes, L.M.H., and Baldwin, T.A. 2016. RasGRP1 and RasGRP3 Are Required for Efficient Generation of Early Thymic Progenitors. J Immunol *197*, 1743-1753
- Golec, D.P., Dower, N.A., Stone, J.C. and Baldwin, T.A. 2013. RasGRP1, but not RasGRP3, is required for efficient thymic β-selection and ERK activation downstream of CXCR4. PLoS One *8*: e53300
- Golec, D.P., Hoeppli, R.E., Caviedes, L.M.H., McCann, J., Levings, M.K. and Baldwin, T.A. 2017. Thymic progenitors of TCRαβ^{+} CD8αα intestinal intraepithelial lymphocytes require RasGRP1 for development. J Exp Med. *214,* 2421-2435

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DEDICATION

This thesis is dedicated to my loving parents Ella and Zbe Golec, whose hard work has made my education possible.

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LIST OF RECURRING ABBREVIATIONS

1KO – RasGRP1$^{-/-}$

3KO – RasGRP3$^{-/-}$

APC – antigen presenting cell

B6 – C57BL/6 wild type mice

Bim – Bcl-2-interacting mediator of death

BKO – Bim$^{-/-}$

BM – bone marrow

CCL19 – chemokine (C-C motif) ligand 19

CCL25 – chemokine (C-C motif) ligand 25

CCR7 – C-C chemokine receptor type 7

CCR9 – C-C chemokine receptor type 9

CD122 – IL-2 receptor β

CLP – common lymphoid progenitor

CXCR4 – C-X-C chemokine receptor type 4

DAG – diacylglycerol

DC – dendritic cell

DKO – double knockout (RasGRP1$^{-/-}$RasGRP3$^{-/-}$ or RasGRP1$^{-/-}$Bim$^{-/-}$)

DN – double negative (CD4^{-}CD8^{-})

DP – double positive (CD4^{+}CD8^{+})

ERK – extracellular signal-regulated kinase

ETP – early thymic progenitor

FSC – forward scatter

HSC – hematopoietic stem cell

IEL – intraepithelial lymphocyte

IELp – intraepithelial lymphocyte progenitor

iNKT – invariant natural killer T

ITAM – immunoreceptor tyrosine-based activation motif

LAT – linker for the activation of T cells

LMPP – lymphoid primed multipotent progenitor

LSK – Lineage^{-}Sca-1^{+}c-Kit^{hi}

MAPK – mitogen-activated protein kinase

MFI – mean or median fluorescent intensity

MHC – major histocompatibility complex

MPP – multipotent progenitor

NK – natural killer

Nur77-GFP – mice expressing green fluorescent protein under control of the Nur77 promoter

PD-1 – programmed death-1

PI3K – phosphoinositide 3-kinase

PLCγ-1 – phospholipase C γ 1

PSGL-1 – P-selectin glycoprotein ligand 1

RAG – recombination-activating gene

RasGEF – Ras guanine nucleotide exchange factor

RasGRP – Ras guanine nucleotide releasing protein

SDF-1α – stromal cell derived factor 1 α

SSC – side scatter

SP – single positive ($CD4^-CD8^+$ CD8SP or $CD4^+CD8^-$ CD4SP)

TCR – T cell receptor

Treg – T regulatory cell

WT – wild type

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CHAPTER 1: INTRODUCTION

This chapter contains content from the following sources, republished with permission:

- Golec, D.P., Caviedes, L.M.H., and Baldwin, T.A. 2016. RasGRP1 and RasGRP3 Are Required for Efficient Generation of Early Thymic Progenitors. J Immunol *197*, 1743-1753
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T cells are critical drivers of adaptive immunity

Establishment of the immune system relies upon a complex network of interactions between hematopoietic progenitor cells and many other non-hematopoietic cell types that regulate its development, functionality and homeostasis. Generally, immune cell types derived from hematopoietic progenitors can be classified into two broad groups; innate and adaptive immune cells. Cells of the innate immune system act as the first line of defense against incoming factors from the external environment and are programmed to rapidly respond to non-specific, highly conserved and widely encountered antigenic

challenges to the host, but lack the ability to mount specific responses to pathogens that escape this initial barrier. In contrast, cells of the adaptive immune system are programmed to mount a slow, but highly antigen specific, response to pathogens that evade the innate immune system and give the host the ability to respond to a plethora of unique antigenic signatures from a wide variety of external factors. The lymphocyte arm of the hematopoietic system includes T and B cells, both of which are critical for adaptive immunity. Central to their role as key drivers of adaptive immunity, T and B cells bear highly antigen-specific receptors on their cell surface that are generated through a stochastic process of somatic recombination, known as VDJ recombination. The product of this process is the generation of clonal populations of lymphocytes that collectively bear a high diversity of antigen specific receptors, which enable the host to respond to a wide array of potential antigens at any given moment. The focus of this thesis will be on the mechanisms that orchestrate the development of the T cell branch of the lymphocyte lineage.

T cell diversity

T cells are heterogeneous in their composition and include a variety of unique subsets, with differing functional characteristics. However, one unifying feature of all T cells is expression of T cell receptors (TCRs) on their cell surface. The TCR gene family includes a number of distinct TCR genes and there are two broad groups of T cells based upon expression of unique TCR α, β, γ and δ chains; αβ and γδ T cells. As indicated by their names, cells of the αβ T cell lineage express TCRs that are heterodimers of TCRα and TCRβ chains, while γδ T cells bear TCRs that are heterodimers of TCRγ and TCRδ

chains. In the majority of adult lymphoid tissues, αβ T cells greatly outnumber γδ T cells and αβ T cells are commonly referred to as 'conventional' T cells. Conventional T cells express αβ-TCRs that, for the most part, recognize peptides presented in the context of MHC molecules and this population includes both $CD4^+$ and $CD8^+$ T cells. CD4 and CD8 are TCR co-receptors that bind to MHC class II and MHC class I molecules, respectively. Likewise, $CD4^+$ T cells recognize peptides in the context of MHC class II and are said to be MHC class II restricted, while $CD8^+$ T cells recognize peptides in the context of MHC class I and are MHC class I restricted. Ultimately, TCR – peptide-MHC interactions lead to the activation of signaling pathways downstream of the TCR that regulate the development and functionality of T cells.

Central to the diversity of the T cell repertoire, is the process of VDJ recombination. During development, VDJ recombination is initiated at the TCR α, β, γ and δ gene loci. The variable regions of the TCR β and δ genes are composed of distinct V, D and J gene segments, while the α and γ chains are made up of V and J gene segments (*3*). Furthermore, there are numerous sequential copies of V, D and/or J gene segments within the TCR gene loci. The process of VDJ recombination is mediated by the VDJ recombinase complex, which is composed of a variety of enzymes that regulate distinct steps in this process. Some key enzymes in this complex include recombination-activating genes 1 and 2 (RAG1 and RAG2), which induce DNA double-stranded breaks and are critical for the initiation of recombination (*3*). During VDJ recombination, the VDJ recombinase complex mediates the sequential joining of random V, D and J gene segments, to produce unique TCR chains. In addition, enzymes present in this complex

can also induce nucleotide additions and deletions during recombination, further promoting TCR chain heterogeneity. As a result, it is estimated that between 10^{15} and 10^{20} unique αβ-TCR structures can be generated (*4*). It is the stochastic nature of this process that endows T cells with the ability to generate TCRs that can respond to a multitude of different antigens in a specific manner, through diverse TCR recognition of specific peptide-MHC complexes. However, this high degree of receptor diversity comes with the caveat of having the ability to generate T cells with self-antigen specific receptors and developmental mechanisms limiting the generation of autoreactive T cells are in place to positively select useful cells and negatively select potentially harmful cells.

T cell progenitors present in bone marrow

The major site of T cell maturation is in the thymus and T cell development requires the mobilization of hematopoietic progenitors out of the bone marrow, into the blood and finally into the thymus (**Fig. 1-1**). Under homeostatic conditions, the thymus does not contain a population of self-renewing progenitors (*5*). Therefore, the development of mature T cells is dependent on the import of bone marrow derived progenitors into the thymus from circulation. The most immature progenitors with T cell potential reside within the Lineage $(Lin)^-Sca1^+cKit^{hi}$ (LSK) fraction of the bone marrow (Lin^- refers to the lack of expression of mature differentiated hematopoietic lineage markers) (*6, 7*). LSK progenitors can be further resolved into hematopoietic stem cells (HSCs), multipotent progenitors (MPPs) and lymphoid primed multipotent progenitors (LMPPs), based on expression of fms-like tyrosine kinase receptor-3 (Flt3). HSCs ($Flt3^-$LSK) are self-

renewing, multipotent stem cells that give rise to all lineages of hematopoietic cells. HSCs differentiate into MPPs ($Flt3^{lo}$LSK), and following this transition these progenitors lose their self-renewing capacity (*8, 9*). MPPs can differentiate into all hematopoietic lineage cell types, however their differentiation into LMPPs ($Flt3^{hi}$LSK) is characterized by their loss of erythroid/megakaryocyte lineage potential (*10*). LMPPs can further differentiate into the largely lymphoid lineage restricted common lymphoid progenitors (CLPs), which do not reside within the LSK fraction and instead have a Lin^{-} $Sca1^{lo}cKit^{lo}IL\text{-}7R\alpha^{+}Flt3^{hi}$ surface phenotype (*11*). In terms of lineage potential, both LMPPs and CLPs are progenitors of lymphoid lineage cells; T cells, B cells and natural killer (NK) cells.

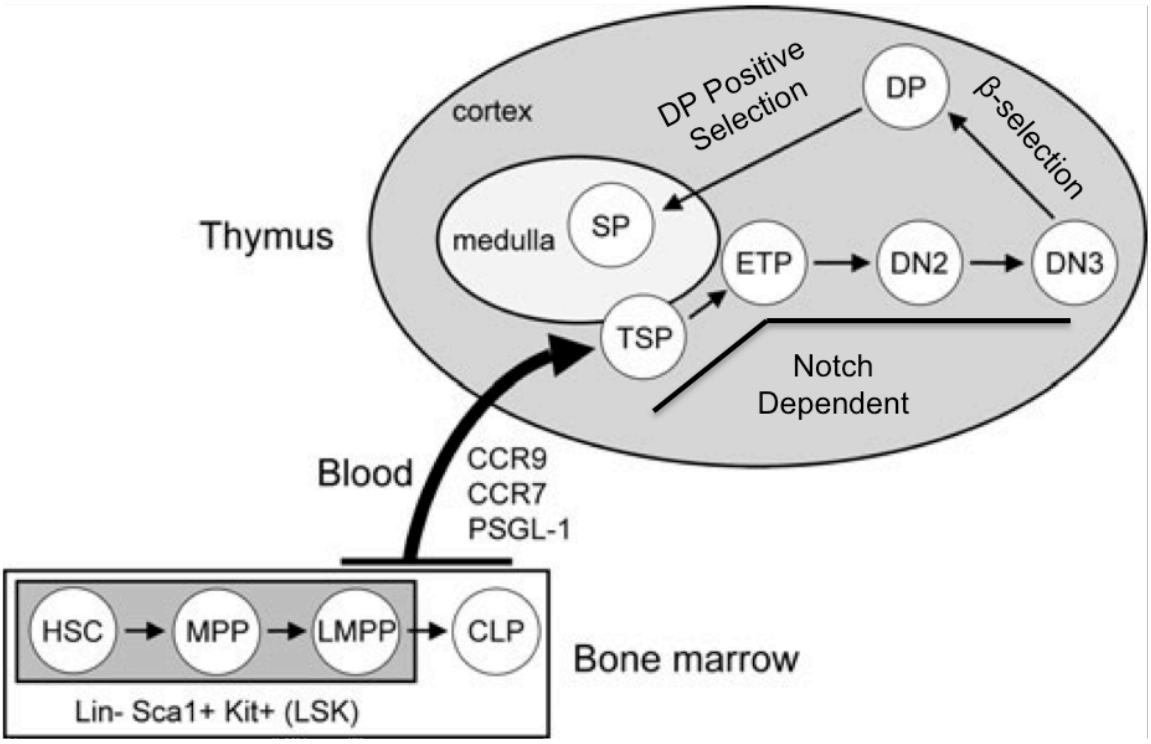

Figure 1-1. **An overview of T cell development**. The most immature progenitors with T cell lineage potential reside in the bone marrow. Developing T cell progenitors differentiate through the HSC, MPP, LMPP and CLP stages in the bone marrow. The final site of T cell development is in the thymus and thymic T cell maturation requires the migration of progenitors from the bone marrow into the thymus. It remains unclear which progenitors migrate to the thymus, however it is thought that subsets of LMPPs and CLPs are the most likely candidates. The migration of progenitors from the bone marrow into the thymus is dependent on the chemokine receptors CCR7 and CCR9 and the P-selectin ligand PSGL-1. Upon entering the thymus through the cortico-medullary junction, TSPs begin to receive Notch signals that result in the development of the ETP compartment. Continued Notch signaling drives development onward to the DN3 stage. DN3 thymocytes experience an important developmental checkpoint called β-selection. The result of successful β-selection is the development of the DP pool of thymocytes. DP thymocytes undergo positive selection and mature into SP thymocytes, which can exit the thymus and traffic to peripheral lymphoid tissues. Figure adapted from Zlotoff and Bhandoola, Annals of the New York Academy of Sciences, 2011 (*1*)

Although the precise identity of the progenitors that migrate to the thymus from circulation remains elusive, it is known that these progenitors require expression of the P-selectin glycoprotein ligand-1 (PSGL-1) and the chemokine receptors CCR7 and CCR9 to gain entry into the thymus (**Fig. 1-1**) (*12-16*). Interactions between P-selectin expressed on vascular endothelium and progenitor PSGL-1 are thought to mediate progenitor rolling along endothelial cells in circulation, while signaling through CCR7 and CCR9 are thought to be required for integrin activation and subsequent progenitor transmigration through the thymic endothelium, into the thymus (*1, 17*). Notably, following adoptive transfer of sorted bone marrow progenitor populations into recipient mice, only LMPPs and CLPs can transit to the thymus (*18*). Therefore, it appears that PSGL-1$^+$CCR7$^+$CCR9$^+$ populations of LMPPs and CLPs likely contain the thymus settling progenitors (TSPs) (*1, 18-20*).

Thymic Architecture

Progenitors that enter the thymus from circulation begin to navigate their way through the distinct architecture of the thymus. Broadly, the thymus is composed of two main compartments, the outer cortex and the inner medulla (**Fig. 1-1**). Progenitors enter the thymus through the cortico-medullary junction and migrate outward within the thymic cortex as they mature through the early stages of T cell development (**Fig. 1-1**). The later stages of thymocyte development occur in the medulla and upon completing development in this compartment, mature CD4$^+$ and CD8$^+$ T cells may egress from the thymus (**Fig. 1-1**). Thymic epithelial cells are critical to the structure of the thymus such that the cortex is

made up of cortical thymic epithelial cells (cTECs), while the medulla contains medullary thymic epithelial cells (mTECs). Both cTECs and mTECs act as antigen presenting cells (APCs) in the thymus and interact with developing thymocytes extensively. Furthermore, dendritic cells (DCs) can be found throughout the thymus, but are especially prevalent in the medulla where they have also been shown to act as APCs and interact with thymocytes during development.

Early events of T cell development

Immediately upon entering the thymus through the cortico-medullary junction, TSPs begin to receive signals that direct their specification into the T cell lineage. Perhaps the most critical of these early cues are signals transduced through the Notch signaling pathway (**Fig. 1-1**). Cortical thymic epithelial cells (cTECs) express the Notch ligand, Delta-like 4, which stimulates Notch1 receptor signaling on the surface of incoming T cell progenitors. Signaling downstream of Notch results in the proteolytic cleavage of the intracellular domain of the receptor, which, following its liberation, can transit to the nucleus to regulate gene expression in developing progenitors. The result of the activation of Notch signaling is the transcriptional regulation of genes that specify development into the T cell lineage. Following the entry of TSPs into the thymus, these cells receive Notch signals that are required for the development of ETPs (*21-23*). ETPs ($Lin^-CD44^+cKit^{hi}CD25^-$) represent the most immature T cell progenitors found in the thymus and continued stimulation of ETPs through Notch is thought to drive their subsequent development into $CD4^-CD8^-$ 'double negative' 2 (DN2) thymocytes (*22*). DN2 thymocytes ($Lin^-CD44^+cKit^{hi}CD25^+$) continue to require Notch signaling for their

subsequent development through the DN2a ($cKit^{+++}$) and DN2b ($cKit^{++}$) stages. In addition to Notch, signaling through the Wnt/β-catenin axis has also proved to be an important regulator of early T cell development. More specifically, mice lacking a T cell specific mediator of Wnt signaling, called TCF-1, show drastically decreased numbers of ETPs and DN2 cells, accompanied by increased apoptosis within the ETP population (*24*). Furthermore, TCF-1 expression is associated with the upregulation of the transcription factors GATA-3 and Bcl11b (*25, 26*). DN2 thymocytes ultimately differentiate into DN3 thymocytes and it is thought that the activity of the transcription factors GATA-3, TCF-1 and Bcl11b are important for driving the development from the ETP stage to the DN3 stage (*26*).Within the DN2 stages, VDJ recombination occurs at the TCRβ, γ and δ gene loci (*27, 28*), with completion of rearrangement occurring at the $CD44^{-}CD25^{+}$ DN3 stage. Cells that successfully undergo rearrangement at the TCRγ and TCRδ loci, but not TCRβ, may adopt the γδ T cell lineage and it is thought that development of the αβ and γδ T cell lineages bifurcates at the DN3 thymocyte stage. In contrast, DN3 thymocytes that undergo successful recombination at the TCRβ locus may adopt the αβ T cell lineage. Pairing of TCRβ with the invariant pre-Tα produces the pre-TCR, which signals DN3 thymocytes to undergo a process termed 'β-selection' (**Fig. 1-1**). The characteristic features of β-selection include commitment to the αβ-T cell lineage, continued differentiation, proliferation, survival and cessation of recombination at the TCRβ locus. Thymocytes that pass the β-selection checkpoint initiate recombination at the TCRα loci and enter the $CD44^{-}CD25^{-}$ DN4 stage, before CD4 and CD8 are upregulated to generate 'double positive' (DP) thymocytes (*29*). Recombination at the TCRα locus continues within DP thymocytes and following a productive TCRα

rearrangement and pairing with TCRβ to produce a mature αβTCR, DP thymocytes can experience a number of different fates based upon the specificity of the mature TCR for self-peptide MHC complexes presented within the thymus (*30*).

The early events of αβ T cell development culminate with rearrangement at the TCRβ locus. Progenitors that have undergone productive rearrangement at the TCRβ locus pass through the β-selection checkpoint by integrating signals from multiple signaling pathways. Currently it is thought that signals downstream of the pre-TCR, Notch and chemokine receptor CXCR4 drive DN3 thymocytes through β-selection (**Fig. 1-2**) (*31*). Additionally, it is known that signals downstream of the pre-TCR can activate the canonical Ras/Erk pathway (*32*). While Notch signaling is critically important at earlier stages of DN thymocyte development, at the β-selection checkpoint Notch appears to cooperate with pre-TCR signals to promote survival and metabolic activity through the phosphoinositide 3-kinase (PI3K) pathway (*33-36*). Furthermore, the chemokine receptor CXCR4 has also been shown to be required as a 'co-stimulatory' receptor for β-selection (*37, 38*). Again, the pre-TCR appears to cooperate with CXCR4 possibly through a PI3K mediated program of survival. Altogether, the signals driving β-selection result in the development of DP thymocytes, which may ultimately differentiate into mature T cells following stimulation through the TCR.

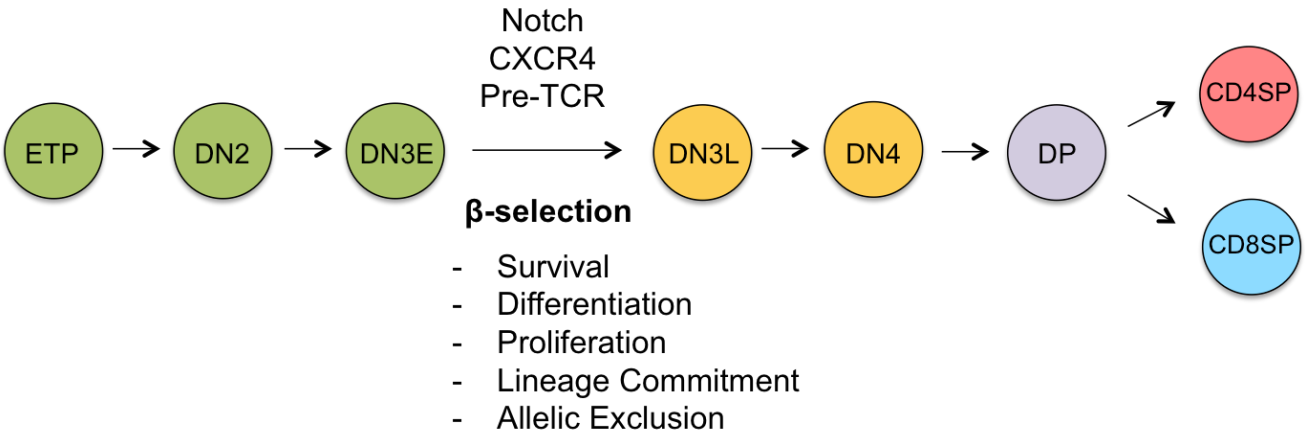

Figure 1-2. **Defining features of thymocyte β-selection.** Thymocytes develop through the early ETP and DN2 stages, during which VDJ recombination is initiated at the TCRβ, γ and δ loci. At the DN3 stage, cells that have successfully undergone recombination at the TCRβ locus may pass through the critical β-selection checkpoint. Functionally rearranged TCRβ molecules can form heterodimeric complexes with the germline encoded invariant pre-Tα chain to form the pre-TCR. It has been shown that signals transduced through the pre-TCR, Notch and chemokine receptor CXCR4 are required for β-selection to occur. The result of β-selection is continued survival, differentiation, proliferation and initiation of VDJ recombination at the TCRα loci, allowing the generation of a robust DP thymocyte population. Furthermore, thymocytes passing through β-selection lose their γδ lineage potential and are committed to the αβ developmental pathway. In addition, further rearrangements at the TCRβ loci are repressed.

Fates of DP Thymocytes

Although all DP thymocytes that have successfully undergone recombination at the TCRβ and TCRα loci may express mature αβ-TCRs, not all of these cells experience the same developmental fate. Due to the random nature of VDJ recombination, each DP thymocyte potentially expresses a unique mature TCR. Although this property of VDJ recombination is useful for generating a T cell repertoire capable of recognizing a wide array of antigens from incoming pathogens, it also unleashes the potential to generate T cells that strongly interact with self. At the DP stage, the subsequent development of these thymocytes is dictated by the affinity with which they bind self-peptide MHC complexes presented within the thymus (*39*). Despite the complexity of T cell development, many thymocytes express TCRs that are unable to recognize self-peptide-MHC and these cells undergo a process termed death by neglect. Since these cells are unable to interact with self-peptide-MHC presenting cells in the thymus, these thymocytes are unable to receive the signals required for further survival and differentiation and experience a passive process of cell death. In contrast, DP thymocytes expressing TCRs that bind to self-peptide-MHC with relatively low affinity undergo positive selection and subsequently develop into CD4 or CD8 'single positive' (SP) thymocytes, depending on whether they recognize antigen in the context of MHC class II or MHC class I, respectively (**Fig. 1-3**). However, DP thymocytes that bear TCRs that strongly bind to self-peptide-MHC experience negative selection, whereby these highly self-reactive cells are physically or functionally removed from the T cell repertoire. Negative selection occurs through multiple mechanisms including clonal deletion, receptor editing and the induction of anergy (*30*). Despite numerous mechanisms of

negative selection having been described, clonal deletion appears to play the most significant role. In particular, pro-apoptotic members of the Bcl-2 family, such as Bim, have been shown to induce thymocyte clonal deletion following high affinity antigen stimulation (*40, 41*). Therefore, cells that are overtly self-reactive are removed from the T cell repertoire, while cells that weakly recognize self are permitted to complete their maturation into $CD4^+$ or $CD8^+$ T cells (**Fig. 1-3**).

Although the positive/negative selection paradigm has been critical to our understanding of T cell development, more recently it has been appreciated that high affinity, or agonist, stimulation through the TCR is also required for positive selection of several alternatively selected thymocyte lineages (**Fig. 1-3**). This process has been termed "agonist selection" and instructs the development of numerous non-conventional T cell lineages such as invariant natural killer T cells (iNKT), natural regulatory T cells (nTreg) and natural T_H17 cells (nT_H17), within the thymus (*39*). In addition, agonist selection guides the development of thymic precursor cells that give rise to a subset of intestinal $TCR\alpha\beta^+$ intraepithelial lymphocytes (IEL) characterized by their unique expression of unconventional CD8αα homodimers ($TCR\alpha\beta^+CD8\alpha\alpha$ IEL), known as IEL progenitors (IELp) (**Fig. 1-3, 4**). Interestingly, mice overexpressing anti-apoptotic Bcl-2 (*42*) and mice deficient in pro-apoptotic Bim (*43*) have been shown to contain increased numbers of IELp cells, due to diversion of cells normally destined for clonal deletion, into the IELp lineage. Therefore, although negative selection serves to remove self-reactive cells from the T cell pool, there are also mechanisms in place to nurture the development of some self-reactive T cell subsets.

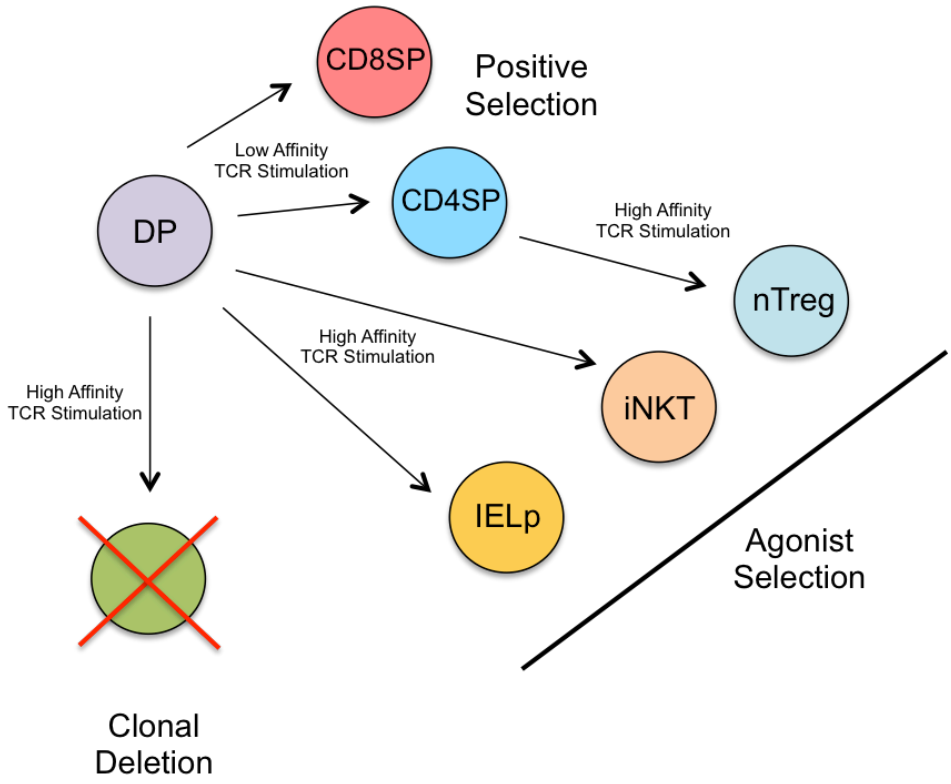

Figure 1-3. **TCR signal strength and DP thymocyte selection.** Following productive β-selection, developing thymocytes expressing a functionally rearranged αβTCR upregulate expression of CD4 and CD8 to form the 'double positive' (DP) thymocyte population. DP thymocytes can experience a number of different fates depending on the affinity with which they bind to self-peptide-MHC complexes presented by a number of different cell types within the thymus. The majority of thymocytes do not express a TCR capable of binding self-peptide-MHC and undergo a passive cell death process known as death by neglect. In contrast, DP thymocytes that weakly bind self-peptide-MHC undergo positive selection and develop into either CD4 or CD8 'single positive' (SP), depending on whether they recognize antigen in the context MHC class II or MHC class I, respectively. Classically, it has been thought that DP thymocytes expressing TCRs that strongly recognize self-peptide-MHC complexes undergo a process of programmed cell death called clonal deletion, as a means of removing T cell clones from the T cell repertoire which strongly recognize self. However, it has more recently been appreciated that some thymocytes that strongly recognize self-peptide-MHC can undergo an alternative positive selection process referred to as agonist selection. Some T cell subsets known to be derived from agonist selection include nTreg, nT_H17, iNKT and IEL progenitor cells. Agonist selected IELp cells can traffic to small intestine to further develop into TCR$\alpha\beta^+$CD8$\alpha\alpha$ IEL and will be extensively studied in this thesis.

Intraepithelial Lymphocyte Development

The intestinal epithelium contains distinct populations of intraepithelial lymphocytes (IELs) that are thought to regulate intestinal homeostasis and whose dysregulation has been implicated in numerous disease models (*44*). Intestinal IELs are heterogeneous, reflecting their distinct developmental origins. Broadly, the intestinal IEL pool includes both the γδ- and αβ-T cell lineages. TCRαβ$^+$ IEL can be further classified into "natural" and "induced" subsets (*44*). Induced IEL arise from mature naïve CD4$^+$ or CD8$^+$ T cells following peripheral activation. Natural IELs are generated from thymic precursors that migrate to the intestine where they complete their development (**Fig. 1-4**). Furthermore, natural IELs are characterized by expression of unconventional CD8αα homodimers and lack expression of conventional CD8αβ heterodimers. Local IL-15 signals induce expression of the transcription factor T-bet and the CD8αα homodimer (**Fig. 1-4**) (*45-48*). Historically, the identity of the thymic precursor of TCRαβ$^+$CD8αα IEL has been contentious. While early work suggested pre-selection DN thymocytes as the direct IEL precursor, more recent studies have conclusively shown IEL progenitors (IELp) arise from DP thymocytes following a high affinity TCR signal (*49-51*). Part of the confusion likely stemmed from the fact that following a high affinity TCR signal, DP thymocytes downregulate expression of CD4 and CD8 and IELp cells reside within the DP^{dull} and DN thymocyte fractions (*43, 49*). Within this DN + DP^{dull} population, IELp cells display high expression of TCRβ and CD5, but only a fraction of this population expresses markers of high affinity Ag encounter, such as CD122, PD-1 and Helios (*43, 45, 49, 52*). Whether the entire TCRβ$^+$CD5$^+$ population, or only a sub fraction thereof, is truly an IELp is unclear. IELp cells are also thought to upregulate expression of gut-homing molecules

like the chemokine receptor CCR9 (*53, 54*) and the integrins $\alpha_4\beta_7$ and CD103 within the thymus (*55, 56*). However, the role precise role of these trafficking molecules in TCRαβ$^+$CD8αα IEL generation remains unknown.

Following the migration of IELp cells from the thymus to the small intestine, these agonist selected cells develop into TCRαβ$^+$CD8αα IEL. Although the pathways that regulate the development of TCRαβ$^+$CD8αα IELs are beginning to emerge, the precise function of these cells remains unclear. Despite bearing self-specific TCRs, under homeostatic conditions TCRαβ$^+$CD8αα IELs appear to be quiescent. However, these cells show high expression of molecules associated with cytotoxic T cells, such as granzyme B and Fas ligand, but show relatively low expression of the effector cytokine IFNγ (*44, 57, 58*). Given the location of TCRαβ$^+$CD8αα IELs within the gut epithelium, these cells may potentially utilize their cytotoxic capacity to regulate the clearance of damaged or cancerous intestinal epithelial cells (*44*). In contrast, TCRαβ$^+$CD8αα IELs have also been shown to express molecules associated with suppressing immune responses such as TGF-β and LAG3 and have also been shown to express NK cell receptors (*44, 58*). Supporting a regulatory role for TCRαβ$^+$CD8αα IELs, it was shown that TCRαβ$^+$CD8αα IELs could suppress inflammation in a cell-transfer model of colitis, when adoptively transferred into recipient animals at high numbers (*44, 59*).

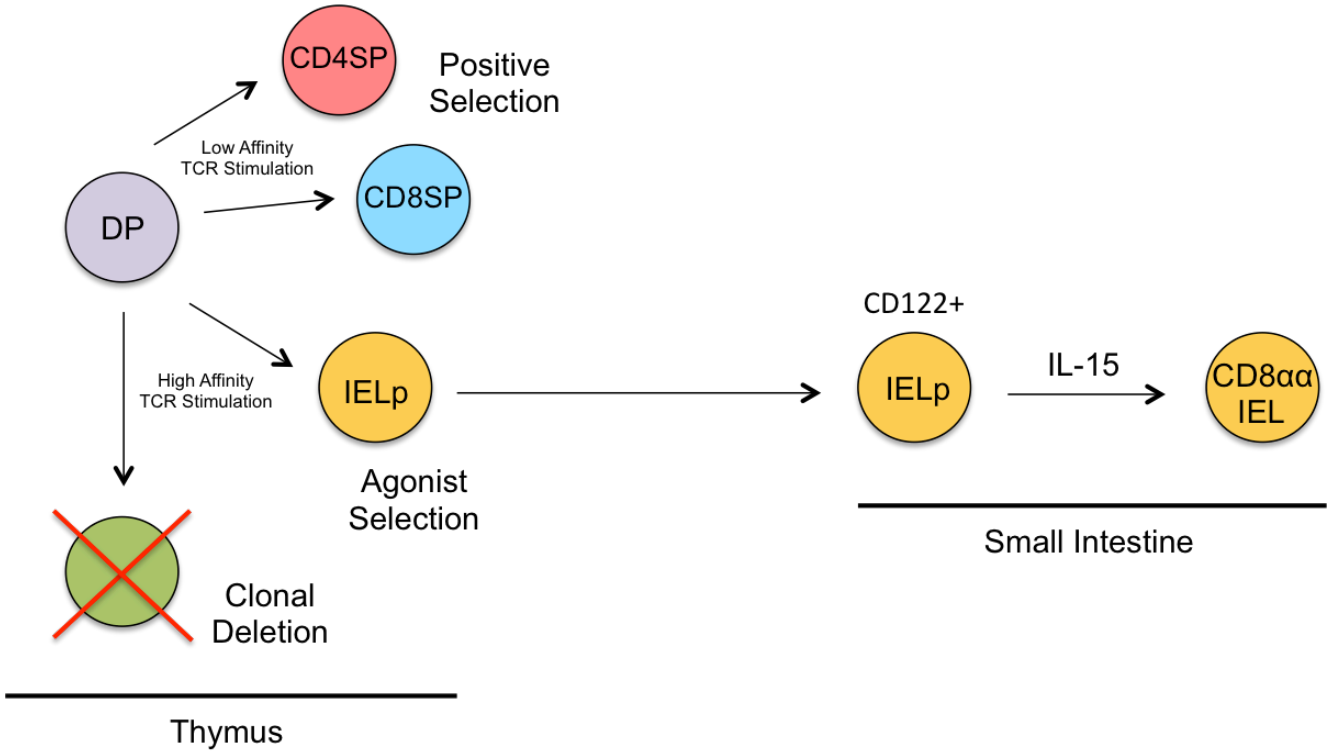

Figure 1-4. **TCRαβ$^+$CD8αα IELs develop from agonist selected IELp cells.** Although the development of TCRαβ$^+$CD8αα IEL occurs in the small intestine, these cells ultimately require the generation of agonist selected IEL progenitor (IELp) cells for their development. Following agonist selection of IELp cells, these progenitors are able to transit out of the thymus and traffic to the small intestine via circulation. Upon reaching the gut, these cells rely upon IL-15 for their differentiation into TCRαβ$^+$CD8αα IEL. IELp cells express IL-2Rβ (CD122), which they require to respond to IL-15 provided within the small intestine. Signaling through this axis results in the upregulation of the critical lineage specifying transcription factor T-bet, which is required for TCRαβ$^+$CD8αα IEL development. Furthermore, IL-15 signals have been shown to induce upregulation of CD8αα homodimers, which is a key defining characteristic of this population. To date, the precise function of the TCRαβ$^+$CD8αα IEL population remains unclear. However, given the requirement of agonist selection signals for the development of TCRαβ$^+$CD8αα IEL, it is clear that this population is enriched with T cells bearing TCRs with a high degree of self-reactivity.

TCR signaling and thymocyte selection

Signaling initiated downstream of the TCR ultimately dictates the developmental fate of immature DP thymocytes. Although the αβ-TCR specifically binds to antigens presented in the context of MHC molecules, the TCR complex also includes numerous other accessory molecules that control the functionality of this critical receptor. Notably, the intracellular tails of the TCR α and β chains lack the ability to initiate downstream signaling alone and the TCR complex also includes members of the CD3 gene family to transduce signals received through the TCR (*60*). Specifically, the CD3 co-receptor complex is made up of γ, δ, ε and ζ chains, which are organized as εγ, δε and ζζ dimers. The γ, δ and ε chains have prominent extracellular domains and are closely associated with the αβ-TCR on the cell surface. In contrast, the CD3ζ chains lack significant extracellular regions and instead feature prominent intracellular domains that serve as key sites for the initiation of TCR signaling. The ability of CD3 molecules to transduce signals detected through the TCR is mediated by immunoreceptor tyrosine-based activation motifs (ITAMs) present in the intracellular tails of the CD3 chains. Each of the CD3 γ, δ and ε chains contain one ITAM, while each of the CD3ζ chains contain three ITAMs. Immediately following TCR engagement by peptide-MHC binding, the TCR signaling pathway is initiated through phosphorylation of ITAM tyrosine residues by the Src family kinase Lck (*61*). The cytoplasmic tails of CD4 and CD8 are closely associated with Lck and engagement of the CD4/CD8 co-receptors with MHC molecules during TCR stimulation brings Lck in close proximity to the CD3 ITAMs (**Fig. 1-5**). Phosphorylation of cytoplasmic ITAMs results in the SH2 domain mediated recruitment and activation of the Syk family kinase ZAP-70 (**Fig. 1-5**). Activated ZAP-70 kinase

activity drives the TCR signaling cascade forward by phosphorylating numerous tyrosine residues on the important adaptor molecule linker of activated T cells (LAT) (*62*). Phosphorylated tyrosine residues present on LAT serve as docking sites for a variety of other signaling proteins that mediate the activation of numerous downstream signaling pathways (**Fig. 1-5**). One such protein is phospholipase C γ 1 (PLCγ1), which binds phosphorylated LAT at Y136. Regulating its activation state, PLCγ1 requires phosphorylation for its full activation, which is dependent on the recruitment of the GADS-SLP-76 adaptor to LAT. Following GADS-SLP-76 binding to LAT, the kinase ITK is recruited to the adaptor complex and can phosphorylate and activate PLCγ1. Upon activation, PLCγ1 lipase activity results in the cleavage of cell membrane phosphatidylinositol-4,5-bisphosphate (PIP_2), generating diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). These products of PLCγ1 activity are potent signaling second messengers that activate numerous downstream signaling molecules. In particular, DAG regulates the cell membrane recruitment and activation of numerous signaling molecules that bear DAG binding C1 domains. Some of the signaling pathways regulated by DAG include the highly conserved Ras and protein kinase C (PKC) pathways, both of which are critical drivers of T cell development (*63, 64*). In addition, IP_3 regulates Ca^{++} flux into the cytoplasm during T cell activation, which regulates numerous Ca^{++} dependent-signaling pathways that also influence T cell development (*65*).

The strength of signals transduced through the TCR machinery is central to the outcome of thymocyte selection. Likewise, disruption of the TCR signaling machinery causes impairments in thymocyte selection. Given the important role of CD3 in initiating

TCR signaling, it is perhaps not surprising that deletion of members of the CD3 complex or mutation of CD3-ITAMs in thymocytes results in impaired thymocyte positive and negative selection (*66-68*). Furthermore, analysis of ZAP-70 deficient thymocytes has shown that ZAP-70 activity is also critical during thymocyte positive and negative selection (*69-71*). The adaptor LAT is the downstream target of ZAP-70 kinase activity and has also proven to be critical in regulating positive and negative selection (*72, 73*). In particular, phosphorylated tyrosine residues present on LAT serve as docking sites for a variety of other signaling molecules including PLCγ1, GADS and SLP-76, all of which are required for both positive and negative selection (*74-76*). Furthermore, the signal transduction pathway downstream of the pre-TCR is thought to be similar to that of the mature αβ-TCR and mice deficient in kinases Zap70 or Syk, or the adaptors LAT or SLP-76, also show a profound block in T cell development at the β-selection checkpoint (*77-81*). Therefore, in addition to regulating the selection of DP thymocytes, the TCR signaling pathway also regulates thymocyte β-selection. One critical signaling pathway activated downstream of LAT is the highly conserved Ras pathway and the role of Ras in regulating T cell development will be the focus of this thesis.

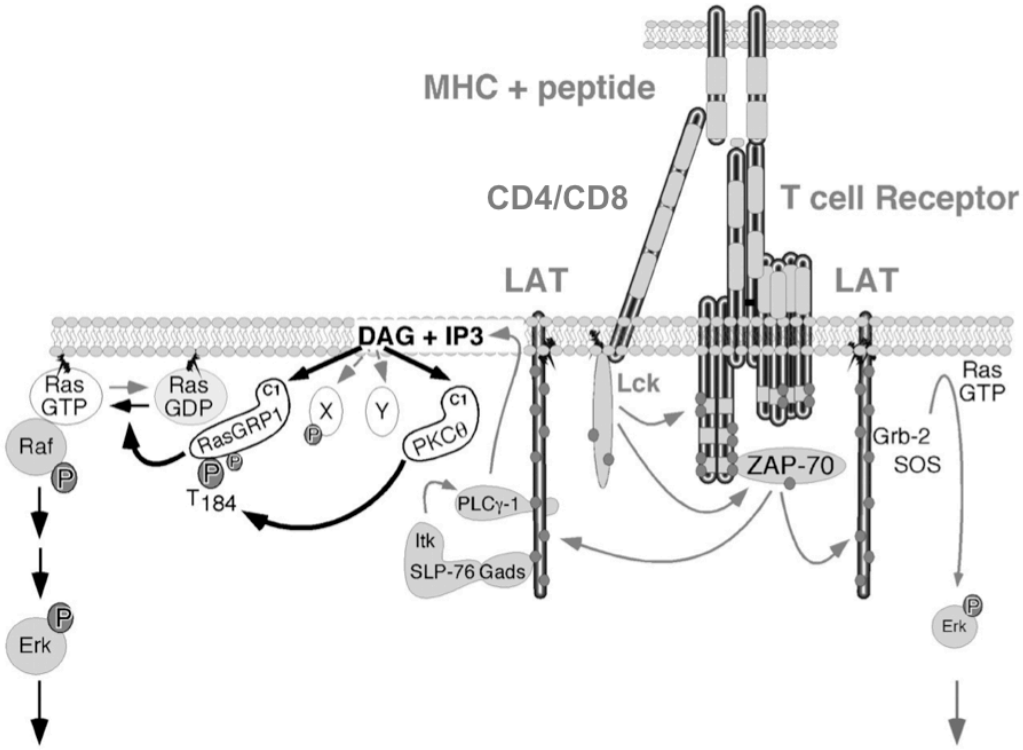

Figure 1-5. **An overview of the TCR signaling pathway**. Signaling through the T cell receptor (TCR) is initiated upon binding of peptide-MHC to the TCR. Following antigen recognition, ITAM residues present in the intracellular tails of the CD3 molecules are phosphorylated by Lck. Phosphorylation of CD3 ITAMs results in the recruitment of Zap-70 to the TCR complex. The recruitment of Zap-70 results in the extensive phosphorylation of the critical adaptor molecule LAT. The LAT adaptor contains numerous tyrosine residues, which serve as docking sites for the recruitment of several important signaling molecules. One particularly important molecule that is recruited to this complex is the lipase PLCγ1. Activation of PLCγ1 is dependent on its recruitment to this complex, where it is phosphorylated and activated by the Itk kinase. The recruitment of Itk to LAT is dependent on its association with the GADS-SLP-76 adaptor complex, which directly binds to LAT. The activation of PLCγ1 leads to the production of DAG and IP_3, which regulate numerous downstream signaling pathways. In particular, DAG regulates the activation of the Ras-ERK signaling pathway through C1 domain dependent activation of RasGRP1. In addition, DAG also mediates the activation of PKCθ, again in a C1 domain dependent manner. Interestingly, PKCθ has been shown to phosphorylate RasGRP1, regulating its activation. Another activator of Ras-ERK signaling downstream of the TCR is Sos1. Sos1 is recruited to LAT via its adaptor Grb2, which directly binds to LAT. Figure adapted from Roose et al., Molecular and Cellular Biology, 2005 (*2*)

Ras pathway in thymocytes

Ras proteins are small membrane anchored G-proteins that control the activation of numerous cellular signaling pathways. Notably, Ras controls the activation of the Raf-MEK-ERK signaling cascade, which results in the phosphorylation and activation of extracellular signal-related kinase (ERK) (**Fig. 1-6A**). Signaling through this pathway induces cellular activities such as differentiation, survival and proliferation, all of which are important in many different developmental contexts (*82*). The activation of Ras is regulated by its guanine nucleotide binding state such that GTP-Ras is in a catalytically active state, while GDP-Ras is inactive. The association of Ras with guanine nucleotides is regulated by two classes of proteins: Ras guanine nucleotide exchange factors (RasGEFs) and Ras GTPase activating proteins (RasGAPs). RasGEFs regulate the activation of Ras through promoting the dissociation of GDP from GDP-Ras, allowing the more cellularly abundant GTP to associate with Ras. In contrast, RasGAPs negatively regulate Ras through activating the intrinsic GTPase activity of Ras, promoting the hydrolysis of GTP on GTP-Ras, resulting in Ras inactivation.

An early study examining the role of Ras activity in T cell development using mice expressing a dominant negative Ras (dnRas) in thymocytes showed defects in DP positive selection (**Fig. 1-6B**), suggesting that the Ras pathway plays an important role during T cell development (*83*). Subsequent work elegantly demonstrated that the activation kinetics of the Ras-ERK cascade is differentially regulated during thymocyte positive and negative selection (*84-86*). During positive selection, thymocytes show a sustained increase in ERK activation while thymocytes undergoing negative selection

show a strong, but transient, burst of ERK activation. Notably, numerous studies have shown that ERK activity is dispensable during thymocyte negative selection, but is required for efficient thymocyte positive selection (*84, 87, 88*).

Two different classes of RasGEFs are known to regulate Ras activation in T cells during development. One of these RasGEFs is called son of sevenless 1 (Sos1) and it is thought that Sos1 is involved in regulating Ras activation during β-selection (**Fig. 1-6B**). The catalytic domains of Sos1 that are responsible for Ras activation are the Ras exchange motif (REM) and Cdc25 domains. A study from Kortum et al. demonstrated that Sos1 deficient mice showed increased frequencies of DN3 thymocytes relative to wild type mice, suggesting that Sos1 activity regulates β-selection (*89*). The findings of this study revealed a critical role for RasGEF activity during early T cell development and prompt the examination of mechanisms of Ras activation during these immature stages. Another class of RasGEFs known to be active during T cell development are called Ras guanine nucleotide releasing proteins or RasGRPs. The focus of this thesis will be on the roles of RasGRPs during T cell development.

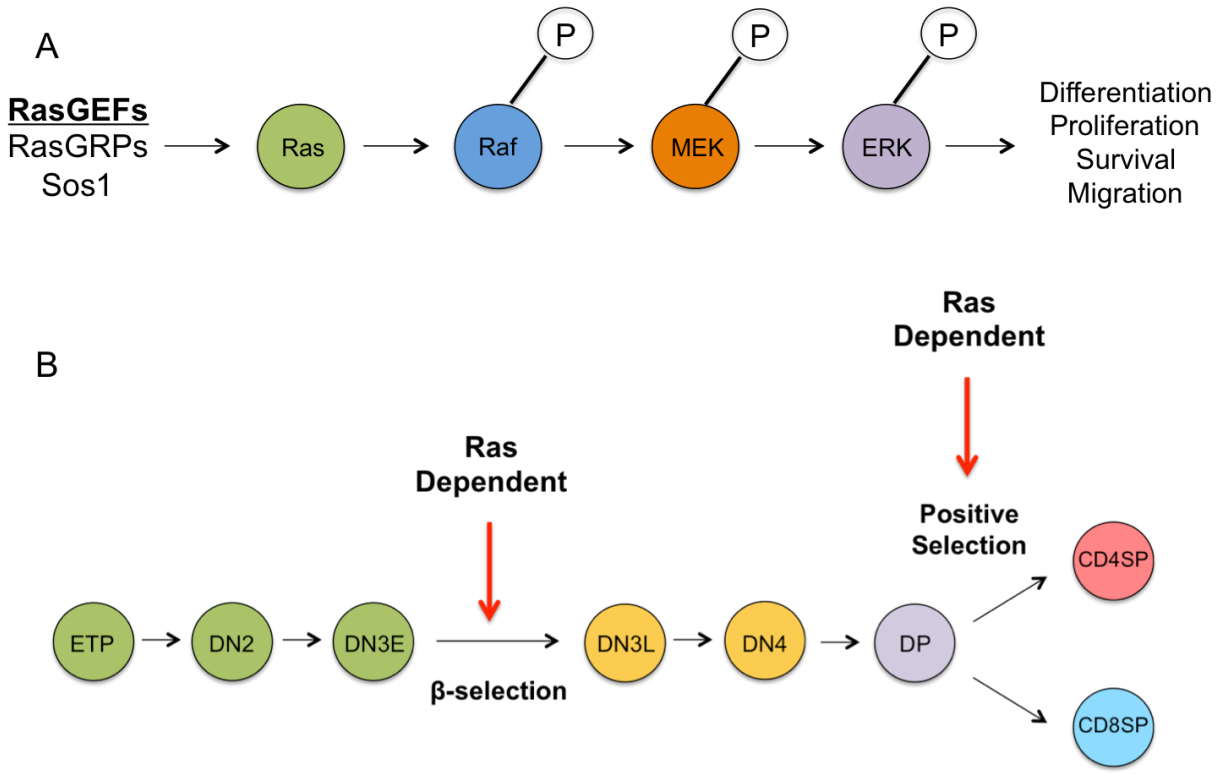

Figure 1-6. **Ras signaling and T cell development.** A) Signaling through the Ras pathway in T cells is promoted by multiple RasGEFs, including members of the RasGRP family and Sos1. RasGEF activity results in the association of GTP with Ras, leading to its activation. The result of Ras activation is the initiation of the downstream Raf-MEK-ERK signaling cascade. Ras activity promotes the kinase activity of Raf, leading to the phosphorylation and activation of MEK. Activated MEK can phosphorylate and activate ERK, which regulates numerous downstream pathways involved in numerous cellular processes, such as differentiation, proliferation, survival and migration. B) Previous studies examining the role of Ras activity during T cell development have implicated Ras signaling at multiple stages of T cell development. To date, Ras activity has been shown to regulate development at the critical β-selection and positive selection transitions. However, Ras activity has not been extensively studied at all stages of development and may play additional roles in the generation of T lineage cells.

RasGRP Family

RasGRPs are a family of RasGEFs expressed in numerous hematopoietic lineage cells. The RasGRP family consists of RasGRP1, RasGRP2, RasGRP3 and RasGRP4. All RasGRP family members contain REM and Cdc25 domains, similar to Sos1, that are responsible for mediating Ras activation. RasGRP2 has been reported to be important in controlling platelet adhesion, while RasGRP4 has been shown to control Ras activation in mast cells (*90, 91*). The remaining RasGRP family members, RasGRPs 1 and 3, function prominently in lymphocytes and will be the focus of this thesis. RasGRP1 is a critical part of the TCR signaling machinery and is required for ERK activation following stimulation of both thymocytes and peripheral T cells (*92, 93*). RasGRP3 is expressed most prominently in B cells and is thought to function downstream of the B cell receptor (BCR) (*94, 95*). However, low levels of RasGRP3 expression are detectable in T cells throughout T cell development (*96*). In addition to the previously described catalytic domains, RasGRPs 1 and 3 contain several similar regulatory domains that are important in modulating RasGRP1/3 activity during signaling (**Fig. 1-7**). Both RasGRPs 1 and 3 contain C1 domains, which bind DAG and recruit RasGRP1/3 to cell membranes, bringing them in close proximity to membrane anchored Ras (*97*). Likewise, RasGRPs depend on the generation of DAG by PLC family members for their activity. Also, RasGRPs 1 and 3 are phosphorylated on threonine residues T184 and T133, respectively, by PKC (*2, 98*). It is thought that PKC phosphorylation of RasGRPs 1 and 3 is required for full activation of these proteins, however the physiological role of RasGRP1/3 phosphorylation is still unclear. Despite similarities in regulatory domain structures, the C-terminal regions of RasGRPs 1 and 3 are less conserved. RasGRP1 contains a C-

terminal phosphoinositide-targeting (PT) domain that is thought to bind highly phosphorylated phosphoinositide residues generated by PI3K activity and may be important for recruiting RasGRP1 to the cell membrane (*99*). In contrast, the C-terminal region of RasGRP3 contains a domain found to interact with dynein light chain, however the physiological relevance of this interaction remains unclear (*100*).

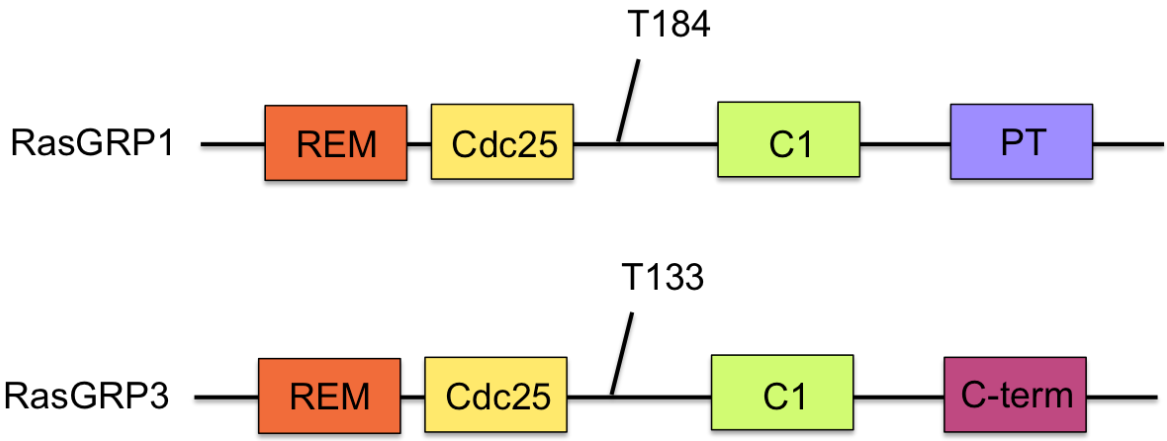

Figure 1-7. **RasGRP1 and RasGRP3 regulatory domains.** Both RasGRPs 1 and 3 contain REM and Cdc25 domains, which make up the catalytic core of these proteins. RasGRPs 1 and 3 have also both been shown to undergo regulatory phosphorylation by PKC at threonine residues 184 and 133, respectively. Furthermore, RasGRPs 1 and 3 both contain C1 domains that have been shown to bind diacylglycerol and recruit RasGRPs to cellular membranes. The C-termini of RasGRPs 1 and 3 are much less conserved than the rest of these proteins. RasGRP1 contains a C-terminal phosphoinositide-targeting (PT) domain that is though to recruit RasGRP1 to cell membranes through binding highly phosphorylated phosphoinositide membrane lipids. In contrast, RasGRP3 has a C-terminal domain that has been shown to interact with dynein light chain.

RasGRPs and T cell Development

RasGRP1 is highly expressed in thymocytes (*96*) and has been implicated in multiple events during T cell development. Similar to dnRas expressing thymi, RasGRP1 deficient thymi show reduced frequencies of SP thymocytes (*101*), demonstrating that RasGRP1 activity is critical for thymocyte positive selection. In contrast, numerous studies have shown that RasGRP1 activity is dispensable for thymocyte negative selection (*92, 102*). This differential requirement of RasGRP1 during thymocyte selection events remains unclear and warrants further study. In addition, the role of RasGRP1 during thymocyte agonist selection has not been extensively studied and may prove useful in dissecting the various roles of RasGRP1 during thymocyte selection. Also, similar to Sos1 deficient thymi, RasGRP1 deficient thymi show increased numbers and frequencies of DN3 thymocytes, suggesting that RasGRP1 also regulates β-selection (*103*). However, the precise mechanism through which RasGRP1 regulates β-selection has not been determined. Interestingly, RasGRP family members other than RasGRP1 have recently been implicated in T cell development. A report from Zhu et al. examined the contributions of RasGRP1 and RasGRP4 to T cell development and found that RasGRP1/4 double deficient thymi showed more dramatic impairment of β-selection than RasGRP1 deficient thymi, suggesting that RasGRP4 also contributes to T cell development (*103*). Although RasGRP1 has been classically thought to be the only RasGRP family member involved in T cell development, the finding that RasGRP4 is also involved in T cell development has prompted us to examine the involvement of other RasGRP family members in T cell development. Given the structural similarities between

RasGRP1 and RasGRP3, we propose to examine the role of RasGRP3 during T cell development.

Rationale and Objectives

1) Although RasGRPs have been implicated early on in T cell development, in particular at the β-selection checkpoint, RasGRP activity has not been studied at the earliest stages of development within the thymus. Furthermore, while much is known regarding the cell surface receptors and transcription factors that are important for early T cell development events, the intracellular signaling events that promote this developmental program are less clear. Notably, data provided by the Immgen consortium indicate that a number of putative transcriptional targets of the Ras pathway are induced in ETPs (*104*). We hypothesize that RasGRP-mediated regulation of Ras activity is involved in the early stages of T cell development in the thymus and we will seek to determine the potential roles of RasGRPs 1 and 3 in regulating the development of immature thymocyte populations.

2) RasGRP activity appears to be an important regulator of thymocyte β-selection. Although previously published data have shown that RasGRP1 knock-out (1KO) and RasGRP1/4 double KO (DKO) mice display a block in thymocyte β-selection, an in depth analysis of which β-selection events were RasGRP dependent was not performed. Additionally, since there was a more profound β-selection block in DKO mice relative to 1KO, there is evidence that multiple

RasGEFs regulate early T cell development. Therefore, it is possible that RasGRP family members other than RasGRP1 and RasGRP4 also regulate β-selection. Again, given the structural similarities between RasGRP1 and RasGRP3, we hypothesized that RasGRP3 may also contribute to thymocyte β-selection. We will examine the potential roles of RasGRPs 1 and 3 at the β-selection checkpoint and we will seek to determine the mechanism through which RasGRPs regulate this process.

3) RasGRP1 activity during thymocyte positive and negative selection has been extensively studied, however the role of this critical Ras activator in agonist selection remains unclear. Recent work suggests that RasGRP1 regulates agonist selection of the iNKT lineage (*105*), but the role of RasGRP1 in regulating other agonist selected lineages has not been examined. We hypothesize that RasGRP1 regulates agonist selection of the intestinal TCRαβ^{+}CD8αα IEL lineage and we will examine the role of RasGRP1 in regulating the development of this population.

Overall Theses

1) RasGRPs 1 and 3 are required for the efficient development of the ETP population. In particular, RasGRPs 1 and 3 regulate the ability of circulating progenitors to establish the ETP pool within the thymus. RasGRP1/3 DKO progenitors show impaired migration towards the CCR9 ligand CCL25 and likely regulate progenitor entry into the thymus through this axis.

2) RasGRP1, but not RasGRP3, is required for thymic β-selection. RasGRP1 deficient thymocytes show impaired transition through β-selection, which is associated with a reduction in cellular proliferation at this stage. Furthermore, RasGRP1 deficient thymocytes show impaired ERK activation following CXCR4 stimulation of DN3 cells, describing a potential mechanism through which RasGRP1 regulates this developmental process.

3) RasGRP1 regulates agonist selection of TCRαβ^{+}CD8αα IEL cells through control of thymic IELp generation. In addition, RasGRP1 is differentially involved during thymocyte agonist selection and clonal deletion, highlighting the different signaling requirements for these opposing fates, which both rely upon strong TCR stimulation.

CHAPTER 2: MATERIALS AND METHODS

This chapter contains content from the following sources, republished with permission:

- Golec, D.P., Caviedes, L.M.H., and Baldwin, T.A. 2016. RasGRP1 and RasGRP3 Are Required for Efficient Generation of Early Thymic Progenitors. J Immunol *197*, 1743-1753
- Golec, D.P., Dower, N.A., Stone, J.C. and Baldwin, T.A. 2013. RasGRP1, but not RasGRP3, is required for efficient thymic β-selection and ERK activation downstream of CXCR4. PLoS One *8*: e53300
- Golec, D.P., Hoeppli, R.E., Caviedes, L.M.H., McCann, J., Levings, M.K. and Baldwin, T.A. 2017. Thymic progenitors of TCRαβ^{+} CD8αα intestinal intraepithelial lymphocytes require RasGRP1 for development. J Exp Med. *214,* 2421-2435.

Mice

C57BL/6 mice were purchased from The Jackson Laboratory. The generation of RasGRP1 KO, RasGRP3 KO and RasGRP1/3 DKO has been previously described (*94, 101*). In addition, the generation of Bim KO(*106*), HY^{cd4} (*107*), Nur77^{GFP} (*108*), RAG2p-GFP(*109*) and RAG1$^{-/-}$(*110*) mice has been previously described. WT CD45.1^{+} and CD45.1/2^{+} mice were maintained in our colony. RAG2p-GFP and RAG1$^{-/-}$ mice were kindly provided by Dr. Colin Anderson. All mice were maintained on the C57BL/6 background. For all strains, mice of both sexes were used between 4 weeks and 6 months of age. All mice were treated in accordance with protocols approved by the University of Alberta Animal Care and Use Committee.

BrdU

Mice were injected i.p. with 1 mg of BrdU and were euthanized 2h after injection. Thymocyte BrdU incorporation was assessed by flow cytometry using a protocol adapted from the BD APC BrdU Flow Kit (BD Biosciences). Thymocytes were first stained for surface markers in FACS buffer as described above. Following surface staining, thymocytes were resuspended in BD Cytofix (BD Fix/Perm kit), incubated on ice for 20 min and washed twice in 1X Fix/Perm buffer (BD Fix/Perm kit). Cells were then treated with a solution of 90% FCS and 10% DMSO on ice for 10 min and washed twice in 1X Fix/Perm buffer. Thymocytes were again treated with BD Cytofix on ice for 5 min and washed twice in 1X Fix/Perm buffer. Cells then underwent DNase treatment for 1 h at 37°C and were washed twice in 1X Fix/Perm buffer. Thymocytes were then stained with anti-BrdU in 1X Fix/Perm buffer for 30 min on ice, washed twice in FACS buffer and analyzed by flow cytometry.

Competitive BM Chimeras

Wild type ($CD45.1^+$) and RasGRP1/3 DKO ($CD45.2^+$) BM cells were mixed 1:1 and at least 5 $x10^6$ cells were transplanted into lethally irradiated (1000 Gy) wild type mice ($CD45.1^+$) by tail vein injection. T cells were depleted from BM donors by i.p. administration of 100 μg anti-Thy1.2 (clone 30H12) on days -1 and -2 prior to BM harvest. Mice were provided with antibiotic water (40 mg/L neomycin and 15 mg/L polymyxin or novotrimel) for 4 weeks post-injection. Mice were allowed to reconstitute for at least 12 weeks before analysis.

Chemotaxis transwell Assays

BM cells were harvested from wild type and DKO mice and cultured in opti-MEM (Life Technologies) at 37°C for 1.5 hours. 2 x 10^6 cells were placed in the top well of chemotaxis plates (Corning – 5μm pore), with media controls or chemokines placed in the bottom well. Chemotaxis plates were incubated at 37°C for 5 hours. For inhibitor experiments, DMSO, LY294002 (20 μM) or U0126 (20 μM) were added to the upper well of chemotaxis plates prior to incubation with chemokines. Cells were harvested from the bottom well of chemotaxis plates and analyzed by flow cytometry. 2 x 10^6 cells input BM cells were analyzed by flow cytometry as a control. CountBright™ beads (Life Technologies) were included in all samples to determine cell numbers and progenitor migration was measured as % migration ((# migrating cells/# input cells) x 100).

OP9-DL1 Culture

OP9-DL1 cells were maintained as previously described (*111*). A total of 4x10^4 DN3E were cultured in DM20 (DMEM, 5 mM Hepes, 50 units (mg/mL) penicillin/streptomycin, 2 mM L-glutamine, 50 mM 2-mercaptoethanol, 50 mg/mL gentamicin sulfate) supplemented with 1 ng/mL IL-7 in 96 wells plates previously seeded with 1.2x10^4 OP9-DL1 cells at 37°C. Co-cultures were harvested through a 70μm cell strainer after 24 h and 48 h of culture and analyzed by flow cytometry.

SDF-1α Stimulations

Total thymocytes were resuspended at $1x10^7$ cells/mL in DMEM with 0.1% BSA. Cells were pre-treated or not with 20μM of the PI3K inhibitor, LY294002, for 30 min at 37°C. Cells were stimulated with 10nM SDF-1α (PeproTech) for various time points, immediately fixed in 1X BD Phosflow Lyse/Fix Buffer (BD Biosciences) and washed once with FACS buffer. Thymocytes were then permeabilized in 90% methanol on ice for a minimum of 30 min and washed twice in FACS buffer. AKT activation was detected with anti-pAKT (1/100) followed by incubation with an Alexa fluor 647 conjugated anti-rabbit secondary antibody. ERK activation was detected with anti-pERK (1/400) followed by incubation with biotin conjugated anti-mouse anitbody and APC conjugated streptavidin. Cells were also stained with Abs to surface markers and analyzed by flow cytometry. ERK/AKT phosphorylation was quantified as fold induction of mean fluorescent intensities of stimulated samples/unstimulated samples.

IEL Preparations

Small intestines were removed from mice, cleaned and Peyer's patches were excised. Intestines were opened longitudinally, rinsed of contents and cut into ~0.5 cm pieces. Intestinal pieces were placed in Ca^{2+}/Mg^{2+} free HBSS supplemented with 5% FCS, 5 mM EDTA and 2 mM DTT and shaken at 37°C for 30 min. Following incubation, cell-containing media was passed through 70 μm cell strainers and cells were pelleted. Cell pellets were resuspended in 40% Percoll, layered on top of an 80% Percoll solution and the gradient was centrifuged at 900g for 20 min. IEL were extracted from the 40%/80%

interface, washed in PBS and used for analysis. Flow cytometry and CountBright™ beads (Life Technologies) were used for quantification.

Adoptive Transfer Experiments

Thymocytes were obtained from congenic CD45.1$^+$ and CD45.1/2$^+$ donor mice and stained as described above in PBS supplemented with 2% FCS. Five IELp populations were sorted from donor mice: CD4$^{-/dull}$CD8$^{-/dull}$NK1.1$^-$TCRβ$^+$CD5$^+$CD122$^-$, CD4$^{-/dull}$CD8$^{-/dull}$NK1.1$^-$TCRβ$^+$CD5$^+$CD122$^+$, CD4$^{-/dull}$CD8$^{-/dull}$NK1.1$^-$TCRβ$^+$CD5$^+$CD122$^+$PD-1$^+$α$_4$β$_7$$^+$CD103$^-$, CD4$^{-/dull}$CD8$^{-/dull}$NK1.1$^-$TCRβ$^+$CD5$^+$CD122$^+$PD-1$^+$α$_4$β$_7$$^-$CD103$^-$ and CD4$^{-/dull}$CD8$^{-/dull}$NK1.1$^-$TCRβ$^+$CD5$^+$CD122$^+$PD-1$^-$α$_4$β$_7$$^-$CD103$^+$. Congenically marked, sorted IELp were mixed 1:1 (1.5 – 10.0 x 10^4 cells of each donor population per recipient) and injected i.v. into RAG1$^{-/-}$ mice (CD45.2$^+$). Recipient mice were analyzed for the presence of IEL populations 6 weeks later. Cell sorting was performed using a FACSAriaIII (BD Biosciences) instrument.

Antibodies and Flow Cytometry

Fluorochrome-conjugated and biotinylated Ab were purchased from eBioSciences, BioLegend or BD Pharmingen. Active caspase 3 (Asp-175) and anti-pAKT (pS473) Ab was purchased from Cell Signaling Technologies. Anti-pERK (pT202/pY204) and Mouse P-selectin - Human IgG fusion were purchased from BD Biosciences. Anti-BrdU Ab (Alexa 647 and FITC) were purchased from Phoenix Flow Systems. The mCD1d/PBS57 tetramer was kindly provided by the NIH Tetramer Facility. Cells were stained with Ab

cocktails in FACS buffer (PBS, 1% FCS, 0.02% sodium azide) for 30 min on ice. Cells were washed twice with FACS buffer following primary and secondary Ab staining. In the case of CCR7 staining, anti-CCR7 (Clone 4B12-eBioSciences) staining was performed at room temperature in FACS buffer for 30 minutes. For intracellular Ag staining, cells were treated with BD Cytofix/Cytoperm^{TM} (BD Bioscience) or the Foxp3 Staining Buffer Set (eBioScience). S1P1 antibody was purchased from R&D Systems and surface staining was performed as previously described (*112, 113*). Cell events were collected on FACSCanto II, LSRFortessa or LSR II (all BD Pharmingen) analyzers and data was analyzed with FlowJo software (Tree Star).

Statistics

For the majority of data sets, mean, SD and P values were calculated using Prism software (Graphpad) using a two-tailed, unpaired or paired Student's t-tests. For statistical analysis of competitive BM chimeras, we performed one-way ANOVA including Bonferroni's multiple comparison test. In all data sets, statistical significance is represented as *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

CHAPTER 3: RESULTS - RasGRP1 and RasGRP3 are required for efficient ETP development

This chapter contains content from the following source, republished with permission:

- Golec, D.P., Caviedes, L.M.H., and Baldwin, T.A. 2016. RasGRP1 and RasGRP3 Are Required for Efficient Generation of Early Thymic Progenitors. J Immunol *197*, 1743-1753

Experiments shown in **Figure 3-9** were designed by D.P.G and T.A.B and performed by D.P.G and L.M.H.C.

Introduction

Although members of the RasGRP family have previously been implicated during thymocyte β-selection and positive selection, the contributions of these proteins have not been evaluated during the earliest stages of development within the thymus. Furthermore, RasGRP1 has previously been studied in the context of T cell development, however related family member RasGRP3 has yet to be examined. Development through the early ETP stage drives the thymic generation of both αβ and γδ T cells and is dependent on the import of circulating BM derived T cell progenitors into the thymus. Notably, signaling through the Notch pathway has proven to be a critical regulator of the early stages of T cell development. Furthermore, signals transduced through the chemokine receptors CCR7 and CCR9 are required for progenitor entry into the thymus. However, the intracellular signaling events that occur downstream of these chemotactic receptors are not clear. Given the importance of Ras signaling during T cell development, we

hypothesized that RasGRPs 1 and 3 were involved in the regulation of early T cell development. Here we examine early T cell development in wild-type (B6), RasGRP1 KO (1KO), RasGRP3 KO (3KO) and RasGRP1/3 double KO (DKO) mice to investigate the roles of RasGRPs 1 and 3 in regulating the early stages of T cell development.

RasGRP1 and RasGRP3 are required for efficient generation of ETPs

To assess the contributions of RasGRP1 and RasGRP3 to the early stages of T cell development, we examined thymi from wildtype (B6), RasGRP1 KO (1KO), RasGRP3 KO (3KO) and RasGRP1/3 DKO (DKO) mice. 1KO thymi showed a significant reduction in thymocyte numbers compared to B6, while DKO thymi showed reduced cellularity compared to both 1KO and 3KO thymi (**Fig. 3-1A**). The most immature T cell progenitors present in the thymus reside within the $Lin^{-}CD44^{+}cKit^{hi}$ fraction and this population includes the ETP ($CD25^{-}$), transitional DN1-2 ($CD25^{int}$) and DN2a and b ($CD25^{hi}$ and $cKit^{++}$ or $cKit^{+}$ respectively) thymocyte subsets. We observed significantly reduced frequencies of $CD44^{+}cKit^{hi}$ progenitors within the Lin^{-} fraction of 1KO, 3KO and DKO thymi relative to B6 (**Fig. 3-1B, C**), suggesting RasGRPs 1 and 3 may regulate early T cell development events. Due to changes in both total thymic cellularity and frequency of $Lin^{-}CD44^{+}cKit^{hi}$, we found significantly reduced numbers of ETPs in 1KO and 3KO thymi compared to B6 thymi (**Fig. 1D**). Furthermore, mice deficient in both RasGRP1 and RasGRP3 showed significant reductions in ETP numbers compared to either single knockout alone, suggesting that the activities of both RasGRP1 and RasGRP3 are involved in regulating ETP generation. Given that DN1-2 and DN2 progenitors are the downstream progeny of ETPs, we expected that reductions in ETP

numbers would limit numbers of downstream developmental progeny. Accordingly, both 1KO and 3KO thymi showed modest reductions in numbers of transitional DN1-2 cells and DN2b thymocytes compared to B6, while DKO mice show a more pronounced reduction in DN1-2 and DN2b numbers (**Fig. 1D**). Also, thymi from 1KO and 3KO mice showed significantly decreased numbers of DN2a thymocytes compared to B6, while DKO thymi show significantly reduced DN2a numbers compared to either single knockout. Altogether, these data suggested that both RasGRP1 and RasGRP3 regulate the development of ETPs and their downstream progeny and that combined RasGRP1/3 deficiency results in a more pronounced deficit in the ETP pool.

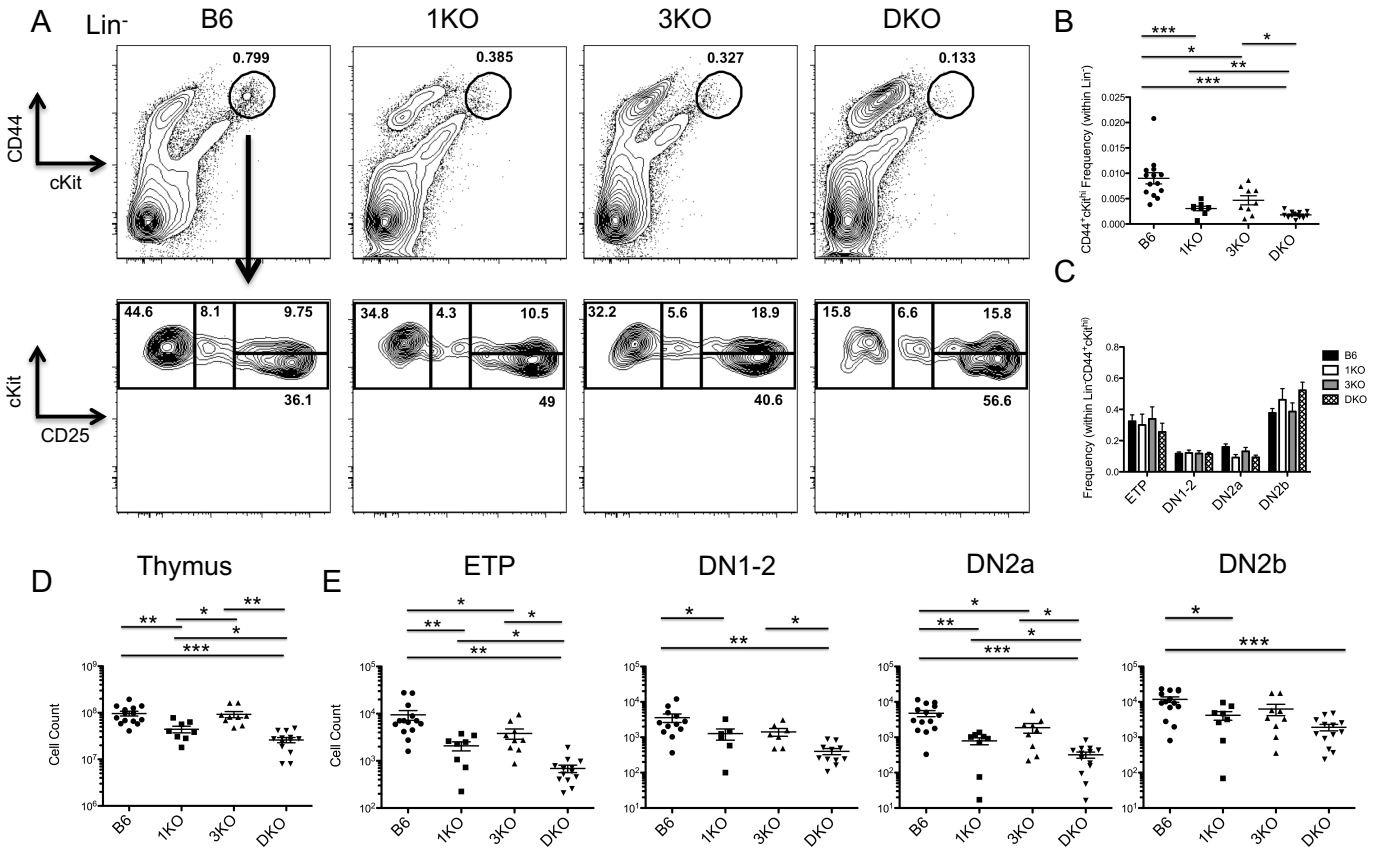

Figure 3-1. **RasGRP1 and RasGRP3 are required for efficient early thymic progenitor development.** Thymi were harvested from wild type (B6), RasGRP1 KO (1KO), RasGRP3 KO (3KO) and RasGRP1/3 DKO (DKO) mice and analyzed by flow cytometry. A) Lin$^-$ thymocytes (NK1.1$^-$, CD3$^-$, CD8$^-$, Gr-1$^-$, CD11b$^-$, TER119$^-$ and CD19$^-$) were examined for expression of CD44 and cKit. Lin$^-$CD44$^+$cKit^{hi} thymocytes were further analyzed for expression of cKit and CD25 to resolve this fraction into the ETP (Lin$^-$CD44$^+$cKit^{hi}CD25$^-$), DN1-2 (Lin$^-$CD44$^+$cKit^{hi}CD25^{int}), DN2a (Lin$^-$CD44$^+$cKit^{++}CD25^{hi}) and DN2b (Lin$^-$CD44$^+$cKit$^+$CD25^{hi}) populations. B) Frequencies of CD44$^+$cKit^{hi} progenitors within the Lin$^-$ fraction of the thymus. C) Frequencies of ETPs, DN1-2, DN2a and DN2b cells within the Lin$^-$CD44$^+$cKit^{hi} fraction of the thymus. D),E) Numbers of total thymocytes (D), ETPs, DN1-2, DN2a and DN2b thymocytes (E) from B6, 1KO, 3KO and DKO thymi. For all experiments shown: B6 (n=14), 1KO (n=8), 3KO (n=9), DKO (n=13). *p<0.05, **p<0.01, ***p<0.001

RasGRP1 and RasGRP3 deficient progenitors show intact proliferation, survival and differentiation

Given that RasGRP1/3 deficient thymi showed reductions in the frequency and number of $Lin^-CD44^+cKit^{hi}$ progenitors, we sought to determine the mechanism(s) underlying these reductions. Work from our lab demonstrated that RasGRP1 regulates DN3 thymocyte proliferation during β-selection, highlighting a role for RasGRP1 in regulating thymocyte proliferation (*114*). Since decreased proliferation of $Lin^-CD44^+cKit^{hi}$ progenitors could explain the reductions in progenitor numbers we observed in RasGRP1/3 deficient thymi, we evaluated progenitor proliferation in our panel of mice. Specifically, we measured BrdU incorporation in $Lin^-CD44^+cKit^{hi}$ thymocytes two hours after i.p. BrdU injection. We observed no significant differences between B6, 1KO, 3KO and DKO progenitors with respect to frequencies of $BrdU^+$ cells, indicating that RasGRP1 and RasGRP3 do not regulate progenitor proliferation (**Fig. 3-2A**). Another possible explanation for reduced numbers of $Lin^-CD44^+cKit^{hi}$ cells in RasGRP1/3 deficient mice was increased apoptosis within this progenitor population. We examined apoptosis of $Lin^-CD44^+cKit^{hi}$ thymocytes in B6, 1KO, 3KO and DKO mice using AnnexinV staining as a measure of cell death. Again, we observed no major differences in frequencies of $AnnexinV^+$ $Lin^-CD44^+cKit^{hi}$ progenitors, suggesting that RasGRPs 1 and 3 do not regulate progenitor apoptosis (**Fig. 3-2B**). Our data show that neither alterations to the proliferative ability nor to the apoptotic activity of $Lin^-CD44^+cKit^{hi}$ cells contributes to reductions in these progenitors in RasGRP1/3 deficient thymi, suggesting that RasGRP1 and RasGRP3 regulate progenitor development independently of these important developmental processes. Another developmental process that RasGRPs 1 and

3 could potentially regulate is differentiation through the $Lin^{-}CD44^{+}cKit^{hi}$ thymocyte stages. Given that RasGRP1 and RasGRP3 deficient thymi showed reduced numbers and frequencies of $Lin^{-}CD44^{+}cKit^{hi}$ cells, we investigated differentiation through these immature progenitor stages. While there were differences in the frequencies of $Lin^{-}CD44^{+}cKit^{hi}$ cells in various strains, within the $Lin^{-}CD44^{+}cKit^{hi}$ fraction, similar frequencies of ETP, DN1-2, DN2a and DN2b cells were observed in all strains (**Fig. 3-1C**). To more directly quantify steady state progenitor differentiation in B6, 1KO, 3KO and DKO thymi *in vivo*, we calculated the number of total DN2 thymocytes generated per ETP (DN2/ETP) in all strains. In DKO thymi, we observed significantly increased ratios of DN2/ETP compared to B6 (**Fig. 3-2C**). To further dissect differentiation through these early stages, we also examined numbers of DN2b thymocytes generated per DN2a (DN2b/DN2a). Similar to the trend seen in DN2/ETP, 1KO and DKO thymi showed significantly increased ratios of DN2b/DN2a relative to B6 (**Fig. 3-2D)**. These results suggest that the progeny populations downstream of ETP actually develop more efficiently in DKO thymi and ruled out the possibility that reductions in numbers of $Lin^{-}CD44^{+}cKit^{hi}$ progenitors in RasGRP1/3 deficient thymi was due to deficiencies in progenitor differentiation.

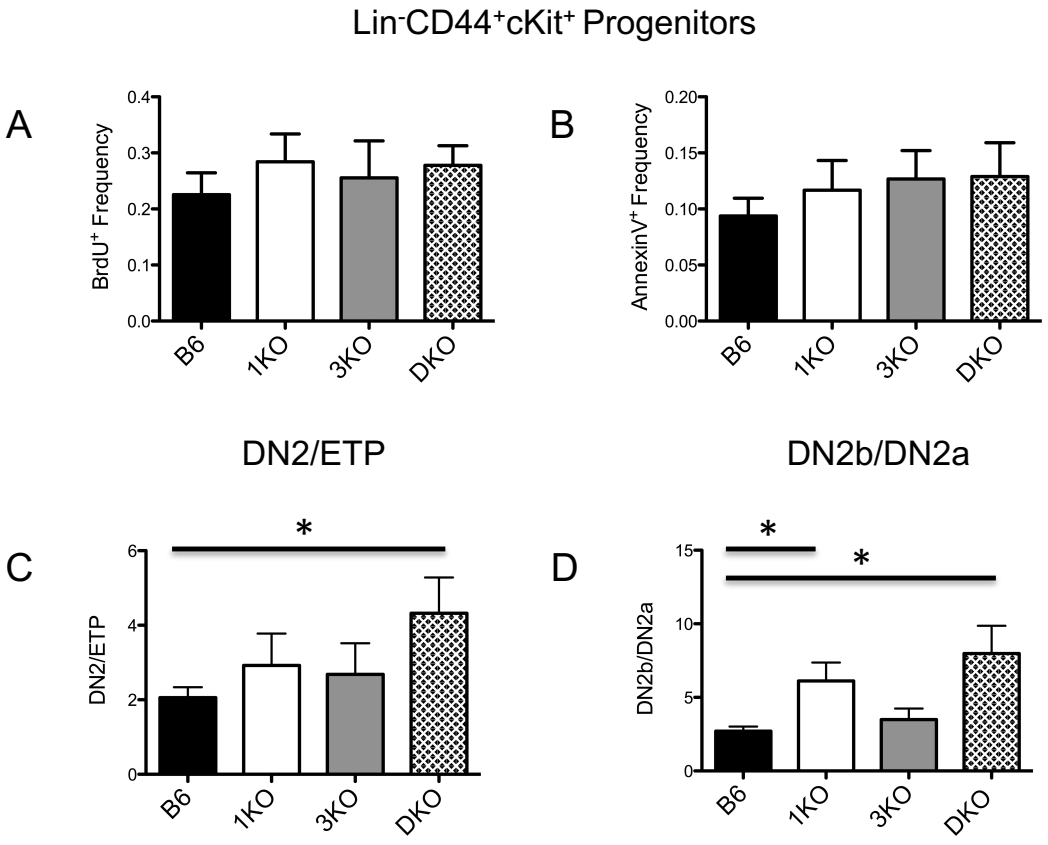

Figure 3-2. **RasGRP1 and RasGRP3 deficient thymi show intact proliferation, survival and differentiation.** Wild type (B6), RasGRP1 KO (1KO) , RasGRP3 KO (3KO) and RasGRP1/3 DKO (DKO) mice were injected i.p. with 100µg BrdU two hours prior to euthanasia, thymi were harvested and analyzed by flow cytometry. A) Frequencies of $BrdU^{+}$ cells within the $Lin^{-}CD44^{+}ckit^{hi}$ progenitor population. B) Frequencies of AnnexinV binding cells within the $Lin^{-}CD44^{+}ckit^{hi}$ progenitor population. C) Ratio of DN2 thymocytes generated per ETP (DN2/ETP) within the indicated thymi. D) Ratio of DN2b thymocytes generated per DN2a (DN2b/DN2a) within the indicated thymi. For all experiments shown: B6 (n≥5), 1KO (n≥5), 3KO (n≥5), DKO (n≥5). *$p<0.05$

RasGRP1 and RasGRP3 deficient mice exhibit normal bone marrow progenitor development

Decreased numbers of $Lin^{-}CD44^{+}cKit^{hi}$ progenitors in RasGRP1/3 deficient thymi could not be explained by differences in progenitor proliferation, apoptosis or differentiation within the thymus. However, progenitors capable of seeding the thymus (TSPs) are derived from the bone marrow and must transit out of the bone marrow into circulation in order to reach the thymus. Therefore, impaired development of bone marrow progenitors with the ability to migrate to the thymus could limit numbers of ETPs and their downstream developmental progeny. We examined progenitor populations present in bone marrow from B6, 1KO, 3KO and DKO mice. The key progenitor subsets in the bone marrow are HSCs, MPPs, LMPPs and CLPs and we were able to detect robust populations of each of these progenitor populations in B6, 1KO, 3KO and DKO bone marrow (**Fig. 3-3A, B**). We found that our panel of mice showed similar numbers of bone marrow HSCs, MPPs, LMPPs and CLPs, as well as similar total numbers of bone marrow cells (**Fig. 3-3C, D**). These results indicate that RasGRP1 and RasGRP3 do not regulate the development of T cell progenitors within the bone marrow.

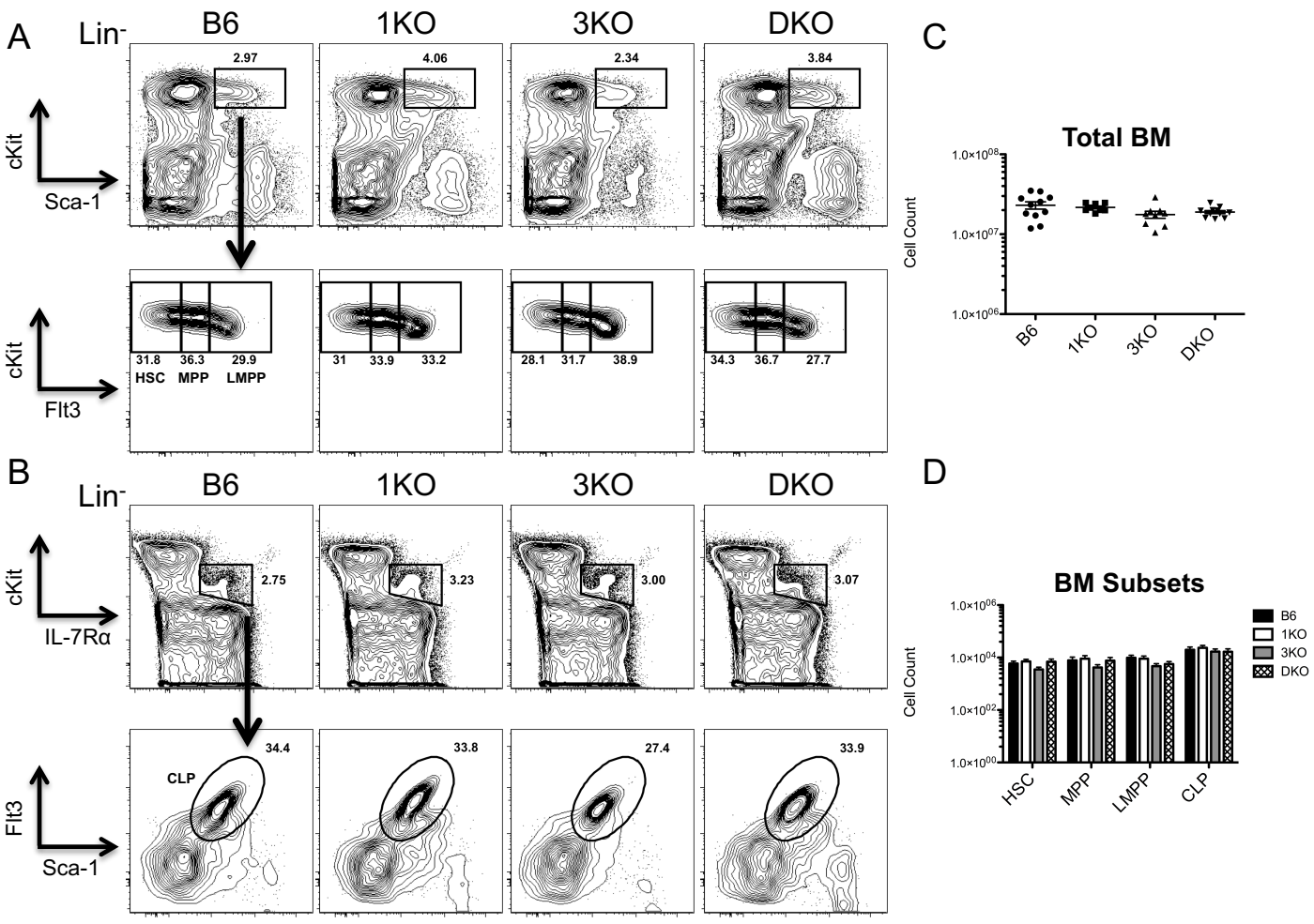

Figure 3-3. **RasGRP1 and RasGRP3 deficient thymi show intact bone marrow progenitor development.** Bone marrow cells were obtained from one femur and one tibia of wild type (B6), RasGRP1 KO (1KO) , RasGRP3 KO (3KO) and RasGRP1/3 DKO (DKO) mice and were analyzed by flow cytometry. A) Lin^- bone marrow cells were examined for expression of cKit and Sca1. Lin^- $Sca1^+cKit^{hi}$ (LSK) progenitors were gated on and subsequently analyzed for expression of Flt3. Levels of Flt3 expression on LSK progenitors identified the HSC ($Flt3^-$LSK), MPP ($Flt3^{lo}$LSK) and LMPP ($Flt3^{hi}$LSK) progenitor populations. B) Lin^- bone marrow cells were examined for expression of IL-7Rα and cKit. We gated on Lin^-IL-7Rα^+cKit^{lo} cells and further analyzed these cells for expression of Flt3 and Sca1. We identified CLPs as having a Lin^-IL-7R$\alpha^+cKit^{lo}Flt3^{hi}Sca1^{lo}$ surface phenotype. C),D) Numbers of bone marrow cells (C), HSCs, MPPs, LMPPs and CLPs (D) from the indicated mice. For all experiments shown: B6 (n=9), 1KO (n=8), 3KO (n=9), DKO (n=9).

RasGRP1 and RasGRP3 deficient progenitors show normal expression of chemokine receptors CCR7 and CCR9

Although HSCs, MPPs, LMPPs and CLPs all have a high degree of T cell potential in vitro, only LMPPs and CLPs are able to migrate to the thymus from circulation *in vivo* (*18*). Furthermore, it is known that the P-selectin ligand PSGL-1, as well as the chemokine receptors CCR7 and CCR9 are required for progenitor migration to the thymus. Our analysis of bone marrow T cell progenitor development suggested that LMPP and CLP development does not require RasGRPs 1 and 3. However, it remained possible that RasGRP1 and RasGRP3 may regulate expression of trafficking molecules needed for LMPPs and CLPs to reach the thymus. We examined cell surface expression of chemokine receptors CCR7 and CCR9 on LMPPs and CLPs from B6, 1KO, 3KO and DKO bone marrow. We found that LMPPs from 3KO and DKO animals showed slightly elevated frequencies of $CCR7^{+}CCR9^{+}$ cells compared to WT and 1KO LMPPs (**Fig. 3-4A, B**), however this difference was not statistically significant. In addition, CLPs from WT, 1KO, 3KO and DKO animals all showed similar frequencies of $CCR7^{+}CCR9^{+}$ cells (**Fig. 3-4C**). Together, these results indicate that RasGRPs 1 and 3 are not required for CCR7 and CCR9 expression on LMPPs and CLPs.

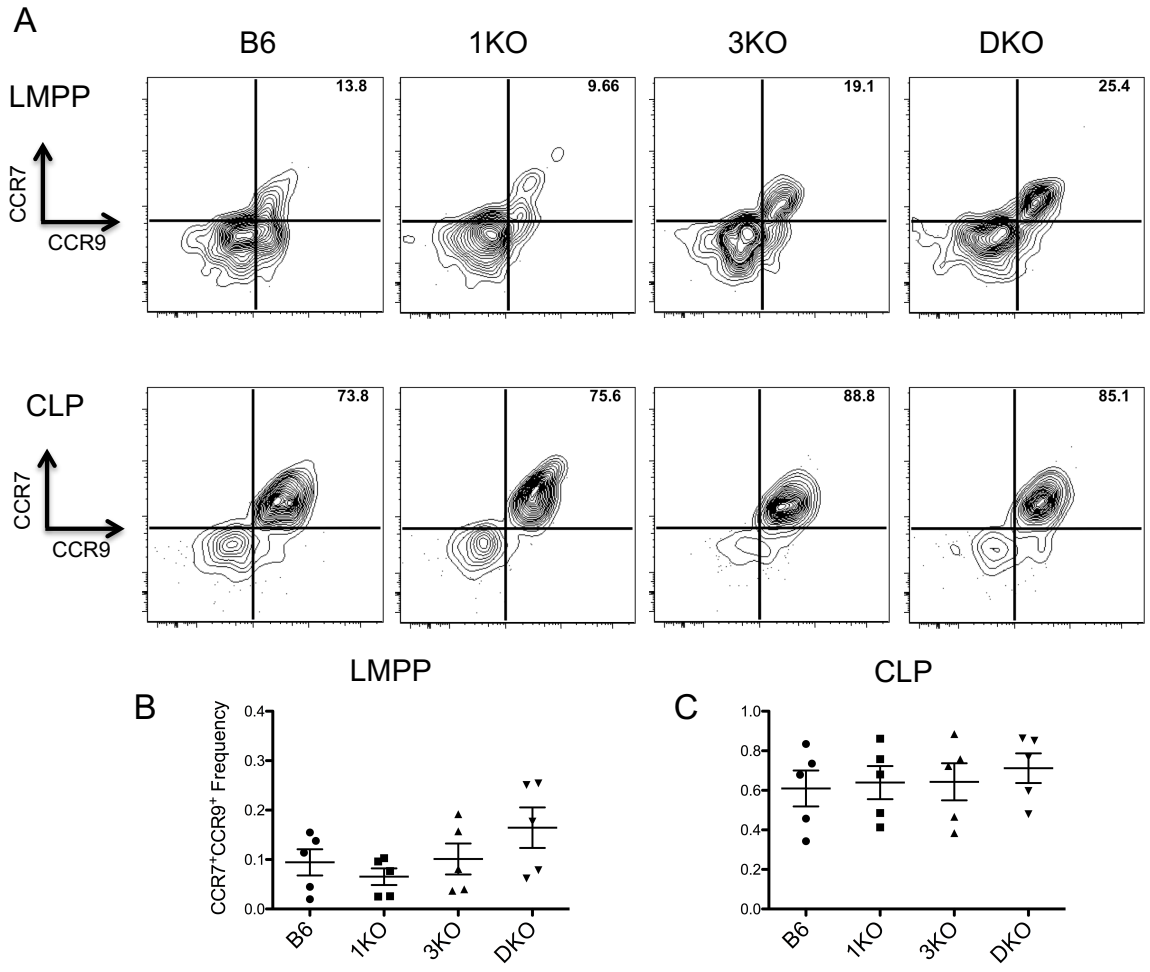

Figure 3-4. **RasGRP1 and RasGRP3 deficient bone marrow progenitors show normal expression of chemokine receptors CCR7 and CCR9.** LMPPs ($Lin^{-}Sca1^{+}cKit^{hi}Flt3^{hi}$) and CLPs ($Lin^{-}IL\text{-}7R\alpha^{+}cKit^{lo}Flt3^{hi}Sca1^{lo}$) from wild type (B6), RasGRP1 KO (1KO) , RasGRP3 KO (3KO) and RasGRP1/3 DKO (DKO) mice were analyzed for expression of thymus homing molecules by flow cytometry. A) Expression profiles of chemokine receptors CCR7 and CCR9 on LMPPs and CLPs from the indicated mice. B),C) Frequencies of $CCR7^{+}CCR9^{+}$ LMPPs (B) and CLPs (C) from the indicated mice. For all experiments shown: B6 (n=5), 1KO (n=5), 3KO (n=5), DKO (n=5).

RasGRP1 and RasGRP3 deficient progenitors show intact PSGL-1 functionality

Although, RasGRP1 and RasGRP3 did not appear to regulate chemokine receptor expression, it remained possible that they could regulate other molecules involved in progenitor homing. To further investigate the roles of RasGRPs 1 and 3 in regulating progenitor trafficking, we also examined PSGL-1 functionality on bone marrow LMPPs and CLPs from our panel of mice using recombinant P-selectin binding as a readout of PSGL-1 activity. We found modestly elevated levels of P-selectin binding on LMPPs from 1KO, 3KO and DKO mice compared to B6 (**Fig. 3-5A, B**). Similarly, 1KO and DKO CLPs showed mildly increased levels of P-selectin binding compared to B6, while 3KO CLPs showed near equal P-selectin binding as B6 cells (**Fig3-5A, B**). Altogether, these results demonstrated that LMPPs and CLPs were able to express molecules critical for progenitor migration into the thymus. As a result, our observed reductions in immature thymic progenitors in RasGRP1/3 deficient mice could not be explained by impaired thymic homing molecule expression on bone marrow T cell progenitors. However, it remains possible that RasGRP1 and RasGRP3 act in signaling pathways downstream of PSGL-1, CCR7 or CCR9 to regulate progenitor migration to the thymus and subsequent ETP development.

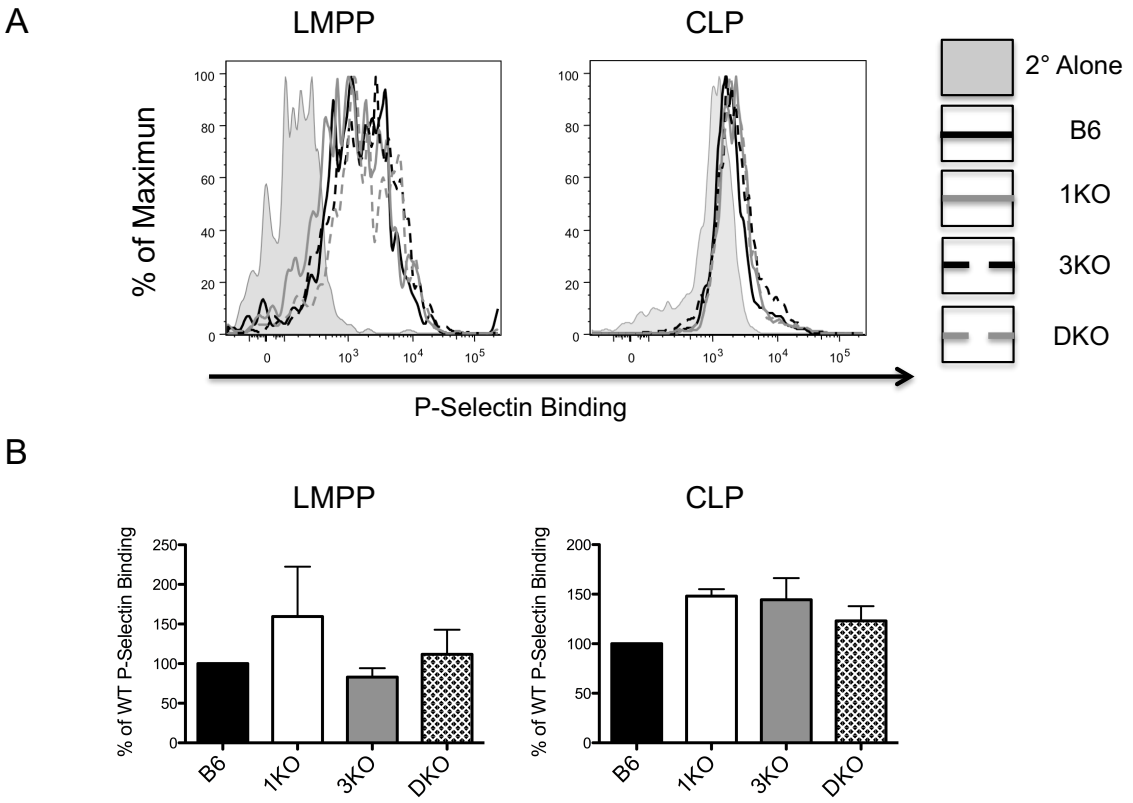

Figure 3-5. **RasGRP1 and RasGRP3 deficient bone marrow progenitors show intact PSGL-1 functionality.** LMPPs ($Lin^{-}Sca1^{+}cKit^{hi}Flt3^{hi}$) and CLPs ($Lin^{-}IL\text{-}7R\alpha^{+}cKit^{lo}Flt3^{hi}Sca1^{lo}$) from wild type (B6), RasGRP1 KO (1KO) , RasGRP3 KO (3KO) and RasGRP1/3 DKO (DKO) mice were analyzed for binding to recombinant P-selectin. A) Histogram overlays showing P-selectin binding by LMPPs and CLPs from the indicated mice. B) Mean fluorescent frequencies (MFIs) of P-selectin binding on LMPPs (left) and CLPs (right) expressed as a percentage of WT values. For all experiments shown: B6 (n=4), 1KO (n=3), 3KO (n=3), DKO (n=4).

RasGRP1 and RasGRP3 deficient mice contain increased frequencies of circulating T cell progenitors

In order for bone marrow progenitors to migrate to the thymus, they must first transit out of the bone marrow into the blood. To determine if RasGRP1 and RasGRP3 regulated progenitor abundance in circulation, we examined blood from B6 and DKO mice for the presence of bone marrow derived progenitors. HSCs, MPPs, LMPPs and CLPs have all been found to reside within blood in mice (*20, 115*). However, these progenitor populations are incredibly rare in circulation, making them extremely difficult to study. Given the rarity of these progenitors, we took the approach of examining blood for heterogeneous cell populations that included multiple progenitor populations of interest. The Lin^-cKit^+ fraction of blood includes HSCs, MPPs, LMPPs and CLPs, as well as other cell types. We found similar frequencies of Lin^-cKit^+ cells in DKO blood compared to B6 (**Fig. 3-6A, B**), suggesting that bone marrow derived progenitors were able to migrate out of the bone marrow into circulation in DKO animals. Given that LMPPs and CLPs are the only progenitors able to enter the thymus from circulation, we further examined the Lin^-cKit^+ fraction more closely for expression of Sca1 and Flt3 to focus our analysis on these progenitors (**Fig. 3-6A**). The $Lin^-cKit^+Sca1^+Flt3^{hi}$ fraction of blood includes LMPPs and CLPs, but does not include HSCs and MPPs. We observed significantly increased frequencies of both $Sca1^+Flt3^{hi}$ cells within the Lin^-cKit^+ fraction as well as total $Lin^-cKit^+Sca1^+Flt3^{hi}$ progenitors present in circulation (**Fig. 3-6C**). Since CCR7 and CCR9 have been established as required for efficient progenitor migration to the thymus, we also examined $Lin^-cKit^+Sca1^+Flt3^{hi}$ blood progenitors for cell surface expression of these important chemokine receptors. We found that within the $Lin^-cKit^+Sca1^+Flt3^{hi}$ blood cell

fraction, B6 and DKO blood showed similar frequencies of $CCR7^{+}CCR9^{+}$ progenitors (**Fig. 3-6D**). However, when looking at total frequencies of $CCR7^{+}CCR9^{+}Lin^{-}$ $cKit^{+}Sca1^{+}Flt3^{hi}$ cells, we observed significantly increased frequencies of progenitors present in DKO blood compared to B6 (**Fig. 3-6E**). This accumulation of progenitors capable of colonizing the thymus in DKO blood again suggested that circulating progenitors lacking RasGRP1 and RasGRP3 might be impaired in their ability to traffic into tissues such as the thymus.

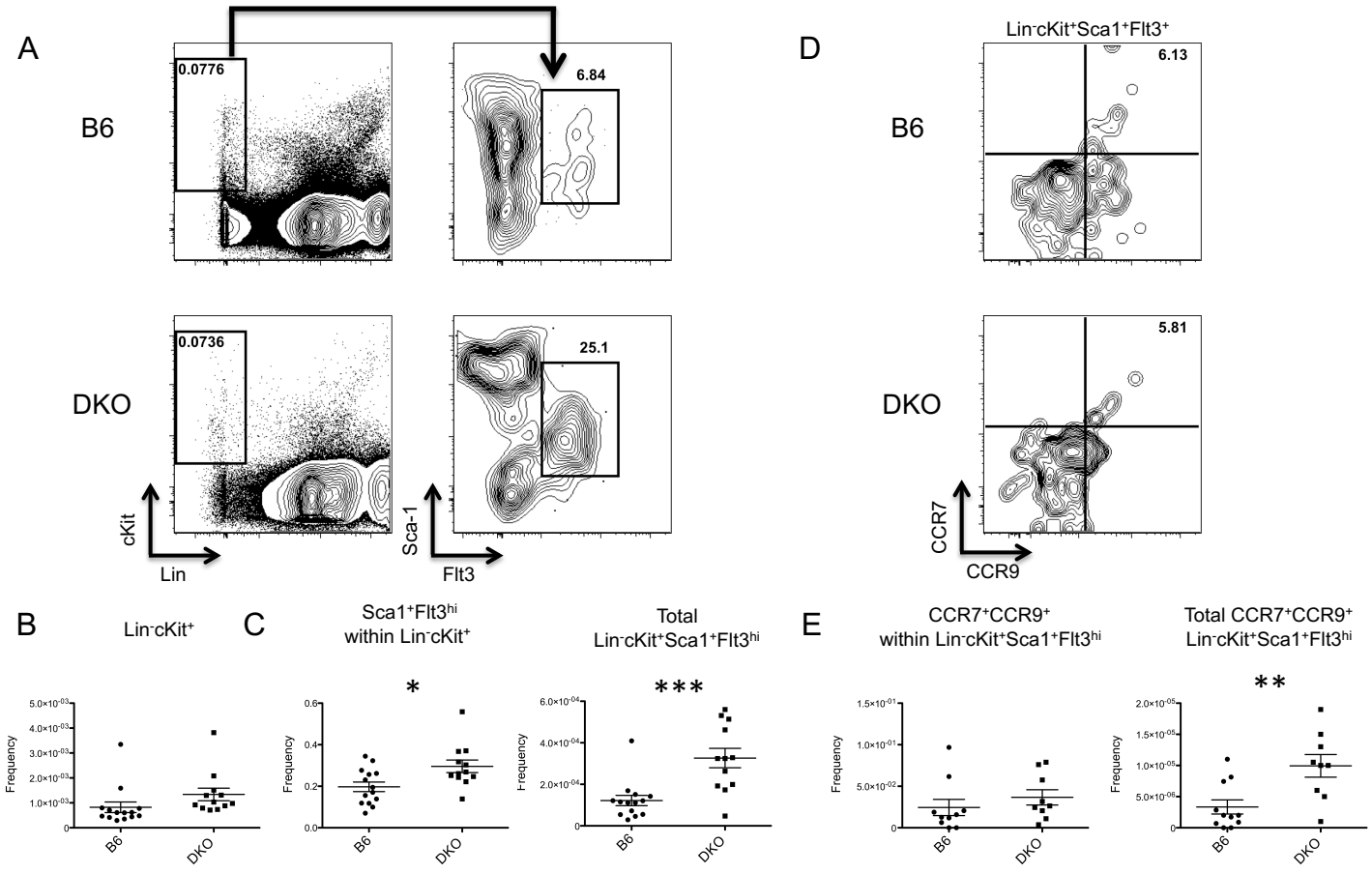

Figure 3-6. **RasGRP1 and RasGRP3 deficient mice show elevated frequencies of circulating T cell progenitors.** Blood was harvested from wild type (B6) and RasGRP1/3 DKO (DKO) mice and analyzed for the presence of circulating T cell progenitors by flow cytometry. A) Blood cells were analyzed for expression of lineage markers and cKit. Progenitors with a $Lin^{-}cKit^{+}$ surface phenotype were gated on and subsequently analyzed for expression of Sca1 and Flt3. We identified circulating T cell progenitors as $Lin^{-}cKit^{+}Sca1^{+}Flt3^{hi}$ cells. B) Frequencies of $Lin^{-}cKit^{+}$ cells present in blood. C) Frequencies of $Sca1^{+}Flt3^{hi}$ cells within the $Lin^{-}cKit^{+}$ fraction of blood (left) and total frequencies of $Lin^{-}cKit^{+}Sca1^{+}Flt3^{hi}$ cells present within blood (right). D) CCR7 and CCR9 expression on $Lin^{-}cKit^{+}Sca1^{+}Flt3^{+}$, blood progenitors. E) Frequencies of $CCR7^{+}CCR9^{+}$ cells present within the $Lin^{-}cKit^{+}Sca1^{+}Flt3^{hi}$ blood fraction (left) and total frequencies of $CCR7^{+}CCR9^{+}Lin^{-}cKit^{+}Sca1^{+}Flt3^{hi}$ blood progenitors (right). For all experiments shown: B6 ($n \geq 11$) and DKO ($n \geq 9$). *$p<0.05$, **$p<0.01$, ***$p<0.001$

Competitive bone marrow chimeras reveal multiple defects in T cell development from RasGRP1 and RaGRP3 deficient progenitors

Analysis of intact 1KO, 3KO and DKO mice provided us with valuable insight into the roles of RasGRP1 and RasGRP3 in regulating early thymocyte development events, but this approach has several limitations. One caveat to this approach is that RasGRP1/3 ablation is not restricted to T cell progenitors alone, but rather RasGRP1/3 deficiency is global in nature. As such, impairments in T cell development in RasGRP1/3 deficient animals may not strictly be due to progenitor specific RasGRP1/3 deficiency, but rather to RasGRP1/3 loss in other cell types that support T cell development. Another caveat to this approach is that we cannot evaluate development of B6 and DKO progenitors in a competitive manner. To address these limitations we generated mixed BM chimeras using congenically labeled B6 ($CD45.1^+$) and DKO ($CD45.2^+$) donor BM mixed at a 1:1 ratio to reconstitute lethally irradiated B6 recipients ($CD45.1^+$). Three months after reconstitution, the thymus from chimeric animals was examined for contributions by B6 and DKO donor cells. To examine the contributions of RasGRP1 and RasGRP3 to ETP development specifically, we utilized absolute chimerism values to analyze B6 and DKO progenitor development through the early T cell progenitor populations (**Fig. 3-7A**). One-way analysis of variance of absolute chimerism values from these various T cell progenitor populations indicated a statistically significant ($p<0.0001$) difference in means of absolute chimerism, suggesting that B6 and DKO progenitors differentially contribute to development through these progenitor populations. We also applied Bonferroni's multiple comparison test to this data set to determine precisely which progenitor subsets significantly differed in absolute chimerism values. We found that ETP absolute

chimerism was significantly higher than absolute chimerism found in the blood progenitor, CLP and HSC populations (**Fig 3-7A**). These results strongly suggest that B6 progenitors more efficiently contributed to the ETP population relative to DKO progenitors, and did so in a cell-intrinsic manner. Also, given that circulating progenitors are the direct precursor of ETPs and that absolute chimerism was significantly higher in ETPs compared to blood progenitors, it appears that circulating DKO progenitors are specifically impaired in their ability to generate ETPs in the thymus.

To evaluate B6 and DKO progenitor development through more downstream T cell development events, we examined HSC normalized chimerism values (relative chimerism) in a variety of T cell populations found within the thymus (**Fig. 3-7B**). Similar to the trend seen with absolute chimerism, we found increased ETP chimerism compared to BM and blood progenitors (**Fig. 3-7B**). Unexpectedly, we also observed differences in MPP, LMPP and CLP development between WT and DKO cells. MPP and LMPP cells showed increased absolute and relative chimerism values compared to HSCs (**Fig. 3-7A, B**), suggesting that RasGRPs 1 and 3 may play a minor role in MPP and LMPP generation. Furthermore, CLP cells showed reduced absolute and relative chimerism values compared to HSCs, suggesting that DKO cells outnumber WT cells within the CLP compartment (**Fig. 3-7A, B**). Consistent with previously reported roles of RasGRP1 in thymocyte β-selection and positive selection, we found that B6 cells greatly outcompeted DKO cells during these developmental transitions. We found large increases in relative chimerism in DN3b and DP thymocytes relative to DN3a cells, suggesting that DKO cells less efficiently passed through the β-selection checkpoint

compared to B6 cells. In accordance with a role for RasGRP1 during positive selection, we observed over 15 fold increases in relative chimerism within the mature CD4SP and CD8SP populations relative to DP thymocytes (**Fig. 3-7B**). Altogether, these results confirm previously established roles for RasGRP1 during T cell development and again highlight novel roles for RasGRP1 and RasGRP3 in regulating ETP generation.

Apart from residing within the $Lin^{-}CD44^{+}ckit^{hi}$ fraction of the thymus, ETPs are also found within the canonical DN1 thymocyte subset. DN1 thymocytes are heterogeneous group of cells and this group includes DN1a, DN1b, DN1c, DN1d and DN1e cells. ETPs correspond to the $cKit^{hi}$ DN1a and DN1b subsets of DN1 cells, while DN1c, DN1d and DN1e cells show a $cKit^{lo/-}$ surface phenotype. Since RasGRP1 and RasGRP3 regulate ETP development, we also examined the contributions of B6 and DKO progenitors to the development of the remaining DN1 cell subsets in our competitive bone marrow chimeras. We found that B6 progenitors greatly outnumbered DKO cells in the DN1c, DN1d and DN1e subsets (**Fig. 3-7C**). Furthermore, the relative chimerism values observed within the DN1c, DN1d and DN1e subsets were even greater than those observed in ETPs. In fact, the relative chimerism values obtained for the DN1c, DN1d and DN1e subsets more closely resembled those of DN3 and DP thymocytes than they did ETPs, suggesting that, despite all being members of the DN1 fraction, the developmental pathways of these thymocytes are distinct from that of ETPs.

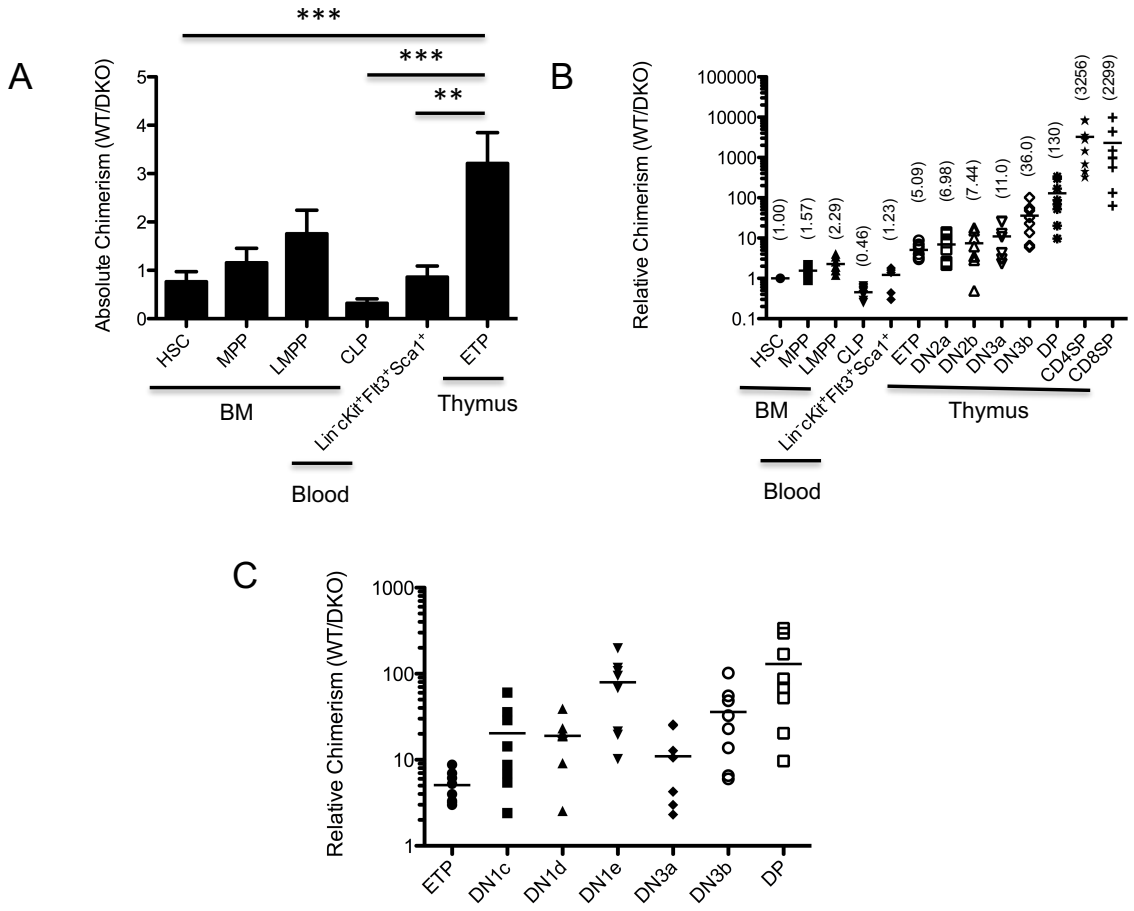

Figure 3-7. **Competitive bone marrow chimeras reveal multiple defects in T cell development from RasGRP1 and RasGRP3 deficient progenitors.** Wild type (WT - CD45.1^{+}) and RasGRP1/3 DKO (DKO - CD45.2^{+}) BM cells were mixed 1:1 and transplanted into lethally irradiated WT (CD45.1^{+}) recipient mice. Three months following transplant, bone marrow, blood and thymi were harvested from chimeric mice and analyzed by flow cytometry. A) Absolute chimerism (WT/DKO) values of T cell progenitor subsets from bone marrow (BM), blood and thymi of chimeric mice. B) Relative chimerism (WT/DKO normalized to HSC) values of T cell progenitor subsets from bone marrow (BM), blood and thymi of chimeric mice. Mean relative chimerism values are indicated above each data column in parenthesis. C) Relative chimerism (WT/DKO normalized to HSC) values of the indicated thymocyte subsets. For all populations analyzed a minimum of 6 chimeric mice derived from a minimum of 4 independent BM donors of both genotypes were examined. **p<0.01, ***p<0.001

RasGRP1 and RasGRP3 deficient progenitors show impaired migration towards the CCR9 ligand CCL25

Our data suggested that RasGRP1/3 deficient progenitors are intrinsically less efficient in their ability to generate ETPs relative to B6 progenitors. However, the basis for this impaired progenitor development was still unclear. Given that we observed significantly increased frequencies of blood progenitors in DKO mice and that our mixed bone marrow chimeras clearly showed that B6 progenitors significantly outnumber DKO progenitors at the ETP stage, we hypothesized that RasGRP1 and RasGRP3 may regulate progenitor entry into the thymus from circulation through regulating the function of chemokine receptors. The expression of CCR7 and CCR9 is required for progenitor migration into the thymus (*12-16*). In addition, CXCR4 has been shown to be involved in progenitor retention in the bone marrow and has also been implicated in progenitor migration to the embryonic mouse thymus (*16, 116*). To evaluate progenitor migration in response to chemokine receptor ligands, we performed transwell assays using bone marrow cells from B6 and DKO mice. Specifically, we measured HSC, MPP, LMPP and CLP migration towards SDF1α (CXCR4 ligand), CCL19 (CCR7 ligand) and CCL25 (CCR9 ligand) as a percentage of migrated progenitors relative to input controls. SDF1α has previously been shown to be a potent inducer of bone marrow progenitor migration and as expected we found that progenitor migration towards SDF1α was most robust of all the chemokines examined (**Fig. 3-8**). Furthermore, we found similar percentages of migrated progenitors from B6 and DKO mice in response to SDF1α, with LMPPs and CLPs from DKO bone marrow showing modestly higher levels of migration than their B6 counterparts (**Fig. 3-8**). Examining progenitor migration towards the CCR7 ligand,

CCL19, we found no significant differences between B6 and DKO progenitor populations. However, we did observe slight reductions in DKO LMPP and CLP migration relative to B6 cells (**Fig. 3-8**). Analysis of progenitor migration towards CCR9 ligand, CCL25, demonstrated a significant reduction in the percentages of migrating LMPPs and CLPs from DKO bone marrow compared to B6 (**Fig. 3-8**). DKO progenitor migration towards CCL19 and CCL25 together was reduced compared to B6, but this difference was not statistically significant (**Fig. 3-8**). These results suggest a role for RasGRP1 and RasGRP3 downstream of CCR9 and provide insight into RasGRP-mediated control of progenitor migration into the thymus from the circulation.

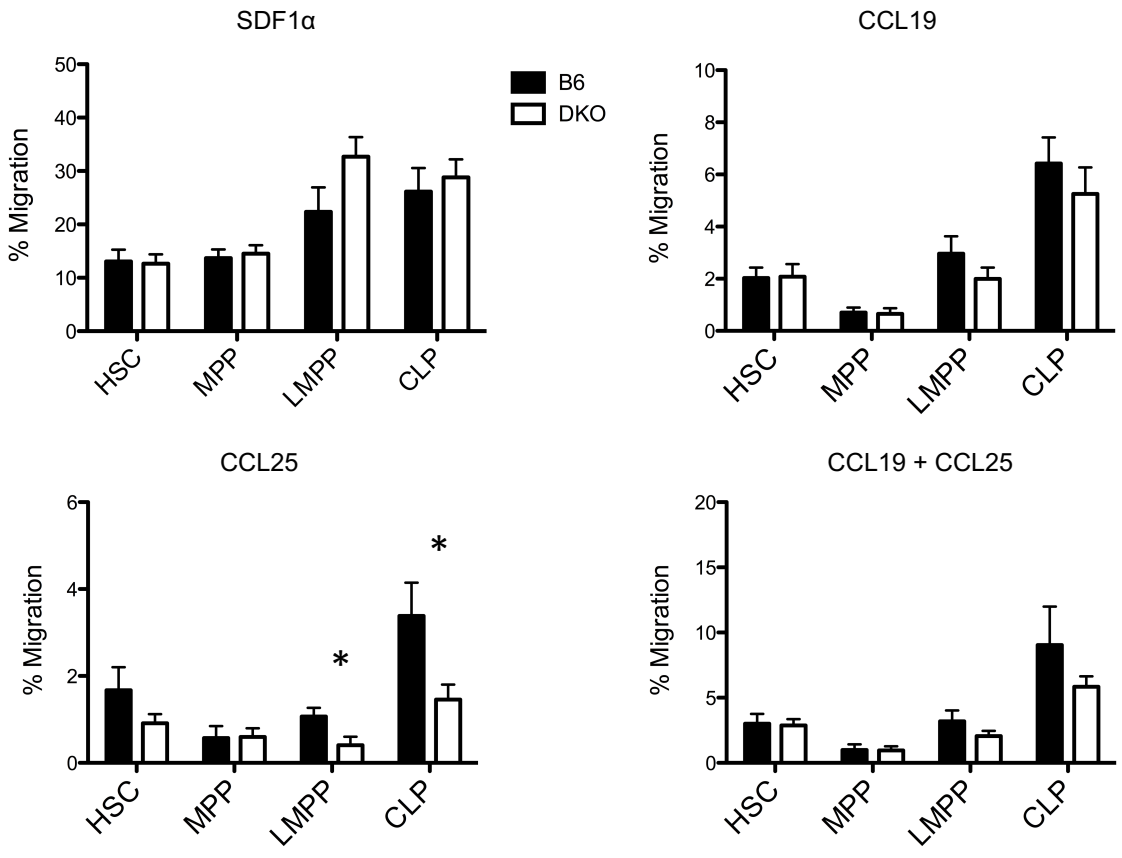

Figure 3-8. ***In vitro*** **migration assays reveal impaired responsiveness to CCR9 ligand CCL25 by RasGRP1 and RasGRP3 deficient T cell progenitors.** $2x10^6$ bone marrow cells from wild type (B6) and RasGRP1/3 DKO (DKO) mice were placed in the upper chamber of a transwell insert (5μm pore). Bone marrow cell migration was measured towards media alone (not shown), SDF1α (20 nM), CCL19 (500 ng/mL), CCL25 (500 ng/mL) and CCL19 + CCL25 (500 ng/mL each). Migrating cells were collected from the bottom chamber of transwells and were analyzed by flow cytometry. HSC ($Lin^-Sca1^+cKit^{hi}Flt3^-$), MPP ($Lin^-Sca1^+cKit^{hi}Flt3^{lo}$), LMPP ($Lin^-Sca1^+cKit^{hi}Flt3^{hi}$) and CLP ($Lin^-IL\text{-}7R\alpha^+cKit^{lo}Flt3^{hi}Sca1^{lo}$) migration was measured as percentage of cells migrating relative to input cells (% Migration). For all experiments: B6 (n≥6) and DKO (n≥6). *$p<0.05$

Inhibition of Ras regulated signaling pathways impairs progenitor migration

Our results suggested that RasGRPs 1 and 3 regulate progenitor migration into the thymus in a CCR9 dependent manner. To further interrogate the signaling pathways involved in regulating progenitor migration downstream of CXCR4, CCR7 and CCR9 we used inhibitors of Ras regulated signaling pathways within in our previous transwell assay. RasGRPs 1 and 3 regulate the activation of Ras and two major signaling pathways activated downstream of Ras are the Raf-MEK-ERK and PI3K pathways. To test the role of these pathways in progenitor migration, bulk BM cells were placed in transwells in the presence of DMSO (control), LY294002 (PI3K inhibitor) or U0126 (MEK inhibitor) and migration of LMPPs and CLPs towards the chemokines SDF1α, CCL19 and CCL25 was evaluated as a percentage of migrated progenitors relative to input controls. BM cells treated with either LY294002 or U0126 showed similar percentages of migrating LMPPs and CLPs as DMSO treated cells in response to SDF1α (**Fig. 3-9**), suggesting that PI3K and MEK-ERK signals are not involved in regulating progenitor migration towards SDF1α. LY294002 treated BM cells showed modest reductions in percentages of migrating LMPPs and CLPs in response to CCL19 compared to DMSO treated cells, while U0126 treated cells showed similar percentages of migrating LMPPs in response to CCL19 and minimally reduced CLP migration relative to control cells (**Fig. 3-9**). Our data suggested that RasGRPs 1 and 3 regulate progenitor migration in response to CCL25 and we predicted that Ras regulated signaling pathways would likely be involved in mediating progenitor chemotaxis towards CCL25. LY294002 treated BM cells showed minor reductions in percentages of migrating LMPPs in response to CCL25 compared to control cells (**Fig. 3-9**), while LY294002 treated CLPs showed a more substantial 2-fold

reduction in percentage migration compared to control (**Fig. 3-9**). U0126 treated BM cells showed significantly reduced percentages of migrating LMPPs in response to CCL25 relative to DMSO treated cells (Figure 7E), suggesting that MEK-ERK signaling regulates LMPP migration in response to CCL25. U0126 treated CLP cells showed a 2-fold reduction in the percentage of migrating cells compared to control cells (**Fig. 3-9**), suggesting that MEK-ERK signals may also contribute to CLP migration in response CCL25. These results highlight the roles of the PI3K and MEK-ERK signaling pathways in regulating progenitor migration and provide a basis for the roles of RasGRPs 1 and 3 downstream of CCR9.

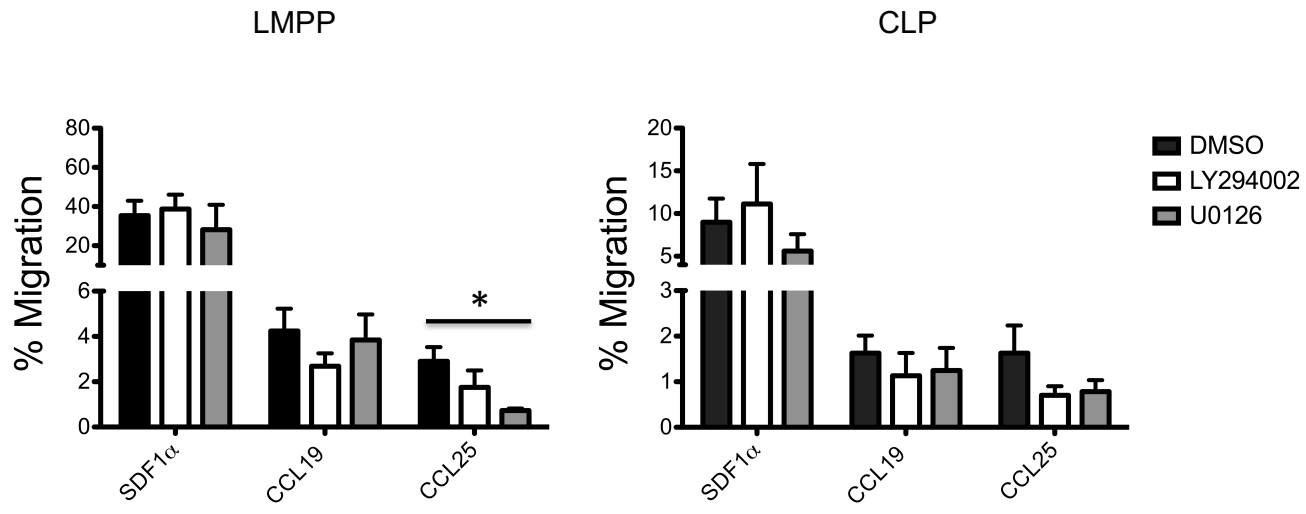

Figure 3-9. **Inhibition of Ras regulated signaling pathways impairs progenitor migration.** $2x10^6$ bone marrow cells from wild type (B6) and RasGRP1/3 DKO (DKO) mice were placed in the upper chamber of a transwell insert (5μm pore). Bone marrow cell migration was measured towards media alone (not shown), SDF1α (20 nM), CCL19 (500 ng/mL), CCL25 (500 ng/mL) and CCL19 + CCL25 (500 ng/mL each). DMSO (control), LY294002 (20 mM), or U0126 (20 mM) was added to the upper chamber of transwell inserts before incubation of cells with chemokines Migrating cells were collected from the bottom chamber of transwells and were analyzed by flow cytometry. LMPP (Lin^- $Sca1^+$ $cKit^{hi}$ $Flt3^{hi}$) and CLP (Lin^- IL-7Rα $cKit^+$ $Flt3^{hi}$ $Sca1^{lo}$) migration was measured as percentage of cells migrating relative to input cells (% Migration). For all experiments: B6 (n=4) and DKO (n=4). *$p<0.05$

Conclusion

The data presented in this chapter describe novel roles for RasGRP1 and RasGRP3 in regulating the earliest thymic stages of T cell development. RasGRP1 and/or RasGRP3 deficient animals showed significantly reduced numbers of ETPs, indicating that RasGRPs 1 and 3 are required for efficient ETP development. Analysis of early thymocyte populations showed that RasGRPs 1 and 3 did not regulate the apoptosis or proliferation of these immature thymocytes. Furthermore, RasGRP1 and RasGRP3 were not required for the development of bone marrow progenitors that give rise to T cells. However, examination of circulating progenitor cells showed that DKO animals contained increased frequencies of progenitors with the potential to migrate to the thymus, suggesting that RasGRPs 1 and 3 may regulate progenitor entry to the thymus. Consistent with this result, competitive bone marrow chimera experiments showed that DKO progenitors were impaired in the blood progenitor to ETP transition compared to WT progenitors. Specific examination of T cell progenitor migration demonstrated that DKO progenitors were impaired in their ability to migrate towards CCR9 ligand CCL25, which may represent a potential mechanism through which RasGRP1 and RasGRP3 regulate progenitor entry into the thymus.

CHAPTER 4: RESULTS - RasGRP1, but not RasGRP3, is required for efficient thymocyte β-selection and ERK activation downstream of CXCR4

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- Golec, D.P., Dower, N.A., Stone, J.C. and Baldwin, T.A. 2013. RasGRP1, but not RasGRP3, is required for efficient thymic β-selection and ERK activation downstream of CXCR4. PLoS One *8*: e53300

Introduction

Previous work has demonstrated the importance of RasGRP family members during thymocyte β-selection. The Ras/ERK pathway is activated during β-selection and deletion of the RasGEF Sos1 impairs DN3 differentiation. Reports published by Zhu et al. and Kortum et al. revealed that RasGRP1 and RasGRP1/4 deficient mice showed impaired development beyond DN3, suggesting defects in β-selection (*102, 103*). Although previous studies have suggested that RasGRP1 and RasGRP4 are important during β-selection, the involvement of other RasGRP family members has not been thoroughly examined. In particular, the role of RasGRP3 during T cell development has not been extensively studied. Our analysis of the role of RasGRP3 in ETP development, described in the previous chapter, suggests that RasGRP3 regulates early T cell development and we will seek to explore potential additional roles of RasGRP3 during β-selection and DP thymocyte selection. In addition, a mechanistic description of the role of RasGRPs during β-selection has not been explored in detail and will be examined here. Similar to the approach outlined in the previous chapter, we examined the roles of

RasGRPs 1 and 3 during thymocyte development using wild-type (B6), RasGRP1 KO (1KO), RasGRP3 KO (3KO) and RasGRP1/3 double KO (DKO) mice.

RasGRP1, but not RasGRP3, is required for thymocyte positive selection

Although RasGRP1 has been established as a key driver of thymocyte positive selection, the role of RasGRP3 during positive selection has not been examined. To explore potential roles for RasGRP1 and/or RasGRP3 in thymocyte positive selection, we first examined CD4/CD8 profiles of Thy1.2^{+} thymocytes from wildtype (B6), RasGRP1$^{-/-}$ (1KO), RasGRP3$^{-/-}$ (3KO) and RasGRP1$^{-/-}$; 3$^{-/-}$ (DKO) mice. As has been previously reported, 1KO mice showed significantly reduced frequencies and numbers of CD4SP and CD8SP thymocytes (**Fig. 4-1A,B**) due to defects in thymocyte positive selection (*92, 101*). Likewise, DKO mice also showed significant reductions in CD4SP and CD8SP frequencies and numbers. However, double deficiency of RasGRP1 and RasGRP3 does not appear to further abrogate positive selection compared to RasGRP1 deficiency alone. Finally, 3KO mice did not show significant alterations in numbers or frequencies of any major thymic subsets, suggesting that RasGRP3 does not regulate thymocyte positive selection.

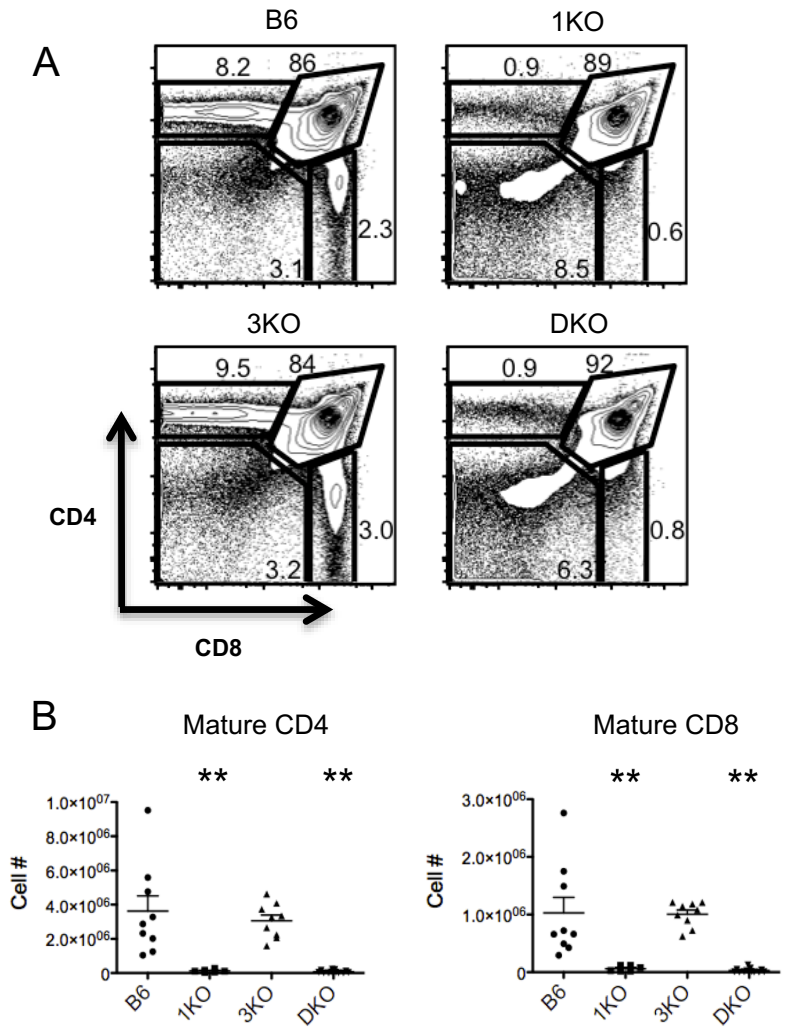

Figure 4-1. RasGRP1, but not RasGRP3, is required for thymocyte positive selection. A) CD4 by CD8 profiles of Thy1.2^{+} cells from B6 (n=9), 1KO (n=6), 3KO (n=9) and DKO thymi (n=13). B) Numbers of mature Thy1.2^{+}CD3^{+}CD44^{-} CD4 and CD8 SP thymocytes. **p<0.01

RasGRP1, but not RasGRP3, is required for the development of iNKT cells

Our previous results suggested that RasGRP3 does not regulate thymocyte positive selection, but it remained possible that it could regulate other selection processes. In addition to defects in positive selection, 1KO thymi have recently been reported to show impaired iNKT cell development (*105*). To assess a potential additional role for RasGRP3 in thymic iNKT cell development we examined B6 and RasGRP1/3 deficient thymi for the presence of mature CD1d(PBS57) tetramer binding $CD3^+$ iNKT cells. As expected, 1KO and DKO thymi showed statistically significant reductions in iNKT cell frequencies and numbers compared to B6 (**Fig. 4-2A,B**). 3KO thymi showed similar numbers and frequencies of iNKT cells as B6 and iNKT cell selection did not appear further abrogated by RasGRP1/3 double deficiency compared to RasGRP1 loss alone (**Fig. 4-2A,B**). Altogether, these results confirm the critical role of RasGRP1 in iNKT cell development and demonstrate that RasGRP3 activity is dispensable for the development of this population.

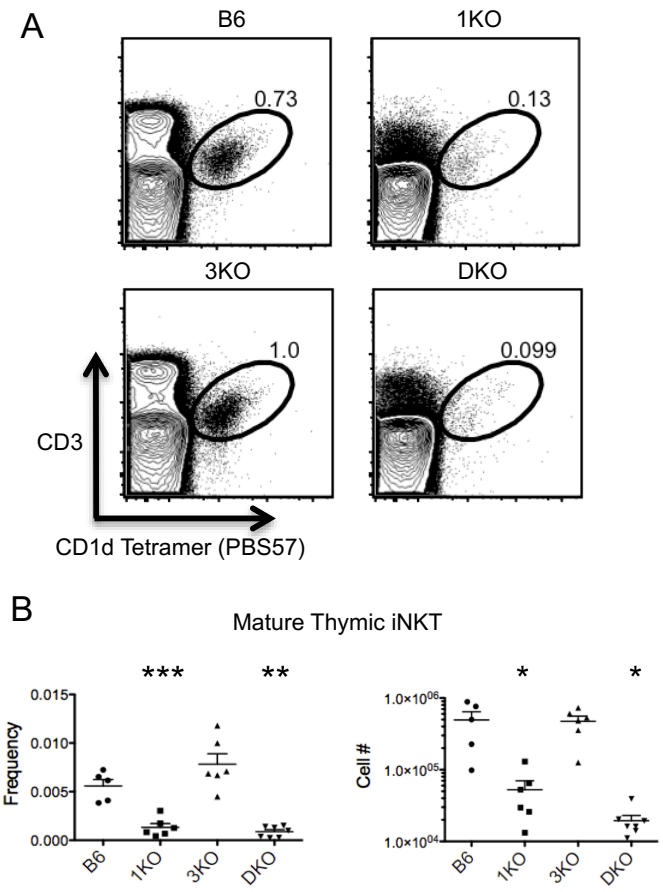

Figure 4-2. RasGRP1, but not RasGRP3, is required for thymocyte positive selection. A) CD3 by CD1d Tetramer (PBS57) profiles of bulk thymocytes from B6 (n=5), 1KO (n=6), 3KO (n=6) and DKO (n=7) thymi. B) Frequencies and numbers of mature $CD3^{+}CD1d(PBS57)$ $Tetramer^{+}$ iNKT cells. *p<0.05, **p<0.01 and ***p<0.001.

RasGRP1 KO and RasGRP1/3 DKO mice display inefficient generation of DP thymocytes

To gain a better understanding of the influence of RasGRP1, RasGRP3 and RasGRP1/3 deficiency on β-selection, we examined total thymic cellularity as the proliferative burst that accompanies β-selection is largely responsible for the total number of thymocytes present in the thymus. As a result of inefficient β-selection, DKO thymi showed a significant reduction in total thymic cellularity compared to B6 (**Fig. 4-3A**). 1KO and 3KO thymi showed a reduction in total thymocyte numbers compared to B6, however this was not statistically significant (**Fig. 4-3A**). An important outcome of β-selection is the development of DP thymocytes from DN progenitors. Likewise, defects in β-selection disrupt the normal balance of DN to DP in the thymus. Interestingly, 1KO and DKO mice showed significantly elevated frequencies and increased, although not statistically significant, numbers of DN thymocytes compared to B6 (**Fig. 4-3B**). In addition to having an increased pool of DN, 1KO and DKO thymi also showed decreased numbers of DP compared to B6 (**Fig. 4-3C**). Since the DP compartment is generated from DN progenitors, analyzing the ratio of DP to DN (DP/DN) provides insight into the efficiency with which DN thymocytes develop into DP. Strikingly, 1KO and DKO mice show significant reductions in DP/DN, suggesting inefficient developmental progression through the DN stages, resulting in fewer DP (**Fig. 4-3C**). In contrast, 3KO mice showed a modestly increased DP/DN, which may represent more efficient development of DP due to predominantly RasGRP1 driven signaling during development. No statistical difference in DP/DN ratio was observed between 1KO and DKO mice. Therefore, it

appears that in addition to regulating positive selection, RasGRP1 also regulates the generation of DP thymocytes.

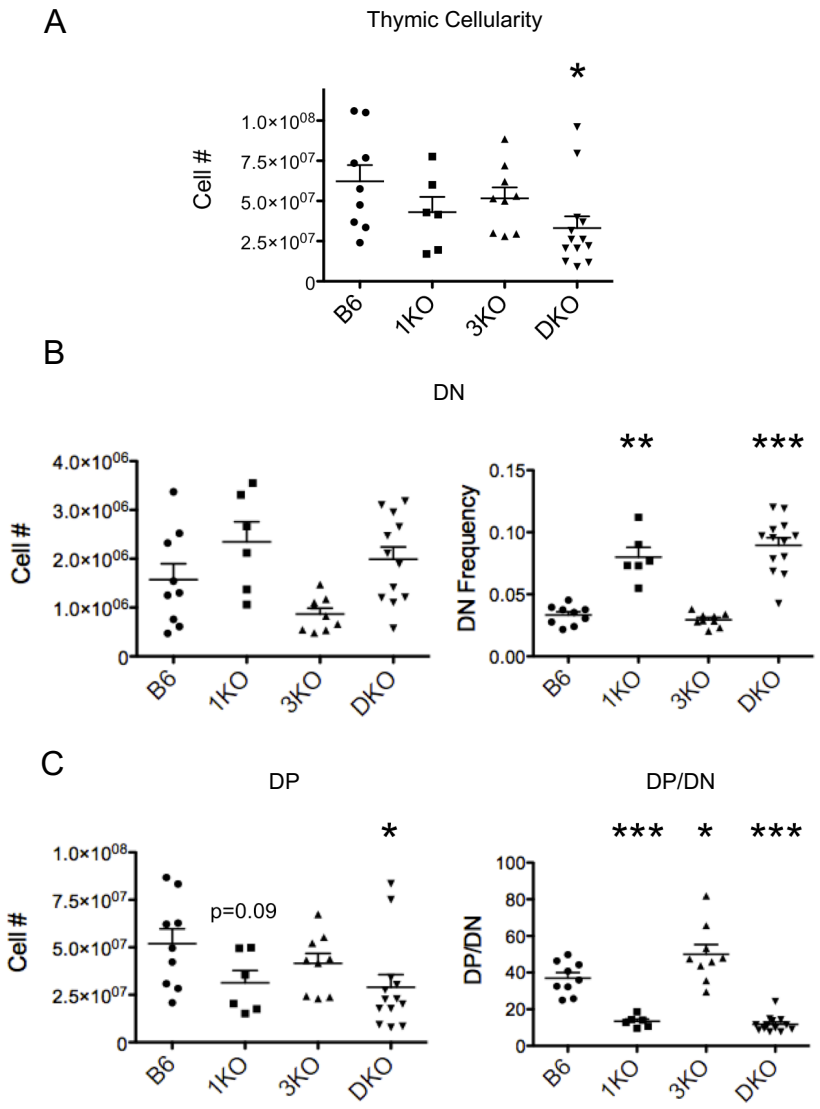

Figure 4-3. RasGRP1 KO and RasGRP1/3 DKO mice display inefficient generation of DP thymocytes. A) Numbers of thymocytes from B6 (n=9), 1KO (n=6), 3KO (n=9) and DKO (n=13) thymi. B) Numbers and frequencies of DN ($CD4^{-}CD8^{-}Thy1.2^{+}CD3^{lo}$) from B6 (n=9), 1KO (n=6), 3KO (n=9) and DKO (n=13) thymi. C) left, numbers of DP ($CD4^{+}CD8^{+}Thy1.2^{+}$); right, ratio of frequencies of DP/DN. *p<0.05, **p<0.01 and ***p<0.001.

RasGRP1 KO and RasGRP 1/3 DKO animals show inefficient thymocyte development beyond DN3

Since RasGRP1 deficiency results in inefficient DN to DP development, we next examined the DN compartment of wildtype and RasGRP1/3 deficient thymi through examining CD44/CD25 profiles of DN ($CD4^-CD8^-Thy1.2^+CD3^{lo}$) thymocytes. We found that 1KO and DKO thymi showed significantly increased frequencies and numbers of DN3 thymocytes ($CD44^-CD25^+$) relative to B6 (**Fig. 4-4A,B**), suggesting defects in β-selection. One important result of β-selection is the differentiation of DN3 thymocytes into DN4 thymocytes ($CD44^-CD25^-$). To evaluate this critical differentiation step in the thymocyte developmental program, we examined the ratio of frequencies of DN3 to DN4 thymocytes (DN3/DN4). Interestingly, 1KO and DKO mice showed significant increases in DN3/DN4, providing further evidence of impaired β-selection in the absence of RasGRPs (**Fig. 4-4B**). The DN3/DN4 appears to be modestly higher in DKO thymi compared to 1KO, although not statistically significant (**Fig. 4-4B**). Loss of RasGRP3 alone does not appear to influence the development of DN3 into DN4 and the contribution of RasGRP1 to β-selection appears to be dominant to RasGRP3. Altogether, these results confirm that RasGRP1 is required for efficient thymocyte β-selection, while RasGRP3 does not appear to contribute at this developmental checkpoint.

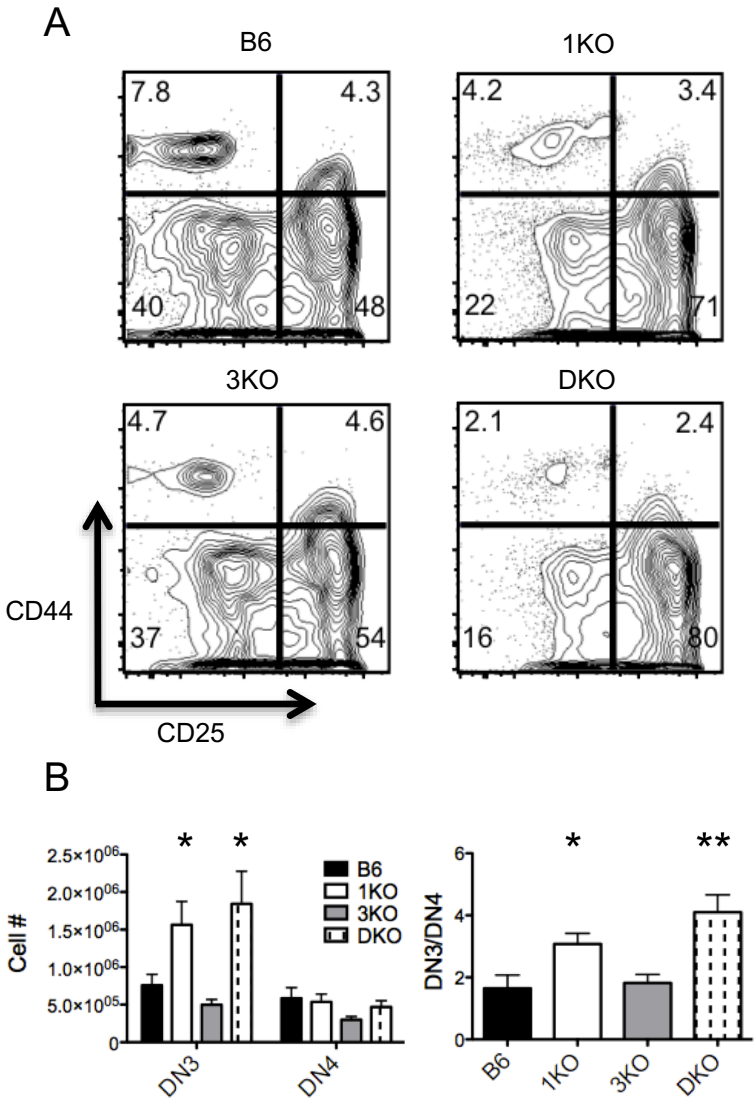

Figure 4-4. RasGRP1 KO and RasGRP 1/3 DKO show inefficient thymocyte development beyond DN3. A) CD44 by CD25 profiles of DN thymocytes ($CD4^-CD8^-Thy1.2^+CD3^{lo}$) from B6 (n=9), 1KO (n=6), 3KO (n=9) and DKO (n=13) thymi. B) left, numbers of DN3 ($CD4^-CD8^-CD3^{lo}CD44^-CD25^+$) thymocytes and DN4 ($CD4^-CD8^-CD3^{lo}CD44^-CD25^-$) thymocytes; right, ratio of frequencies of DN3/DN4. *p<0.05 and **p<0.01

RasGRP1 and RasGRP3 are dispensable for bulk γδ T cell development

The bifurcation of αβ and γδ T cell development is thought to occur at the DN3 stage. Since we saw a significant increase in DN3 numbers in RasGRP1/3 deficient thymi and DN3 can give rise to both αβ and γδ T cells, we wanted to address possible alterations in γδ T cell development as a result of RasGRP1/3 deficiency. To examine thymic γδ development we looked for the presence of mature γδTCR^{+}CD3^{+} thymocytes in B6 and RasGRP1/3 deficient thymi. We found that 1KO, 3KO and DKO thymi showed similar numbers and frequencies of mature γδ thymocytes as B6 (**Fig. 4-5A,B**). These results suggested that the increased DN3/DN4 seen in RasGRP1/3 deficient thymi was likely due to defects in αβ development and not γδ development. Therefore, we next focused on αβ development from DN3 in RasGRP1/3 deficient mice to further study the basis of impaired β-selection in these animals.

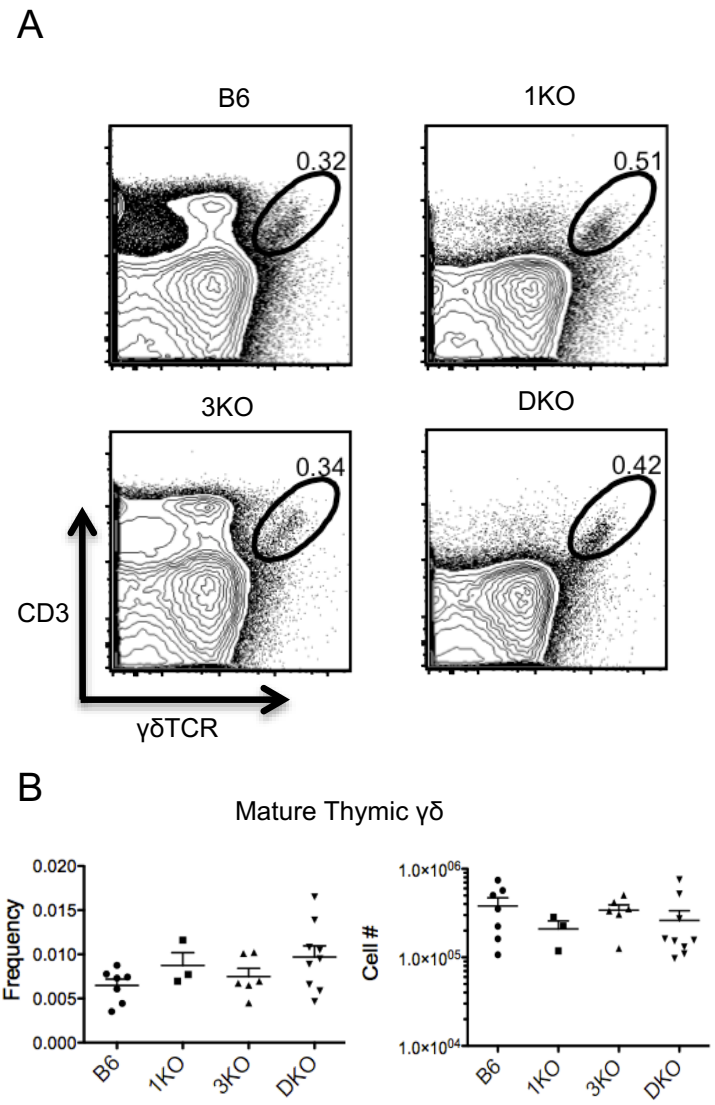

Figure 4-5. RasGRP1 KO and RasGRP1/3 DKO DN4 thymocytes show an increased frequency of γδ T cells, despite normal numbers and frequencies of mature thymic γδ T cells. A) CD3 by γδTCR profiles of bulk thymocytes from B6 (n=7), 1KO (n=3), 3KO (n=6) and DKO (n=9) thymi. B) Frequencies (left) and numbers (right) of mature $CD3^{+}\gamma\delta TCR^{+}$ γδ T cells.

RasGRP1 deficient DN3 show impaired transition from DN3E to DN3L

To evaluate the passage of DN3 through β-selection more closely, we examined early DN3 (DN3E) and late DN3 (DN3L) populations of B6, 1KO, 3KO and DKO thymocytes. $DN3E^{TCR\beta i.c.+}$ are DN3 that have undergone successful VDJ recombination, but have not yet undergone pre-TCR driven proliferation. In contrast, $DN3L^{TCR\beta i.c.+}$ are DN3 that have received a pre-TCR signal and as a result undergo blastogenesis, which can be detected as changes in cell size (*117, 118*). To focus our analysis on the DN3E to DN3L transition specifically, we examined intracellular $TCR\beta^+$ ($TCR\beta^+_{i.c.}$) by forward scatter (FSC) profiles of DN3 thymocytes. The DN3 compartment of 1KO and DKO thymi showed increased frequencies of $DN3E^{TCR\beta i.c.+}$ and decreased frequencies of $DN3L^{TCR\beta i.c.+}$ compared to B6 and 3KO DN3 (**Fig. 4-6A**), despite showing similar frequencies of total $TCR\beta_{i.c.}^+$ DN3 (**Fig. 4-6C**), suggesting defective TCRβ rearrangement does not underlie impaired β-selection in these mice. Of note, 1KO and DKO thymi showed significantly reduced frequencies of $TCR\beta_{i.c.}^+$ DN4 compared to B6 (**Fig. 4-6C**), suggesting that the composition of the DN4 compartment is altered in RasGRP1 and RasGRP1/3-deficient thymi. To gain more insight into progression through β-selection, we calculated the ratio of frequencies of $DN3E^{TCR\beta i.c.+}$ to $DN3L^{TCR\beta i.c.+}$ over multiple experiments. 1KO and DKO thymi showed a significant increase in (DN3E/DN3L)$TCR\beta_{i.c.}^+$ ratios compared to B6 (**Fig. 4-6B**), strongly suggesting DN3E are unable to undergo efficient blastogenesis in the absence of RasGRPs. Furthermore, DKO DN3 showed significantly higher (DN3E/DN3L)$TCR\beta_{i.c.}^+$ ratios than 1KO mice (**Fig. 4-6B**). Consistent with our previous data, 3KO mice appeared to undergo normal β-selection and showed similar (DN3E/DN3L)$TCR\beta_{i.c.}^+$ ratios as B6. The results presented here suggest that 1KO and

DKO thymocytes are specifically impaired in the transition between the DN3E and DN3L stages, despite showing normal frequencies of post VDJ recombination TCR$\beta_{i.c.}^{+}$ DN3 thymocytes. Again, our results also indicate that RasGRP3 is not required for thymocyte β-selection.

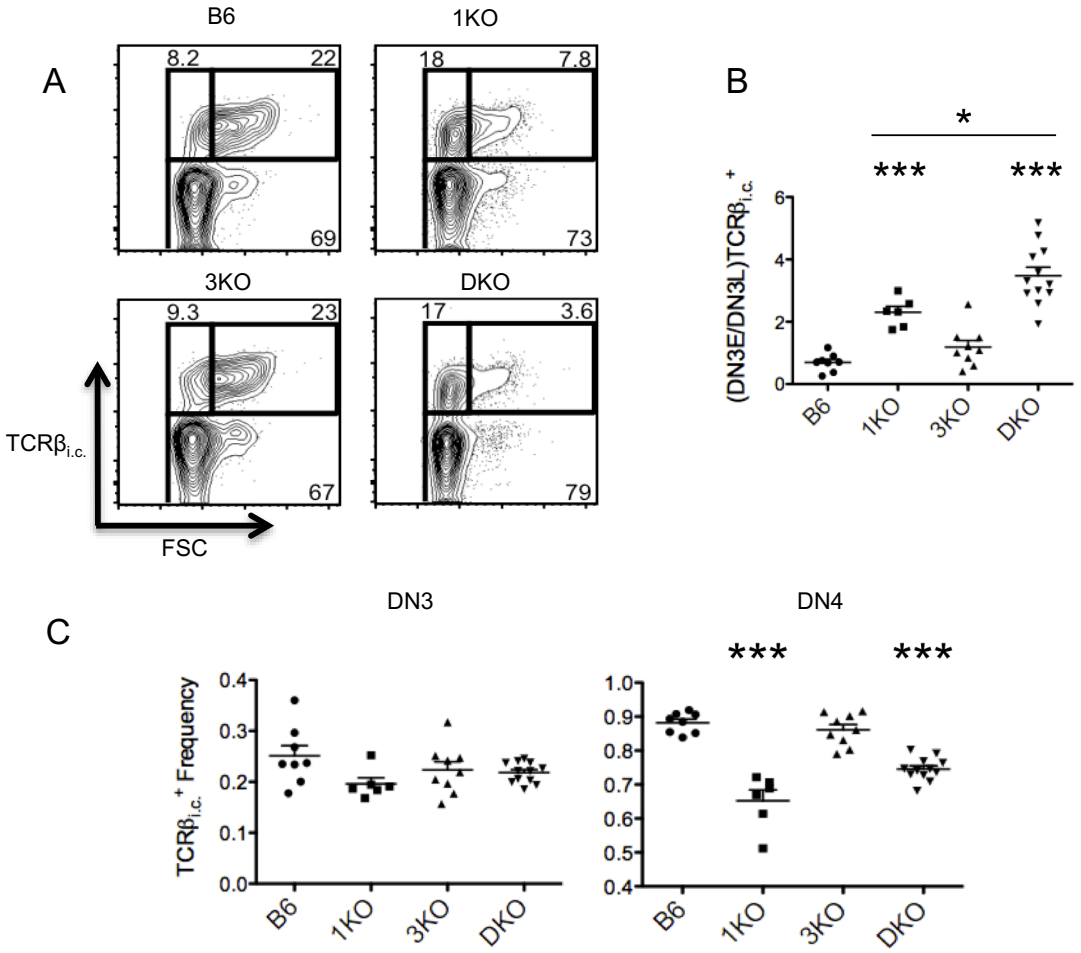

Figure 4-6. RasGRP1 deficient DN3 show impaired transition from DN3E to DN3L

A) Intracellular TCRβ (TCRβ$_{i.c.}$) by forward scatter (FSC) profiles of DN3 (CD4$^-$CD8$^-$ Thy1.2$^+$CD44$^-$CD25$^+$) from B6 (n=8), 1KO (n=6), 3KO (n=9) and DKO (n=12) thymi. B) Ratio of frequencies of TCRβ$_{i.c.}^+$ DN3E/DN3L ((DN3E/DN3L)TCRβ$_{i.c.}^+$) from the indicated mice. C) Frequencies of DN3 (CD4$^-$CD8$^-$Thy1.2$^+$CD44$^-$CD25$^+$) and DN4 (CD4$^-$CD8$^-$ Thy1.2$^+$CD44$^-$CD25$^-$) expressing intracellular TCRβ (TCRβ$_{i.c.}$).

RasGRP1 and RasGRP3 deficient DN3E show impaired differentiation on OP9-DL1 monolayers *in vitro*

Our previous results indicated that RasGRP1 deficient DN3 are impaired in the DN3E to DN3L transition *in* vivo. To obtain further confirmation that RasGRP1/3 deficient thymocytes were specifically impaired in the DN3E to DN3L transition, we tested the ability of B6 and DKO DN3E to mature using the *in vitro* OP9-DL1 model of T cell development. DN3E ($CD25^{+}CD98^{lo}$) were isolated from B6 and DKO thymi by FACS, seeded on OP9-DL1 monolayers and $Thy1.2^{+}CD44^{lo}$ DN were analyzed after 1 and 2 days of co-culture for the expression of surface markers CD98 and CD25. Consistent with our previous *in vivo* data, DKO DN3E ($CD25^{+}CD98^{lo}$) were unable to transition to DN3L ($CD25^{+}CD98^{hi}$) after 2 days of co-culture, while B6 DN3E underwent extensive maturation to DN3L (**Fig. 4-7**). Altogether, our *in vivo* and *in vitro* data strongly suggest that RasGRPs are required for efficient β-selection of DN3E and subsequent differentiation to DN3L. However, we next wanted to address the impact of RasGRP1/3 ablation on other aspects of β-selection.

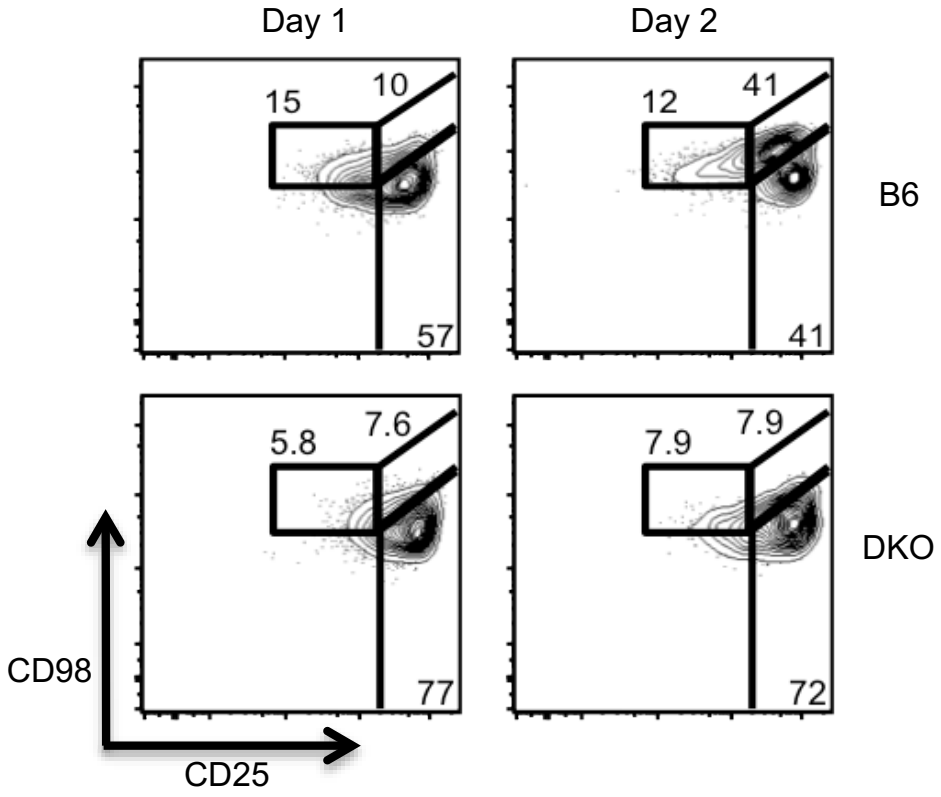

Figure 4-7. RasGRP1 and RasGRP3 deficient DN3E show impaired differentiation on OP9-DL1 monolayers *in vitro*. DN3E thymocytes ($CD4^{-}CD8^{-}Thy1.2^{+}CD3^{-}CD25^{+}CD98^{lo}$) were sorted from B6 and DKO thymi and seeded on OP9-DL1 monolayers. Cells were cultured for 1 or 2 days and subsequently analyzed for differentiation beyond DN3E. Data are presented as CD98 by CD25 profiles of $Thy1.2^{+}CD44^{-}$ cells from 1 and 2 day DN3E-OP9-DL1 co-cultures; data are representative of 3 independent experiments.

RasGRP1 deficient DN3 show impaired proliferation, but intact cell survival

One important result of β-selection is the extensive proliferation of DN3s expressing a functionally rearranged TCRβ. The lack of DN3L in RasGRP1/3 deficient mice suggested defects in DN3 proliferation. To assess proliferation in RasGRP1/3 deficient thymi more directly, we injected mice with BrdU i.p. 2 hours prior to euthanasia and assayed BrdU incorporation in DN3 and DN4 thymocytes. 1KO and DKO thymi showed a significant decrease in the frequency of $BrdU^+$ DN3 compared to B6, but not compared to each other, while 3KO DN3 showed no significant difference in BrdU incorporation compared to B6 (**Fig. 4-8A**). There were no significant differences in DN4 BrdU incorporation between any of the mice examined, suggesting DN4 proliferation occurs independently of RasGRP1/3 activity (**Fig. 4-8A**). Altogether, these data suggest that defective β-selection driven proliferation of DN3 is a major consequence of RasGRP1 loss in thymocytes.

Another important result of β-selection is the survival of developing thymocytes as they differentiate from DN3 to DN4. Therefore, changes in the apoptotic activity of developing DN3/DN4 may result in changes in β-selection efficiency. To address potential alterations in thymocyte apoptosis, we examined caspase 3 activation in DN3, DN4 and DP thymocytes from B6 and RasGRP1/3 deficient mice. We found that 1KO, 3KO and DKO thymi showed no significant differences in the percentages of active caspase 3^+ DN3, DN4 or DP compared to B6 (**Fig. 4-8B**). In fact, it appears that defective β-selection in 1KO and DKO thymi mildly reduced the frequency of active caspase 3^+ DN3 and DN4, suggesting that RasGRP1/3 deficiency does not alter thymocyte

apoptosis. Furthermore, normal frequencies of active caspase 3^+ DP thymocytes from RasGRP1/3 deficient thymi suggest that reduced DP/DN ratios observed in RasGRP1/3 deficient thymi are a result of inefficient DN to DP development, rather than increased DP apoptosis.

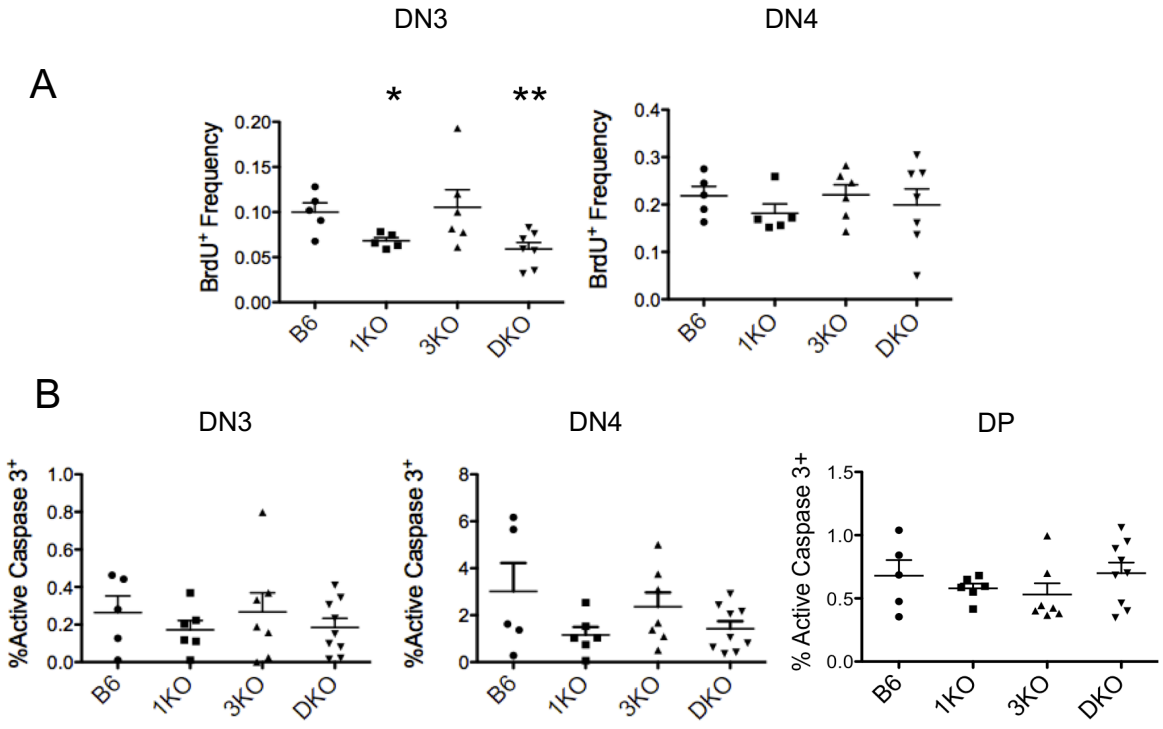

Figure 4-8. RasGRP1 deficient DN3 show impaired proliferation, but intact cell survival. A) Frequencies of BrdU$^+$ DN3 (CD4$^-$CD8$^-$Thy1.2$^+$CD44$^-$CD25$^+$) and DN4 (CD4$^-$CD8$^-$Thy1.2$^+$CD44$^-$CD25$^-$) from B6 (n=5), 1KO (n=5), 3KO (n=6) and DKO (n=7) mice injected with BrdU i.p. 2h prior to euthanasia. B) Percentages of DN3 (CD4$^-$CD8$^-$Thy1.2$^+$CD44$^-$CD25$^+$), DN4 (CD4$^-$CD8$^-$Thy1.2$^+$CD44$^-$CD25$^-$) and DP (CD4$^+$CD8$^+$Thy1.2$^+$) showing active caspase 3.

RasGRP1 is required for ERK activation in DN3 thymocytes in response to CXCR4 stimulation

Signaling through CXCR4 has recently been shown to be an important co-stimulator of pre-TCR signaling and is required for efficient β-selection (*37*). Stimulating DN3 through CXCR4 with SDF1α results in the phosphorylation and activation of ERK and AKT, two kinases that are thought to be important during β-selection. To address whether RasGRP1/3 are involved in CXCR4 dependent ERK and AKT activation, we stimulated B6 and DKO thymocytes with 10 nM SDF1α and examined the phosphorylation of ERK and AKT in DN3. DKO DN3 showed an almost complete loss of ERK phosphorylation after 1 min and 2 min of SDF1α stimulation compared to B6 (**Fig. 4-9A**). In contrast, B6 and DKO DN3 showed similar fold inductions of AKT activation at all time points (**Fig. 4-9B**). These results suggested that RasGRP1/3 are required for ERK activation in response to SDF1α/CXCR4 signaling, but RasGRP1/3 deficiency did not impair PI3K/AKT signaling in response to SDF1α. Interestingly, RasGRP1 contains a C-terminal plasma membrane targeting (PT) domain that includes a basic/hydrophobic cluster of amino acids shown to interact with phosphoinositides (*99, 119*). Therefore, targeting of RasGRP1/3 to the plasma membrane and subsequent Ras activation may be dependent on PI3K activity. To address whether PI3K may be regulating RasGRP1/3 during CXCR4 signaling, we carried out the same stimulations in presence of 20 μM of the PI3K inhibibitor, LY294002. We found that inhibition of PI3K had little to no effect on ERK activation in B6 and DKO DN3 (**Fig. 4-9A**). LY294002 treated DN3 from B6 and DKO thymi showed similar fold inductions of pERK in the presence or absence of inhibitor and B6 DN3 maintained significantly higher fold inductions of pERK compared

to DKO. Also, as a negative control we measured pAKT fold induction in LY294002 treated DN3 and saw a dramatic loss of AKT activation in both mice as expected (**Fig. 4-9B**). Therefore, although RasGRP1/3 are required for ERK activation in response to SDF1α, PI3K activity does not regulate RasGRP1/3 mediated ERK activation during CXCR4 signaling.

Although RasGRP1/3 deficient DN3 were unable to activate ERK in response to CXCR4 signaling, analysis of DKO DN3 could not distinguish the individual contributions of RasGRP1 and RasGRP3 to ERK activation downstream of CXCR4. To gain insight into the individual roles of RasGRP1 and RasGRP3 during CXCR4 mediated ERK activation, we carried out the previously described SDF1α stimulations using 1KO and 3KO DN3 (**Fig. 4-9C**). 3KO DN3 showed similar fold inductions of pERK as B6 in response to SDF1α stimulation, at all time points. In contrast, 1KO and DKO DN3 were equally impaired in their ability to activate ERK following SDF1α stimulation **Fig. 4-9C**. Therefore, RasGRP1, but not RasGRP3, is required for ERK activation downstream of CXCR4. In addition, activation of ERK downstream of CXCR4/pre-TCR signaling represents a potential mechanism by which RasGRP1 regulates thymocyte β-selection.

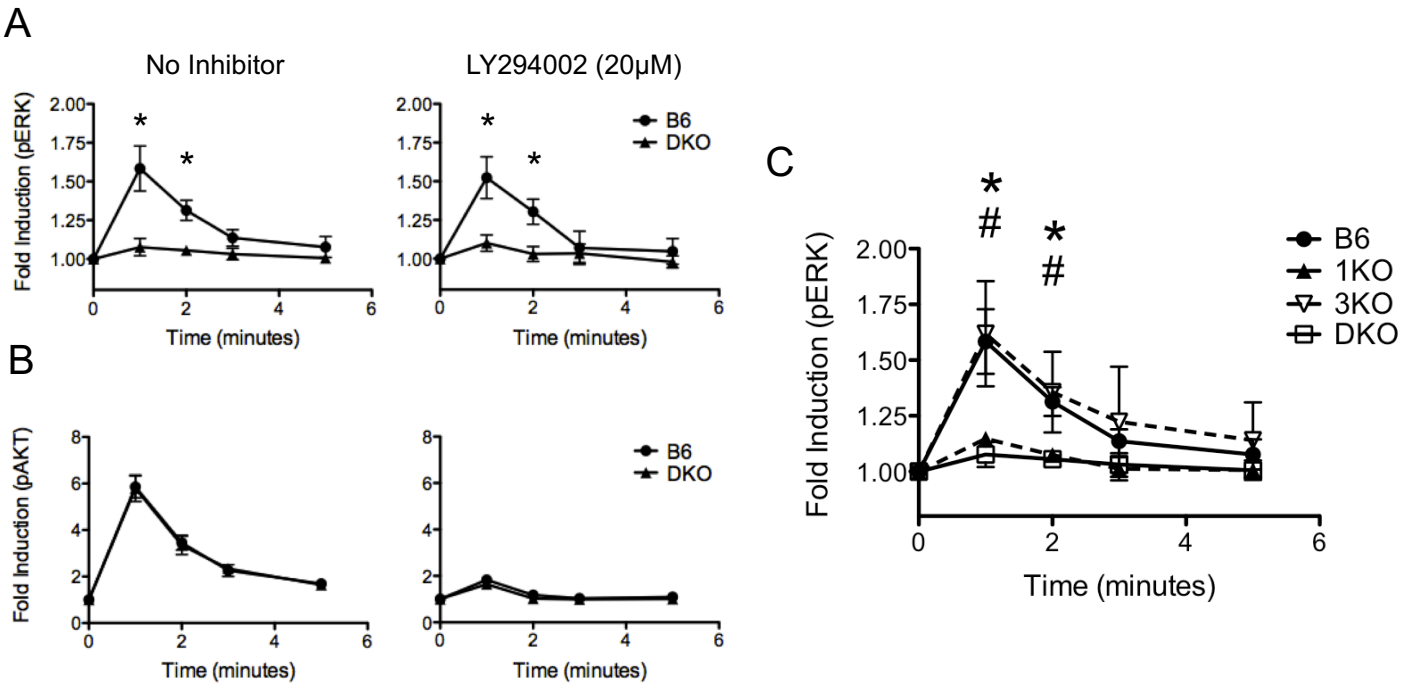

Figure 4-9. DN3 require RasGRP1 for ERK phosphorylation in response to CXCR4 activation. A, B) Bulk thymocytes were pre-treated with (right panels) or without (left panels) 20μM of the PI3K inhibitor LY294002 and were stimulated with 10nM SDF1α for 0, 1, 2, 3 or 5 minutes. A) Induction of p-ERK (pT202/pY204) was measured in DN3 ($CD4^-CD8^-Thy1.2^+CD3^{lo}$ $Thy1.2^+CD44^-CD25^+$) from B6 and DKO thymi. B) Induction of p-AKT (pS473) was measured in DN3 ($CD4^-CD8^-Thy1.2^+CD3^{lo}$ $Thy1.2^+CD44^-CD25^+$) from B6 and DKO thymi. **C.** Bulk thymocytes were stimulated with 10nM SDF1α for 0, 1, 2, 3 or 5 minutes and the induction of p-ERK (pT202/pY204) was measured in DN3 ($CD4^-CD8^-Thy1.2^+CD3^{lo}$ $Thy1.2^+CD44^-CD25^+$) from B6, 1KO, 3KO and DKO thymi. Data are representative of between 3 and 7 independent experiments. * represents a significant statistical comparison between B6 and DKO. # represents a significant statistical comparison between B6 and 1KO. */# $p<0.05$.

Conclusion

The data presented in this chapter highlight the importance of RasGRP1 in thymocyte β-selection and describe various roles of RasGRP1 in regulating this critical developmental checkpoint. Although our previous work described a role for RasGRP3 in regulating ETP development, it appears that RasGRP3 is dispensable for T cell development events downstream of ETPs. In particular, RasGRP3 is not required for β-selection, positive selection and iNKT cell agonist selection, while RasGRP1 activity is required for all of these developmental events. Examination of RasGRP1/3 deficient mice revealed that 1KO and DKO animals show inefficient development of DP thymocytes from DN, suggesting impairments in β-selection. Detailed analysis of thymocyte β-selection revealed that RasGRP1 regulates thymocyte differentiation and proliferation during β-selection. Both 1KO and DKO mice showed increased numbers of DN3 thymocytes, accompanied by impaired transition between the DN3E and DN3L stages, both *in vivo* and *in vitro*. Finally, co-stimulation of pre-TCR signaling by the chemokine receptor CXCR4 has been shown to be a critical regulator of β-selection. Our data show that RasGRP1 is required for ERK activation downstream of CXCR4, describing a potential mechanism through which RasGRP1 regulates passage through β-selection.

CHAPTER 5: RESULTS - RasGRP1 is required for agonist selection of a heterogeneous population of TCRαβ$^+$CD8αα IEL progenitors

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Experiments shown in **Figure 5-3** and **Figure 5-4** were designed by D.P.G and T.A.B and performed by D.P.G and L.M.H.C.

Introduction

Previous work aimed at understanding the role of RasGRP1 during thymocyte positive and negative selection has shown that RasGRP1 is critical for transducing low-affinity TCR signals during thymocyte positive selection, while RasGRP1 activity is dispensable for high-affinity TCR signaling during clonal deletion. However, it has recently emerged that RasGRP1 activity may also regulate thymocyte agonist selection. In particular, RasGRP1 is critical for the development of the agonist selected iNKT lineage (*120*). Another lineage that requires agonist signals for its selection is the TCRαβ$^+$CD8αα IEL lineage. To date, the role of RasGRP1 in regulating agonist selection of the TCRαβ$^+$CD8αα IEL lineage has not been examined. Given that RasGRP1 regulates agonist selection of iNKT cells, we predict that RasGRP1 is also required for agonist

selection of TCRαβ$^+$CD8αα IEL. More specifically, we hypothesize that RasGRP1 regulates the development of IELp cells that develop following agonist stimulation of DP thymocytes. These agonist selected cells ultimately traffic out of the thymus and move into the small intestine, where they develop into TCRαβ$^+$CD8αα IEL in an IL-15 dependent manner. Here, we explore the role of RasGRP1 in regulating the development of agonist selected IELp and downstream TCRαβ$^+$CD8αα IEL progeny.

RasGRP1 regulates TCRαβ$^+$CD8αα IEL development

To investigate a potential role for RasGRP1 in TCRαβ$^+$CD8αα IEL generation, we examined IEL populations present in small intestines from wild type (WT) and RasGRP1 knockout (1KO) mice. The IEL compartment includes both γδ and αβ IEL populations that express CD8αα homodimers, which we defined as γδTCR$^+$CD8αα (CD45$^+$CD4$^-$ TCRβ$^-$gdTCR$^+$CD8α$^+$CD8β$^-$) and TCRαβ$^+$CD8αα IEL (CD45$^+$CD4$^-$TCRβ$^+$gdTCR$^-$ CD8α$^+$CD8β$^-$). Within the CD45$^+$CD4$^-$ compartment, 1KO mice showed dramatically reduced frequencies of TCRβ$^+$ cells, accompanied by increased frequencies of γδTCR$^+$ cells, relative to WT animals (**Fig. 5-1A**). Further analysis of the TCRβ$^+$ and γδTCR$^+$ compartments revealed a differential requirement of RasGRP1 in γδ and αβ IEL development. Specifically, 1KO animals showed significantly reduced numbers and frequencies of TCRαβ$^+$CD8αα IEL compared to WT (**Fig. 5-1A, B**), suggesting that RasGRP1 is required for TCRαβ$^+$CD8αα IEL development. Furthermore, accompanying reductions to the TCRαβ$^+$CD8αα compartment, 1KO small intestines showed significantly increased numbers and frequencies of γδTCR$^+$CD8αα relative to WT (**Fig. 5-1A, B**). Altogether, these results suggest that RasGRP1 deficiency alters the

composition of the IEL compartment and highlight the requirement of RasGRP1 in TCRαβ^{+}CD8αα IEL development.

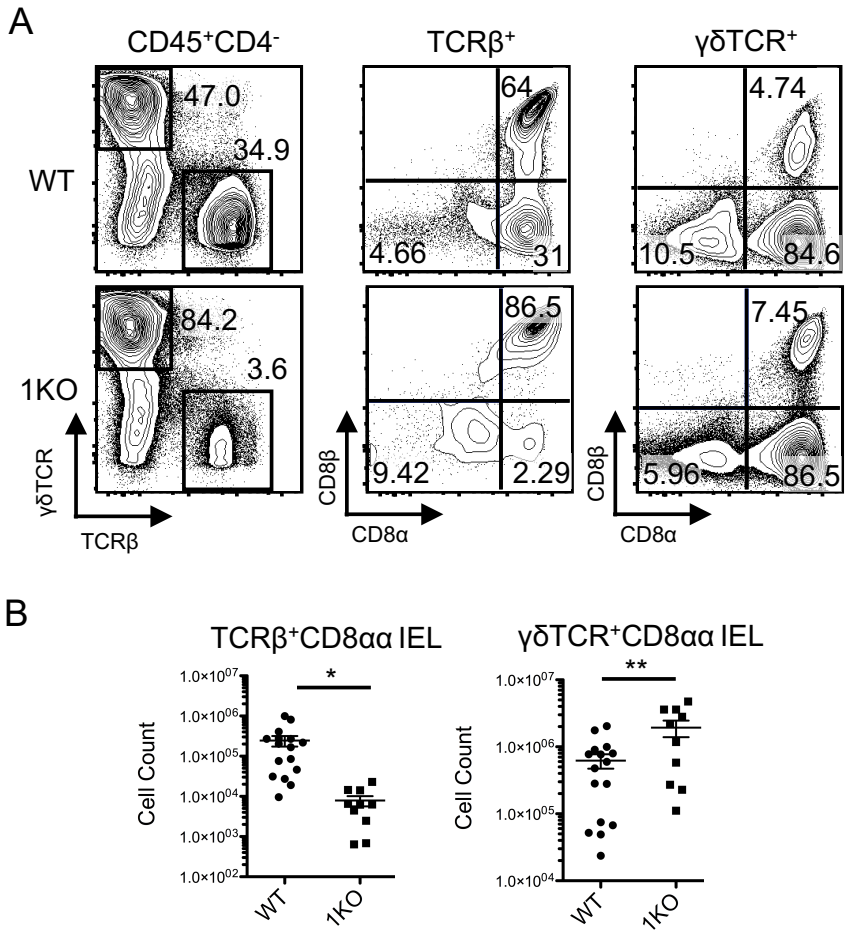

Figure 5-1. **RasGRP1 regulates TCRαβ$^+$CD8αα IEL development**. IELs were isolated from small intestines of WT and 1KO animals and analyzed by flow cytometry. A) CD45$^+$CD4$^-$ cells were gated and analyzed for expression of TCRβ and TCRγδ (left). TCRβ$^+$ (middle) and γδTCR$^+$ (right) IELs were analyzed for expression of CD8α and CD8β. B) Numbers of TCRαβ$^+$ CD8αα IEL (left) and γδTCR$^+$CD8αα (right) isolated from the indicated mice. Data were obtained from indicated number of mice over 14 individual experiments; WT (n=16), 1KO (n=10). *$p<0.05$ and **$p<0.01$

RasGRP1 is required for the development of agonist selected thymic IEL progenitors

Since thymic progenitors are known to give rise to TCRαβ^{+}CD8αα IEL, we examined thymocytes from WT and 1KO mice for the presence of agonist selected IELp cells. Based on a number of previous studies (*43, 45, 49*), we identified IELp cells as having a CD4$^{-/dull}$CD8$^{-/dull}$NK1.1^{-}TCRβ^{+}CD5^{+} phenotype (**Fig 5-2A**). Compared to WT mice, 1KO mice contained higher frequencies of CD4$^{-/dull}$CD8$^{-/dull}$ thymocytes, but showed dramatically lower frequencies of NK1.1^{-}TCRβ^{+}CD5^{+} cells, indicative of impaired IELp development. Similar to the pattern observed in TCRαβ^{+}CD8αα IEL, 1KO thymi showed significantly reduced numbers of IELp compared to WT (**Fig 5-2B**). Altogether, these data suggest that RasGRP1 regulates TCRαβ^{+}CD8αα IEL generation through regulating the development of agonist selected IELp cells.

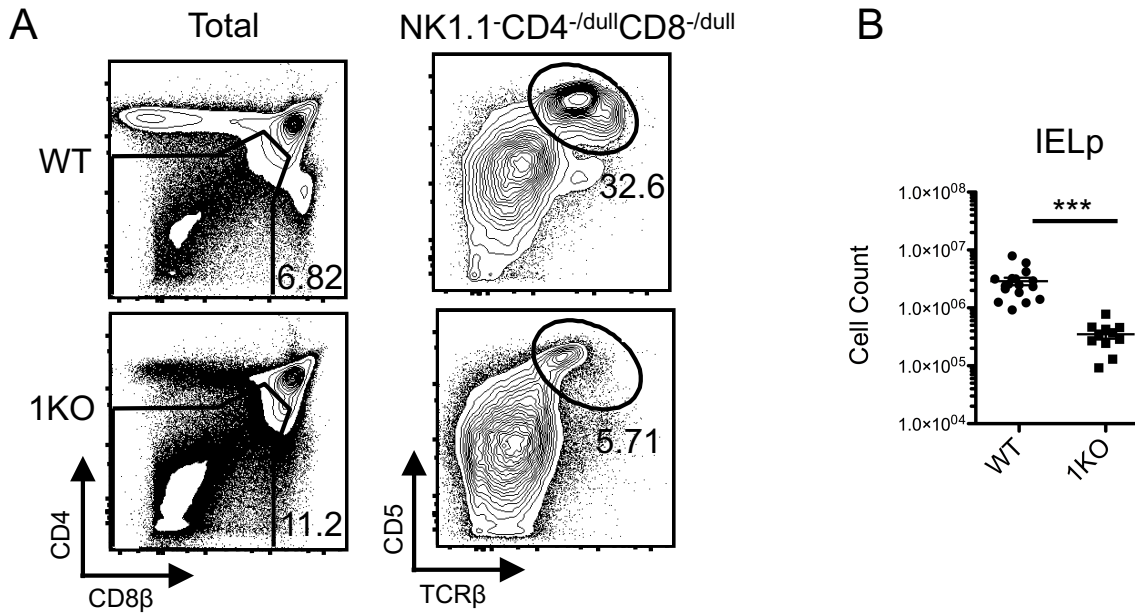

Figure 5-2. **RasGRP1 is required for the development of agonist selected thymic IEL progenitors**. Thymi were harvested from the indicated mice and analyzed for the presence of IELp cells. A) Total thymocytes were analyzed for expression of CD4 and CD8 and $CD4^{-/dull}CD8^{-/dull}$ were gated (left). $NK1.1^{-}$ cells were subsequently analyzed for expression of TCRβ and CD5 (right). B) Number of IELp cells ($CD4^{-/dull}CD8^{-/dull}NK1.1^{-}TCR\beta^{+}CD5^{+}$) from the indicated mice. Data were obtained from the indicated number of mice over 17 individual experiments; WT (n=17), 1KO (n=11). ***$p<0.001$.

RasGRP1-deficient IELp cells show signs of impaired TCR signaling

Given that IELp cells ultimately rely upon high affinity TCR stimulation for their development, we next examined IELp cells for the expression of molecules associated with high affinity antigen encounter. Signaling through CD122, expressed on the cell surface of IELp cells that have reached the small intestine, is required for the IL-15 dependent generation of TCRαβ$^+$CD8αα IEL within the gut. Furthermore, CD122 has been shown to be upregulated following high affinity antigen encounter in the thymus and is expressed on the surface of a fraction of IELp cells within the thymus (*52*). Therefore, we wished to determine if RasGRP1 influenced the frequency of IELp that express CD122. Despite finding markedly reduced numbers of IELp in 1KO thymi, we observed similar frequencies of CD122$^-$ and CD122$^+$ IELp within WT and 1KO animals (**Fig. 5-3A**). Given that CD122 expression is required for TCRαβ$^+$CD8αα IEL development and that we observed two distinct populations of IELp based on expression of CD122, we divided the IELp population into CD122$^-$ and CD122$^+$ fractions for subsequent analysis of IELp development.

Since IELp are generated following strong TCR signaling (*43*) and RasGRP1 is a key regulator of TCR signaling at various stages of thymocyte development (*92, 105, 114*), we examined the expression of additional molecules regulated by strong TCR signals. Both the co-inhibitory molecule PD-1 and transcription factor Helios are induced following high affinity TCR signaling (*121, 122*). In both WT and 1KO IELp, we observed significantly increased frequencies of PD-1$^+$Helios$^+$ cells within CD122$^+$ IELp compared to CD122$^-$ IELp, suggesting that high affinity signaled IELp were enriched

within the CD122^{+} fraction. However, there was a significant reduction in the frequency of PD-1^{+}Helios^{+} cells in 1KO mice compared to WT mice in both the CD122^{-} and CD122^{+} IELp fractions (**Fig. 5-3B, C**). Collectively, these data suggest that RasGRP1-deficiency impairs the generation of high affinity signaled IELp, which may contribute to impaired IELp generation in 1KO thymi.

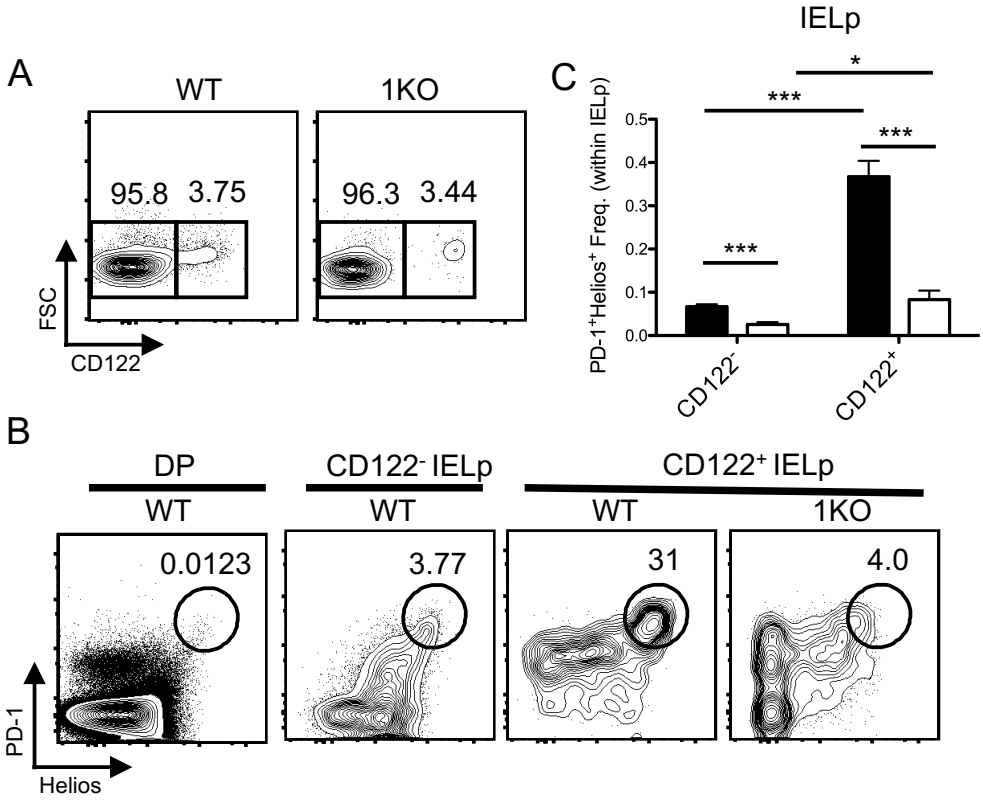

Figure 5-3. **IELp from RasGRP1-deficient animals show signs of impaired TCR signaling**. A) Representative expression of CD122 on IELp cells ($CD4^{-/dull}CD8^{-/dull}NK1.1^{-}TCR\beta^{+}CD5^{+}$) from WT and 1KO mice. Data were obtained from the indicated number of mice over 17 individual experiments; WT (n=17), 1KO (n=11). B) Representative PD-1 and Helios expression on DP thymocytes and $CD122^{-}$ IELp from WT mice and $CD122^{+}$ IELp cells from WT and 1KO mice. C) Frequencies of $PD\text{-}1^{+}Helios^{+}$ cells within $CD122^{-}$ and $CD122^{+}$ IELp cells from the indicated mice. Data were obtained from the indicated number of mice over 9 individual experiments; WT (n=9), 1KO (n=5). *$p<0.05$, **$p<0.01$, ***$p<0.001$.

RasGRP1 is dispensable for thymocyte clonal deletion, but is required for positive selection

To further dissect the involvement of RasGRP1 during thymic selection events, we crossed RasGRP1-deficient mice to the physiological HY^{cd4} TCR transgenic mouse model to allow examination of thymocytes with a fixed TCR specificity (*107*). HY^{cd4} mice express a MHCI-restricted, male Ag-specific, TCR that is recognized by the mAb T3.70. Female HY^{cd4} mice are a model of positive selection due to a lack of high affinity Ag expression, while male mice provide a model to examine responses to high affinity peptide. Consistent with a role for RasGRP1 during thymocyte positive selection, HY^{cd4} female mice showed a robust Ag specific ($T3.70^{+}$) CD8SP thymocyte population, which was severely reduced in the absence of RasGRP1 (**Fig. 5-4A**). Thymocytes from HY^{cd4} $RasGRP1^{-/-}$ male mice showed a similar reduction of Ag specific CD8SP thymocytes compared to their RasGRP1-sufficient counterparts, suggesting that clonal deletion remains intact in the absence of RasGRP1 (**Fig. 5-4A)**. To directly evaluate clonal deletion, we examined caspase 3 activation within male HY^{cd4} and HY^{cd4} $RasGRP1^{-/-}$ $T3.70^{+}$ thymocytes. While $T3.70^{+}$ DP^{bright} thymocytes showed little caspase 3 activation in both HY^{cd4} and HY^{cd4} $RasGRP1^{-/-}$ male mice, $T3.70^{+}$ DP^{dull} thymocytes from both strains showed a similar, elevated percentages of active caspase 3^{+} cells (**Fig. 5-4B**). These data confirm that while RasGRP1 is required for positive selection, it is dispensable for thymocyte clonal deletion.

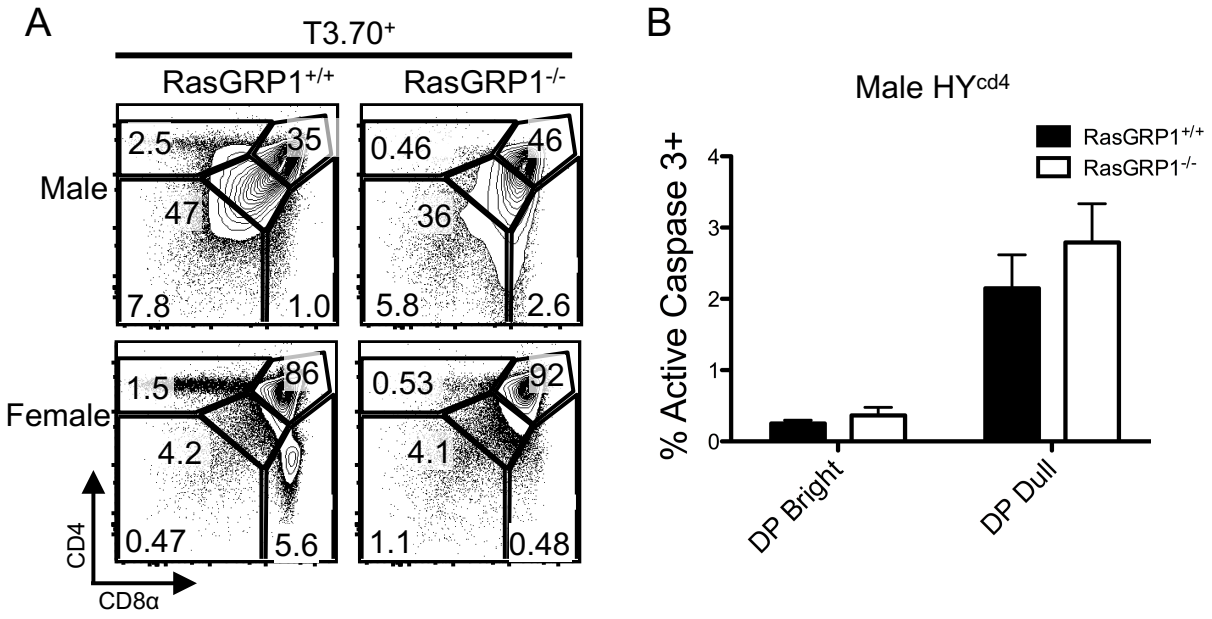

Figure 5-4. **RasGRP1 is dispensable for thymocyte clonal deletion, but is required for positive selection.** HY^{cd4} mice were crossed onto the RasGRP1 KO background and thymi from male and female HY^{cd4} animals were examined. A) CD4 and CD8 profiles of $T3.70^{+}$ thymocytes from the indicated mice. B) Percentages of active caspase 3^{+} DP^{bright} and DP^{dull} cells. Data were obtained from the indicated number of mice over 4 individual experiments; WT (n=4), 1KO (n=4).

RasGRP1 is required for transducing TCR signals in response to high affinity antigen stimulation

Although RasGRP1 activity is dispensable for thymocyte clonal deletion, it remains unclear whether RasGRP1 deficiency impacts high affinity TCR signaling. We next sought to evaluate the impact of RasGRP1 deficiency on high affinity TCR signaling using male HY^{cd4} TCR transgenic mice. As expected, we observed higher frequencies of $PD\text{-}1^{+}Helios^{+}$ thymocytes in $T3.70^{+}$ DP^{dull} compared to DP^{bright} populations in HY^{cd4} male mice (**Fig. 5-5A**)(*123*). However, $T3.70^{+}$ DP^{bright} and DP^{dull} fractions from HY^{cd4} $RasGRP1^{-/-}$ male mice showed few $PD\text{-}1^{+}Helios^{+}$ cells (**Fig. 5-5A**). Additionally, we examined the $CD4^{-/dull}CD8^{-/dull}$ compartment of HY^{cd4} male mice to examine the expression of other proteins induced by strong TCR signaling. We found a prominent population of $T3.70^{+}CD5^{+}$ thymocytes within the $CD4^{-/dull}CD8^{-/dull}$ population that was substantially reduced in HY^{cd4} $RasGRP1^{-/-}$ male mice (**Fig. 5-5B**). Furthermore, within the $CD4^{-/dull}CD8^{-/dull}T3.70^{+}CD5^{+}$ thymocyte compartment, HY^{cd4} $RasGRP1^{-/-}$ male mice showed reduced frequencies of $CD122^{+}$ cells relative to their WT counterparts (**Fig. 5-5B**). Altogether, these results demonstrate that RasGRP1 deficient thymocytes show reduced expression of multiple markers of strong TCR signaling, suggesting that RasGRP1 regulates TCR signaling strength following high affinity antigen encounter.

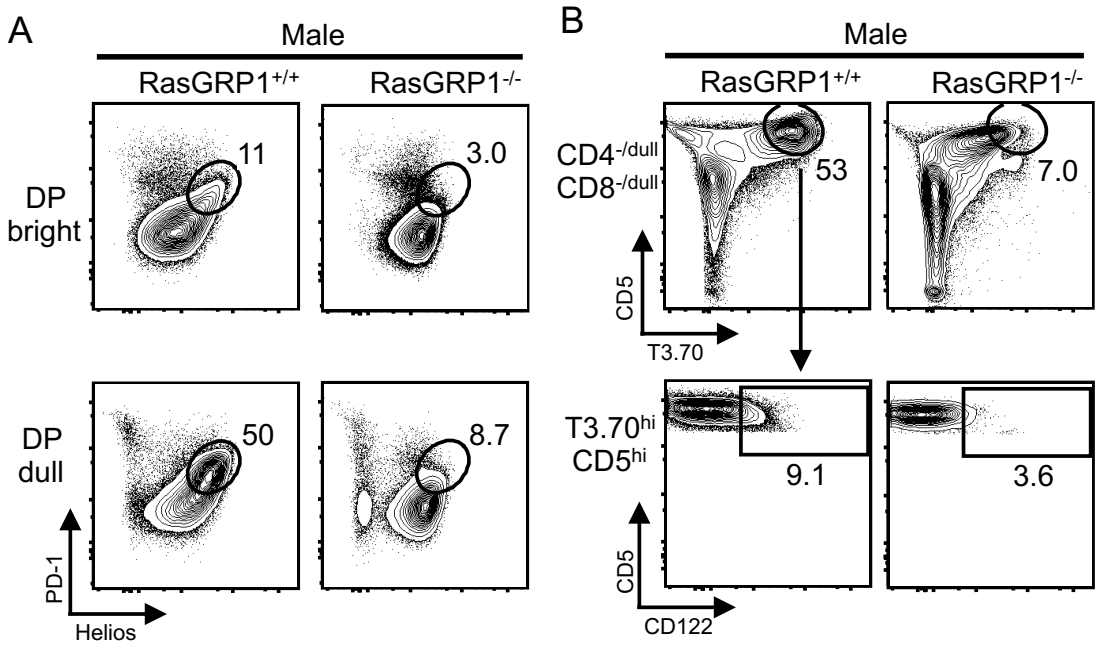

Figure 5-5. **RasGRP1 is required for transducing TCR signals in response to high affinity antigen stimulation.** HY^{cd4} mice were crossed onto the RasGRP1 KO background and thymi from male HY^{cd4} animals were examined for markers of high affinity antigen signaling. A) PD-1 and Helios profiles of DP^{bright} (top) and DP^{dull} (bottom) cells from the indicated mice. Data are representative of 4 individual experiments; n=4. B) CD4$^{-/dull}$CD8$^{-/dull}$ cells from the indicated mice were examined for expression of CD5 and T3.70 (top). T3.70^{hi}CD5^{hi} thymocytes were analyzed for expression of CD5 and CD122 (bottom). Data are representative of 3 individual experiments; n=3.

Bim deletion rescues the generation of IELp in RasGRP1-deficient mice

Either impairment in the transmission of differentiation signals required for selection and/or an inability of IELp to survive could underlie the defect in IELp generation in 1KO mice. Mice deficient in the pro-apoptotic Bcl-2 family member Bim have previously been shown to exhibit defects in clonal deletion, due to impaired ability of these cells to undergo apoptosis (*40*). In addition, Bim deficient mice contain increased numbers of IELp due to clonal diversion of cell normally destined for clonal deletion into the IELp cell lineage (*42, 43*). To determine whether increased apoptosis caused the defect in IELp generation in 1KO animals, we intercrossed RasGRP1$^{-/-}$ and Bim$^{-/-}$ (BKO) mice to create RasGRP1$^{-/-}$ Bim$^{-/-}$ double knockout (DKO) mice. As previously reported, BKO thymocytes were impaired in apoptosis induction and displayed enhanced selection of IELp (*43*) compared to WT (**Fig. 5-6A, B**). Furthermore, deletion of Bim in the 1KO background resulted in the generation of IELp to levels greater than WT (**Fig. 5-6A, B**). Therefore, the loss of Bim, in the context of RasGRP1 deficiency, rescues the development of IELp cells in 1KO animal and these results strongly suggest that RasGRP1 regulates IELp cell survival during agonist selection in a Bim dependent manner. However, it should be noted that DKO animals showed significantly reduced numbers of IELp cells compared to BKO animals (**Fig. 5-6B**), suggesting that RasGRP1 may also contribute to the regulation of IELp development independently of Bim.

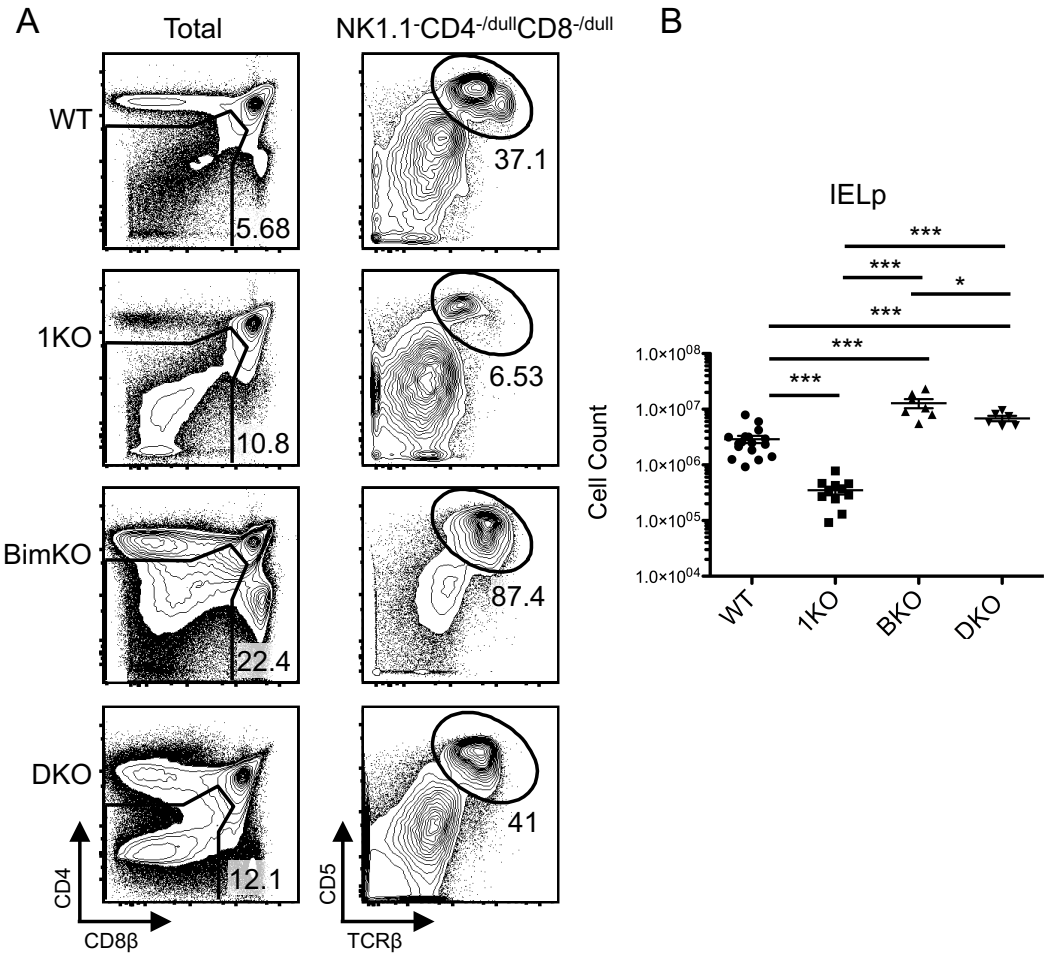

Figure 5-6. **Bim deletion rescues the generation of IELp in RasGRP1-deficient mice.** RasGRP1$^{-/-}$ mice were crossed onto the Bim$^{-/-}$ background to create DKO mice. A) Thymi were harvested from the indicated mice and analyzed for the presence of IELp cells. Total thymocytes were analyzed for expression of CD4 and CD8 and CD4$^{-/dull}$CD8$^{-/dull}$ were gated (left). NK1.1^{-} cells were subsequently analyzed for expression of TCRβ and CD5 (right). B) Number of IELp cells (CD4$^{-/dull}$CD8$^{-/dull}$NK1.1^{-}TCRβ^{+}CD5^{+}) from the indicated mice. Data were obtained from the indicated number of mice over 17 individual experiments; WT (n=17), 1KO (n=11), BKO (n=7), DKO (n=6).

Rescued IELp cells in DKO animals show signs of high affinity antigen stimulation

We determined that Bim deletion rescues IELp development in 1KO animals and sought to further study the basis of this finding. Similar to the approach taken in **Figure 5-3**, we examined expression of markers induced by high affinity TCR signaling in IELp cells. Again, we examined CD122 expression on IELp cells from our panel of mice and found that BKO IELp showed dramatically higher frequencies of $CD122^+$ cells compared to WT mice (**Fig. 5-7A**). In contrast, DKO thymi showed similar frequencies of $CD122^+$ IELp as WT (**Fig. 5-7A**). In addition to CD122 expression, we also examined IELp for expression of PD-1 and Helios. We found that $CD122^+$ IELp from BKO thymi showed significantly higher frequencies of $PD\text{-}1^+Helios^+$ cells compared to WT (**Fig. 5-7A, B**), suggesting that high affinity signaled thymocytes robustly accumulate in BKO animals. Furthermore, DKO thymi showed significantly higher frequencies of $PD\text{-}1^+Helios^+$ cells compared to 1KO (**Fig. 5-7A, B**), suggesting that the accumulation of high affinity signaled IELp cells is restored in these mice.

Given the intimate relationship between high affinity TCR signaling and clonal deletion, we further analyzed IELp for markers of apoptosis. Examination of apoptosis directly ex vivo through active caspase 3 staining revealed consistently higher levels of apoptosis in 1KO IELp in both $CD122^-$ and $CD122^+$ fractions compared to WT, but this was not statistically significant (**Fig. 5-7C**). However, it is important to note that the transition from $CD122^-$ to $CD122^+$ resulted in a statistically significant decrease in the percentage of active caspase 3^+ cells in both WT and 1KO mice, suggesting that $CD122^+$

IELp are protected from apoptosis (**Fig. 5-7C**). As expected, both BKO and DKO cells showed very low percentages of active caspase 3^+ cells.

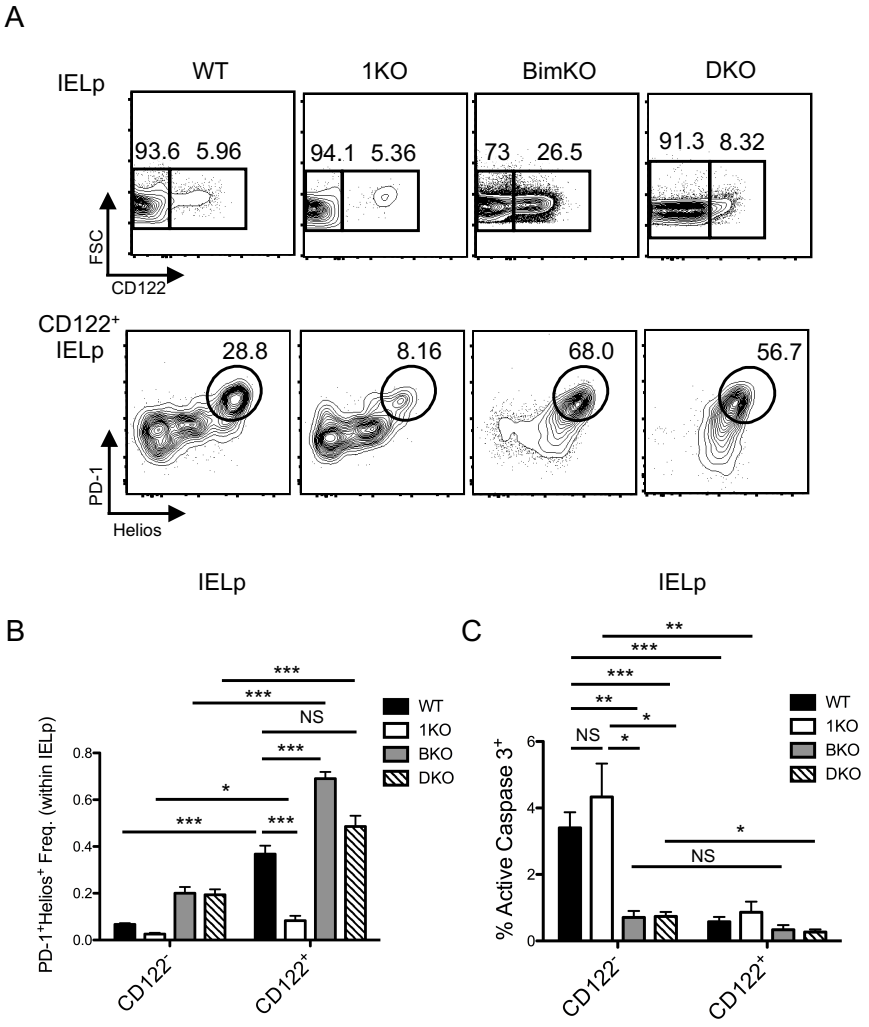

Figure 5-7. **Rescued IELp cells in DKO animals show signs of high affinity antigen stimulation.** RasGRP1$^{-/-}$ mice were crossed onto the Bim$^{-/-}$ background to create DKO mice. A) Representative expression of CD122 on IELp cells (CD4$^{-/dull}$CD8$^{-/dull}$NK1.1^{-}TCRβ^{+}CD5^{+}) (top) and representative PD-1 and Helios expression on CD122^{+} IELp cells (bottom) from the indicated mice. B) Frequencies of PD-1^{+}Helios^{+} cells within CD122^{-} and CD122^{+} IELp fractions from the indicated mice. Data were obtained from the indicated number of mice over 9 individual experiments; WT (n=9), 1KO (n=5), BKO (n=5), DKO (n=6). C) Percentages of active caspase 3^{+} cells within the CD122^{-} and CD122^{+} IELp fractions from the indicated mice. Data were obtained from the indicated number of mice over 12 individual experiments; WT (n=11), 1KO (n=8), BKO (n=4), DKO (n=6). *p<0.05, **p<0.01, ***p<0.001.

Bim deletion rescues the generation TCRαβ$^+$ CD8αα IEL in RasGRP1-deficient mice

Our previous data demonstrated that loss of Bim in 1KO animals restores IELp development, however it remained unclear whether these rescued progenitors were functional in their ability to develop into TCRαβ$^+$ CD8αα IEL. To further study the interplay between Bim and RasGRP1 in regulating the development of TCRαβ$^+$ CD8αα IEL, we analyzed small intestines from WT, 1KO, BKO and DKO animals for the presence of IEL cells. Consistent with containing increased numbers of IELp cells, BKO animals showed significantly increased numbers of TCRαβ$^+$ CD8αα IEL cells compared to WT mice (**Fig 5-8A, B**). Notably, increased numbers of TCRαβ$^+$ CD8αα IEL in BKO animals are accompanied by significantly reduced numbers of γδTCR$^+$ CD8αα IEL cells relative to WT (**Fig 5-8A, B**). Similar to the trend seen in the IELp compartment, DKO animals showed significantly higher numbers of TCRαβ$^+$ CD8αα IEL cells compared to 1KO and showed similar numbers of TCRαβ$^+$ CD8αα IEL as WT animals (**Fig 5-8A, B**). These results strongly suggest that the IELp rescue observed in DKO thymi leads to a functional restoration of the TCRαβ$^+$ CD8αα IEL compartment and further confirms the importance of RasGRP1 and Bim in regulating the development of this population.

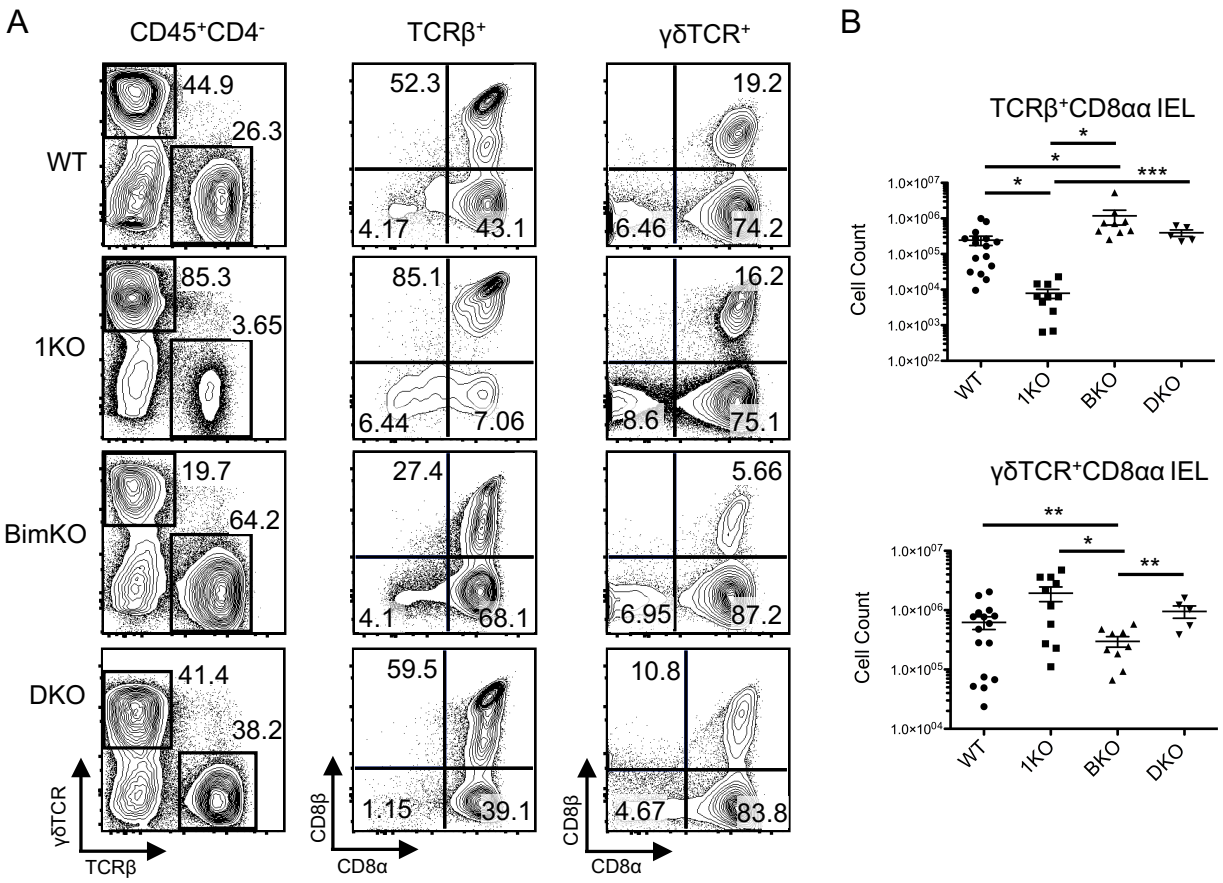

Figure 5-8. **Bim deletion rescues the generation TCRαβ$^+$ CD8αα IEL in RasGRP1-deficient mice.** IELs were isolated from small intestines of WT, 1KO, BKO and DKO animals and analyzed by flow cytometry. A) CD45$^+$CD4$^-$ cells were gated and analyzed for expression of TCRβ and TCRγδ (left). TCRβ$^+$ (middle) and γδTCR$^+$ (right) IELs were analyzed for expression of CD8α and CD8β. B) Numbers of TCRαβ$^+$ CD8αα IEL (top) and γδTCR$^+$CD8αα (bottom) isolated from the indicated mice over 15 independent experiments; WT (n=16), 1KO (n=10), BKO (n=9), DKO (n=5). *p<0.05, **p<0.01, ***p<0.001.

RasGRP1 deficient IELp show altered expression of adhesion molecules $\alpha_4\beta_7$ and CD103

To further examine the role of RasGRP1 in IELp development, we analyzed IELp cells for the expression of adhesion molecules $\alpha_4\beta_7$ and CD103. Both $\alpha_4\beta_7$ and CD103 have been shown to be induced on IELp within the thymus and may regulate progenitor migration to the gut (*55, 56*). Most $CD122^-$ IELp cells did not express either $\alpha_4\beta_7$ or CD103 (**Fig. 5-9A**). In contrast, $CD122^+$ IELp contained four distinct populations of $\alpha_4\beta_7$ and CD103 expressing cells, with the $\alpha_4\beta_7^-CD103^-$, $\alpha_4\beta_7^+CD103^-$ and $\alpha_4\beta_7^-CD103^+$ populations being most prominent and $\alpha_4\beta_7^+CD103^+$ cells constituting a minor fraction (**Fig. 5-9A**). To determine if the absence of RasGRP1 and/or Bim regulated these IELp subsets, we examined adhesion molecule expression on $CD122^+$ IELp from 1KO and BKO mice. RasGRP1 deficient $CD122^+$ IELp showed increased frequencies, but reduced numbers of the $\alpha_4\beta_7^-CD103^-$ subset, and reduced frequencies and numbers of all other subsets, relative to WT (**Fig. 5-9A, B**). In contrast, BKO IELp showed significantly increased frequencies and numbers of $\alpha_4\beta_7^+CD103^-$ cells relative to WT and reduced frequencies, but similar numbers, of $\alpha_4\beta_7^-CD103^+$ IELp (**Fig. 5-9A, B**). Taken together, these results suggest the composition of the $CD122^+$ IELp pool is altered in 1KO and BKO thymi, which may account for altered $TCR\alpha\beta^+CD8\alpha\alpha$ IEL development in these animals.

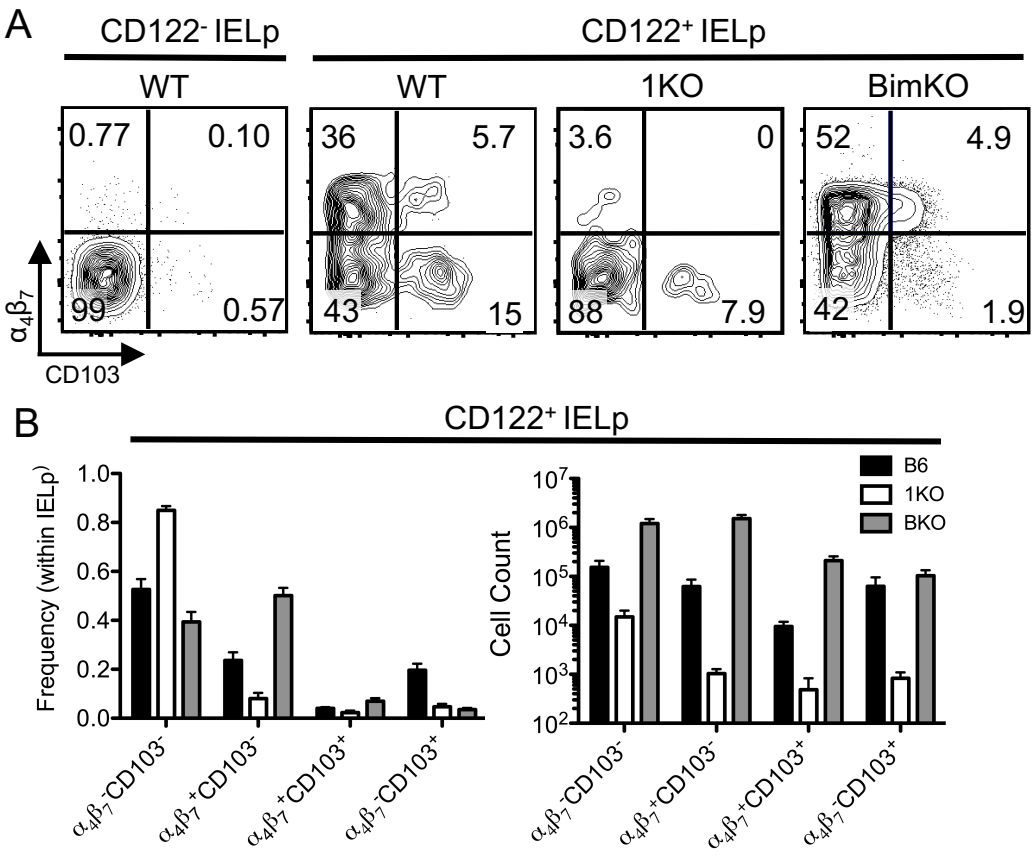

Figure 5-9. **RasGRP1 deficient IELp show altered expression of adhesion molecules $\alpha_4\beta_7$ and CD103.** Thymocytes were analyzed for expression of adhesion molecules $\alpha_4\beta_7$ and CD103 on the surface of IELp ($CD4^{-/dull}CD8^{-/dull}NK1.1^{-}TCR\beta^{+}CD5^{+}$) cells. A) $CD122^{-}$ and $CD122^{+}$ IELp cells from the indicated mice were analyzed for expression of $\alpha_4\beta_7$ and CD103. Data are representative of 6 individual experiments; WT (n=6), 1KO (n=6), BKO (n=6). B) Frequencies (left) and numbers (right) of $\alpha_4\beta_7$ and CD103 expressing subpopulations of $CD122^{+}$ IELp from the indicated mice.

Heterogeneity within the IELp population is highlighted by differences in expression of adhesion molecules and proteins regulated by TCR signal strength

Since we observed significant differences in $\alpha_4\beta_7$ and CD103 expression between $CD122^-$ and $CD122^+$ IELp, we further analyzed these distinct fractions to gain insight into the relationship between TCR signaling and adhesion molecule expression in these cells. To examine the strength of TCR signaling in $CD122^-$ and $CD122^+$ IELp cells, we utilized the $Nur77^{GFP}$ reporter mouse (*108*). Levels of Nur77 expression downstream of TCR stimulation are proportional to the strength of TCR signaling experienced by thymocytes, making this a useful model for studying TCR signal strength. Similar to previously published data using $Nur77^{GFP}$ mice, we saw that both mature CD4SP and CD8SP thymocytes expressed increased levels of GFP relative to DP thymocytes, with CD4SP thymocytes displaying elevated levels of GFP compared to CD8SP thymocytes (**Fig. 5-10A**). $CD122^-$ IELp cells showed a relatively uniform pattern of GFP expression that overlapped both CD4SP and CD8SP thymocytes. $CD122^+$ IELp pool displayed a bimodal pattern of GFP expression, such that one fraction showed similar levels of GFP as DP thymocytes, while the other showed higher levels of GFP than CD4SP thymocytes (**Fig. 5-10A**). Given that most $CD122^-$ IELp expressed GFP to levels similar to conventionally selected SP thymocytes, it appears this IELp fraction is enriched in cells that have not received agonist signals. However, $CD122^+$ IELp showed a prominent population of cells with greater GFP expression than SP thymocytes (**Fig. 5-10A**), indicating that only the $CD122^+$ IELp pool contained cells that have recently received agonist signals.

Given that we observed two distinct populations of $CD122^+$ IELp in $Nur77^{GFP}$ mice based on expression GFP, we wanted to examine these fractions for other phenotypic differences, namely expression of $\alpha_4\beta_7$ and CD103. $CD122^+GFP^{hi}$ IELp cells had prominent populations of $\alpha_4\beta_7^-CD103^-$ cells and $\alpha_4\beta_7^+CD103^-$ cells with few $\alpha_4\beta_7^+CD103^+$ cells (**Fig. 5-10B**). The $CD122^+GFP^{lo}$ IELp fraction also contained a prominent population of $\alpha_4\beta_7^-CD103^-$, but few $\alpha_4\beta_7^+CD103^-$ cells (**Fig. 5-10B**). Rather, many $CD122^+GFP^{lo}$ IELp were $\alpha_4\beta_7^-CD103^+$. Therefore, $CD122^+GFP^{lo}$ and $CD122^+GFP^{hi}$ IELp from $Nur77^{GFP}$ mice show unique expression patterns of $\alpha_4\beta_7$ and CD103 that may impact their ability to generate $TCR\alpha\beta^+CD8\alpha\alpha$ IEL. This adhesion molecule expression pattern was confirmed using PD-1 expression as an additional marker of high affinity Ag signaling (**Fig. 5-10C**). Altogether, these results indicate that IELp cells are heterogeneous with respect to expression of markers of high affinity TCR signaling and that IELp cells that have recently received agonist signals show a distinct expression pattern of adhesion molecules $\alpha_4\beta_7$ and CD103 from those IELp cells that have not recently received TCR signals.

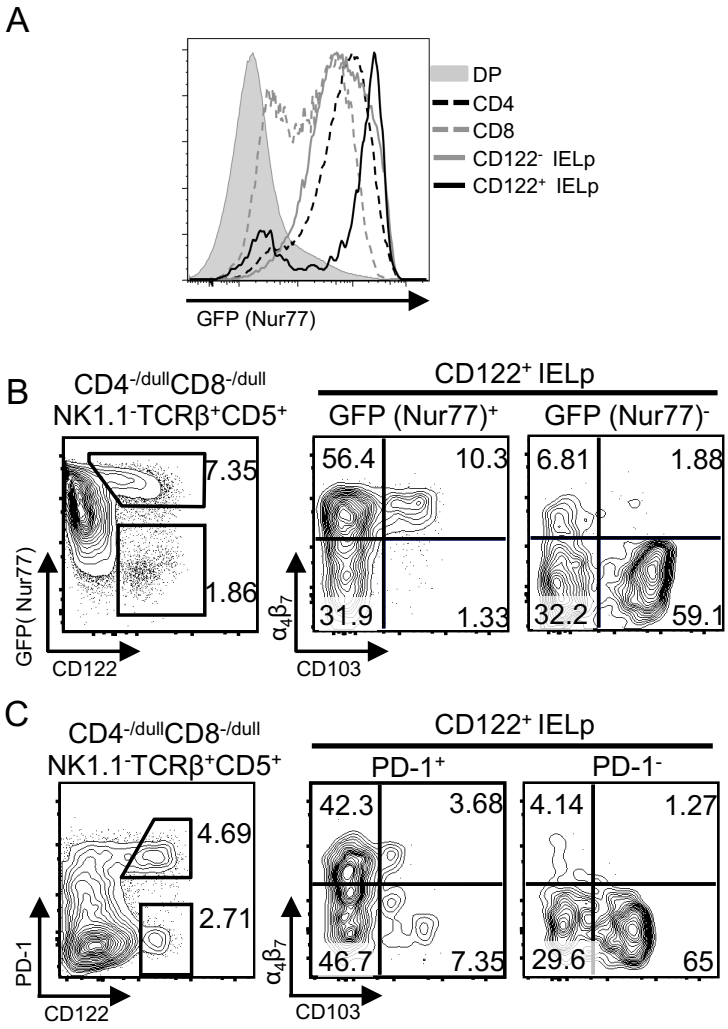

Figure 5-10. **Heterogeneity within the IELp population is highlighted by differences in expression of adhesion molecules and proteins regulated by TCR signal strength.**
A) GFP expression in DP, CD4SP, CD8SP, CD122$^-$ IELp and CD122$^+$ IELp thymocyte populations from Nur77^{GFP} reporter mice. Data are representative of 5 individual experiments; n=5. B) IELp cells were analyzed for expression of GFP and CD122. GFP^{hi}CD122$^+$ and GFP^{lo}CD122$^+$ thymocytes from Nur77^{GFP} mice were subsequently examined for expression of $\alpha_4\beta_7$ and CD103 (right). Data are representative of 5 individual experiments; n=5. C) IELp cells were analyzed for expression of PD-1 and CD122 (left). PD-1$^+$CD122$^+$ and PD-1$^-$CD122$^+$ were examined for expression of $\alpha_4\beta_7$ and CD103 (right). Data are representative of 4 individual experiments; n=4.

CD122, $\alpha_4\beta_7$ and CD103 expressing subpopulations of IELp display distinct temporal patterns of development

To gain further insight into the development of the heterogeneous IELp pool, we made use of RAG2p-GFP reporter mice(*109*). Since developing thymocytes extinguish RAG expression at the DP stage, GFP expression in mature thymocytes identifies cells that were recently derived from DP. Furthermore, we can measure the time elapsed from precursor DP thymocytes to the progeny cell of interest based on decay of the GFP signal (~54h half-life) (*124*). Similar to previous reports, most CD4SP and CD8SP thymocytes were GFP^+, with CD8SP thymocytes expressing less GFP (MFI) $\alpha_4\beta_7$ and CD103 (**Fig. 5-11A**). For agonist selected lineages, approximately 50% of Treg and 20% of iNKT cells were GFP^+ and nascent nTreg and iNKT were similar in age to CD4SP thymocytes (**Fig. 5-11A**). Similar to nTreg, approximately 50% of $CD122^+$ IELp were GFP^+, again suggesting heterogeneity within this population (**Fig. 5-11A**). Furthermore, nascent $CD122^+$ IELp displayed similar MFIs of GFP expression as CD8SP thymocytes, suggesting similar developmental kinetics (**Fig. 5-11A**). In contrast, $CD122^-$ IELp showed similar frequencies of GFP^+ cells and similar GFP MFIs as DP thymocytes (**Fig. 5-11A**). These data suggest that the $CD122^-$ IELp fraction is very recently derived from DP and appears to be immature relative to other post-selection populations, while $CD122^+$ IELp cells show similar kinetics as post-selection CD8SP thymocytes.

Our results showed that $CD122^+$ IELp were evenly split between cells that were recently derived from DP thymocytes (GFP^+) and cells that were not recently derived from DP (GFP^-). Given the presence of two distinct $CD122^+$ IELp based upon Rag2p-

GFP expression, we next examined $\alpha_4\beta_7$ and CD103 expression within these subsets (**Fig. 5-11A, B**). The majority of $\alpha_4\beta_7^+$CD103$^-$ and $\alpha_4\beta_7^+$CD103$^+$ cells were GFP$^+$, approximately 50% of $\alpha_4\beta_7^-$CD103$^-$ cells were GFP$^+$ and only about 10% of $\alpha_4\beta_7^-$CD103$^+$ cells were GFP$^+$ (**Fig. 5-11B**). These results indicated that the majority of $\alpha_4\beta_7^+$CD103$^-$ and $\alpha_4\beta_7^+$CD103$^+$ CD122$^+$ IELp are recently derived from DP thymocytes, while only half of $\alpha_4\beta_7^-$CD103$^-$ cells are nascent and the majority of $\alpha_4\beta_7^-$CD103$^+$ cells are aged. Within the GFP$^+$ populations, we further analyzed GFP MFIs to determine temporal patterns of development. GFP$^+$ $\alpha_4\beta_7^-$CD103$^-$ cells were the youngest in the CD122$^+$ IELp pool, followed by $\alpha_4\beta_7^+$CD103$^-$ cells, $\alpha_4\beta_7^+$CD103$^+$ cells, and finally $\alpha_4\beta_7^-$CD103$^+$ cells (**Fig. 5-11A**). Taken together, these results suggest that the CD122$^+$ IELp pool contains distinct subpopulations of cells with unique temporal patterns of development.

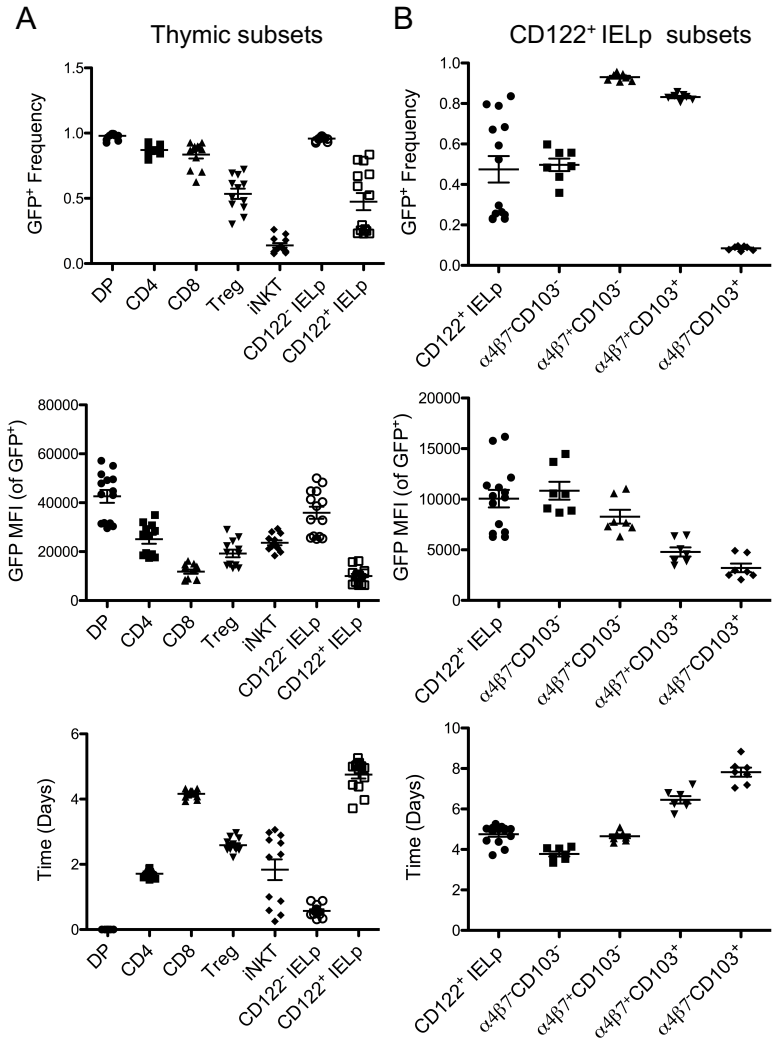

Figure 5-11. **CD122, $\alpha_4\beta_7$ and CD103 expressing subpopulations of IELp display distinct temporal patterns of development.** Numerous thymocyte populations from RAG2p-GFP mice were analyzed for frequencies of GFP^+ cells (top), and MFI of GFP expression within GFP^+ cells (middle), to study the temporal dynamics of development from DP progenitors. GFP MFI within GFP^+ cells were used to calculate the time elapsed from the DP stage to the time of interrogation using a GFP half-life of 54h (bottom). A) Analysis of conventionally selected lineages (CD4 and CD8) and agonist selected lineages (Treg, iNKT and $CD122^{-/+}$ IELp). B) Analysis of $\alpha_4\beta_7$ and CD103 expressing subpopulations of $CD122^+$ IELp. DP, CD4, CD8, Treg, iNKT and IELp data were obtained over 4 individual experiments (n=14). $\alpha_4\beta_7$ and CD103 subpopulation data were obtained over 2 individual experiments (n=7).

CD122, $\alpha_4\beta_7$ and CD103 expressing subpopulations of IELp show differential expression of egress receptor S1P1

Using the RAG2p-GFP model we defined distinct sub-populations of IELp cells with unique temporal signatures that may represent cells at various stages of maturity. Ultimately, for IELp cells to traffic to intestinal tissues, they must first exit the thymus. Prior to export, conventional thymocytes upregulate the S1P receptor, S1P1, to promote thymocyte egress (*125, 126*). Therefore, we measured the expression of S1P1 on the surface of IELp subsets by flow cytometry (**Fig. 5-12**). We observed significantly increased levels of S1P1 expression on $CD122^+$ IELp cells relative to $CD122^-$ IELp (**Fig. 5-12**). Furthermore, $CD122^-$ IELp had a significantly lower MFI of S1P1 expression relative to DP thymocytes (**Fig. 5-12**), which themselves do not exit the thymus under physiological conditions. We next examined S1P1 expression on $\alpha_4\beta_7$ and CD103 expressing sub-populations of $CD122^+$ IELp. We found that $\alpha_4\beta_7^+CD103^-$ and $\alpha_4\beta_7^+CD103^+$ cells both showed significantly higher S1P1 MFIs compared to either of their $\alpha_4\beta_7^-CD103^-$ or $\alpha_4\beta_7^-CD103^+$ counterparts (**Fig. 5-12**). However, all of the $CD122^+$ IELp sub-populations examined showed substantially higher S1P1 expression than DP thymocytes or $CD122^-$ IELp, suggesting that all of these populations may have the potential to egress from the thymus.

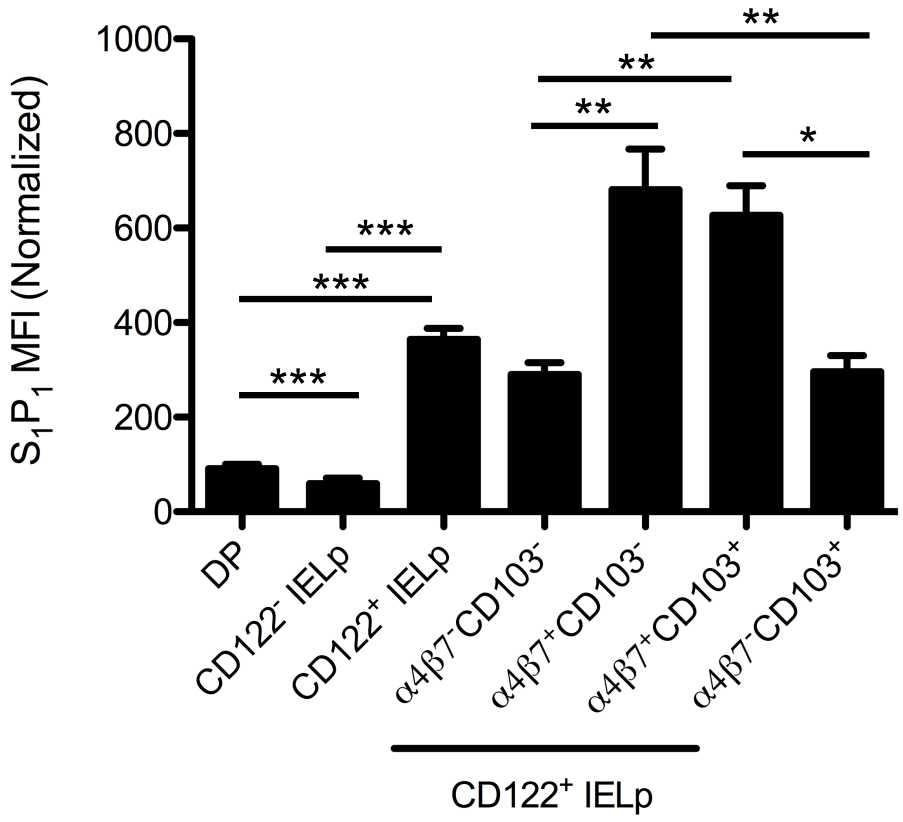

Figure 5-12. **CD122, $\alpha_4\beta_7$ and CD103 expressing subpopulations of IELp show differential expression of egress receptor S1P1.** Normalized MFIs of S1P1 expression on the indicated thymocyte populations. S1P1 expression was normalized using control cells lacking primary antibody staining (Normalized MFI = S1P1 MFI – Control MFI). For DP, $CD122^-$ IELp and $CD122^+$ IELp, data are compiled from 7 mice over four individual experiments. For $\alpha_4\beta_7$ and CD103 expressing subpopulations of $CD122^+$ IELp, data are compiled from 5 mice over 2 independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

CD122$^-$ and CD122$^+$ IELp subpopulations show differing abilities to generate IELs *in vivo*

Our previous data indicated that there were major differences between CD122$^-$ and CD122$^+$ IELp cells with respect to TCR signaling (**Fig. 5-3, 10**), adhesion molecule expression (**Fig. 5-9**), age measurements (**Fig. 5-11**) and S1P1 expression (**Fig. 5-12**). To determine if these differing characteristics of CD122$^-$ and CD122$^+$ IELp corresponded to differing abilities to generate TCRαβ$^+$CD8αα IEL, we examined IEL development using an *in vivo* model. We sorted the CD122$^-$ and CD122$^+$ IELp populations from CD45.1$^+$ or CD45.1/2$^+$ donors, mixed these populations at a 1:1 ratio, co-adoptively transferred them into T cell deficient RAG1$^{-/-}$ recipient mice (CD45.2$^+$) and examined the IEL compartment 6 weeks post-transfer. We examined total lymphocytes obtained from recipient small intestines for expression of CD45.1 and CD45.2 and observed cells derived from both CD122$^-$ (CD45.1$^+$) and CD122$^+$ (CD45.1/2$^+$) IELp donors (**Fig. 5-13A**). However, we detected significantly increased frequencies of CD122$^+$ donor derived cells compared to CD122$^-$ donors (**Fig. 5-13A, B**). Within Donor$^+$ cells, we subsequently gated on TCRβ$^+$CD4$^-$ cells and examined expression of CD8α and CD8β to further assess TCRαβ$^+$CD8αα IEL development in recipient mice. We observed significantly increased frequencies of TCRαβ$^+$CD8αα IEL within CD122$^+$ IELp donor derived cells compared to CD122$^-$ IELp donor cells (**Fig. 5-13A, C**), strongly suggesting that CD122$^+$ IELp are better able to generate TCRαβ$^+$CD8αα IEL than CD122$^-$ IELp. However, it should be noted that substantial frequencies of TCRαβ$^+$CD8αα IEL were observed within the CD122$^-$ IELp derived population, suggesting that these cells can generate TCRαβ$^+$CD8αα IEL to some degree. Furthermore, CD122$^-$ IELp donor derived

cells showed significantly increased frequencies of TCRαβ^{+}CD8αβ IEL relative to CD122^{+} donors (**Fig. 5-13A, C**). Altogether, these results demonstrate that CD122 expression marks distinct fractions within the IELp pool, which show differential patterns of IEL development *in vivo*.

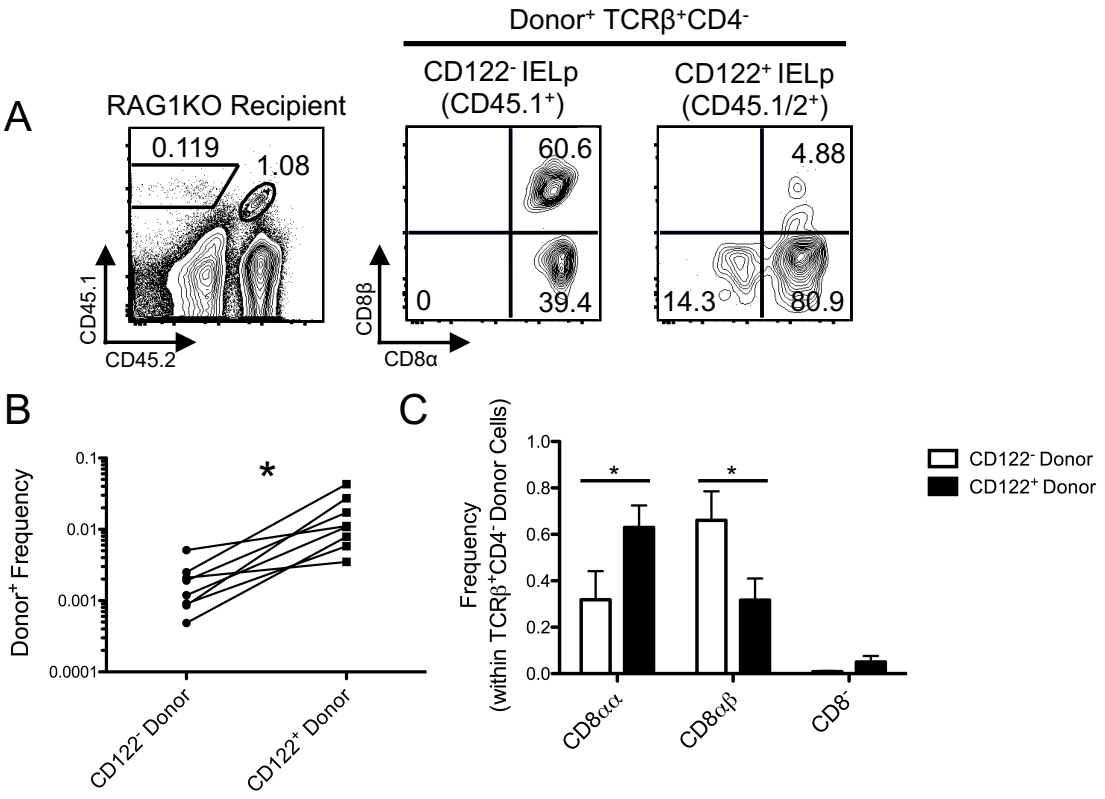

Figure 5-13. **CD122$^-$ and CD122$^+$ IELp subpopulations show differing abilities to generate IEL *in vivo.*** A) Congenically marked CD122$^-$ (CD45.1 $^+$) and CD122$^+$ (CD45.1/2$^+$) IELp (CD4$^{-/dull}$CD8$^{-/dull}$NK1.1$^-$TCRβ$^+$CD5$^+$) were sorted and adoptively transferred into RAG1-deficient animals at a 1:1 ratio. Small intestines were harvested from recipient mice 6 weeks later and analyzed for the presence of IEL populations. A) CD45.1 and CD45.2 expression profiles of total IEL cells from recipient mice (left). TCRβ$^+$CD4$^-$ cells were gated on within each Donor$^+$ population and were analyzed for expression of CD8β and CD8α (right). B) Frequencies of Donor$^+$ cells within total IEL derived the indicated donors C) Frequencies of CD8αα, CD8αβ and CD8$^-$ cells within TCRβ$^+$CD4$^-$ from the indicated donor derived populations (right). Data were collected from n=6 recipient mice over 2 individual experiments. *p<0.05

$\alpha_4\beta_7$ and CD103 expressing subpopulations of IELp show distinct patterns of IEL development *in vivo*

Given that the IELp pool is heterogeneous in nature, we sought to determine if different IELp subpopulations had differing abilities to generate TCR$\alpha\beta^+$CD8$\alpha\alpha$ IEL. Since the CD122$^+$ IELp population showed protection from apoptosis (**Fig. 5-7C**), expressed proteins induced by high affinity TCR signaling (**Fig. 5-3B, C**), contained subsets that expressed the adhesion molecules $\alpha_4\beta_7$ and CD103 (**Fig. 5-9A**) and expressed higher levels of egress receptor S1P1 than CD122$^-$ IELp (**Fig. 5-12**) and was better able to generate TCR$\alpha\beta^+$CD8$\alpha\alpha$ IEL *in vivo* (**Fig. 5-13**), we focused our analysis on prominent sub-populations within the CD122$^+$ IELp fraction. We chose to examine CD122$^+$PD-1$^+$ $\alpha_4\beta_7{}^+$CD103$^-$ IELp and CD122$^+$PD-1$^-\alpha_4\beta_7{}^-$CD103$^+$ IELp since they represented two of the major subsets of IELp, were mostly homogeneous from an 'age' perspective and were polar opposites with respect to 'age' and cell surface phenotype. Similar to previously, we sorted these IELp populations from CD45.1$^+$ or CD45.1/2$^+$ donors, mixed these populations at a 1:1 ratio, co-adoptively transferred them into RAG1$^{-/-}$ recipient mice (CD45.2$^+$) and examined the IEL compartment 6 weeks post-transfer. At the time of harvest, TCR$\alpha\beta^+$ IEL from both CD122$^+$PD-1$^+\alpha_4\beta_7{}^+$CD103$^-$ and CD122$^+$PD-1$^-\alpha_4\beta_7{}^-$ CD103$^+$ IELp donors were clearly detectable in recipient mice (**Fig. 5-14A**). However, the PD-1$^+\alpha_4\beta_7{}^+$CD103$^-$ subset showed almost complete skewing towards TCR$\alpha\beta^+$CD8$\alpha\alpha$ IEL development, while PD-1$^-\alpha_4\beta_7{}^-$CD103$^+$ cells showed nearly exclusive development of TCR$\alpha\beta^+$CD8$\alpha\beta$ IEL (**Fig. 5-14A**). Therefore, it appears that despite both residing within the CD122$^+$ IELp fraction, PD-1$^+\alpha_4\beta_7{}^+$CD103$^-$ and PD-1$^-\alpha_4\beta_7{}^-$CD103$^+$ cells showed distinct patterns of IEL development. Furthermore, these results suggest that PD-

$1^-\alpha_4\beta_7^-CD103^+$ $CD122^+$ IELp are not significant progenitors of $TCR\alpha\beta^+CD8\alpha\alpha$ IEL, while $PD\text{-}1^+\alpha_4\beta_7^+CD103^-$ IELp showed robust development into $TCR\alpha\beta^+CD8\alpha\alpha$ IEL.

Since we found that $CD122^+PD\text{-}1^-\alpha_4\beta_7^-CD103^+$ IELp cells did not give rise to $TCR\alpha\beta^+CD8\alpha\alpha$ IEL, we tested the ability of another $CD122^+$ IELp subset to give rise to intestinal IEL populations *in vivo.* We chose $PD\text{-}1^+\alpha_4\beta_7^-CD103^-$ cells to compete against the $PD\text{-}1^+\alpha_4\beta_7^+CD103^-$ fraction because they constituted a relatively major fraction of IELp cells (**Fig 5-10C**) and were "younger" than $\alpha_4\beta_7^+CD103^-$ cells (**Fig. 5-11B**). Both $PD\text{-}1^+\alpha_4\beta_7^+CD103^-$ and $PD\text{-}1^+\alpha_4\beta_7^-CD103^-$ donor cells predominantly gave rise to $TCR\alpha\beta^+CD8\alpha\alpha$ IEL (**Fig. 5-14B**), suggesting that $TCR\alpha\beta^+CD8\alpha\alpha$ IEL lineage potential is not limited to the $CD122^+PD\text{-}1^+\alpha_4\beta_7^+CD103^-$ IELp fraction alone.

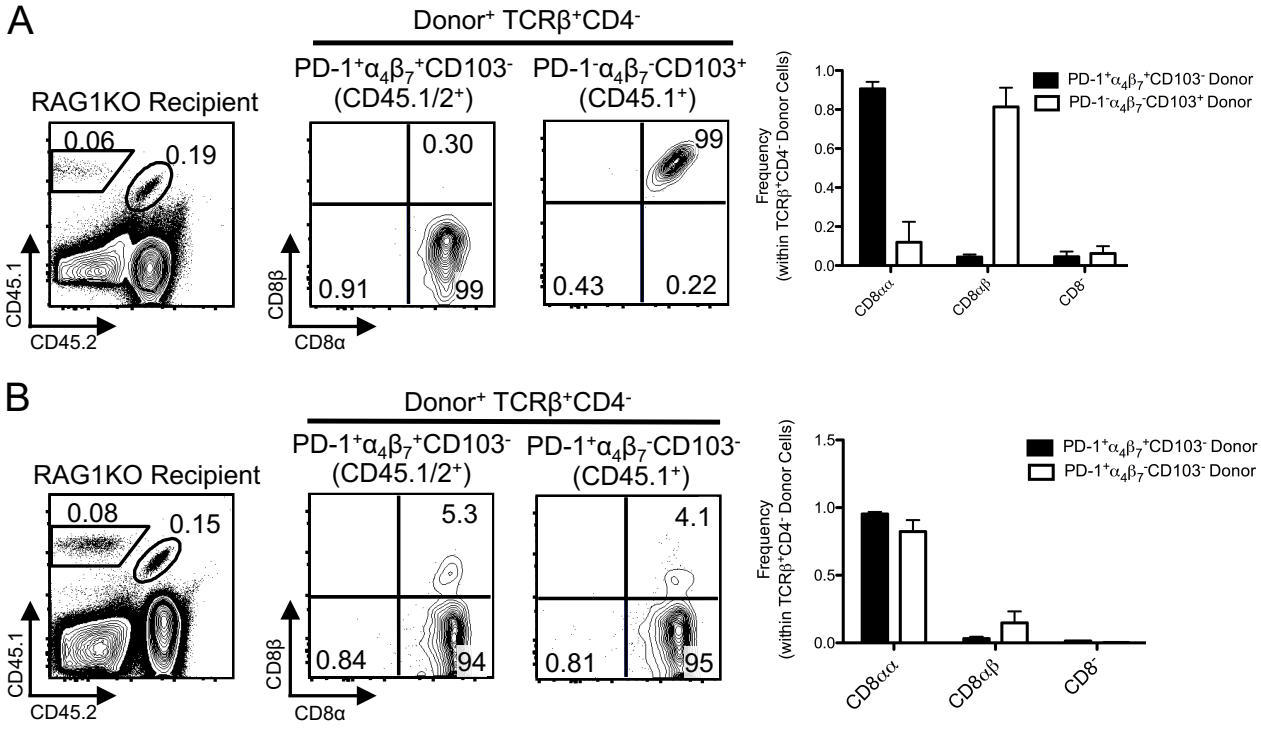

Figure 5-14. **$\alpha_4\beta_7$ and CD103 expressing subpopulations of IELp show distinct patterns of IEL development *in vivo.*** A) Congenically marked PD-1$^+\alpha_4\beta_7^+$CD103$^-$ (CD45.1/2$^+$) and PD-1$^-\alpha_4\beta_7^-$CD103$^+$ (CD45.1$^+$) CD122$^+$ IELp (CD4$^{-/dull}$CD8$^{-/dull}$NK1.1$^-$TCRβ$^+$CD5$^+$CD122$^+$) or B) congenically marked PD-1$^+\alpha_4\beta_7^+$CD103$^-$ (CD45.1/2$^+$) and PD-1$^+\alpha_4\beta_7^-$CD103$^-$ (CD45.1$^+$) CD122$^+$ IELp were sorted and adoptively transferred into RAG1-deficient animals at a 1:1 ratio. Small intestines were harvested from recipient mice 6 weeks later and analyzed for the presence of IEL populations. A, B) CD45.1 and CD45.2 expression profiles of total IEL cells from recipient mice (left). TCRβ$^+$CD4$^-$ cells were gated on within each Donor$^+$ population and were analyzed for expression of CD8β and CD8α (middle). Frequencies of CD8αα, CD8αβ and CD8$^-$ cells within TCRβ$^+$CD4$^-$ from the indicated donor derived populations (right). For A), data were collected from n=5 recipient mice over 4 individual experiments. For B), data were collected from n=5 recipient mice over 3 individual experiments.

Conclusion

The data presented in this chapter demonstrate the critical role of RasGRP1 in regulating the development of agonist selected TCRαβ$^+$CD8αα IELs. In addition, our analysis of RasGRP1's role during TCRαβ$^+$CD8αα IELs development uncovered many layers of heterogeneity within the IELp population, which was previously unappreciated. Specifically, we found that RasGRP1 is required for TCRαβ$^+$CD8αα IEL development through control of agonist selection of IELp cells. We found signs of impaired high affinity TCR signaling in IELp cells from 1KO mice, despite normal expression of CD122. Furthermore, we confirmed that RasGRP1 is required for thymocyte positive selection, but is dispensable for clonal deletion, highlighting the different roles of RasGRP1 in thymocyte positive, negative and agonist selection. Interestingly, deletion of Bim in 1KO mice restored IELp selection, suggesting that RasGRP1 and Bim cooperate in regulating IELp development. Furthermore, we found that RasGRP1 was required for the expression of the putative gut-homing integrins $\alpha_4\beta_7$ and CD103. Additionally, we found that IELp cells showing recent signs of high affinity antigen encounter were skewed towards expression of $\alpha_4\beta_7$, while cells lacking signs of recent TCR stimulation favored expression of CD103. Analysis of these distinct $\alpha_4\beta_7$ and CD103 expressing populations revealed that they bear unique time signatures, show different levels of egress receptor S1P1 expression and differentially generate TCRαβ$^+$CD8αα IEL cells *in vivo*. Altogether, these results highlight the importance of RasGRP1 in IELp agonist selection and uncover the diversity within the IELp population.

CHAPTER 6: DISCUSSION

This chapter contains content from the following sources, republished with permission:

- Golec, D.P., Caviedes, L.M.H., and Baldwin, T.A. 2016. RasGRP1 and RasGRP3 Are Required for Efficient Generation of Early Thymic Progenitors. J Immunol *197*, 1743-1753
- Golec, D.P., Dower, N.A., Stone, J.C. and Baldwin, T.A. 2013. RasGRP1, but not RasGRP3, is required for efficient thymic β-selection and ERK activation downstream of CXCR4. PLoS One *8*: e53300
- Golec, D.P., Hoeppli, R.E., Caviedes, L.M.H., McCann, J., Levings, M.K. and Baldwin, T.A. 2017. Thymic progenitors of TCRαβ$^+$ CD8αα intestinal intraepithelial lymphocytes require RasGRP1 for development. J Exp Med. *214,* 2421-2435.

Overview

The data presented in this thesis examined the roles of RasGRPs 1 and 3 throughout T cell development and clearly demonstrate the importance of the RasGRP family in T cells. ETPs are the most immature population of progenitors within the thymus and RasGRPs 1 and 3 are required for the efficient development of this early progenitor subset. Just downstream of ETPs, DN3 thymocytes show impaired passage through the β-selection checkpoint in the absence of RasGRP1, while RasGRP3 activity is dispensable during β-selection and RasGRP1 is required for ERK activation at this developmental stage. Finally, RasGRP1 deficient thymocytes show defective TCRαβ$^+$CD8αα IEL

development, suggesting that RasGRP1 activity is critical for thymocyte agonist selection. Altogether, these data provide novel insight into the roles of RasGRP proteins during T cell development and highlight the broad importance of RasGRP mediated signaling throughout this complex process.

I. Summary of the roles of RasGRPs 1 and 3 in ETP development

Previously, the roles for RasGRP1 and RasGRP3 during the earliest stages of thymocyte development had not been examined. Through this work, we have identified new roles for RasGRP1 and RasGRP3 in regulating T cell development though these early stages (**Fig. 6-1**). Reductions in numbers of ETPs in 1KO, 3KO and DKO thymi, relative to wild type, strongly suggest that both RasGRP1 and RasGRP3 are involved in regulating the development of this immature T cell progenitor population. Analysis of blood from wild type and DKO mice showed increased frequencies of circulating T cell progenitors in RasGRP1/3 deficient mice, suggesting that DKO progenitors are impaired in their ability to traffic out of the blood and that RasGRP1 and RasGRP3 may regulate progenitor mobilization into the thymus. Accordingly, competitive bone marrow chimeras showed that wild type progenitors strongly outcompeted DKO progenitors at the ETP stage compared to T cell progenitors present in blood, and did so in a cell-intrinsic manner. Furthermore, *in vitro* analysis of progenitor migration showed that LMPPs and CLPs from DKO mice were impaired in their ability to migrate towards CCR9 ligand CCL25, providing a basis for the reduced efficiency of ETP development in RasGRP1 and RasGRP3 deficient animals. Altogether, our data suggest a model whereby

RasGRP1 and RasGRP3 regulate ETP development through control of progenitor migration out of circulation, into the thymus, via a CCR9 dependent mechanism (**Fig. 6-1**).

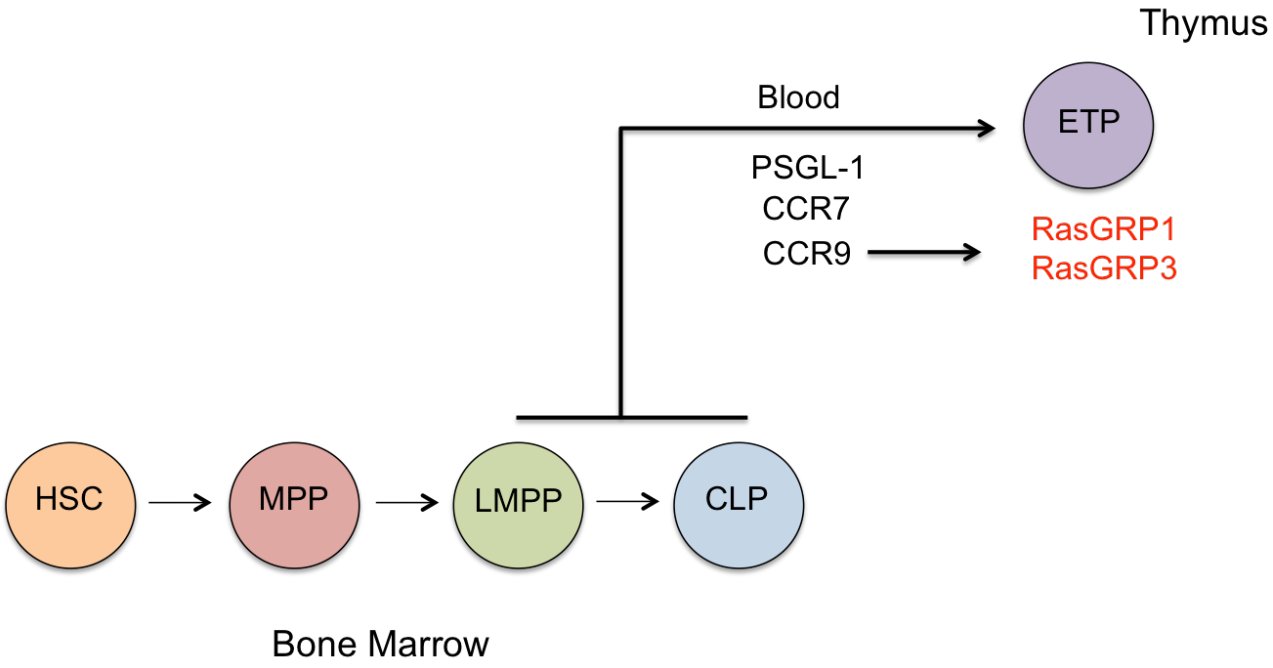

Figure 6-1. **RasGR1 and RasGRP3 regulate ETP development through a CCR9 dependent mechanism**. The bone marrow harbors numerous hematopoietic progenitors with T cell lineage potential. However, only LMPPs and CLPs have been shown to migrate to the thymus, through the combined activities of CCR7, CCR9 and PSGL-1. Progenitor migration to the thymus results in the development of ETPs, through Notch signals received by incoming progenitors. Our results showed that RasGRP 1 and 3 deficient thymi contained reduced numbers of ETPs, indicating that RasGRP1 and RasGRP3 regulate ETP development. In particular, analysis bone marrow chimeric animals revealed that RasGRPs 1 and 3 regulate the transition between the circulating progenitor and ETP stages. Analysis of progenitor migration *in vitro* revealed that RasGRP1 and RasGRP3 double deficient were impaired in their migration in response to CCR9 ligand CCL25. Therefore, our results indicate that RasGRP1 and RasGRP3 regulate the earliest events of T cell development, through controlling progenitor migration into the thymus.

I-a. RasGRPs 1 and 3 regulate ETP generation through controlling progenitor migration to the thymus

Our analysis of immature thymocyte populations in wild type, 1KO, 3KO and DKO thymi revealed that RasGRP1 and RasGRP3 regulate the development of ETPs (**Fig. 6-1**). Specifically, we saw that both single RasGRP1 and RasGRP3 deficiency resulted in significant reductions in numbers of ETPs and downstream progeny. Also, we observed that RasGRP1/3 double deficiency resulted in further reductions in ETP numbers compared to either single knockout. These results clearly demonstrate that both RasGRP1 and RasGRP3 regulate ETP development. Furthermore, since both single knockouts show impaired ETP development and since double knockout animals show even more dramatically disrupted ETP development, it appears that RasGRP1 and RasGRP3 regulate ETP development in an additive manner. RasGRP1 has been previously shown to regulate thymocyte β-selection, positive selection and iNKT development (*92, 101, 105, 114*), however these results provide the first evidence of RasGRP1 involvement in ETP development. To date, RasGRP3 has not been shown to be involved in T lymphopoiesis and our results provide the first evidence of a role for RasGRP3 in regulating T cell development.

Consistent with impaired progenitor migration to the thymus, we found significantly increased frequencies of circulating T cell progenitors in RasGRP1/3 deficient thymi relative to wild type thymi. Increased frequencies of circulating progenitors can be explained by increased trafficking of progenitors out of the bone marrow into blood or by decreased importation of progenitors out of the blood into the

thymus. Our competitive bone marrow experiments showed that DKO progenitors performed similarly to wild type cells in the transition between the bone marrow and blood progenitor stages. In contrast, between the blood progenitor and ETP stages, wild type progenitors strongly outcompeted DKO cells, suggesting that DKO progenitors are impaired in the blood progenitor to thymus transition. Also, given that SDF1α signaling is thought to mediate progenitor retention in the bone marrow (*1*) and that our *in vitro* transwell experiments show that DKO progenitors are able to respond to SDF1α similarly to wild type, this provides further evidence against a role for RasGRP1 and RasGRP3 in regulating bone marrow progenitor mobilization into blood.

Mechanistically, it appears that RasGRP1 and RasGRP3 may regulate ETP development in a CCR9 dependent fashion. Our *in vitro* migration experiments showed that lower frequencies of DKO LMPPs and CLPs migrate towards the CCR9 ligand CCL25 relative to wild type cells, suggesting that RasGRPs 1 and 3 are involved in regulating progenitor migration in response to CCL25. In addition to CCR9, CCR7 is also involved in progenitor trafficking to the thymus. However, DKO LMPPs and CLPs showed only mild reductions in frequencies of migrating cells in response to CCR7 ligand CCL19. Interestingly, thymi from CCR9 deficient mice show significantly reduced numbers of ETPs, while CCR7 deficient thymi show similar numbers of ETPs as wild type mice (*13*). Consistent with roles for RasGRP1 and RasGRP3 downstream of CCR9, thymi from RasGRP1/3 deficient mice show similar reductions in ETP numbers as those observed in CCR9 deficient animals. Also, since CCR9 is a G-protein coupled receptor and RasGRP family members have been implicated in signaling events downstream of

several such receptors of this family (*114, 127, 128*), it is reasonable to speculate that RasGRPs 1 and 3 may operate downstream of CCR9.

I-b. Which signaling pathways do RasGRPs 1 and 3 regulate during ETP development?

Given that RasGRP1 and RasGRP3 have been shown regulate Ras activation in other contexts, it is tempting to speculate that RasGRP1 and RasGRP3 regulate the Ras-ERK signaling cascade following CCR9 stimulation. In support of this idea, our transwell experiments showed that LMPPs and CLPs treated with MEK inhibitor are impaired in their ability to migrate towards CCL25, suggesting that Ras-ERK signaling downstream of CCR9 regulates progenitor migration. Furthermore, transcriptome analyses performed by the Immgen consortium indicate a number of putative ERK responsive genes being induced in the ETP population in comparison to bone marrow resident CLP (*104*). In addition, RasGRP3 has recently been identified as regulating glioma cell migration through interaction with Arp3 (*129*), a key component of the actin remodeling machinery. Given that RasGRP family members can potentially modulate the function of this migration regulating complex, it is plausible that RasGRPs 1 and 3 could regulate chemotaxis in T cell progenitors by a similar mechanism.

Although work aimed at understanding how progenitors reach the thymus from their origin in the bone marrow has been instrumental in identifying cell surface molecules involved in this process, the specific intracellular signaling events that occur

downstream of these molecules remain poorly understood. In this study, we have identified RasGRP1 and RasGRP3 as intracellular signaling molecules involved in regulating progenitor migration to the thymus. However, given the rarity of these progenitor populations, interrogation of the precise signaling events regulated by RasGRP 1 and 3 is incredibly difficult using conventional approaches and the precise signaling pathways involved in regulating progenitor migration remain unknown. Developing assays that are sufficiently sensitive to study cell signaling events in incredibly rare populations, such as these immature T cell progenitors, will be critical for further understanding the precise intracellular signaling events that occur during these early stages.

I-c. Do RasGRPs 1 and 3 regulate the development of other immature thymocyte populations?

ETPs express high levels of cKit and reside within the DN1 fraction of thymocytes, which also includes a number of other distinct cell subsets. In particular, our competitive bone marrow chimera experiments revealed that, in addition to regulating ETP development, RasGRP1/3 are also involved in regulating the development of the $cKit^{lo/-}$ DN1c, DN1d, and DN1e thymocyte populations. However, the relative chimerism values observed within these populations more closely resembled DN3 and DP thymocytes than they did ETPs and DN2 thymocytes. These results suggest that the developmental origin of DN1c, DN1d, and DN1e progenitors is unique from that of ETPs, despite the grouping of ETPs within the DN1 fraction of the thymus. In agreement with our results, elegant work from Benz et al. clearly shows that newly arriving progenitors that reach the thymus

differentiate through the ETP stage of development early after arrival and do not adopt the DN1c, DN1d and DN1e lineages with the same kinetics (*130*). Furthermore, work from several groups has illustrated that the DN1c, DN1d and DN1e populations are not significant progenitors of T cells, but rather give rise to other lineages of cells (*131, 132*). These results, along with our data, suggest that the development of DN1c, DN1d and DN1e cells is temporally distinct from that of ETPs and likely occurs downstream of ETP generation or through progenitor populations distinct from those that form the ETP pool and give rise to T cells.

I-d. Conclusion

Our data demonstrate, for the first time, that RasGRPs 1 and 3 control the development of the ETP pool through regulation of progenitor migration to the thymus. Given the established role of RasGRPs as signal transduction proteins, these results provide novel insight into the signaling molecules involved in regulating progenitor entry into the thymus. Furthermore, since T lineage reconstitution has proven to be a limiting factor in success following BM transplantation (BMT) (*133-135*) and that progenitor migration to the thymus is impaired following BMT (*136*), better understanding the signaling pathways underlying this process could provide valuable insight into the design of new approaches to improve T cell development following BMT. Altogether, the results presented here show novel roles for RasGRPs 1 and 3 in regulating the earliest stages of T cell development and provide mechanistic insight into the control of progenitor migration to the thymus.

II. Summary of the roles of RasGRPs 1 and 3 in thymocyte β-selection

Through using a comprehensive analysis of thymocyte β-selection, we were able to elucidate the contributions of RasGRPs 1 and 3 during this important developmental checkpoint (**Fig. 6-2**). Importantly, we found that RasGRP1 plays a significant role in regulating β-selection, while RasGRP3 activity appeared to be largely dispensable. Previous work had described increased frequencies of DN3 thymocytes in 1KO animals, but the mechanism through which RasGRP1 regulates β-selection was not explored. We found that RasGRP1 controls the differentiation and proliferation of DN3 thymocytes as they transition from DN3E to DN3L, despite intact intracellular expression of TCRβ within DN3. As a result of impaired β-selection and developmental arrest at DN3E, RasGRP1 deficient thymi showed inefficient development of DN into DP. Of note, we found that RasGRP1 deficient mice showed no overall change in αβ vs γδ lineage commitment. Finally, we found that RasGRP1 deficient DN3 were unable to activate ERK in response to CXCR4 signaling, which may contribute to impaired β-selection in RasGRP1 deficient thymi. Our findings provide a basis for understanding RasGRP1 mediated control of the β-selection checkpoint.

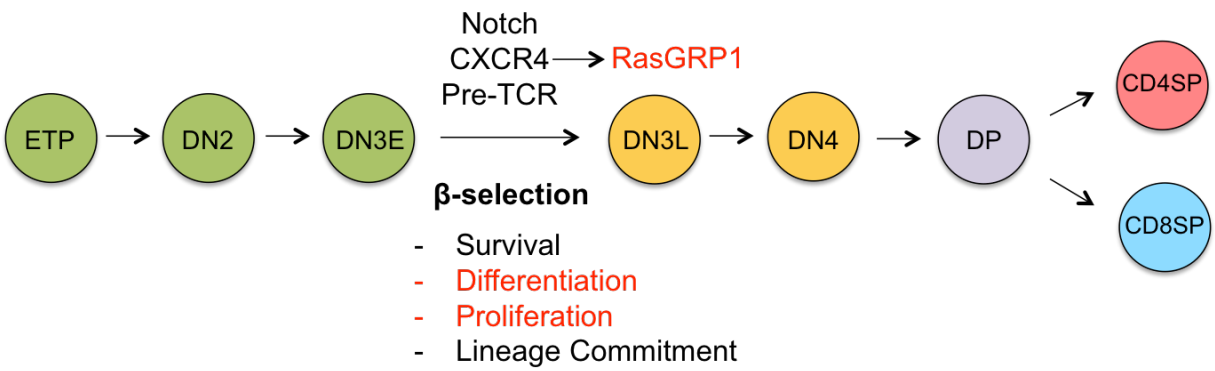

Figure 6-2. **RasGRP1 is required for thymocyte β-selection through control of thymocyte differentiation, proliferation and ERK activation downstream of CXCR4 stimulation.** Transition between the DN3E and DN3L marks passage through the β-selection checkpoint. Development through β-selection is characterized by continued survival of developing thymocytes, differentiation, proliferation and commitment to the αβ T cell lineage. Our results indicate that RasGRP1 is required for efficient passage through β-selection, by controlling DN3 thymocyte proliferation and differentiation between the DN3E and DN3L stages. Furthermore, DN3 thymocytes stimulated with CXCR4 ligand SDF-1α show impaired activation of ERK, providing a mechanistic basis through which RasGRP1 may regulate β-selection.

II-a. What are the relative contributions of RasGRPs 1 and 3 during β-selection?

In most cases, RasGRP1 and RasGRP1/3-deficient thymocytes displayed equivalent deficiencies in β-selection, while 3KO mice appeared largely similar to controls. Therefore, we attribute most of the deficiencies in β-selection observed in DKO mice to a loss of RasGRP1 and suggest that RasGRP3 cannot compensate for the loss of RasGRP1. Indeed, it has been shown that RasGRP1 is the most highly expressed RasGRP member in DN3a thymocytes [34]. The lack of a difference between RasGRP1 KO and RasGRP1/3 DKO mice contrasts the finding of the Zhang group where RasGRP4-defient mice showed no impairment in β-selection, but the combined loss of RasGRP1 and 4 showed a more profound phenotype than RasGRP1 deficiency alone. This suggests that RasGRP4 could compensate somewhat for the loss of RasGRP1 [24]. The difference observed between RasGRP1/3 DKO and RasGRP1/4 DKO is likely due to relatively higher expression of RasGRP4 than RasGRP3 in DN3 thymocytes as reported by the Immunological Genome Project (*96, 103*).

II-b. How does RasGRP1 regulate thymocyte β-selection?

The development of DN into DP is a complex multi-stage program involving interactions between developing thymocytes and the diverse elements that make up the thymic microenvironment. RasGRP1 ablation results in inefficient development of DN thymocytes into DP cells. Signaling downstream of the pre-TCR is known to involve the signaling molecules Zap70, Syk, LAT and SLP76, as well as activation of the Ras/ERK signaling pathway (*32, 77-81*). Given that RasGRP1 contains a physiologically relevant C1 domain that binds DAG, it is likely that LAT mediated PLCγ recruitment, activation

and subsequent DAG production in response to pre-TCR signaling recruits RasGRP1 to the plasma membrane, resulting in Ras activation (*29, 137*). In support of this mode of RasGRP1 regulation, although not extensively studied, mice with a LAT Y136F mutation that abrogates PLCγ recruitment and activation show impaired DN to DP development, suggesting impaired β-selection (*73, 138*). However, RasGRP1 regulation downstream of the pre-TCR remains poorly understood.

We have identified a novel role for RasGRP1 downstream of CXCR4 activation in DN3 thymocytes. RasGRP1 deficient DN3 cells are unable to activate ERK in response to SDF1α stimulation of CXCR4. However, RasGRP1 deficient DN3 are able to activate AKT downstream of CXCR4 activation. Interestingly, CXCR4 deficient thymi show impaired β-selection and signals transduced through CXCR4 are important during early T cell development (*37*). The mechanism of RasGRP1 activation downstream of CXCR4 remains unclear. To address one possible mechanism of RasGRP1 activation in response to CXCR4 signaling we carried out SDF1α stimulations of DN3 thymocytes in the presence of PI3K inhibitor, LY294002. RasGRP1 contains a C-terminal PT domain that has been shown to bind phosphoinositides and recruit RasGRP1 to the plasma membrane (*99, 119*). However, we found that inhibition of PI3K activity had no effect on ERK activation in response to SDF1α stimulation, suggesting PI3K mediated phoshoinositide generation is not required for RasGRP1 mediated ERK activation downstream of CXCR4. Both RasGRP1 and RasGRP3 contain DAG binding C1 domains that have been classically thought to recruit RasGRPs to Ras containing membranes (*97*). Therefore, it is likely that the mechanism of RasGRP1 activation downstream of CXCR4

is PLCγ dependent and involves RasGRP1 binding membrane DAG through its C1 domain. Interestingly, ERK activation downstream of CXCR4 is also defective in RAG2$^{-/-}$ DN3, unable to express a pre-TCR (*139*). Since CXCR4 mediated ERK activation is pre-TCR dependent, RasGRP1 may activate ERK downstream of the pre-TCR. It is known that LAT is part of the pre-TCR signaling complex and that LAT can recruit and activate PLCγ (*140*). Given the potential for PLCγ mediated DAG production downstream of the pre-TCR, we predict that RasGRP1 activation during β-selection is PLCγ dependent. However, more experiments are needed to address the precise mechanism of RasGRP1/3 activation in thymocytes.

II-c. Multiple RasGEFs operate to coordinate thymocyte β-selection

Although RasGRP1 deficient and targeted Sos1 KO thymi both show impaired thymocyte β-selection, there are some subtle, yet important, differences in the phenotypes of these different mouse models. Deceptively, both RasGRP1 deficient and Sos1 KO thymi show significantly elevated DN3/DN4 ratios (*89*). However, Sos1 KO thymi show a significant reduction in DN4 numbers accompanied by modestly reduced DN3 numbers, while RasGRP1/3 deficient thymi show significantly elevated DN3 numbers and mildly reduced DN4 numbers. Therefore, it appears that the Sos1 KO phenotype lies within the DN4 population rather than within the DN3 population. Furthermore, RasGRP1 and Sos1 deficient DN3 and DN4 show opposing proliferation phenotypes. RasGRP1 deficient DN3 show significantly reduced proliferation, while Sos1 KO DN3 cells proliferate normally. In contrast, Sos1 KO DN4 show significantly reduced proliferation, while RasGRP1 deficient DN4 show very modest reductions in

proliferation. Therefore, Sos1 seems to control the proliferation of DN4 and as a result Sos1 deficiency results in significantly reduced DN4 numbers. It should be noted, complicating this interpretation is the fact that the BrdU pulse times varied between the two studies and this difference may account for the observed differences in proliferation. Interestingly, RasGRP1 deficient thymi show increased DN3 numbers despite significant reductions in DN3 proliferation. This finding clearly demonstrates that increased DN3 cell numbers in RasGRP1 thymi are due to developmental arrest and highlights the intimate connection between DN3 proliferation and their subsequent differentiation into DN4. Altogether these findings suggest that RasGRP1 and Sos1 may regulate temporally distinct events in αβ T cell development.

In addition to its classical role as a RasGEF, Sos1 has also been shown to be a Rac activator (*141, 142*). Similar to Dbl family members, Sos1 contains tandem Dbl homology (DH) and pleckstrin homology (PH) domains, which control the localization and RacGEF activity of Sos1. Interestingly, Rac1/2 deficient thymocytes show impaired early T cell development, including β-selection (*143, 144*). Therefore, it is possible that Sos1 may regulate Rac activation directly during early T cell development, which may account for the different developmental phenotypes of RasGRP1 deficient and Sos1 deficient thymi. However, there is no evidence of Sos1 regulating Rac activation during T cell development and further studies are required to address this possibility.

II-d. Conclusion

In this study, we found that RasGRP1 controls the differentiation of DN3E into DN3L and regulates proliferation of DN3 thymocytes. Additionally, RasGRP1 is required for the activation of ERK, downstream of CXCR4. It has become clear that control of the Ras pathway is an important facet of early thymic T cell development. However, many questions remained unanswered. The precise mechanism of RasGRP1 activation during pre-TCR signaling and signaling downstream of CXCR4 remains a mystery. Most importantly, the downstream consequences of Ras activation are poorly understood in the context of T cell development. Much work has been done linking Ras/MAPK signaling to T cell development, providing valuable insight into understanding developmental signaling in thymocytes. However, Ras regulates a diverse array of pathways and uncovering the network of Ras regulated signaling pathways in T cells will shed light onto the precise mechanisms by which Ras regulates T cell development.

III. Summary of the role of RasGRP1 in regulating the agonist selection of thymic progenitors of TCRαβ$^+$CD8αα IEL

We have identified RasGRP1 as a critical regulator of TCRαβ$^+$CD8αα IEL development through its control of agonist selection and IELp generation in the thymus (**Fig. 6-3**). Given that RasGRP1 is required for conventional thymocyte positive selection in response to low affinity TCR signaling and is also required for iNKT(*105*) and TCRαβ$^+$CD8αα IEL development in response to high affinity TCR stimulus, there is clearly an overlap between the signal transduction molecules that regulate positive selection and agonist selection. However, the differential requirements of RasGRP1 in

thymocyte agonist selection and clonal deletion highlight the different signaling requirements that drive these opposing fates that derive from a high affinity TCR stimulus. These data suggest a global requirement for RasGRP1 signaling in generating a diverse T cell repertoire stemming from either weak or strong TCR selection signals in the thymus. In addition to our analysis of the role of RasGRP1 in regulating TCR$\alpha\beta^+$CD8$\alpha\alpha$ IEL development, we also uncovered a significant degree of heterogeneity within the IELp population. In particular, expression of CD122, PD-1, $\alpha_4\beta_7$ and CD103 mark distinct fractions within the IELp fraction that have differing abilities to generate TCR$\alpha\beta^+$CD8$\alpha\alpha$ IEL *in vivo*.

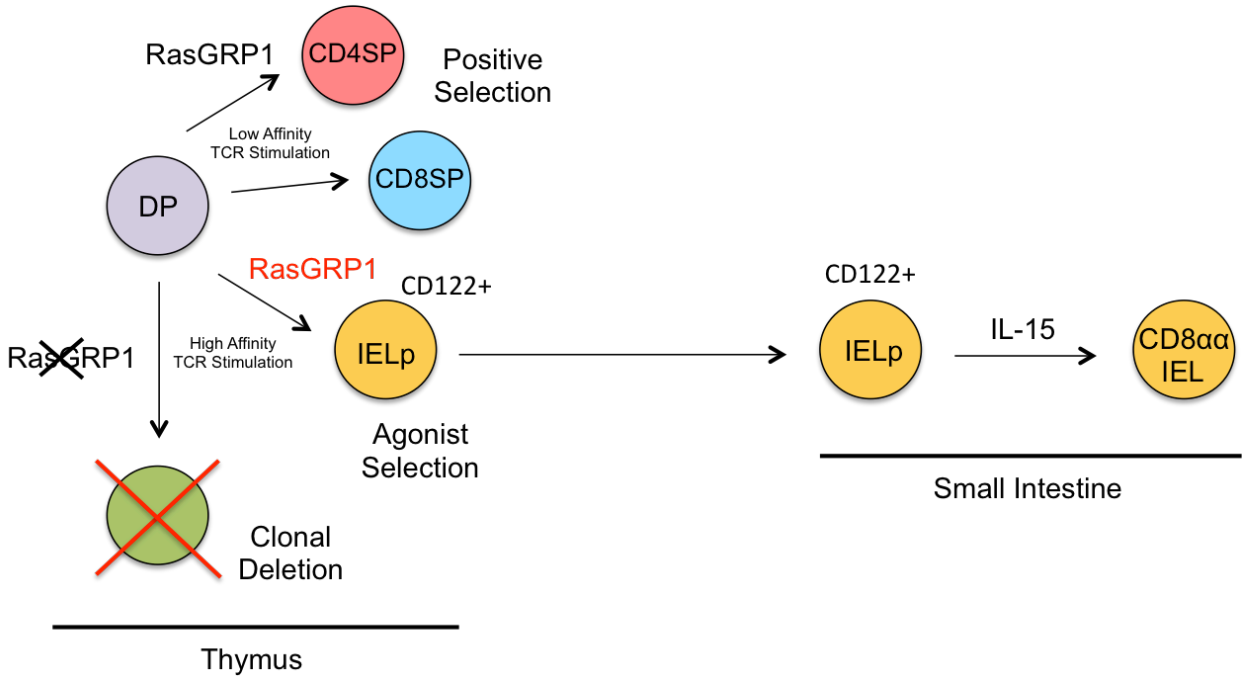

Figure 6-3. **RasGRP1 is dispensable for thymocyte clonal deletion, but is required for thymocyte positive selection and agonist selection of IELp cells.** The strength of TCR signals received by developing DP thymocytes is a critical determinant of thymocyte fate. Previous work has established that RasGRP1 is critical for thymocyte positive selection, but is not required for thymocyte clonal deletion. Our results confirm that RasGRP1 is not required for thymocyte clonal deletion, but also reveal that RasGRP1 activity is important for agonist selection of IELp cells and, as a result, downstream development of TCRαβ^{+}CD8αα IEL. In particular, we found that RasGRP1 deficient IELp cells showed signs of impaired high affinity TCR signaling and regulated IELp survival through a Bim dependent mechanism.

III-a. How does RasGRP1 activity influence DP thymocyte fate and the generation of TCRαβ^{+}CD8αα IEL?

In contrast to agonist selection, RasGRP1 activity is not required for thymocyte clonal deletion. The finding that Bim deficiency rescues IELp development in RasGRP1$^{-/-}$ mice suggests that RasGRP1 signaling is pro-survival rather than fate specifying in this context. As such, these data provide a possible explanation for the differential requirement of RasGRP1 in clonal deletion versus IELp generation, both of which require strong TCR signals. For clonal deletion, an active apoptotic program is initiated and therefore pro-survival signaling mediated by RasGRP1 would be detrimental to this outcome. On the other hand, IELp generation requires survival of the cells that receive a strong TCR signal and under these conditions RasGRP1 appears to be required to deliver these signals. However, it should be noted that RasGRP1$^{-/-}$ Bim$^{-/-}$ mice show reduced numbers of IELps compared with Bim$^{-/-}$ mice, suggesting a role for RasGRP1 signaling outside of survival.

The primary function of RasGRP1 is to activate the highly conserved signal transduction protein Ras. The activation kinetics of the Ras-ERK cascade are differentially regulated during thymocyte positive and negative selection. During positive selection, thymocytes show a sustained increase in ERK activation while thymocytes undergoing negative selection show a strong, but transient, burst of ERK activation (*84-86*). Importantly, numerous studies have shown that ERK activity is dispensable during thymocyte negative selection, but is required for efficient thymocyte positive selection (*84, 87, 88*). Given the requirement for RasGRP1 in agonist selection of both iNKT (*105*)

and IELp, ERK activity is likely important for thymocyte agonist selection generally. However, the kinetics of ERK activation during agonist selection is unknown and further studies are required to dissect the role and kinetics of ERK signaling in agonist selection.

Consistent with the above described pattern of Erk activation, thymocytes undergoing positive selection show numerous, brief serial interactions with stromal cells over the course of days, while thymocytes undergoing negative selection show relatively stable interactions with stromal cells over the course of hours (*145*). Since the strength of signal that drives clonal deletion and agonist selection are similar, one might predict that those cells undergoing agonist selection also engage in stable interactions with APC. However, given that IELp have a relative 'age' similar to CD8SP there must be mechanisms in place to regulate the outcome of the interactions with APC, that being differentiation or death. One such regulatory mechanism could be the CD28-B7 signaling pathway (*42*). Alternatively, or in addition to, it is possible that it is the nature of the APC that regulates the outcome of these interactions. For example, in the HY^{cd4} model, thymocytes undergoing apoptosis are positioned closely to DCs in the thymus and restricting male Ag expression to cTEC or radio-resistant APC impaired apoptosis induction (*146*). Given that agonist selected cells do not undergo apoptosis during development, it is likely that thymocyte agonist selection does not involve stable interactions with apoptosis-inducing DCs but rather involves interactions with cTEC cells that are less potent inducers of clonal deletion. Whether the nature of the APC presenting high affinity Ag regulates IELp development requires investigation.

III-b. Deciphering heterogeneity within the IELp fraction

Distinct CD122$^-$ and CD122$^+$ populations make up the "IELp" fraction and highlight the diversity of cells present within this pool. CD122$^-$ IELp lacked expression of proteins induced by strong TCR signaling and showed high frequencies of cell death while CD122$^+$ IELp were enriched in cells expressing markers of high affinity antigen encounter and were largely protected from apoptosis. Furthermore, using RAG2p-GFP animals, we found that CD122$^-$ IELp were similar in age to immature DP cells, while CD122$^+$ IELp showed a similar age as post-selection CD8SP thymocytes. In addition, CD122$^-$ IELp lacked expression of egress receptor S1P1 while CD122$^+$ IELp showed robust expression of S1P1, consistent with the known phenotype of mature post-selection cells (*125*). Our *in vivo* analysis of TCRαβ$^+$CD8αα IEL development from CD122$^-$ and CD122$^+$ IELp revealed that CD122$^+$ IELp are more efficient progenitors of TCRαβ$^+$CD8αα IEL than their CD122$^-$ counterparts. However, it should be noted CD122$^-$ were able to generate TCRαβ$^+$CD8αα IEL to some degree, indicating that these cells are capable of giving rise to TCRαβ$^+$CD8αα IEL. Although this was the case in our adoptive transfer experiments, CD122$^-$ IELp cells expressed low levels of egress receptor S1P1 and would likely be unable to traffic out of the thymus under physiological conditions. Of note, McDonald *et al.* recently determined that in TCR transgenic mice that express a TCR cloned from TCRαβ$^+$ CD8αα IEL, almost all recent thymic emigrants expressed CD122 and most were $\alpha_4\beta_7^+$ (*43*). Collectively, these data suggest that CD122 expression marks an important transition in the development and maturation of IELp. Of note, CD122 expression *per se* may not be important for IELp thymic development as mice

with IL-15-deficient thymi contain similar numbers of IELp compared to WT (*45*) and are able to reconstitute the IEL compartment of nude mice (*46*).

Although CD122 expression appears to separate distinct IELp fractions, expression of $\alpha_4\beta_7$ and CD103 revealed an additional layer of diversity within the IELp. Notably, $CD122^-$ IELp cells lacked $\alpha_4\beta_7$ and CD103 expressing cells altogether, while $CD122^+$ IELp showed four distinct populations of $\alpha_4\beta_7$ and CD103 expressing progenitors. Using PD-1 and Nur77-GFP as readouts of TCR signaling, we found that $\alpha_4\beta_7$ expression was associated with $CD122^+$ cells showing recent high affinity TCR signaling, while CD103 expression was enriched on cells lacking signs of recent TCR signaling. Additionally, analysis of Rag2p-GFP animals revealed that nascent $\alpha_4\beta_7^-$ $CD103^-$ were the youngest of the $CD122^+$ IELp pool, while $\alpha_4\beta_7^+CD103^-$ and $\alpha_4\beta_7^+CD103^+$ were slightly older and $\alpha_4\beta_7^-CD103^+$ cells were especially aged. Testing the significance of $\alpha_4\beta_7$ and CD103 expression *in vivo* revealed that PD-$1^-\alpha_4\beta_7^-CD103^+$ gave rise to TCR$\alpha\beta^+$CD8$\alpha\alpha$ IEL, while both PD-$1^+\alpha_4\beta_7^+CD103^-$ and PD-$1^+\alpha_4\beta_7^-CD103^-$ developed into TCR$\alpha\beta^+$CD8$\alpha\alpha$ IEL almost exclusively. However, S1P1 expression analysis showed that $\alpha_4\beta_7^+CD103^-$ cells expressed significantly higher levels of S1P1 than $\alpha_4\beta_7^-CD103^-$ cells, making it tempting to speculate that the $CD122^+$PD-$1^+\alpha_4\beta_7^+CD103^-$ IELp subset is the population that emigrates out of the thymus under physiological conditions. Previously described data from McDonald *et al*. support this notion (*43*).

III-c. Debating the functional significance of the various IELp subsets

A recently published study from Ruscher *et al. (147)* also examined heterogeneity within the IELp fraction and corroborated many of the findings of our work. In particular, both studies concluded that $CD122^+\alpha_4\beta_7^+$ IELp cells showed increased S1P1 expression relative to $CD122^+$ $CD103^+$ IELp cells, suggesting that $CD122^+\alpha_4\beta_7^+$ IELp are better able to egress from the thymus compared to their $CD122^+CD103^+$ counterparts. In addition, both studies confirmed that $CD122^+CD103^+$ IELp cells lack signs of recent TCR signaling and appear aged, while $CD122^+\alpha_4\beta_7^+$ IELp cells show signs of recent agonist TCR stimulation and are recently derived from DP. In contrast to our findings, Ruscher *et al.* demonstrated that both PD-1^+CD122^+ IELp (type A), which lack expression of T-bet and show high $\alpha_4\beta_7$ expression, and PD-1^-CD122^+ IELp (type B), which showed high expression of T-bet and CD103, could generate TCR$\alpha\beta^+$CD8$\alpha\alpha$ IEL *in vivo*. However, the approach used in these experiments differed significantly from those described here, which likely contributed to the different outcomes in the two studies. In particular, Ruscher and colleagues adoptively transferred sorted IELp progenitors alongside a general DN thymocyte competitor, while in our study we directly competed specific IELp populations against one another. In addition, our sorting strategy utilized PD-1, CD122, $\alpha_4\beta_7$ and CD103 to specifically gate on rare IELp sub-populations, while Ruscher *et al.* favored sorting broader IELp populations using expression of PD-1 and CD122. Furthermore, in our experiments we used NK1.1 to exclude NK lineage cells from our IELp gate, while Ruscher *et al.* used CD1d tetramers to specifically exluced. As a result, Ruscher *et al.* found that PD-1^-CD122^+ IELp (type B) can generate TCR$\alpha\beta^+$CD8$\alpha\alpha$ IEL *in vivo*, in the absence of another specific IELp competitor, suggesting that this

population must posses TCRαβ^{+}CD8αα IEL lineage potential. However, our results indicate that when PD-1^{-}CD122^{+}$\alpha_4\beta_7^{-}$CD103^{+} IELp are in placed in direct competition with PD-1^{+}CD122^{+}$\alpha_4\beta_7^{+}$CD103^{-} IELp within the TCRαβ^{+}CD8αα IEL niche, they are outcompeted by PD-1^{+}CD122^{+}$\alpha_4\beta_7^{+}$CD103^{-} IELp cells and instead differentiate into TCRαβ^{+}CD8αb IEL. Altogether, these data highlight the heterogeneity of the IELp population and provide insight into the mechanisms governing the development of this diverse population.

III-d. Conclusion

The functional heterogeneity of the IELp population is defined by the TCR signaling events experienced by developing thymocytes. RasGRP1-mediated signals are clearly required for efficient generation of IELp and RasGRP1-deficient IELp show signs of impaired high affinity TCR signaling. Furthermore, the expression of markers of TCR signaling strength by IELp cells was associated with their thymic expression of CD122 and adhesion molecules $\alpha_4\beta_7$ and CD103. IELp cells showing signs of recent high affinity TCR signaling are skewed towards expression of $\alpha_4\beta_7$, and proved to be high fidelity progenitors of TCRαβ^{+}CD8αα IEL. The requirement of RasGRP1 activity during agonist selection separates the signaling requirements during agonist selection and clonal deletion. These results provide critical insight into the signaling events that drive TCRαβ^{+}CD8αα IEL development, but the precise outcome of RasGRP1-mediated signals in agonist-selected thymocytes that specify the development of IELp is unclear. One possibility is the induction of a lineage specifying transcription factor(s). Future work

examining the transcriptional landscape of the specific IELp that gives rise to TCRαβ^{+}CD8αα IEL will be required.

IV. Final Conclusions

The data presented in this thesis clearly demonstrate the importance of RasGRPs 1 and 3 throughout the T cell developmental program. Although we analyzed the impact of RasGRP1/3 deficiency in a stage-wise manner to focus our analyses, it is possible that the sequential impairments in T cell development observed in RasGRP1/3 deficient animals were not isolated to one particular developmental stage, such that impairment at one developmental stage may have impacted development at a downstream stage. For example, Notch dependent development from the ETP to DN3 stages ultimately sets the stage for thymocyte β-selection. Since RasGRP1/3 activity was required for efficient ETP development, alterations to the ETP pool in RasGP1/3 deficient animals may have impacted the quality of DN3 thymocytes developing in these mice and may have indirectly influenced the outcome of β-selection. Furthermore, given that β-selection generates the DP thymocyte population and that 1KO mice were impaired in β-selection, it is possible that the nature of DP thymocyte development in these animals was impacted. Notably, 1KO animals showed defects in DP positive and agonist selection, which may have been indirectly influenced by impairments to thymocyte β-selection. All of this being said, our data clearly indicate stage specific contributions of RasGRPs 1 and 3 to the described developmental processes and any potential interplay between these stage specific impairments only adds an additional layer of complexity to our understanding of the roles of RasGRP1 and RasGRP3 during T cell development.

Given the ubiquitous nature of Ras signaling, it is fitting that the regulation of this highly conserved signaling pathway is so important throughout T cell development. The search for factors that regulate Ras activation in T cells has proven to be a useful means of uncovering the involvement of this signaling pathway during T cell development. To date, the Ras activators Sos1 and RasGRP1 have been shown to make the most substantial contributions during T cell development. However, Sos1 activity has only been shown to be strictly required during β-selection, while RasGRP1 plays numerous roles during development. Notably, a model of Ras regulation in T cells has been proposed, whereby RasGRP1 and Sos1 cooperate to mediate Ras activation (*148*). Sos1 contains an allosteric Ras-GTP binding pocket that, when engaged by Ras-GTP, facilitates the priming of Sos1 and enhances its RasGEF activity (*148*). It is thought that RasGRP1 activity is required to induce the initial Ras activation needed to prime Sos1 activity. However, to date Sos1 has been shown not to be required for thymocyte positive selection (*102*) and has not been implicated in ETP development or agonist selection, suggesting that RasGRP1 acts independently of Sos1 during these developmental stages. Furthermore, despite the fact that RasGRP1 and Sos1 both operate to regulate β-selection, the β-selection phenotypes of 1KO and Sos1 KO thymi are substantially different from one another, again indicating a divergence in the roles of these RasGEFs during development. Therefore, RasGRP1 and Sos1 do not appear to synergize in their regulation of T cell development. However, this cooperative model may still be relevant in other contexts, such as the activation of peripheral T cells.

The experiments outlined in this thesis utilized global RasGRP deficient animals to study the role of these molecules in the development of T cells. Although these mice proved incredibly useful for studying relatively early developmental events, the numerous defects to T cell development they exhibit, especially in RasGRP1 deficient animals, makes them unsuitable for studying RasGRPs in the context of mature peripheral T cells. Despite a wealth of data examining the roles of RasGRP1 in mature lymphocyte derived cell lines, very little work has been done examining the role of RasGRP1 in regulating the functionality of mature T cells, *in vivo*. To advance the study of RasGRP1 into this area of T cell biology, the generation of RasGRP1 floxed mice is necessary to achieve specific deletion of RasGRP1 in mature lymphocyte populations experiencing normal development. This tool would unravel new possibilities, allowing the assessment of RasGRP1 in the context of mature T cell signaling events, pathogenic challenge experiments, vaccination responses and peripheral tolerance. Therefore, although RasGRP activity has been relatively well studied throughout T cell development, many questions remain regarding the role of these signaling molecules in T cell biology. Further studies of RasGRPs may reveal novel contributions of these signaling molecules in regulating the functionality of T cells and the immune system as a whole.

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