Analysis of TCR Signaling and Erk Activation in T Cell Development and Autoimmunity

by

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Dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of
Philosophy in the Department of
Immunology in the Graduate School
of Duke University

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ABSTRACT

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Abstract

LAT is a transmembrane adaptor protein that is critical for the emanation of signals downstream of the TCR. Following TCR engagement, LAT is phosphorylated on multiple tyrosine residues, allowing it to serve as a scaffold for a multi-protein signaling complex. Mutation of tyrosine 136 on LAT abrogates binding of PLC-γ1. The disruption of this interaction has severe consequences on TCR-mediated calcium signaling and MAPK activation. Mice harboring a mutation at this tyrosine, LATY136F ($LAT^{m/m}$) mice, have drastically impaired thymocyte development; however, $CD4^+$ T cells in the periphery rapidly expand and instigate a fatal lymphoproliferative syndrome. In order to bypass the severe developmental defects exhibited in $LAT^{m/m}$ mice, our laboratory previously developed a conditional knock-in mouse line in which the mutated LAT allele is expressed in mature T cells following deletion of a floxed wildtype LAT allele ($ERCre^+LAT^{f/m}$ mice). $LAT^{f/m}$ mice develop a similar lymphoproliferative syndrome as $LAT^{m/m}$ mice. We used both of these mouse models to analyze the contribution of two other proteins that are essential for TCR-mediated signaling, RasGRP1 and Gads, in LAT-mediated autoimmunity.

Analysis of $LAT^{m/m}RasGRP1^{-/-}$ mice demonstrated that the additional deletion of RasGRP1 increased the thymocyte development block and, as a result, young mice contained markedly reduced T cell populations. However, by four months of age, a

lymphoproliferative disease had developed in these mice. To bypass the severe developmental block, we analyzed $LAT^{f/m}RasGRP1^{-/-}$ mice and observed that they developed disease similarly to $LAT^{f/m}$ mice. We also assessed the effect of Gads deletion in both mouse models of LAT disease. $LAT^{m/m}Gads^{-/-}$ mice had an even more dramatic block in the DN stage of thymocyte development compared to $LAT^{m/m}$ controls, although by four months of age $CD4^{+}$ T cells had expanded. Following deletion of the wildtype LAT allele, $LAT^{f/m}Gads^{-/-}$ mice also developed disease. Our results indicated that LAT-mediated autoimmunity can occur independently of the critical T cell signaling components RasGRP1 and Gads.

In addition, we more closely examined RasGRP1-mediated Erk activation in T cells. RasGRP1 is a Ras-guanyl nucleotide exchange factor that is required for positive selection of thymocytes, activation of T cells, and control of T cell mediated-autoimmunity. While the importance of various RasGRP1 structural domains has previously been explored, RasGRP1 also contains a tail domain of unknown function. To elucidate the physiological role of this domain, we generated knock-in mice expressing RasGRP1 without the tail domain, $RasGRP1^{d/d}$ mice. Analysis of these mice demonstrated that deletion of the tail domain led to impaired T cell development but, with age, $CD4^{+}$ T cells expanded and auto-antibodies were produced. $RasGRP1^{d/d}$ thymocytes were unable to activate Erk and underwent aberrant thymic selection

processes. Mechanistically, the tail-deleted form of RasGRP1 was not able to traffic to the cell membrane following stimulation, indicating a potential reason for its inability to activate Erk. While the DAG-binding C1 domain of RasGRP1 has long been recognized as an important factor mediating Erk activation, our data revealed the physiological relevance of the tail domain of RasGRP1 in the control of Erk signaling.

Dedication

I would like to dedicate this to my mom and dad, who have been my biggest supporters for the past twenty-seven years. I owe all of my successes to them and can only hope to repay them one day.

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1. Introduction

1.1 Thymocyte development

In order to become mature T cells that are able to participate in immune responses, thymocytes must first undergo a tightly regulated development process. In the bone marrow, hematopoietic stem cells (HSCs) give rise to early T cell progenitors (ETPs), which migrate from the bone marrow to the thymus. In the thymus, these cells develop into double negative (DN) thymocytes ($CD4^-CD8^-$) and proceed through multiple developmental steps (Figure 1) to ultimately die or populate the periphery as T cells (Godfrey et al., 1993).

DN thymocyte differentiation is marked by the surface expression of CD4, CD8, CD44, and CD25. The first subset, DN1 thymocytes ($CD44^+CD25^-$), enter the thymus in the corticomedullary junction and move through the cortex during the DN1 and DN2 stages. DN1 cells retain a certain amount of lineage plasticity, as they are able to differentiate into natural killer (NK), dendritic (DC), myeloid, T, and B cell lineages (Ardavin et al., 1993; Matsuzaki et al., 1993; Moore and Zlotnik, 1995). As cells move to the DN2 stage ($CD44^+CD25^+$), recombinating activating genes 1 (RAG-1) and 2 (RAG-2) are activated, beginning the process of β, γ, and δ rearrangement (Godfrey et al., 1994; Wilson et al., 1994). While DN2 cells are still able to differentiate into NK and DC lineages, this plasticity is lost as they mature into DN3 cells, moving to the subcapsular

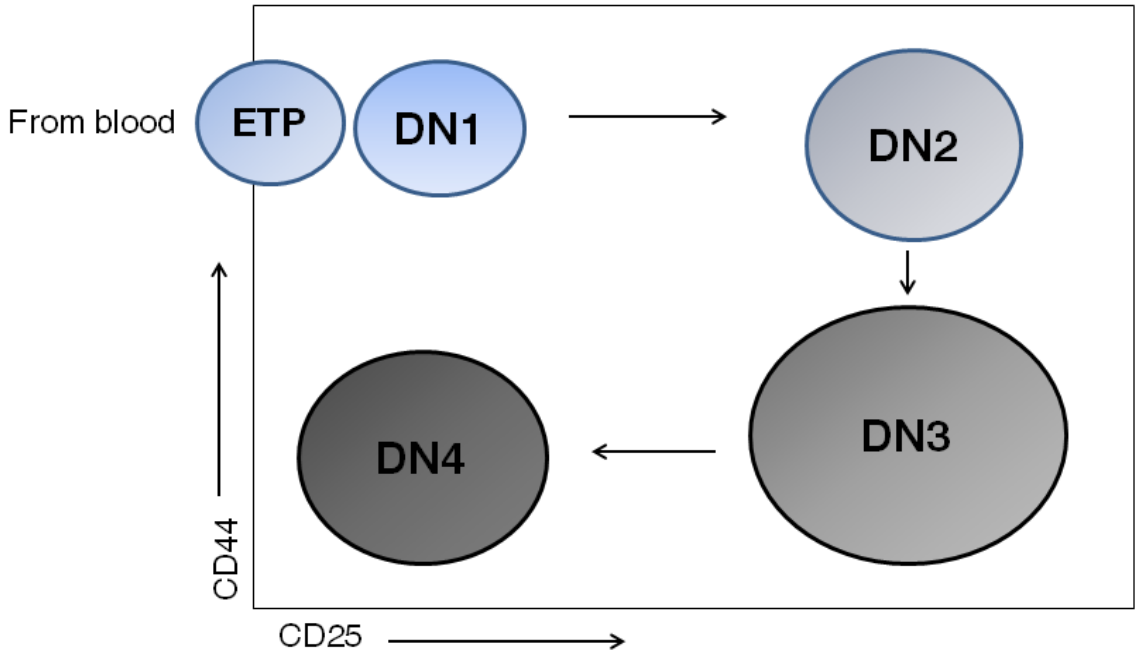


Figure 1: Early thymocyte differentiation

Early T cell progenitors (ETPs) travel from the bone marrow through the blood to the thymus where they are developmentally equivalent to the double negative 1 (DN1) compartment. These $CD25^{-}CD44^{+}$ cells transition into the DN2 ($CD25^{+}CD44^{+}$) stage. From the DN2 subset emerges DN3 ($CD25^{+}CD44^{-}$) cells, which are committed to the T cell lineage. Following β-selection, DN3 cells become DN4 ($CD25^{-}CD44^{-}$) thymocytes. Adapted from (Bhandoola and Sambandam, 2006).

zone of the thymus and becoming committed T cells (Bhandoola and Sambandam, 2006; Schmitt et al., 2004; Wu et al., 1996).

Before committing to the T cell lineage, immature thymocytes receive T cell receptor (TCR)-independent signals from cells in the thymic environment, namely cortical thymic epithelial cells (cTECs), to drive their differentiation (Petrie et al., 1993; Rothenberg and Taghon, 2005). Upon progression into the DN3 subset ($CD44^{-}CD25^{+}$), a small population of cells rearrange their γ and δ loci to comprise a unique T cell population, $\gamma\delta$ T cells (MacDonald et al., 2001). However, the majority of DN3 thymocytes follow the $\alpha\beta$ lineage path, rearranging the TCRβ chain. This rearrangement is proceeded by the assembly of the pre-TCR complex, containing the TCRβ chain, CD3, and the invariant pre-Tα chain (Groettrup and von Boehmer, 1993; Saint-Ruf et al., 1994). Cells that successfully express a functional pre-TCR become DN4 ($CD44^{-}CD25^{-}$) thymocytes. At this point, cells are able to transmit signals through the pre-TCR, resulting in allelic exclusion, cell survival, proliferation, and the initiation of TCRα rearrangement (O'Shea et al., 1997).

Upon the transmission of signals through the pre-TCR, cells transition into DP ($CD4^{+}CD8^{+}$) thymocytes (Figure 2). DP thymocytes undergo α chain rearrangement, ultimately expressing a functional $\alpha\beta$ TCR (Hernandez-Munain et al., 1999). The vast majority (~95%) of thymocytes will fail to express a functional $\alpha\beta$ TCR, causing them to

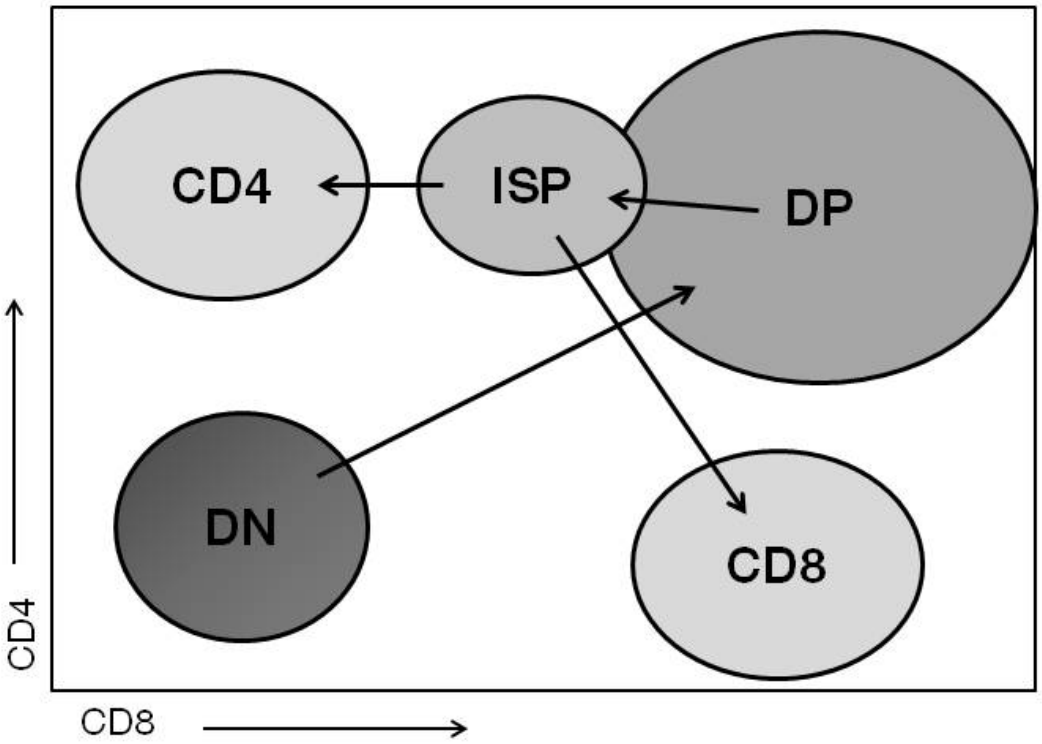


Figure 2: Thymocyte development

DN ($CD4^-CD8^-$) cells progress to the DP ($CD4^+CD8^+$) stage. Following positive selection, DP cells transition into intermediate single positive (ISP) cells, which have low CD8 expression while maintaining CD4 expression. From the ISP stage, thymocytes become either CD4 SP or CD8 SP cells. Adapted from (Singer et al., 2008).

undergo death by neglect, a form of programmed cell death (Huesmann et al., 1991; Shortman et al., 1991; Surh and Sprent, 1994). The remaining thymocytes must pass through the positive selection checkpoint, in which cells expressing TCRs that can bind self peptide-MHC molecules receive survival signals (Surh and Sprent, 1994).

From the DP stage, thymocytes must develop into either CD4 or CD8 single positive (SP) cells. Immediately following positive selection, DP thymocytes stop transcribing *cd8* (Brugnera et al., 2000) and become intermediate single positive (ISP) thymocytes expressing high levels of CD4 and low levels of CD8 (Lundberg et al., 1995; Suzuki et al., 1995). While many hypotheses have been formulated as to how a DP cell chooses its lineage fate, a more recent model, the kinetic signaling model, is likely an accurate representation of this process (Figure 3) (Singer, 2002; Singer et al., 2008). According to the kinetic signaling model, if TCR signaling is disrupted during the ISP stage, thymocytes develop into a CD8 SP cell (Singer et al., 2008). In addition to TCR signaling, thymocytes rely upon other signals for their differentiation. In particular, IL-7 signaling through the IL-7 receptor (IL-7R) plays a crucial role in guiding CD8 lineage commitment (Brugnera et al., 2000; Park et al., 2010).

As opposed to CD8 SP development, CD4 lineage commitment requires sustained signaling through MHC class II-restricted TCRs. Also, CD4 SP development depends upon TCR, but not IL-7R, signaling (Singer et al., 2008). Sustained TCR signals

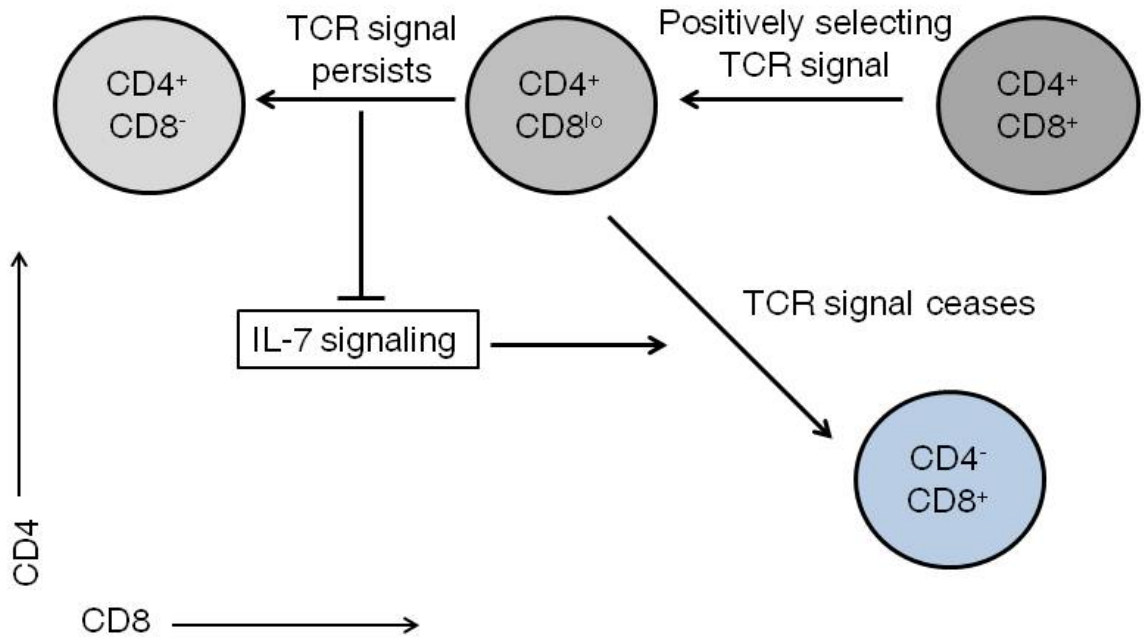


Figure 3: The kinetic signaling model

Positively-selected DP thymocytes cease *cd8* gene transcription to become $CD4^{+}CD8^{lo}$ intermediate single positive thymocytes. A persistence of TCR signaling in these cells blocks IL-7-mediated signaling, inducing CD4 SP T cell maturation. Disruption of TCR signaling allows for IL-7 signaling, which initiates co-receptor reversal. These cells gain CD8 expression, allowing their differentiation into CD8 SP thymocytes. Adapted from (Singer et al., 2008).

during the ISP stage, in which *cd8* transcription has already been disrupted, causes the continued silencing of *cd8* and increased expression of *cd4* (He et al., 2008).

Following CD4/CD8 lineage commitment, SP thymocytes migrate to the medulla of the thymus. In the medulla, medullary thymic epithelial cells (mTECs) and thymic DCs present self-antigen to the newly-arrived SP cells (Klein et al., 2009). Central tolerance is described as the process by which SP cells that bind strongly to self-antigen undergo negative selection, which serves to prevent the development of autoreactive T cells. After this final checkpoint, SP cells are able to exit the thymus and populate the periphery, standing sentinel for immune reactions (Hogquist et al., 2005).

1.2 TCR-mediated signaling

1.2.1 Early events in TCR-mediated signaling

The immune system depends upon a number of finely tuned signals to maintain homeostasis and to drive the development, differentiation, and activation of its many cellular components. Most of these processes are regulated by antigen receptors, such as T and B cell receptors (TCR and BCR) (Freitas and Rocha, 2000). These antigen receptors, located on the surface of lymphocytes, are critical to initiate downstream signaling events that dictate immune responses (Samelson, 2002).

Following the engagement of an antigen receptor, immunoreceptor tyrosine-based activation motifs (ITAMs), which are located in the cytoplasmic tails of receptor-

associated subunits, are phosphorylated by Src family kinases, such as Lck (lymphocyte-specific protein tyrosine kinase), Lyn, and Fyn (Cambier, 1992; Cambier, 1995; Reth, 1989). The phosphorylated tyrosines within ITAMs serve as docking sites for the Syk (spleen tyrosine kinase) family kinases, Syk and Zap-70 (ζ-chain-associated protein kinase of 70 kDa) in B and T cells, respectively. Syk and Zap-70 are then phosphorylated and activated by Src kinases, leading to the activation of downstream pathways through the phosphorylation of numerous proteins in close proximity to the receptor complex (Shaw and Thomas, 1991). These signals can be amplified by the presence of multiple ITAMs within antigen receptors and by the concentrated localization of receptors, co-receptors, and co-stimulatory molecules within specialized regions of the plasma membrane called lipid rafts (Rolli et al., 2002; Shores et al., 1997; van Oers et al., 1998).

1.2.2 The role of TRAPs in signal transduction through the TCR

Transmembrane adaptor proteins (TRAPs) are essential for the dissemination of antigen receptor signals by organizing multi-molecular protein complexes at the plasma membrane. Although they lack kinase activity, TRAPs are phosphorylated and serve as scaffolds to recruit critical downstream effector proteins (Horejsi et al., 2004; Kliche et al., 2004; Lindquist et al., 2003). Many TRAPs have been recently identified and their crucial roles in antigen receptor-mediated signaling have been highlighted in studies using cell lines and genetically-deficient mice. LAT (linker for activation of T cells), a

TRAP which serves as an anchor for a large signalosome upon TCR engagement, is essential for T cell development and function (Wange, 2000; Zhang et al., 1998a). While LAT plays an indispensable role in TCR- and FcεRI-mediated signaling, two other LAT family adaptor proteins, LAB (linker for activation of B cells)/NTAL (non-T cell activation linker) and LAX (linker for activation of X cells) are needed to guide lymphocyte responses (Zhu et al., 2005a; Zhu et al., 2006). Together, these proteins are able to exert both positive and negative effects on the complex signaling pathways that regulate immune responses.

In addition to the LAT family of adaptor proteins, other TRAPs also play important roles in the immune system. For example, SIT (SH2 domain-containing phosphatase 2-interacting TRAP) and TRIM (TCR-interacting molecule) dampen TCR-mediated signaling. Upon the deletion of both of these adaptor proteins, positive selection is enhanced, resulting in up-regulation of CD5, CD69, and TCRβ surface expression and strong MAPK (mitogen activated protein kinase) activation (Koelsch et al., 2008). PAG (phosphoprotein associated with glycosphingolipid-enriched domains) recruits Csk (C-terminal Src kinase) and RasGAP (RasGTPase activating protein) to the membrane, thus inhibiting Src kinase activity and Ras activation (Brdicka et al., 2000; Davidson et al., 2003; Smida et al., 2007). Another adaptor protein, LIME (Lck-interacting molecule), has been shown to associate with Lck, Gads (Grb2-related adaptor

downstream of Shc), and Grb2 (growth factor receptor-bound protein 2), contributing to Erk1/2 (extracellular-signal regulated kinase) and JNK (Jun N-terminal kinase) activation (Hur et al., 2003).

1.2.3 Linker for activation of T cells

As mentioned, LAT is one of the most crucial adaptor proteins involved in TCR signaling. Before its discovery, researchers sought to identify a molecule that linked TCR engagement to downstream signaling pathways. They focused their attention on a 36-38 kDa protein that is heavily tyrosine-phosphorylated after TCR stimulation (June et al., 1990). This protein was shown to localize to the plasma membrane and to bind to the SH2 domains of Grb2, Grap (Grb2-related adaptor protein), PLC (phospholipase C) -γ1, and the p85 subunit of PI3K (phosphatidylinositol 3-kinase) (Buday et al., 1994; Fukazawa et al., 1995; Gilliland et al., 1992; Sieh et al., 1994; Trub et al., 1997). This protein, which was cloned and named LAT, seemed to be an ideal candidate for linking initial TCR engagement to a complex orchestration of downstream signaling pathways (Figure 4).

Cloning of LAT revealed that it is a transmembrane protein containing a short extracellular domain, transmembrane domain, and cytoplasmic tail with nine conserved tyrosine motifs. LAT is expressed in thymocytes, mature T cells, NK cells, mast cells,

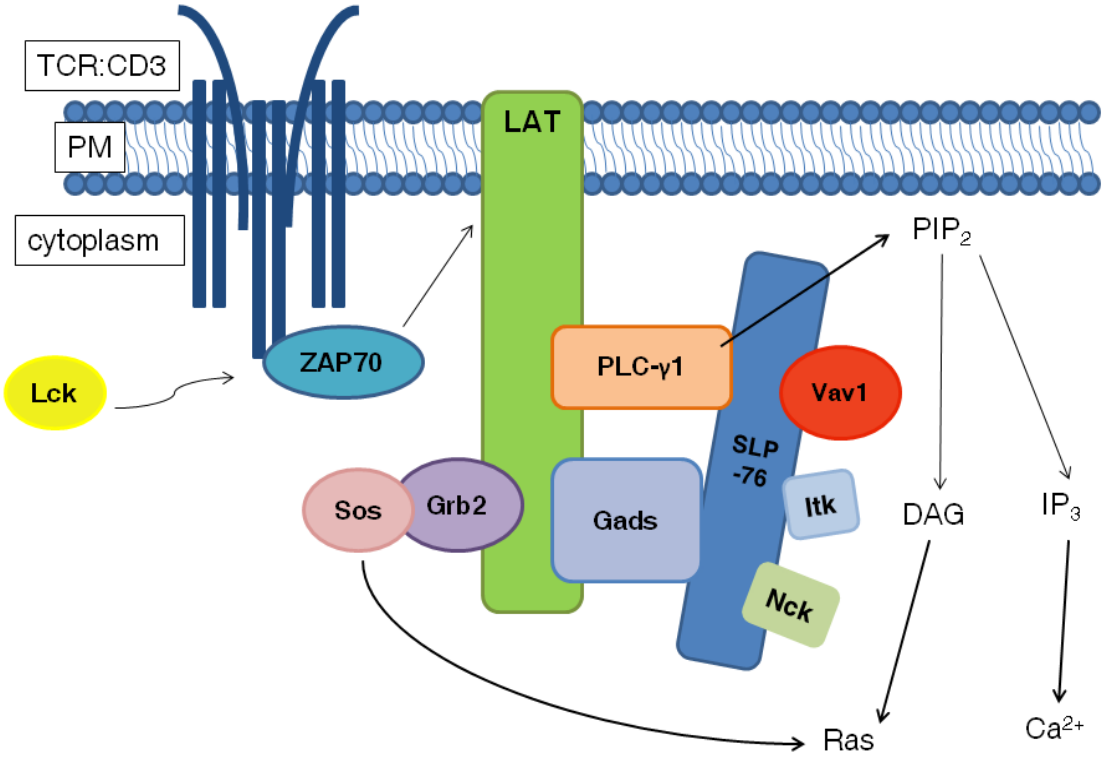


Figure 4: Assembly of the LAT-mediated signalosome

TCR engagement leads to ZAP-70 phosphorylation, allowing it to phosphorylate LAT on its multiple tyrosine residues. Phosphorylated LAT recruits Grb2, Gads, and PLC-γ1. Grb2 interacts with Sos, a Ras nucleotide exchange factor, to activate the Ras-Erk pathway. The recruitment of Gads, which is constitutively associated with SLP-76, and activation of PLC-γ1 also lead to Ras activation, in addition to Ca^{2+} flux.

megakaryoctes, and pre-B cells (Facchetti et al., 1999; Su and Jumaa, 2003; Weber et al., 1998; Zhang et al., 1998a). The juxtamembrane region of LAT contains two cysteine residues, which are necessary for LAT palmitoylation, raft localization, and function. Upon mutation of these cysteines, both LAT localization to lipid rafts and LAT phosphorylation following TCR ligation are severely impaired (Zhang et al., 1998b). While the physiological relevance of LAT localization to lipid rafts is unresolved (Zhu et al., 2005b), palmitoylation is critical for proper LAT function. Studies have shown that LAT palmitoylation is impaired in antigen-primed anergic T cells, causing a decrease in the activation of downstream signaling components. However, signaling events upstream of LAT, such as Zap-70 phosphorylation, are unperturbed in these cells, implying that the LAT palmitoylation defect is the initiating event in the establishment of anergy in T cells (Hundt et al., 2006).

The indispensable function of LAT in TCR-mediated signaling has been shown in LAT-deficient Jurkat T cells, in which calcium mobilization, Erk activation, CD69 upregulation, and AP (activator protein)-1- and NFAT (nuclear factor of activated T cells)-mediated gene transcription are severely impaired (Zhang et al., 1999a). Further evidence supporting the crucial role of LAT comes from LAT-deficient mice, which completely lack peripheral T cells. LAT is essential for the generation of DP and SP thymocytes as these mice have a block at the DN3 stage of thymocyte development.

Additionally, LAT-deficient mice contain no γδ T cells, further demonstrating the critical role of LAT in pre-TCR signaling (Zhang et al., 1999b).

Because LAT$^{-/-}$ thymocytes cannot progress past the DN stage, the role of LAT in the later stages of thymic development could not be elucidated from analysis of these mice. To study the potential role of LAT during the DP and SP stages, our lab generated LAT knock-in mice in which the *lat* gene can be deleted by the Cre recombinase (Shen et al., 2009). Deletion of LAT by Cre under the control of the CD4 proximal promoter allows for the generation of DP thymocytes but the transition from DP to SP thymocytes is severely blocked. Therefore, LAT is also irreplaceable during late stages of thymic development.

LAT does not have intrinsic enzymatic activity; hence, the ability of LAT to transmit signals depends entirely upon its phosphorylation. Following TCR engagement, LAT is phosphorylated by Zap70, allowing it to interact with SH2 domain-containing proteins, such as Grb2 and PLC-γ1 (Zhang et al., 1998a). A study examining the importance of LAT tyrosine phosphorylation generated LAT-deficient Jurkat cells reconstituted with LAT mutants unable to bind PLC-γ1 and Grb2. Results show that the disruption of these interactions inhibits TCR-mediated Ras activation, calcium flux, and NFAT activation. Also, LAT contains two binding sites for Gads. LAT association with Gads is needed for full T cell activation, although LAT-deficient Jurkat cells expressing a

LAT mutant unable to bind Gads show a minimal amount of calcium flux and NFAT activation (Zhu et al., 2003). Furthermore, the association of LAT and Gads allows LAT to indirectly bind to the cytosolic adaptor protein SLP-76 (SH2 domain-containing leukocyte protein of 76 kDa) (Liu and Zhang, 2008; Weber et al., 1998; Zhang et al., 1998a). SLP-76 is essential for TCR signaling through its regulation of actin polymerization; consequently, SLP-76-deficient mice demonstrate a thymic block at the DN3 stage of development (Koretzky et al., 2006).

Although the interaction of LAT with the aforementioned proteins occurs at specific tyrosine residues, data indicate that Grb2, Gads, and PLC-γ1 may bind cooperatively to LAT (Zhang et al., 2000). Only LAT mutants that are capable of binding Grb2 and PLC-γ1 are able to reconstitute T cell activation and thymocyte development, emphasizing the importance of these tyrosine residues in LAT function (Zhu et al., 2003). Mutating the four distal tyrosine residues on LAT, which mediate binding to Gads, Grb2, and PLC-γ1, renders Jurkat T cells completely unresponsive to TCR ligation (Zhang et al., 2000). Accordingly, knock-in mice harboring mutations at the four distal tyrosines – Y136, Y175, Y195, and Y235 in mice – have an identical phenotype as $LAT^{-/-}$ mice (Sommers et al., 2001).

1.2.4 PLC-γ1 and calcium mobilization

One of the most critical molecules involved in TCR signaling immediately downstream of LAT phosphorylation is PLC-γ1, which can bind LAT, SLP-76, and Vav. Upon the recruitment of PLC-γ1 to the LAT signalosome, Itk (IL2-inducible T cell kinase) phosphorylates and activates PLC-γ1. Activated PLC-γ1 is able to hydrolyze PIP_2 (phospholipid phosphatidylinositol 4,5 biphosphate) to IP_3 (inositol triphosphate) and DAG (diacylglycerol) (Rhee, 2001). While DAG serves to activate the Ras-MAPK pathway, IP_3 binds to IP_3R (IP_3 receptor) on the endoplasmic reticulum (ER) membrane, triggering the release of calcium. The depletion of calcium from the ER lumen is sensed by clusters of STIM (stromal interaction molecules), which are ER transmembrane proteins (Liou et al., 2005; Roos et al., 2005). STIM1 causes the initial rise in SOCE (store-operated Ca^{2+} entry) while STIM2 maintains basal Ca^{2+} and drives late-stage SOCE (Brandman et al., 2007; Oh-Hora et al., 2008). Furthermore, the oligomerization of STIM1 in extremely close proximity to the plasma membrane allows for Orai1 binding, which induces the formation of a CRAC (Ca^{2+} release-activated Ca^{2+}) channel and calcium influx into the cell (Feske et al., 2006; Luik et al., 2008; Luik et al., 2006).

The resulting elevation in intracellular Ca^{2+} concentration causes Ca^{2+}-calmodulin to bind calcineurin, displacing the autoinhibitory subunit of this enzyme. Active calcineurin is able to then dephosphorylate NFAT. NFAT is normally anchored in the

cytoplasm; however, dephosphorylation of NFAT exposes its nuclear localization sequence, thus driving its translocation into the nucleus (Hogan et al., 2003). NFAT, along with other transcription factors, initiates the transcription of many genes necessary for T cell development, differentiation, and function. The critical role of this pathway has been demonstrated in humans as patients with defects in SOCE and CRAC channel function develop severe combined immunodeficiency (SCID), marked by defective lymphocyte function and cytokine production (Feske et al., 1996; Le Deist et al., 1995; Partiseti et al., 1994).

The essential role of PLC-γ1 in T cell signaling has also been demonstrated *in vivo*. Although the complete ablation of PLC-γ1 is embryonic lethal, its selective deletion in T cells through the use of a Cre recombinase allows for analysis of its function specifically in T cells. As anticipated, these T cells are unable to flux Ca^{2+}, activate MAPKs, produce IL (interleukin)-2, and proliferate following TCR stimulation. Furthermore, these mice demonstrate impaired thymocyte development and develop an autoimmune disease with age (Fu et al., 2010).

1.2.5 Ras-MAPK signaling

In addition to PLC-γ1 activation and calcium mobilization, the phosphorylation of LAT leads to the activation of the MAPK pathway. Phosphorylated LAT recruits two different Ras GEFs (guanine exchange factors), which catalyze the activation of Ras.

These factors convert Ras from an inactive to active form by inducing the release of GDP (guanosine diphosphate) bound to Ras, thus allowing GTP (guanosine-5′-triphosphate) to bind. Ras-GTP assumes a different conformation which enables its association with critical MAPK signaling components (Vetter and Wittinghofer, 2001). One of the Ras GEFs, Sos (Son of sevenless), is brought to the LAT signalosome through its association with Grb2 (Finco et al., 1998; Zhang et al., 1999a). The other Ras GEF, RasGRP1 (Ras guanyl releasing protein 1), is recruited to cell membranes upon the production of DAG following PLC-γ1 activation, thus initiating Ras-MAPK signaling (Ebinu et al., 1998).

Following Ras activation by Sos and/or RasGRP1, Ras is able to active Raf-1, a serine-threonine kinase. Raf-1 then activates MEK (MAPK/Erk kinase), which in turn phosphorylates Erk1/2 (extracellular signal-regulated kinases 1 and 2) (Izquierdo et al., 1993). Phosphorylated Erk dimerizes and translocates to the nucleus, where it contributes to the assembly of the AP-1 transcription complex. Erk is necessary for thymocyte development, as well as the differentiation, survival, and activation of mature T cells (Dong et al., 2002).

Similar to Erk, p38 and JNK (Jun N-terminal kinase) are critical MAP kinases involved in T cell signaling. However, unlike Erk, the activation of p38 and JNK relies upon the Rho family of GTPases, rather than the Raf MAPKKK (MAPK kinase kinase) family. The Rho proteins, such as Rac and Cdc42, activate MKK3, MKK4, and MKK6,

which in turn activate p38, a critical pathway in early thymocyte development (Diehl et al., 2000; Sugawara et al., 1998). In contrast to Erk, which is mainly thought to play a role in the positive selection of thymocytes, p38 seems to be critical for negative selection (Sugawara et al., 1998). p38 may also contribute to mature T cell function, particularly the production of IFN-γ in Th1 cells (Rincon et al., 1998).

The Rho proteins also activate MKK4 and MKK7, which serve to activate JNK (Dong et al., 2002). JNK is essential for T cell activation, proliferation, and differentiation. Interestingly, JNK is encoded by two different genes, *jnk1* and *jnk2*, and significantly different outcomes result from signaling through either of these proteins. The absence of JNK1 promotes the production of IL-4, a Th2-type cytokine, but has no effect on IFN (interferon)-γ production, a Th1-type cytokine (Dong et al., 1998). In contrast, the loss of JNK2 expression has a marked effect on IFN-γ production (Yang et al., 1998). The two also seem to play contrasting roles in c-Jun phosphorylation, a critical event in negative selection (Sabapathy et al., 1999). While JNK1 induces c-Jun activity, JNK2 targets this transcription factor for degradation (Derijard et al., 1994; Fuchs et al., 1996; Fuchs et al., 1997). Together, all three MAPK families in T cells are critical for the emanation of signals through the T cell receptor, leading to a number of different cellular outcomes.

1.2.6 RasGRP1 in T cell signaling

As mentioned, the Ras protein family is crucial for T cell development and activation. The most well characterized signaling pathway regulated by Ras is the Erk1/2 MAPK pathway. In T cells, Ras is activated by Ras GEFs, such as RasGRP1 or Sos (Figure 5). Upon activation, Ras recruits Raf-1, a serine/threonine kinase, to phosphorylate MEK, in turn activating Erk1 and Erk2 (Genot and Cantrell, 2000; Hogquist, 2001; Mor and Philips, 2006).

It was recently demonstrated in T cells that Sos contains an allosteric Ras-GTP binding pocket that, upon binding to Ras-GTP, enhances Sos activity, suggesting that RasGRP1 may be necessary to prime Sos (Roose et al., 2007). However, RasGRP1 cannot fully compensate for Sos1 as the deletion of Sos1 in early thymocyte development leads to a partial developmental block. Also, the ratio of Sos1 to RasGRP1 expression changes dramatically upon the DN3 to DP transition, indicating non-overlapping functions for these GEFs (Kortum et al., 2011).

The importance of RasGRP1 in TCR signaling was first suggested in Jurkat T cells overexpressing RasGRP1 which display enhanced Ras/Erk signaling (Ebinu et al., 2000). Moreover, analysis of a RasGRP1-deficient Jurkat cell line illustrated the role of RasGRP1 in antigen receptor- and PMA (phorbol 12-myristate 13-acetate)-triggered Erk activation. Specifically, RasGRP1-dependent Erk activation has been shown to rely upon

its DAG-binding C1 domain (Roose et al., 2005). RasGRP1-deficient mice, in which B cells develop normally, have severely reduced numbers of single positive thymocytes and, consequently, very few mature T cells in the periphery. Further analysis of RasGRP1$^{-/-}$ thymocytes reveals their inability to activate Erk after stimulation with PMA, a DAG-analog (Dower et al., 2000). RasGRP1 has also been shown to be essential for the weak TCR signals necessary for Erk activation during positive selection. However, stronger signals that induce JNK and p38 activation, ultimately leading to negative selection, can be transmitted independently of RasGRP1 (Priatel et al., 2002).

As RasGRP1$^{-/-}$ mice age, they develop an autoimmune disorder marked by splenomegaly and auto-antibody production. This disease is primarily mediated by T cells that exhibit numerous functional defects (Coughlin et al., 2005; Coughlin et al., 2006). Interestingly, this phenotype arises despite reports that regulatory T cell function is intact and may be enhanced in RasGRP1$^{-/-}$ mice (Chen et al., 2008). In contrast to this finding, Priatel et al. assert that this autoimmune condition does not develop upon backcrossing RasGRP1$^{-/-}$ mice onto a B6 background (Priatel et al., 2007). The reason for these disparate results is yet unknown but it remains clear that RasGRP1 is a critical factor involved in T cell development, activation, and homeostasis.

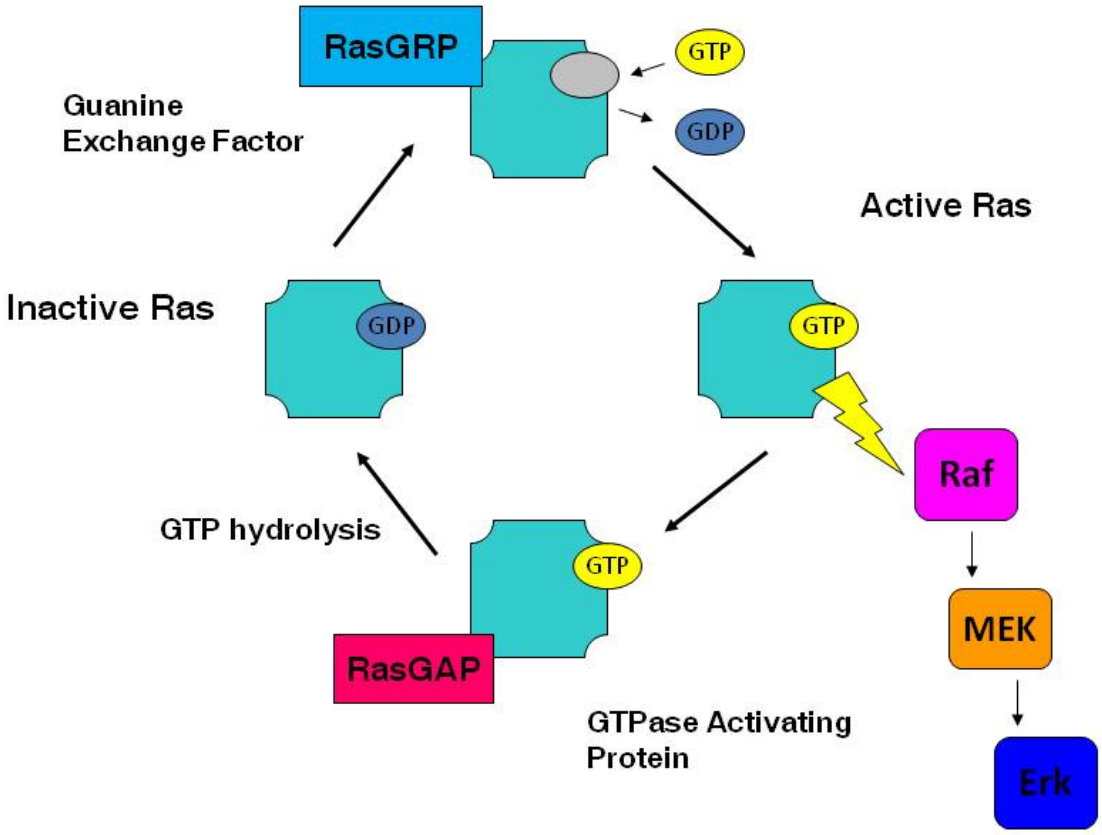


Figure 5: Ras cycles as GTP-bound active and GDP-bound inactive forms

The control of Ras activation is critical for T cell function. A RasGEF, such as RasGRP1, will catalyze the release of GDP from Ras, thus allowing GTP to bind. Ras-GTP undergoes a conformational change, allowing Ras to interact with Raf, a component of the MAPK signaling cascade. A RasGAP hydrolyzes GTP to GDP, returning Ras to its inactive state.

1.3 T helper cells

1.3.1 Regulatory T cells

The idea that suppressor T cells must exist in the body to negatively regulate immune responses is not a new concept but has recently re-emerged as a main focus of study. Termed regulatory T cells, or T_{regs}, $CD4^+CD25^+Foxp3^+$ cells have been identified as a crucial subset of helper T cells whose absence in humans leads to a rare X-linked fatal autoimmune disease referred to as IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome and causes a similar disease in "scurfy" mice (Bennett et al., 2001; Brunkow et al., 2001; Wildin et al., 2001). Indeed, data show that the expression of the transcription factor Foxp3 is limited to naturally occurring and peripherally induced regulatory $CD4^+CD25^+$ T cells but not in TCR-activated conventional $CD25^+$ T cells. In fact, the autoimmune disease observed in scurfy mice, characterized by lymphoproliferation in the skin, lymph nodes, and spleen, is a direct result of a Foxp3 mutation. Furthermore, ectopic expression of Foxp3 in $CD4^+CD25^-$ T cells imparts suppressor function onto these cells (Fontenot et al., 2003).

Although the indispensable role of Foxp3 has been well characterized, the mechanisms by which regulatory T cells suppress immune responses are not entirely known. T_{reg} cells are able to produce suppressive cytokines, such as IL-10 and TGF-β, although the physiological relevance of cytokine production by T_{regs} remains unclear

(Joetham et al., 2007; Kursar et al., 2007; Li et al., 2007; Rubtsov et al., 2008; Shevach, 2009). Additionally, the high expression of the IL-2 receptor α subunit (CD25) on T_{regs} has led to studies demonstrating that regulatory T cells are able to act as an IL-2 "sink," thus denying this critical cytokine to conventional T cells and inhibiting their proliferation (Pandiyan et al., 2007). However, a contrary report suggests that the consumption of IL-2 by T_{regs} does not impair conventional T cell function (Shevach, 2009). Regulatory T cells also express high levels of CTLA-4, a molecule which disrupts stimulation of conventional T cells by competing with the co-stimulation molecule CD28 for binding of CD80 and CD86 on APCs (antigen presenting cells) (Wing et al., 2008). The study of T_{reg} suppression is a dynamic field that is continually changing; therefore, new mechanisms detailing potential modes of suppression will inevitably be revealed in the coming years.

1.3.2 Helper T cell differentiation

Following thymocyte development and entry into the periphery, mature $CD4^+$ T cells can assume various phenotypes upon antigen recognition. $CD4^+$ cells were initially subdivided into two classes, Th1 and Th2, characterized by their disparate cytokine production of IFN-γ and IL-4, respectively (Mosmann et al., 1986). Recently, another subset, Th17, was identified based on its ability to produce the cytokine IL-17 (Harrington et al., 2005; Park et al., 2005). Further studies have revealed the existence of an inducible regulatory T cell (iT_{reg}) subset, critical for dampening autoimmune

responses in the periphery (Chen et al., 2003; Fantini et al., 2004). Lastly, a new subset termed follicular helper T cells (Tfh) has been recently described (King et al., 2008); however, the mechanism driving their differentiation remains unclear.

A naïve T cell in the periphery is only able to differentiate into one of the aforementioned subsets if a number of requirements are met (Figure 6). Crucial to this process are APCs, namely dendritic cells, B cells, and macrophages. APCs are able to present antigen on surface MHC class II molecules to naïve CD4 T cells. They also provide important secondary stimulation through the expression of co-receptors, co-stimulatory molecules, and cytokines (Kapsenberg, 2003). Of these, cytokines prove to be the most important signals driving T cell differentiation. Upon binding to their specific receptors on the surface of $CD4^+$ T cells, cytokine receptors are able to dimerize and activate the JAK (Janus kinase) and STAT (signal transducers and activators of transcription) pathways. Dimerization of STAT proteins leads to nuclear translocation where they serve to activate a number of gene transcription programs. The particular cytokine signaling received by a cell directs its differentiation. The production of IFN-γ and IL-12 by APCs drives Th1 differentiation, while IL-4 and IL-2 promote Th2 lineage commitment. TGF-β and IL-6 induce Th17 differentiation; however, the presence of TGF-β and IL-2 in the absence of IL-6 will allow naïve T cells to become iT_{regs}. These cytokines also help to stabilize the assumption of a particular T helper cell fate through

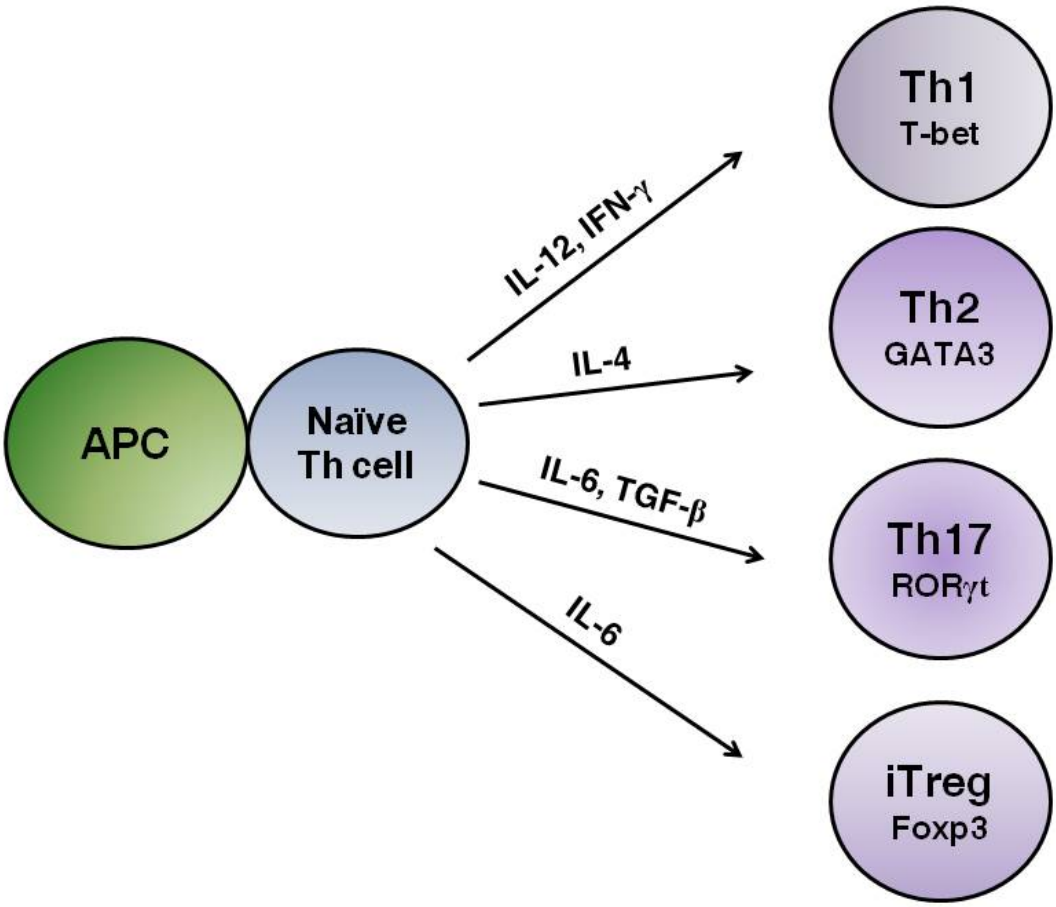


Figure 6: T helper cell differentiation

Following activation by an antigen presenting cell (APC), $CD4^+$ T cells can differentiate into multiple lineages depending upon environmental signals. The four main T helper subsets are shown above, as well as the main transcription factors driving their differentiation.

the active suppression of opposing genes.

Th1 cells are responsible for the control of intracellular infections caused by viral or bacterial entry into the host. Th1 cells are marked by their production of IFN-γ, IL-2, and TNF-α. Upon IL-12 binding to the IL-12R on $CD4^+$ T cells, Jak2 and Tyk2 kinases lead to the dimerization of STAT4 (Gately et al., 1998). STAT4 dimerization drives the increased expression of T-bet (T-box expressed in T cells), the hallmark transcription factor of Th1 cells (Szabo et al., 2000). Together, STAT4 and T-bet bind to the *ifng* promoter (Zhu et al., 2010). In response to IFN-γ binding to its receptor on T cells, Jak1 and Jak2 activate STAT1, causing further upregulation of T-bet expression (Afkarian et al., 2002). This positive feedback loop effectively increases the expression of T-bet, which is able to negatively regulate Th2 differentiation through the silencing of the transcription factor GATA3 (GATA binding protein 3) (Usui et al., 2006).

In contrast to Th1 cells, the Th2 lineage serves to defend the body against invading parasites, such as helminthes. However, they also are key mediators of asthma and allergic disease. Th2 cells are defined by their ability to produce IL-4, IL-5, and IL-13, ultimately leading to B cell activation and mast cell degranulation. IL-4 is the most critical factor initiating and maintaining Th2 differentiation. Binding of IL-4 to its receptor on T cells leads to Jak1 and Jak3 activation, causing the phosphorylation of

STAT6 (Hou et al., 1994; Quelle et al., 1995). As a result, expression of GATA3, the master regulator of Th2 differentiation, is increased (Zheng and Flavell, 1997). GATA3 is able to bind to the *il4*, *il5*, and *il13* genes, allowing for conformational changes that enable the binding of other transcription factors to drive expression (Ho et al., 2009). In addition to STAT6, STAT5 is critical for Th2 lineage commitment. Following IL-2 binding to the IL-2R, STAT5 is activated, enhancing IL-4Rα expression on the T cell surface and ultimately leading to increased GATA3 expression (Liao et al., 2008). Upon deletion of STAT5a, one of two STAT5 isoforms, Th2 differentiation is impaired (Kagami et al., 2001). Similar to T-bet expression, the upregulation of GATA3 effectively stymies other T lineage potential.

Another helper T cell subset, Th17, is critical in the defense against fungal and bacterial infections, especially at barrier surfaces where the body is constantly challenged by extracellular pathogens. Th17 cells, which produce IL-17, IL-17F, IL-21, and IL-22, have emerged as key components involved in autoimmune diseases, such as inflammatory bowel disease, arthritis, and multiple sclerosis (Korn et al., 2009). Th17 cells develop in the presence of IL-6 and TGF-β, although IL-21 and TGF-β can also drive their differentiation in the absence of IL-6 (Korn et al., 2007). These cytokoines serve to activate STAT3, which enhances expression of the main Th17 transcription factor, RORγt (retinoic acid related orphan receptor) (Ivanov et al., 2006; Korn et al.,

2009). RORγt increases IL-17 production and IL-23R surface expression, which maintains the Th17 lineage (Korn et al., 2009).

In addition to being a key cytokine driving Th17 development, TGF-β is also critical for iT_{reg} differentiation (Chen et al., 2003; Kretschmer et al., 2005). Similar to their thymic-derived counterparts, natural T_{regs}, inducible T_{regs} are marked by their surface expression of CD25 and expression of the transcription factor Foxp3. Through their disruption of DC maturation, production of inhibitory cytokines, and potential ability to act as an "IL-2 sink," both subsets of regulatory T cells are critical for immune tolerance and the prevention of autoimmunity (Vignali et al., 2008).

1.4 LAT-mediated autoimmunity

As one could expect in such a complex network as the immune system, there are instances in which the ability to distinguish between "self" and "non-self" fails, thus resulting in an autoimmune condition. One such case occurs upon the abrogation of the LAT-PLC-γ1 interaction. Experiments using Jurkat T cells expressing LAT with a tyrosine-to-phenylalanine mutation at the PLC-γ1 binding site (Y136) show the necessity of this residue in calcium mobilization and NFAT activation. Therefore, the LAT-PLC-γ1 interaction is critical for the emanation of signals originating from the TCR (Lin and Weiss, 2001; Paz et al., 2001). To demonstrate the importance of the LAT-PLC-γ1 interaction *in vivo*, two groups independently generated LATY136F knock-in mice,

which harbor a point mutation in the PLC-γ1 binding site of LAT (Aguado et al., 2002; Sommers et al., 2002). LATY136F mice have a partial block at the DN3 stage of thymocyte development but a small percentage of cells are able to mature into DP and SP cells. After maturation and entry into the periphery, these T cells become hyperactive and quickly initiate a fatal disease (Aguado et al., 2002; Genton et al., 2006; Sommers et al., 2002).

The lymphoproliferative disease observed in LATY136F mice is Th2-skewed, characterized by tissue eosinophilia and the massive production of IgE and IgG_1. These mice have severe tissue infiltration that causes lymphadenopathy, splenomegaly, and lesions in the lung, liver, and kidney. The infiltrating T cells harbor an effector/memory phenotype of $CD44^{hi}CD62L^{lo}$ and are resistant to TCR-mediated cell death. B cells from these mice are hyperactivated, resulting in elevated autoantibody serum titers (Aguado et al., 2002; Genton et al., 2006; Sommers et al., 2002). These findings indicate that, in addition to its established role as a critical positive regulator of the immune response, LAT is essential for the control of T cell homeostasis.

In order to elucidate the cause of the severe autoimmune-like disease in LATY136F mice, they were crossed onto the HY-TCR transgenic system, which is routinely used to examine positive and negative thymic selection events. Analysis of these mice showed that both thymic selection processes are severely impaired in

LATY136F mice. Thus, autoreactive T cells that would normally be deleted are able to escape central tolerance, perhaps driving their uncontrolled expansion in the periphery (Sommers et al., 2005). However, adoptive transfer of LATY136F T cells into MHC-deficient hosts does not affect the slow and sustained proliferation of the mutant T cells. Therefore, LATY136F T cells are able to expand in the absence of MHC molecules and without presentation of self-ligands by APCs (Wang et al., 2008).

As noted, LATY136F mice have impaired central tolerance, but they also have defective peripheral tolerance as they fail to develop natural $CD4^+CD25^+$ regulatory T cells. Foxp3 expression is significantly reduced at the RNA level and Foxp3 protein expression is not detectable by intracellular antibody staining in mutant thymocytes and mature T cells. Adoptive transfer of normal T_{reg} cells into neonatal LATY136F mice prevents the development of the lymphoproliferative disease. Furthermore, ectopic expression of Foxp3, which converts conventional $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ regulatory T cells (Fontenot et al., 2003), confers suppressor function onto LATY136F T cells (Koonpaew et al., 2006).

Contrary to our results, Wang et al. utilized Foxp3EGFP reporter mice to determine the contribution of T_{regs} to the LATY136F phenotype (Wang et al., 2008). These results demonstrate that $Foxp3^+$ T cells are actually present in LATY136F mice but are nonfunctional. Moreover, the authors contend that conventional LATY136F T cells are

able to escape the control of wildtype regulatory T cells. However, whether natural regulatory T cells are able to suppress the autoimmune syndrome in a long-term situation in these mice remains to be determined.

As described, the LATY136F disease is characterized by tremendous amounts of IL-4 and Th2-type skewing. However, a recent study indicates that this disease is not dependent upon the assumption of a Th2 lineage fate. In the absence of STAT6, which plays a major role in Th2 differentiation, STAT6$^{-/-}$LATY136F mice develop a lymphoproliferative disease of the same timing and magnitude as LATY136F mice. However, in contrast to the LATY136F disease, the autoimmunity that develops in these mice is mediated by CD8^{+} and Th1 cells, culminating in IgG_{2a} and IgG_{2b} hyperagammaglobulinemia (Archambaud et al., 2009).

Similar to LATY136F mice, LATY7/8/9F mice, which harbor mutations at tyrosines 175, 195, and 235, express a form of LAT with partial docking function. While PLC-γ1 may potentially bind to the intact Y136 residue upon phosphorylation, the mutation of tyrosines 175, 195, and 235 abolishes association with Gads and Grb2. LATY7/8/9F mice have a complete block in $\alpha\beta$ T cell development but accumulate $\gamma\delta$ T cells in the periphery, suggesting a differential requirement of LAT in $\alpha\beta$ and $\gamma\delta$ T cell development. Interestingly, these mice develop a severe lymphoproliferative disease characterized by Th2-like $\gamma\delta$ T cells and IgG_1/IgE hyperagammaglobulinemia (Nunez-

Cruz et al., 2003). Similar to LATY136F mice, LATY7/8/9F mice lack T_{regs}. However, disease onset cannot be attributed solely to the absence of this population as TCR$\beta^{-/-}$ and $E_{\alpha}^{-/-}$ mice also lack all $\alpha\beta$ T cell lineages but do not develop a lymphoproliferative disease (Leduc et al., 2000; Mombaerts et al., 1992).

Our laboratory has recently been working to further characterize T cell function in LATY136F mice. In order to bypass the severe developmental defects associated with the LATY136F mutation, we have developed an inducible deletion system in which a wildtype LAT allele allows for proper T cell development in the thymus but can be deleted in the periphery upon injection of tamoxifen (ERCre^{+}LAT$^{f/m}$ mice, f=floxed, m=Y136F). After deletion of the floxed wildtype allele, T cells are marked by expression of GFP and are left with only the mutated LATY136F allele. Our data show that treatment with tamoxifen for 3-4 weeks allows for the development of an autoimmune disease similar, although less severe, to that seen in LATY136F mice. Thus, the Y136F mutation in mature T cells alone can drive the lymphoproliferative disease (Chuck et al., 2010). Interestingly, using the deletion system described above, mice containing LAT deleted in mature T cells (ERCre^{+}LAT$^{f/-}$ mice) develop a similar autoimmune syndrome, although the disease observed is much less severe (Shen et al., 2010). Together, the data from analyses of these LAT mutant mice clearly indicate the critical role of LAT in the control of T cell activation and homeostasis.

2. Materials and Methods

2.1 Mouse models

2.1.1 $LAT^{m/m}$ and $ERCre^{+}LAT^{f/m}$ studies

$ERCre^{+}LAT^{f/+}$ and $ERCre^{+}LAT^{f/m}$ mice were generated as described previously (Chuck et al., 2010; Shen et al., 2009). For all experiments, age-matched $LAT^{m/+}$ or $ERCre^{+}LAT^{f/+}$ mice were used as controls and are referred to as wildtype (WT). $Gads^{-/-}$ mice were from Alec Cheng (Washington University) and $RasGRP1^{-/-}$ mice were obtained from James Stone (University of Elberta). Mice were housed in specific pathogen-free conditions and were used in accordance with National Institutes of Health guidelines. The experiments described here were reviewed and approved by the Duke University Institutional Animal Care Committee.

2.1.2 Generation of $RasGRP1^{d/d}$ knock-in mice

RasGRP1 genomic fragments were amplified from embryonic stem (ES) cells by PCR, sequenced, and cloned into the targeting plasmid (Figure 19). The short arm contained a 1.8-kb sequence including exon 14 and a modified exon 15 with a premature stop codon to delete amino acids 669-852. The long arm was comprised of a 4.2-kb sequence including exon 16 and surrounding introns. After transfection, G418-resistant ES cells were screened by PCR. The correctly targeted ES cells were injected into blastocysts to generate chimeric mice. To delete the PGK-Neo cassette, chimeric mice

were crossed with β-actin-Cre mice to produce RasGRP1$^{d/+}$ mice. These mice were subsequently backcrossed with C57BL/6 mice for ten generations. Genotyping of littermates was done by PCR using the following primers: 5′-CAT GAA AGC CAT CGG GTA CT-3′ and 5′-AAG ATC CTT CTT CGG GTG CT-3′. To confirm the insertion of a premature stop codon in exon 15, RasGRP1 RNA was amplified by reverse transcription (RT)-PCR using two primers: 5′-TGA CAA CTG TGC TGG CTT TC-3′ and 5′-ACG ATT CTG TTT GGG TGC TC-3′. C57/BL6 mice, OT-II mice, and β-actin-Cre mice were purchased from Jackson Laboratory. HY-TCR transgenic mice were purchased from Taconic Farms. Mice were housed in specific pathogen-free conditions and were used in accordance with National Institutes of Health guidelines. The experiments described here were reviewed and approved by the Duke University Institutional Animal Care Committee.

2.1.3 Confirming RasGRP1 gene targeting

For Western blot analyses, $5x10^7$ thymocytes or splenocytes were lysed in 1mL of RIPA buffer. The lysates were resolved on SDS-PAGE and probed with a RasGRP1 monoclonal antibody (Santa Cruz) or J32, a polyclonal antibody directed against the GRP1 CDC25 domain (Ebinu et al., 2000). To confirm equal expression of RasGRP1 mRNA, DP thymocytes were sorted and used for total RNA extraction using Trizol reagents. cDNAs were synthesized using SuperScript reverse transcriptase (Invitrogen)

with oligo-dT as the primer. Quantification of RasGRP1 RNAs was performed by RT-PCR with SYBR Green Super Mix (Bio-Rad) using the following primers: 5′-AGG ATG CCC TGG AAA AGA AT-3′ and 5′-AGC TGG CAT CCA TCT TGA AC-3′.

2.2 Inducible deletion of LAT

To delete the floxed *lat* allele *in vivo*, 1.5mg of tamoxifen (Sigma) was dissolved in corn oil. Mice were initially injected intraperitoneally for two consecutive days. Following the initial deletion, mice were administered the same amount of tamoxifen once every other week for four to eight weeks.

2.3 Antibodies

2.3.1 Antibodies for flow cytometry

For use in flow cytometry, the cell viability marker 7AAD was purchased from Invitrogen (Carlsbad, CA). The following antibodies were used to stain cell surface markers and were acquired from eBioscience (San Diego, CA): CD4, CD8α, B220, CD11c, CD25, MHC class II, CD44, CD62L, CD69, CD25, CD5, HY TCR, Gr-1, CD11b, vα2, TCRβ, IL-2, IL-4, IFN-γ, and Foxp3.

2.3.2 Antibodies for biochemical studies

Monoclonal phospho-Erk1/2 antibody was obtained from Cell Signaling Technology. Monoclonal anti-phosphotyrosine antibody (4G10) was purchased from Upstate Biotechnology. Polyclonal anti-Erk2 and monoclonal anti-RasGRP1 antibodies

were acquired from Santa Cruz Biotechnology. Secondary antibodies, such as goat anti-mouse and anti-rabbit Ig conjugated with Alexa Fluor 680, were purchased from Molecular Probes. IRDye 800 anti-rabbit-IgG was bought from Rockland Immunochemicals.

2.4 Cell isolation

Thymuses, spleens, and lymph nodes were extracted from sacrificed mice. Single cell suspensions were created by homogenizing organs with frosted glass slides. Splenocytes were then resuspended in ACK lysis buffer (0.15M NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA pH7.4) for one minute at room temperature to lyse red blood cells. They were then washed with PBS. Cell suspensions were filtered through mesh and resuspended to the desired concentration following cell counting using a hemocytometer.

2.5 Flow cytometry

2.5.1 Cell surface staining

In order to stain cell surface markers, cells were incubated with 2.4G2 (anti-FcγII/III receptor) for 15 minutes on ice before staining with different fluorochrome-conjugated antibody mixtures. Cells were then washed with FACS buffer (2% FBS in PBS) and analyzed on FACSCanto II (BD Biosciences). Flow cytometry data were analyzed with FlowJo software.

2.5.2 Intracellular staining

To detect transcription factors, splenocytes were isolated and stained for cell surface markers as described above before being fixed and permeabilized according to the manufacturer's instructions (BD Biosciences). Cells were then stained on ice for 30 minutes in the dark with anti-T-bet, -GATA3, and/or -Foxp3 antibodies (eBioscience). For intracellular staining of cytokines, splenocytes were isolated and stimulated with PMA (20ng/mL) and ionomycin (0.5μg/mL) for one hour before the addition of monensin (BioLegend). Cells were left for an additional three hours before being washed and stained with cell surface antibodies. Samples were then fixed, permeabilized, and stained with anti-IFN-γ, -IL-2, and -IL-4 antibodies per the manufacturer's instructions (eBioscience). Flow cytometry data were acquired on FACSCanto II (BD Biosciences) and analyzed with FlowJo software.

2.6 Cell activation and CFSE staining

Splenocytes were isolated as above and stimulated with plate-coated anti-CD3 (3μg/mL 2C11) or with PMA (20ng/mL) and ionomycin (0.5μg/mL) overnight to assess upregulation of activation markers. Cells were then stained with antibodies against CD4, CD8, CD25, CD69, and 7AAD and analyzed via flow cytometry. To determine cell proliferation, splenocytes were loaded with 5μM CFDA-SE and stimulated with 2C11 or

PMA and ionomycin as above. After forty-eight hours, cells were harvested, stained, and analyzed by flow cytometry for CFSE dilution.

2.7 ELISA and autoimmunity analysis

2.7.1 TCR-mediated IL-2 production

For IL-2 production, at twenty-four hours after the stimulation described in Section 2.6, 50μL of supernatants were collected and analyzed by ELISA. Samples were analyzed in triplicate using IL-2 capture and detection antibodies according to the manufacturer's instructions (eBioscience).

2.7.2 Serum immunoglobulin and autoimmunity analysis

For serum antibody detection, sera were serially diluted as shown in Figure 21. 96-well plates were coated with anti-mouse IgE and Ig(H+L) before incubation with sera and then detection antibodies. For autoantibody detection, 96-well plates were coated with NIH3T3 cells and incubated with sera followed by anti-mouse IgG-HRP. Absorbance at 405 nm (anti-IgE and autoantibody) or 450 nm (all other isotypes) was determined using a precision microplate reader (Molecular Devices). For anti-nuclear antibody fluorescent staining, NIH3T3 cells were blocked with 2.4G2 antibody and then incubated with sera (1:50) followed by FITC goat anti-mouse IgG. Immune complex deposition in mice was detected by staining cryosections of kidneys with FITC-conjugated goat anti-mouse IgG. Slides were visualized by fluorescent microscopy.

2.8 DP thymocyte isolation, activation, and Western blot analysis

DP thymocytes were enriched using the EasySep biotin selection kit (StemCell Technologies) in which thymocytes were blocked using anti-mouse FcR antibody and then incubated with biotin-conjugated H2-D^b antibody (BioLegend). Cells were mixed with biotin selection cocktail and magnetic beads, per the manufacturer's instructions, to remove SP and DN cells, both of which express high levels of this MHC class I molecule. Selected thymocytes were then stimulated directly with 20ng/mL PMA or were incubated with 10μg/mL biotin-αCD3 and 2.5μg/mL biotin-αCD4/CD8 and activated by cross-linking with 25μg/mL streptavidin (Sigma). The cells were lysed at the indicated time points in RIPA lysis buffer. Cell lysates were resolved using SDS-PAGE and transferred onto nitrocellulose membranes (BioRad). Membranes were blotted with anti-pTyr (Upstate Biotechnology), anti-LAT, anti-pErk (Cell Signaling Technology), and anti-Erk2 (Santa Cruz Biotechnology). For secondary antibodies, AlexaFluor 680 anti-mouse or anti-rabbit IgG (Molecular Probes) or IRDye 800 anti-rabbit (Rockland) were used. The membranes were scanned using the LI-COR Odyssey infrared imaging system.

2.9 GFP-RasGRP1 imaging

The cDNA sequences of mouse *rasgrp1* and *rasgrp1Δtail* (lacking amino acids 665-852) fused to GFP were cloned into the pHSIB retroviral vector. Jurkat cells were

electroporated with 10μg of plasmid DNA. Live cell imaging was performed on a Zeiss Observer D1 station equipped with a CoolSNAP$_{HQ}$ charge-coupled device camera (Roper Scientific) and a high-speed automatic objective stage. Images were collected with a 40x objective lens and 2.5x camera zoom at 45 second intervals. For each cell at each time point, GFP data were collected over 21 continuous vertical Z positions. After 45 seconds, 3μg/mL OKT3 or 20ng/mL PMA were added to the samples. Images were processed by 3D deconvolution using AutoQuant X software (Media Cybernetics). Imaging manipulation and analyses were done using the MetaMorph software suite (Molecular Probes).

3. The Importance of the Erk Pathway in the Development of LAT-Mediated Disease

3.1 Introduction

The phosphorylation of LAT following receptor ligation is indispensable for its function. Upon TCR ligation, phosphorylation by Zap-70 allows LAT to serve as a scaffold at the plasma membrane for the assembly of a multi-molecular signaling complex. LAT-deficient Jurkat cells have severely impaired TCR-mediated calcium mobilization, Erk activation, CD69 upregulation, and AP- and NFAT-mediated gene transcription (Zhang et al., 1999a). Correspondingly, LAT-deficient mice have a complete block at the DN3 stage of thymocyte development and lack peripheral T cells (Zhang et al., 1999b).

Similar to the complete deletion of LAT, the mutation of its four distal tyrosines, thus abrogating interaction with Grb2, Gads/SLP-76, and PLC-γ1, blocks thymocyte development and renders Jurkat T cells completely unresponsive to receptor engagement (Sommers et al., 2001; Zhang et al., 2000). Specifically, the binding of PLC-γ1 to LAT depends upon tyrosine 132 in humans and 136 in mice. Mutation of this site in Jurkat cells disrupts calcium mobilization and NFAT activation (Lin and Weiss, 2001; Paz et al., 2001). Analyses of mice harboring a mutation at tyrosine 136, LATY136F mice, reveal a regulatory role for the LAT-PLC-γ1 interaction in thymocyte development (Aguado et al., 2002; Sommers et al., 2002). LATY136F mice have a severe but incomplete

block at the DN3 stage, allowing only a small percentage of cells to mature into DP and SP cells. The $CD4^+$ T cells that populate the periphery in these mice are hyperactivated and expand vigorously, causing a fatal autoimmune disease. This disease is Th2-skewed, characterized by tissue eosinophilia and extremely high levels of serum IgE and IgG_1. These mice exhibit splenomegaly, lymphadenopathy, and lymphocyte infiltration into the lung, liver, and kidney. While there are virtually no $CD8^+$ T cells in these mice, the $CD4^+$ population is marked by an effector/memory phenotype of $CD44^{hi}CD62L^{lo}$ with severely decreased levels of surface TCRβ. Resultant of this mutation, TCR-mediated LAT and PLC-γ1 phosphorylation and calcium flux are also impaired.

A striking characteristic of the LATY136F mutant T cells is the tremendous amount of IL-4 they are able to produce. However, the development of the LATY136F disease is not dependent upon the assumption of a Th2 lineage fate. In the absence of STAT6, a transcription factor necessary for IL-4 production and Th2 differentiation, $STAT6^{-/-}$LATY136F mice develop a lymphoproliferative disease of the same timing and severity as LATY136F mice. Interestingly, this disease is mediated by $CD8^+$ and Th1 cells (Archambaud et al., 2009).

Our laboratory has recently been working to further characterize T cell function in LATY136F mice. In order to bypass the severe developmental defects associated with the LATY136F mutation, we developed an inducible deletion system using the ERCre

recombinase in which a wildtype LAT allele allows for proper T cell development but can be deleted in the periphery upon the injection of tamoxifen (ERCre^{+}LAT$^{f/m}$ mice). After deletion of the floxed wildtype allele, T cells are left with only a copy of the mutated LATY136F allele. Our data show that treatment with tamoxifen for 3-4 weeks incites the development of an autoimmune disease similar, although less severe, to that seen in LATY136F mice. Thus, the Y136F mutation in mature T cells alone can drive the lymphoproliferative disease (Chuck et al., 2010). Together, data from analyses of these LAT mutant mice clearly indicate a role for this adaptor protein in controlling CD4^{+} T cell activation and proliferation.

Furthermore, a previous report shows that adoptive transfer of LATY136F T cells into MHC-deficient hosts does not affect the slow and sustained proliferation of the mutant T cells (Wang et al., 2008). However, another study examining the disease mediated by mature mutant T cells demonstrated that LAT$^{fl\text{-}dtr/Y136F}$ cells, which are similar to ERCre^{+}LAT$^{f/m}$ cells, require MHC class II for their proliferation (Mingueneau et al., 2009). These data suggest that LAT-mediated disease onset may require TCR engagement whereas the perpetuation of the disease may be TCR-independent. Thus, it seems probable that continuous tonic signaling through the TCR could be inciting the hyperproliferation of mutant T cells.

In this study, we undertook the task of further illuminating the signaling components involved in LAT-mediated disease, using both LATY136F ($LAT^{m/m}$) and $ERCre^{+}LAT^{f/m}$ ($LAT^{f/m}$) mouse models (Roncagalli et al., 2010). We tested if the deletion of the LAT signalosome components Gads, whose interaction with LAT recruits the essential cytoplasmic adaptor protein SLP-76, and RasGRP1, a critical Ras GEF (guanine exchange factor) in T cells, would lessen disease severity. We subsequently restored the signaling pathways that are disrupted upon the abolishment of the LAT-PLC-γ1 interaction, namely NFAT, NF-κB, and Erk activation, to try to ameliorate disease development.

3.2 Results

3.2.1 Ablation of RasGRP1 does not prevent disease development

We first evaluated if the disruption of Ras/Erk signaling through the deletion of RasGRP1 would ameliorate disease progression. RasGRP1, a Ras guanine exchange factor, serves to activate Ras following PLC-γ1 activation and DAG production (Genot and Cantrell, 2000; Mor and Philips, 2006). The deletion of RasGRP1 in T cells leads to impaired thymocyte development, Erk activation, and mature T cell function (Dower et al., 2000; Layer et al., 2003; Priatel et al., 2006). Because RasGRP1 plays such a critical role in mediating Ras/Erk activation in T cells, we hypothesized that its deletion in

LAT$^{m/m}$ T cells could stymie disease development. To this end, we crossed LAT$^{m/m}$ mice with RasGRP1$^{-/-}$ mice.

As seen previously, LAT$^{m/m}$ thymocytes were severely blocked during the DN stage of development (Figure 7A). RasGRP1$^{-/-}$ mice had increased DN and DP compartments with very few cells progressing to either the CD4 or CD8 SP stage. Analysis of LAT$^{m/m}$RasGRP1$^{-/-}$ mice showed that thymocytes had an increasingly severe block in the DN stage, with only ~7% of thymocytes becoming DP cells compared to ~27% of LAT$^{m/m}$ thymocytes. We further analyzed the DN compartment of these mice. LAT$^{m/m}$ mice displayed a severe block in the DN3 stage, with ~60% of DN thymocytes being CD25^{+}CD44^{-} and only 12.7% CD25^{-}CD44^{-} DN4 cells (Figure 7B). RasGRP1$^{-/-}$ thymocytes were similarly blocked in the DN3 stage but had an increased proportion of DN4 cells. LAT$^{m/m}$RasGRP1$^{-/-}$ thymocytes had the most severe block with 79.3% of thymocytes being DN3 and only 5.6% DN4. Despite having a developmental block, by about one month of age, LAT$^{m/m}$ spleens already contained a significant percentage of CD4^{+} T cells (Figure 7C). In contrast, and likely due to the more dramatic developmental block, LAT$^{m/m}$RasGRP1$^{-/-}$ mice contained virtually no splenic T cells.

To observe whether LAT$^{m/m}$RasGRP1$^{-/-}$ T cells would eventually become hyperproliferative, we analyzed mice at four months of age. LAT$^{m/m}$ mice had striking splenomegaly and drastically increased numbers of splenocytes (Figure 8A). RasGRP1$^{-/-}$

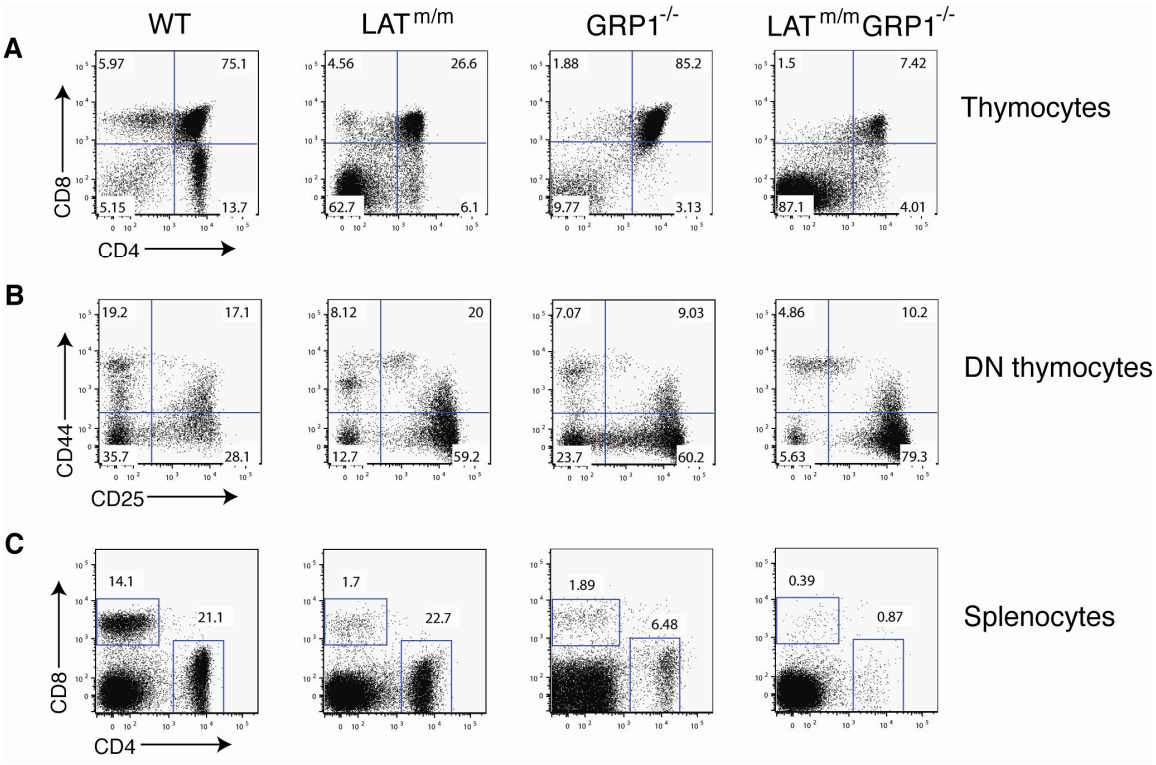


Figure 7: Impaired thymocyte development in LAT$^{m/m}$GRP1$^{-/-}$ mice

(A-C) Mice were analyzed at 4 weeks of age. (A) Representative FACS plots of CD4 and CD8 expression on thymocytes. (B) CD25 and CD44 expression on DN thymocytes. (C) Surface expression of CD4 and CD8 on splenocytes. Data are representative of 2 independent experiments using 2-4 mice per genotype.

mice had slightly increased spleen weights and cell numbers compared to wildtype mice. Despite the developmental block, LAT$^{m/m}$RasGRP1$^{-/-}$ mice developed a similar disease as LAT$^{m/m}$ mice, although severity varied tremendously in individual mice. While some mice had only moderately increased spleen weight and cellularity compared to wildtype mice, other littermates had striking splenomegaly, similar to that of age-matched LAT$^{m/m}$ mice. Furthermore, LAT$^{m/m}$RasGRP1$^{-/-}$ spleens consisted of extremely high numbers of CD4^{+} splenocytes compared to wildtype and GRP1$^{-/-}$ spleens. The spleens of LAT$^{m/m}$ mice were ~60-80% CD4^{+} T cells and contained virtually no CD8^{+} T cells (Figure 8B). By four months of age, RasGRP1$^{-/-}$ spleens contained a slightly decreased percentage of CD4^{+} T cells compared to wildtype spleens and a much reduced CD8^{+} compartment. LAT$^{m/m}$RasGRP1$^{-/-}$ mice all had increased, but variable, percentages of CD4^{+} cells, from ~23-70%. Again, none of these mice contained significant numbers of CD8^{+} cells. Additionally, almost all CD4^{+} T cells in LAT$^{m/m}$, RasGRP1$^{-/-}$, and LAT$^{m/m}$RasGRP1$^{-/-}$ mice were CD62L^{lo} and CD44^{hi}. LAT$^{m/m}$ CD4^{+} splenocytes had much reduced levels of surface TCRβ (Figure 8C). Interestingly, LAT$^{m/m}$RasGRP1$^{-/-}$ CD4^{+} T cells had even further decreased TCRβ expression compared to both LAT$^{m/m}$ and RasGRP1$^{-/-}$ T cells. It is important to note, however, that TCRβ staining on these cells was still higher than that on B cells (data not shown), indicating that TCR was not absent from the cell surface.

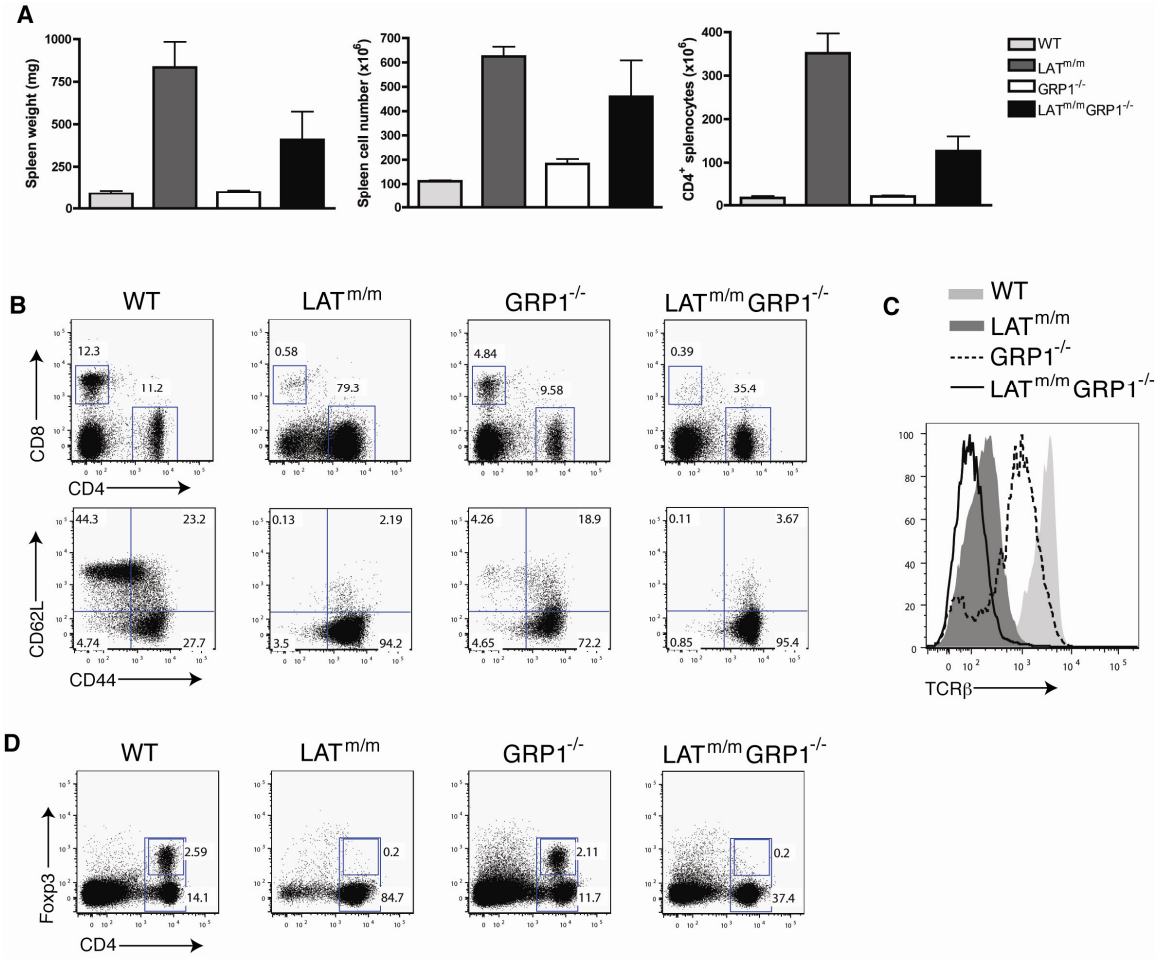


Figure 8: Development of LAT-mediated disease upon the loss of RasGRP1

(A-D) Mice were analyzed at 4 months of age. (A) Spleen weights (left), total numbers (middle), and total CD4⁺ numbers (right). (B) FACS plots of CD4 and CD8 expression on splenocytes (top) or CD44 and CD62L expression on CD4⁺ splenocytes (bottom). (C) TCRβ expression on CD4⁺ splenocytes. (D) CD4 and Foxp3 expression on live splenocytes. Top number corresponds to the percentage of Foxp3⁺ cells while bottom number is the percentage of total CD4⁺ cells. Data are representative of 3 independent experiments using at least 2 mice per genotype.

We also assessed whether the deletion of RasGRP1 could affect T_{reg} cell populations. The regulatory T cell population was obviously absent in $LAT^{m/m}$ mice (Figure 8D). $RasGRP1^{-/-}$ mice had similar and even slightly increased percentages of $CD4^{+}Foxp3^{+}$ regulatory T cells compared to wildtype controls. $Foxp3^{+}$ T cells were clearly unable to develop in $LAT^{m/m}RasGRP1^{-/-}$ spleens. From these data, we were able to conclude that the deletion of RasGRP1 on a $LAT^{m/m}$ background significantly impaired thymocyte development but was unable to suppress peripheral $CD4^{+}$ T cell proliferation.

Upon assessing the ability of $CD4^{+}$ T cells from these mice to produce cytokines, we made some interesting observations. While a small percentage of wildtype $CD4^{+}$ T cells produced IL-4 and IFN-γ upon stimulation, an overwhelming percentage of $LAT^{m/m}$ T cells (~90%) produced IL-4 and a minimal amount of IFN-γ (Figure 9A). $RasGRP1^{-/-}$ $CD4^{+}$ T cells produced IFN-γ but no IL-4. However, nearly 70% of $LAT^{m/m}RasGRP1^{-/-}$ T cells produced IL-4 while almost no cells produced IFN-γ alone. Furthermore, we assessed IL-2 production in these cells. A significant amount of wildtype $CD4^{+}$ T cells made IL-2 upon stimulation. There were fewer $LAT^{m/m}$ $IL\text{-}2^{+}$ T cells and slightly more $IL\text{-}2^{+}$ $RasGRP1^{-/-}$ T cells compared to wildtype controls. While only ~14% of $LAT^{m/m}$ $CD4^{+}$ cells made IL-2, ~74% of $LAT^{m/m}RasGRP1^{-/-}$ CD4 T cells produced this cytokine either alone or in conjunction with IL-4. These results indicated that the deletion of RasGRP1

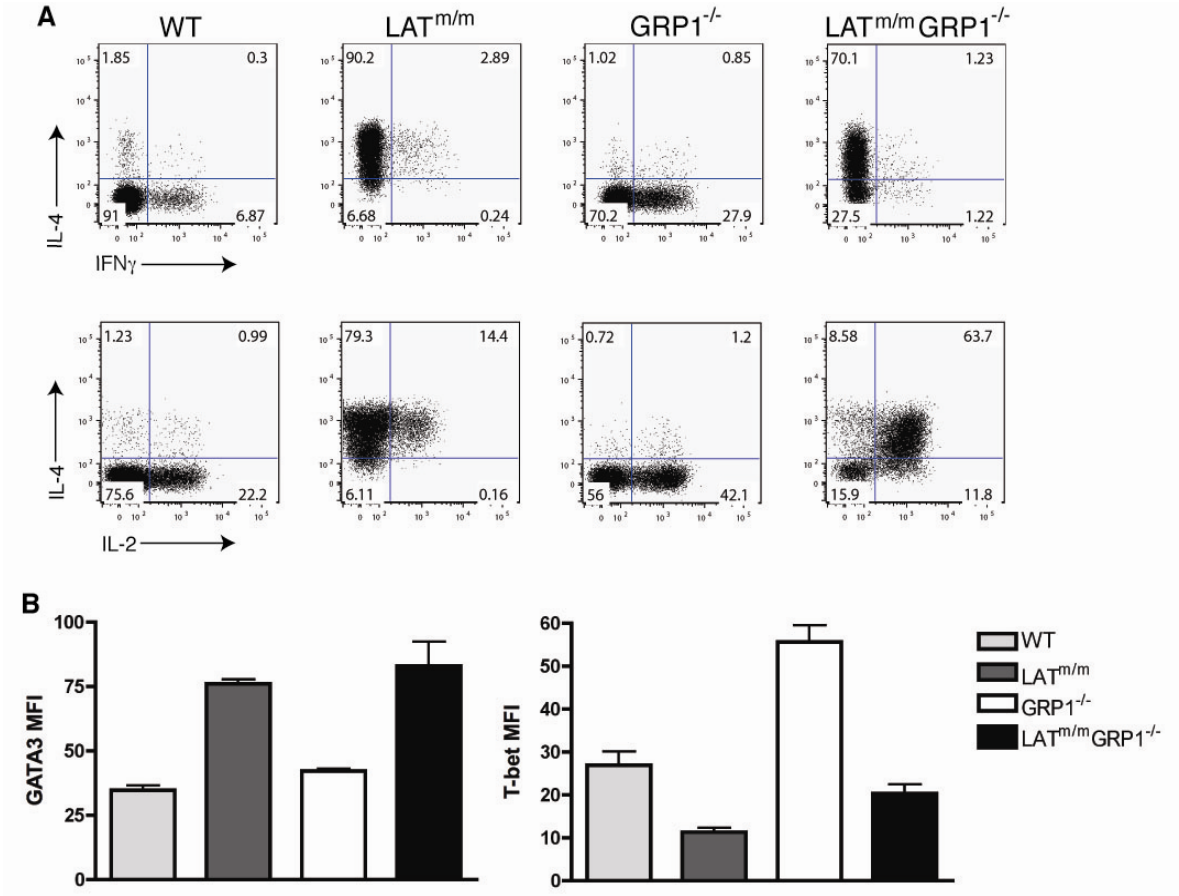


Figure 9: LAT$^{m/m}$GRP1$^{-/-}$ CD4^{+} T cells produce large amounts of IL-4 and IL-2 and have enhanced expression of GATA3

(A) Representative FACS plots of cytokine production by CD4^{+} cells. Splenocytes from 4-month-old mice were stimulated with PMA and ionomycin in the presence of monensin for 4 hours. Cells were then harvested, stained for cell surface markers, fixed, permeabilized, and stained for intracellular cytokines. (B) GATA3 (left) and T-bet (right) MFIs in CD4^{+} splenocytes from 4-month-old mice. Data shown are representative of 2 independent experiments using 2-5 mice per genotype.

impacted the cytokine potential of LAT$^{m/m}$ T cells, although the mechanism by which the loss of RasGRP1 would enhance IL-2 production is unknown.

To further investigate the mechanisms underlying cytokine production in these cells, we assessed the expression of two key transcription factors, GATA3 and T-bet. GATA3 guides Th2 lineage commitment and IL-4 production, while T-bet is responsible for Th1 differentiation and IFN-γ production. Intracellular FACS staining of splenocytes with antibodies against these transcription factors allowed us to gauge the expression of these proteins (Figure 9B). The mean fluorescent intensity (MFI) of GATA3 was comparably high in LAT$^{m/m}$ and LAT$^{m/m}$RasGRP1$^{-/-}$ CD4^{+} T cells, corresponding to the high production of IL-4 in these cells. We found increased T-bet MFI levels in RasGRP1$^{-/-}$ T cells, which produced more IFN-γ and IL-2 upon *in vitro* stimulation than wildtype cells. Predictably, LAT$^{m/m}$ cells, which produced little IL-2 and no IFN-γ, had extremely low T-bet expression. LAT$^{m/m}$RasGRP1$^{-/-}$ T cells, which produced lots of IL-2 and no IFN-γ, expressed only slightly increased amounts of T-bet compared to LAT$^{m/m}$ cells and had lower T-bet expression than wildtype cells. The mechanisms guiding expression of these transcription factors and production of cytokines in LAT$^{m/m}$ and LAT$^{m/m}$RasGRP1$^{-/-}$ T cells are intriguing questions but remain unknown.

3.2.2 RasGRP1 deletion does not inhibit hyperproliferation of LAT$^{f/m}$ T cells

Since LAT$^{m/m}$RasGRP1$^{-/-}$ thymocytes exhibited such a severe developmental defect, we bypassed this block by employing the ERCre^{+}LAT$^{f/m}$ (LAT$^{f/m}$) mouse model. In these mice, a wildtype LAT allele allows for normal development but is deleted upon tamoxifen treatment. Following eight weeks of tamoxifen treatment, the expression of mutant LAT resulted in splenomegaly and increased spleen cell numbers in LAT$^{f/m}$ mice (Figure 10A). RasGRP1$^{-/-}$ spleens were again slightly increased in size and cellularity compared to wildtype mice. LAT$^{f/m}$RasGRP1$^{-/-}$ spleens were comparable to those of LAT$^{f/m}$ mice, indicating that the loss of RasGRP1 did not slow disease development as mediated by mature T cells. Similarly, LAT$^{f/m}$ and LAT$^{f/m}$RasGRP1$^{-/-}$ spleens contained similarly high percentages of CD4^{+} T cells, all of which bore an effector/memory phenotype (Figure 10B). Also, LAT$^{f/m}$RasGRP1$^{-/-}$ T cells expressed even lower levels of surface TCR than LAT$^{f/m}$ and RasGRP1$^{-/-}$ T cells (Figure 10C). From these data, we were able to conclude that the absence of RasGRP1 in LAT$^{f/m}$ T cells had no effect on the ability of these cells to instigate autoimmunity.

We next wanted to asses if the RasGRP1 deletion in mature mutant T cells would have an effect on cytokine production and transcription factor expression. A high percentage of LAT$^{f/m}$ cells, in contrast to LAT$^{m/m}$ cells, retained the ability to produce IFN-γ, in addition to IL-4. Deletion of RasGRP1 enhanced IFN-γ production compared

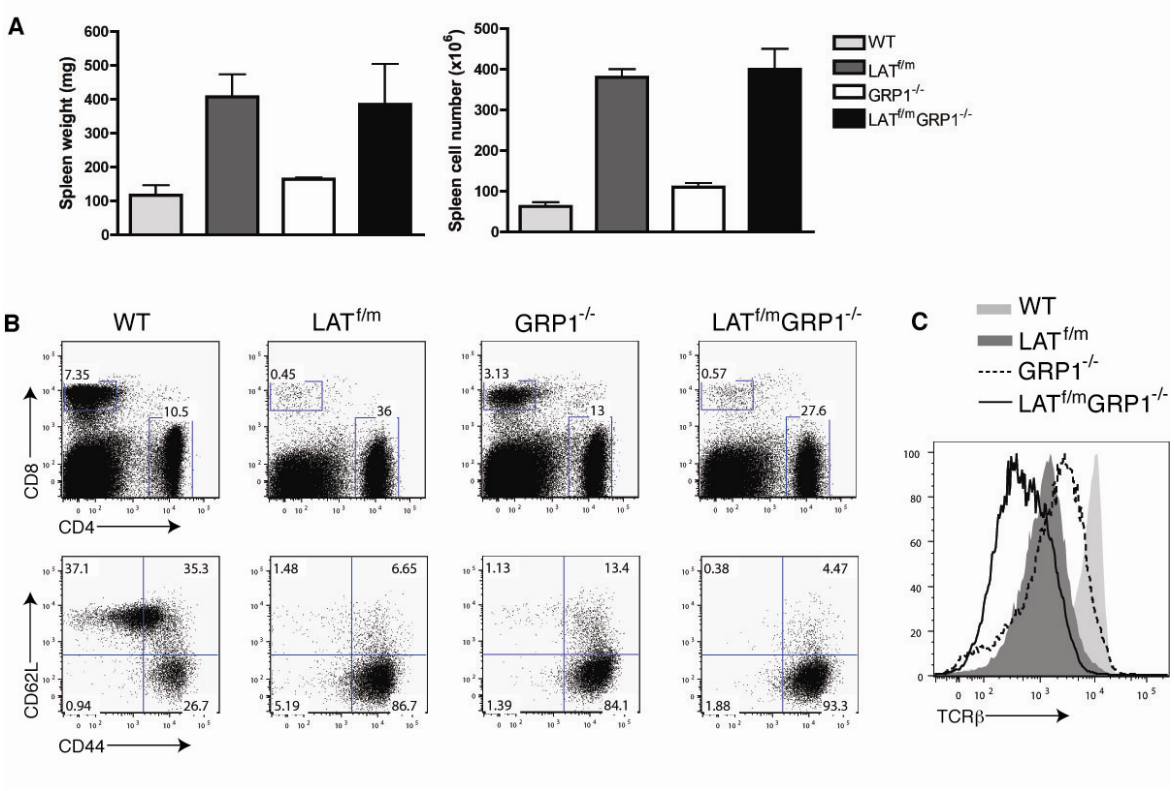


Figure 10: Development of disease in LAT$^{f/m}$GRP1$^{-/-}$ mice

(A-C) Mice were treated with tamoxifen for eight weeks before being analyzed. (A) Spleen weights and total cell numbers. (B) Representative FACS plots of CD4 and CD8 expression on splenocytes, as well as CD44 and CD62L expression on CD4$^+$ splenocytes. (C) TCRβ surface expression on CD4$^+$ T cells from the indicated genotypes. Data are representative of 4 independent experiments using 2-4 mice per genotype.

to wildtype cells, while IL-4 production was not affected (Figure 11A, top panel). However, LAT$^{f/m}$RasGRP1$^{-/-}$ T cells completely lost the ability to produce IFN-γ, making only vast amounts of IL-4. Again, CD4^{+} T cells from wildtype and RasGRP1$^{-/-}$ mice were able to produce substantial amounts of IL-2 (Figure 11A, bottom panel). About 60% of LAT$^{f/m}$ T cells stained positive for IL-2 expression, which was significantly increased compared to the percentage of LAT$^{m/m}$ T cells able to produce this cytokine. The loss of RasGRP1 again enhanced the ability of mutant T cells to produce IL-2 with ~80% of LAT$^{f/m}$RasGRP1$^{-/-}$ CD4^{+} T cells staining positive for IL-2. Over 70% of LAT$^{f/m}$RasGRP1$^{-/-}$ CD4^{+} T cells produced both IL-2 and IL-4. This was in stark contrast to LAT$^{f/m}$ CD4^{+} T cells, of which ~33% produced solely IL-2 and ~30% produced both IL-2 and IL-4. Again, this was an unexpected finding implicating a previously unknown role for RasGRP1 impacting cytokine production.

To ascertain the effect on transcription factors, we compared the MFI values for GATA3 and T-bet in CD4^{+} T cells (Figure 11B). LAT$^{f/m}$ T cells had increased GATA3 MFIs compared to wildtype and RasGRP1$^{-/-}$ T cells. In correspondence with IL-4 production, LAT$^{f/m}$RasGRP1$^{-/-}$ cells had drastically increased expression of GATA3, constituting over a 50% increase compared to LAT$^{f/m}$ cells. In contrast, LAT$^{f/m}$ and RasGRP1$^{-/-}$ T cells had increased T-bet expression compared to wildtype cells. LAT$^{f/m}$RasGRP1$^{-/-}$ T cells were similar to wildtype cells in their expression of T-bet. From these studies we concluded

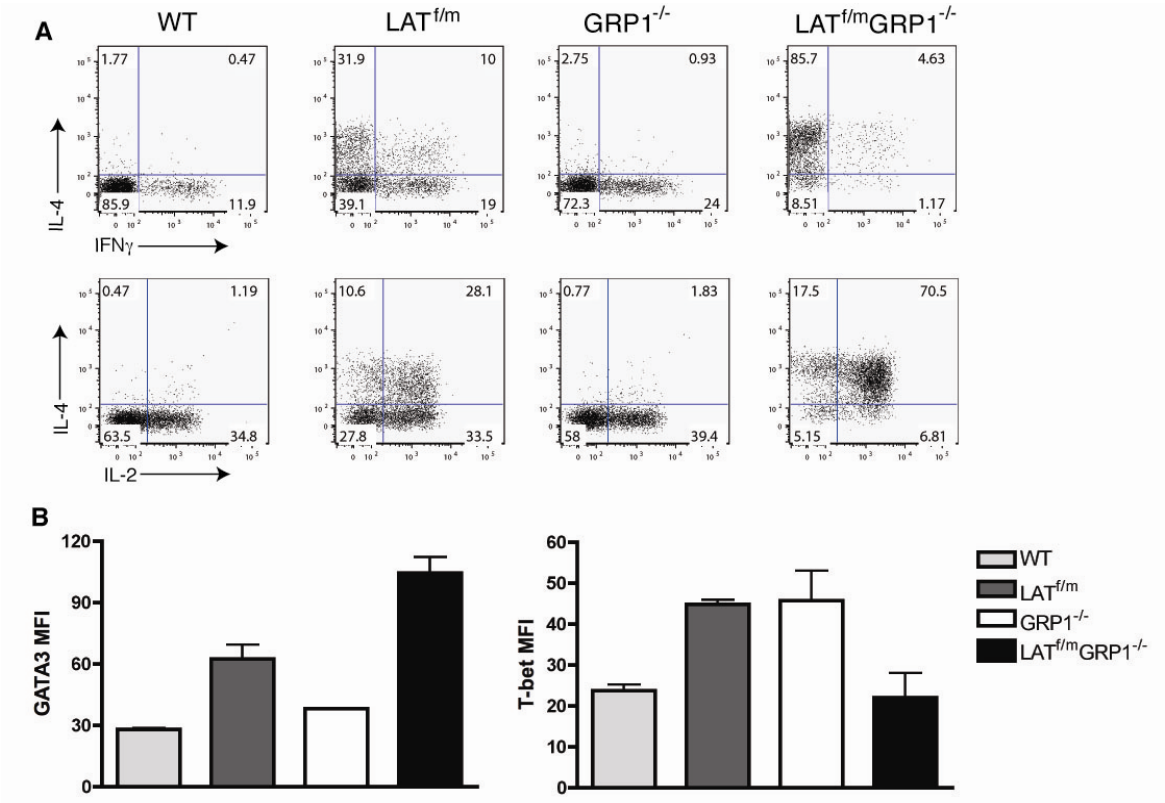


Figure 11: Enhanced IL-4 production and GATA3 expression in LAT$^{f/m}$GRP1$^{-/-}$ T cells

(A) Splenocytes from tamoxifen-treated mice were cultured for 4 hours with PMA, ionomycin, and monensin. Cells were fixed, permeabilized, and stained for intracellular cytokines. (B) Splenocytes were stained for intracellular transcription factors. MFIs shown are gated on CD4^{+} cells. The data shown are representative of 3 independent experiments using 2-4 mice per genotype.

that $LAT^{f/m}$ T cells expressed high levels of both GATA3 and T-bet, reflecting their ability to produce both cytokines. The deletion of RasGRP1 in these cells had a dramatic effect by increasing GATA3 expression and decreasing T-bet expression, effectively abolishing the ability of $LAT^{f/m}$ cells to produce IFN-γ.

3.2.3 LAT-mediated disease development in the absence of Gads

As the deletion of RasGRP1 did not inhibit the development of LAT-mediated disease, we decided to investigate the role of another critical signaling molecule, Gads. Because Gads is still able to bind to LAT following TCR engagement upon the mutation of tyrosine 136 (Zhang et al., 2000), we hypothesized that perhaps LAT-mediated phosphorylation of PLC-γ1 through Gads-SLP-76 could be driving thymocyte development and T cell proliferation. Upon LAT phosphorylation, Gads, which is constitutively bound to the adaptor protein SLP-76, is recruited to the cell membrane (Asada et al., 1999; Law et al., 1999). SLP-76 contains a proline-rich P1 domain which mediates its interaction with PLC-γ1 (Singer et al., 2004; Yablonski et al., 2001). The indispensable role of SLP-76 has been previously documented as its deletion leads to a complete block in thymocyte development (Clements et al., 1998; Pivniouk et al., 1998). In contrast, the deletion of Gads in mice leads to significantly fewer thymocytes; however, T cells are able to develop and populate the periphery (Yoder et al., 2001). We

thus decided to investigate the role of the Gads-SLP-76 complex in the development of LAT-dependent autoimmunity.

We first crossed Gads$^{-/-}$ mice with LAT$^{m/m}$ mice to assess the contribution of Gads to disease development. As shown in Figure 12A, LAT$^{m/m}$ thymocytes had a partial block in development, with a small percentage of cells escaping the DN compartment and maturing to the DP stage. The deletion of Gads also led to a marked defect in thymocyte development, with an increased percentage of DN cells and reduced DP and SP compartments compared to wildtype mice, as published previously (Yoder et al., 2001). In contrast, LAT$^{m/m}$Gads$^{-/-}$ thymocytes had a near-complete block at the DN stage of thymocyte development and contained only miniscule percentages of DP or SP cells. Furthermore, we examined DN thymocyte development as marked by the expression of CD44 and CD25. LAT$^{m/m}$ and Gads$^{-/-}$ thymuses exhibited a block in the DN3 (CD44^{-}CD25^{+}) compartment with much reduced DN4 (CD44^{-}CD25^{-}) populations (Figure 12B). LAT$^{m/m}$Gads$^{-/-}$ thymocytes were more severely blocked in the DN3 stage with virtually no DN4 cells, highlighting the dramatic developmental block in these mice. By 1 month of age, LAT$^{m/m}$ CD4^{+} T cells had undergone expansion to account for 23% of splenocytes (Figure 12C). Gads$^{-/-}$ mice were T cell lymphopenic as spleens were 4% CD8^{+} and ~2% CD4^{+} T cells. And, due to the severe thymocyte block, LAT$^{m/m}$Gads$^{-/-}$ mice contained almost no CD8^{+} or CD4^{+} cells.

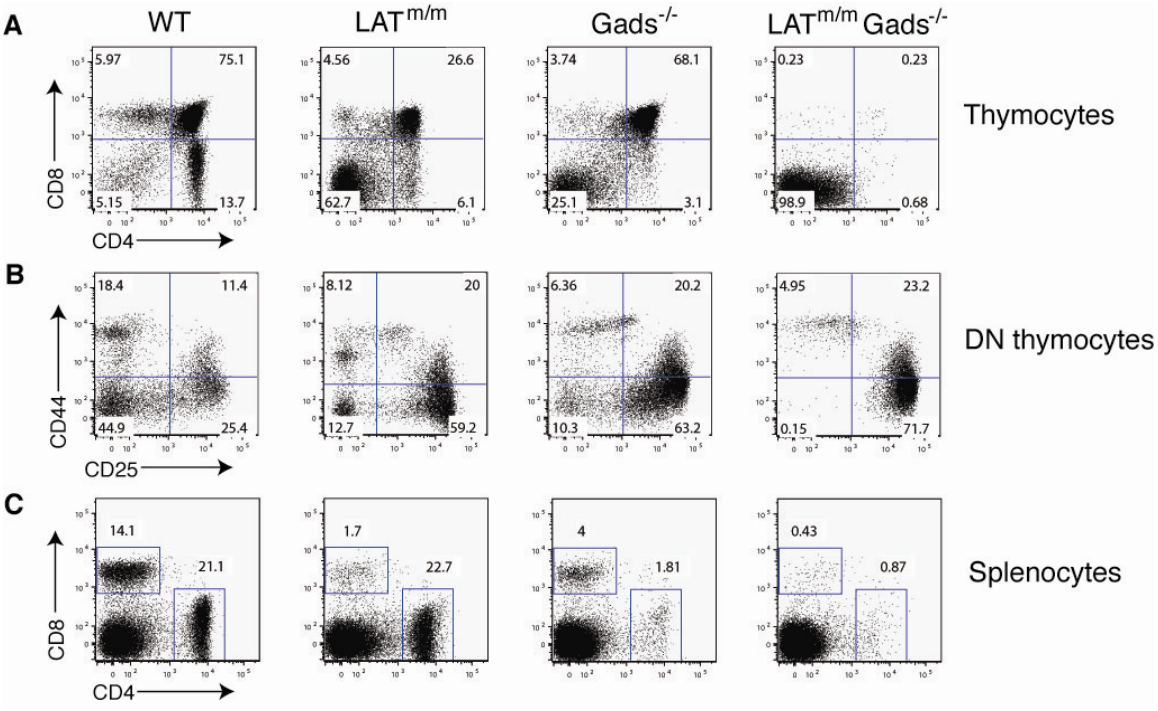


Figure 12: Thymocyte development is severely blocked in LAT$^{m/m}$Gads$^{-/-}$ mice

(A-C) Mice were analyzed at 4 weeks of age. (A) Thymocytes were examined for surface expression of CD4 and CD8. (B) Staining with CD25 and CD44 on DN thymocytes reveals DN1-DN4 populations. (C) Representative FACS plots of CD4 and CD8 expression on splenocytes. Data are representative of 2 independent experiments analyzing 1-3 mice per genotype.

To observe whether LAT$^{m/m}$Gads$^{-/-}$ mice would develop autoimmunity over time, we analyzed mice at 4 months of age. By this time point, LAT$^{m/m}$ mice contained greatly increased spleen sizes and cellularity (Figure 13A). Gads$^{-/-}$ spleens were comparable to wildtype spleens. LAT$^{m/m}$Gads$^{-/-}$ spleens varied with individual mice but overall were demonstrably larger than wildtype and Gads$^{-/-}$ spleens. Furthermore, LAT$^{m/m}$ spleens contained a tremendously high number of CD4^{+} cells. While LAT$^{m/m}$Gads$^{-/-}$ spleens contained only a fraction of this number, they still had an increased amount of CD4^{+} splenocytes compared to wildtype and Gads$^{-/-}$ mice. At 4 months of age, LAT$^{m/m}$ spleens were ~70% CD4^{+} T cells and contained no CD8^{+} T cells (Figure 13B). Gads$^{-/-}$ spleens remained T cell lymphopenic with slightly reduced CD8^{+} numbers and much reduced CD4^{+} populations. LAT$^{m/m}$Gads$^{-/-}$ spleens contained no CD8^{+} T cells and variable CD4^{+} percentages, ranging from 7-30% of splenocytes. Additionally, the majority of CD4^{+} T cells in LAT$^{m/m}$, Gads$^{-/-}$, and LAT$^{m/m}$Gads$^{-/-}$ mice were CD62L^{lo} and CD44^{hi}. LAT$^{m/m}$ CD4^{+} splenocytes again had much reduced levels of surface TCRβ (Figure 13C). Gads$^{-/-}$ T cells had decreased TCR surface expression compared to wildtype cells. LAT$^{m/m}$Gads$^{-/-}$ CD4^{+} T cells had even further decreased TCRβ expression compared to both LAT$^{m/m}$ and Gads$^{-/-}$ T cells. However, TCRβ surface staining on these cells was still higher than that on B cells (data not shown), implying that TCR was still expressed on the cell surface.

We also assessed the effect of the Gads deletion on T_{reg} cells. The regulatory T cell

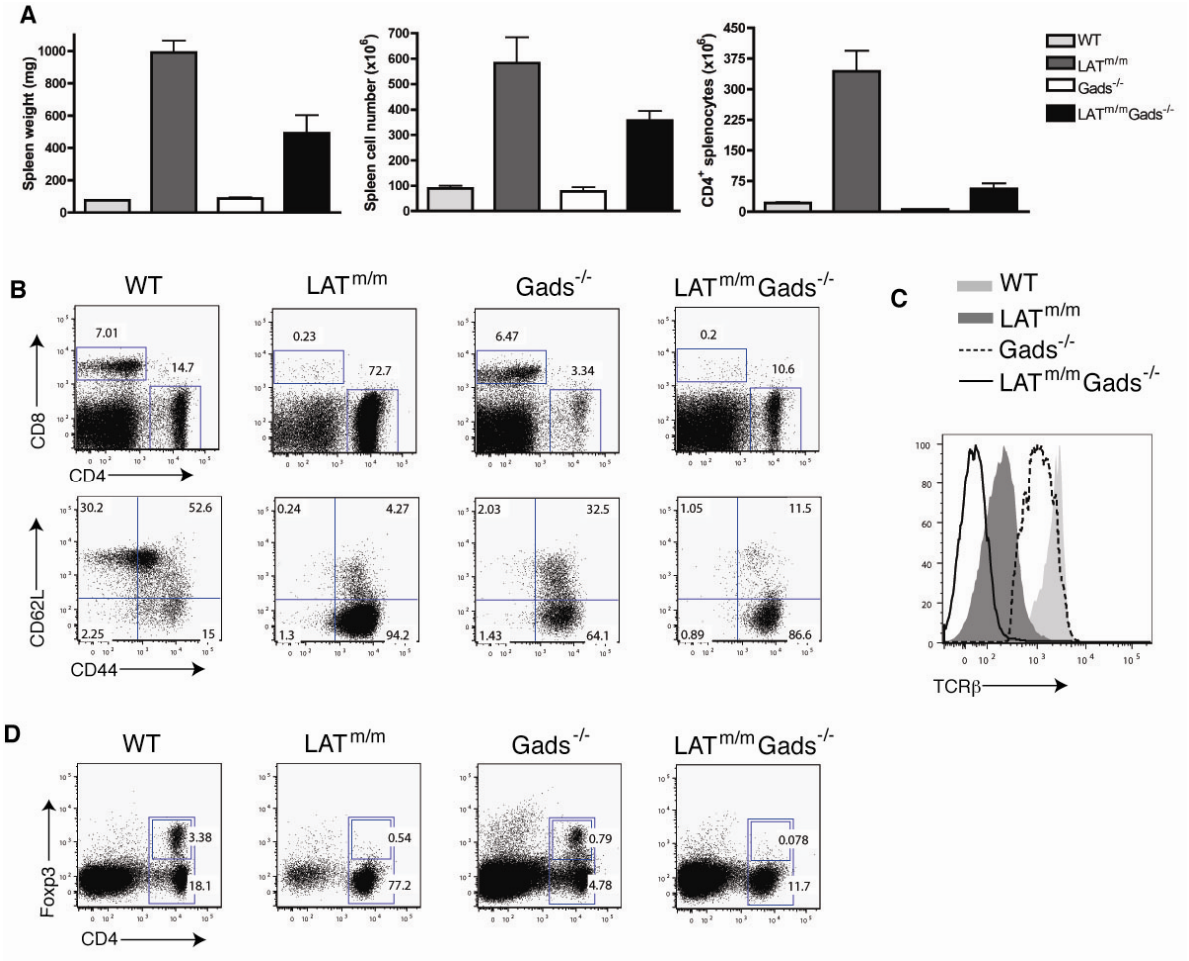


Figure 13: Development of LAT-mediated disease upon the deletion of Gads

(A-D) Mice were sacrificed at 4 months of age. (A) Spleen weights, cellularity, and number of CD4+ splenocytes from the indicated genotypes. (B) Representative FACS staining of CD4 and CD8 expression on splenocytes (top) and of CD62L and CD44 expression on CD4+ splenocytes (bottom). (C) TCRβ surface expression on CD4+ splenocytes. (D) Splenocytes were stained with CD4 and intracellularly stained with Foxp3 to reveal regulatory T cell populations. Data are representative of 2 independent experiments analyzing 2-4 mice per genotype.

population was conspicuously absent in LAT$^{m/m}$ mice. Foxp3^{+} cells accounted for ~14% of CD4^{+} splenocytes in Gads$^{-/-}$ mice, similar to the wildtype proportion. In stark contrast, LAT$^{m/m}$Gads$^{-/-}$ spleens contained no Foxp3^{+}CD4^{+} T_{reg} cells. From these data, we were able to conclude that the deletion of Gads drastically impaired LAT$^{m/m}$ thymocyte development but over time was unable to suppress mutant T cell proliferation.

We were interested in examining the effect of the Gads deletion on cytokine production in LAT$^{m/m}$ T cells. Again, ~7% of wildtype CD4^{+} T cells produced IFN-γ upon stimulation with PMA and ionomycin *in vitro* and an overwhelming percentage of LAT$^{m/m}$ T cells (~90%) produced IL-4 and a minimal amount of IFN-γ (Figure 14A, top panel). Gads$^{-/-}$ CD4^{+} T cells produced an increased amount of both cytokines compared to wildtype controls. However, ~90% of LAT$^{m/m}$Gads$^{-/-}$ T cells produced IL-4 while almost no cells produced IFN-γ. Furthermore, we assessed IL-2 production in these cells (Figure 14A, bottom panel). Compared with wildtype T cells, a smaller percentage of LAT$^{m/m}$ T cells produced IL-2, while slightly more Gads$^{-/-}$ CD4^{+} T cells made IL-2. While only ~30% of LAT$^{m/m}$ CD4^{+} cells made IL-2, ~80% of LAT$^{m/m}$Gads$^{-/-}$ CD4 T cells produced this cytokine either alone or in conjunction with IL-4. These results indicated that the deletion of Gads enhanced IL-2 production by LAT$^{m/m}$ T cells.

We next assessed the expression of GATA3 and T-bet, which guide Th2 and Th1 commitment, respectively. Intracellular FACS staining of splenocytes with antibodies

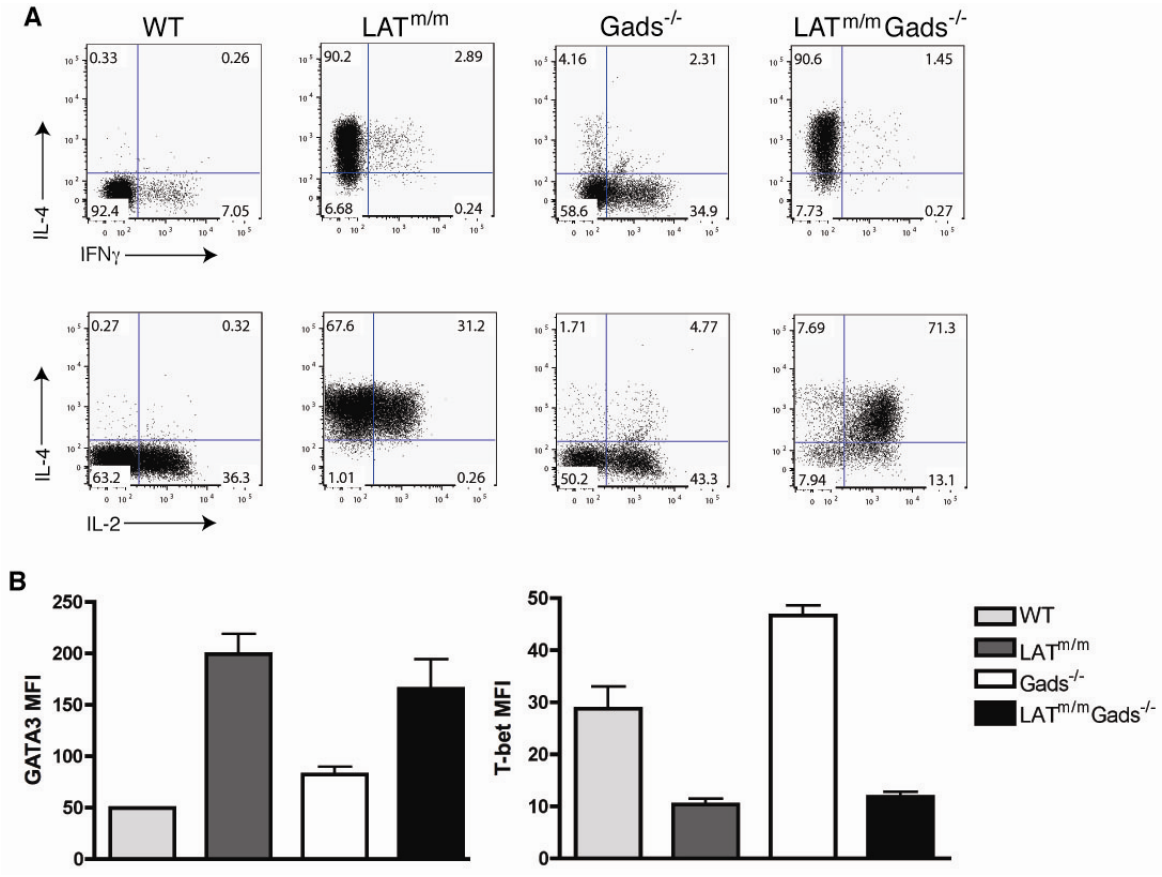


Figure 14: Cytokine production and transcription factor expression in LAT$^{m/m}$Gads$^{-/-}$ T cells

(A) Splenocytes were stimulated with PMA and ionomycin in the presence of monensin for 4 hours before being stained for intracellular cytokine production. Representative plots shown are gated on CD4^{+} cells. (B) Splenocytes were intracellularly stained for GATA3 and T-bet expression. MFIs of each protein in CD4^{+} cells are shown. Data shown are representative of four independent experiments examining 2-4 mice per genotype.

against these transcription factors allowed us to evaluate the expression of these proteins within our different genotypes (Figure 14B). The MFI of GATA3 was comparably high in $LAT^{m/m}$ and $LAT^{m/m}Gads^{-/-}$ $CD4^+$ T cells, supporting the Th2-skewed nature of these cells. We found increased T-bet MFI levels in $Gads^{-/-}$ T cells, which produced more IFN-γ upon *in vitro* stimulation than wildtype cells. Predictably, $LAT^{m/m}$ and $LAT^{m/m}Gads^{-/-}$ cells, which produced little IFN-γ, had extremely low T-bet protein. From these results, we were able to conclude that the deletion of Gads did not alter transcription factor expression in $LAT^{m/m}$ cells.

3.2.4 The deletion of Gads in an inducible model of LAT disease does not suppress lymphoproliferation

To bypass the severe thymocyte developmental block, we also analyzed $ERCre^+LAT^{f/m}Gads^{-/-}$ ($LAT^{f/m}Gads^{-/-}$) mice in which the wildtype LAT allele can be deleted upon tamoxifen injection. The deletion of LAT *in vivo* for 8 weeks led to splenomegaly and hyperproliferation in $LAT^{f/m}$ mice (Figure 15A). $Gads^{-/-}$ spleens, on the other hand, looked similar to wildtype spleens yet contained reduced percentages of $CD4^+$ and $CD8^+$ T cells. $LAT^{f/m}Gads^{-/-}$ mice also displayed severe splenomegaly, with only slightly reduced spleen weights and cell numbers compared to $LAT^{f/m}$ mice. While $LAT^{f/m}$ spleens were ~50% $CD4^+$ cells, $LAT^{f/m}Gads^{-/-}$ spleens contained ~25% $CD4^+$ T cells (Figure 15B). However, like $LAT^{f/m}$ mice, $LAT^{f/m}Gads^{-/-}$ mice had virtually no $CD8^+$ T cells. Also, almost all $CD4^+$ T cells in $LAT^{f/m}$, $Gads^{-/-}$, and $LAT^{f/m}Gads^{-/-}$ spleens bore an

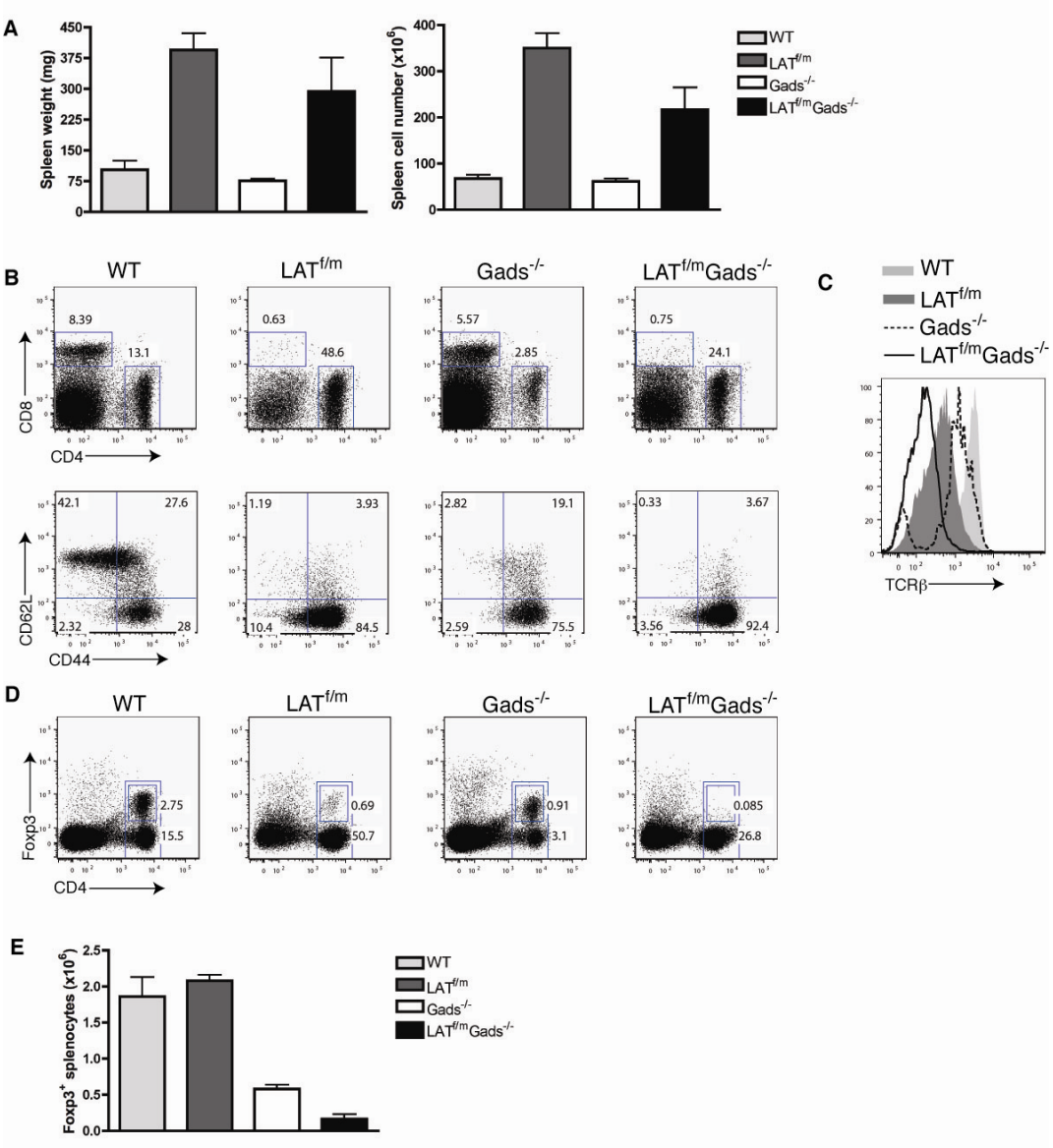


Figure 15: LAT$^{f/m}$Gads$^{-/-}$ mice develop disease comparably to LAT$^{f/m}$ mice

(A-D) Mice were treated with tamoxifen for 8 weeks. (A) Spleen weights and total cellularity. (B) Representative FACS plots of CD4 and CD8 expression on splenocytes (top) and CD62L and CD44 expression on CD4$^+$ splenocytes (bottom). (C) TCRβ surface expression on CD4$^+$ splenocytes. (D) Representative plots of CD4 and Foxp3 expression in splenocytes. (E) Total numbers of Foxp3$^+$ splenocytes. Data are representative of 2 experiments using 2-3 mice per genotype.

effector/memory phenotype of $CD44^{hi}CD62L^{lo}$. The $CD4^+$ cells in $Gads^{-/-}$ mice expressed lower levels of surface TCR than wildtype cells and the TCR levels of $LAT^{f/m}Gads^{-/-}$ $CD4^+$ cells were even further decreased compared to $LAT^{f/m}$ T cells (Figure 15C).

We also wanted to assess if the deletion of Gads had any impact on regulatory T cells. Following 8 weeks of tamoxifen treatment, $LAT^{f/m}$ mice contained a small but distinct population of $Foxp3^+$ CD4 T cells (Figure 15D). Furthermore, the total number of $LAT^{f/m}$ $Foxp3^+$ splenocytes was similar to the number in wildtype mice (Figure 15E). Despite having decreased $CD4^+$ T cell percentages, $Gads^{-/-}$ spleens actually contained a higher percentage of $Foxp3^+$ regulatory T cells in the $CD4^+$ compartment compared to wildtype controls. However, $LAT^{f/m}Gads^{-/-}$ mice completely lacked $Foxp3^+$ T cells, indicating that the loss of Gads in mutant T cells had a negative effect on regulatory T cell survival and/or development. From the data accumulated here, we were able to conclude that the deletion of Gads significantly inhibited thymocyte development but was not able to prevent the proliferation of conventional mutant $CD4^+$ T cells.

Interestingly, the deletion of Gads had a profound effect on the cytokines produced by $LAT^{f/m}$ $CD4^+$ T cells. Upon P+I stimulation, ~30% of $LAT^{f/m}$ $CD4^+$ T cells produced IL-4 or IFN-γ alone and about 10% produced both cytokines (Figure 16A). In contrast, a small percentage of wildtype cells produced IFN-γ while an increased percentage of $Gads^{-/-}$ $CD4^+$ T cells stained positive for IFN-γ and IL-4. Noticeably, T cells

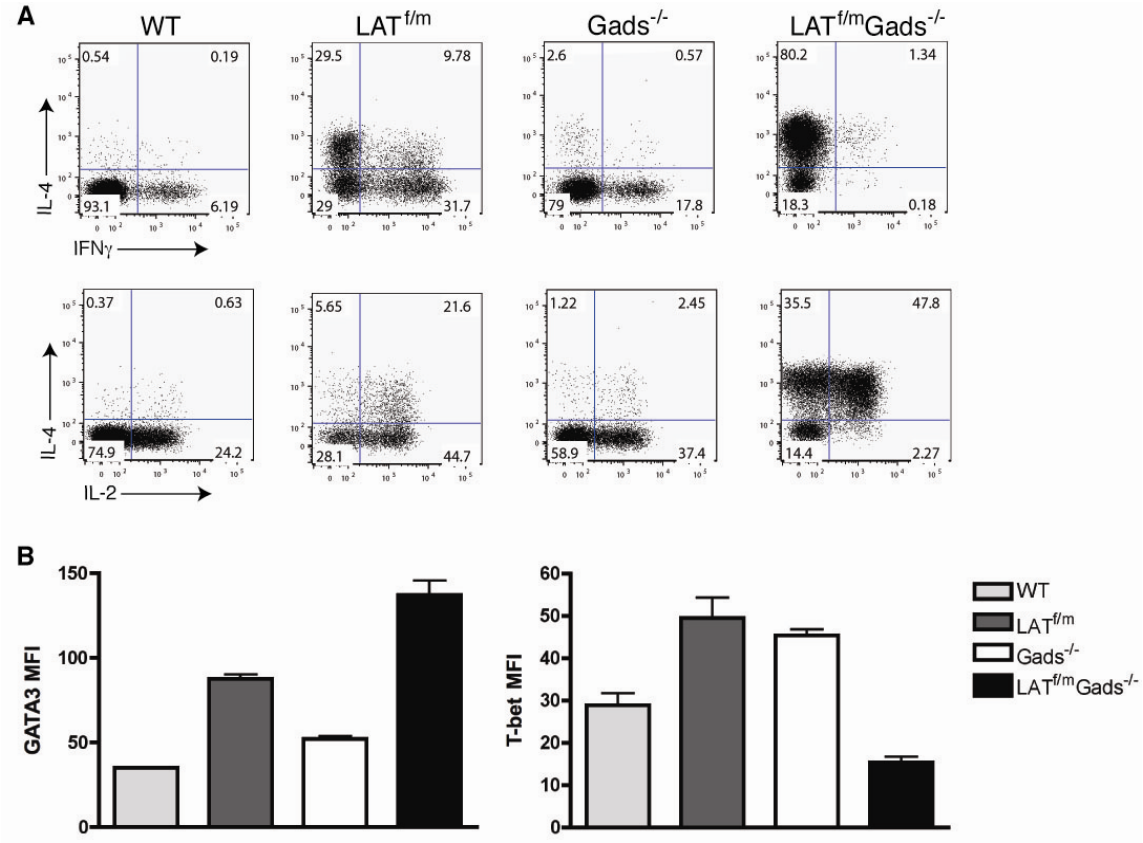


Figure 16: Increased IL-4 production and GATA3 expression in LAT$^{f/m}$Gads$^{-/-}$ T cells

(A) Splenocytes were stimulated *in vitro* for 4 hours in the presence of PMA, ionomycin, and monensin. They were then stained for CD4 expression, as well as fixed and permeabilized before being intracellularly stained for cytokines. (B) GATA3 and T-bet MFIs gated on CD4^{+} spenocytes. The data depicted here are representative of 3 experiments using 2-4 mice per genotype.

From $LAT^{f/m}Gads^{-/-}$ mice completely lost the ability to produce IFN-γ with about 80% producing only IL-4. Furthermore, we assessed IL-2 production in $CD4^+$ splenocytes. WT and $Gads^{-/-}$ T cells produced a significant amount of IL-2, while ~20% of $LAT^{f/m}$ T cells were IL-2/IL-4^+ and ~45% were IL-2^+. About 50% of $LAT^{f/m}Gads^{-/-}$ $CD4^+$ T cells produced IL-2 as well, although there were no cells producing solely IL-2 as there were in $LAT^{f/m}$ mice. Rather, almost all $LAT^{f/m}Gads^{-/-}$ cells producing cytokines made either IL-4 alone or IL-4 in conjunction with IL-2. Thus, while $LAT^{f/m}Gads^{-/-}$ T cells were similar phenotypically to $LAT^{f/m}$ T cells, they harbored distinct differences in their ability to produce cytokines.

A closer look at transcription factor expression in these mice revealed that $LAT^{f/m}$ T cells had increased GATA3 MFI levels compared to wildtype and $Gads^{-/-}$ $CD4^+$ T cells (Figure 16B). $LAT^{f/m}Gads^{-/-}$ T cells had markedly increased GATA3 MFI levels compared to $LAT^{f/m}$ cells, indicative of their increased propensity to produce IL-4. Strikingly, $LAT^{f/m}Gads^{-/-}$ T cells had substantially decreased T-bet levels in comparison to wildtype, $LAT^{f/m}$, and $Gads^{-/-}$ $CD4^+$ T cells. $LAT^{f/m}Gads^{-/-}$ T cells seemed to have about a two-fold decrease in T-bet MFI compared to wildtype cells and about a three-fold decrease compared to $LAT^{f/m}$ and $Gads^{-/-}$ cells. From these data, it is clear that Gads, in the context of the $LAT^{f/m}$ disease, affects the upregulation of GATA3 and the severe downregulation of T-bet.

3.2.5 Expression of constitutively active calcineurin does not rescue LAT-mediated disease

Since disease development was not impeded by the deletion of Gads or RasGRP1, we next determined whether reconstituting the signaling pathways downstream of the LAT-PLC-γ1 interaction could help restore proper T cell function. We first assessed the role of calcineurin signaling in LAT-mediated disease as perturbation of the LAT-PLC-γ1 interaction causes $LAT^{m/m}$ T cells to have a severe defect in calcium mobilization and NFAT activation (Sommers et al., 2002). Calcineurin is a calcium/calmodulin-dependent phosphatase whose activity is required for the translocation of NFAT to the nucleus and, ultimately, T cell cytokine production and proliferation (Clipstone and Crabtree, 1992). Thus, we wanted to assess if the expression of constitutively active calcineurin could restore proper T cell development and activation in the LAT-mediated disease.

In order to test if the enforced expression of calcineurin could ameliorate disease development, we generated transgenic mice expressing constitutively active calcineurin under the human CD2 promoter. These mice (CnA^+) contained a form of calcineurin in which the calmodulin binding domain and autoinhibitory domain were deleted. These mice were then crossed with $LAT^{m/m}$ mice to produce $LAT^{m/m}CnA^+$ mice. As previously reported, expression of constitutively active calcineurin impairs thymocyte development (Hayden-Martinez et al., 2000). Therefore, $LAT^{m/m}CnA^+$ mice did not develop disease by

three months of age due to a severe block in thymocyte development that prevented T cells from populating the periphery in these mice (data not shown). We crossed mice expressing constitutively active calcineurin onto the $LAT^{f/m}$ background to eliminate this developmental defect. Mice were then analyzed by a former member of our lab, Mariana (Chuck) Nelson.

Despite the severe block in thymocyte development observed in $LAT^{f/m}CnA^{+}$ thymuses (Figure 17A), these mice developed disease after four weeks of tamoxifen treatment comparably to $LAT^{f/m}$ mice. Analysis of lymph nodes showed that wildtype mice had normal percentages of $CD4^{+}$ and $CD8^{+}$ cells, while $LAT^{f/m}$ mice contained only $CD4^{+}$ T cells (Figure 17B). Expression of the CnA transgene led to reduced percentages of peripheral T cells, although the CD4:CD8 ratio was relatively normal. $LAT^{f/m}CnA^{+}$ mice contained predominantly $CD4^{+}$ T cells, indicating that restoring NFAT signaling did not improve the hyperproliferation of mutant T cells. In addition, $CD4^{+}CD25^{+}$ T_{reg} cells were absent in these mice (data not shown). Together, these data provided sufficient evidence that restoring NFAT signaling did not prevent disease development.

3.2.6 Canonical NF-κB activation does not rescue LAT-mediated autoimmunity

While enforced activation of calcineurin did not lessen disease severity, we also examined the role of another critical pathway downstream of the LAT-PLC-γ1 interaction, canonical NF-κB signaling. The NF-κB family members are normally

sequestered in the cytoplasm through association with the inhibitory IκB family. Signaling through the TCR, among other stimuli, induces the IKK (IκB kinase) complex to phosphorylate IκB, which is then ubiquitinated and degraded, allowing for nuclear translocation of NF-κB (Karin and Ben-Neriah, 2000; Li and Verma, 2002). The IKK complex consists of two kinases, IKK1/α and IKK2/β, as well as a regulatory subunit, NF-κB essential modulator (NEMO)/IKKγ. We generated LAT$^{f/m}$IKK2ca (constitutively active calcineurin) mice by crossing LAT$^{f/m}$ mice to a strain generated by Sasaki et al. in which a constitutively active form of IKK2 preceded by a *loxP*-flanked STOP cassette is inserted into the ROSA26 locus. In the presence of Cre, the floxed stop codon is excised, allowing for the expression of constitutively active-IKK2, as well as the bicistronic expression of eGFP (Sasaki et al., 2006).

Minghua Zhu of our laboratory performed experiments in which mice were injected with tamoxifen for four weeks to induce both the deletion of the floxed wildtype LAT allele and the expression of active IKK2. Analysis of thymuses showed that the expression of constitutively active IKK2 did not visibly affect thymocyte development and did not rescue thymocyte development in LAT$^{f/m}$ mice (data not shown). Analysis of disease development clearly showed that expression of constitutively active IKK2 did nothing to ameliorate splenomegaly, as LAT$^{f/m}$IKK2ca spleens were similar in size to LAT$^{f/m}$ spleens (data not shown). In the periphery, IKK2ca mice contained similar

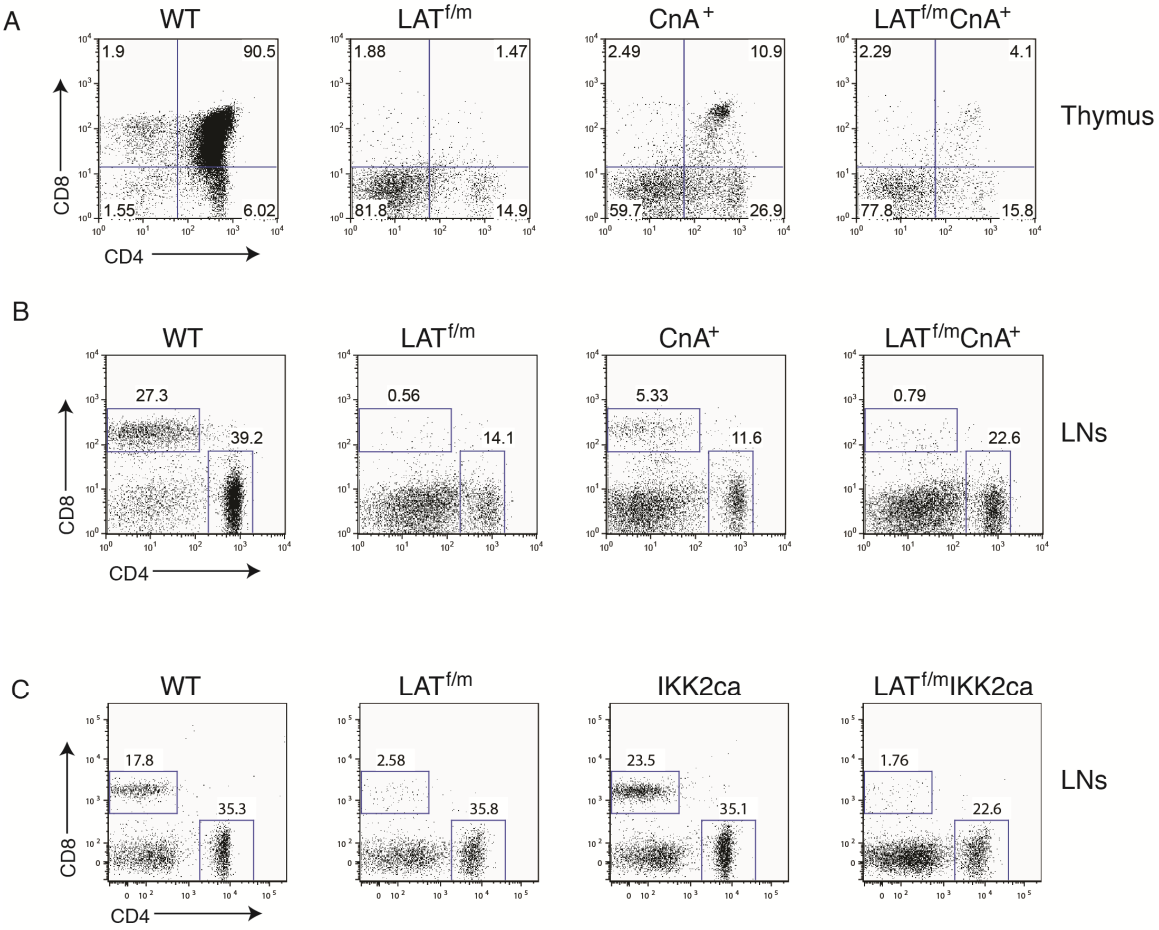


Figure 17: Restoration of NFAT and NF-κB signaling cannot suppress the LAT-mediated disease

(A-C) Mice were treated with tamoxifen for 4 weeks. (A) Thymocytes were harvested and stained for CD4 and CD8 expression. (B) Representative FACS plots of CD4 vs CD8 expression on live LN cells. (C) Surface expression of CD4 and CD8 on live LN cells. Data shown are representative of at least two independent experiments using 1-2 mice per genotype.

percentages of $CD4^+$ and $CD8^+$ T cells compared to WT mice. $LAT^{f/m}$ and $LAT^{f/m}$IKK2ca lymph nodes consisted of very few $CD8^+$ cells and large percentages of $CD4^+$ T cells, most of which expressed an effector-memory phenotype (Figure 17C and data not shown). In sum, our data demonstrated that T cell hyperproliferation cannot be corrected by restoration of NFAT or NF-κB signaling.

3.2.7 Constitutive activation of Raf augments LATY136F disease

As the activation of the Ras/Raf/MEK/Erk signaling cascade is a crucial component of TCR-mediated signaling downstream of PLC-γ1 activation, we decided to investigate the role of this pathway in LAT disease. We employed mice expressing a constitutively active form of Raf, which harbors a CAAX farnesylation sequence at its C-terminus. The farnesylation sequence directs Raf to cell membranes, imitating Ras-mediated Raf activation, thus causing constitutive activation of Raf/MEK/Erk (Iritani et al., 1997).

A previous lab member, Surapong Koonpaew, crossed constitutively active Raf (Raf^{Tg}) mice onto the $LAT^{m/m}$ background. While expression of the Raf transgene minimally affected thymocyte development, its expression on the mutant LAT background caused abnormal development in $LAT^{m/m}Raf^{Tg}$ mice (Figure 18A). While there are very few DP and SP thymocytes in $LAT^{m/m}$ mice, we observed virtually no DP cells and ~70% $CD4^+$ cells in $LAT^{m/m}Raf^{Tg}$ thymuses. Furthermore, splenocyte numbers in

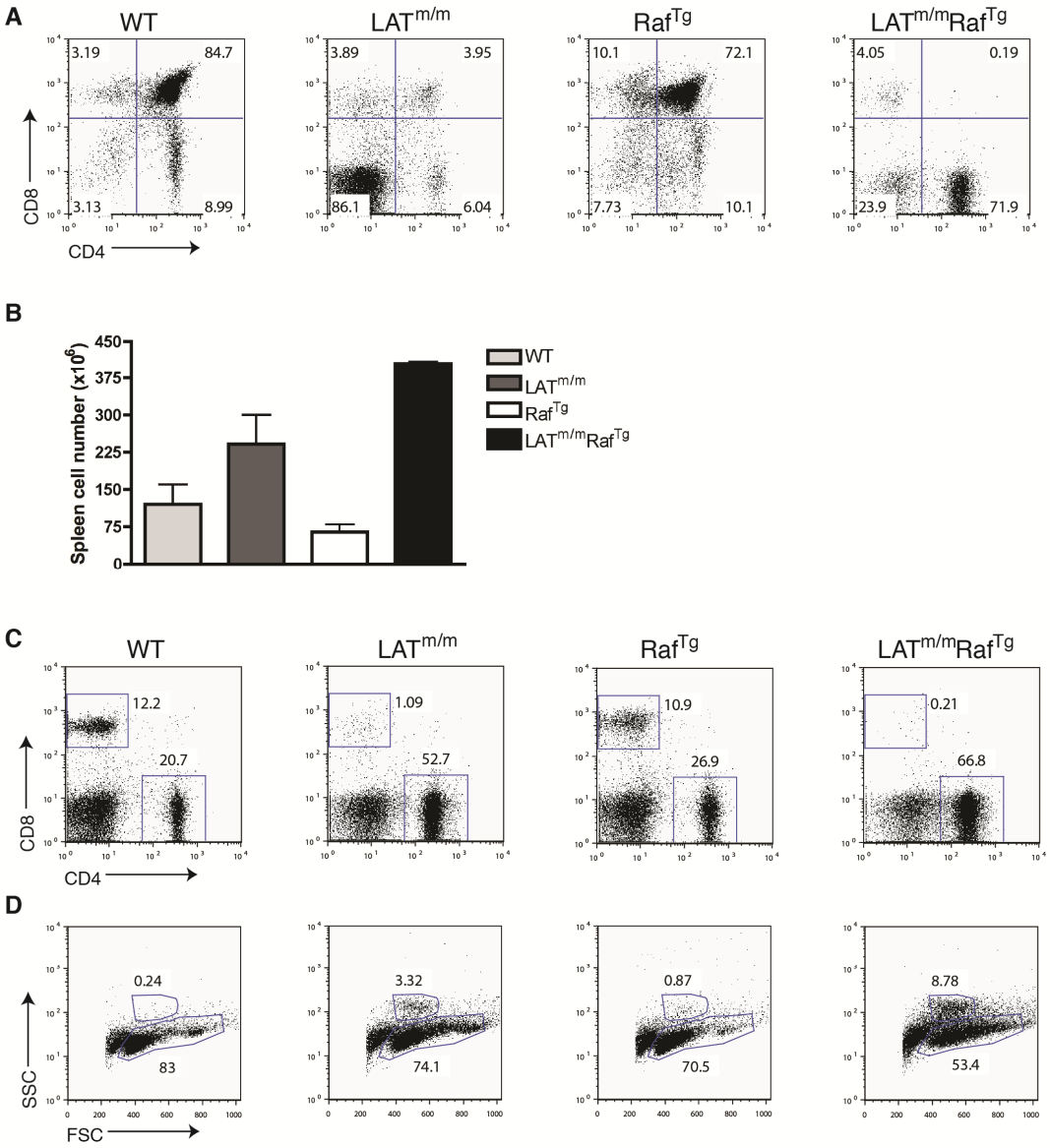


Figure 18: Constitutively active Raf augments disease severity

(A) FACS plots on live thymocytes. (B) Total splenocyte numbers. (C) CD4 vs CD8 expression on live splenocytes. (D) Gating on forward scatter vs side scatter reveals an increased eosinophil population in $LAT^{m/m}Raf^{Tg}$ mice.

LAT$^{m/m}$Raf^{Tg} mice were greatly increased compared to LAT$^{m/m}$ mice (Figure 18B). LAT$^{m/m}$Raf^{Tg} spleens consisted of incredibly high percentages of CD4^{+} T cells, similar to and even exceeding the percentage of CD4^{+} T cells seen in LAT$^{m/m}$ spleens (Figure 18C). Another mark of the LATY136F disease is severe eosinophilia (Sommers et al., 2002), as seen in Figure 18D. LAT$^{m/m}$Raf^{Tg} lymph nodes had an extremely high percentage of eosinophils as compared to control and even LAT$^{m/m}$ LNs, indicating aggressive disease.These data demonstrated that the transgenic expression of Raf in the context of the LATY136F mutation actually served to exacerbate disease development, implying an important role for the Ras/Erk pathway in LAT-mediated disease.

3.3 Discussion

From the data gathered here, it was obvious that the absence of Gads or RasGRP1 had a significant effect on LAT$^{m/m}$ thymocyte development. Normally, LAT$^{m/m}$ thymocytes exhibit an incomplete block in the DN3 stage (Aguado et al., 2002; Sommers et al., 2002). In these mice, the DP population constitutes ~20-30% of thymocytes and SP compartments are reduced yet distinct. LAT$^{m/m}$Gads$^{-/-}$ mice displayed a near-complete block in the DN stage of thymocyte development, with virtually no DP or SP compartments. LAT$^{m/m}$RasGRP1$^{-/-}$ mice also had a more severe block than LAT$^{m/m}$ mice with only ~7% of thymocytes progressing to the DP stage. Because LAT retains partial function upon mutation of tyrosine 136, a number of signaling pathways remain intact

following the loss of the LAT-PLC-γ1 interaction and allow for a minimal amount of thymocyte differentiation. These pathways seem to act predominantly through Gads, as the ablation of this protein severely hindered thymocyte development. As the deletion of RasGRP1 also increased the developmental block of mutant thymocytes, one or more of these signaling pathways might also utilize RasGRP1. Perhaps the deletion of these proteins diminished or abolished the residual calcium flux and/or MAPK activation in $LAT^{m/m}$ thymocytes. Interestingly, despite having virtually no peripheral T cells by about 1 month of age, $LAT^{m/m}RasGRP1^{-/-}$ and $LAT^{m/m}Gads^{-/-}$ mice developed disease comparably to $LAT^{m/m}$ mice by 4 months of age, indicating that the few T cells able to populate the periphery were sufficient to instigate autoimmunity.

Despite the dramatic effect on thymocyte development, LAT-mediated disease as driven by mature T cells in the $LAT^{f/m}$ model was not significantly affected by the loss of Gads or RasGRP1. A previous report demonstrated that the uncontrolled hyperproliferation and Th2 effector function of LATY136F T cells is not dependent on MHC class II (Wang et al., 2008). However, another study examining the disease mediated by mature mutant T cells demonstrated that $LAT^{fl\text{-}dtr/Y136F}$ cells, which are similar to $ERCre^{+}LAT^{f/m}$ cells, require MHC class II to initiate disease following wildtype LAT deletion (Mingueneau et al., 2009). These data suggest that LAT-mediated disease onset may require TCR engagement whereas the perpetuation of the disease may be

TCR-independent. Thus, it seemed probable that continuous tonic signaling through the TCR could be driving the hyperproliferation of mutant T cells. Through the disruption of Gads and RasGRP1, we were able to effectively disrupt TCR-proximal signaling and observe the effect on mutant T cells. Both $LAT^{f/m}Gads^{-/-}$ and $LAT^{f/m}RasGRP1^{-/-}$ $CD4^{+}$ T cells had decreased surface expression of TCRβ compared to $LAT^{f/m}$ cells. Yet, $LAT^{f/m}Gads^{-/-}$ and $LAT^{f/m}RasGRP1^{-/-}$ mice developed disease similarly to $LAT^{f/m}$ mice following eight weeks of tamoxifen treatment, suggesting that tonic signaling through the TCR is not inciting the lymphoproliferative disease.

In addition to the impaired central tolerance seen in $LAT^{m/m}$ mice, results from our lab show that these mice fail to develop peripheral tolerance and indeed lack natural $CD4^{+}CD25^{+}$ T regulatory cells (Koonpaew et al., 2006). Adoptive transfer of wildtype T_{reg} cells into neonatal $LAT^{m/m}$ mice prevents the development of the autoimmune disease, highlighting the importance of natural T_{regs} in the suppression of the LATY136F autoimmune syndrome. Studies have demonstrated that c-Rel, a member of the NF-κB family, is needed for thymic T_{reg} differentiation and regulates Foxp3 transcription by directly binding to cis-regulatory elements at the Foxp3 locus (Long et al., 2009; Ruan et al., 2009). Additionally, NFAT may bind to the *foxp3* promoter and play a role in TCR-induced Foxp3 expression (Mantel et al., 2006). However, restoration of these signaling

pathways in $LAT^{f/m}$ mice had no effect on disease development, indicating that regulatory T cells were unable to suppress the hyperproliferation.

Further study of the LAT-PLC-γ1 interaction in regulatory T cells revealed that $ERCre^{+}LAT^{f/m}$ mice contain $Foxp3^{+}$ cells following four weeks of tamoxifen treatment; however, these regulatory T cells are non-functional (Chuck et al., 2010). True to form, we observed that $LAT^{m/m}$ mice completely lacked regulatory T cells while $LAT^{f/m}$ mice had severely reduced percentages of $Foxp3^{+}$ cells, as a result of the massive conventional T cell expansion. Yet, $LAT^{f/m}$ mice had similar total numbers of regulatory T cells compared to wildtype mice. Although $Gads^{-/-}$ mice contained normal and perhaps slightly elevated percentages of $Foxp3^{+}CD4^{+}$ cells, $LAT^{f/m}Gads^{-/-}$ mice completely lacked regulatory T cells. Therefore, in the presence of mutant LAT, the absence of Gads negatively affected regulatory T cell development or survival in the periphery. This finding may correlate to observations made during thymic development, during which regulatory T cells perceive stronger TCR signals than conventional T cells (Jordan et al., 2001). Perhaps the residual signaling in $LAT^{f/m}$ T cells was sufficient to maintain Foxp3 expression; however, the more severely decreased signaling resultant of the additional Gads deletion led to the complete absence of Foxp3 expression in $LAT^{f/m}Gads^{-/-}$ $CD4^{+}$ cells.

From the data detailed in this report, it is apparent that LAT-mediated autoimmunity was able to occur independently of TCR-proximal signaling events. A previous study reported that decreasing Erk activation through the deletion of Bam32, an adaptor protein known to activate MAPK in B cells, lessens the severity of LATY136F disease (Miyaji et al., 2009). In contrast to this, the deletion of RasGRP1, despite increasing the thymocyte developmental block, did not prevent the progression of disease over time. Perhaps, in the context of this disease, the Grb2-Sos complex, which is able to bind directly to the phosphorylated TCR-ζ chain to activate Ras (Chau and Madrenas, 1999), may be able to compensate for the RasGRP1 deficiency and drive T cell expansion. Notably, expression of constitutively active Raf increased disease severity, as indicated by the tremendous spleen size, $CD4^+$ proliferation, and eosinophilia observed in $LAT^{m/m}Raf^{Tg}$ mice. These data support the idea that the Ras/MAPK pathway plays a significant role in guiding $LAT^{m/m}$ T cell hyperproliferation. In this context, Raf may serve to enhance positive selection of mutant thymocytes, as Erk activation is known to contribute to positive selection (Alberola-Ila et al., 1996; Fischer et al., 2005; Sugawara et al., 1998). Also, the presence of Raf-CAAX likely led to persistent Erk activation, causing increased proliferation of mutant T cells. Additionally, if T cell proliferation in $LAT^{m/m}$ mice is driven by cytokine signaling, it is possible that Raf-CAAX enhanced Ras-MAPK

activation downstream of cytokine receptor signaling and, consequently, increased $LAT^{m/m}$ T cell proliferation.

In sum, we have shown that deletion of critical TCR signaling components, Gads and RasGRP1, did not ameliorate the LAT-dependent lymphoproliferative syndrome. Moreover, the restoration of signaling pathways immediately downstream of LAT could not rescue the development of the LATY136F disease. In fact, by enhancing MAPK signaling through the expression of constitutively active Raf, we significantly increased the severity of the disease, indicating that the Erk pathway does indeed contribute to the uncontrolled T cell expansion that occurs upon abrogation of the LAT-PLC-γ1 interaction.

4. Regulation of RasGRP1 Function in T Cell Development and Activation by its Unique Tail Domain

4.1 Introduction

The Ras family proteins are critical components of T cell signaling and serve to regulate a variety of cell functions, such as proliferation and differentiation. The most well-characterized signaling pathway regulated by Ras is the Erk (extracellular signal-regulated kinases)-MAPK (mitogen activated protein kinase) pathway. Upon activation, Ras recruits Raf-1, a serine/threonine kinase, to phosphorylate MEK (MAPK/Erk kinase), which in turn activates Erk1 and Erk2. In T cells, Ras is activated by two families of Ras GEFs (guanine exchange factors), RasGRP (Ras guanyl releasing protein) and Sos (son of sevenless). It was recently shown that Sos contains an allosteric Ras-GTP binding pocket that binds to Ras-GTP and enhances Sos activity, suggesting that RasGRP1 may be necessary to prime Sos (Roose et al., 2007). Studies analyzing mice deficient in either RasGRP1 or Sos1 demonstrate that both GEFs are indispensable for proper thymocyte development (Dower et al., 2000; Kortum et al., 2011). These proteins probably have disparate roles during the stages of thymocyte development as the ratio of Sos1 to RasGRP1 expression changes dramatically upon the DN3 to DP transition (Kortum et al., 2011).

RasGRP1 is most highly expressed in T cells but is also detected in B cells, neuronal cells, and mast cells (Ebinu et al., 2000; Liu et al., 2007). Like the other members

of the RasGRP family, RasGRP1 contains a catalytic region, which consists of a REM (Ras exchange motif) and a CDC25 (cell division cycle 25) domain, a pair of EF hands (calcium binding), and a C1 domain (DAG binding) (Ebinu et al., 1998). Analyses of the structural domains of RasGRP1 have shown that deletion of the C1 domain impairs Ras activation through the abrogation of RasGRP1-DAG binding (Johnson et al., 2007; Roose et al., 2005; Tognon et al., 1998). However, RasGRP1 also has a C-terminal tail domain, consisting of about two hundred amino acid residues, of unknown function (Ebinu et al., 1998). Recently, studies have implicated two domains, the PT (plasma membrane targeting) and SuPT (suppressor of PT), located in the C-terminus of RasGRP1 as important mediators of protein localization (Beaulieu et al., 2007; Zahedi et al., 2011). However, the physiological relevance of this tail domain is yet unknown.

The importance of RasGRP1 in TCR signaling was first suggested in Jurkat T cells, in which overexpression of RasGRP1 enhances Ras/Erk signaling (Ebinu et al., 2000). Correspondingly, analysis of a RasGRP1-deficient Jurkat line demonstrated that RasGRP1 is indispensable for antigen receptor- and PMA-triggered Erk activation. RasGRP1-dependent Erk activation relies upon its DAG-binding C1 domain (Roose et al., 2005). RasGRP1-deficient mice have severely reduced numbers of single positive thymocytes and, consequently, very few mature T cells in the periphery, while B cell development is not affected. Further analysis of RasGRP1$^{-/-}$ thymocytes reveals their

inability to activate Erk after stimulation with the DAG-analog PMA (Dower et al., 2000). Another study has shown that RasGRP1 is essential for the weak TCR signals that drive Erk activation during positive selection. However, stronger signals that induce JNK and p38 activation leading to negative selection can be transmitted independently of RasGRP1 (Priatel et al., 2002).

Interestingly, RasGRP1$^{-/-}$ mice develop an autoimmune disorder marked by splenomegaly and auto-antibody production. This disease is primarily mediated by T cells that exhibit several functional defects (Coughlin et al., 2005; Coughlin et al., 2006). Furthermore, this phenotype arises despite reports that regulatory T cell function is intact and may be enhanced in RasGRP1$^{-/-}$ mice (Chen et al.). Contrary to these results, Priatel et al. assert that this autoimmune condition does not develop upon backcrossing RasGRP1$^{-/-}$ mice onto a B6 background (Priatel et al., 2007). The reason for these disparate results is unknown but could be due to differences in the ages of the mice used or in the environmental conditions in which the mice were housed.

In accordance with the idea that RasGRP1 regulates autoimmunity in mice, a study analyzing a cohort of patients with systemic lupus erythematosus (SLE) discovered 13 new splice variants of RasGRP1 transcripts, resulting in diminished RasGRP1 activity in these patients. Also, two SLE patients have T cells containing very little, if any, RasGRP1 protein, supporting a role for RasGRP1 in the onset of

autoimmunity (Yasuda et al., 2007). Another study examining microRNA expression in lupus $CD4^+$ T cells reveals that RasGRP1 can be targeted by miR-21, ultimately contributing to the DNA hypomethylation seen in patients with SLE (Pan et al., 2010). Moreover, RasGRP1 is one potential protein whose structural mutation or deregulated expression could mimic oncogenic Ras mutations (Quilliam et al., 2002), which are detected in many solid tumors and a subset of T lymphocytic leukemia (Kim et al., 2003; Malumbres and Barbacid, 2003; Vassiliou et al., 2011). Therefore, understanding the regulation of RasGRP1 and its role in Erk activation is essential to comprehend the establishment of T cell tolerance, autoimmunity, and transformation.

Knowing the importance of RasGRP1 in T cell signaling and that RasGRP1 contains a unique tail domain, we decided to elucidate the role of this domain in RasGRP1 function. To this end, we generated $RasGRP1^{d/d}$ knock-in mice, which express a form of RasGRP1 lacking the tail domain. While previous studies have highlighted the irreplaceable role of the C1 domain in RasGRP1-mediated Erk activation, our findings reveal a novel means by which the tail domain of RasGRP1 regulates TCR-mediated Ras signaling.

4.2 Results

4.2.1 Generation of RasGRP1$^{d/d}$ mice

To assess the function of the RasGRP1 tail domain, we designed a targeting construct to generate knock-in mice with the tail domain deleted, which we designated as RasGRP1$^{d/d}$ (d=deleted) mice. *Rasgrp1* consists of seventeen exons; we inserted a stop codon, TGA, into exon 15 to ensure retention of the C1 domain but deletion of the tail. This insertion site, in addition to being immediately downstream of a *Bgl II* restriction site, is located between the C1 domain and the SuPT domain. Our targeting vector, as depicted in Figure 19A, was used to transfect ES cells. Positive clones were then used to generate chimeric mice. To remove the PGK-Neo cassette, chimeric mice were crossed with β-actin-Cre transgenic mice to generate RasGRP1$^{d/+}$ mice. These mice were then backcrossed onto a B6 background for ten generations. Intercrossing RasGRP1$^{d/+}$ mice led to RasGRP1$^{d/d}$ offspring at an expected ratio with no visible defects in viability, fertility, or behavior.

Next, we confirmed correct targeting by sequencing a PCR fragment of RasGRP1 amplified from thymocyte cDNA. Sequencing results indicated that a TGA stop codon was correctly inserted towards the 3′ end of exon 15 (data not shown). We also performed real-time PCR analysis to demonstrate comparable transcript expression (Figure 19B). We further analyzed the expression of the truncated RasGRP1 protein by

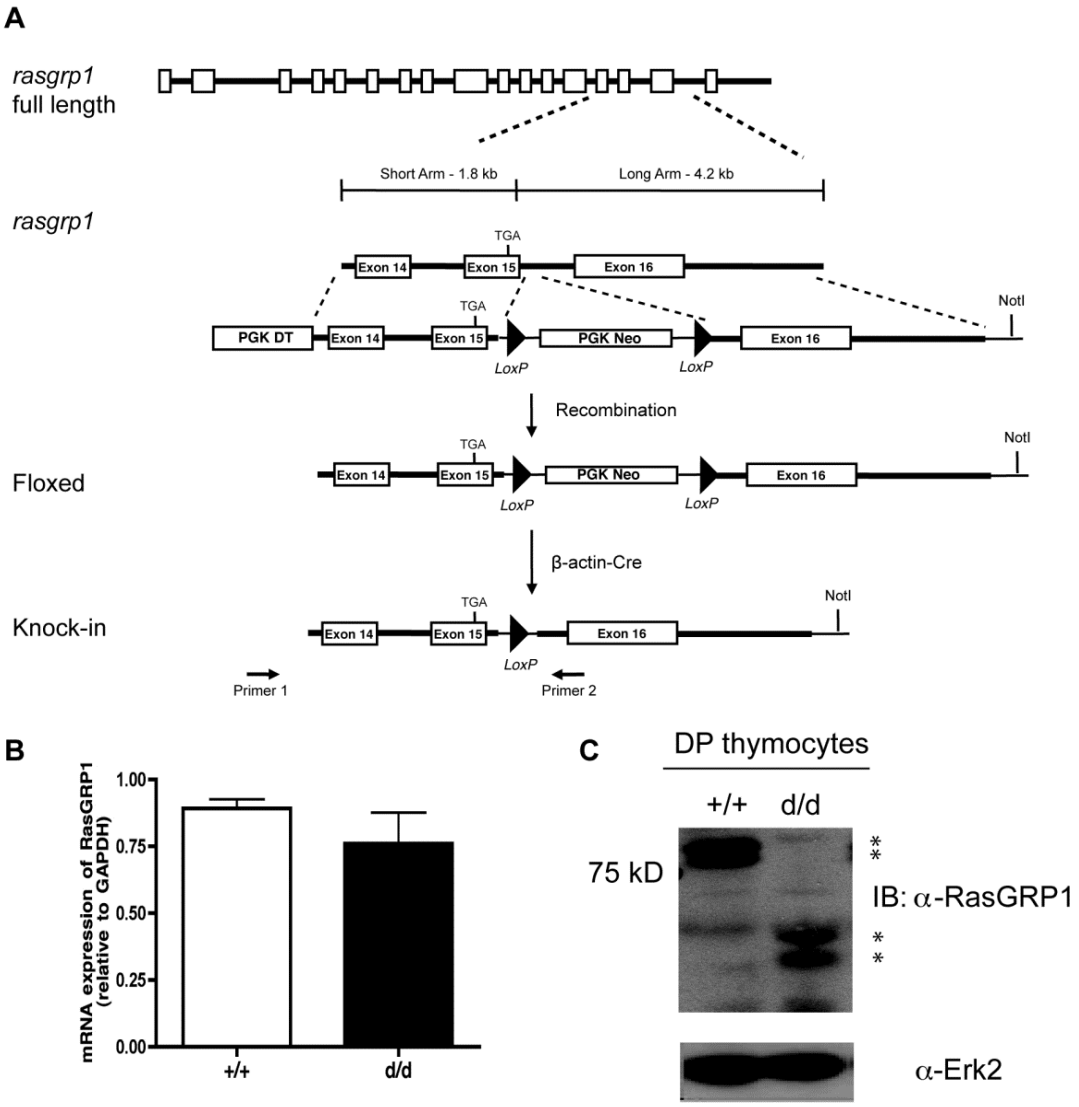


Figure 19: Generation of RasGRP1$^{d/d}$ mice

(A) Targeting construct showing insertion of a stop codon into exon 15 of *rasgrp1*. (B) Real-time PCR analysis of RasGRP1 transcript in DP thymocytes from RasGRP1$^{+/+}$ and RasGRP1$^{d/d}$ mice. (C) Western blot analysis of RasGRP1 expression in DP thymocyte lysate from RasGRP1$^{+/+}$ and RasGRP1$^{d/d}$ mice. * indicates two RasGRP1 protein isoforms on the SDS-PAGE.

Western blotting to confirm successful gene targeting. Western blot analysis of thymocyte lysates using a monoclonal antibody against the C-terminal portion of RasGRP1 revealed the presence of endogenous RasGRP1 in wildtype but not RasGRP1$^{d/d}$ or RasGRP1$^{-/-}$ lysates (data not shown). Blotting with a polyclonal antibody against the CDC25 domain of RasGRP1 revealed the presence of faster migrating bands in RasGRP1$^{d/d}$ thymocyte lysates than in wildtype samples (Figure 19C). Expression of the mutant RasGRP1 protein was reduced to ~70% of wildtype protein expression, suggesting that the deletion of the tail domain slightly affected RasGRP1 protein stability. Notably, RasGRP1$^{+/-}$ T cells express the full-length protein at a reduced level compared to wildtype cells; however, this reduction has no impact on T cell function (Dower et al., 2000).

4.2.2 Defective thymic and peripheral development in RasGRP1$^{d/d}$ mice

Analysis of 4-week-old RasGRP1$^{d/d}$ mice revealed a defect in thymocyte development. Unlike RasGRP1$^{-/-}$ mice, which had a severe decrease in both SP populations, the percentages of RasGRP1$^{d/d}$ SP thymocytes were only moderately decreased to 1.3% CD8 SP, compared to 2.4% in wildtype mice, and 4.6% CD4 SP, relative to 8.7% in wildtype thymuses (Figure 20A). Whereas total thymic cellularity was not affected in RasGRP1$^{d/d}$ mice, the numbers of CD4 and CD8 SP thymocytes were significantly reduced (Figure 20B). In RasGRP1$^{-/-}$ mice, total thymocyte numbers, along

with both SP compartments, were appreciably decreased. Correspondingly, upregulation of TCRβ and CD69, both known indicators of positive selection, was impaired in RasGRP1$^{d/d}$ and RasGRP1$^{-/-}$ DP thymocytes (Figure 20C). While ~6% of wildtype DP thymocytes upregulated TCRβ and/or CD69, that percentage was reduced to ~2.5% and ~1.5%, respectively, in RasGRP1$^{d/d}$ thymocytes. RasGRP1$^{-/-}$ thymocytes had an even greater reduction, only 0.2% upregulated TCRβ and/or CD69. However, TCRβ

and CD69 expression on RasGRP1$^{d/d}$ CD4 and CD8 SP thymocytes was not drastically reduced, as it was on RasGRP1$^{-/-}$ CD4 and CD8 SP cells (Figure 20D). In the periphery, the percentages of CD4^{+} and CD8^{+} T cells in RasGRP1$^{d/d}$ mice were decreased, although not to the extent seen in RasGRP1$^{-/-}$ mice (Figure 20E). Interestingly, 40% of RasGRP1$^{d/d}$ CD4^{+} T cells in these young mice had upregulated CD44 and downregulated CD62L, while only 15% of wildtype cells exhibited this activated/memory phenotype (Figure 20F). In contrast, 66% of RasGRP1$^{-/-}$ CD4^{+} T cells were CD62L^{lo}CD44^{hi}, likely a result of homeostatic proliferation following the entry of T cells into the lymphopenic periphery seen in these mice. Furthermore, the surface expression of TCRβ on CD4^{+} and CD8^{+} RasGRP1$^{d/d}$ T cells was slightly reduced, although higher than that on RasGRP1$^{-/-}$ T cells (Figure 20G). Despite the defect in conventional T cell development, RasGRP1$^{d/d}$ and RasGRP1$^{-/-}$ spleens contained distinct populations of Foxp3^{+}CD4^{+} regulatory T cells (Figure 20H). In fact, the percent of Foxp3^{+} cells in the CD4^{+} splenic compartment was

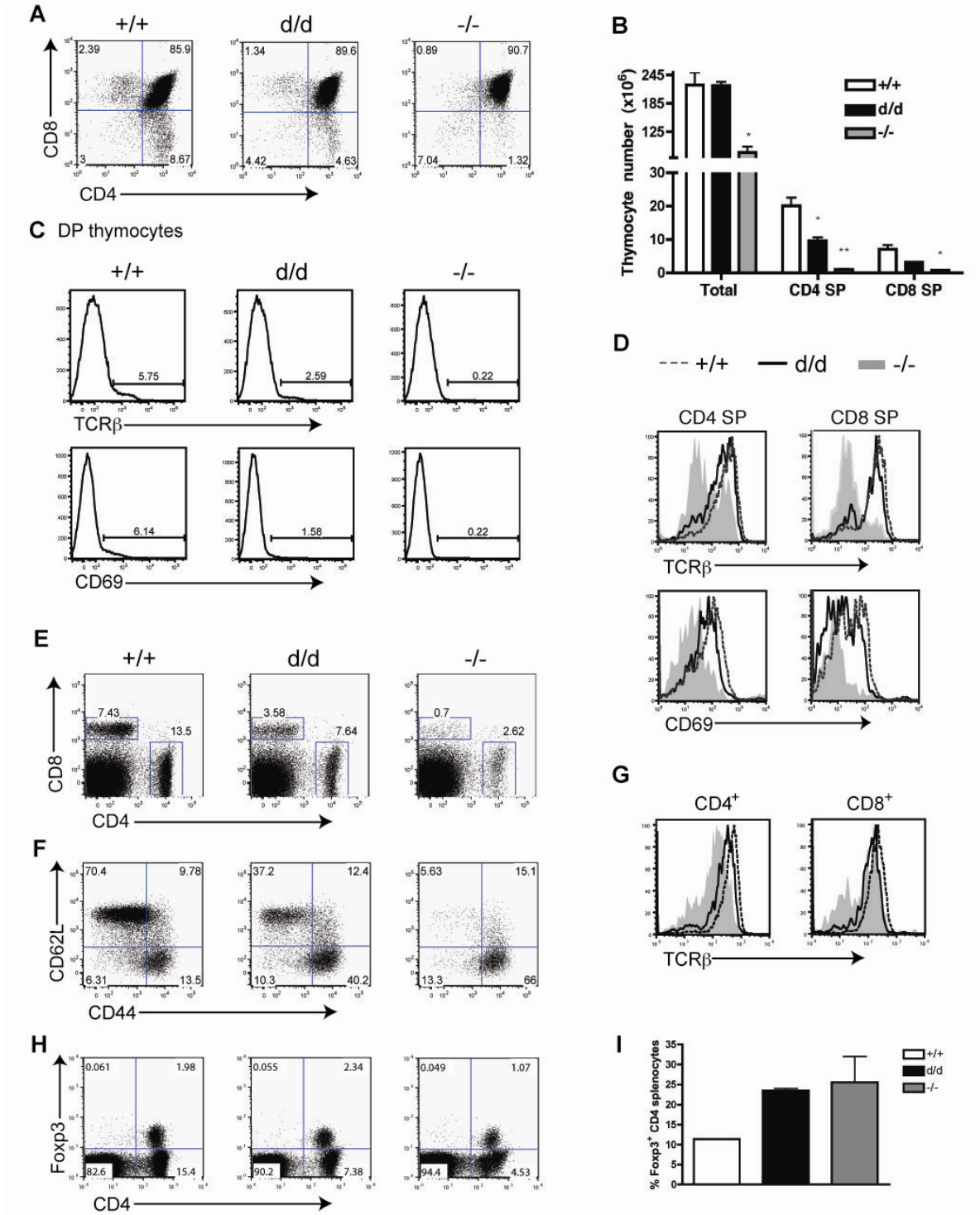

A
+/+
d/d
-/-
CD8
CD4
2.39
85.9
8.67
1.34
89.6
4.42
4.63
0.89
90.7
7.04
1.32
B
Thymocyte number (x10^6)
+/+
d/d
-/-
Total
CD4 SP
CD8 SP
C DP thymocytes
TCRβ
CD69
5.75
2.59
0.22
6.14
1.58
0.22
D
+/+
d/d
-/-
CD4 SP
CD8 SP
TCRβ
CD69
E
CD8
CD4
7.43
13.5
3.58
7.64
0.7
2.62
F
CD62L
CD44
70.4
9.78
6.31
13.5
37.2
12.4
10.3
40.2
5.63
15.1
13.3
66
G
CD4+
CD8+
TCRβ
H
Foxp3
CD4
0.061
1.98
82.6
15.4
0.055
2.34
90.2
7.38
0.049
1.07
94.4
4.53
I
% Foxp3+ CD4 splenocytes
+/+
d/d
-/-

Figure 20: T cell development is impaired in RasGRP1$^{d/d}$ mice

(A) Representative FACS plots of CD8 versus CD4 expression gated on total live thymocytes from 4- week-old mice. (B) Total numbers of thymocytes, CD4 SP, and CD8 SP populations. (C) Expression of TCRβ (top) and CD69 (bottom) on DP thymocytes. Numbers indicate the percentage of surface marker positive cells. (D) Cell surface expression of TCRβ (top) and CD69 (bottom) on CD4 SP (left) and CD8 SP (right) thymocytes. (E) Representative FACS plot of CD8 versus CD4 on splenocytes from 4-week-old mice. (F) Surface expression of CD44 and CD62L on live CD4^{+} splenocytes. (G) TCRβ expression on CD4^{+} (left) and CD8^{+} (right) splenocytes. Shaded histogram represents RasGRP1$^{-/-}$, solid black line represents RasGRP1$^{d/d}$, and dashed black line represents RasGRP1$^{+/+}$ thymocytes. (H) Surface expression of CD4 and intracellular expression of Foxp3. (I) The percent of Foxp3^{+} cells in the CD4 splenic compartment was calculated by dividing the percent of Foxp3^{+}CD4^{+} cells by the total percent of CD4^{+} cells. Two-tailed *t* test; *, $p < 0.05$; **, $p < 0.005$. Data are representative of at least four independent experiments.

increased from about 11% in wildtype mice to 20-25% in RasGRP1$^{d/d}$ and RasGRP1$^{-/-}$ mice. (Figure 20I) This finding was in agreement with a previous study which demonstrated normal regulatory T cell development and suppressor function in RasGRP1$^{-/-}$ mice (Chen et al., 2008). These data showed that the deletion of the tail domain of RasGRP1 led to impaired T cell development, causing reduced numbers of SP thymocytes and mature conventional T cells.

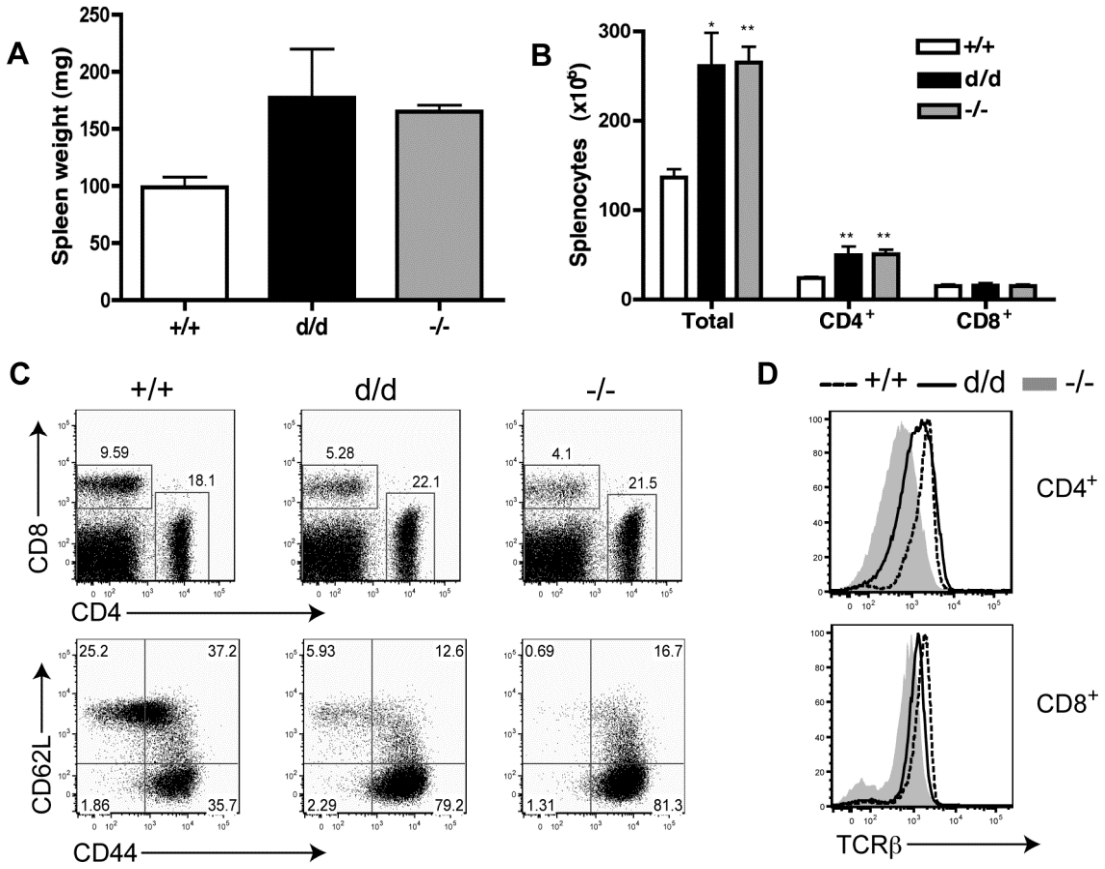
4.2.3 Splenomegaly and autoimmunity in aged RasGRP1$^{d/d}$ mice

Although RasGRP1$^{d/d}$ mice did not have as severe defects in T cell development as RasGRP1$^{-/-}$ mice, they exhibited splenomegaly at ~3-4 months of age. At six months of age, spleen weights were greatly increased in RasGRP1$^{d/d}$ and RasGRP1$^{-/-}$ mice, along with overall cell numbers (Figures 21A and 21B). Despite the decreased percentages of CD4^{+} T cells in young RasGRP1$^{d/d}$ and RasGRP1$^{-/-}$ mice (Figure 20E), total numbers of CD4^{+} T cells were significantly increased compared to wildtype mice. CD4^{+} T cells preferentially expanded over CD8^{+} splenocytes, which were comparable in total numbers but decreased in percentages compared to wildtype splenocytes. In wildtype mice, ~35% of CD4^{+} cells were CD62L^{lo}CD44^{hi}, whereas ~80% of RasGRP1$^{d/d}$ and RasGRP1$^{-/-}$ CD4^{+} T cells were CD62L^{lo}CD44^{hi} (Figure 21C). Surface expression of TCRβ was also reduced on RasGRP1$^{d/d}$ T cells and further reduced on RasGRP1$^{-/-}$ T cells (Figure 21D).

To evaluate systemic irregularities in these mice, we assayed their serum antibody levels. Although the percentages and activation status of B cells were similar (data not shown), serum from RasGRP1$^{d/d}$ and RasGRP1$^{-/-}$ mice contained higher levels of IgE and IgG_1, while other isotypes measured, including IgG_{2a}, IgG_{2b}, and IgM, were comparable (Figure 21E and data not shown). Also, we performed auto-antibody ELISAs using NIH3T3-coated plates to observe if the RasGRP1 mutation caused an autoimmune-like disorder. Serum from RasGRP1$^{d/d}$ and RasGRP1$^{-/-}$ mice contained a higher amount of auto-antibodies than that from wildtype mice (Figure 21F). Furthermore, staining NIH3T3 cells with serum from RasGRP1$^{d/d}$ and RasGRP1$^{-/-}$ mice revealed distinct nuclear staining (Figure 21G, top panel). We also detected immune complexes in the kidneys of RasGRP1$^{d/d}$ and RasGRP1$^{-/-}$ mice (Figure 21G, bottom panel). These data indicated that T cell tolerance and/or homeostasis were disrupted upon the loss of the RasGRP1 tail domain, culminating in auto-antibody production and immune complex deposition.

4.2.4 Impaired function in RasGRP1$^{d/d}$ T cells

To determine the role of the RasGRP1 tail domain in T cell activation, we assayed the ability of RasGRP1$^{d/d}$ splenic T cells to respond to stimulation. To assess proliferative capacity, we labeled splenocytes with CFSE *in vitro*, stimulated them with PMA and ionomycin (P+I), as well as through the TCR using 2C11, and harvested the cells after 48


A
Spleen weight (mg)
250
200
150
100
50
0
+/+
d/d
-/-
B
Splenocytes (x10⁶)
300
200
100
0
*
**
**
**
+/+
d/d
-/-
Total
CD4+
CD8+
C
+/+
d/d
-/-
9.59
18.1
5.28
22.1
4.1
21.5
CD8
CD4
25.2
37.2
1.86
35.7
5.93
12.6
2.29
79.2
0.69
16.7
1.31
81.3
CD62L
CD44
D
+/+
d/d
-/-
CD4+
CD8+
TCRβ

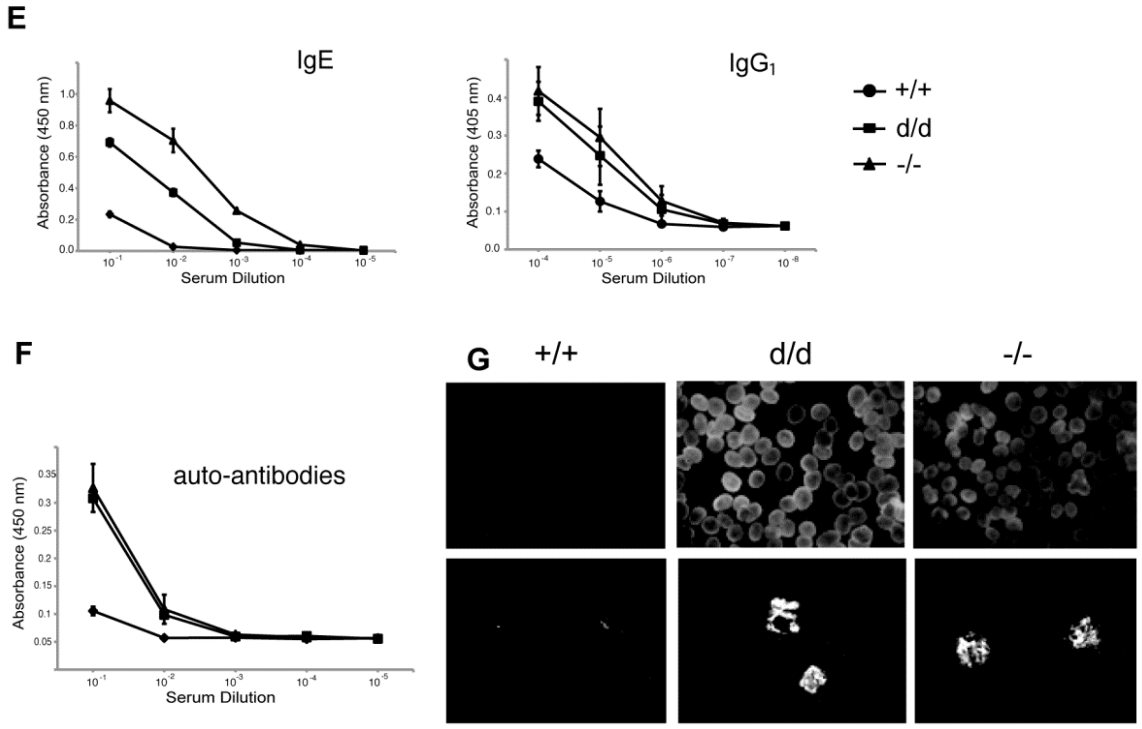

E
IgE
Absorbance (450 nm)
Serum Dilution
IgG1
Absorbance (405 nm)
Serum Dilution
+/+
d/d
-/-
F
auto-antibodies
Absorbance (450 nm)
Serum Dilution
G
+/+
d/d
-/-

Figure 21: Development of splenomegaly and autoimmunity in aged RasGRP1$^{d/d}$ mice

(A-G) Mice were analyzed at 6 months of age. (A) Spleen weights. (B) Numbers of total, CD4^{+}, and CD8^{+} splenocytes. (C) Representative FACS plots of CD4 versus CD8 on live splenocytes (top), as well as CD62L and CD44 expression on CD4^{+} splenocytes (bottom). (D) TCRβ expression on RasGRP1$^{-/-}$ (shaded histogram), RasGRP1$^{d/d}$ (solid black line), and RasGRP1$^{+/+}$ (dashed black line) splenocytes (left). (E-F) Blood serum levels of IgE, IgG_1 (E), and anti-nuclear antibodies (F) from RasGRP1$^{-/-}$ (triangles), RasGRP1$^{d/d}$ (squares), and RasGRP1$^{+/+}$ (circles) mice. (G) Top panel, auto-antibodies were detected by immunostaining slides with fixed NIH3T3 cells with mouse serum, followed by FITC-conjugated anti-mouse IgG. Bottom panel, immune complex deposition in the glomeruli of mice was visualized by staining kidney cryosections with FITC-conjugated anti-mouse IgG. Imaging was performed using light or fluorescent microscopy at 10x magnification. Two-tailed *t* test; *, $p < 0.05$; **, $p < 0.005$. Data are representative of at least three independent experiments.

hours. RasGRP1$^{d/d}$ and RasGRP1$^{-/-}$ CD4^{+} T cells had slightly impaired proliferation as measured by CFSE dilution in response to 2C11 stimulation (Figure 22A). Upon the addition of P+I, RasGRP1$^{d/d}$ CD4^{+} T cells, but not RasGRP1$^{-/-}$ T cells, were able to proliferate comparably to wildtype cells. ELISA analysis of secreted IL-2 revealed a marked decrease in the presence of IL-2 in RasGRP1$^{d/d}$ and RasGRP1$^{-/-}$ splenocyte cultures, particularly following 2C11 stimulation (Figure 22B). Furthermore, the addition of IL-2 in the presence of 2C11 did not rescue RasGRP1$^{d/d}$ and RasGRP1$^{-/-}$ CD4^{+} T cell proliferation (data not shown).

We next assessed the ability of RasGRP1$^{d/d}$ and RasGRP1$^{-/-}$ T cells to upregulate activation markers. As seen in Figure 22C, RasGRP1$^{d/d}$ cells increased CD25 expression following 2C11 stimulation, although to a lesser extent than wildtype cells. RasGRP1$^{-/-}$ CD4^{+} T cells displayed a significantly impaired ability to upregulate CD25 following 2C11 stimulation. However, in response to P+I stimulation, cells from all 3 genotypes were able to upregulate CD25. While CD69 upregulation was only slightly defective in RasGRP1$^{d/d}$ CD4^{+} T cells following both types of stimulation, RasGRP1$^{-/-}$ CD4^{+} T cells had a marked defect in their ability to upregulate CD69. CD8^{+} T cells from RasGRP1$^{d/d}$ and RasGRP1$^{-/-}$ mice behaved similarly to CD4^{+} cells but with a less severe defect in their proliferation and activation marker upregulation (data not shown). We further determined the ability of these T cells to produce cytokines by stimulating splenocytes

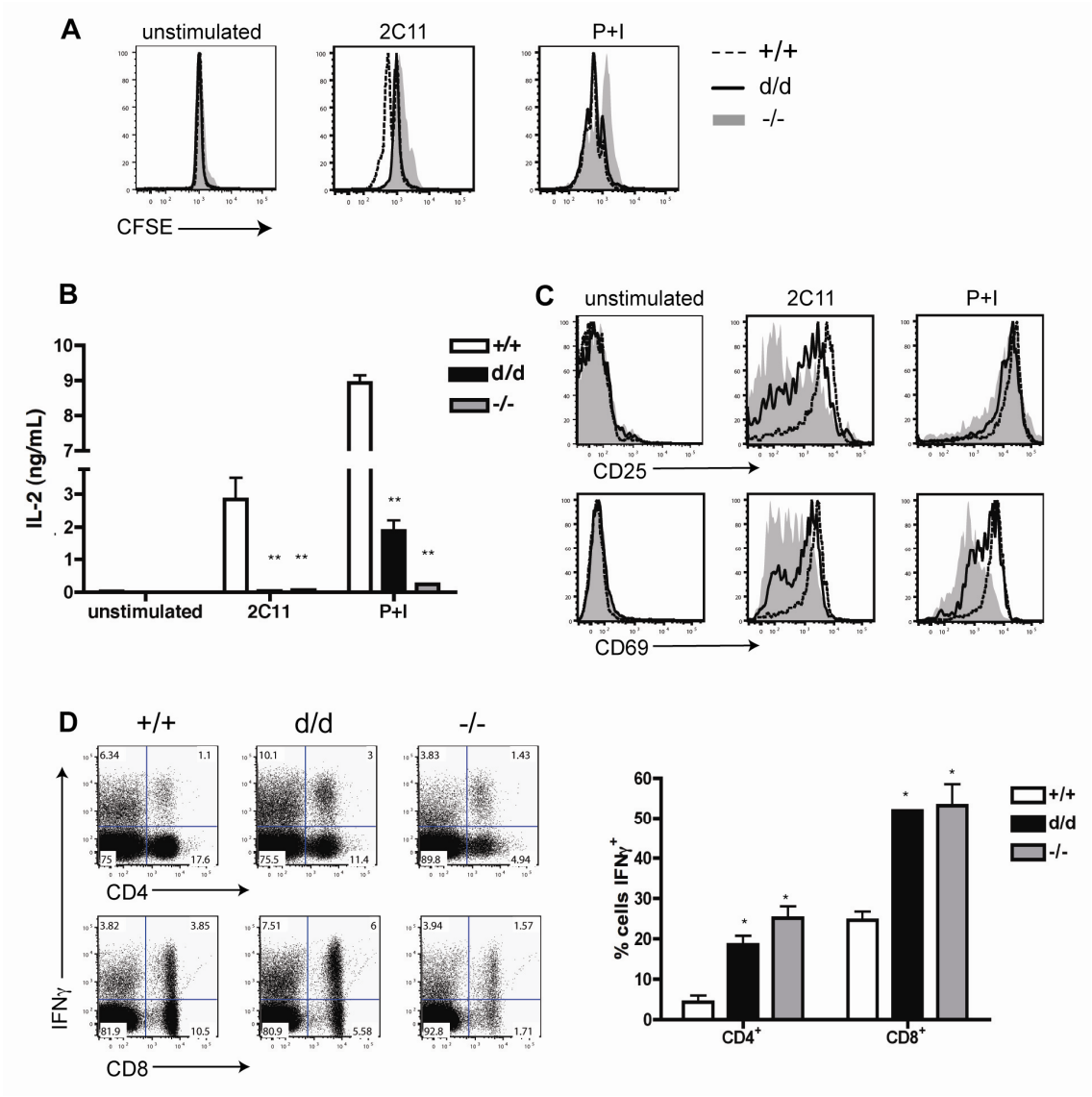

A
unstimulated
2C11
P+I
+/+
d/d
-/-
CFSE
B
IL-2 (ng/mL)
+/+
d/d
-/-
unstimulated
2C11
P+I
C
unstimulated
2C11
P+I
CD25
CD69
D
+/+
d/d
-/-
CD4
IFNγ
CD8
% cells IFNγ+
CD4+
CD8+

Figure 22: Aberrant proliferation, activation, and cytokine production in RasGRP1$^{d/d}$ T cells

(A) Splenocytes were isolated from six to eight-week-old mice and stained with 5μM CFSE and stimulated as shown. After forty-eight hours, cells were harvested and stained for CD4 and CD8. Histograms shown are gated on CD4^{+} cells. Shaded histograms represent RasGRP1$^{-/-}$, solid black lines represent RasGRP1$^{d/d}$, and dashed black lines represent RasGRP1$^{+/+}$ cells. (B) Supernatants from the CFSE cultures were collected after 24 hours and used to perform an IL-2 ELISA. (C) Cells were stimulated as in (A) but harvested after 24 hours and analyzed by FACS for CD4, CD8, CD69, and CD25 expression. Shaded histograms represent RasGRP1$^{-/-}$, solid black lines represent RasGRP1$^{d/d}$, and dashed black lines represent RasGRP1$^{+/+}$ CD4^{+} splenocytes. (D)Splenocytes from six-week-old mice were harvested and stimulated with P+I with monensin for 4 hours. Left: Representative FACS plots of splenocytes stained with 7AAD, CD4, CD8, and IFN-γ. Right: Quantitative representation of the percent of CD4 and CD8 cells from each genotype that made IFN-γ following *in vitro* P+I stimulation. Two-tailed *t* test; *, $p < 0.05$; **, $p < 0.005$. Data are representative of at least three independent experiments.

for 4 hours with P+I before intracellular staining. Interestingly, ~20% of $CD4^+$ and over 50% of $CD8^+$ T cells from $RasGRP1^{d/d}$ and $RasGRP1^{-/-}$ mice produced IFN-γ. In comparison, ~5% of $CD4^+$ and ~25% of $CD8^+$ T cells from wildtype mice were IFN-γ^+ (Figure 22D). None of the T cells made a significant amount of IL-4 (data not shown).

4.2.5 Diminished Erk activation in $RasGRP1^{d/d}$ thymocytes

As shown in Figure 20C, $RasGRP1^{d/d}$ DP thymocytes did not upregulate TCRβ and CD69 to the extent of wildtype cells, indicating a defect in positive selection. Because Erk activation is known to be essential for positive selection (Alberola-Ila et al., 1996; Fischer et al., 2005; Sugawara et al., 1998), we analyzed Erk phosphorylation in DP thymocytes. Thymocytes were isolated from three-week-old mice and enriched for DP populations to eliminate any differences that might arise from unequal numbers of SP cells. DP thymocytes were then stimulated using anti-CD3/CD4/CD8 antibodies or PMA. Western blots performed on cell lysates revealed similar levels of tyrosine phosphorylation of proteins, with slightly reduced levels of LAT phosphorylation in $RasGRP1^{d/d}$ and $RasGRP1^{-/-}$ thymocytes (Figure 23A). $RasGRP1^{-/-}$ thymocytes could not activate Erk following both types of stimulation (Figure 23B). Unexpectedly, despite having only a slight defect in thymocyte development, $RasGRP1^{d/d}$ DP thymocytes had a severely diminished ability to activate the Erk pathway. These data demonstrated that the tail domain of RasGRP1 is critical for TCR-mediated Erk activation.

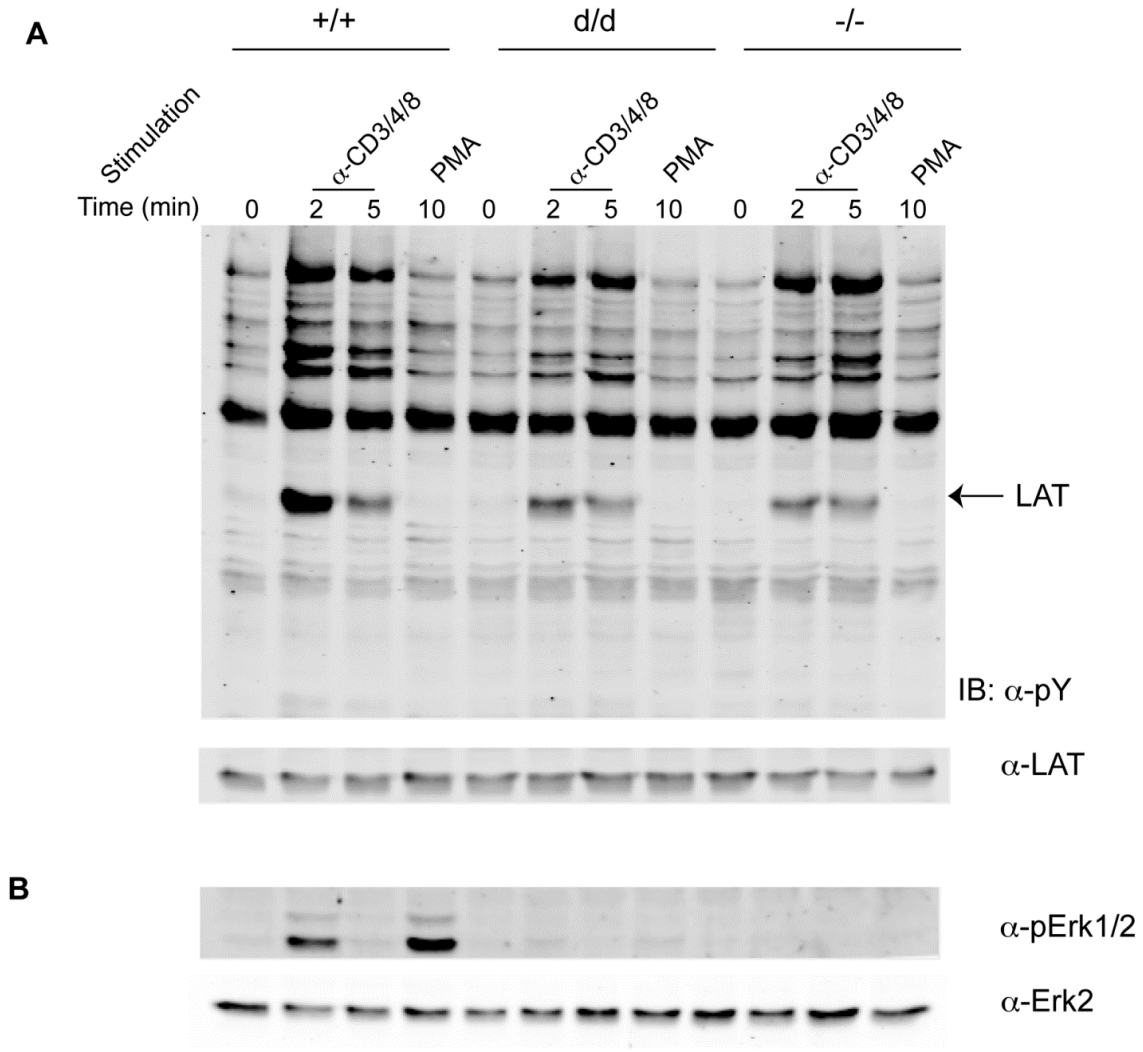
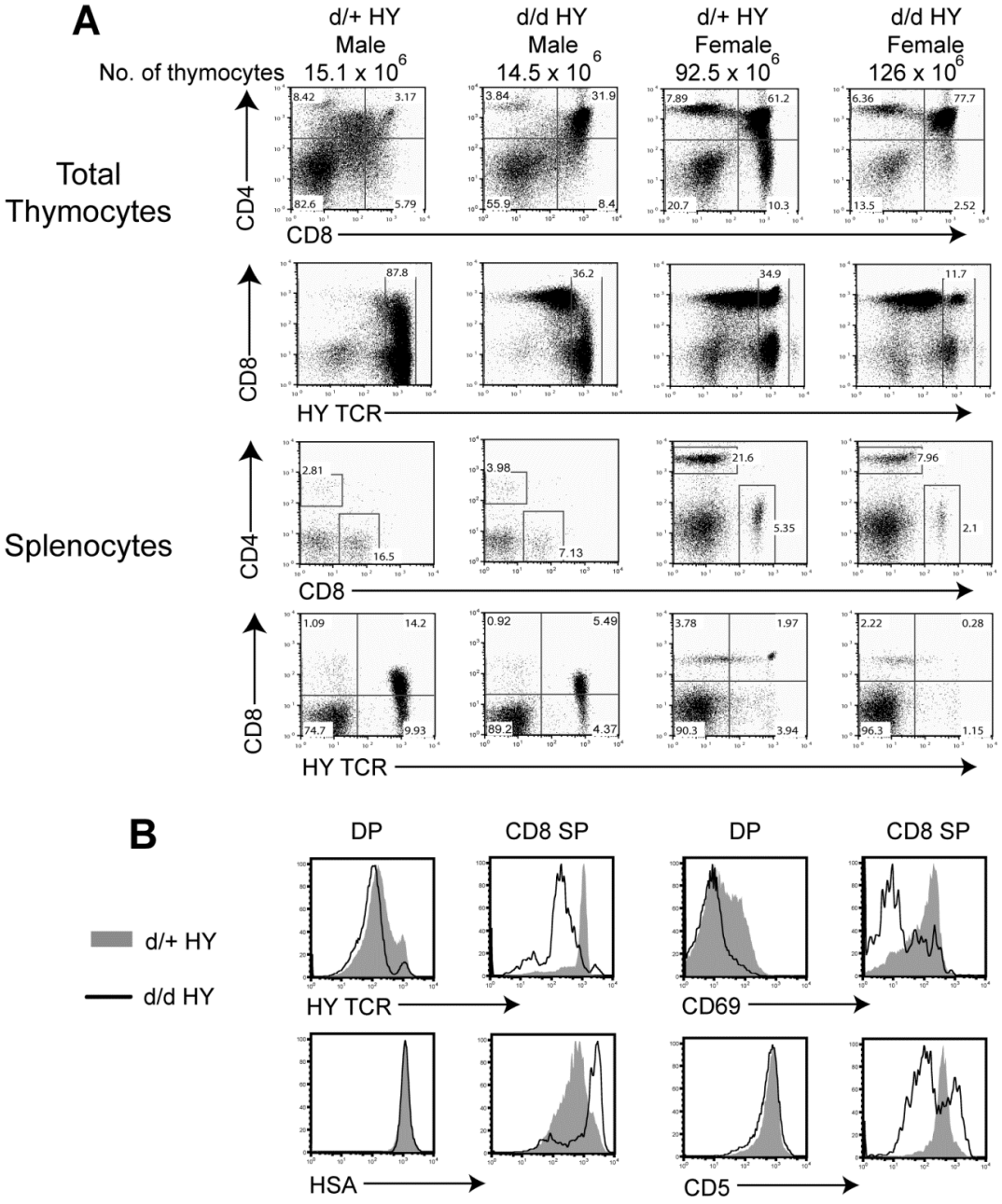


Figure 23: Impaired Erk activation in RasGRP1$^{d/d}$ thymocytes

(A-B) Enriched DP thymocytes were stimulated as shown. Lysates were analyzed via SDS-PAGE and immunoblotting for overall tyrosine phosphorylation (pY) and LAT (A) or pErk and Erk2 (B).

4.2.6 Defective thymic selection in RasGRP1$^{d/d}$ mice

Considering the impaired thymic development and autoimmune disorder seen in our mice, we decided to look more closely at thymic selection processes in RasGRP1$^{d/d}$ mice. Therefore, we analyzed RasGRP1$^{d/d}$ mice on the MHC class I-restricted HY TCR transgenic background. The HY system allows for the examination of negative selection in males which express the male-specific HY antigen, thus causing massive deletion of antigen-specific DP thymocytes. Similar numbers of thymocytes were recovered from wildtype HY and RasGRP1$^{d/d}$ HY mice (Figure 24A, top left). RasGRP1$^{-/-}$ mice have also been examined on the HY background with the conclusion that the loss of RasGRP1 does not affect negative selection (Priatel et al., 2002). However, staining of thymocytes revealed the presence of a significant DP population in RasGRP1$^{d/d}$ HY thymuses that was not seen in wildtype HY controls. While only 3% of thymocytes in wildtype mice were double positive, ~30% of thymocytes in RasGRP1$^{d/d}$ HY male mice were CD4^{+}CD8^{+}. However, the CD8 SP compartment was not substantially increased in male RasGRP1$^{d/d}$ mice; there was also no increase in peripheral CD8^{+} T cells in the spleens of these mice. We hypothesized that the lower levels of HY TCR expressed on RasGRP1$^{d/d}$ thymocytes accounted for the increased DP compartment, although this population did not successfully develop into CD8 SP cells.


A
d/+ HY Male
d/d HY Male
d/+ HY Female
d/d HY Female
No. of thymocytes
15.1 x 10^6
14.5 x 10^6
92.5 x 10^6
126 x 10^6
Total Thymocytes
CD4
CD8
8.42
3.17
82.6
5.79
3.84
31.9
55.9
8.4
7.89
61.2
20.7
10.3
6.36
77.7
13.5
2.52
CD8
HY TCR
87.8
36.2
34.9
11.7
Splenocytes
CD4
CD8
2.81
16.5
3.98
7.13
21.6
5.35
7.96
2.1
CD8
HY TCR
1.09
14.2
74.7
9.93
0.92
5.49
89.2
4.37
3.78
1.97
90.3
3.94
2.22
0.28
96.3
1.15
B
DP
CD8 SP
DP
CD8 SP
d/+ HY
d/d HY
HY TCR
CD69
HSA
CD5

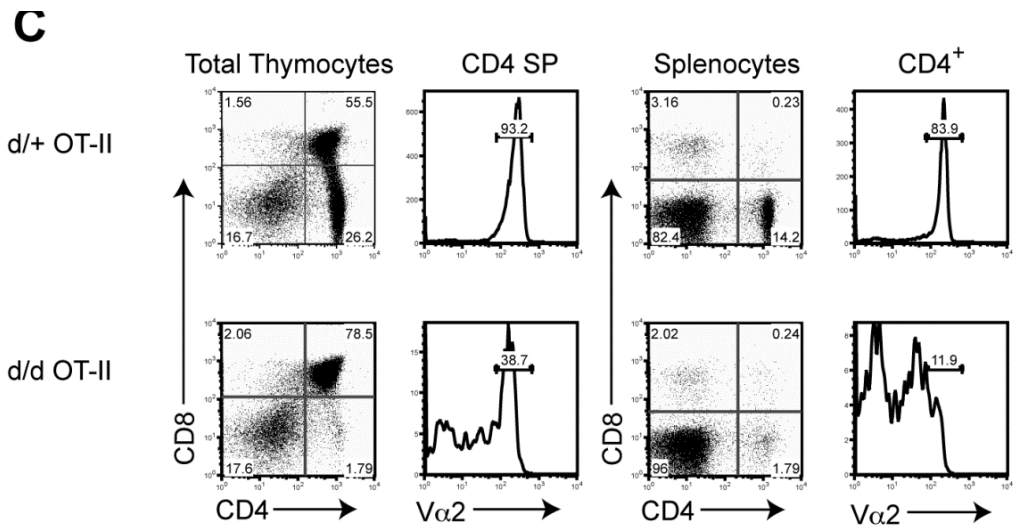


Figure 24: Negative and positive selection defects in RasGRP1$^{d/d}$ mice

(A) Total thymocytes and splenocytes from wildtype and RasGRP1$^{d/d}$ male and female mice expressing the HY TCR transgene were analyzed for the expression of CD4, CD8, and the transgenic TCR chain used by the HY TCR. Numbers above the plots indicate the total number of thymocytes. N = 6-9 mice per group. (B) DP and CD8 SP thymocytes from female mice expressing the HY TCR were analyzed for expression of the HY TCR, HSA, CD69, and CD5. The shaded histogram represents wildtype cells while the solid line indicates RasGRP1$^{d/d}$ thymocytes. (C) Thymocytes and splenocytes from wildtype and RasGRP1$^{d/d}$ mice expressing the OT-II TCR were analyzed for the expression of CD4 and CD8. Representative histograms show the levels of Vα2 on either CD4 SP or CD4^{+} splenocytes. The data are representative of four independent experiments.

Upon analysis of RasGRP1$^{d/d}$ HY female mice, we found a marked defect in positive selection. In wildtype HY females, thymocytes expressing the antigen-specific TCR are positively selected, as indicated by an increased percentage of CD8 SP cells. Female RasGRP1$^{d/d}$ HY mice, however, contained far fewer CD8 SP thymocytes, decreased from ~10% in wildtype to 2.5% in knock-in mice (Figure 24A, right). Fewer CD8^{+} HY TCR^{+} T cells were also found in the periphery of RasGRP1$^{d/d}$ HY mice. Also, closer examination of DP thymocytes showed aberrant expression of the HY TCR, CD69, and CD5, while maintaining high expression of HSA, a marker typically downregulated on mature SP cells (Figure 24B).

In order to assess the requirement of the tail domain during positive selection of MHC class II-restricted TCRs, we crossed RasGRP1$^{d/d}$ mice onto the OT-II TCR transgenic background. Positive selection of OT-II^{+} T cells on an I-A^{b}-background results in the accumulation of CD4 SP thymocytes that are Vβ5^{hi}Vα2^{hi}. Analysis of RasGRP1$^{d/d}$ mice expressing the OT-II-specific TCR revealed a severe defect in the percentage of CD4 SP thymocytes (Figure 24C). While 26% of RasGRP1$^{d/+}$ OT-II^{+} thymocytes were CD4 SP cells, RasGRP1$^{d/d}$ OT-II^{+} thymuses contained only ~2% CD4 SP cells. Furthermore, while over 90% of RasGRP1$^{d/+}$ OT-II^{+} CD4 SP cells stained positive for the Vα2 TCR, only 38.7% of RasGRP1$^{d/d}$ OT-II^{+} CD4 SP cells expressed this TCR. This diminution could be accounted for by either decreased TCR surface expression or the presence of

endogenously rearranged TCRs. Likewise, the spleens of RasGRP1$^{d/d}$ OT-II^{+} mice contained far fewer CD4^{+} and Vα2^{+} T cells (Figure 24C, right). Collectively, our data from these two different TCR transgenic lines indicated that the tail domain of RasGRP1 is critical for proper positive and negative selection.

4.2.7 Impaired localization of RasGRP1Δtail in T cells

In an attempt to elucidate the mechanism by which the tail domain regulates RasGRP1 function and Erk activation, we investigated RasGRP1 translocation following stimulation. Different studies have focused on the localization of RasGRP1 and the ramifications this may have on Ras activation (Beaulieu et al., 2007; Bivona et al., 2003; Carrasco and Merida, 2004; Daniels et al., 2006; Johnson et al., 2007). We wanted to determine if the loss of the tail domain would impact RasGRP1 localization. We electroporated Jurkat cells with GFP-tagged *rasgrp1* wildtype or Δtail construct and performed live cell imaging. The GFP-RasGRP1 wildtype protein was initially found either in the cytoplasm or on the plasma membrane depending upon the level of protein expression (Figure 25). Following stimulation through the TCR via OKT3 or through the addition of PMA, GFP fluorescence moved to or intensified at the plasma membrane. In contrast, GFP-RasGRP1Δtail was initially seen throughout the entire cell, including the nucleus. Following OKT3 stimulation, GFP fluorescence failed to move to the membrane and remained dispersed throughout the cells. Interestingly, after the addition of PMA,

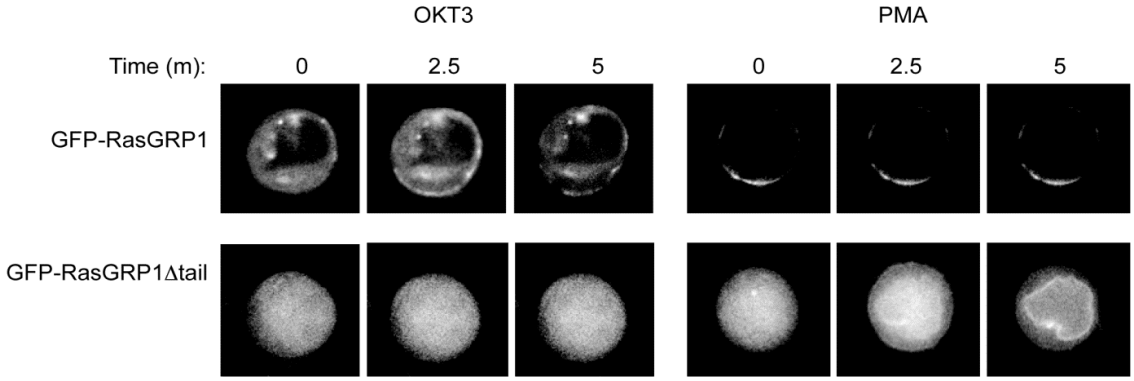


Figure 25: Impaired localization of the RasGRP1Δtail construct in Jurkat T cells.

Jurkat cells were electroporated with GFP-RasGRP1 or GFP-RasGRP1Δtail plasmids. Live cell imaging was performed on a Zeiss Observer D1 station equipped with a CoolSNAP$_{HQ}$ charge-coupled device camera (Roper Scientific) and a high-speed automatic objective stage for multiple Z stack recording. The images were collected with a 40x objective lens and 2.5x camera zoom at 45 second intervals at 37ºC. For each cell at each time point, GFP data were collected over 21 continuous vertical Z positions. After the first time frame, OKT3 or PMA were added to the samples. Images were processed by 3D deconvolution using AutoQuant X software (Media Cybernetics). All imaging manipulation and analysis were done using the MetaMorph software suite (Molecular Probes). Data are representative of at least six independent experiments analyzing at least five cells per experiment.

GFP-RasGRP1Δtail consistently localized in a perinuclear pattern. This aberrant localization was not driven by the GFP tag seeing as similar experiments using Flag-tagged RasGRP1 and RasGRP1Δtail proteins yielded identical results (data not shown). This defect in protein translocation could account for the inability of the truncated RasGRP1 protein to activate the Ras-Erk pathway.

4.3 Discussion

Recent publications have investigated the roles of the PT (Plasma membrane Targeter) and SuPT (Suppressor of PT) domains of RasGRP1, both of which are included in the tail domain. The PT domain was found to be sufficient and necessary for antigen receptor- mediated plasma membrane translocation of RasGRP1 in several B cell lines (Beaulieu et al., 2007). A follow-up study defined the precise mechanism by which the PT domain regulates plasma membrane targeting. The authors demonstrate that a small segment of the PT domain, which is enriched in basic/hydrophobic residues, binds directly to PI3K-generated products (Zahedi et al., 2011). In this report, we demonstrated that the tail domain of RasGRP1 was essential for its function in T cell development and activation *in vivo*. The deletion of the tail domain significantly affected thymocyte development and T cell function. RasGRP1$^{d/d}$ T cells were intermediary between wildtype and knockout profiles in terms of thymocyte subset percentages and surface marker expression. Despite only a slight defect in T cell development, Erk

activation was drastically impaired in RasGRP1$^{d/d}$ DP cells. Furthermore, crossing upon the OT-II and HY transgenic TCR backgrounds led to a near-complete block in positive selection. And, as RasGRP1$^{d/d}$ mice aged, they more closely resembled RasGRP1$^{-/-}$ mice in CD4^{+} T cell percentages, ability of those T cells to produce IFN-γ, and high levels of serum IgE.

In RasGRP1$^{d/d}$ T cells, the remaining RasGRP1 domains may have been able to bypass the need for the tail domain and account for some level of development and activation. The GEF and REM domains are likely required for membrane localization through their binding to Ras, which is anchored in endomembranes or the plasma membrane (Gureasko et al., 2008; Tazmini et al., 2009). Also, the calcium-binding EF hands and the SuPT domain are able to affect plasma membrane targeting of RasGRP1 (Beaulieu et al., 2007; Tazmini et al., 2009). Furthermore, the C1 domain mediates both internal membrane and plasma membrane targeting (Beaulieu et al., 2007; Carrasco and Merida, 2004). When the C1 domain is deleted in B cell lines, plasma membrane targeting of RasGRP1 still occurs, albeit with significantly reduced efficiency (Beaulieu et al., 2007). In some circumstances, signal strength may be enough to recruit RasGRP1 through its remaining domains, but, with a lower level or different form of activation, the tail domain may be indispensable. In this manner, we speculate that RasGRP1$^{d/d}$ T

cells bearing TCRs with strong affinity (or stronger than normal) for self-antigen were able to bypass the need for intact RasGRP1 activity and develop into mature T cells.

Additional evidence supporting our claim that the tail domain is essential for RasGRP1 function came from our assessment of T cell function and autoimmunity in RasGRP1$^{d/d}$ mice. RasGRP1$^{d/d}$ T cells exhibited defects in their proliferative capacity, IL-2 production, and upregulation of activation markers, particularly in response to TCR stimulation. Interestingly, despite high levels of serum IgE, T cells from RasGRP1$^{d/d}$ and RasGRP1$^{-/-}$ mice had an increased propensity to produce IFN-γ, but not IL-4, upon stimulation. A report examining TCR-stimulated human cells asserts that naïve CD45RO^{-} T cells stimulated briefly via the TCR produce high levels of IFN-γ, but no IL-4, inducing B cells to generate IgE (Brinkmann and Kristofic, 1995). Alternatively, as seen in Itk$^{-/-}$ mice (Qi et al., 2009), γδ T cells could be inducing IgE production by B cells in RasGRP1$^{d/d}$ and RasGRP1$^{-/-}$ mice.

While autoimmunity in RasGRP1$^{-/-}$ mice has been demonstrated previously (Coughlin et al., 2005; Coughlin et al., 2006), a conflicting report proposes that those results stem from the mixed background of the mice analyzed (Priatel et al., 2007). The mice used in the studies described here were backcrossed onto the B6 background for ten generations. By six months of age, the CD4 compartment had expanded significantly and serum from these mice contained high levels of auto-antibodies. From our results,

we concluded that the deletion or truncation of RasGRP1 protein impaired thymic selection and/or T cell homeostasis such that these mice indeed developed an autoimmune disorder. The issue of how disrupting RasGRP1 function seems to selectively affect $CD4^+$ T cells remains unknown. It has been shown that the $CD4^+$ T cell lineage relies more heavily upon Erk signaling (Fischer et al., 2005; Priatel et al., 2006) and perhaps the loss of RasGRP1 function had more severe ramifications on $CD4^+$ rather than $CD8^+$ T cells.

Surprisingly, it seems as though peripheral tolerance in these mice was not affected. A previous study documents the functional suppressive nature of $RasGRP1^{-/-}$ regulatory T cells (Chen et al., 2008). In accordance with this work, unpublished studies in our lab showed that $RasGRP1^{-/-}$ T cells were able to suppress $LAT^{m/m}$ T cell expansion upon co-transfer into lymphopenic mice. Also, the percentage of $Foxp3^+$ T cells in $CD4^+$ T cell populations from both $RasGRP1^{-/-}$ and $RasGRP1^{d/d}$ thymuses and spleens were actually increased compared to wildtype mice (data not shown and Figure 18I). Regulatory T cells are thought to be selected based on their intermediate affinity for self-ligands in the thymus (Jordan et al., 2001; Sakaguchi, 2004). The signaling pathways guiding conventional and regulatory T cell development may be intrinsically different and the regulatory T cell lineage may be able to bypass the need for RasGRP1 during its maturation.

Our data showed that the deletion of the tail domain mildly impaired RasGRP1 protein expression. A similar effect was seen in human SLE patients (Yasuda et al., 2007). In this study, it was shown that 9 of 13 new splice variants of the human RasGRP1 gene contain a form of RasGRP1 that lacks exons 16 and 17, which comprise most of the tail domain, either alone or in conjunction with other RasGRP1 exons. Moreover, this report shows that the RasGRP1 protein level is reduced in these patients. Thus, our RasGRP1$^{d/d}$ line should be an excellent model to study the human diseases caused by splice variations in the human RasGRP1 gene. We cannot totally exclude the possibility that the functional defect in RasGRP1$^{d/d}$ mice is caused by the reduced expression of RasGRP1 protein. However, the proper function of RasGRP1$^{+/-}$ cells, which express a much reduced level of protein, indicates that the defect observed in RasGRP1$^{d/d}$ mice was a true consequence of the tail domain deletion. Moreover, our data with Jurkat cells clearly showed that deletion of this tail domain impaired RasGRP1 membrane localization. While wildtype GFP-RasGRP1 was concentrated on the plasma membrane, GFP-RasGRP1Δtail was seen throughout the cell and failed to move following TCR engagement. After PMA stimulation, GFP-RasGRP1Δtail moved into a perinuclear pattern, perhaps reflecting the tendency of the C1 domain to accumulate at endomembranes (Beaulieu et al., 2007; Carrasco and Merida, 2004; Johnson et al., 2007). Thus, our data *in vivo* and *in vitro* implicate an important role for the tail domain in

controlling T cell development and autoimmunity. Our study reveals the novel findings that Erk activation in T cells is dependent upon the tail domain of RasGRP1 and that the loss of the RasGRP1 tail domain has severe consequences on thymocyte selection and T cell function.

5. General Discussion and Future Directions

5.1 LAT-mediated autoimmunity

The mechanism(s) guiding the uncontrollable proliferation of $CD4^+$ T cells carrying the LATY136F mutation remains unknown. Through our studies, we were able to conclude that restoring signaling through NFAT, NF-κB, and Raf, all major signaling pathways that are disrupted upon the abrogation of the LAT-PLCγ-1 interaction, were unable to inhibit disease development. In fact, enhancing Erk signaling through the use of a constitutively active form of Raf exacerbated autoimmunity. However, further disrupting the formation of the LAT signalosome through the deletion of Gads and RasGRP1 did not prevent LAT-mediated autoimmunity. Although RasGRP1 plays a major role in Erk activation in T cells, the possibility remains that the Grb2/Sos complex in $LAT^{m/m}RasGRP1^{-/-}$ T cells is driving the activation of the Ras/Erk pathway. If this is the case, perhaps a required negative signal from the LAT signalosome is absent, allowing for aberrant Erk activation and causing hyperproliferation. Thus, it would be interesting to use MEK1 inhibitors in the $LAT^{f/m}$ model to determine if the further inactivation of Erk can inhibit disease onset.

However, it seems more likely that a number of pathways must be altered in order to completely quell $CD4^+$ hyperproliferation. We are currently examining the role of cytokine signaling in promoting LAT-mediated disease. We are specifically interested

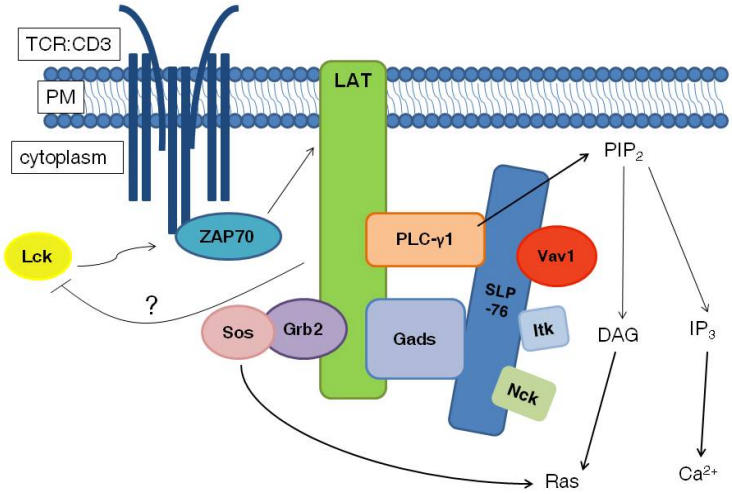
in the potential role of common gamma chain cytokines, such as IL-2, IL-4, IL-7, and IL-15, and will investigate the effect of inhibiting gamma chain signaling in mutant T cell proliferation. In particular, IL-7 is dispensable for fast, spontaneous T cell proliferation in lymphopenic hosts but is required for the slow, continuous proliferation of T cells (Kieper et al., 2005; Min et al., 2005). Notably, T cells containing no or mutated LAT undergo the slow sustained proliferation reminiscent of IL-7-dependent proliferation (Shen et al., 2010; Wang et al., 2008). A previous study demonstrates that the transfer of $LAT^{m/m}$ $CD4^{+}$ cells into Rag-$2^{-/-}$IL-$7^{-/-}$ mice show reduced proliferation compared to their transfer into $Rag2^{-/-}$ or $Rag2^{-/-}$MHC-deficient mice (Wang et al., 2008). These data indicate that IL-7 is critical for mutant T cell proliferation while TCR-MHC interaction is dispensable. However, $LAT^{fl\text{-}dtr/Y136F}$ T cells, which are similar to $ERCre^{+}LAT^{f/m}$ T cells, that are transferred into MHC-deficient hosts do not initiate autoimmunity upon wildtype LAT deletion, suggesting that the initial conversion of these cells into pathogenic effectors is dependent upon TCR engagement with MHC (Mingueneau et al., 2009). The severity of the hyperproliferation suggests that the LAT mutation could have severe ramifications on a spectrum of diverse signaling pathways, such that no one solution may be possible. Certainly, our studies, in conjunction with previous reports examining the intricacies of this disease, demonstrate how critical LAT is to the function and homeostasis of $CD4^{+}$ T cells.

In addition to potentially activating many cellular pathways, LAT could also serve a critical role as a central mediator of a negative feedback loop necessary to dampen T cell signaling and proliferation. LAT has been shown to play a negative role in T cell signaling through its recruitment of Gab2 to lipid rafts where Gab2 is able to constitutively associate with Gads/Grb2. Gab2 exerts a negative effect on T cell signaling through its recruitment of SHP (Src homology 2 domain-containing tyrosine phosphatase)-2, which induces the de-phosphorylation of crucial signaling components, such as CD3ζ. Additionally, Gab2 may negatively impact signaling by competing with SLP-76 for Gads/Grb2 binding (Yamasaki et al., 2001; Yamasaki et al., 2003). LAT is also known to associate with a complex containing Grb2, SHIP (Src homology 2 domain-containing inositol polyphosphate 5'-phosphatase)-1, and Dok (downstream of kinase)-2. This interaction leads to the tyrosine phosphorylation of SHIP-1 and Dok-2, the latter of which serves as a negative regulator of TCR-mediated IL-2 production and Zap-70 activation (Dong et al., 2006). Therefore, it is possible that LAT serves as a source from which negative signals can propagate in T cells, presumably to dampen T cell responses and avoid hyperactivation.

In addition to the interactions between LAT and SHP2- and SHIP-containing complexes, the recruitment of the tyrosine phosphatase SHP (Src homology 2 domain-containing tyrosine phosphatase)-1 to lipid rafts and its interaction with LAT are

markedly enhanced after TCR ligation (Kosugi et al., 2001). Another interesting study indicates that Erk and JNK are able to phosphorylate LAT at its Thr155 residue following TCR engagement. The result of this phosphorylation event is a defect in the ability of LAT to recruit PLC-γ1 and SLP-76, ultimately resulting in a decrease in calcium mobilization and MAP kinase activation (Matsuda et al., 2004). However, this threonine residue is only found in human LAT and a homologous phenomenon has not been discovered in mice. Nevertheless, these studies imply a role for LAT in the negative regulation of T cell signaling and lead to the possibility that blocking PLC-γ1 binding to LAT perturbs a negative function of the LAT signalosome.

In light of these findings, it is apparent that in the absence or mutation of LAT, T cells are converted into a hyperproliferative state where they are able to undergo sustained proliferation. Perhaps, the TCR-MHC interaction is initially needed to activate non-conventional or LAT-independent signaling pathways, converting T cells into pathogenic effectors. Once T cells have undergone this conversion, they may expand independent of TCR signaling. Or, the disruption of the LAT signalosome may abrogate a critical negative regulatory pathway, without which proliferation cannot be stopped. Also, the converted T cells may require external cues, such as IL-7, to maintain proliferation. Therefore, gamma-chain cytokines, particularly IL-7, will next be evaluated for their role in driving LAT-mediated disease (Figure 26). Here, we have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PM
cytoplasm
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ZAP70
PLC-γ1
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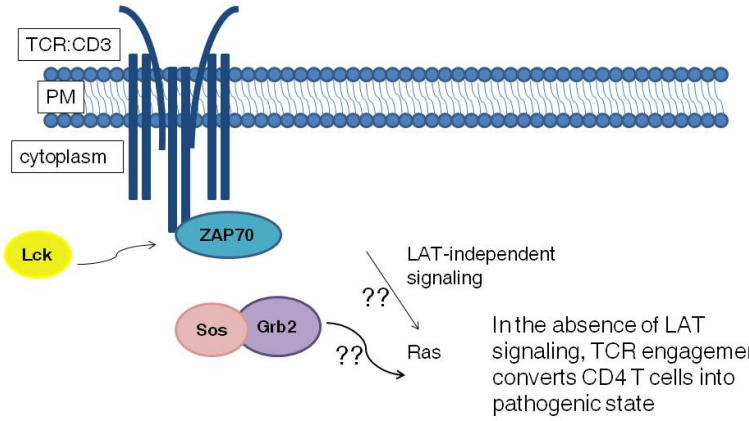
TCR:CD3
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LAT-independent signaling
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In the absence of LAT signaling, TCR engagement converts CD4 T cells into pathogenic state

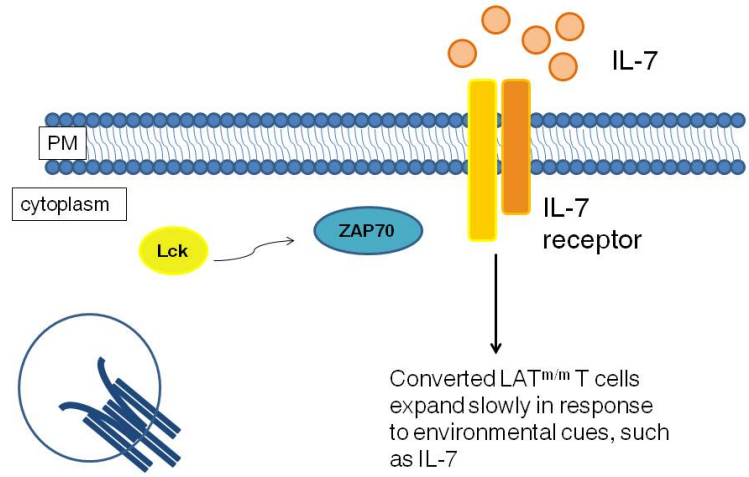
IL-7
PM
cytoplasm
IL-7 receptor
ZAP70
Lck
Converted LATm/m T cells expand slowly in response to environmental cues, such as IL-7

Figure 26: A model of LAT-mediated disease conversion and propagation.

(Top) In the presence of LAT, signaling through the TCR leads to MAPK activation and calcium flux. (Middle) In the absence of an intact LAT signalosome, TCR signaling through non-conventional or LAT-independent pathways may convert T cells into pathogenic effector cells. LAT may also be necessary for negative feedback on Lck activity. (Bottom) Following their conversion, mutant T cells may rely upon extrinsic signals, such as cytokine signaling, to maintain their sustained expansion.

shown that further obstructing TCR signaling through the deletion of Gads or RasGRP1 does not inhibit disease development, indicating that the hyperproliferation stems from non-conventional T cell signaling, the loss of LAT-dependent negative regulation, or an unknown signaling component downstream of the TCR.

5.2 The role of LAT in controlling cytokine production

Another interesting observation made in our laboratory was the differences in the helper T cell profiles of $LAT^{f/m}$ and $LAT^{m/m}$ T cells. Because $LAT^{m/m}$ mice undergo defective thymocyte development, unlike $LAT^{f/m}$ T cells in which the wildtype LAT allele is present until tamoxifen administration, it is not surprising that disease severity is much more pronounced in the $LAT^{m/m}$ model. However, the fact that $LAT^{f/m}$ T cells retained the ability to produce IFN-γ, even after two months of tamoxifen treatment, was an unexpected finding. $LAT^{m/m}$ T cells were unable to make this cytokine, instead producing vast amounts of IL-4. Furthermore, the deletion of additional LAT-binding partners, Gads and RasGRP1, in $LAT^{f/m}$ cells led to an identical phenotype, in which $LAT^{f/m}GRP1^{-/-}$ and $LAT^{f/m}Gads^{-/-}$ cells produced excessive amounts of IL-4 only. Also, the deletion of RasGRP1 and Gads in $LAT^{m/m}$ and $LAT^{f/m}$ T cells considerably increased their ability to produce IL-2. These results indicated that the presence of normal LAT during T cell development instills the ability to make IFN-γ and that this ability is not completely lost upon the deletion of wildtype LAT. Because $RasGRP1^{-/-}$ and $Gads^{-/-}$ T

cells produced significant amounts of IFN-γ, it seems that only in the context of the LAT mutation are these proteins needed to maintain the ability to produce IFN-γ.

As mentioned previously, the transcription factor T-bet plays a major role in IFN-γ production (Gately et al., 1998; Szabo et al., 2000; Szabo et al., 2002), while GATA3 is the master regulator of IL-4 (Ho et al., 2009; Zheng and Flavell, 1997). As anticipated by their ability to produce IFN-γ, $LAT^{f/m}$ CD4 T cells contain higher levels of T-bet, as indicated by an increased MFI, compared to wildtype cells. $Gads^{-/-}$ T cells have T-bet levels comparable to $LAT^{f/m}$ cells, while $GRP1^{-/-}$ cells contain slightly increased T-bet MFIs. In both $LAT^{f/m}Gads^{-/-}$ and $LAT^{f/m}GRP1^{-/-}$ T cells, T-bet MFIs are considerably decreased, reflecting the inability of these cells to make IFN-γ. Similarly, levels of GATA3 expression correspond to a genotype's propensity to make IL-4. In this vein, $LAT^{m/m}$ and $LAT^{f/m}$ cells both have appreciably increased GATA3 expression compared to wildtype cells. $LAT^{m/m}Gads^{-/-}$ and $LAT^{m/m}GRP1^{-/-}$ T cells show similar GATA3 MFIs compared to $LAT^{m/m}$ T cells. However, $LAT^{f/m}Gads^{-/-}$ and $LAT^{f/m}GRP1^{-/-}$ T cells show an increased GATA3 expression level, suggesting that perhaps the severe signaling defects seen in these mice inhibit T-bet expression and/or promote GATA3 upregulation.

In addition to promoting Th2 lineage development, GATA3 has been well-established as a main mediator of CD4 SP differentiation during thymocyte development (Hernandez-Hoyos et al., 2003). Furthermore, ectopic expression of

GATA3 in adult mice inhibits $CD8^+$ development (Nawijn et al., 2001) and retroviral expression of GATA3 in fetal thymocytes promotes $CD4^+$ development at the expense of $CD8^+$ T cells (Pai et al., 2008). In light of these results, the upregulation of GATA3 in $LAT^{m/m}$ amd $LAT^{f/m}$ cells could be the cause of the dramatic Th2 skewing. On the other hand, the increased expression of GATA3 could simply be a result of the increased Th2-skewing and IL-4 production by these cells. It will be interesting to determine if the Notch signaling pathway, which can directly regulate GATA3 expression (Fang et al., 2007), is dysregulated upon disruption of the LAT-PLC-γ1 interaction.

Further experiments in our lab will focus on the ability of LAT to affect the methylation status of particular cytokine loci or determine if LAT has a global impact on transcription. Indeed, preliminary work shows that the LAT mutation increases the level of T-bet and GATA3 transcripts. To look more closely at what is occurring at the transcriptional level, we will perform methylation analyses of the *ifng* and *il4* loci, as well as examine the differences in global methylation patterns following LAT mutation. We will also assess the effect of the LAT mutation on different STAT proteins and other pathways, such as Wnt and Notch, which may be responsible for Th2 skewing and IL-4 production in $LAT^{m/m}$ T cells.

5.3 The control of RasGRP1 activity by the tail domain

We were able to provide a novel mechanism by which RasGRP1 controls Erk activation through its tail domain. Through the use of our knock-in mouse model, we demonstrated that the loss of the tail domain has severe consequences on RasGRP1 localization, thereby hindering Erk activation, thymocyte development, and peripheral T cell function. However, in order to have a comprehensive understanding of how the tail domain affects Erk activation, multiple things remain to be determined. Thus far, all localization studies looking specifically at the role of the tail domain have been performed in cell lines. It would be ideal if we could employ an antibody that cleanly recognizes the N-terminal portion of RasGRP1 to do live imaging in thymocytes and mature T cells. Through this method, we would be able to definitively conclude that the tail domain is essential for proper protein movement following T cell stimulation.

Also, early studies in our lab were aimed at finding a protein that bound to the tail domain and whose interaction with RasGRP1 may have been essential for proper Erk activation. Following ^{35}S labeling and pull-down, we performed mass spectrometry to identify potential binding partners. Unfortunately, the only protein we were able to identify as specifically binding to the tail domain of RasGRP1 was HSP70. We believed this interaction to be a byproduct of the artificial expression of our truncated RasGRP1

protein. However, seeing as the deletion of the tail domain affects protein stability *in vivo*, perhaps this interaction is indeed necessary for RasGRP1 function.

Our data demonstrate that the tail domain is necessary for stable RasGRP1 protein expression. A similar observation has been made in human SLE patients (Yasuda et al., 2007). This study showed that 9 of 13 splice variants of the human *rasgrp1* gene contain a form of RasGRP1 that lacks exons 16 and 17, which comprise most of the tail domain, either alone or in conjunction with other RasGRP1 exons. Furthermore, the authors reveal that the RasGRP1 protein level is reduced in these patients. Thus, RasGRP1$^{d/d}$ mice could serve as a useful model to more closely study human disease caused by splice variations in the human *rasgrp1* gene. Also, as the "personalization" of cancer treatment becomes more common and as Ras is commonly mutated in many types of human cancers, manipulation of RasGRP1 could become an important mechanism to be considered in cancer therapy.

We cannot totally exclude the possibility that the functional defect in RasGRP1$^{d/d}$ mice is caused by the slightly reduced expression of RasGRP1 protein. But, we found further evidence supporting a role for the tail domain in Jurkat cells, in which the deletion of the tail domain impaired RasGRP1 membrane localization. While wildtype GFP-RasGRP1 was located on the plasma membrane, GFP-RasGRP1Δtail could be seen throughout the cell or in a perinuclear pattern, failing to move to the plasma membrane

following TCR or PMA engagement. Thus, we revealed the novel finding that Erk activation in T cells is dependent upon the tail domain of RasGRP1 and that the loss of this domain has severe consequences on Erk activation and T cell function.

5.4 Conclusions

The immune system consists of highly controlled and regulated networks to ensure protection against invading organisms. It is known that the mutation of a single tyrosine in the adaptor protein LAT causes unregulated T cell expansion and the development of an autoimmune-like disease. Here, we demonstrate that the deletion of the LAT signalosome components, RasGRP1 and Gads, could not suppress mutant T cell proliferation but did affect their cytokine potential. We further analyzed the role of the RasGRP1 tail domain in controlling Erk activation in T cells. The loss of this domain led to impaired thymic development, T cell function, and an autoimmune-like condition. Imaging studies further pointed to a role for the tail domain in mediating protein translocation following TCR stimulation. In both of our models, we clearly show how slight perturbations of TCR-mediated signaling pathways have severe ramifications on T cell function. These data also highlight the delicate balance that is constantly required by the immune system to allow for protection while preventing autoimmunity within the host.

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Biography

I was born on August 9, 1984 in Mobile, Alabama. After short stops in Maryland and Tennessee, we settled in Cranston, Rhode Island. I attended the College of William and Mary as a James Monroe Scholar, earning a Bachelor of Science in Biology in May 2006. Following my love of immunology, I then joined the Department of Immunology at Duke University. For the past 6 years, I have been fortunate to study T cell signaling and autoimmunity under the guidance of Dr. Weiguo Zhang.

Publications:

- **Fuller DM,** Zhu M, Koonpaew S, Nelson M, Zhang W. *The importance of the Erk pathway in the development of LAT-mediated disease.* Manuscript submitted.
- **Fuller DM**, Zhu M, Song X, Ou-Yang CW, Sullivan SA, Stone JC, Zhang W. *Regulation of RasGRP1 function in T cell development and activation by its unique tail domain.* Manuscript accepted. PLoS ONE.
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